Analysis and behaviour of bioactive pollutants in wastewater and reuse for irrigation: Addressing country specific issues in Mexico and the UK

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ABSTRACT

Treated and untreated municipal wastewater reuse for agricultural irrigation is a widespread worldwide practice that has many benefits. These include reusing a waste stream, its year-round availability and the presence of nutrients in wastewater. However, municipal wastewater has been identified as one of the main sources of bioactive chemical pollutants or BACs (also known as emerging pollutants) in the environment. Many unknowns remain in terms of the extent of the removal of these pollutants using conventional wastewater treatment, seasonal and geographical variation on the prevalence of these pollutants, the extent of natural attenuation and the behaviour of these pollutants once in the environment. The aim of this thesis was to address some of these knowledge gaps by developing environmentally-relevant targeted and untargeted analytical methods, modelling tools, and undertaking an evaluation of wastewater treatment plants and the aquatic environment in the UK and Mexico.

The two wastewater treatment plants monitored in the UK were Stoke Bardolph wastewater treatment plant (PE~650,000) which has advanced biological treatment and Sutton Bonington wastewater treatment plant (PE~2,000) which has conventional trickling filter and a tertiary sand filtration step. Both of these plants were monitored using targeted methods, Stoke Bardolph for a year and Sutton Bonington for seven months. It was found that sulfamethoxazole, acetaminophen, acetyl-sulfamethoxazole and ibuprofen were better removed by Stoke Bardolph, while the estrogen hormones, diclofenac, morphine, erythromycin, trimethoprim, o-desmethylvenlafaxine, propranolol and codeine were better removed by Sutton Bonington. Despite their differences in removal efficiencies, both plants have risk coefficients consistently above 1 for the same 8 out of 36 pollutants; namely, codeine, diclofenac, erythromycin, morphine, oxytetracycline, propranolol, salicylic acid, simvastatin, sulfamethoxazole, tramadol and trimethoprim. Furthermore, the equivalent estradiol concentrations were found to be at least 8 times above the predicted no effect concentration. Significant ($p<0.05$) seasonal variations in removal for Stoke Bardolph wastewater treatment plant for 12 pollutants were identified, including diclofenac, sulfamethoxazole and carbamazepine. Eight strong and significant correlations were identified among several pollutants and between pollutants and water quality parameters, these are important in pinpointing removal efficiencies in relation to process operating conditions. For example, a correlation between temperatures >15°C and increased removal for o-desmethylvenlafaxine in Sutton Bonington treatment plant was identified.

In order to address the knowledge gaps in relation to metabolites, transformation products and unknown BACs, an untargeted method was developed and used to analyse samples from Stoke Bardolph and Sutton Bonington wastewater treatment plants. Twenty-five compounds not previously identified in relation to wastewater pollution were identified, as well as 10 biomarkers (including caffeine, nicotine, saccharin and acesulfame), 2 illicit drugs rarely reported in wastewater, and 3 pollutants identified in relation to potential disease monitoring. Partial least square analysis provided insights on the removal efficiencies of both plants for these pollutants. This work helps to address the knowledge gap on the environmental toxicity which remains unmonitored using targeted approaches, as well as identifying pollutants relevant for wastewater-based epidemiology.

Both targeted and untargeted methods developed were used to study an agricultural area in Mexico known as the Tula Valley, which has been irrigated using wastewater from Mexico City for over 100 years. Seven BACs were quantified which had not been previously studied in the Tula Valley, of which 4-tert-octylphenol, levonorgestrel,

simvastatin and tramadol were found to have environmental risk quotients above one. Five BACs were found with measurable concentrations in spring groundwater, indicating prevalence and poor natural attenuation, with sulfamethoxazole found with an environmental risk quotient above one. The total estradiol equivalent was found to have environmental risk quotient above 1, with influence from estriol and its sulfate conjugate which have not been previously measured in the Tula Valley. Using the untargeted method, over 10 pollutants which have not been previously detected in the aquatic environment and with likely bioactive effects were detected in the Tula Valley. For example, 4-hydroxyindole which is used in the hair dyes and banned by ASEAN. More than 20 pollutants known for their toxicity and bioactivity, but not previously detected in the Tula Valley were identified. For example, the benzotriazole 5-methylbenzotriazole, cocaine metabolite benzoylecgonine and the antibiotic, ciprofloxacin. Potential biomarkers (e.g., caffeine and nicotine), which were also found in the UK wastewaters were detected. It is significant that common biomarkers were found in two locations using an untargeted method, because this makes them strong candidates to use in future international wastewater-based epidemiology studies.

The data gathered using targeted and untargeted methods in field studies in Mexico and the UK was used to identify pollutants of environmental significance for further study. These were then shortlisted as sulfamethoxazole, carbamazepine and tramadol. The first modelling investigation of BAC sorption in soil using data obtained from the literature enabled identification of gaps in the literature and unveiled experimental biases about these pollutants once they reach the soil compartment following water application. Six specific gaps which require further study were identified. 1) Further data is required for understudied pollutants such as tramadol and for pollutants which present inconsistencies across the literature like sulfamethoxazole. 2) Sorption studies are needed with environmentally-relevant concentrations, as they are usually carried out at higher concentrations. 3) In order to rule out biodegradation, different biodegradation quenching methods are used. These methods were found to influence sorption, with sodium azide having a greater influence than autoclaving, pointing towards the need for further study. 4) Sorption studies at different temperatures that account for variations which occur seasonally and geographically need to be carried out. 5) Sorption studies using treated and untreated liquid phase (realistic irrigation water) are needed. 6) Untargeted analysis of soil and liquid phases in sorption studies could elucidate competitive sorption effects as well as transformation process which may account for discrepancies in the literature.

PUBLICATIONS AND PRESENTATIONS

Publications

Angelakis, A.N., Zaccaria, D., Krasilnikoff, J., Salgot, M., Bazza, M., Roccaro, P., Jimenez, B., Kumar, A., Yinghua, W., Baba, A., Harrison, J.A., **Garduno-Jimenez, A**., Fereres, E., 2020. Irrigation of world agricultural lands: Evolution through the Millennia. Water (Switzerland) 12. https://doi.org/10.3390/W12051285
This paper contributed to the introduction.

Garduño-Jiménez AG, Ortori C, Barrett DA, Duran-Alvarez JC, Gomes RL. Understanding and addressing issues in quantifying emerging bioactive pollutants in soil: ageing time and soil type. Chemosphere. (Submitted February 2021).
This manuscript corresponds to the soil method development reported in section 4.1.5 of this thesis.

Garduño-Jiménez AG, Ortori C, Barrett DA, Vale P, Gomes RL. Seasonal evaluation, treatment efficiency and correlations of emerging pollutants in two wastewater treatment plants. Environmental Science Process & Impacts (In review with industial collaborator, due to submit February 2021).
This manuscript corresponds to the targeted LC-MS method and aqueous matrix validation from Chapter 4 and to Chapter 5 of this thesis.

Acheampong E, **Garduño-Jiménez AG**, Dryden IL, Wattis JAD, Gomes RL. Classification of the removal of emerging pollutants to inform on sustainable wastewater treatment policies. *Chemical Engineering Journal.* (In review with industrial collaborator, due to submit April 2021)
This manuscript was written using the data collected from Chapter 5 of this thesis.

Garduño-Jiménez AG, Ortori C, Barrett DA, Gomes RL. Screening of emerging pollutants and environmental risk evaluation in two wastewater treatment plants. *Journal of Hazardous Materials*. (In review with supervisors with plan to submit April 2021).
This paper corresponds to the untargeted method development from Chapter 4 and to Chapter 6.

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This paper corresponds to Chapter 8.

Presentations

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Garduño-Jiménez AG, Ortori C, Barrett DA, Gomes RL. Plants on the Pill: We are what we eat, but do we know what we are eating? *Nottingham Environment Conference.* Nottingham, UK, 25th June 2018.

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pollutants in wastewater for reuse in agricultural irrigation. *Engineering Research Showcase, University of Nottingham*. Nottingham, UK, 2nd May 2018.

Garduño-Jiménez AG, Dodsworth T, Ortori C, Duran-Álvarez JC, Barrett DA, Gomes RL. *28th Annual Meeting of the Society of Environmental Toxicology and Chemistry (SETAC).* Rome, Italy 13th -17th May 2018.

Garduño-Jiménez AG, Ortori C, Duran-Álvarez JC, Barrett DA, Gomes RL**.** Development of method to quantify bioactive pollutants due to wastewater irrigation in environmental matrices*. 15th International Conference on Environmental Science and Technology (CEST)***.** Rhodes, Greece, 13th August- 2 September 2017

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LIST OF ABBREVIATIONS

Abbreviation	Meaning
AMU	Atomic mass units
APCI	Atmospheric pressure chemical ionization
APPI	Atmospheric pressure photo ionization
BACs	Bioactive chemicals
BLD	Below limit of detection
BOD	Biological oxygen demand
CBZ-EP	Carbamazepine10,11-epoxide
CEC	Cation exchange capacity
COD	Chemical oxygen demand
CV	Coefficient of variation
DIOH-CBZ	10,11-dihydro-10,11-dihydroxycarbamazepine
DLLME	Dispersive solid-liquid liquid microextraction
E1	Estrone
E1-3S	Estrone-3-sulfate
E2	Estradiol
E2-3S	Estradiol-3-sulfate
E3	Estriol
E3-3S	Estriol-3-sulfate
EE2	Ethinyl estradiol
EI	Electron impact ionization
ESI	Electrospray ionization
FAB	Fast atom bombardment
FPSE	Fabric phase sportive extraction
FTIC	Fourier transform Ion Cyclotron Resonance
GC	Gas chromatography
HGT	Horizontal gene transfer
HPLC	High performance liquid chromatography
HRT	Hydraulic retention time
LOD	Limit of detection
iLOD	Instrumental limit of detection
LC	Liquid Chromatography
LLE	liquid-liquid extraction
MAE	Microwave-assisted extraction
MALDI	Matrix-assisted laser-desorption ionisation
MEC	Measured environmental concentration
MIC	Minimum inhibitory concentration
mLOD	Method limit of detection
MRM	Multiple reaction monitoring
MS	Mass spectrometry
NDT	N-Desmethyltramadol
NSAID	Non-steroidal anti-inflammatory drug
ODT	O-Desmethyltramadol
P.S.	Particle size
PCA	Principal component analysis
PE	Population equivalent
PEC	Predicted environmental concentration
PLE	Pressurized liquid extraction
PLS-DA	Partial least squares differential analysis
PNEC	Predicted no effect concentration
QIT	Quadrupole Ion Trap
QqQ	Triple quadrupole

Abbreviation	Meaning
QTrap	Linear quadrupole ion trap
QUECHERS	Quick Easy Cheap Rugged and Safe
RPLC	Reversed-phase liquid chromatography
RQ	Risk quotient
S/N	Signal to noise ratio
SD	Standard deviation
SLE	Solid-liquid extraction
SPE	Solid phase extraction
SPME	Solid phase microextraction
SRM	Single reaction monitoring
SRT	Sludge retention time
SS	Suspended solids
SSS	System suitability sample
Stk	Stoke Bardolph wastewater treatment plant
SuB	Sutton Bonington wastewater treatment plant
TNO	Tramadol-N oxide
TOF	Time-of-Flight
TSS	Total suspended solids
UAE	Ultrasound assisted extraction
UHPLC	Ultra-high pressure liquid chromatography
WWD	Water downstream of a wastewater treatment plant
WWE	Wastewater effluent
WWI	Wastewater influent
WWTP	Wastewater treatment plant

1 INTRODUCTION

1.1 Thesis overview

This introduction presents the rationale of research answersing the following questions:

1. Why focus on wastewater reuse in the context of water scarcity and pollution?

2. Why are bioactive chemical pollutants in the environment an issue worthy of study?

3. Why study bioactive chemical pollutants from an engineering perspective underpinned by analytical chemistry?

The answers to these questions act as an introduction to the topic studied, as well as a justification for its relevance. From this section the reader should understand the rationale behind focusing on the study of bioactive chemical pollutants (BACs) in the agricultural environment. Within the agricultural environment, the scope of this thesis is in studying BACs in the aqueous and soil matrices in terms of environmental monitoring and data modelling. The current knowledge and technologies used for studying the environmental prevalence, fate and remediation of bioactive chemical pollutants (BACs) is presented in the literature review chapter. This chapter includes background on the three locations used as case studies. Two of these locations are UK municipal wastewater treatment plants (WWTPs) from which the influent and effluent were monitored. The third location is an area in Mexico where treated and untreated wastewater has been used for agricultural irrigation for over 100 years. A small section in the literature review deals with some important considerations when dealing with environmental pollution in a global context.

Methods to detect and quantify BACs in environmental matrices have been previously developed and reported in the literature, however it is necessary to validate them for the instruments used and the matrices and analytes studied (United States Environmental Protection Agency, 1999). The analytical chemistry methods are detailed in Chapter 3. The development and validation of these methods is then described in Chapter 4. When developing the method to quantify BACs in soil matrices an important gap in the literature was addressed (Michael, et al., 2017). The effect of ageing (the time between spiking and extraction), which is often overlooked when new methods are developed (Michael, et al., 2017). This effect is studied in detail and the optimum ageing time for the pollutants studied is presented.

In the context of wastewater reuse for agriculture, it is necessary to identify and quantify the BACs found in treated and untreated wastewater as well as their seasonal and spatial variability (Carter, et al., 2019; Fernández, et al., 2014; Höhne, et al., 2008; Zhou, et al., 2017). This is important because findings for a particular geographical area or season will likely be different for another and actions determined from the findings must be consistent with the local context (Carter, et al., 2019; Madikizela, et al., 2017; Peña-Guzmán, et al., 2019). That is why a large part of this thesis is devoted to monitoring in the three case study areas. A long-term targeted monitoring campaign was undertaken in two UK WWTPs and the findings in terms of removal efficiencies depending on the treatment technology used, the effect of seasonality and the toxicity of the effluents discharged to the environment are presented in Chapter 5. This work adds to the few long-term monitoring efforts of WWTPs (Fernández, et al., 2014; Li, et al., 2014; Silva, et al., 2014; Zhang, et al., 2015; Zhao, et al., 2017; Zhou, et al., 2017). It adds to the knowledge gaps in terms of frequency of detection of certain BACs, the

removal efficiency of different WWTPs and the seasonal changes of these pollutants (Carter, et al., 2019). Targeted methods, as the one used to obtain the results in Chapter 5, are valuable in terms of confidence and quantification, however their main drawback is that analytes which are not in the method (such as metabolites or transformation products of BACs) are overlooked (Petrie, et al., 2015). Therefore, some samples from these two UK WWTPs were analysed using the untargeted method developed and are presented in Chapter 6. The value of this work lies in the putative identification of BACs which are not in the targeted method. For example, new drugs of abuse, metabolites and transformation products.

The results obtained from the Mexico case study are detailed in Chapter 7. This site is very interesting as wastewater has been reused for irrigation for over 100 yearsm (Chávez-Mejía, et al., 2019). This use of water, in an arid region, has resulted in the recharge of an artificial aquifer, used as a water source by the local population (Chávez-Mejía, et al., 2019). For practical reasons it was only possible to do a single time point sampling campaign, however it includes several geographical sampling locations, giving an indication of the fate and prevalence of BACs in an agricultural context. It also contributes to bridging the knowledge gap in terms of the geographical distribution of current knowledge on the presence of BACs in the environment which is mostly focused on the global north (Carter, et al., 2019; Madikizela, et al., 2017; Peña-Guzmán, et al., 2019). More importantly it is a first step for an international collaboration, which is of great importance when addressing pollution issues (Bernhardt, et al., 2019).

From the case studies and literature review, it was possible to identify important BACs on which to focus for modelling work on BAC soil sorption. Fate and behaviour of BACs in soil matrices is very important as it determines how much of the pollutants are leached into groundwater after irrigation and how much is available for plant uptake (Drillia, et al., 2005a; Li, et al., 2020; Paz, et al., 2016). Modelling of results reported in the literature in terms of sorption of BACs to different soils was carried out to identify literature gaps in our understanding of the relationship between soil properties and physicochemical characteristics of BACs in the extent of sorption. The modelling undertaken was also adequate for identifying biases in experimental set-ups across the literature studied. These results are presented in Chapter 8.

In the last chapter the main conclusions are presented as well as recommendations for future work.

1.2 Rationale for research

1.2.1 Wastewater reuse in the context of water scarcity and pollution

One of the most pressing global problems of our time is water scarcity and pollution. Water scarcity may refer to economic or physical scarcity. Economic water scarcity means that even if there is water in an area, the economic resources to make it available to cover the needs of the population are not available. Physical water scarcity means there is not enough water to meet the demands of the population (Food Agriculture Organization and UN Water, 2007). Figure 1.2.1 illustrates water availability worldwide.

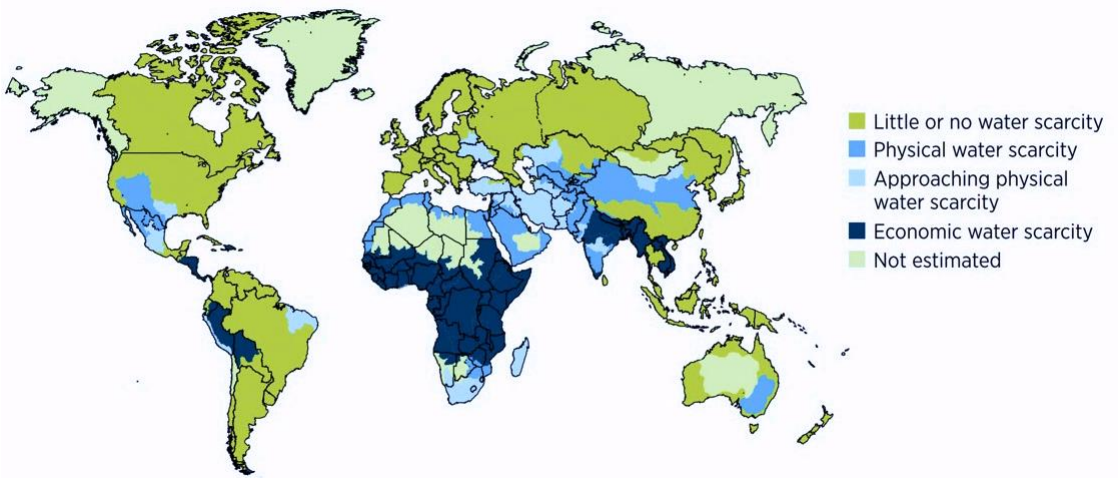


Figure 1.2-1 Global physical and water scarcity (World Water Assessment Programme, 2012)

The World Economic Forum has ranked Water Crisis as a top five risk in terms of impact every year since 2012 (World Economic Forum, 2017). Around 1.2 billion (almost a fifth of the world's population) live in areas of physical water scarcity and 1.6 billion (almost a quarter of the world's population) face economic water shortage (Food Agriculture Organization and UN Water, 2007). By 2050 world water demand is projected to increase by 55% (UN-WWAP, 2015). These figures are worrying given that adequate management of water is necessary for humans and the environment (WWAP, 2017) This idea is reflected in the Sustainable Development Goals (Figure 1.2.2), which include SDG 6: Clean Water and Sanitation and is also strongly linked with several other SDGs including 2, 3 and 14:



Figure 1.2-2 Sustainable Development Goals (United Nations, 2015)

In this context it is key to assess the agricultural sector, given that 70% of the global water use is for irrigation (WWAP, 2017) However only 20% of farmlands are irrigated and this 20% produce 40% of the world's food (Lazarte, 2017). This is a testament to the increase in agricultural productivity when the land is irrigated. Statistics indicate that the current population increase means that by 2050, 60% more food will need to be produced globally and double the current amount in certain countries (UN-WWAP, 2015).These statistics may be deceiving as it is not only an issue of productivity. It has been shown that the current global food production is enough to feed everyone, but hunger is also a matter of poverty and unequal distribution (Holt-Giménez, et al., 2012).

We do not only need a more efficient use of water for agriculture, but also a fairer one. For example, highly profitable crops with high water demand are sometimes grown in arid regions to export to other parts of the world. This practice reduces the agricultural resources of the local population. Irrigation practices therefore are an issue that needs to be addressed from a political, social and economic perspective as well as an engineering one (Williams, et al., 2019).

Water reuse is a worldwide practice that has been undertaken since ancient times. In particular, the reuse of wastewater for agricultural irrigation goes back to at least 3000 BC in Greece (Metcalf & Eddy, et al., 2007). In more recent history, a prominent example are the drainage canals built in 1890 to take untreated wastewater from Mexico City to irrigate agricultural areas outside the city (Metcalf & Eddy, et al., 2007), a practice which, to some extent, remains today (Jimenez-Cisneros, et al., 2012).

Wastewater reuse represents a relief to the environmental and economic pressures of agriculture and an increase in the accessibility of water for irrigation. This has the potential to create more sustainable and efficient food systems. Wastewater is available all year round and so it reduces the demand on potable water resources (Jovanovic, 2008). This is becoming increasingly impotant as we account for the increasing extreme and uncertain weather patterns caused by climate change (Holt-Giménez, 2012). The high nutrient content of wastewater means that the use of commercially bought fertilisers can be reduced, bringing economic and environmental benefits. Wastewater reuse also represents an economic advantage for farmers as they are using what others may consider an undesirable waste stream (Jimenez-Cisneros, 2006). Finally, this practice rarely has the same high pumping costs associated to sources like groundwater (Jaramillo, 2017).

Wastewater reuse requires attention not only for the benefits mentioned, but also for the scale of the practice. A recent study based on a Geographical Information System (GIS) model produced an estimate of the global agricultural croplands irrigated using water influenced by urban wastewater flows. This paper found that 65% of irrigated croplands are located in wastewater dependent catchments of which 82% are located in countries where less than 75% of wastewater is treated. Most of these irrigated croplands are found in: China, India, Pakistan, Mexico and Iran. This study found that the practice of wastewater irrigation is 50% higher than previous high estimates extrapolated from case studies and government statistics (Thebo, et al., 2017). This practice is prominent in arid regions where rainfall or groundwater availability does not cover the necessities of the population (Shenker, et al., 2011). One tenth of the world's population consumes food that has been irrigated with wastewater (Metcalf & Eddy, et al., 2007).

The global magnitude, necessity and benefits of direct or indirect wastewater reuse have led to national and international standards and laws on the quality of the water necessary for specific uses (Metcalf & Eddy, et al., 2007). The main concerns are the effects of pathogens, nutrients, inorganic salts, heavy metals, and BACs (pharmaceuticals, carcinogenic substances and endocrine disruptors (Jimenez-Cisneros, et al., 2008; Jovanovic, 2008). Pollutants such as pathogens, nutrients, inorganic salts and metals are better understood. The concentrations at which they present a risk to the environment and human health, as well as technologies for their removal are well defined (Metcalf & Eddy, et al., 2007). The same is not true for BACs, which in the past two decades have become of increasing concern. This is mainly due to the increasing negative effects they have been found to have in the environment, as well as their persistence due to their incomplete removal from wastewater treatment plants (Boshir Ahmed, et al., 2017; Halling-Sorensen, et al., 1998).

1.2.2 Why are bioactive chemical pollutants an issue worth studying?

1.2.2.1 BAC as pollutants and drivers for antimicrobial resistance

Antibiotics have saved millions of lives since the modern era of antibiotic use, however antibiotic resistance has reduced their effectiveness which is already claiming lives and making many bacterial infections more difficult and expensive to control (Ventola, 2015). For these reasons the World Health Organization has identified antibiotic resistance as a threat to global health, food security and development (Wolrd Health Organization, 2018). Evidence shows that antibiotics act as promoters of antibiotic resistance, therefore more antibiotics in the environment drive an increase in antibiotic resistance (Marti, et al., 2014). Therefore, the main identified causes of antibiotic resistance are the overuse of antibiotics (due to incorrect or over-prescription and the lack of antibiotic regulation in some places) and the extensive use in agriculture. Furthermore, there has been a decrease in research for new antibiotics as it is not profitable to spend time and money on a drug that will eventually become ineffective (Ventola, 2015).

Bacteria have naturally developed the ability to synthesise bioactive molecules to cooperate or antagonise other members of their community. They, therefore, also have a collection of genetic encoding to defend themselves from these molecules called resistome. These genetic sequencings can eventually participate in the development of antimicrobial resistance (Marti, et al., 2014). Bacteria can either inherit antibiotic resistance genes or horizontal gene transfer (HGT) can occur among non-related bacteria and even among different species (Ventola, 2015) through processes such as plasmid exchange or through bacteriophages (Marti, et al., 2014).

Only a small proportion of antibiotics consumed are metabolised *in vivo* and between 20-90% are excreted unchanged through animal or human urine or faecal excretion (Sabri, et al., 2020; Ventola, 2015). This results in antibiotics from farming being widely dispersed through fertiliser, groundwater and surface runoff (Ventola, 2015). As well as their ubiquitous presence in wastewater and subsequently surface waters due to their incomplete removal in wastewater treatment plants (Sabri, et al., 2020). Furthermore, there is evidence that antibiotic resistance bacteria from human and animal guts make their way to WWTPs (Becerra-Castro, et al., 2015). WWTPs have been identified as ideal environments for HGT to occur, due to the high bacteria density coupled with high oxygen and nutrient content as well as the presence of antibiotics and antibiotic resistance bacteria (Marti, et al., 2014). BACs which are not antibiotics, such as preservatives (sodium nitrite, sodium benzoate and triclocarban) have been found to promote HGT through mechanisms such as increasing cell permeability. This shows the potential of non-antibiotic BACs in spreading antimicrobial genes (Cen, et al., 2020). The favourable conditions for antibiotic resistance and HGT in WWTPs as well as the increased antibiotic and other BACs concentrations in the environment are factors in the increase of antibiotic resistance (Marti, et al., 2014).

1.2.2.2 Effects of BACs on aquatic and terrestrial life

It is expected that BACs would have an effect on other species as they are designed to interact with specific molecular targets in humans and these targets have orthologs that are conserved in other species e.g. 20–25% of the drug targets in humans had predicted orthologs in plants (Carter, et al., 2019). Field studies have revealed some of the long-term effects that exposure to these pollutants has on different life forms. One of the better-known examples is that of female oestrogens at very low ngL^{-1} concentrations in aquatic environments being linked with the feminisation of fish (Burkhardt-Holm, 2010; Sumpter, et al., 1995). Other examples show the effect of

bioaccumulation such as the decline of vulture populations which fed on carcasses of animals recently treated with diclofenac in Pakistan (Oaks, et al., 2004).

Effects on plants have also been observed such as the inhibition of photosynthesis in algae caused by exposure to β-blockers (Escher, et al., 2006) as well as plant biomass being affected (Carter, et al., 2019).Changes in biomass may be due to the potential pharmaceuticals have to affect in-plant homeostasis, such as changes in phytohormones, cellular metabolism, nutrient uptake and signalling without phenotypic change (Carter, et al., 2019).

Irrigation with untreated or partially treated wastewater has been shown to have effects on soil microbial community as well as effecting changes on soil physicochemical properties (Becerra-Castro, et al., 2015). For example, antibiotics have been shown to affect soil microbial communities and in doing so affecting processes such as nitrification and denitrification (Cycoń, et al., 2019). Despite some studied effects of antibiotic in soil communities, much still remains unknown about the effects other types of BACs can have as well as mixture effects (Carter, et al., 2019). For example, atenolol has been shown to be of concern due to possible synergistic effects with other β-blockers (Xu, et al., 2017). The proven uptake of BACs by plants through irrigation (Calderón-Preciado, et al., 2009b; Christou, et al., 2017; Eggen, et al., 2011; Hurtado, et al., 2016; Shenker, et al., 2011; Chenix Wu, et al., 2012; Wu, et al., 2015) has another concerning implication in that agricultural produce consumption may prove a route of BAC exposure to animals and humans (Calderón-Preciado, et al., 2011).

Despite the known negative effects, many unknowns remain. These are mainly due to the wide variety and large number of BACs and the labour-intensive nature of determining toxicity. Of the approximately 1500 pharmaceuticals in use in the United Kingdom acute ecotoxicity data are available for only a small proportion, and chronic data are even scarcer (Burns, et al., 2017). In addition, many variables remain near impossible to account for simultaneously in order to gain a holistic perspective when determining toxicity, namely: exposure route (e.g., dermal, enteral, pulmonary), exposure timing (windows of vulnerability, e.g., developmental stage), prior exposure history, prior exposure duration (e.g., acute or short-term, sequential, intermittent, episodic or chronic), nutrition, age, sex, genetics, and nonchemical co-stressors (temperature, physical/metabolic stress, noise, electro-magnetic radiation, pathogens) (Daughton, 2003). If taken individually, many BACs do not present a risk at the concentrations in which they are detected in the environment (Di Nica, et al., 2017), but these pollutants are found in complex mixtures and can potentially cause additive and even synergist effects (Petrie, et al., 2015). Furthermore, data on toxicity from transformation products and metabolites is even scarcer, and these pollutants can be found at higher concentrations than the parent compound or can be converted back to the bioactive parent compound (Carter, et al., 2019).These uncertainties are likely why negative effects have been first identified from field studies compared to laboratory scale studies.

1.2.2.3 Legislation, policy drivers and precautionary principle

Consensus on the need to address the issue of BACs in the environment has led to related legislation being drafted and approved. Within the European Union (EU) the Water Framework Directive (WFD) is the legislation which ensures the biological and chemical quality of water in river catchments across Europe. The Watch List was published under this legislation in 2015. This is a list of potential water pollutants which EU Member States should monitor in order to determine the risk they pose. This list is reviewed every 2 years and currently includes several BACs, such as one of the active ingredients in birth control pills: 17-α-ethinylestradiol (EE2), natural oestrogens 17-β-

estradiol (E2), and estrone (E1), macrolide antibiotics: erythromycin, clarithromycin and azithromycin, among others (Directive 2008/105/EC, 2008).

In 2005, the European Commission funded the NORMAN project which improves data quality and comparability through common analytical and sampling approaches as well as being an independent organisation at the interface between science and policy (Dulio, et al., 2018). In the United Kingdom the UK Water Industry Research, the body responsible for facilitating the shaping of the water industry's research agenda invested £25 million between 2010-2013 through the Chemical Investigation Programme (CIP) in an effort to better understand the prevalence of priority substances and inform policies responding to new environmental quality standards. This programme has been extended to a second phase CIP 2 (UK Water Industry Research, 2017).

Further research is needed to determine if and which other pollutants should be added the European Watch list and which are relevant for other parts of the world where no relevant legislation exists yet (Peña-Guzmán, et al., 2019; Yang, et al., 2014).

Despite the advances in analytical chemistry, it is highly likely that the pollutants we can currently measure are only a small fraction of what is actually found in the environment (Daughton, 2003). The precautionary principle states that: *in cases of serious or irreversible threats to the health of humans or ecosystems, acknowledged scientific uncertainty should not be used as a reason to postpone preventive measures* (Martuzzi, et al., 2004). This principle exists primarily to encourage political and legislative action to prevent environmental damage before it is too late (Cameron, et al., 1991). The remaining unknowns in relation to BACs should be a driver for further research to be able to quantify the risk they pose and develop the technologies and legislative frameworks to reduce or eliminate their negative effects.

1.2.3 Why work from an engineering perspective underpinned by analytical chemistry and modelling tools?

The ideal solution would be to avoid the production of BACs, produce them in such a way in which they are not harmful to the environment or prevent them from entering the environment. However, pharmaceuticals and antibiotics save many lives and our societies are highly dependent on them (Braund, et al., 2016a). The pharmaceutical and personal care product industry is a very significant economic activity globally (Braund, et al., 2016a) driven by the high demand on pharmaceuticals for human and animal health. This means that it is not possible in the short-term to fully eliminate their production (Daughton, 2003), therefore we could consider manufacturing BACs in such a way in which they are not harmful to the environment. However, their effectiveness relies on them being bioactive and often persistent, so even if current pharmaceuticals were replaced with different ones, they would need to have similar properties (Jones O., 2014). Furthermore, some bioactive chemicals are naturally secreted hormones (e.g. steroid hormones) (Gomes, et al., 2005) and so it is not possible to prevent them from being excreted. In order to prevent them from entering the environment it would be necessary to change our wastewater management practices, as currently they are constantly entering the environment, giving them a 'pseudo-persistence' even when some of them may be more degradable (Daughton, 2003). Despite these reasons on why it is not currently practical to prevent BAC pollution from an earlier stage, it should be considered that where or when possible, BAC production should be minimised and their harm to the environment considered from manufacture as well as their safe disposal (Daughton, 2003). Other ways in which to reduce BAC pollution are education and legislation (Directive, 2012; Kotchen, et al., 2009)

A survey conducted in Southern California in the United States of America, concluded that respondents who are aware of pharmaceuticals and personal care products being found in wastewater are three times more likely to dispose of their unused pharmaceuticals by returning them to a pharmacy or through a hazardous waste management centre rather than disposing of them through the sink, toilet or trash (Kotchen, et al., 2009). Despite this being only a single survey, conducted in a very specific location the trends shown are encouraging. However, this solution can only reduce, but not eliminate the number of pharmaceuticals, antibiotics and personal care products entering the environment.

Legislation is undoubtedly another useful tool to prevent and control the release of pollutants into the environment However, regulation alone cannot solve environmental protection problems. Environmental pollution tends to be a transboundary issue, in the case of water pollution, rivers and catchments do not adhere to politically constructed boundaries (Jones O., 2014). Therefore, even if legislation is implemented in one country, pollutants can still make their way into the environment, even in countries where the pollutants are banned. An example is the case of nonylphenol and nonylphenol ethoxylates which have been banned in Europe but are still found in European wastewaters and surface waters. This is due to these pollutants being used in clothes which are manufactured elsewhere, when these clothes are washed in the EU the pollutants enter wastewater (Jones O., 2014). Another issue is the implementation of legislation. Whereas in some countries there may be effective mechanisms to implement environmental laws there are others where such mechanisms do not exist or other factors such as scarce economic resources or corruption may hinder the application of environmental laws (Jones O., 2014).

Legislation and education alone cannot solve the challenges that BACs pose to human health and the environment because the demand and dependency on these chemicals remains too great. Their use is currently coupled to the existing wastewater management and treatment practices which are not able to reduce BACs to levels that may not cause harm (Petrie, et al., 2015). This makes it a process engineering challenge in that WWTPs act as the gateway for these pollutants entering the environment. In order to determine process removal efficiency and to understand the behaviour of these pollutants in the environment it is essential to have adequate robust analytical methods to be able to measure them (Dimpe, et al., 2016; Ort, et al., 2010; Richardson, et al., 2018). As is further explained in Chapter 2, knowledge gaps remain in the analytical methods to measure BACs in the environment (Michael, et al., 2017) as well as in their removal efficiency in WWTPs and their environmental fate (Carter, et al., 2019). These unknowns must be addressed in order to prevent the spread of antimicrobial resistance and damage to the environment and the animal and human food chain. With adequate and reliable analytical chemistry methods it is possible to obtain data which along with modelling tools are helpful in further understanding BAC presence, fate and prevalence (Acheampong, et al., 2019; Comber, et al., 2019; M. Conde-Cid, et al., 2020; Li, et al., 2020; Pomiès, et al., 2013; Villa, et al., 2009) and even making predictions (Delli Compagni, et al., 2020; Han, et al., 2017; He, et al., 2020) in order to address these issues from an engineering perceptive. With an engineering perspective it is possible to improve wastewater treatment process as well as to understand and implement natural attenuation process, addressing threats posed by BACs in the environment.

1.3 Aim and Objectives

The aim of this work was to develop the skills and build on the current analytical chemistry knowledge and modelling approaches in order to have the tools to address

the wastewater engineering treatment and environmental fate challenges of BACs in the environment. The objectives were formed after the literature review was carried out and were adapted in line with emerging literature during the course of this PhD. The objectives of this thesis, addressed from Chapter 4 to 8, are as follows:

1. Develop robust and environmentally relevant analytical methods to allow the detection and quantification of bioactive chemical pollutants in environmental matrices related to the agricultural sector (namely water and soil).

2. Carry out long-term targeted monitoring of two UK WWTPs to address knowledge gaps in terms of removal efficiencies of different treatment technologies and study the seasonal variation of this removal. Use this information to determine the environmental risk posed by the effluent entering the environment from these WWTPs

3. Perform untargeted analysis of two UK WWTPs to identify metabolites, transformation products and other BACs which go undetected when using targeted methods.

4. Analyse different areas of an agricultural zone in Mexico where wastewater is reused for irrigation to contribute to the understanding of BACs presence in a real agricultural environment and to be able to compare BAC presence in two different countries.

5. Carry out modelling work to further understand the behaviour and therefore fate of BACs in soil (as it is the first point of contact of irrigation water and will determine how much of the pollutants are leached into groundwater and how much is available for plant uptake). Use the modelling work to pinpoint necessary experimental work in this area.

2 LITERATURE REVIEW

2.1 What are bioactive chemicals (BACs) and where do they come from?

Awareness of the effects of chemicals made by humans on the environment has increased over the last decades. In the past attention and control measures have been given to large-scale chemical waste such as those associated with industry (Braund, et al., 2016b). The term emerging pollutants refers to a wide range of pollutants which started to gain public attention approximately three decades ago (Petrie, et al., 2015). These pollutants became relevant as more advanced techniques capable of measuring them in the low concentrations at which they are found have been developed (Snow, et al., 2019). Furthermore, some of their negative effects started to be identified (Petrie, et al., 2015), as discussed in Section 1.2.2. These pollutants encompass a very wide variety of chemicals and their definition changes with increasing research on them, according to UNESCO's International Initiative on Water Quality:

'Emerging pollutants can be understood in a broad sense as any synthetic or naturally-occurring chemical or any microorganism that is not commonly monitored or regulated in the environment with potentially known or suspected adverse ecological and human health effects. *These contaminants include mainly chemicals found in pharmaceuticals, personal care products, pesticides, industrial and household products, metals, surfactants, industrial additives and solvents. Many of them are used and released continuously into the environment even in very low quantities and some may cause chronic toxicity, endocrine disruption in humans and aquatic wildlife and the development of bacterial pathogen resistance'* (UNESCO, 2019).

In this thesis the term bioactive chemicals (BACs) is used as it refers to their nature (Carpenter, 2013; Ludewig, et al., 2013), as opposed to emerging pollutants, which refers to their novelty at the time they started being addressed. BACs are found in wastewater mainly due to human consumption and subsequent excretion as well as topical use of personal care products and the natural excretion of hormones (Petrie, et al., 2015). All of these products are used domestically and therefore can be found in domestic wastewater (Carpenter, 2013). Higher concentrations of pharmaceuticals are often found in hospital and long-term care facilities (Gavrilescu, et al., 2015). Different industries produce wastewater containing different types of micropollutants which enter the environment through wastewater (Kim, et al., 2016). Municipal, industrial and hospital wastewater is typically treated in WWTPs. WWTPs were not originally designed to remove BACs from wastewater. Therefore, the main sources of BACs in the environment are raw wastewater discharges in the environment and the effluent from WWTPs (Ashton, et al., 2004; Boshir Ahmed, et al., 2017; Chávez, et al., 2011; Halling-Sorensen, et al., 1998; Kolpin, et al., 2002; Petrie, et al., 2015; Ternes, et al., 1999).

Other sources include amendment of agricultural soils using sludges or livestock manures(Carter, et al., 2019), pharmaceuticals and hormones given to livestock and other animals (Gavrilescu, et al., 2015), wash out from agricultural fields (Ventola, 2015) and landfill leachate (Gavrilescu, et al., 2015). The relationships between these different sources and pathways are illustrated in Figure 2.1.1:

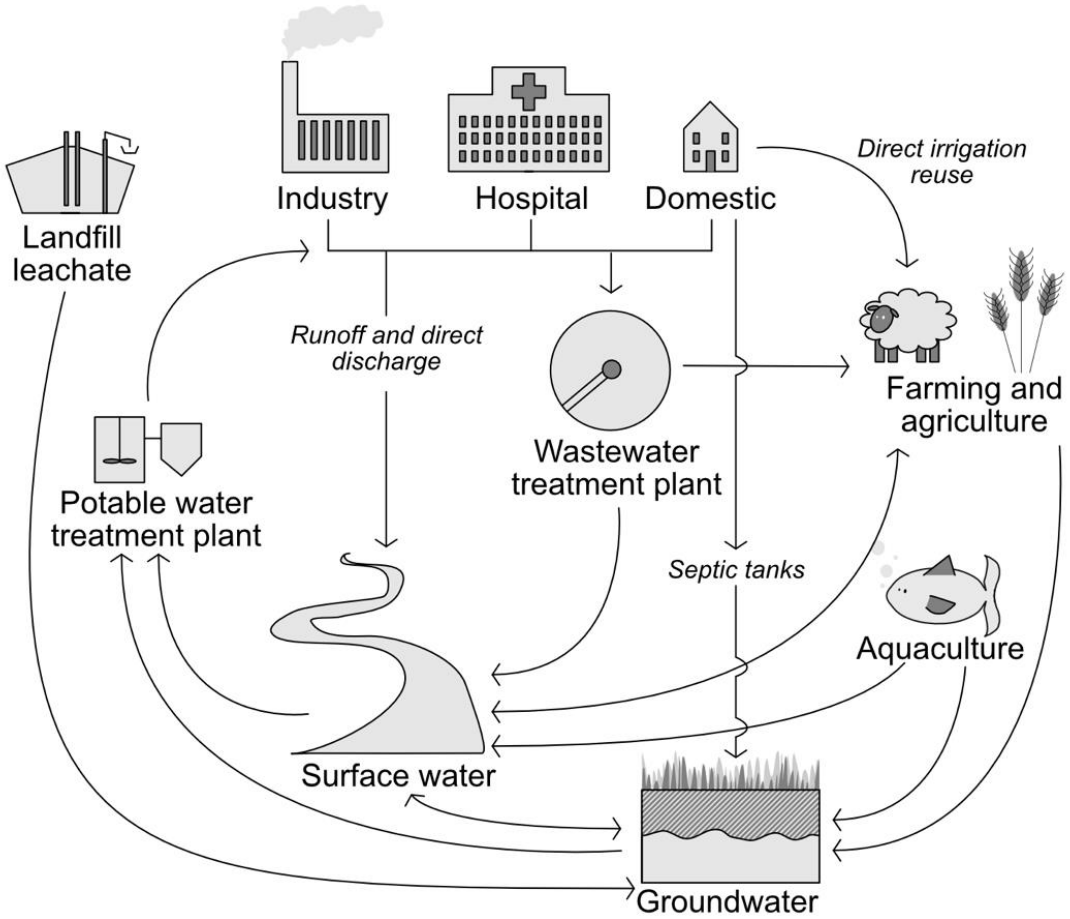


Figure 2.1-1 Sources and pathways of BACs adapted from (Gavrilescu, et al., 2015; Kim, et al., 2016; Tran, et al., 2019)

A less studied source of BACs are those which are airborne, this may occur as direct atmospheric emissions during manufacturing or incineration or from volatilization from surface waters or WWTPs (Wilkinson, et al., 2017). Once airborne they may enter the aquatic environment, but this process is not well characterised and consequently it remains unknown whether it is an influential source of BACs. Examples of BACs which have been measured in outdoor air include dibutyl phthalate (DBP): 55 ngm^{-3}, di(2-ethylhexyl)phthalate (DEHP): 45 ngm^{-3} and nonylphenol (NP): 30.5 ngm^{-3}. BACs may also be airborne due to agricultural spray drift such as that used on fruit trees (Wilkinson, et al., 2017), however this is not in the scope of this work.

2.1.1 What are the different forms in which BACs can be found in the environment?

In the human or animal body pharmaceuticals are often converted to polar metabolites to facilitate excretion. Metabolism is divided into Phase I and Phase II. Phase I is the formation of more polar compounds through reactions such as oxidation, reduction, hydrolysis, cyclization or decyclization. The resulting metabolite is either directly excreted in urine or is further converted in Phase II reactions. Phase II involves conjugation with sulphates, glucuronides, glutathione or amino acids (Shehata, 2010). Metabolites can be just as bioactive, or even more so, than the parent compound (Petrie, et al., 2015). Important examples include the urinary excretion of steroids mainly as glucuronide conjugates (Gomes, et al., 2009) and the excretion of 70-75% of the ingested dose of carbamazepine being excreted as 10,11-epoxy carbamazepine (which is therapeutically active) and 10-11 dihydro-10,11-trans-dihydro-carbamazepine (Paz, et al., 2016). Once in the environment, metabolites can be converted back to the bioactive parent compound in WWTPs or in the environment.

Furthermore parent compounds can undergo transformations which may result in more toxic pollutants (Petrie, et al., 2015). Metabolites and transformation products can often go undetected due to the lack of analytical reference standards, this is a knowledge gap which requires attention due to their similarly bioactive effects as well as their potential to transform back to the parent compound (Petrie, et al., 2015).

Another understudied and newly emerging aspect is the chirality of bioactive chemical pollutants (Evans, et al., 2017). Chiral molecules are found in racemic mixtures and it has been shown that biodegradation of chiral drugs is stereoselective (Evans, et al., 2017). The wide range of physicochemical properties and chirality of BACs has analytical implications. It has been challenging to develop methods which can extract, separate and detect such a wide range of pollutants, and it is only recently that this has started to become more common (Petrie, et al., 2016). Developing stereoselective methods requires different chromatographic columns, longer run times and more specialised techniques making it less commonplace (Petrie, et al., 2015). Even though chirality is outside the reach of this thesis, it is an important aspect to be aware of.

Bioactive chemical pollutants present a wide range of physicochemical properties. They cover the full spectrum of pK_a values, which, along with environmental pH determine whether they will be in the neutral, ionised or partially ionised form (Lorphensri, et al., 2007). In general, at environmentally relevant pH values, bases (pK_a > 8.6) will be fully ionised, weak bases (8 > pK_a > 4.8) will be partially ionised and acids (3.2 < pK_a < 6.8) will be partially or fully ionised depending on the environmental pH (Li, et al., 2020). Another important property is the hydrophobicity of the molecules (Yasong Li, et al., 2018a). Molecules with high hydrophobicity are more likely to be found adsorbed to solids such as sludge in wastewater treatment plants (Çeçen, et al., 2020; Kim, et al., 2016) and soils in the environment (Ramil, et al., 2010). Conversely highly hydrophilic compounds are more likely to be found in the aqueous phase and therefore present behaviours such as leaching into groundwater from soils (McCance, et al., 2018; Pan, et al., 2017). Hydrophobicity can be quantified using the octanol/water partitioning coefficient (Log_{Kow}), a high Log_{Kow} indicates a hydrophobic compound as it preferentially partitions to octanol (Cumming, et al., 2017). Log_{Kow} increases with increasing number of chlorinated benzenes and number of rings (Piwoni, et al., 1990). Other molecular properties such as functional groups and calculated dipole moments will play an important role on the partitioning, interaction with molecules in environmental matrices such as soils (Paz, et al., 2016) and degradability (Martínez-Hernández, et al., 2016). Physicochemical properties are important in predicting and understating the fate and transformation of BACs in the environment (Al-Khazrajy, et al., 2016; Barron, et al., 2009; Li, et al., 2020). For the most commonly monitored and studied BACs physicochemical properties are well known (S. Kim, et al., 2019; Wishart, et al., 2017). These tend to be parent compounds, however, for metabolites and transformation products physicochemical properties are often unknown (Picó, et al., 2015). Section 2.3 provides further explanation on the relationship between physicochemical properties of BACs and their fate in the environment.

2.1.2 BACs of interest for this thesis

For this thesis a list of 35 analytes were chosen for targeted analysis, they were chosen because of their ubiquity in the environment, their toxicity and their legal status, as detailed in Table 2.1.1. In addition to these reasons, natural steroids and their metabolites were added because they cannot be prevented from being excreted (Gomes, et al., 2009). A range of classes was chosen to include estrogens, antibiotics, non-steroidal anti-inflammatory drugs (NSAID), lipid modifiers and β-blockers. Antibiotics were chosen based on their ubiquity as well as their minimum inhibitory

concentration (MIC) which is calculated in order to prevent antimicrobial resistance development (AMR Industry Alliance, 2018). MICs were compared with environmental concentrations and this was an important factor in choosing the antibiotics studied. With this diversity of classes comes an important diversity in physicochemical properties ranging from acidic to basic analytes as well as hydrophobic and hydrophilic compounds, as can be seen in Table 2.1.1. Untargeted methods were used to complement the targeted analysis, particularly in terms of metabolites and transformation products. The difference between targeted and untargeted analysis is further detailed in Section 2.6.3.4.

Log D, the liphophiclic (Log_{know}) partitioning corrected for ionisation of the pollutant dependent on the environmental pH was calculated for the analytes studied at the pH of the soil used for this thesis (Section 3.3.1). When the pollutant is expeteced to be ionised at the environmental pH the LogD will differ from the Log_{know}, however if the pollutant is expetected to be found in the neutral form the LogD should be the same as the Log_{know}(Cunningham, 2008).

BAC analyte	Class	Water solubility (mgL^{-1})	pK_a	Log_{Kow}	Log D_{ow} at pH 6.2	Toxicity, legal status and usage
4-tert-octylphenol *(4TO)*	Estrogenic alkylbenzene	7.0[50]	NA	3.87[21]	3.8	Reproductive toxicity to aquatic life at 330 ngL^{-1} [3] and it has been measured in wastewater effluent in the range 1-351 ngL^{-1} [49].
Acetaminophen *(ACE)*	Non-steroidal anti-inflammatory drug	14,000[15]	9.38-9.5[1,2]	1.3-1.65[7,22]	3.3	Between 390.9 and 3303.08 tons consumed annually in several European countries[44]. Found in both wastewater influent and effluent at thousands of ngL^{-1} [37]. Reproductive toxicity to aquatic life at 9,580 ngL^{-1} [38]. Classified as harmful to aquatic organisms[33]. Environmental risk quotients have been found above 10[46].
Acetyl-sulfamethoxazole (*ASMX)*	Antibiotic metabolite	NA	5.6-6.03[3,4]	NA.	-0.39	Main sulfamethoxazole metabolite[33] and can be converted back to sulfamethoxazole in the environment[58].
Atenolol *(ATE)*	β -blocker	13,300[15]	9.4 -9.6[4-7]	0.61[6],1.82[22],2.2-2.47 [5,7]	3.4	One of the most important β-blockers, due to its ease of administration as a liquid formulation[48]. In top 10 of most commonly reported pharmaceuticals in wastewaters[44]. Toxic to aquatic life[33] and has been predicted as carcinogenic by some models[51].
Bezafibrate *(BEZ)*	Lipid modifying	1.55[17]	3.6 [8]	0.8 ± 0.3 [23]	-2.6	Classified as harmful to aquatic organisms [33]. In top 10 of most commonly reported pharmaceuticals in wastewaters[44].
Carbamazepine *(CBZ)*	Antipsychotic	17.7[15]	7 [6],2.3 [5] ,13.9[7]	1 ± 0.2 [23], 2.23-2.70[6,7],3.6 [5,9]	Same as Log_{Know}	In top 10 of most commonly reported pharmaceuticals in wastewaters[44]. Very persistent in the environment with little or no removal in WWTPs[37]. Harmful to aquatic organisms[33].
Clofibric acid *(CLO)*	Metabolite of fibrate drug clofibrate	582.5[11]	2.5-3.2[2,9,10,11]	-1.46[24],1.6 [9]	-3.7	Classified as harmful to aquatic organisms [33]. Has very variable removal in WWTPs, form (<50 - >80%)[37].

BAC analyte	Class	Water solubility (mgL^{-1})	pK_a	Log_{Kow}	Log D_{ow} at pH 6.2	Toxicity, legal status and usage
Codeine *(COD)*	Opioid	9,000[15]	13.78	2.5[15]	2.0	Reproductive toxicity to aquatic life at 60 ngL^{-1} [28] and in wastewater effluents it has been reported in the range 372-5,271 ngL^{-1} and in surface waters <1.5-347 ngL^{-1} [37].
Diclofenac *(DCF)*	Non-steroidal anti-inflammatory drug	2.37[15]	4.1-4.2[8,9,13]	1.0 ± 0.3 [23],2.66-2.95[,22 ,]10[25]	-2.1	In top 10 of most commonly reported pharmaceuticals in wastewaters[44]. Classified as harmful to aquatic organisms[33]. Reproductive toxicity to aquatic life at 20 ngL^{-1} [28] and has been measured in surface waters in the range <0.5-154 ngL^{-1} [37].
Erythromycin *(ERY)*	Antibiotic (macrolide)	2,000[15]	8.9[5]	1.0[5],2.76-2.80[7,15],10[25]	Same as Log_{Know}	EU countries are legislatively required to monitor erythromycin[27].Classified as toxic to aquatic organisms[33]. Minimum inhibitory concentration of 1,000 ngL^{-1} [34] and has been measured in wastewater effluent in the range 109-1,385 ngL^{-1} [37].
Estradiol *(E2)*	Natural oestrogen	3.9[15]	10.3-10.71[7,13]	2.94-4.48 [7,14,15,21,26]	4.2	Reproductive toxicity to aquatic life at 0.4 ngL^{-1} [29] and has been found in surface waters at 1,422.2 ngL^{-1} [54]. EU countries are legislatively required to monitor it[27].
Estradiol-3-sulfate *(E2-3S)*	Natural oestrogen	NA	NA	NA	Neutral	Excreted in this conjugated form and can be transformed into E2 in WWTPs [36]
Estriol *(E3)*	Natural oestrogen	13.25[15]	10.4-10.54[13,15]	3.08-3.15[15,21,] 4.11[14]	4.3	Reproductive toxicity to aquatic life at 60 ngL^{-1} [30] Has been found at concentrations as high as 45,550 ngL^{-1} in the aquatic environment[30].
Estriol-3-sulfate *(E3-3S)*	Natural oestrogen	NA	NA	NA	Neutral	Excreted in this conjugated form and can be transformed into E3 in WWTPs [36].
Estrone *(E1)*	Natural oestrogen	12.42[15]	10.5[14]	3.3-3.45[15,21,26], 4.17-4.6[14]	4.3	Reproductive toxicity to aquatic life at 3.6 ngL^{-1} [29] and found in wastewater effluents in the range1.04-4.99 ngL^{-1} [52]. EU countries are legislatively required to monitor it[27].

BAC analyte	Class	Water solubility (mgL^{-1})	pK_a	Log_{Kow}	Log D_{ow} at pH 6.2	Toxicity, legal status and usage
Estrone-3-sulfate *(E1-3S)*	Natural oestrogen	3.6[15]	2.2 [16]	3.2[26],4.48[15]	Neutral	Excreted in this conjugated form and can be transformed into E1 in WWTPs[36].
Ethinylestradiol *(EE2)*	Birth control pill active ingredient	11.3[15]	10.33-10.4[14,17]	3.45-5.52 [7,21,22,27]	4.2	Active ingredient in the birth control pill. Toxic at 0.1 ngL^{-1} [33] and has been found in surface waters at 876.94[54]. EU countries are legislatively required to monitor it[27].
Gemfibrozil *(GEM)*	Lipid modifying	11.0[15]	4.42-4.75 [5,9,17]	2.6-2.7[5,15]	-1.5	In top 10 of most commonly reported pharmaceuticals in wastewaters [44]. Relatively long degradation rate in soil with a half-life of 18-94 days[8].
Ibuprofen *(IBU)*	Non-steroidal anti-inflammatory drug	21.0[53]	4.91[53]	3.97[53]	-0.9	In top 10 of most commonly reported pharmaceuticals in wastewaters [44]. Classified as harmful to aquatic organisms [33]. Reproductive toxicity to aquatic life at 8,610 ngL^{-1} [38] and reported in surface waters in Latin America in the range 2-37,000 [54].
Ketoprofen *(KTP)*	Non-steroidal anti-inflammatory drug	51[15]	3.88-4.45[15,17,18]	0.2 [28] ,2.59[18]	-2.2	In top 10 of most commonly reported pharmaceuticals in wastewaters [44].
Levonorgestrel *(LEV)*	Birth control pill active ingredient	1.73[15]	-1.5, 17.91 [17]	3.91[22]	Neutral	Toxic to fish larvae at 0.01 ngL^{-1} [39] and has been found in surface waters at 38 ngL^{-1} [57].
Metoprolol *(MET)*	β -blocker	16,900[15]	9.6 [8]	1.4 ± 0.5 [23],2.05[22]	3.4	In top 10 of most commonly reported pharmaceuticals in wastewaters [44].
Morphine *(MOR)*	Opioid	149[15]	8.21[17]	3.0[15]	2.0	Reproductive toxicity to aquatic life at 50 ngL^{-1} [28] and found in influent wastewaters at 2,552.83 ngL^{-1} [54]
Naproxen *(NPX)*	Non-steroidal anti-inflammatory drug	15.9[53]	4.45[11]	2.5 [15]4.45 [8]	-1.7	In top 10 of most commonly reported pharmaceuticals in wastewaters [44]. Classified as harmful to aquatic organisms [33]

BAC analyte	Class	Water solubility (mgL^{-1})	pK_a	Log_{Kow}	Log D_{ow} at pH 6.2	Toxicity, legal status and usage
Oxytetracycline *(OXY)*	Antibiotic	313[15]	9.5[15]	4.7 [15]	Neutral	Classified as toxic to aquatic organisms [33]. MIC of 500 ngL^{-1} [34] and found in surface waters in concentrations very close to this value, 428 ngL^{-1} [54].
Paroxetine *(PXT)*	Antidepressant	1,131[15]	9.60-9.77[7,17]	4.00[7],5.2[15]	Neutral	Extensively used pharmaceutical [45].
Phenazone *(PHE)*	Non-steroidal anti-inflammatory drug	51,900[15]	1.4[6],9.77[17]	1.07[6]	3.6	Found in surface waters in the range 5.9-40.27 ngL^{-1} [55] and has been detected in groundwater [56].
Propranolol *(PRO)*	β -blocker	61.7[15]	9.2 [9]	3.1[9]	3.0	Commonly detected β -blocker[44]. Found in hundreds of ngL^{-1} in wastewater influent and effluent[37].
Salicylic Acid *(SAL)*	Non-steroidal anti-inflammatory drug	2,240[15]	2.97-2.98[7,17,18]	1.336[18],2.6 [15]	-3.2	Drug metabolite, frequently identified in thousands of ngL^{-1} in wastewater influent and hundreds of ngL^{-1} in wastewater effluent and surface waters in the UK [37], found in Mexico in groundwater and surface water at hundreds of ngL^{-1} [43].
Simvastatin *(SIM)*	Lipid modifier	0.765[15]	14.91[17]	2.97[15]	Neutral	Toxic to *Daphnia magna* at 3.2 ngL^{-1} [40] and has been reported in final effluents in the range <3- 5ngL^{-1} [37].
Sulfadiazine *(SDZ)*	Antibiotic	77[15]	6.36[19]	2.09[19]	-0.3	One of three most commonly measured sulfonamides in some areas[59]. Used in aquaculture [42]
Sulfamethoxazole *(SMX)*	Antibiotic	610[53]	5.6-5.94[6,7,8,19] 1.4-1.8[5,8,19]	1.90[7],2.34-2.41[19,22], 3.2[5]	-0.04	Classified as toxicity to aquatic organisms[33.] Reproductive toxicity to aquatic life at 27 ngL^{-1} [31] and has been found in wastewater effluent at 660.62 ngL^{-1} [54].
Sulfapyridine *(SPY)*	Antibiotic	268[15]	4.25[6] 2.3[8], 6.39, 8.4[8]	1.41[6]	0.04	Sulfonamide, widely prescribed veterinary antibiotic [47].

BAC analyte	Class	Water solubility (mgL^{-1})	pK_a	Log_{Kow}	Log D_{ow} at pH 6.2	Toxicity, legal status and usage
Tramadol *(TRA)*	Opioid	1,151[15]	9.4[17]	2.79[15]	Neutral	Toxic to fish, *Daphnia magna* and algae at 130 ngL^{-1} and has been measured in surface water between <30-5,970[37].
Trimethoprim *(TMP)*	Antibiotic	400[53]	6.4-7.8[6,20]	1.90[6],2.48 - 2.85[19,22]	1.1	Reproductive toxicity to aquatic life at 50 ngL^{-1} [31] and minimum inhibitory concentration for antibiotic resistance is 500 ngL^{-1} [34]. Has been found in surface waters at 183[37] and in some drinking waters between 470-2,330 ngL^{-1} [54].

Table 2.1-12.1-2 Chosen analytes classes, physicochemical properties, toxicity, legal status and usage.

Superscripts refer to the following references: ***1*** (Wishart, et al., 2017), ***2*** (Zhou, et al., 2017), ***3*** (Mullen, et al., 2017), ***4*** (Aurore Andriamalala, et al., 2018), ***5*** (Muñoz, et al., 2009), ***6*** (Qiao, et al., 2011), ***7*** (McEachran, et al., 2017), ***8*** (Grossberger, et al., 2014), ***9*** (Cerqueira, et al., 2018), ***10*** (Drillia, et al., 2005b), ***11*** (Goldstein, et al., 2014), ***12*** (Wishart, et al., 2017), ***13*** (Chefetz, et al., 2008), ***14*** (Huang, et al., 2019), ***15*** (S. Kim, et al., 2019), ***16*** (Gram, et al., 2009), ***17*** (Wishart, et al., 2017) , ***18*** (Hijosa-Valsero, et al., 2016), ***19*** (Nguyen Dang Giang, et al., 2015), ***20*** (Williams-Nguyen, et al., 2016), ***21*** (Ying, et al., 2005), ***22*** (dos Santos, et al., 2019), ***23*** (Aminot, et al., 2018), ***24*** (Gao, et al., 2011), ***25*** (Cycoń, et al., 2019), ***26*** (Casey, et al., 2005), ***27*** (Directive, 2012), ***28*** (Daouk, et al., 2016), ***29*** (Burkhardt-Holm, 2010), ***30*** (Fekadu, et al., 2019), ***31*** (Phonsiri, et al., 2019), ***32*** (Thomaidi, et al., 2015), ***33*** (Jurado, et al., 2014), ***34*** (AMR Industry Alliance, 2018), ***35*** (Quednow, et al., 2008),***36*** (Gomes, et al., 2009), ***37*** (Petrie, et al., 2015), ***38*** (Han, et al., 2017), ***39*** (Yu, et al., 2019a), ***40*** (Pereira, et al., 2017), ***41*** (Biel-Maeso, et al., 2017), ***42*** (Binh, et al., 2018), ***43*** (Félix–Cañedo, et al., 2013), ***44*** (Wilkinson, et al., 2017), ***45*** (Duarte, et al., 2019), ***46*** (Wielens Becker, et al., 2020), ***47*** (Le-Minh, et al., 2012), ***48*** (Karaman, 2015), ***49***(Gorga, et al., 2013), ***50****(Sigma-Aldrich, n.d.)*, ***51*** (Wielens Becker, et al., 2020), ***52*** (Mohagheghian, et al., 2014), ***53*** (Lin Jay, 2011), ***54*** (Peña-Guzmán, et al., 2019), ***55***(Lopez-Serna, et al., 2010), ***56,***(Stuart, et al., 2012), ***57***(Al-Odaini, et al., 2010), ***58*** (Martínez-Hernández, et al., 2016), ***59*** (Zhao, et al., 2017).

2.2 The role of wastewater treatment technologies to remediate BACs

2.2.1 Conventional wastewater treatment plants

Conventional wastewater treatment plants were not originally designed to remove BACs (Petrie, et al., 2015). Current WWTPs are mostly conventional, therefore it is important to understand them in order to understand why and how some BACs are partially removed, some pass unchanged through the treatment processes and some can be converted into other pollutants which may be as hazardous or more hazardous than the parent compound (Metcalf & Eddy, et al., 2007). Conventional wastewater treatment plants were designed to remove the pollutants detailed in Table 2.2.1:

Pollutants	Description	Reason for removal
Suspended solids (SS)	Include suspended and colloidal matter, mainly composed of silt and clay, microorganisms and particulate organic matter.	If it is not removed properly it can affect the efficiency of the disinfection process.
Organic matter	Includes dissolved and particulate matter, composed mainly of proteins, carbohydrates and fats. It is usually measured in terms of biological oxygen demand (BOD) and chemical oxygen demand (COD). Organic compounds which are not effectively removed by biological treatment are termed refractory pollutants, examples include surfactants, phenols and agricultural pesticides.	Poor stabilization of organic matter can lead to septic conditions and the formation of hydrogen sulphide gas and odours.
Inorganic matter	Includes constituents such as calcium, sodium and sulphate as well as heavy metals	Can have toxic effects on humans and animals.
Pathogens	The main types are bacteria, protozoa, helminths and viruses.	Can cause diseases to animals and humans.
Nutrients	the main ones are nitrogen and phosphorus in various forms.	Excessive nutrients in the aquatic environment can lead to eutrophication.
Total dissolved solids:	These are inorganic and organic pollutants found in the dissolved phase	

Table 2.2-1 Pollutants conventional treatment wastewater treatment plants were designed to remove. Content from (Metcalf & Eddy, et al., 2007)*.*

Conventional wastewater treatment is composed of preliminary, primary and secondary treatment. Preliminary treatment uses physical methods to remove larger particles, by implementing screens and settling grit chambers. Primary treatment is mainly targeted at removing larger suspended solids using sedimentation (clarification) or fine screens to further remove organic and inorganic suspended solids (Metcalf & Eddy, et al., 2007). Preliminary and primary stages have low BAC removal (Wang, et al., 2016). Secondary treatment is mainly designed for the removal of organic matter and nutrients (Metcalf & Eddy, et al., 2007). Secondary treatment has variable BAC removal depending on the specific configurations, hydraulic retention times and other operating procedure such as pH and temperature (Wang, et al., 2016). Depending on the country and the application, secondary treatment may be followed by tertiary treatment to target specific pollutants treatment such as pathogens, inorganic pollutants and residual dissolved and suspended solids (Metcalf & Eddy, et al., 2007).

These treatment technologies are namely, filtration or adsorption, advanced oxidation and disinfection (Green, et al., 2008).

Secondary treatment, also known as biological treatment, is the heart of the WWTP and is composed of a bacteria community which under adequate environmental conditions (pH, temperature, nutrient level) acts to degrade pollutants(Green, et al., 2008):

Waste (electron donor) + Biomass + Electron acceptor → More biomass + end products

Electron acceptors	End product
O_2	H_2O, CO_2, oxidised nitrogen
Oxidised nitrogen	N_2, N_2O, NO, CO_2, H_2O
Oxidised sulphur	H_2S, S, CO_2, H_2O
CO_2, acetic acid, formic acid	CH_4, CO_2, H_2
Complex organics	H_2, simple organics, CO_2, H_2O

Table 2.2-2 Electron acceptors and end products for biological reactors (Green, et al., 2008)

There are different types of biological treatment, the most widely used is activated sludge process in Western countries (Green, et al., 2008). The biomass is suspended in the water in a mass known as biological floc. Removal of soluble organic and inorganic colloidal and particulates is achieved through biological metabolism and through adsorption and entrapment in the biological floc. Many non-biodegradable pollutants get removed in this second way; this process is mainly governed by the pollutant's hydrophobicity which can be quantified with their Log_{Kow}. Molecular oxygen is pumped into the water to maintain aerated conditions in aerobic activated sludge. This method will consistently produce effluent with BOD and SS of 20-30 mgL^{-1}. Activated sludge can also be run in anaerobic (total absence of free (O_2) and bound (NO_2, NO_3) oxygen) or anoxic (absence of free oxygen, but presence of bound oxygen) conditions in order to remove nitrate (Green, et al., 2008). The efficiency of activated sludge in removing BACs depends on the physicochemical properties of the specific pollutant and on the process conditions of the activated sludge unit (Barbosa, et al., 2016). The operational process conditions which affect BAC removal the most are the temperature, the hydraulic retention time (HRT) and the sludge retention time (SRT). Higher HRT and SRT allow for the removal of more persistent BACs, due to a higher diversity of microorganisms. Typical SRT values lie between 7-20 days and HRTs range from 2-24 hours (Barbosa, et al., 2016). Another factor affecting BAC removal is the biomass concentration, for activated sludge this usually ranges between 3-5 kgm^{-3} (Barbosa, et al., 2016).

Oxidation ditches are a modification of the activated sludge process which uses long sludge retention times (SRTs). It can be adapted to have cycled anoxic and anaerobic phases (Shammas, et al., 2009), an example is the orbal oxidation ditch. An inexpensive version of activated sludge are lagoons. These are basically a suspended-grow no recycle reactor with variable degree of mixing. Solids settle and remain at the bottom and periodic removal of the solids must take place. Aeration occurs by gas released from biological processes and surface aeration through wind. Lagoons design parameters include the depth, based on the available sunlight and they can also be supplied with aeration, if necessary for the treatment (Green, et al., 2008).

Other types of biological treatment commonly used are fixed-film reactor systems. The biomass is fixed, and their main advantage is their simplicity of design. However, it is harder to achieve the same BOD removal as with suspended growth systems because

of the reduced contact (Metcalf & Eddy, et al., 2007). Fixed-film reactors can be used in aerobic, anoxic and anaerobic conditions. Aerobic systems are operated in non-flooded mode and the main type for municipal wastewaters is trickling filters. Trickling filters are being replaced by activated sludge as they provide superior pollutant removal to meet the increasingly stringent legislation over the past years. In trickling filters, the wastewater is spread over a solid medium where the microbes are growing. For better effluent quality trickling filters must be operated at low hydraulic-loading and low organic-loading rates. Low -rate trickling filters can remove > 90% BOD_5 removal, while high-rate systems will produce 65-75 % BOD_5 removal (Green, et al., 2008; Metcalf & Eddy, et al., 2007)

Other aerobic, anaerobic and anoxic systems are operated in flooded reactor form and the main type used in municipal wastewater treatment are biological fluidized beds (Green, et al., 2008). Biological fluidized beds consist of a fluidized bed of solid particles such as sand or activated carbon to which active biological mass is attached. This bed is placed in a reactor through which the wastewater is passed in up-flow motion with sufficient velocity to fluidize the bed. Removal of pollutants occurs by biological metabolism as well as by adsorption to the solid carrier. When granular activated carbon is used this can provide added removal of BACs (Green, et al., 2008).

Rotational biological reactors (RBCs) are another type of fixed-film reactor, in which the microbial community is attached to rotating disks which are mounted in a horizontal shaft which makes the disks rotate. The disks are partially submerged in the reactor through which wastewater flows. The process is optimised by adjusting the rotating speed and depth of submersion (Metcalf & Eddy, et al., 2007).

Membrane bioreactors (MBR) combine a biological reactor with a membrane (approx. 0.04 µm pore size). This membrane replaces sedimentation and depth filtration (Metcalf & Eddy, et al., 2007), which must follow after activated sludge and fixed-film reactors. The specific configuration of an MBR depends on the manufacturer. Along with activated sludge, MBR are one of the most commonly used processes in secondary treatment (Barbosa, et al., 2016). MBRs have slightly better removal of certain BACs compared to activated sludge. SRT for MBRs is usually between 15-80 days, and HRT between 7-15 hours. MBRs have typically higher biomass concentration than activated sludge, ranging from 8-10 kgm^{-3} (Barbosa, et al., 2016).

Secondary treatment technologies can be configured in certain ways to achieve nutrient removal. These configurations result in different microorganism colonies and process conditions which can have variable effects on BAC removal (Barbosa, et al., 2016). Nitrogen removal requires sequential aerobic and anoxic steps. The nitrification step takes place in aerobic conditions where ammonium (the main form in which nitrogen is found in wastewater) is oxidised into nitrate. During the denitrification step nitrate is reduced to molecular nitrogen under anaerobic conditions. Phosphorus removal can occur under specific redox conditions as part of the activated sludge process. Phosphorus removing bacteria must be removed after phosphorus uptake has occurred to complete phosphorus removal. Several modifications on biological treatment configurations to achieve nitrogen and phosphorus removal have been developed. Some include sequential anaerobic/aerobic sections (e.g. modified Ludzack-Ettinger process), while others have sequential anaerobic/anoxic/aerobic (Metcalf & Eddy, et al., 2007) (e.g. the Bardenpho process for both nitrogen and phosphorus removal) (Banayan Esfahani, et al., 2019). Phosphorus removal can also be achieved by chemical action. This is mainly accomplished by incorporation of phosphate into the total suspended solids (TSS). This is achieved by the addition of salts of multivalent ions that form precipitates of phosphates. The salts may be added in the primary sedimentation tank (pre-precipitation), during secondary treatment for

removal with the secondary sludge (co-precipitation) and addition of chemicals to the secondary effluent and removal using filtration (post-precipitation) (Metcalf & Eddy, et al., 2007).

2.2.2 Advanced treatment processes

After secondary treatment there may be a tertiary step (Peake, et al., 2016a). These processes comprise of filtration/membrane separation processes, adsorption and advanced oxidation (Peake, et al., 2016a). These processes may also target pollutants such as pathogens, colloidal matter, nutrients or trace pollutants such as BACs (Peake, et al., 2016a).

2.2.2.1 Filtration and membrane separation

Filtration may be driven by gravity (depth and particle filtration, such as sand filters), or for membrane processes (microfiltration, ultrafiltration, nanofiltration and reverse osmosis) where pressure may be applied. The most common adsorption material used in WWTPs is activated carbon (Metcalf & Eddy, et al., 2007; Rodriguez-Narvaez, et al., 2017). These processes act as phase-changing processes in the removal of BACs and summarised in Figure 2.2.1.

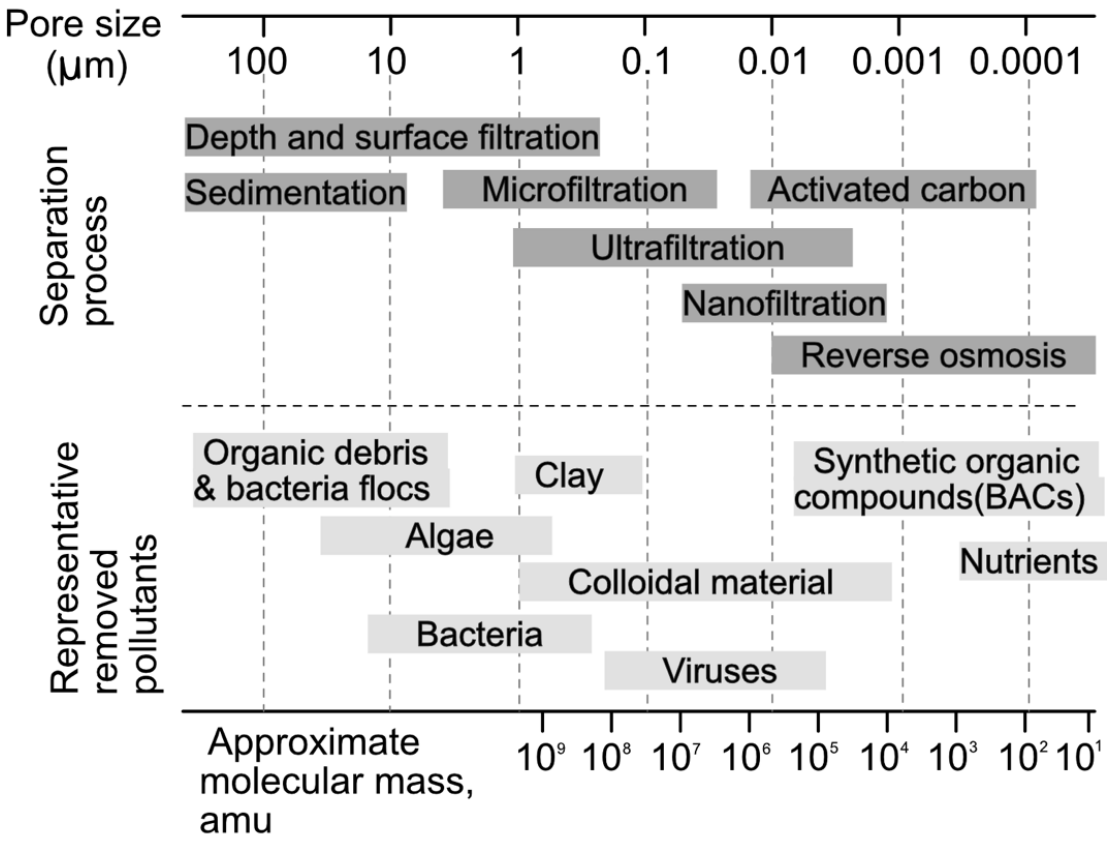


Figure 2.2-1 Types of filtration processes and pollutants removed adapted from (Metcalf & Eddy, et al., 2007)

Membrane filtration technologies, from ultrafiltration to reverse osmosis, in general have increasingly good BAC removal with decreasing pore size (Rodriguez-Narvaez, et al., 2017). Reverse osmosis can remove very recalcitrant pollutants such as carbamazepine with 65- 99% removal. Nanofiltration has shown removal rates of 74-98% for the same pollutant, while 15% has been observed using ultrafiltration. However, this trend is also dependent on the type of membrane used and, on the

pollutant. For example, estrone had high removal using ultrafiltration (98%), while the removal using nanofiltration was 79-97%. Despite the high removals observed the main drawback of these technologies is that BACs are not degraded, they are just in another phase and they still need to be dealt with. Ways to deal with this are still under investigation (Rodriguez-Narvaez, et al., 2017).

2.2.2.2 Sorption

Sorption materials used specifically for BAC removal include activated carbon, biochar, carbon nanotubes, clay minerals and materials such as zeolites, meso and micropores materials, resins and metal oxides. Activated carbon is the most common sorption process in WWTPs (Peake, et al., 2016a). Activated carbon has been found to have removal efficiencies above 80% for lincomycin, levofloxacin and diclofenac and above 60% for ibuprofen, trimethoprim, ciprofloxacin, erythromycin and carbamazepine. However, caffeine and DEET removal has been found to range from 0-80% and -20-100% for each pollutant respectively (Rodriguez-Narvaez, et al., 2017). The source of the material used to produce the activated carbon has been found to have a high impact on the removal efficiency and selectivity. Most adsorption-based systems still have knowledge gaps in terms of scale-up, the sustainability on the production of some of the adsorption materials (like biochar) (Rodriguez-Narvaez, et al., 2017).

2.2.2.3 Oxidation processes

Oxidation process are normally used for potable water treatment, however they can also be used as a tertiary step for wastewater treatment (Peake, et al., 2016a). These processes may be used with different objectives: odour control, hydrogen sulfide control, colour removal, iron and manganese removal, disinfection, control of biofilm growth and biofouling and oxidation of BACs. Commonly used oxidants in descending electrical potential are hydroxyl radicals, ozone, hydrogen peroxide, permanganate, free chlorine, combined chlorine and chlorine dioxide (Metcalf & Eddy, et al., 2007). The main concern with these processes is the formation of hazardous by-products which may need to be removed using other technologies such as adsorption (Richardson, 2003). Advanced oxidation process specifically targeting BAC removal include UV irradiation, photocatalysis, ozone, hydrogen peroxide, Fenton process, Photo-Fenton process, sonochemical, titanium dioxide and combinations of these (Rodriguez-Narvaez, et al., 2017). These processes have very good BAC removal, above 80% for a wide variety of BACs. Their main drawback is the use of toxic chemicals for chemical synthesis. Research is being carried out to overcome this drawback, an example is the use of biological processes to create oxidants (Rodriguez-Narvaez, et al., 2017).

2.2.3 Comparison of BAC removal of different treatment technologies

Table 2.2.3 presents a comparison of the removal efficiencies of different configurations of technologies in use in various WWTPs. However, it is important to keep in mind that there are challenges when trying to compare the removal of BACs between different processes. One of the challenges is that there are different operational process parameters which cannot be kept constant in order to obtain a fair comparison. The type and frequency of sampling, as well as the BACs studied and their influent concentrations also varies between different research works, making comparison challenging (Barbosa, et al., 2016). Influent BAC concentrations as well as removal efficiencies in WWTPs have been found to have diurnal, weekly and seasonal variation (Peake, et al., 2016b) which is not always accounted for in sampling strategies. The HRT of the WWTP or the unit operator being assessed must be taken into account during the sampling campaign; if just one influent and effluent sample is

taken and they are not spaced in time according to the HRT the removal calculated from them would not be accurate as they do not correspond to each other (Comber, et al., 2019). Finally, most sampling campaigns in WWTPs are targeted. This means that some BACs may be converted to metabolites in the biological processes or other by-products which could convert back to the parent compound in the environment or which may be more bioactive themselves (Petrie, et al., 2015)

Removals are usually calculated using Eq. 1:

$$R = \frac{C_i - C_e}{C_i} \qquad \textit{Eq. 1}$$

Where R= removal (%), C_i= Influent concentration and C_e= Effluent concentration. For SuB, WWD was considered as C_e as it is effectively what is coming out of the plant and entering the environment. For BACs removals have been classified as Same removal classification as Petrie was used: Low (<50%), medium (50-80%), high (>80%) (Petrie, et al., 2015).

Table 2.2-3 shows that there is variability in the removal for each pollutant even among similar plants. Some of these variations are due to factors mentioned previously such as sampling strategies not considering HRT, diurnal and seasonal variability as well as different process operating conditions (Comber, et al., 2019). There is a knowledge gaps in terms of the removal of BACs in different WWTPs (Carter, et al., 2019).

BAC	Initial concentration (ngL^{-1})	Treatment process	Removal efficiency (%)	Reference
4-nonylphenol	Not specified	Anaerobic digestion activated sludge WWTP in the UK	0	1
	Not specified	Aerobic activated sludge WWTP in Mexico	53	1
Atenolol	255	Grit tanks + primary sedimentation + bioreactor + clarifiers	47.1	3
Bezafibrate	Not specified	Anaerobic and anaerobic activated sludge WWTP in Beijing	19-80	1
	Not specified	Aerobic activated sludge WWTP in Germany	>90	1
Bisphenol-A	Not specified	Aerobic activated sludge WWTP in Mexico	84	1
Caffeine	Not specified	Anaerobic and anaerobic activated sludge WWTP in China	78-100	1
Carbamazepine	Not specified	Aerobic activated sludge WWTP in Mexico	9	1
	Not specified	Aerobic activated sludge WWTP in Germany	0	1
	589	Grit tanks + primary sedimentation + bioreactor + clarifiers	-16.3	3
	350	Oxidation dich	81	3
	10,000,000	Grit channels + primary clarifies + conventional activated sludge	-92.4	3
Ciprofloxacin	2,200	Grit channels + primary clarifies + conventional activated sludge	-88.6	3
Clofibric Acid	420	Oxidation ditch	81	3
Diclofenac	12.7 (max)	Primary treatment + conventional activated sludge	79	2
	38 (max)	Primary treatment + membrane bioreactor + UF or MF	54-71	2
	220 (after primary clarifier)	Primary treatment + conventional activated sludge +microfiltration + granular activated carbon	51-80	2
	507	Primary treatment + parallel conventional activated sludge and membrane bioreactor	~0	2
	1,450	Primary treatment + conventional activated sludge	~0	2
	Not specified	Aerobic activated sludge WWTP in Mexico	75	1
	Not specified	Aerobic activated sludge WWTP in Germany	>90	1
	Not specified	Aerobic activated sludge WWTP in Finland	0-26	1
	580	Bardenpho processes	80	3
	20,000,000-70,000,000	Primary treatment + Orbal oxidation ditch + UV disinfection	10-60	3
Estriol	60	Grit channels + primary clarifiers + conventional activated sludge	66.8	3

	Not specified	Anaerobic digestion activated sludge WWTP in the UK	45	1
	Not specified	Aerobic and anaerobic activated sludge WWTP in France	0-1.67	1
BAC	Initial concentration (ngL^{-1})	Treatment process	Removal efficiency (%)	Reference
Estrone	57	Grit channels + primary clarifiers + conventional activated sludge	93.7	3
	Not specified	Anaerobic digestion activated sludge WWTP in the UK	79	1
	Not specified	Aerobic and anaerobic activated sludge WWTP in France	0-36	1
	Not specified	Aerobic activated sludge WWTP in Mexico	>95	1
Estrone-3-sulfate	Not specified	Anaerobic digestion activated sludge WWTP in the UK	36	1
17- β estradiol	Not specified	Anaerobic digestion activated sludge WWTP in the UK	0	1
17- β ethynyl estradiol	Not specified	Anaerobic digestion activated sludge WWTP in the UK	34	1
	Not specified	Aerobic activated sludge WWTP in Mexico	>93	1
Gemfibrozil	Not specified	Aerobic activated sludge WWTP in Mexico	70	1
	190	Grit channels + primary clarifies + conventional activated sludge	50.8	3
Ibuprofen	Not specified	Aerobic activated sludge WWTP in Mexico	83	1
	Not specified	Aerobic activated sludge WWTP in Germany	>90	1
	Not specified	Aerobic activated sludge WWTP in Malaysia	90	1
	5650	Modified Bardenpho process	98	3
	4500	Grit channels + primary clarifies + conventional activated sludge	99.7	3
	130-450	Primary treatment + Orbal oxidation ditch + UV disinfection	60-90	3
Ketoprofen	Not specified	Aerobic activated sludge WWTP in Mexico	71	1
	Not specified	Aerobic activated sludge WWTP in Malaysia	92	1
Metoprolol	379	Grit tanks + primary sedimentation + bioreactor + clarifiers	52.9	3
Naproxen	Not specified	Anaerobic and anaerobic activated sludge WWTP in Beijing	97-100	1
	Not specified	Aerobic activated sludge WWTP in Mexico	75	1
	Not specified	Aerobic activated sludge WWTP in Germany	>90	1
	Not specified	Aerobic activated sludge WWTP in Finland	97-100	1
	870	Modified Bardenpho process	94	3
	3000	Grit channels + primary clarifies + conventional activated sludge	96.2	3

BAC	Initial concentration (ngL^{-1})	Treatment process	Removal efficiency (%)	Reference
Paracetamol	2,180,000	Modified Bardenpho process	99	3
Propranolol	151	Grit tanks + primary sedimentation + bioreactor + clarifiers	49.9	3
Salicylic acid	Not specified	Aerobic activated sludge WWTP in Mexico	-6.8	1
Sotalol	711	Grit tanks + primary sedimentation + bioreactor + clarifiers	47.1	3
Sulfamethoxazole	7400	Grit channels + primary clarifiers + conventional activated sludge	-35.8	3
Sulfadiazine	20	Grit channels + primary clarifiers + conventional activated sludge	-64.1	3
	10-22	Primary treatment + orbal oxidation ditch + UV disinfection	40-100	3
Triclosan	Not specified	Aerobic activated sludge WWTP in Mexico	41	1
Trimethoprim	570	Grit channels + primary clarifiers + conventional activated sludge	-53.1	3
	Not specified	Anaerobic and anaerobic activated sludge WWTP in Beijing	38-55	1

Table 2.2-3 Removal efficiency comparison across different conventional wastewater treatment plants.

Reference column numbers refer to the following references: **1** (Rodriguez-Narvaez, et al., 2017), **2** (Barbosa, et al., 2016), **3** (Wang, et al., 2016).

2.2.4 New technologies being developed

Newer technologies, targeted at removing BACs, are being developed (Bilal, et al., 2019; Rodriguez-Narvaez, et al., 2017; Rojas, et al., 2020). These are not the focus of this thesis; however, an awareness is important as in the future they may be used for certain applications, however for many of these they are currently too energy-inetnsive or too expensive for use with the large volumes of municipal wastewater. . These include metal organic frameworks (MOFs) (Rojas, et al., 2020) and layered double hydroxides (LDHs)(Zubair, et al., 2017), which are materials with promising qualities such as BAC selectivity, reusability and the potential to degrade the pollutants (Rojas, et al., 2020). However, challenges still remain before they can be used in scale-up WWTPs, such as toxic by-products, scale-up parameters, stability in water (Rojas, et al., 2020). Another important removal technology being developed is enzyme-assisted biodegradation. This includes the use of oxidoreductases which catalyse oxidation/removal of many BACs. Their main advantages include their high removal of BACs in a cost-effective way (no need for advanced equipment and only hydrogen peroxide and molecular oxygen are needed). Nevertheless, research is still needed for their implementation in WWTPs, particularly in the area of enzyme immobilization and scale-up parameters in real reaction conditions (Bilal, et al., 2019).

There are also advancements on biological treatment being developed. For example, bio-electrochemical systems, which can produce electricity alongside the removal of pollutants. In this process there is an anode cover in a biofilm where biological oxidation of BACs occurs, this process transfers electrons through an external circuit to the cathode where reduction reactions may occur. This and other advanced biological treatment processes are promising but are still in the development stage. An important remaining unknown is the production of transformation products and by-products from these process which may be equally or more bioactive than the BACs targeted (Rodriguez-Narvaez, et al., 2017).

Finally, other treatment technologies which utilise natural processes such as wetlands treatment and soil aquifer treatment are also being researched for BAC removal. These technologies have the advantages of being very cost-effective (Delgadillo, et al., 2010). BACs removal is based on the combined effect of plant uptake and degradation, soil sorption processes and microbial degradation. The processes taking place are further detailed in the next section as they are those also occurring naturally in the environment.

2.3 The behaviour of bioactive pollutants in the agricultural environment

One study identified reclaimed wastewater as the main source of BACs in the agricultural environment (Carter, et al., 2019). This results in the following environmental compartments being contaminated: soil, surface water and groundwater, as is summarised in Figure 2.3.1. The receptors identified are terrestrial plants, wildlife, aquatic species, people, soil fauna and livestock. These receptors can then act as secondary sources through the food chain (Carter, et al., 2019). This thesis focuses on the processes taking place in the aquatic matrix, in terms of water used for irrigation water and agricultural soil matrices.

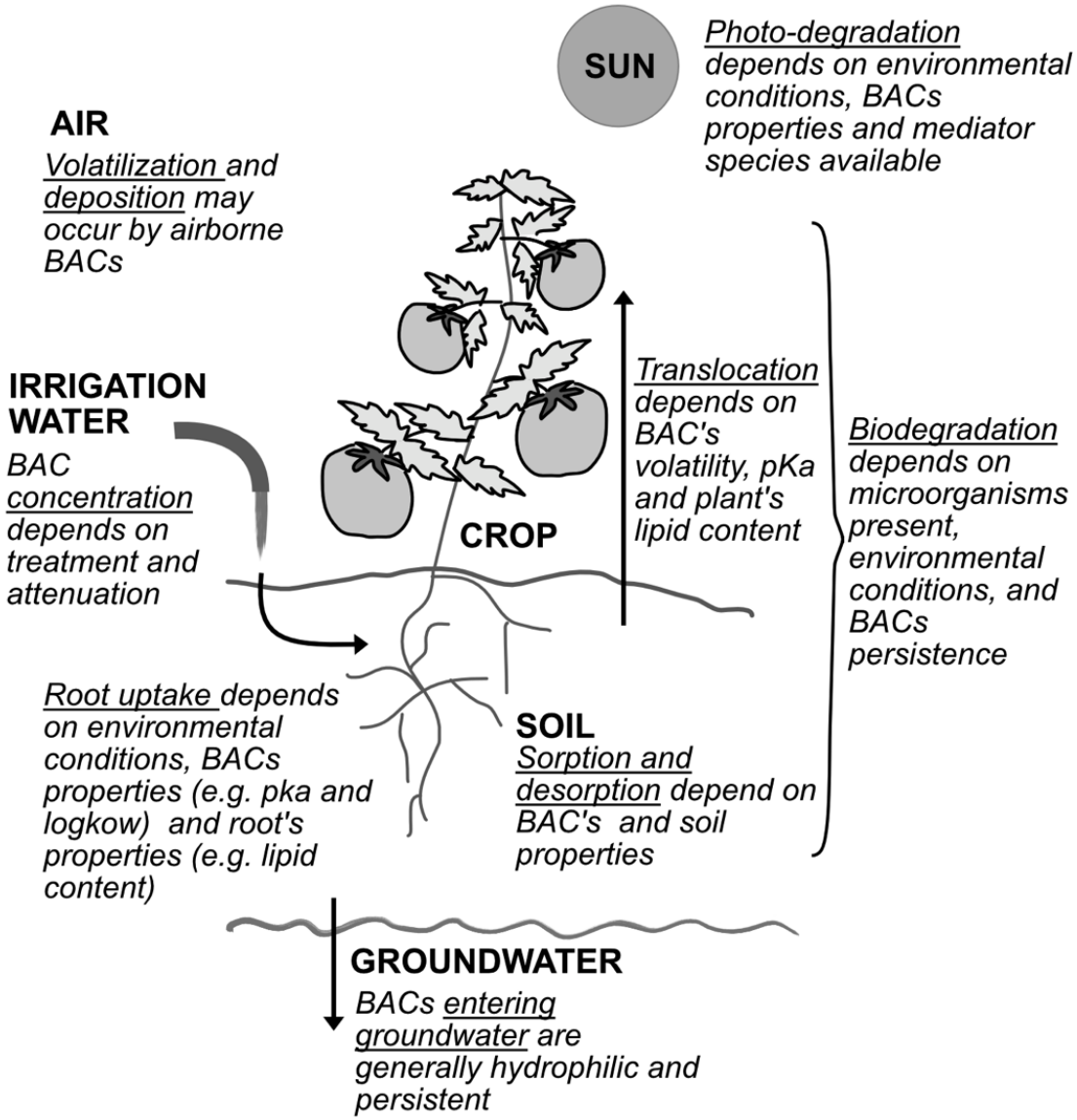


Figure 2.3-1 Summary of processes BACs can undergo in the agricultural environment.

2.3.1 The aquatic environment

The first step in being able to deal with the issue of BACs in the environment is having a comprehensive understanding of the pollutants entering it. As previously detailed, municipal wastewater (treated and untreated) is the main source of BACs in the environment (Petrie, et al., 2015). Gaps remain in this area in terms of metabolite presence, removal efficiency of WWTPs and data on certain geographical regions. There is also limited information on the fate of BACs during piping, such as anaerobic conditions, biofilm processes, chlorination, reservoir storage and effects of temperature (Carter, et al., 2019).

The ten most commonly detected BACs in influent and effluent wastewater have been: atenolol, bezafibrate, carbamazepine, diclofenac, gemfibrozil, ibuprofen, ketoprofen, metoprolol, naproxen and sotalol. Of these carbamazepine and gemfibrozil have been reported to have negative removals in WWTPs (Wilkinson, et al., 2017). Table 2.3-1 shows examples of common BACs and their concentrations found in the aquatic environment.

The concentrations of BACs will vary depending on the region and on seasonality (Carter, et al., 2019; Madikizela, et al., 2017; Peña-Guzmán, et al., 2019; Petrie, et al., 2015; Wilkinson, et al., 2017). In many countries such as Israel, Spain and the United States of America, wastewater is treated prior to reuse (Goldestein et al, 2014). In other countries wastewater sometimes undergoes no treatment prior to reuse for irrigation, some examples can be found in Mexico (Durán-Álvarez *et.al.*, 2015) and Pakistan (Uzma, et al. 2016). Studies have shown that wastewater effluent contains BACs in concentrations that can reach tens of thousands of ngL^{-1} of the most persistent pollutants such as tramadol and paracetamol (Petrie et. al., 2017). After dilution and attenuation, they can be found in surface waters in concentrations ranging from less than <1 -10 ngL^{-1} for the less persistent pollutants such as ketoprofen and up to thousands of ngL^{-1} for more persistent BACs such as tramadol and carbamazepine (Petrie et. al., 2017). These ranges can be higher in countries where wastewater is discharged without previous treatment. For example, raw wastewater from Mexico City can have concentrations of salicylic acid as high as 72,979 $\pm$ 3 910 ngL^{-1} in the dry season (Chavez et. al., 2011).

Once in the aquatic environment the fate as bioaccumulation, spatial distribution, partitioning between dissolved and bound to suspended particulate material phases is not well known, other than for a few BACs such as bisphenol A (BPA) and ethinyl estradiol (EE2) (Wilkinson, et al., 2017). Attenuation and fate of BACs are highly dependent on the geographical region, the hydrogeological characteristics, the season and the pollutant's properties (Wilkinson, et al., 2017).

BACs entering the aquatic environment from wastewater effluent have been observed to reduce in concertation, this is like largely due to dilution effect (Wilkinson, et al., 2017). Attenuation in surface waters may also be due to sorption to suspended solids or sediments, however this adsorption may also aid in the transportation of BACs and not actually mean removal of the pollutant (Wilkinson, et al., 2017). The extent of adsorption to solid matter is compound dependent, for example the antibiotic tetracycline has been shown to bind more to the solid fraction than sulfonamide, quinolone and macrolide antibiotics (Gregg, et al., 2015). Some pollutants, such as nonylphenol, have been found in suspended particles at concentrations up to 21.5 than those in the river water, on the other hand pollutants such as erythromycin, gemfibrozil and metoprolol have been found mostly, if not only, in the aqueous fraction rather than the solid (Wilkinson, et al., 2017). BACs may undergo sorption to microplastics which is of particular concern as these have very low biodegradability meaning they will remain in the environment (Avio, et al., 2017). Aquatic species may ingest microplastics, which is another route for BAC exposure, in a Brazilian study 18-33% of fish from an estuary were found to have microplastics in their stomachs (Wilkinson, et al., 2017).

BACs biodegrade into other compounds by the action of human and animal metabolism and by microorganisms present in the WWTP, in suspended solids or in sediments (Rodriguez-Narvaez, et al., 2017). The reactions which take place in biodegradation are hydrolysis, reduction, oxidation and mineralization (Wilkinson, et al., 2017). Humans and animals mostly tend to only transform BACs via conjugation with glucuronides and sulfates, while microorganisms (bacteria, fungi, microalgae, protozoa) degrade them to greater degree some even to mineralization (Wilkinson, et al., 2017). Biodegradation has been shown to occur at greater rates in the interphase between the aquatic and the soil phase and is dependent on factors such as the BAC species, temperature, water flow, pH, nutrient availability and salinity (Wilkinson, et al., 2017).

BACs may also be degraded by photochemical transformations, either by direct absorption of solar irradiation or by reactions with photosensitized species. BACs species which are more prone to direct photodegradation are those with functional groups that can donate additional π- electrons such as -CH=CH_2, -NO_2, -NH_2, -N=N and -OH. Species which act as photosensitized mediator species in BAC photodegradation include dissolved organic matter and NO_2^- and NO_3^- (Wilkinson, et al., 2017). Photochemical transformation is dependent on factors such as the BAC species, temperature, pH, cloud coverage, altitude, latitude, time of the day and depth below the water surface (the majority of UV light is absorbed within the top 2 m of water). Some examples of photodegradation of BACs include diclofenac into 1-(8-chlorocarbazolyl) acetic acid. Research is still lacking in terms of the toxicity of photodegradation products and real environmental reactions as compared to those observed in laboratory conditions (Wilkinson, et al., 2017).

2.3.2 The soil environment and soil characterization

The concentration of BACs in soil is generally lower than their concertation in water. The six most common pollutants found in soil are the antibiotics: trimethoprim, sulfadiazine and triclosan, the analgesics: ibuprofen and diclofenac and the antiepileptic carbamazepine (Li, 2014).
BACs have been found in soils irrigated with secondary treated wastewater in the range: <1-5 ngg^{-1} wet weight (McEachran et. al., 2017). Similarly, in Spain seven out of 64 pharmaceuticals and personal care products (diclofenac, metoprolol, fenofibrate, carbamazepine, clarithromycin, famotidine and hydrochlorothiazide) were detected occasionally at concentrations ranging from 0.1 to 1.3 ngg^{-1}(Corada-Fernández, et al., 2015). In Mexico, in a region under 85 - 100 years of irrigation with untreated wastewater carbamazepine (1.4-6.2 ngg^{-1}), naproxen (0.7-2.7 ngg^{-1}) and triclosan (6.3-9.7 ngg^{-1}) were detected (Durán-Álvarez, et al., 2012). Antibiotics have been detected in agricultural soils in China ranging from 0.1- 8400 ng g^{-1} (Snow, et al., 2017).

Once in the soil sorption and desorption behaviour determines the availability of the pollutant for other processes; mainly biodegradation, plant uptake and entering groundwater (Alvarino, et al., 2018; Barron, et al., 2009). For example tetracyclines are strongly adsorbed to soil due to their high sorption coefficient (Boxall et al., 2006; Tolls, 2001), and hence less bioavailable for plant uptake (Azanu, et al., 2018). Photodegradation can occur in soils but is limited by low light penetration. However, for some pollutants such as diclofenac and salicylic acid photolysis has been identified as the main attenuation mechanism but it occurs mainly in the aqueous phase (Chávez-Mejía, et al., 2019). Volatilization from soils is mostly negligible given the low vapour pressures of most BACs (Jechalke, et al., 2014).
Soil processes are vulnerable to climate change associated with global warming, including increased extreme rainfall and draught events, soil erosion, and a rise in sea level. There is evidence these changes can affect the mobility, transport, and storage of pollutants in soil, potentially increasing human exposure to soil contaminants (Biswas, et al., 2018). These processes as well as the toxicity and availability of the pollutants is not fully understood (Biswas, et al., 2018). Soil process are highly dependent on the type of soil. Soils are mainly characterized in terms of their texture: percentage of clay, silt and sand (OECD, 2000). Soil texture influences water and nutrient retention capacity as well as the mobility of pollutants in the soil (M. Conde-Cid, et al., 2020). Soil texture is a very standard measurement and is always reported on studies dealing with sorption of pollutants in different soils (OECD, 2000).

Soil pH is another important parameter which determines sorption of pollutants (Lorphensri, et al., 2007). Organic matter content is also a determining factor in nutrient availability as well as pollutant sorption and desorption behaviour (M. Conde-Cid, et

al., 2020). Organic matter is the fraction of soil made of animal and plant tissue in several stages of decomposition (Fenton, et al., 2008). The measurable component of organic matter is organic carbon, usually determined using the Walkley-Black with an adaptation from Mebius, in order to ensure complete digestion of the organic carbon (Schumacher, 2002). Organic matter contains approximately 58% of organic carbon and so a factor of 1.72 is used to convert between total organic carbon and organic matter (Fenton, et al., 2008). However, this can be affected by type of organic matter, soil type and soil depth (Fenton, et al., 2008).

Cation exchange capacity (CEC) is another important parameter which influences the soil's ability to hold on to essential nutrients (Mg^{2+}, Ca^{2+}, Na^{+}, K^{+}) and which provides a buffer against soil acidification. It is defined as the total capacity of a soil to hold exchangeable cations. Normally a higher clay and organic matter content means a higher CEC content (Reganold, et al., 1985). CEC has an impact on the sorption of cationic pollutants in soil (Yasong Li, et al., 2018a). CEC is usually determined by measuring the sum of Mg^{2+}, Ca^{2+}, Na^{+}, K^{+} present in the soil, however these may be replaced with Mn^{2+}, H^{+}, Al^{+3} and so different methods may be used to measure these cations (Reganold, et al., 1985). In some publications related to BAC's sorption in soil the quantity of different cations is reported, along with the overall CEC (Li, et al., 2020), this is beneficial as it gives clarity on the cations used for the calculation. Furthermore, some pollutant behaviour, especially for isomers has been reported to be influenced by the minerals found in soils (Yu, et al., 2020). $CaCO_3$ is sometimes reported (Paz, et al., 2016) and it can also affect CEC (Reganold, et al., 1985).

Other parameters which are occasionally reported are point of zero charge (PZC) (Martínez-Hernández, et al., 2014), electrical conductivity (Durán-Álvarez, et al., 2012; Wojsławski, et al., 2019), and bulk density (Durán-Álvarez, et al., 2012). PZC refers to the pH at which the net charge of the soil is zero, given that charge is affected by H^{+} and OH^{-} ions (Appel, et al., 2003). Electrical conductivity is an indirect measurement which relates to other soil properties (Barbosa, R.N.,& Overstreet, 2011). Soil bulk density affects soil and root structure, in particular for the initial development of seeds (Tracy, et al., 2013). It is an important parameter in models of pollutant mobility (Lorphensri, et al., 2007).

2.3.2.1 BACs sorption to soil

Sorption encompasses processes such as adsorption, surface precipitation and polymerization. Adsorption is the accumulation of a sorbate at the interphase between the solid surface and the bathing solution. It may be driven by physical or chemical mechanisms. Physical mechanisms include Van der Waals forces (e.g., attraction governed by polarity) or electrostatic (e.g., ion exchange). Chemical mechanisms include ligand exchange, covalent bonding and H-bonding (Sparks, 2003).

Sorption and desorption are dependent on the compound's physicochemical properties and on the soil texture, pH and available chemical species (Biswas, et al., 2018; Lorphensri, et al., 2007; Martínez-Hernández, et al., 2016). One of the most important properties is the BAC's pK_a, which will determine whether they are found in a non-ionic, anionic or cationic form. The sorption of non-ionic species is mainly related to the organic matter content. Cation sorption is positively related to the cation exchange capacity (CEC), while anion sorption may be negatively related to the CEC. For example, acetaminophen (pK_a = 9.38 and Log_{Kow} ~0.46) is found in the neutral form at most environmental pHs. This coupled with its low Log_{Kow} results in low soil sorption. On the other hand, ethynyl estradiol (EE2) which is also found mostly in the neutral form at environmental pHs (pK_a=10.4) presents higher sorption than acetaminophen due to its higher Log_{Kow} ~3.67. In the case of EE2 sorption is mostly

governed by hydrophobic partitioning and so less sorption to charged media than hydrophobic media is observed. For nalidixic acid, which has a pK_a close to environmental pH (5.95) sorption is governed by soil pH. Depending on slight pH changes nalidixic acid would be found in the ionised or non-ionised form, meaning that electrostatic mechanisms would play a part on the extent of its sorption. Hydrophobic mechanisms also play a part on its soil sorption. Log_{Kow} is dependent on pH ($Log_{Kow,pH=5}$ = 1.54, $Log_{Kow,pH=7}$ = 0.47) and so the extent of sorption is highly dependent on soil pH. The compound is found in the ionised form when pK_a is below soil pH, in those cases electrostatic mechanisms play a larger role in soil sorption (Lorphensri, et al., 2007).

Another important factor which determines the extent of sorption are the functional groups of the BAC. For example, lamotrigine which has triazine rings and amino groups has an enhanced ability to form H-bonds with functional groups of soils surfaces, making it more biodegradable than pollutants such as carbamazepine and its metabolites (Paz, et al., 2016).

The extent of soil sorption can be measured and modelled in different ways. The most commonly reported in literature are the linear distribution coefficient K_d (Eq. 2), the Langmuir isotherm (Eq. 23) and Freundlich isotherm (Eq. 5) (Durán-Álvarez, et al., 2012; Kodešová, et al., 2015; Koroša, et al., 2020; Kumar, et al., 2009; Li, et al., 2020; Ramil, et al., 2010; Peifang Wang, et al., 2019). For the last two the linear form of the equations is presented below:

$$K_d = \frac{m}{(1-m)}\frac{v}{s} \qquad \textit{Eq. 2}$$

Where m is the fraction of BAC sorbed onto soil particles. For batch tests: v is the volume of liquid phase and s the amount of soil (Durán–Álvarez, et al., 2014). K_d is a linear approximation of the sorption behaviour (Kiecak, et al., 2019). This is a simple partitioning ratio of the amount of compound in the soil compared to the water, after a given equilibration period and it is often calculated at a particular concentration point. K_d is sometimes modelled based on the Log_{Kow} (Kiecak, et al., 2019; Ma, et al., 2018a), however as has been previously explained, for ionizable compounds the pH dependent Log_{Kow} should be used (Lorphensri, et al., 2007). K_d can be normalised based on the organic content by divding it by the fraction of organic matter in the soil (f_{oc}):

$$K_{oc} = \frac{K_d}{f_{oc}} \qquad \textit{Eq. 3}$$

K_{oc} can be useful to compare sorption of a pollutant to different kinds of soils. However this assumes the organic matter in all soils has the same thermodynamic characteristics (Ma, et al., 2018a).

The Langmuir model considers limited adsorption capacity of a given soil (Kiecak, et al., 2019). It assumes that each sorbate molecule occupies a single surface site. The sorbate material is homogenous and there is no lateral interaction between adjacent adsorbed molecules (Lingling Liu, et al., 2019):

$$\frac{C_e}{q_e} = \frac{1}{Q^0 b} + \frac{1}{Q^0} C_e \qquad \textit{Eq. 4}$$

Where q_e is equilibrium absorbent-phase concentration of adsorbate (mgL^{-1}), C_e is the equilibrium aqueous-phase concentration of adsorbate (mgL^{-1}), Q^0 is the monolayer adsorption capacity (mg g^{-1}), b is a constant related to the free adsorption

concentration at which half saturation of the adsorbent is reached (Lingling Liu, et al., 2019).

The Freundlich isotherm considers progressive saturation (Kiecak, et al., 2019) but is only is valid up to a certain concertation were it is still linear (Singh, 2016):

$$\log(q_e) = \log K_f + \frac{1}{n}\log(C_e) \qquad \textit{Eq. 5}$$

Where K_f is the Freundlich constant or maximum adsorption capacity, this is temperature dependent. $\frac{1}{n}$ is the slope obtained from plotting $\log(q_e)$ against $\log(C_e)$ in the linear range (Singh, 2016).

These models are useful in understanding and comparing sorption behaviours between pollutants and types of soils. They can be used to build on models which describe the real environmental conditions. For example, the retardation factor (R) which describes the ability of a compound to travel through the soil and reach the aquifer table (Acheampong, et al., 2019; Li, et al., 2020; Lorphensri, et al., 2007):

$$R = 1 + \frac{k_d \rho}{\Theta} \qquad \textit{Eq. 6}$$

Where ρ is the soil bulk density, Θ is the soil porosity. A higher retardation factor means the compound will be accumulated in the top soil layers, while a lower retardation factor means the compound will travel easily through the soil (Paz, et al., 2016). Examples of pollutants with high mobility include carbamazepine, naproxen and diclofenac, due to their low sorption and biodegradability (Chávez-Mejía, et al., 2019; Chefetz, et al., 2008; Martínez-Hernández, et al., 2016). K_d is directly proportional to the retardation factor. Bench-scale sorption and desorption experiments can help determine K_d values, however these processes are more complicated in real environmental soils. For example, desorption hysteresis is the phenomenon by which faster adsorption than desorption may be observed. This can be due to physical entrapment of contaminants in the complex matrix or to specific strong adsorptive interactions leading to irreversible binding of contaminants to the carriers (Paz, et al., 2016).

Another important consideration is the dependence of K_d on concentration (Kiecak, et al., 2019; Singh, 2016). Many bench-scale studies are done at concentrations higher than those environmentally relevant (Paz, et al., 2016), this work is useful in terms of understanding pollutant behaviour, however it can be problematic in that K_d at lower concentrations has been shown to be higher than at higher concentrations. This can be explained by specific interactions between sorbate molecules and functional groups of soil organic matter which are dominant at lower sorbate concentrations (Paz, et al., 2016).

Another consideration is the real-life situation, where multiple BACs may compete sorption sites in the soil (Conde-Cid, et al., 2019; Paz, et al., 2016). For example competitive sorption has been observed for tetracycline antibiotics, where less sorption was observed for tetracycline, oxytetracycline and chlortetracycline in a mixture system as compared to each individually (Conde-Cid, et al., 2019). Competitive sorption has been identified as concentration dependent and so this must also be accounted for, particularly when experiments are not done at environmentally relevant concentrations (Paz, et al., 2016).

Most sorption experiments are carried out with soil sieved at 2 mm and this does not account for higher vertical water velocities in situations such as coarse-gravel unsaturated zones (Koroša, et al., 2020).

Sorption is also affected by previous soil irrigation and amendment (Aurore Andriamalala, et al., 2018; Dalkmann, et al., 2014). For example, in Mexican soils which have been irrigated with wastewaters for 14-100 years, sulfamethoxazole adsorption decreased with increased periods of wastewater irrigation. This is most likely due to competition for sorption sites between the sulfamethoxazole being added and sulfamethoxazole or other organic pollutants accumulated in the soil. Organic matter content also increases with increasing periods under wastewater irrigation and this may also decrease sorption of some pollutants, like sulfamethoxazole. On the other hand, this effect was not observed for ciprofloxacin (Dalkmann, et al., 2014). A study showed that there is higher removal (sorption and biodegradation) of BACs (those tested were: acetaminophen, caffeine, carbamazepine, naproxen and sulfamethoxazole) after the second time they have been spiked on to a soil. This may be due to microorganism adaptation to biodegrade them (Martínez-Hernández, et al., 2016).

2.3.2.2 Biodegradation

Once in the soil BACs are prone to microbial transformation. For example sulfonamide metabolites may be bio-transformed back to the parent compound (Jechalke, et al., 2014). Sulfamethoxazole has been shown to be biodegraded into N_4-acetyl-sulfamethoxazole, and this compound can be transformed back into sulfamethoxazole in the presence of some enzymes generated by various microorganisms (Martínez-Hernández, et al., 2016). Pollutants with high biodegradation in soil include acetaminophen and caffeine, with nearly complete biodegradation being observed after 100 and 150 hours respectively (Martínez-Hernández, et al., 2016). Other examples of BACs reported to be mostly attenuated due to biodegradation or a combination of biodegradation and sorption include: 17α-ethynilestradiol, 17β-estradiol, 2,4-dichlorophenoxyaceric acid, 4-n-noylphenol, Bisphenol-A, and estrone (Chávez-Mejía, et al., 2019).

BACs which have been reported to have very low biodegradation in soil include carbamazepine and naproxen (Chávez-Mejía, et al., 2019; Martínez-Hernández, et al., 2016). Both of these pollutants have 2 aromatic rings, which makes them highly recalcitrant (Martínez-Hernández, et al., 2016). Carbamazepine biodegradation has been seen to result in the formation of epoxy-carbamazepine, the extent of biodegradation or conversion back to carbamazepine of this compound is not clear (Martínez-Hernández, et al., 2016). Although BACs may also be bio-transformed into non-bioactive products, however, for many antibiotics, mineralization frequently accounts for less than 2% of the compound present (Jechalke, et al., 2014).

Biodegradation has been shown not to be only dependent on the microorganisms present, but also on the soil properties such as organic carbon content and soil texture (Cycoń, et al., 2019). Environmental conditions such as temperature and oxygen availability also play a big role (Cycoń, et al., 2019). For example, carbamazepine has been found to be better biodegraded in aerobic conditions compared to anaerobic conditions in some soil types (Thelusmond, et al., 2016). Some microorganism types have been found to increase in numbers in the presence of carbamazepine, while others have been found to decrease, also depending on oxygen, soil type, concentration and duration of exposure (Thelusmond, et al., 2016). Biodegradation is also dependent on the concentration of BACs in soil, several studies show that while at lower concentrations BACs can be degraded in soils at higher concentrations much

lower biodegradation rates are observed (Cycoń, et al., 2019). This has been attributed to inhibition of soil microorganisms at higher BAC concentrations (Cycoń, et al., 2019). However, with repeated introductions of BACs on to soil increased biodegradation has been observed. This has been attributed to the adaptation of soil microorganisms to the pollutants (Cycoń, et al., 2019; Martínez-Hernández, et al., 2016).

It is important to note that the way in which biodegradation is studied in isolation from sorption in laboratory scale studies is by sterilizing the soil by either autoclaving (Li, et al., 2020; Martínez-Hernández, et al., 2016) or by adding sodium azide (Paz 2016, Chefetz 2008, Kiecaka 2020) these methods may change soil characteristics and affect sorption itself (Lotrario, et al., 1995).

2.3.2.3 Plant uptake

The uptake of BACs by plants from water applied to crops and/ or soil has been highlighted as an issue due to its potential for introducing these pollutants in the animal and human food chains (Christou, et al., 2019). In a study comparing the uptake of atenolol, sulfamethoxazole and carbamazepine by 3 leafy and 1 root vegetable in three different soils, carbamazepine and its metabolites showed the greatest accumulation, posing a potential risk for consumers (Kodesova, et al., 2019). BAC uptake mostly depends on the pollutant's availability (persistence and sorption to soil), the plant's transpiration rate, the soil pore water chemistry, the structure of the plant's cell membrane and the physicochemical properties of the pollutant (Christou, et al., 2019; Kodesova, et al., 2019).

It has been observed and theorised that uptake increases with increasing lipophilicity (Log_{Kow}), molecular size, weight and polarity. Neutral (as opposed to anionic or cationic) species present more uptake. This is because negatively charged molecules are repelled by the cell walls of the root apoplast and the uptake and translocation of positively charged molecules is reduced by their attraction to the negatively charged cell walls (Christou, et al., 2019; Kodesova, et al., 2019).

Most studies have been done in hydroponic conditions although there are a few done in field or greenhouse conditions, which although useful to understand certain aspects are not able to give a full picture of what is occurring in the real environment (Christou, et al., 2019). Not many have studied the impact of soil type, metabolite occurrence in plants, environmentally relevant concentrations or mixture effects, when several BACs are applied simultaneously (Kodesova, et al., 2019). However, a trend has been observed in that crops grown in predominantly sandy soils or soils with low organic matter, or soils with low clay (compared to the silt and sand fractions) have higher potential to uptake BACs. This is compared to soils rich in clay or organic matter (Christou, et al., 2019).

Studies done to date show a trend for the uptake and accumulation of BACs depending on plant type as follows: leafy vegetables > root vegetables > cereals and fodder crops > fruit vegetables (Christou, et al., 2019). Once taken up the translocation of BACs within a plant (through the xylem and phloem) depends on their lipophilicity, and electrical charge as well as the plant's physiology and transpiration rate (Christou, et al., 2019). Environmental factors, such as drought stress, which increases the transpiration rate, also play an important role in determining translocation rates (Christou, et al., 2019). Acidic BACs (pK_a<7, Log_{Kow} <3) tend to remain in the phloem and therefore accumulate more in fruits, basic BACs (pK_a > 7) which are hydrophilic (Log_{Kow} < 0) tend to move both in the xylem and phloem and those which are lipophilic (0 < Log_{Kow} < 4) tend to move in the xylem (Christou, et al., 2019). Once in the phloem or sap BACs are available to higher trophic levels, which requires further research (Pufal, et al., 2019).

An important aspect of plant BAC uptake is the processes taking place in the rhizosphere. Plants are capable of changing rhizosphere pH by 2-3 units either way in response to nutrient availability (Nason, et al., 2018). This will have an effect on the ionization of BACs present and therefore on their availability for plant uptake (Nason, et al., 2018). Other processes such as root exudates can also affect BAC uptake (Elizabeth L. Miller, et al., 2016). For example, compounds found in root exudates such as citric, oxalic and malonic acids have been shown to promote polycyclic aromatic hydrocarbons desorption from soil (Elizabeth L. Miller, et al., 2016). Additionally, microorganisms in the rhizosphere such as mycorrhizal fungi can affect BAC availability by altering soil chemistry and mineralogy (Elizabeth L. Miller, et al., 2016). Several aspects of rhizosphere process remain understudied, in particular in relation to BAC uptake (Elizabeth L. Miller, et al., 2016).

There are some examples of negative effects on plants due to BAC uptake. In a study done at environmentally relevant concentrations, sulfadiazine reduced plant growth and increased aphid mortality through reduced host plant biomass. The authors highlight these findings as evidence of the negative effects that BACs can have not only on plants but also on the herbivore insects which depend on them (Pufal, et al., 2019). However, penicillin and tetracycline did not have an effect on plants (or the aphids being studied alongside them) (Pufal, et al., 2019). In another study with concentrations of atenolol, sulfamethoxazole and carbamazepine 1000 times greater than those environmentally relevant, no negative effects on crops (lamb's lettuce, spinach, arugula and radish) in 3 different soils (silt loam, loam and a sand) were observed (Kodesova, et al., 2019). More research is still necessary to determine the concentrations at which BACs can have a negative effect on plants and the herbivores directly dependent on them (Pufal, et al., 2019). Even though studying plants is not part of the scope of this thesis, they are considered because they are an important part of the agricultural environment.

Table 2.3.1 has some exemplary concentrations of BACs found in the main matrices described in this section (irrigation water, agricultural soil and crops) in relation to agriculture.

	BAC	Matrix/ experimental conditions	Concentration (ngL^{-1})	Ref.
Irrigation water/ aquatic environment (ngL^{-1})	Naproxen	Canada rivers and canals	550	1
	Ibuprofen	Canada rivers and canals	200	1
	Triclosan	US rivers and streams	2,300	1
	Trimethoprim	Canada groundwater	2	1
	Diclofenac	Surface water	<0.5-253	1
	Paracetamol	Surface water	110-10,000	1
	17a-Ethynilestradiol	Surface water	<0.98-10.2	2
	Bezafibrate	Surface water	<10-60	2
	Tamoxifen	UK river	27-212	1
	Codeine	Surface water	12-1,000	1
	Atenolol	French groundwater	30	1
		Korean surface water	690	1
		UK surface water	680	1
	Venlafaxine	Surface water	1.1-35	2
	Synthetic musk compounds	Surface water	150-16,700	2
	Sulfamethoxazole	Canada groundwater	<1-46	1
	Ibuprofen	Surface water	1-2,370	2
Soil (μgkg^{-1})	Sulfadiazine	Maximum reported concentration in soil	85.5	4
	Ciprofloxacin	Maximum reported concentration in soil	5,600	4
	Erythromycin	Maximum reported concentration in soil	7.2	4
	Sulfapyridine	Maximum reported concentration in soil	5.11	4
	Oxytetracycline	Maximum reported concentration in soil	50,000	4
	Sulfamethoxazole	Maximum reported concentration in soil	54.5	4
	Sulfamethoxazole	3-year irrigation using tertiary treated water containing diclofenac, sulfamethoxazole and trimethoprim	0.98	3
	Trimethoprim		0.62	3
	Diclofenac		0.35	3
	Carbamazepine	Biosolid-amended soil growing soybean	44.2	3
		Biosolid-amended soil growing cabbage	93.1	
		Biosolid-amended soil growing lettuce	98.3	
		Biosolid-amended soil growing radish	90.3	
		Biosolid-amended soil growing tomato	96.8	

	BAC	Matrix/ experimental conditions	Concentration (ngL^{-1})	Ref.
	Carbamazepine	Reclaimed WW irrigation of tomato in alluvial soil	1.5	3
		Reclaimed WW irrigation of tomato in aeolian soil	2	
		Reclaimed WW irrigation of tomato in sand	2	
	Sulfamethoxazole	Reclaimed WW irrigation of tomato in alluvial soil	2	3
		Reclaimed WW irrigation of tomato in aeolian soil	1	
		Reclaimed WW irrigation of tomato in sand	1	
	Carbamazepine	Reclaimed WW irrigation of cucumber in alluvial soil	8.5	3
		Reclaimed WW irrigation of cucumber in aeolian soil	1.2	
		Reclaimed WW irrigation of cucumber in sand	1.2	
	Sulfamethoxazole	Reclaimed WW irrigation of cucumber in alluvial soil	0.2	3
		Reclaimed WW irrigation of cucumber in aeolian soil	0.2	
		Reclaimed WW irrigation of cucumber in sand	0.2	
Crops (μgkg^{-1})	Norfloxacin	Vegetable and cereal crops in land irrigated with WW for 20 years	4.6-23.6	3
	Chloramphenicol		2.6-22.4	3
	Tetracycline		4.0-10.1	3
	Sulfamethazine		Not detected	3
	Erythromycin		Not detected	3
	Sulfamethoxazole	3-year irrigation using tertiary treated water containing diclofenac, sulfamethoxazole and trimethoprim of tomatoes (fruit analysed)	5.26	3
	Trimethoprim		3.40	
	Diclofenac		11.63	
	Carbamazepine	Chinese cabbage uptake in fortified soil (aerial parts for the cabbage)	255.4	
	Sulfamethoxazole		222.8	
	Salbutamol		108.3	
	Trimethoprim		20.6	
	Carbamazepine	Chinese cabbage uptake in fortified soil (roots of the cabbage)	272.9	
	Sulfamethoxazole		260.3	
	Salbutamol		140.6	
	Trimethoprim		53.7	
	Carbamazepine	Chinese cabbage uptake in biosolid-amended soil (aerial parts for the cabbage)	317.6	
	Salbutamol		21.2	
	Triclosan		22.9	
	Carbamazepine		416.2	

	Salbutamol	Chinese cabbage uptake in biosolid-amended soil (roots of the cabbage)	187.6	
	Triclosan		1220.1	

Table 2.3-1 BAC concentrations found in real agro-ecosystems.

Ref. column refers to the following numbered references: **1** (Wilkinson, et al., 2017), **2** (Petrie, et al., 2015), **3** (Christou, et al., 2019), **4** (Cycoń, et al., 2019)

2.4 Geographical knowledge gaps

Pollution does not always stay in the country where it was produced. It can move in the air, water and through the food chain globally (Bernhardt, et al., 2019). The aim of this section is not to present a detailed discussion with specific conclusions, but to succinctly raise three important socio-political issues that people working in environmental pollution should consider, in particular when dealing with international collaborations.

Firstly, it is important to consider the language and discourse employed. Language shapes the way we perceive the world (Ziai, 2013) and terms such as 'developing' and 'developed' countries can construct a false narrative with detrimental consequences (Cornwall, et al., 2010). Critics have shown how the concept of 'development' has become a buzzword used to justify actions and policies grounded on the assumption that pure economic growth, in the capitalist sense, leads to a reduction of poverty and thus inherently results in better conditions for humans; when in fact the actions done in the name of 'development' have often led to the destruction of the natural environment and social relations (Cornwall, et al., 2010) These arguments call for a careful examination of what is and is not considered to be 'developed' in global power discourse and how this in turn influences how we approach water treatment.

'Developed' countries are considered to be those in North America and Europe, while those in Asia, Africa and Latin America are considered 'less developed' or 'underdeveloped'. The assumption is that the 'global south' needs industrialization and 'modernisation' to become 'developed' and that this can be achieved by expertise help from the 'global north' (Ziai, 2013). This is problematic as it does not acknowledge indigenous knowledge and practices and is colonial in nature (Prior, 2007). In particular in terms of wastewater treatment a more 'developed' or 'advanced' treatment technology does not necessarily mean a better one, the treatment technology used for a certain area should always be adequate for local use of water as well as the geographical and social conditions of the area. Ancient and indigenous water management technologies are often better suited for the region in which they were developed and have less detrimental impacts on the environment (Angelakis, et al., 2020).

Secondly, while some countries are identified as having less stringent environmental regulations or regulations which are not enforced, it is important to take a step back and acknowledge that this is a global problem given that often the processing of the products causing this pollution is driven by demand in countries with more stringent environmental regulations (Bernhardt, et al., 2019).

Thirdly, there is more data available regarding BACs in the environment for the 'global north' (Carter, et al., 2019; Madikizela, et al., 2017; Williams, et al., 2019). From an engineering perspective it is not possible to apply treatment methods or other engineering solutions in one area based on conclusions mainly drawn from data from another.

2.5 Description of the case studies

The two UK WWTP case studies were chosen in terms of their treatment technologies and locations. Stoke Bardolph WWTP is a relatively large (PE~650,000) with advanced secondary treatment (Durose, et al., 2014). Sutton Bonington is a small (PE~2,000) conventional treatment plant with an advanced tertiary filtration step (Office International de l'Eau, 2014). These plants provide an interesting contrast for BAC removal comparison, given their different treatment technologies. In practical terms,

the closeness of these plants to the laboratory allows for regular sampling allowing to bridge knowledge gaps, such as seasonality of removal, as previously detailed. The third case study was an agricultural area in Mexico which has been under wastewater irrigation for over 100 years. Targeted BAC studies have been previously undertaken in this area (Chamizo-Checa, et al., 2020; Chávez-Mejía, et al., 2019; Chávez, et al., 2011; Downs, et al., 2000, 1999; Gibson, et al., 2010; Jimenez-Cisneros, et al., 2012; Lesser-Carrillo, et al., 2011; Murillo-Torres, et al., 2012), but untargeted analysis has not. Furthermore, by doing this case study as a collaboration between a UK and a Mexican university steps were taken in bridging the geographical knowledge gaps described in the previous sections.

2.5.1 Large wastewater treatment plant (Stoke Bardolph, Nottinghamshire, UK)

Stoke Bardolph (Stk) WWTP (PE~650 000) receives the wastewater from the Nottingham City area (Durose, et al., 2014). Nottingham lies 52 m above sea level and has a latitude and longitude of 52°9548' North and 1°1581' West. The average annual temperature is 9.8 ºC with extreme seasonal variation. January is the coldest month with temperatures which can be just below 0 ºC. July is the warmest month with a mean daily temperatures of 22 ºC. The yearly rainfall is 648 mm, with even the driest months being relatively wet (Clima-Data.org, n.d.; Met Office, 2016).

Stk WWTP receives wastewater with a high loading of nutrients (nitrogen and phosphate) due to the dewatering liquor that is a waste stream form the energy production by sludge digestion and to the trade waste received form a neighbouring rendering plant. Therefore, this plant has cutting edge technology for nutrient removal. It is the first UK installation to utilise Phospaq, which recovers phosphate from effluents as struvite (magnesium-ammonium-phosphate). This treatment biologically converts COD into new biomass and CO_2. Magnesium oxide (MgO) is added which precipitates as struvite ($MgNH_4PO_4.6H_2O$) in the form of granules which can be removed from the water phase and can be used as slow fertilizer, complying with EU fertilizer standards (Paques, n.d.). Stk WWTP also uses BIOPAQ® USAB$^+$ (Upflow anaerobic sludge blanket), anaerobic wastewater treatment uses microorganisms to convert organic pollutants into biogas (containing methane and carbon dioxide). The design is based on three-phase separator, which enables the reactor to separate gas, water and sludge mixtures using high turbulence conditions and allowing for compact and cheaper design (International Water Association, 2010). Finally, nitrogen removal is achieved using Anammox®. Anammox bacteria convert ammonium (NH_4^+) and nitrite (NO_2^-) into nitrogen. In 2014 it was one of 20 in the world to use a full scale anammox, which was first trailed in the Netherlands in 2002 (Durose, et al., 2014).

The water leaves the WWTP through an open channel of approximately 1 km in length (Figure 2.5.1) and is then discharge onto the river Trent (Figure 2.5.2). This is one of England's major river and flows through Nottingham and Newark cities as well as the rural Trent Valley (Canal and River Trust, n.d.).





Figure 2.5-1 Open channel from Stoke Bardolph WWTP to Trent River (left). Sampling point in open channel (bottom)



Figure 2.5-2 Stoke Bardolph wastewater plant effluent discharge to the River Trent

2.5.2 Small sewage treatment (Sutton Bonington, Nottinghamshire, UK)

Sutton Bonington (SuB) WWTP is approximately 28 miles south west of Stoke Bardolph WWTP. It has a capacity for PE approx. 2,000 and utilises conventional wastewater treatment technology comprising of primary sedimentation, trickling filter as secondary biological treatment and sand filtration (Figure 2.5.3) as a polishing step (Office International de l'Eau, 2014). The climate conditions are very similar to those

described for Stk WWTP. The effluent from SuB WWTP goes in an underground pipe of about 100 m to a stream which after approximately 1 km reaches the River Soar.



Figure 2.5-3 Sand filters at Sutton Bonington wastewater treatment plant

2.5.3 Agricultural area irrigated with reclaimed wastewater (Tula Valley, Hidalgo, Mexico)

The Mezquital Valley, also known as the 'Tula Valley' is one of the world's largest areas (8500 ha) where untreated wastewater has been used for irrigation. The area lies between latitudes 19º54' and 20º30' North and longitudes 99º22' and 98º56' West. The altitude varies from 1700 m above sea level in the northern part to 2100 m above sea level in the southern part of the valley. The climate is mild with a mean annual temperature of 16.7 ºC. Rainfall ranges from 435 to 618 mm and falls mainly during the rainy season (May-October). The annual evaporation rate is 1750 mm (Chávez-Mejía, et al., 2019). In general the soil in this area has a pH 6.7-8.4, conductivity is 0.41–2.22 μScm^{-1} (with values of up to 40 μScm^{-1} where the aquifer level is very high), clay content is 11–60%, and texture tends to be clay-loamy (Jimenez-Cisneros, et al., 2008)

In 1898 three channels were built from Mexico City to the Tula Valley to carry municipal, industrial and surface runoff water in order to avoid flooding in Mexico City. The Tula valley is approximately 80 km from Mexico City (Chávez-Mejía, et al., 2019). As can be seen in, Figure 2.5-4 the channels built were the *Gran Canal* (Grand Channel), the *Interceptor del Poniente* (Easter Emitter) and the *Emisor Central* (Central Emitter). In 2020 a fourth channel was inaugurated, the *Tunel Emisor del Oriente* (Western Emitter Tunnel). From 1926 to 2010 the volume of water sent from Mexico City to the Tula Valley greatly increased, resulting in a doubling of the area irrigated using water coming from Mexico City every 25 years. Currently this area receives 52 m^3s^{-1} which serve to irrigate three irrigation districts, as can be seen in Figure 2.5.4 (Chávez-Mejía, et al., 2019). Depending on the region in the Tula Valley, the irrigation water has different water quality. The regions closest to Mexico City utilise raw

wastewater diluted with rainwater, which takes between 20-24 h to travel from Mexico City to the irrigation zones (Zone 1). Other zones use wastewater which, depending on flow, has been stored in dams and is mixed with rainwater (Zone 2). Finally (Zone 3) utilises wastewater which has travelled 48 h (if not stored) and is mixed with spring or well water (Chávez-Mejía, et al., 2019). In the past 10 years the construction and use of the Atotonilco WWTP has taken place. This plant treats raw wastewater coming from the *Tunel Emisor Central* and the *Tunel Emisor del Oriente*. The plant has been designed to remove greases, oils and pathogens but to leave most of the nutrients valued by farmers. The plant was built with the aim of maintaing the water flowrates farmers have been using for irrigation. The treatment methods comprise of preliminary screens, primary sedimentation tanks, secondary aerobic biological treatment and disinfection. This plant has a capacity of 35 m^3s^{-1} and an average of 23 m^3s^{-1} (CONAGUA, 2007). Some areas of the Tula Valley are now irrigated using treated water from the Atotonilco WWTP, however others are still irrigated using raw wastewater (Palacios, et al., 2017).

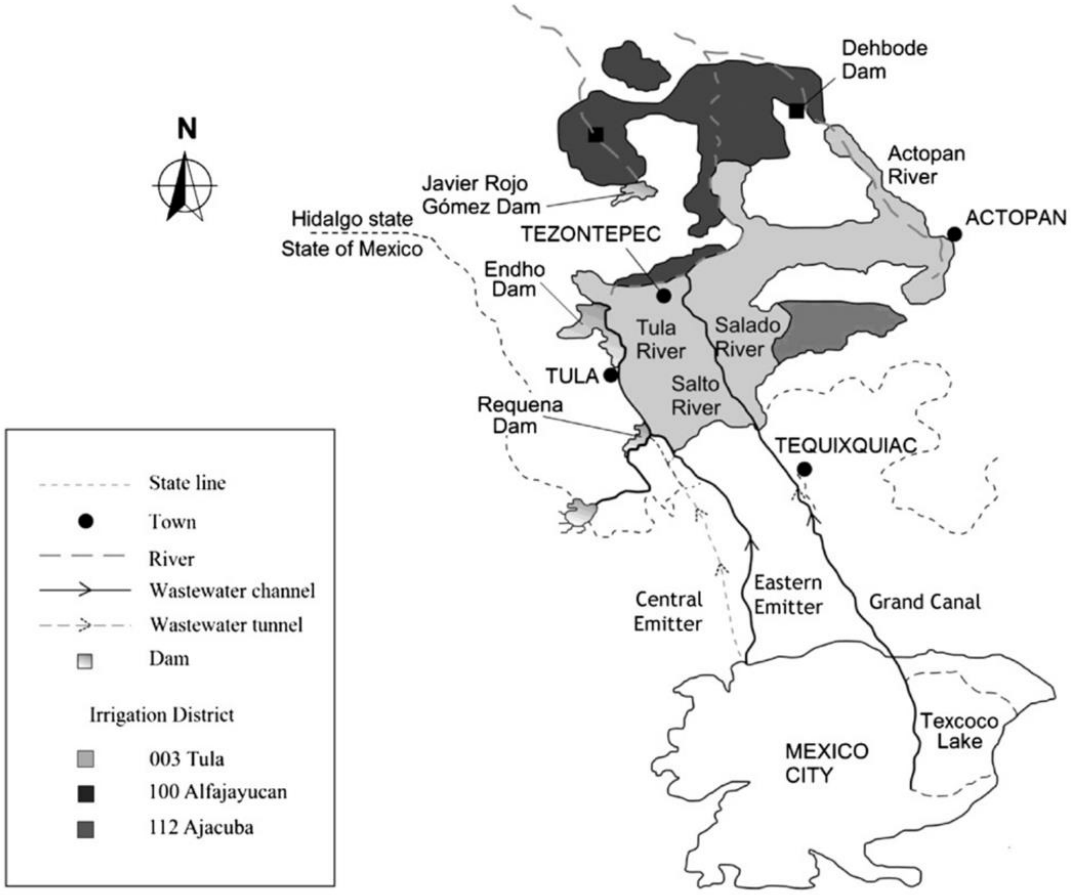


Figure 2.5-4 Tula Valley map from (Chavez et al., 2011)

Crops grown are mostly forage crops, mainly alfalfa and maize, cultivation using untreated wastewater of root vegetables or those eaten raw is prohibited, but has sometimes taken place (Downs, et al., 1999). Flood irrigation is typically used for forage crops consisting of covering the cultivation land with approximately 15 cm of water every 4-6 weeks during each growing cycle. There are two growing cycles per year and so this equates to irrigation rates of 2 $myear^{-1}$ (Chávez, et al., 2011).

The high flowrate the area receives as well as the geological conditions have resulted in an artificial shallow aquifer with recharge rates of around 800 Mm^3year^{-1}. This aquifer is used to supply the local population through 112 wells and 22 springs. One of the

most prominent springs is the *Cerro Colorado* spring which, after chlorine disinfection, supplies water to over 500,000 habitants. Water from this spring is also used to grow crops which are eaten raw (Chávez-Mejía, et al., 2019). The local population has used this water supply for over 40 years and no adverse effects have been noted (Chávez, et al., 2011). Figure 2.5.5 illustrates some of the key areas and processes taking place in the Tula Valley. Further discussion of the bioactive and other pollutants previously found in spring water as well as raw wastewater from this area is given in Chapter 7.





a) Raw wastewater from Mexico City has been used for >100 years for agricultural irrigation in the Tula Valley in Mexico. Water travels for at least 20 hours before it is used for irrigation. used to irrigate some areas. (Left and middle)



b) In the past ten years treated wastewater form Atotonilco WWTP is also used to irrigate some areas in the Tula Valley.



c) The excess treated, and untreated wastewater is stored in two dams, depending on flow. Pictured above is the Endho dam. Natural processes such as sedimentation improve the water quality.



d) The irrigation water has resulted in the recharge of an artificial aquifer. Springs and wells are used to abstract this water and used by local people. Pictured above is the Cerro Colorado Spring. Pollutants are removed by processes such as adsorption and biodegradation.

Figure 2.5-5 a,b,c and d. Illustrates some of the process wastewater goes through in the Tula Valley

2.6 Analytical chemistry and numerical tools to address the knowledge gaps identified

So far, the issue of BACs entering the environment as well as their fate and prevalence in the agricultural environment have been described and gaps in the current knowledge pointed out. In order to address these gaps, it is necessary to have adequate analytical methods to measure these pollutants in the relevant environmental matrices. The matrix, in analytical terms, refers to the material in which the analytes of interest are found (United States Environmental Protection Agency, 1999). For this thesis the relevant matrices are water and soil.

2.6.1 Sampling

The main aim of a sampling strategy is to obtain representative samples which take into account the variation presented by the matrix and to preserve the integrity of the sample until analysis (Wardencki, et al., 2002). When taking samples from a wastewater treatment plant some of the main considerations to achieve this aim are the hydraulic retention time (HRT) of the plant or unit operator being sampled and the daily and seasonal variations (Petrie, et al., 2015). Corresponding influent and effluent grab samples can be obtained to account for the HRT of the plant or unit operator in order to be able to calculate real removal rates, however this can be impractical when HRT are above 6 h, which is common for systems such as activated sludge (Petrie, et al., 2015). In order to account to for daily and seasonal flow variations it is necessary to collect samples at different times of the day and week and at different times of the year (Petrie, et al., 2015).

There are three main types of sampling procedures for bioactive emerging pollutants in the environment, grab/spot samples, composite/automated samplers and passive samples. Grab samples consist of collecting discrete volumes of water at a certain time point. The advantages of this technique are that filtering can be done on each sample when collected and pollutants which may not be stable for long periods are analysed sooner (UKWIR (UK Water Industry Reserach), n.d.). Furthermore, there is no risk of vandalism or theft of the sampling equipment. Their main disadvantage is that a large number of samples need to be collected in order to account for daily and seasonal variation as well as HRT (UKWIR (UK Water Industry Reserach), n.d.).

Composite samples can account for flow variations and reduce the labour intensity, while still collecting samples which account for daily flow variation (Petrie, et al., 2015). However, their main disadvantages are that they require more maintenance, analytes which are prone to degradation may degrade and they are more prone to theft and vandalism (Petrie, et al., 2015). Passive samplers adsorb or absorb pollutants; thus, they present the main advantages of being able to account for pollutant load variation over time and potentially stabilizing some analytes out of the aqueous phase so that they do not degrade (Wardencki, et al., 2002). However, it has not been fully proven that they are able to adsorb all the range of BACs, particularly very polar ones (Petrie, et al., 2015). Furthermore, they are also prone to theft and vandalism. Given the advantages and disadvantages of all the sampling techniques it is necessary to consider the sampling location and resource availability in order to choose the technique which best meets the aims of accounting for the matrix variability, the HRT of WWTP and preserving the integrity of the sample until analysis.

In terms of soil sampling similar principles apply. It is not possible to measure everything of interest and so a subset (or sample) must be chosen for analysis. Ideally the subset should be chosen based on previous knowledge (judgment sapling) or on a design which selects random locations which allows for an estimate of the accuracy and variability of the area sampled. Judgment sample campaigns may be based on

previous geomorphic studies of an area and aligned with the objectives of the sampling campaign (Patterson, et al., 2007).

2.6.2 Sample preparation: BAC extraction from aqueous and soil samples

According to IUPAC, a matrix is defined as the components of the sample other than the analyte (Poole, et al., 2016). Environmental matrices present a wide temporal and spatial variability in terms of their physicochemical properties(Stüber, et al., 2004). It is important to quantify this variation as it can influence the detection and quantification of these pollutants as well as their behaviour in the environment (M. Conde-Cid, et al., 2020). Characterizing the environmental matrices studied allows for results conducted in different conditions to be more comparable. The scope of this thesis includes aqueous (wastewaters and surface waters) and soils.

Standard parameters to characterize water quality include temperature, pH, Total Suspended Solids (TSS) and Total Dissolved Solids (TDS), Chemical Oxygen Demand (COD) (Metcalf & Eddy, et al., 2007). Soil characterization is mainly based on their texture, pH and organic matter content. However, there are other important parameters which are not so commonly reported, as described in Section 2.3.2

For aqueous samples, the procedure is usually filtration followed by solid phase extraction (SPE). The filtration step is usually carried out on the sampling day to reduce degradation of BACs due to biological activity. In literature different pore sizes are used ranging from 0.45 to 1.20 µm (Balakrishnan, et al., 2006; Gibson, et al., 2007; Robert Gurke, et al., 2015b; Petrie, et al., 2016). The pore size refers to the maximum size of particles that will be removed from the water by the filter paper. The aim is to remove interferences from the matrix which may block the SPE cartridge in the following step, or which may intervene with LC-MS/MS analysis later on. Therefore, awareness of the particle sizes of any the following steps are necessary when choosing the filtration pore size. Despite the need to filter aqueous samples for analytical purposes, at least 20% of some BACs may go unquantified as they bind to suspended solids and these are rarely analysed (Petrie et. al., 2015). The extent at which binding to suspended solids occurs is highly compound dependent and is usually highest for basic compounds with a pK_a>7 (Ferreira da Silva et al, 2011). Examples of BACs which have been found in a high percentage in suspended solids of aqueous samples are chloramphenicol, cimetidine, ciprofloxacin, dosulepin, glibenclamide, famotidine, fluoxetine, norfluoxetine, ofloxacin, salbutamol, sulfamethazine, timolol and triclosan, (Baker and Kasprzyk-Hordern, 2011; Ferreira da Silva et al, 2011 and Petrie et. al., 2014). Partitioning of BACs to suspended solids in WWTPs and the environment still remains understudied (Wilkinson, et al., 2017). This is outside the scope of this thesis, but an awareness of this issue is important.

Conventional solid phase extraction (SPE) remains the most common extraction method (Dimpe, et al., 2016). SPE is a chromatographic technique in which the liquid sample is passed through a solid packaging material (usually in a cartridge) to chemically separate the different components of a sample. Compounds that are more chemically attracted to the packaging material will be retained by the cartridge, while those that are not will pass through dissolved in the liquid sample (Arsenault, 2012). The overall procedure for SPE is described in Table 2.6.1 and can be visualised in Figure 2.6.1:

Process	Description	Reason
Conditioning	Passing a solvent followed by an aqueous phase through the cartridge.	Wetting the cartridge is necessary as this allows the aqueous matrix to enter the pores of the solid packaging material allowing the analytes to be retained by the cartridge.
Sample loading	Sample is passed through the cartridge at a slow flow rate to allow enough contact time for the interactions to occur (5-10 $mLmin^{-1}$).	Analytes are retained in the cartridge while the bulk of the matrix passes through.
Washing:	A solution is passed through which gets rid of interfering compounds.	Interfering compounds may be a detriment to analysis.
Drying	Left to dry under vacuum.	To remove any aqueous matrix residue
Elution	A solvent in which analytes are highly soluble is passed through the cartridge to move the analytes from the solid phase (cartridge) to the liquid phase (solvent).	Analytes are in the liquid phase.
Dry down and reconstitution	The liquid solvent in which the analytes are dissolved is evaporated, normally using a gentle stream of nitrogen gas. The analytes are then reconstituted in a small volume (~1mL) of a solution suitable for LC-MS analysis.	Analytes are concentrated and found in a solution amenable for analysis.

Table 2.6-1 Solid phase extraction steps and reasons behind them, content from (Arsenault, 2012; Dimpe, et al., 2016)

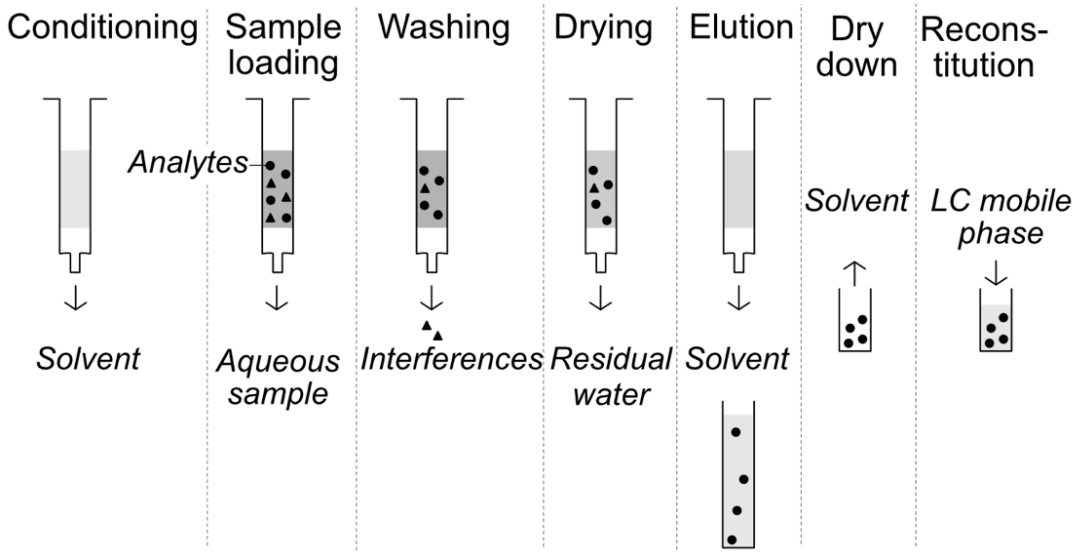


Figure 2.6-1 Summary of Solid Phase Extraction steps

Once these steps have been optimised and the most adequate cartridge has been chosen, the SPE procedure can reduce background matrix interferences and enrich

trace concentrations in environmental samples. Concentration of analytes takes place as the analyte present in a large sample volume (usually 100-500 mL) is reduced to around 1 mL. Finally, it is possible to fractionate the analytes in the sample, in case they need to be analysed separately (Arsenault, 2012). Overall, the main advantages are: simplicity, flexibility, high selectivity, automation, rapidity, higher enrichment factors, and the absence of emulsion and use of different sorbents (Dimpe, et al., 2016). However, the main disadvantages are the relatively high use of solvent, the disposable cartridges and the need of a specialised manifold (Dimpe, et al., 2016).

In order to address these disadvantages several solid phase microextraction (SPME) techniques have been developed. These alternatives move towards reduction of sample preparation times, solvent use and sample volumes required (Dimpe, et al., 2016; Primel, et al., 2017). Some examples are discussed here, however it is not an exhaustive discussion as conventional SPE was used for this work given equipment availability was for conventional SPE.

Fabric phase sportive extraction (FPSE) is an example of a SPME which comprises of small squares of cellulosic (or other) fabric that are coated with an ultrathin sol−gel, which are immersed directly in aqueous environmental samples to sorb and extract analytes. The material can be reduced with minimal cleaning and without cross-contamination. Other improvements on conventional SPE include on-line SPE, which consists of eluents from the SPE entering the chromatographic column directly (Yao et. al., 2016). Another important example is dispersive solid-liquid liquid microextraction (DLLME), which comprises of a mixture of the extractor solvent and the disperser solvent being injected into the liquid sample and forming a cloudy solution which generates a very high contact are between the sample and extractor solvent. Apart from solvent volume and sample preparation time, one of the main advantages of this technique is the easier linkage to analytical methods. However, the main disadvantages are the need for the extractor solvent to be able to form a cloudy solution, which reduces the choice of solvent (Primel, et al., 2017). These methods have been successfully applied to a wide range of matrices such as drinking water, surface water, wastewater, urine, plasma and milk (Dimpe, et al., 2016; Richardson, et al., 2018). Analytes of a wide variety of classes have been recovered with different variations of SPME including: pharmaceuticals and personal care products, hormones, benzotriazoles, for endocrine disruptor compounds, acidic pharmaceuticals, antidepressant drugs and antibiotics (Dimpe, et al., 2016; Richardson, et al., 2018).

In terms of conventional SPE, there are four main types of sorbent: reversed phase, normal phase, ion exchange and adsorption. The most adequate for aqueous samples is reversed phase due to it being non-polar and resistant to pH extremes (Waters Oasis, 2008). Analytes are retained by non-polar-non-polar interactions and van der Waals or dispersion forces. Ion exchange columns also provide benefits for environmental samples such as method recovery improvement though pH adjustments. The most commonly used SPE cartridges are Waters Oasis HLB (Dimpe, et al., 2016; Richardson, et al., 2018), Waters Oasis MCX (Al-Odaini, et al., 2010; Balakrishnan, et al., 2006) and Phenomenex Strata X (Comtois-Marotte, et al., 2017; Hilton, et al., 2003; Santos, et al., 2013; Wilkinson, et al., 2016). Oasis HLB is popular as it retains analytes through hydrophilic and hydrophobic interactions, encompassing a wider range of analytes. Oasis MCX has ion exchange as well as reverse phase, which gives it high stability though the pH range and thus recoveries can be improved by altering the cartridge or sample pH, however this cartridge is particularly selective to bases (Waters Oasis, 2008). Phenomenex Strata-X is one of the main competitors to HLB. It is designed to retain the broad spectrum of neutral, acidic and basic and relies on three action mechanisms: π-π bonding, hydrogen bonding (dipole-dipole interactions), and hydrophobic interaction (Phenomenex, 2020). Given the

manufactures descriptions and the positive results obtained in literature to analyse for BACs in aqueous matrices all three cartridges will be compared in this work. Some studies have used multiple SPE columns in order to extract more analytes (Richardson, et al., 2018).

To extract BACs from soils it is necessary to do a solid to liquid extraction, this is often followed by an SPE clean-up and concentration stage. In broad terms these extraction methods can be classified as Microwave-assisted extraction (MAE), Ultrasound assisted extraction (UAE), Pressurized liquid extraction (PLE), Quick Easy Cheap Rugged and Safe (QuEChERS), liquid-liquid extraction (LLE) and Solid-liquid extraction (SLE). MAE uses microwave energy to contact the sample with the solvent, it can yield good recoveries in relatively short sample preparation times. However the main disadvantages include only a limited amount of sample (0.5 g) can be processed at a time, does not permit the addition of solvents or reagents during operation, the absence of selectivity and long cooling times after pre-treatment (Dimpe, et al., 2016). Similarly, USAE uses ultrasound energy to enhance mass transfer between the sample and the solvent. PLE is also known as accelerated solvent extraction (ASE) and pressure is applied by using solvents at temperatures above their boiling point. These conditions reduce the viscosity and surface tension of the solvent and allow it to enter the matrix more profoundly as well as increasing analyte solubility. PLE reduces the amount of solvent used as well as sample preparation times (Dimpe, et al., 2016). QuEChERS comprises two steps, the first based on the dispersion of salts to extract (salting-out effect) between an aqueous and an organic solvent layer to isolate a wide range of analytes from very complex matrices and the second the clean-up of the extract using dispersive solid phase extraction (Perestrelo, et al., 2019). LLE and SLE contact a liquid or solid sample, respectively, with a solvent in order to extract the analytes. It is an effective technique which does not require specialised equipment, however the main drawback is the large amount of solvent needed (Dimpe, et al., 2016).

2.6.3 Detection and quantification: chromatography and mass spectrometry

Once BACs have been extracted from the environmental matrix under study, they can be analysed for identification and quantification. The most commonly used methods of analysis for BACs in environmental samples are either liquid chromatography-mass spectrometry (LC-MS) or gas chromatography- mass spectrometry (GC-MS) (Petrie, et al., 2015; Richardson, et al., 2018). Both of these techniques work by first separating the analytes using chromatography and then identifying and quantifying them using mass spectrometry (Miller, 2005).

Chromatography is composed of a stationary and a mobile phase. The stationary phase is a solid medium in the form of a column in which the separation is carried out. The mobile phase is an inert fluid that carries the mixture through the system. The mobile and stationary phases are chosen so that the analytes differentially partition between the two phases resulting in different migration rates through the system (Miller, 2005). This means that the various components of the mixture emerge from the column at different times (termed retention times) (Haddad, et al., 2013). In the case of LC more than one mobile phase is commonly used. This allows the separation of analytes with a wide range of physicochemical properties (Alygizakis, et al., 2016; Gorga, et al., 2013; Loos, et al., 2016; Lopardo, et al., 2018; Petrie, et al., 2017; Petrovic, et al., 2014).

LC-MS techniques are more widely used than GC-MS to analyse environmental samples mainly due to the reduced sample preparation required for this technique (Svahn, et al., 2016). GC techniques are more suitable to analyse volatile samples, if

the sample is aqueous there is an extra preparation step called derivatization. This is a process in which the sample has to be vaporized in order for it to by viable for GC analysis (McNair, et al., 2009).

The basic principle of mass spectrometry (MS) is to ionize molecules into what are termed as precursor ions. These charged molecules can be manipulated by external electric and magnetic fields. Precursor ions are then separated according to their mass to charge ratio (*m/z*) and can be quantified using the detected intensity of these ions (Beynon, et al., n.d.). High resolution MS gives the opportunity to obtain the exact mass of a compound to several decimal places of an amu (Atomic Mass Units). This is in contrast to low resolution MS which gives masses to the nearest amu. The following examples illustrates this: 2-octanone, $C_8H_{16}O$, would be shown to have a molecular weight of 128.12018 in a high-resolution MS and naphthalene, $C_{10}H_8$, of 128.06264. In a low resolution MS both would be shown as 128 amu, making them undistinguishable from one another (Schaller, 2020). In low resolution MS, or in order to obtain further confirmation another step can be carried out to distinguish analytes of similar masses. The precursor ions can be fragmented using a technique called tandem mass spectrometry (MS/MS). The procedure to which product ions are subjected to achieve fragmentation is usually Collision Induced Dissociation (CID). This involves the collision of precursor ions with an inert gas, giving them the necessary energy to fragment (RSC Analytical Methods Committee, 2013). Each compound has a specific fragmentation pattern, called a mass spectrum, which will differ even for molecules which may share the same mass to charge (*m/z*) ratio. This allows for a very robust compound identification using three characteristics: their chromatographic retention time, their mass to charge ratio and their mass spectrum (RSC Analytical Methods Committee, 2013). In summary, chromatography ensures that analytes enter the ionization source at a differentiated time, decreasing matrix effects and increasing sensitivity (Loos, et al., 2016). While MS gives a unique fingerprint for each analyte (RSC Analytical Methods Committee, 2013). This can be visualised in Figure 2.6.2.

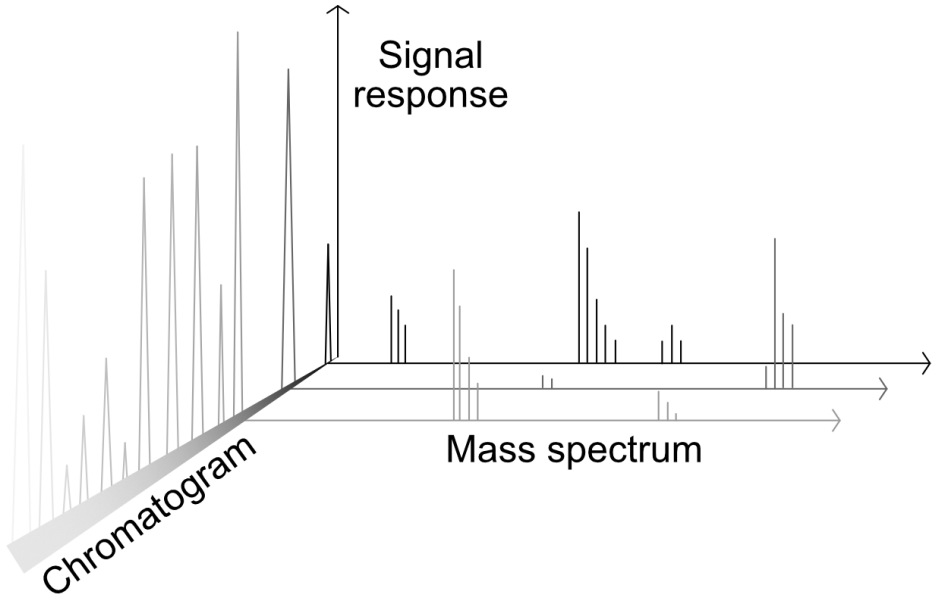


Figure 2.6-2 The power of LC-MS to separate and identify each component of a complex mixture adapted from (RSC Analytical Methods Committee, 2013).

2.6.3.1 Chromatography: basic principles

The efficiency of chromatographic separation can be determined using the van Deemter equation which quantifies the different types of diffusion which determine the

band width of a chromatographic peak H (Han, et al., 2019). H is also known as the theoretical plate height (Nagy, et al., 2008). The aim is for H to be as narrow as possible, in order to maximise sensitivity and resolution.

$$H = A + \frac{B}{\bar{u}} + C\bar{u} \qquad \textit{Eq. 7}$$

The velocity of the mobile phase is denoted by $\bar{u}$. A refers to the multi-flow path or Eddy diffusion. It is a constant which is defined by the packing factor, λ, and the column packing particle diameter d_p (Han, et al., 2019):

$$A = 2\lambda d_p \qquad \textit{Eq. 8}$$

A can be understood as the flowstream before it is changed by the column packing (Moody, 1982). The column packing is determined by the particle size and porosity of the material. The smaller the average particle size the smaller the H and the better the resolution is. Small particle sizes are limited by technological advances, currently it is possible to have columns with a particle size down to 1.5 µm diameter. High porosity means higher surface area and smaller pore diameter, translating into better selectivity. However, if the pore size is too small target molecules cannot get through the pores. Therefore pore size is a compromise; usually the 10–100 nm range is considered best (Nagy, et al., 2008).

B is the longitudinal diffusion constant and it accounts for the effect of a solute undergoing diffusional broadening within the mobile phase (Han, et al., 2019), it is therefore proportional to the mobile phase velocity (Moody, 1982):

$$B = 2\gamma D_m \qquad \textit{Eq. 9}$$

Where γ is a constant for tortuosity, which obstructs the longitudinal spread of the solute molecules (Han, et al., 2019). D_m is the coefficient of the solute in the mobile phase (Moody, 1982).

The C term refers to the mass transfer and it is dependent on K, which is a constant relating to the column geometric factors and capacity. d_f is the liquid film thickness and D_l is the diffusion coefficient of the solution in the liquid phase (Moody, 1982):

$$C = \frac{Kd_f^2}{D_l} \qquad \textit{Eq. 10}$$

The Van Deemter equation is based on theoretical plates (similar to those in distillation), where each theoretical plate is one stage of equilibrium. Therefore H can also be defined by the length of the chromatographic column, L; and N , and the number of theoretical plates in the column can be obtained as follows (Moody, 1982):

$$H = \frac{L}{N} \qquad \textit{Eq. 11}$$

N for a particular solute is defined by the retention time of the solute, t_r, and t_b the width of the chromatographic peak base (Moody, 1982):

$$N = 16\left(\frac{t_r}{t_b}\right)^2 \qquad \textit{Eq. 12}$$

For a given temperature, column and species A, B, and C as well as the optimum mobile phase velocity $\bar{u}$, which can also be defined as in Eq. 13 (Moody, 1982). When this equation is plotted the curve has a minimum which corresponds to the optimal flow rate (Nagy, et al., 2008)

$$\bar{u}_{opt} = \left(\frac{B}{C}\right)^{\frac{1}{2}} \qquad \textit{Eq. 13}$$

The van Deemter equation is useful in understanding the factors affecting chromatographic separation when developing a method. However, it is based on chemical engineering approximations and so recent advances such as the numerical solution of the Navier–Stokes equation and computational advances to simulate the advection–diffusion processes have demonstrated some of the inaccuracies of this equation (Gritti, et al., 2013). Particularly, the van Deemter model overlooks the impact of the retention factor, of the adsorption–desorption kinetics, and of the intra-particle diffusivity on the short-range eddy dispersion HETP term (Gritti, et al., 2013).

Based on the van Deemter equation it is possible to see that higher temperatures and pressure translate to better peak resolution (Han, et al., 2019). This is the reason behind chromatography systems which can be run at higher pressures. Therefore currently, the two main types of chromatography used in environmental sample determination are High Performance Liquid Chromatography (HPLC) and Ultra-High-Performance Liquid Chromatography (UPLC). HPLC works in the 500-6000 psi (50-400 bar) pressure range and UPLC up to 15,000 psi (1034 bar). UPLC has the advantages of faster sample processing and solvent volume decrease (Nováková, et al., 2006).

The stationary phase can be made of different materials depending on the application. Reversed-phased liquid chromatography (RPLC) columns are the most commonly used for environmental sample analysis (Alygizakis, et al., 2016; Blue, et al., 2018; Cirrincione, et al., 2018; Danezis, et al., 2016; Martínez-Piernas, et al., 2018a; Petrie, et al., 2016; Qu, et al., 2016). RPLC uses hydrophobic (non-polar) stationary phase and hydrophilic (polar) mobile phase (Boone, et al., 2016). This means polar analytes are eluted first and non-polar analytes last, as they have a higher affinity for the column material (Boone, et al., 2016). Strongly polar or ionizable analytes can be analysed using RPLC by adjusting the pH to ensure they are in the neutral form (Nagy, et al., 2008).

The stationary phase is commonly composed of porous silica particles linked to alkyl chains (C4, C5, C8, C18) or other inert nonpolar substances such as divinylbenzene (DVB). The shorter the alkyl chain the less retentive they are. Therefore C4-C8 are usually used for protein separation and longer chains (C18) for smaller or hydrophobic analytes (Boone, et al., 2016). A typical mobile phase gradient for RPLC consist of two solvents including a polar solvent and an organic solvent. Two additional steps are also important: removal of any residual analytes from the column in the cleaning step achieved by high organic solvent and column re-equilibration with polar solvent (Boone, et al., 2016).

2.6.3.2 Mass spectrometry: ionization

Mass spectrometry instruments have three main components:

- **Ion source:** Vaporises/ionises the target molecules.
- **Mass analyser:** Separates the gaseous ions by (*m/z*).

- **Detector:** Detects the mass separated ions and measures their relative abundance (RSC Analytical Methods Committee, 2013)

There are several ionization mechanisms which can occur in various ion sources. Protonation and deprotonation are the ionization mechanisms in which there is gain and loss of a proton, respectively. Deprotonation is particularly suitable for acids as they readily loose a hydrogen atom. Another pair of mechanisms are electron ejection and electron absorption/capture, these are the loss and gain of an electron respectively. Electron ejection is suited for relatively nonpolar analytes with low molecular weight. Electron capture is mainly observed for molecules with high electron affinity like halogenated compounds. Finally, cationization is the non-covalent addition of a cation which is not a proton, for example an ammonium, these are called adducts. It is particularly suited to analytes which are not stable with protonation, for example carbohydrates. Another option is that a species may be already charged in solution, in which case it is transferred to the gas phase through desorption or ejection (Siuzdak, 2005).

These different ionization mechanisms can occur in different types of ionization sources and some sources are better suited to perform certain kinds of ionization processes. Electron impact (EI) and fast atom bombardment (FAB) are older techniques which are used with less frequency as they have been surpassed by newer techniques. These newer techniques classify as a 'soft' ionization technique, meaning that little in-source fragmentation occurs. For BACs analysis, this is ideal for the selection of precursor ions and gives maximum sensitivity. (Emroy University, 2017; Pitt, 2009). These newer sources work under continuous introduction of solution, so they are suitable for using as an interface with HPLC (Banerjee, et al., 2012; Emroy University, 2017; Siuzdak, 2005). For these reasons, the main types of sources relevant to BACs analysis are electrospray ionization (ESI), atmospheric pressure chemical ionization (APCI) and matrix-assisted laser-desorption ionization (MALDI) (Figure 2.6.3).

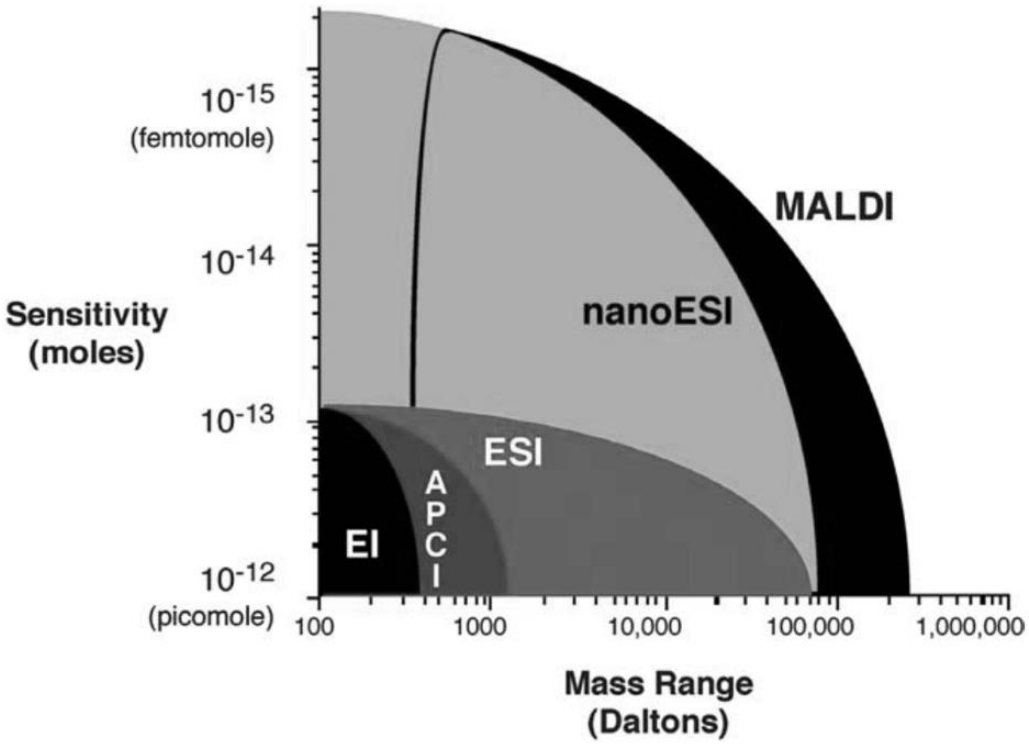


Figure 2.6-3 Ionization sources mass range and sensitivity (Siuzdak, 2005)

ESI can perform all the ionization processes described previously, except for electron capture and ejection. Electron capture and ejection are only observed using electron ionization (EI), which is an older source only suitable for very small molecules (Siuzdak, 2005). ESI is capable of forming single charge as well as multiple charged ions, this can be useful for very larger molecules (up to *m/z* 4000). However, BACs are not usually so large and ESI is not ideal for smaller, neutral and relatively non-polar molecules, such as free steroids. APCI is better for the ionization of such molecules (Pitt, 2009; Ronaldo, 2017). Aside from this disadvantage ESI is still the preferred source for biological molecule analysis as most BAC molecules fall within the polarity and size which ESI can ionise. APCI has been found to have higher matrix effects than ESI in certain applications (De O. Silva, et al., 2019).

ESI operates by creating charged droplets in the presence of an electric field. The electric potential is maintained form 700 – 5000 V, depending on the application. By applying an electric field to the capillary nozzle, a Taylor cone is formed. The capillary voltage can be optimised depending on the analytes studied. The pendant drop emerging form the needle is statically charged form the voltage applied and surface tension should keep these forces balanced, making the droplet round. However, the electric field causes the charges to migrate to the surface of the droplet and making a protrusion appear, which is the Taylor cone (Wang, et al., 2011). As the forces become unbalanced droplets are formed from the Taylor cone. The solvent is evaporated from the charged droplets using a dry gas, heat, or both. Two gases are usually present, the sheath gas and the auxiliary gas as can be seen in Figure 2.6.4 below. The temperature of the gas or heat can be optimised depending on the analytes, as well as the gas flowrate. An additional gas known as nebulizing gas is also used and its pressure can be optimised for optimum ionization. As the solvent evaporates the charges come closer to each other, increasing the charge density. The mutual Coulombic repulsion between like charges exceeds surface tension ejecting the ions form the droplet. Otherwise, the droplet may explode and release the ions. The ions are then directed to the vacuum of the mass analyser through electrostatic lenses (Banerjee, et al., 2012; Siuzdak, 2005). This process is illustrated in Figure 2.6-4, for the formation of positively charged analytes (i.e. under a negative voltage charge). In order to form negatively charged analytes, the same process occurs, but with reverse charges, under a positive voltage charge. The ion source voltage can be operated to switch between negative and positive, forming both analyte polarities (Banerjee, et al., 2012; Siuzdak, 2005).

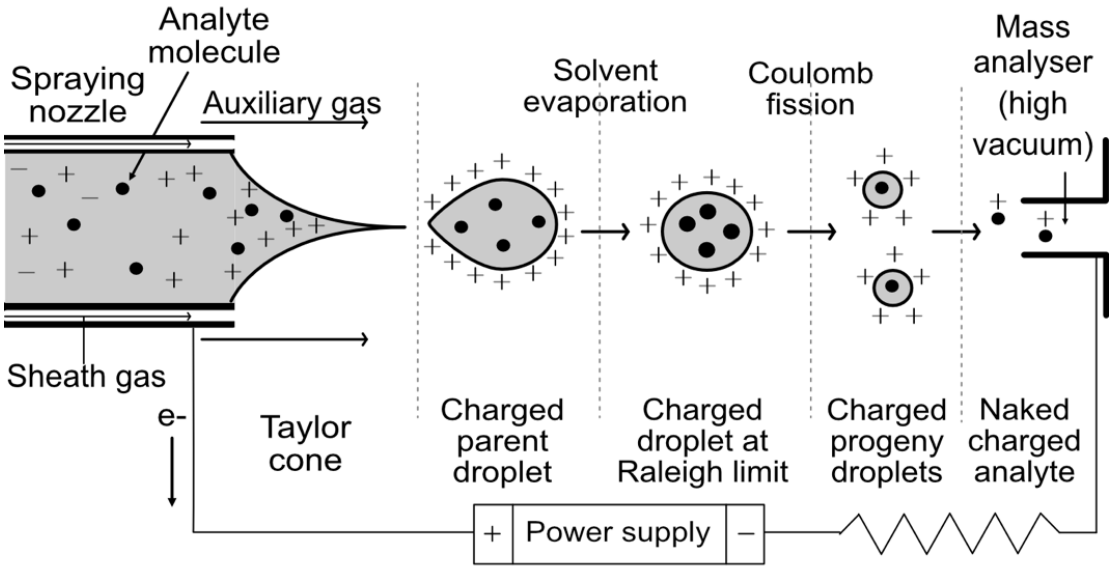


Figure 2.6-4 Electrospray ionisation source schematic adapted from (Banerjee, et al., 2012)

An important consideration for ESI is the volatility of the solvents and modifiers used. Lower volatility decreases sensitivity and so pure water and modifiers such as salts like Na^+ , K^+ and phosphates which lower the volatility are undesirable (Siuzdak, 2005).

Nano ESI is a simple adaptation of ESI, where the spray needle has a smaller orifice and is positioned closer to the mass vacuum. These changes give the technology better sensitivity and lower volumes of sample are required (Siuzdak, 2005). Different manufacturers may have certain adaptations, such as Agilent's Jet Stream technology (AJS) which features superheated nitrogen sheath gas to confine the nebulizer spray and improve desolvation of ions (Agilet, n.d.). When developing a method there are several parameters which can be optimised in order to obtain better ionization of the analytes (Agilet, n.d.).

In APCI droplets are also formed, like in ESI, but they are not charged. The droplets are excited/ionized from a corona discharge, which takes place near the capillary. The discharge first ionises the gas and solvent molecules present in the source, these ions then react with the analyte and ionise it through charge transfer (Pitt, 2009). This process can be seen in Figure 2.6.5 below. Because the solvent ions are present at atmospheric pressure conditions, chemical ionization of analytes occurs easily as molecules frequently collide with the reagent ions. Protonation occurs in the positive mode and, deprotonation or electron loss in the negative mode. In-source fragmentation happens even more rarely in APCI, compared to ESI, this is due to the presence of the solvent clusters which moderate the collisions between the analytes and the reagent ions and of the high gas pressure (Siuzdak, 2005).

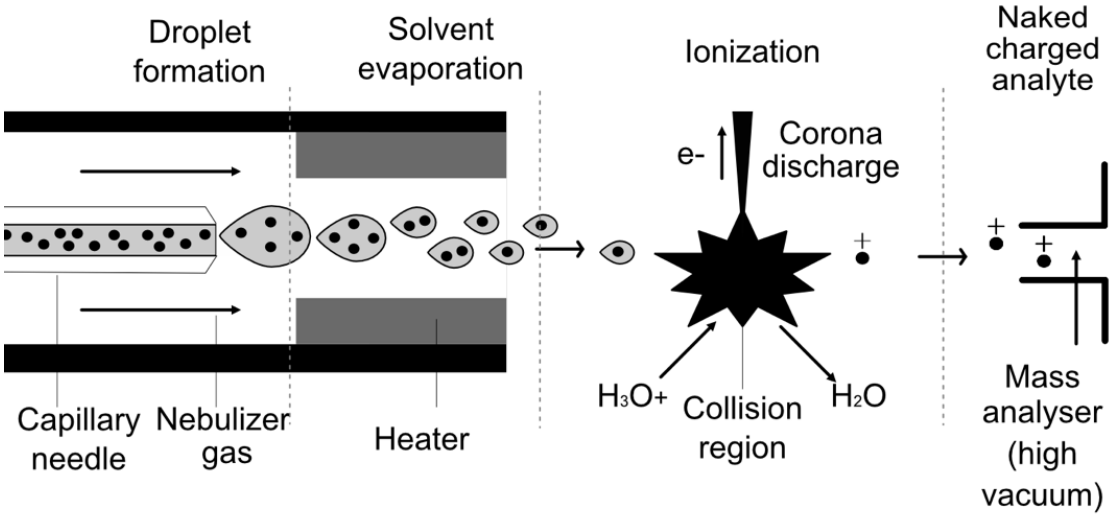


Figure 2.6-5 Schematic of atmospheric pressure chemical ionization source adapted from (Paul J. Gates, 2014; Ronaldo, 2017)

Atmospheric pressure photoionization (APPI) is a very similar technique to APCI, but ionization is done bombarding analytes with photons. These photons normally come from a krypton lamp and have a specific energy targeted to ionise the analytes (Emroy University, 2017; Robb, et al., 2000). APPI has shown to give better results than ESI for low polarity analytes such as sulfur containing hydrocarbons (Richardson, et al., 2018).

Matrix-assisted laser-desorption ionization is a technique mainly used for peptides, proteins, and other biomolecules such as oligonucleotides, carbohydrates, natural products, and lipids (Siuzdak, 2005). This technique works by embedding the sample in a laser absorbing matrix and then bombarding it with a laser to ionise the analytes. MALDI is particularly suited to time-of-flight mass analysers and can also be used in

imaging mass spectrometry (Emroy University, 2017). In environmental fields it has been used to study bacterial communities and their interactions with their environment (Rugova, et al., 2017), for example antimicrobial resistance of bacterial communities in groundwater (Martin, et al., 2018).

2.6.3.3 Mass spectrometry: analysers

Once analytes have been ionised, they enter the mass analyser, which is kept at a high vacuum (around 10^{-6} Torr). All mass analysers make use of the physical properties of ions in the gas phase. When under electric fields, ions can be focussed, steered and bunched. Using magnetic and radio frequency they can be separated. With a combination of electric, magnetic and radio frequency fields they can be trapped. The main types of mass analysers for biological molecules used today are Time-of-Flight (TOF), Quadrupole, Quadrupole Ion Trap (QIT), Fourier transform Ion Cyclotron Resonance (FTIC) (Paul J. Gates, 2014; Pitt, 2009; Ronaldo, 2017) and Orbitrap, which combines characteristics from the previous ones (Thermo Scientific, 2015). All analysers have advantages and disadvantages, and the choice of mass analyser depends on the application. A description of the main types of analysers are compared based on their figures of merit in Table 2.6.2.

Time of flight (TOF) consists in the application of an electric field which gives ions kinetic energy to travel through the field drift zone. Ions with lower *m/z* travel faster, and ions with higher *m/z* travel slower. Figure 2.6.6 shows the equation used to calculate *m/z* based on the energy applied and instrumental configuration parameters. The equation is derived from the potential and kinetic energy of the ion when the energy is applied. The TOF analyser can acquire spectra extremely quickly with high sensitivity and high mass accuracy (Ronaldo, 2017). This allows for molecular formulas to be determined even for small molecules (Paul Gates, 2014; Pitt, 2009).

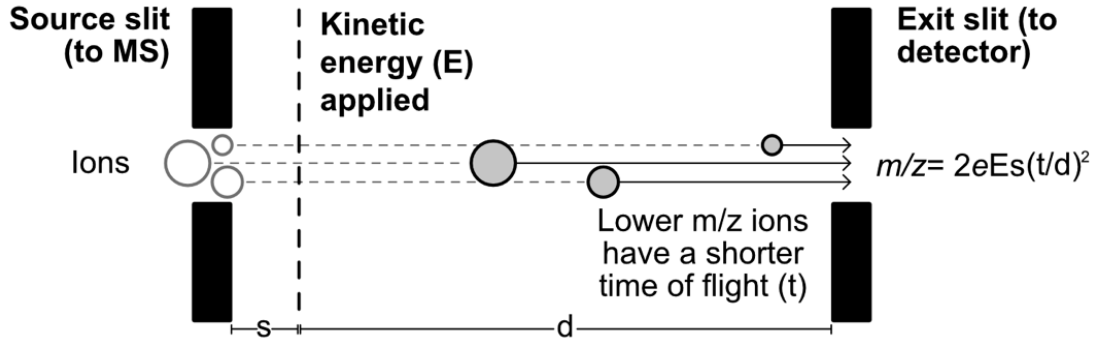


Figure 2.6-6 Time of flight mass spectrometer adapted form (Paul Gates, 2014)

A quadrupole analyser consists of four parallel rods, these rods have a fixed direct current (DC) and an oscillatory radio frequency (RF) applied to them (Figure 2.6.7). Ions are focused to pass through the middle of the four rods. Only ions of a specific *m/z* will be in resonance, and therefore have a stable path, at a particular electric field, allowing them to reach the detector (Ronaldo, 2017), as can be seen in Figure 2.6.7. RF can be varied so ions of different *m/z* can be focused at the detector (Paul Gates, 2014; Pitt, 2009). Quadrupole analysers normally operate at *m/z* <4000 and scan speed up to 1000 m/zs^{-1} or more are common. They usually operate at unit mass resolution meaning, mass accuracy is rarely better than 0.1 *m/z* (Pitt, 2009). Therefore, quadrupoles are very good for selecting very specific *m/z*, but not very good at giving high mass resolution.

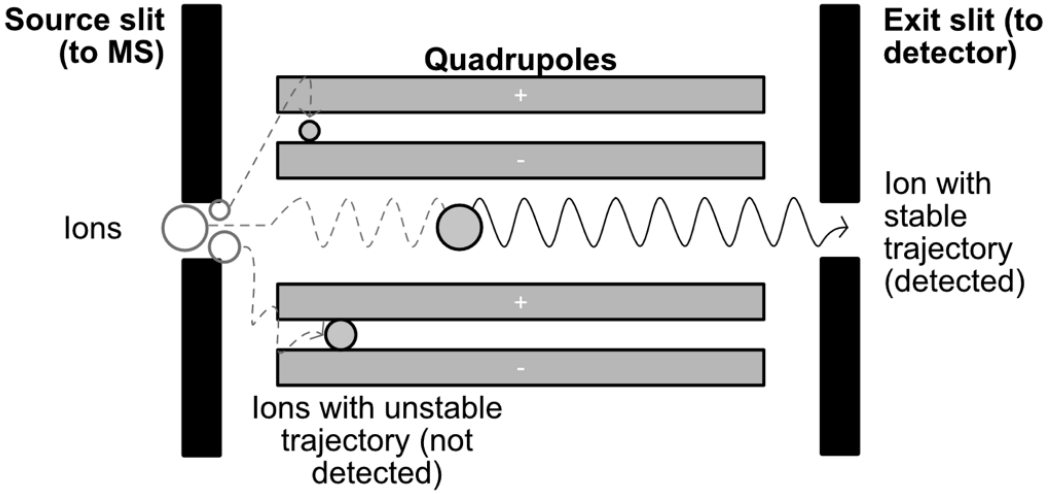


Figure 2.6-7 Quadrupole mass spectrometer adapted from (Paul Gates, 2014)

Ion Trap (Figure 2.6.8) has electrodes wrapped into a circle, with two convex end-cap electrodes and one ring electrode. Different potentials are applied between the ring electrode and the two end caps so that the ions are trapped circling around the middle, can be visualised as a potential well where the potential cannot change (Ronaldo, 2017). Different *m/z* ions have different regions of stability, therefore when the potential is ramped up the ions leave the trap by size (Paul Gates, 2014; Pitt, 2009). An inert gas can be introduced into the trap to induce fragmentation (Pitt, 2009).

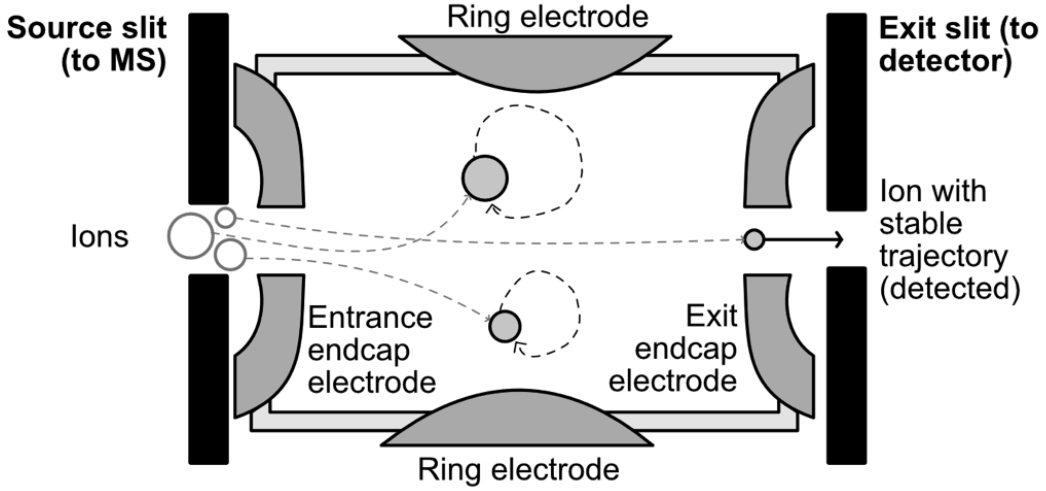


Figure 2.6-8 Ion trap mass spectrometer adapted from (Paul Gates, 2014)

Fourier Transform Ion Cyclotron Resonance (FTIC) is one of the most sensitive methods. The ions are generated at the source and then go through a series of pumping stages with the vacuum increasing progressively. The cell where the analysis takes place has a high vacuum of around 10^{-10} to 10^{-11} and temperatures close to absolute zero, cooled by liquid nitrogen and helium. The cell is inside a uniform static superconducting high field magnet with plates at each end, trapping the ions in. When the ions pass into the magnetic field they are bent into a circular motion. The frequency of rotation of the ions is dependent on their *m/z* ratio. However, their motion cannot be detected because the radius of rotation is too small. Therefore, an RF pulse is applied which excites each individual *m/z* to a higher orbit where they induce an alternating current between the detector plates. The frequency of this current is the same as the cyclotron frequency of the ions and the intensity is proportional to the number of ions. The RF keeps pulsing at different values, exciting different *m/z* ions which get measured correspondingly. Although the RF pulses occur in a series of stepped

frequencies, it can be considered as all frequencies simultaneously. This results in the measurement of all the ions in one go producing a complex frequency vs. time spectrum (the convoluted frequency spectrum or FID) containing all the signals. Deconvolution of this signal is done using Fourier Transform methods resulting in the frequency vs. intensity spectrum which is then converted to the mass vs. intensity spectrum (the mass spectrum). Figure 2.6.9 shows what occurs once the ions are inside the magnetic field cell of the FTIC (Paul Gates, 2014).

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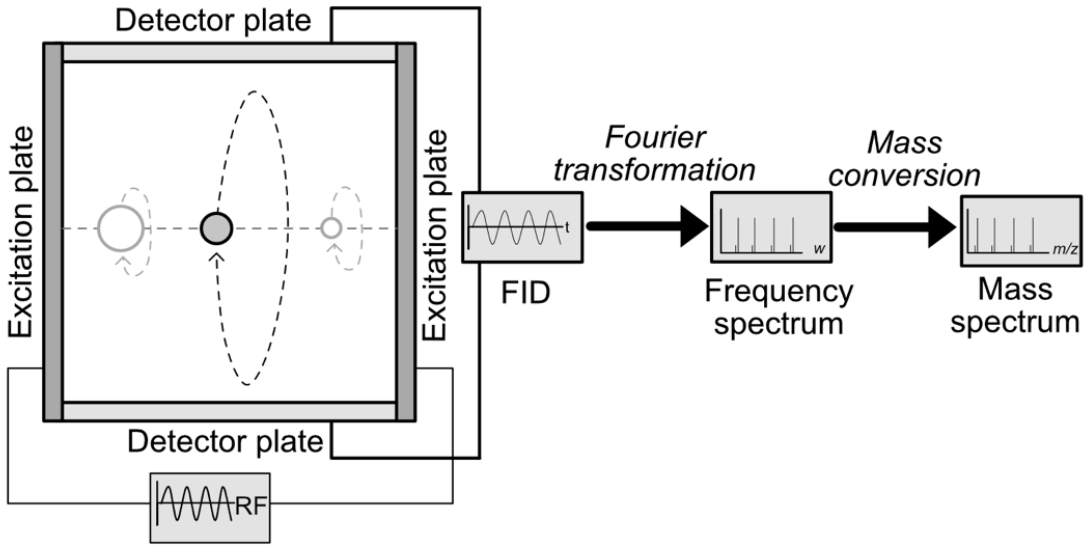


Figure 2.6-9 Fourier transform ion cyclotron mass spectrometer adapted from (Paul Gates, 2014)

Orbitrap (Figure 2.6.10) is a Thermo-Scientific instrument which combines properties of different analysers. It takes ion trapping and shaped electrodes from ion traps, image current detection from FT-ICR and pulsed injection and electrostatic fields from time of flight (Thermo Scientific, 2015). It is one of the newest analysers and it uses the same Fourier Transform principles as the FTIC to interpret frequency signals as mass spectrums. However, it can analyse several *m/z* ions simultaneously. It is made of a large central electrode with two parallel electrodes above and below it. Ions are trapped circling the central electrode, their frequency of rotation is related to their *m/z*. 'Ion packets' of ions with the same *m/z* are formed oscillating with the same frequency. As ion packets oscillate harmonically along the axis, the difference of image currents between electrodes is detected by a differential amplifier. Its output is digitized and subjected to a fast Fourier transform that converts the signal into a mass-to-charge ratio spectrum (Hecht, et al., 2019). Orbitrap is usually coupled to an external pulsing ion source which is a simpler way of ensuring similar *m/z* are grouped together and can be detected increasing sensitivity (Hecht, et al., 2019; Thermo Scientific, 2015).

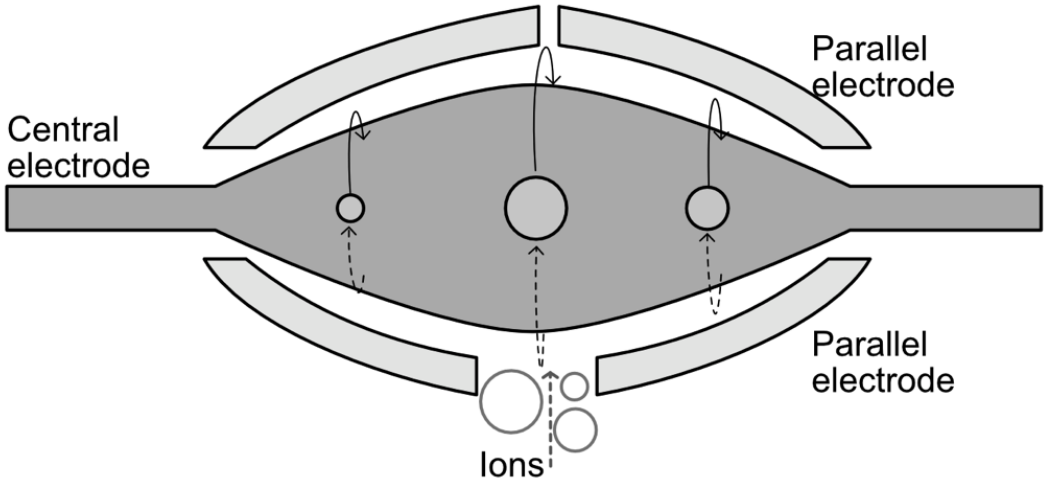


Figure 2.6-10 Orbitrap mass spectrometer adapted form (Chromatography & Mass Spectrometry Solutions, 2016; Qu, et al., 2016)

Two mass analysers can be combined in series with a reaction chamber between them, this is called tandem mass spectrometry. The analysers can be connected in series in time or in space. The sequential analysers are referred to as MS^n, where the *n* refers to their position in the time or space sequence. The reaction chamber is used to fragment the ions to give further indication of their structure for identification. The most widely used method for fragmentation is collisional activation, also known as collision induced dissociation (CID). This technique uses an inert gas such as nitrogen, helium or argon as a collision gas to excite the ions into fragmentation. Different types of analysis can be achieved with this configuration, depending on the objective (Niessen, et al., 2015), the basic types of analysis are outlined below in Figure 2.6-11.

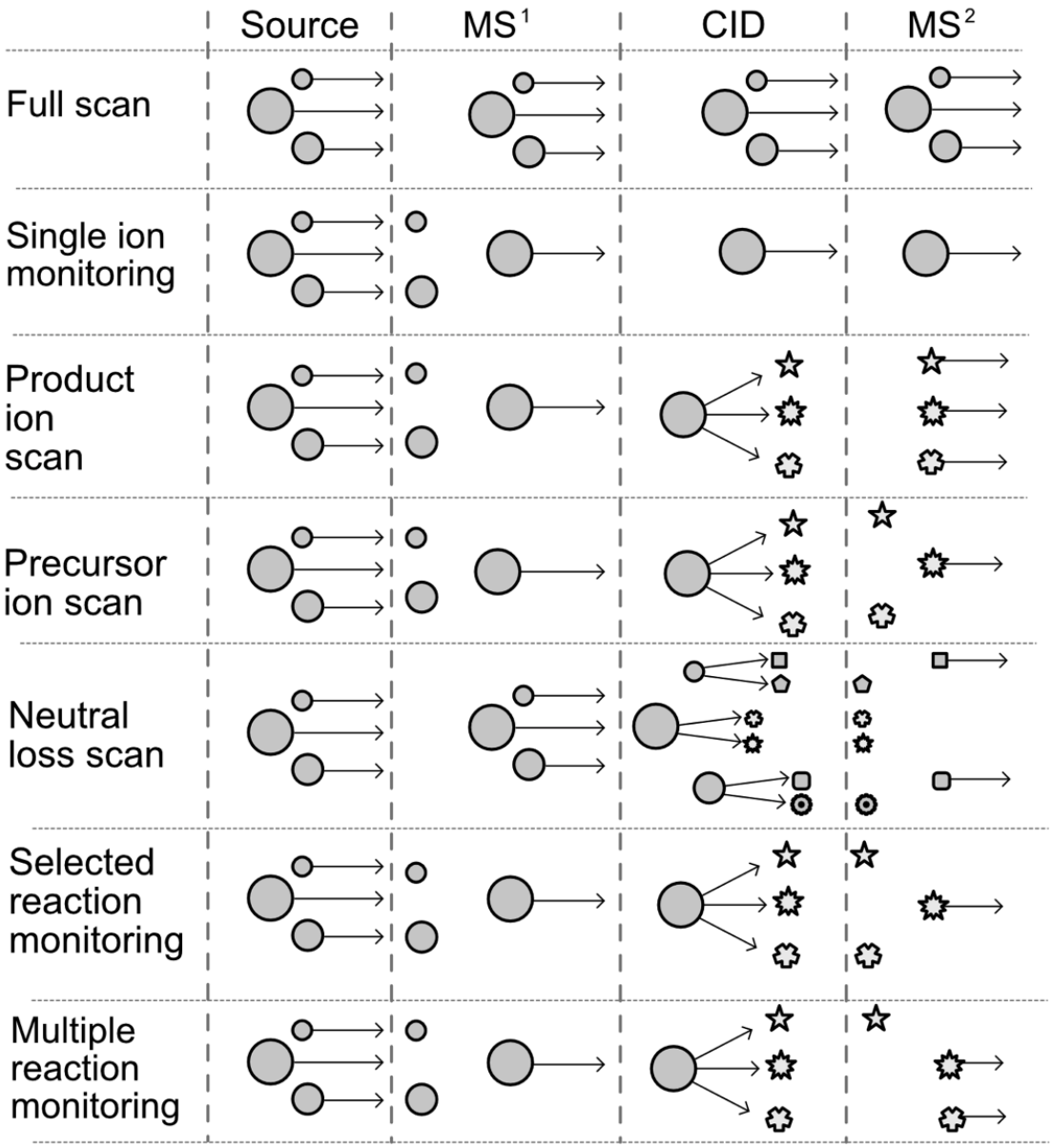


Figure 2.6-11 Tandem mass spectrometry process adapted from (Argoti, 2008; Niessen, et al., 2015; Shih-Yi, et al., 2014)

A full scan can be done over a certain *m/z* range to detect all the ions present. Single ion monitoring is when MS^1 is used to select a specific *m/z* ion to be analysed (Argoti, 2008). Product ion scan is used to select an ion of a given *m/z* in MS^1 and obtain an *m/z* spectrum of its product ions by allowing all product ions through in MS^2 to be scanned. Precursor ion scan is used when the fragment/product ions are known, and the precursor needs to be detected. MS^2 is set to allow a certain fragment through and MS^1 is scanned. Neutral loss scans all ions in MS^1 and selected ions with neutral loss in MS^2 for detection. Selected reaction monitoring (SRM) selects an ion in MS^1 for fragmentation and then MS^2 selects a fragment for scanning. Several SRM stages can be done in series to obtain further fragments from the fragmentation ions (MS^n), this is known as consecutive reaction monitoring (CRM). Multiple reaction monitoring (MRM) is the same as SRM, but more than one fragment is scanned (Niessen, et al., 2015; Shih-Yi, et al., 2014).

The characterises which distinguish analysers from one another (also known as mass spectrometric figures of merit) are:

1) *m/z* range: the range of (*m/z*) that can be measured by the analyser. The linear dynamic range is the range over which ion signal is linear with analyte concentration (Ronaldo, 2017).

2) Resolution (R): ability to produce two distinct signals for ions with a very small mass (*m/z*) difference (Ronaldo, 2017). The IUPAC definition is given by the following equation (Murray, et al., 2013):

$$R = \frac{m/z}{\Delta m/z} \qquad \textit{Eq. 14}$$

Where m/z is that of a specific ion that must be reported along with the resolution value. $\Delta m/z$ for a single peak, is the width measured at a specific hight (usually either 5 or 50% of the peak height). Alternatively it is the spacing between two overlapping peaks of similar magnitudes with a valley between them of no more than 10% (Murray, et al., 2013; Niessen, et al., 2015), shown in Figure 2.6.12. What this means in a practical sense, is that high resolution instruments are able to measure masses to up to ppm (0.000001 Da) while unit-mass resolution instruments can only give *m/z* with ± 0.1 Da (Niessen, et al., 2015).

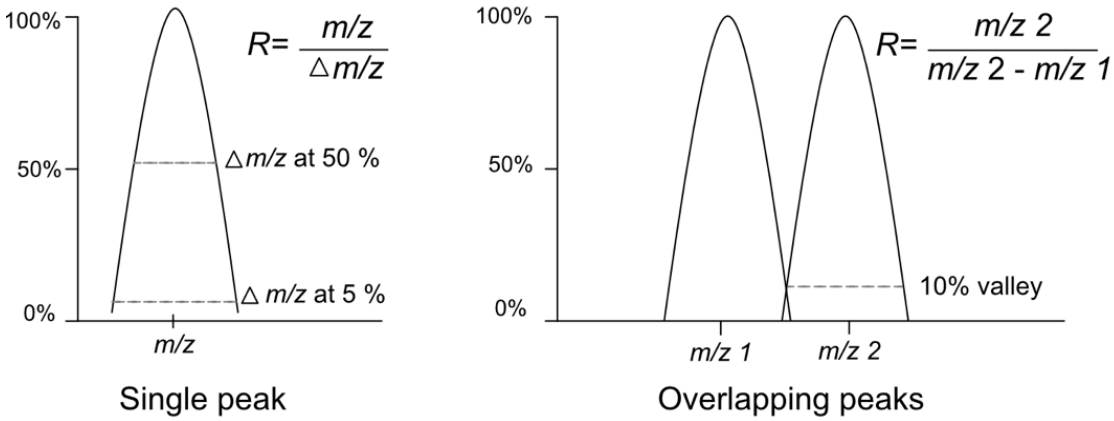


Figure 2.6-12 Mass resolution for single and overlapping peaks adapted from (Waters Corporation, 2018)

3) Accuracy: is the ratio of the mass-to-charge measurement error (i.e. the difference between the measured M and the true M) divided by the true mass-to-charge ratio and is usually stated in parts per million (ppm). Here it is important to note a few definitions in terms of mass terms (Brenton, et al., 2010):

   Nominal mass—the mass of an ion or molecule calculated using the mass of the most abundant isotope of each element rounded to the nearest integer value and equivalent to the sum of the mass numbers of all constituent atoms (Brenton, et al., 2010).

   Accurate mass—the experimentally determined mass of an ion measured to an appropriate degree of accuracy and precision used to determine, or limit the possibilities for, the elemental formula of the ion (Brenton, et al., 2010).

   Exact mass—is the calculated mass of an ion whose elemental formula, isotopic composition and charge state are known, i.e., it is the theoretical mass. The charge

state is relevant as the mass of the electron (0.00055 Da), or multiple charges, may not be negligible in the context of mass measurement (Brenton, et al., 2010).

4) Ionic transmission or duty cycle: This is a measure of the efficiency of the instrument. It is the ratio between the number of ions of interest arriving at the detector and the ions produced at the source (Murray, et al., 2013).

5) Scan speed: Used to determine the number of spectra per unit time that can be generated (measured in Hertz) (Murray, et al., 2013).

6) Reproducibility: The reproducibility with which ion abundances can be determined is called the precision of an instrument (Murray, et al., 2013).

A summary of the figures of merit of the analysers described previously is given in Table 2.6-2 .

	Time of flight	Quadrupole	Ion trap	Fourier transform Ion Cyclotron Resonance	Orbitrap
***m/z* range**	>300,000	4,000	4,000	10,000	50-6,000
Resolution	30,000	4,000	4,000, but up to 20,000 if scan speed is reduced (and reduced accuracy)	>100,000	>140,000
Accuracy	0.02-0.2% (200 ppm)	0.01% (100 ppm)	0.01% (100 ppm)	<0.0005% (<5 ppm)	<0.0001% (<1 ppm)
Ionic transmission/ duty cycle	Resolution is independent of duty cycle and 10 ms can give resolving power of 20,000- 30,000	Dependent on mass range and analyse mode. 10-50 ms for each analyte in SRM, 500 ms for full scan *m/z* range of 100	Faster than quadrupole without compromising sensitivity	Medium, slower than the Orbitrap but able to maintain high speeds with high resolution and good mass accuracy.	100ms (for a resolution of 10,000) 1-1.5 s (for a resolution of 100,000)
Scan speed	ms	~ 1 s	~ 1 s	~ 1 s	cs
Tandem MS	MS^n	MS^2 (triple quadrupole)	MS^n	MS^n	MS^2

Table 2.6-2 Comparison of main mass analysers adapted from (Argoti, 2008; Chromatography & Mass Spectrometry Solutions, 2016; Hopfgartner, 2011; Thermo Scientific, 2015)

2.6.3.4 Accurate quantification vs flexible detection

Targeted mass spectrometry allows for accurate quantification of specific analytes, while non-targeted MS allows for flexible scanning of a range of analytes which are not specifically predetermined (Dudzik, et al., 2018). A common setup for targeted analysis is the triple quadrupole (QqQ). When used in MRM the QqQ has excellent selectivity and sensitivity (RSC Analytical Methods Committee, 2013). In the first quadrupole ions of interest are selected and go through to the second quadrupole (which acts as a collision cell), where the ions are fragmented, these go on to the third quadrupole where selected fragments go through to be quantified in the detector (Ma, et al., 2018b). This allows for maximum selectivity as for each ion there is a characteristic retention time from the LC, a parent ion and typically two fragment ions. One fragment ion is used for quantification (quantifier) and the other as a confirmation (qualifier) (Ma, et al., 2018b). For this thesis quantification work was done using mainly a 1290 Series triple quadrupole (Agilent Scientific, UK) and some work was also carried out using a quadrupole linear ion trap QTRAP 4000 (AB SCIEX, UK). The triple quadrupole is referred to as QqQ and the ion quadrupole as QTrap. The QTrap has the same first two quadrupoles as a QqQ, but the last quadrupole can be configured as a linear ion trap, which gives it further tandem mass analysis functions (Sciex, 2020). The instrument used for this work to do untargeted analysis was a Q-Exactive Plus mass spectrometer (MS) equipped with Dionex U3000 UHPLC system (Thermo Fisher Scientific, Hemel Hempstead, UK) which is hybrid quadrupole-orbitrap. This allows for simultaneous scanning of fragments in MS2.

In targeted MS the chromatographic and MS parameters for the analytes are optimised prior to sample analysis using standards in order to obtain maximum sensitivity and selectivity for each analyte. For QqQ the parameters optimised are: the fragmentor voltage, cell accelerator voltage and collision energy. These voltages act to separate and fragment each of the analytes and so need to be optimised for each analyte. Similarly, for the QTrap the delustering potential (DP), collision cell exit potential (CXP) and fragmentor voltage need to be optimised for each analyte.

Targeted methods must be validated by ensuring the following parameters are suitable: selectivity/specificity, precision, accuracy, linearity, range, and limit of detection (LOD) and limit of quantification (LOQ) (ICH, 2005).

Selectivity: the ability of a method to measure the analyte accurately in the presence of matrix interferences. It is checked by comparing a chromatogram of a blank sample. Targeted methods have 3 ways of confirming an analyte: the retention time, the parent ion and the product ions, which means they have high selectivity (ICH, 2005; Ma, et al., 2018b; Nageswara Rao, 2018).

Precision: the degree of agreement among analytical replicates. It is measured as the relative standard deviation (or coefficient of variation) of a group of at least 3 analytical replicates (ICH, 2005). For multiresidue BACs environmental methods for soil, water and plant matrices precisions, reported as relative standards of deviation can be below 10% (Durán-Alvarez, et al., 2009; Gros, et al., 2006; Petrie, et al., 2016), however higher values are often reported of between 10-28% (Albero, et al., 2014; Aymerich, et al., 2016; Baena-Nogueras, et al., 2016; Comtois-Marotte, et al., 2017; Gibson, et al., 2007; Chenix Wu, et al., 2012), even up to 32 %(Bollmann, et al., 2016), 38% (Chenix Wu, et al., 2012) or 48% (Al-Odaini, et al., 2010).

Accuracy: the degree of agreement between the measured value and the true value. It is measured by spiking the matrix with a known concentration and assessing the closeness of the measured value to the spiked value. This measurement is related to

the recovery of the method. The recovery of the method is the percentage of the spiked amount which can be extracted and quantified (ICH, 2005). In environmental samples there is the added challenge that the analyte may be present in the sample and it is not possible to obtain a matrix that does not contain the analyte (Michael, et al., 2017; Stüber, et al., 2004). The use of ESI and LC-MS/MS is usually associated with significant matrix effects (Ngumba, et al., 2016; Stüber, et al., 2004). Matrix effects are the enhancement or decrease of the analyte signal due to endogenous matrix components which co-elute with the analyte (Ngumba, et al., 2016; Stüber, et al., 2004). Therefore, using external calibration methods were the curve is prepared in pure solvent often leads to under or over estimation of the concentration in the matrix (Ngumba, et al., 2016).To compensate for matrix effects internal standards, matrix-matched standards and standard addition methods have been used. The internal standard method is often used due its simplicity and efficiency. However internal standards are expensive and often not commercially available for all analytes (Ngumba, et al., 2016; Stüber, et al., 2004). Matrix-matched calibration requires the availability of the matrix being studied but without the analytes (Poole, et al., 2016), this is rarely possible with environmental matrices (Stüber, et al., 2004). Standard addition is very labour intensive (Ngumba, et al., 2016), however it has been demonstrated to be a suitable method to compensate for matrix effects (Ngumba, et al., 2016; Rossmann, et al., 2014; Stüber, et al., 2004).

Linearity: the capability of a method to provide signals which increase in a directly proportional way to concentration. It is measured by injecting at least 5 concentration levels and plotting a concentration vs peak area curve of the results. A linear curve is then fitted to this plot and the R^2 value is determined. R^2 value should be >0.99. The equation of this calibration curve is $y = mx + c$, where y is the peak area and x is the concentration. Once the method has been validated this curve can be used to determine the concentration of the analytes in the samples (ICH, 2005).

Range: This is the upper and lower limit concentration levels that have been shown to comply with the precision, accuracy and linearity (ICH, 2005).

Stability: Analytes may decompose through the extraction and analysis procedure, the accuracy test accounts for whether the analytes are stable during the method. If unsatisfactory accuracy results are obtained stability may need to be further investigated (ICH, 2005).

Limit of detection (LOD) and limit of quantification (LOQ): LOD is the lowest concentration at which the instrument can detect but not quantify the analyte, it is determined by a signal to noise ratio of 1:3. The LOQ is the concentration level at which the signal to noise ratio is 1:10 (ICH, 2005).

An important distinction must be made between instrumental metrics and method metrics. The metrics described above may be obtained purely for the instrumental method. This means that standards are used to prepare calibration curves in solvents and are injected directly into the instrument. These metrics are important in order to know the performance of the instrument. However, in terms of environmental analysis overall method metrics are more relevant as they include the extraction procedure. This means that they account for the recovery of the extraction method and for matrix effects. Recovery and matrix effects calculations and further details are provided in Section 3.4.1.2. In order to calculate method metrics, it is necessary to spike the environmental matrix and carry out the extraction and instrumental procedures and compare the concentration obtained to the one spiked. If spiking is done as a calibration curve on the environmental matrix then it is possible to determine the

method limit of detection (mLOD) and all of the above metrics for the overall method (United States Environmental Protection Agency, 1999).

Most environmental BACs analysis is targeted (Petrie, et al., 2015). Targeted methods are very reliable, and it is possibly to accurately quantify environmental concentrations. However, it is important to be aware of the limitations of this approach. Firstly, standards are expensive and the number of known BACs in the environment is huge, therefore methods have to be developed only for a selection of BACs. In addition, new pharmaceuticals are continuously being identified as being of concern, so even if the selection of BACs for analysis is broad it is impossible to cover all that are environmentally relevant. Finally, BACs can exist in several forms as has been discussed in Section 2.1 and standards may not be available for all form (Petrie, et al., 2015). That is why untargeted analysis is a useful tool in identifying other BACs which may be of importance (Archer, et al., 2018; Di Carro, et al., 2018; Gorrochategui, et al., 2016; He, et al., 2020; Richardson, et al., 2018; Wielens Becker, et al., 2020). However, confidence in the identification of analytes using untargeted analysis is lower than targeted as there are no standards to compare the results with (Sumner, et al., 2007). The Metabolomics Standard Initiative (MSI), has determined 4 levels of identification(Sumner, et al., 2007):

1. Identified analytes: with a minimum of two orthogonal data (e.g., retention time and mass spectrum or accurate mass and tandem MS) relative to a standard analysed in identical experimental conditions.
2. Putatively identified analytes: without chemical reference standards, identification is based on physicochemical properties and/or spectral similarity with published data and/or public/commercial spectral libraries.
3. Putatively characterized compound classes: based on characteristic physicochemical properties of a chemical class of compounds of by spectral similarity to known compounds of a chemical class.
4. Unknown compounds: although unidentified or unclassified these analytes can be differentiated and quantified based upon spectral data (Sumner, et al., 2007).

For untargeted data, the amount of data is so large that computational methods are often used for peak identification, noise reduction and the comparison of the obtained results to existing or purpose-built databases of mass spectrums (Richardson, et al., 2018). When comparing to existing databases it must be noted that the drawback is that the signal from one instrument to another in specific conditions will never be the same (Richardson, et al., 2018). However, many databases contain data from a multitude of instruments, and they can be used for putative identification level 2 (Alygizakis, et al., 2019; Di Carro, et al., 2018; Lopardo, et al., 2018; Wielens Becker, et al., 2020). Some commercially available and open access available databases include the NIST library, NORMAN, MZ-cloud, PubChem, MetFrag49, among others (Richardson, et al., 2018). The database used will depend on availability and suitability to the analytes studied (Richardson, et al., 2018).

2.6.4 Data analysis, modelling and interpretation

Once data has been obtained, statistical analysis is necessary to assess its validity (Belouafa, et al., 2017) and to analyse and interpret it (Williams, et al., 2020). The data obtained using targeted and untargeted techniques may be better analysed using univariate or multivariate analysis. Univariate data is that in which there is only one dependent variable. It can be analysed using methods such as variance, hypothesis tests and t-tests of means (Zumbo, 2014). Multivariate analysis is used for datasets with multiple dependent variables (Worley, et al., 2013). Often computational methods

are used (Maggino, et al., 2014). Environmental data can also be modelled in order to compare it to first principle models such as the sorption isotherms described in Section 2.3.2.1 to understand BACs sorption behaviour (Durán-Álvarez, et al., 2012; Kodešová, et al., 2015; Paz, et al., 2016), or it can be used to develop new models to better describe data (Acheampong, et al., 2019; Li, et al., 2020). Data and first principle equations can also be used to predict environmental concentrations (Delli Compagni, et al., 2020; He, et al., 2020) or behaviours in the WWTP(Comber, et al., 2019; Gonzalez-Gil, et al., 2018).

2.6.4.1 Univariate statistical tests

It is recognized by mathematicians that experimental data often exhibits a distribution slightly differing from the classical normal distribution, however the normal distribution is widely used to model experimental data. It is a very useful and simple model which, under certain conditions, can be generally applied as a good approximation to many different types of data (Brenton, et al., 2010). A normal distribution is symmetrical with a central mean (μ) and 99.7% of the data falls above or below three standard deviations ($\pm 3\sigma$) form the mean (Dancey, et al., 2014), as can be seen in Figure 2.6-13:

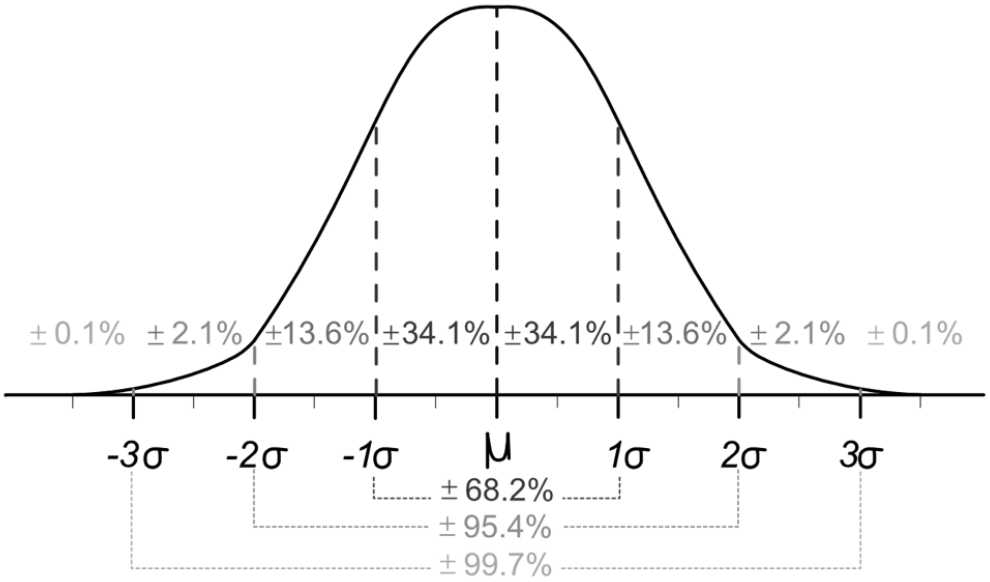


Figure 2.6-13 Normal distribution

The mean of a given dataset ($\bar{x}$), where the values are given by ($x_i - x_n$), and where n is the total number of samples is given by (Dancey, et al., 2014).:

$$\bar{x} = \frac{\sum x_i}{n} \qquad \textit{Eq. 15}$$

The difference between the mean μ of the normal distribution and the mean of the dataset $\bar{x}$, is that $\bar{x}$ is the experimental value obtained from the sample pool, which should approximate the real μ of the real population. Similarly, the standard deviation (s_x) of a given dataset, which is used to assess the spread of the data, is given by the equation below (Dancey, et al., 2014):

$$s_x = \sqrt{\frac{\sum_{i=1}^{n}(x_i - x_n)}{(n-1)}} \qquad \textit{Eq. 16}$$

Any dataset can be normalised so that it has a mean of zero and a standard deviation of one. Normalising data allows datasets of different magnitudes to be compared. The normalised z-value (z_i) is obtained by normalising the sample values (x_i) as below, using the mean ($\bar{x}$) and the standard deviation (s_x) (Dancey, et al., 2014).

$$z_i = \frac{x_i - \bar{x}}{s_x} \qquad \textit{Eq. 17}$$

z-values of a dataset can then be used to check the normality of a dataset, in order to assess whether using the normal distribution for the statistical analysis is adequate. Q-Q plots can be used to visually confirm the normality of the data (StatSoft Inc., 2013). Q-Q plots are a scatter plot of the data obtained in ascending order against the equivalent z-value of the normalised data. The resulting plot should be a straight line. This is a visual test, and as such is slightly subjective, but it gives an indication of whether the data is roughly normal and if there are any outliers they can be easily identified (Ford, 2015).

z-values can also be used in significance tests in order to compare means of different datasets and determine if they are statistically significantly different from each other. This makes use of normal distribution probability tables or z-tables. These tables are constructed from probability theory which states that the area under a normal curve is equal to 1 (or 100%). Using integral calculus these tables give areas under the normal curve which make it possible to calculate the probability of obtaining a certain value. The test statistic to compare two population means: $\bar{y}$ and $\bar{x}$ is given by the equation below (Dancey, et al., 2014):

$$t = \frac{\bar{x} - \bar{y}}{\left(s_x/\sqrt{n_x}\right) + \left(s_y/\sqrt{n_y}\right)} \qquad \textit{Eq. 18}$$

For statistical significance there must be at least 95% confidence in the results. This translates into a probability of 5% that the result is not true, in terms of the normal curve this is an area of 0.05 and it is known as the level of significance (α). These tests are called hypothesis tests and they assume a null hypothesis, H_0, is true. The null hypothesis is that there is no difference between the means, i.e. $\bar{y} = \bar{x}$ unless it can be proven not to be, in which case H_0 can be rejected and H_1 would be true. H_1 depends on what is being tested (Dancey, et al., 2014). This is illustrated in Figure 2.6.14:

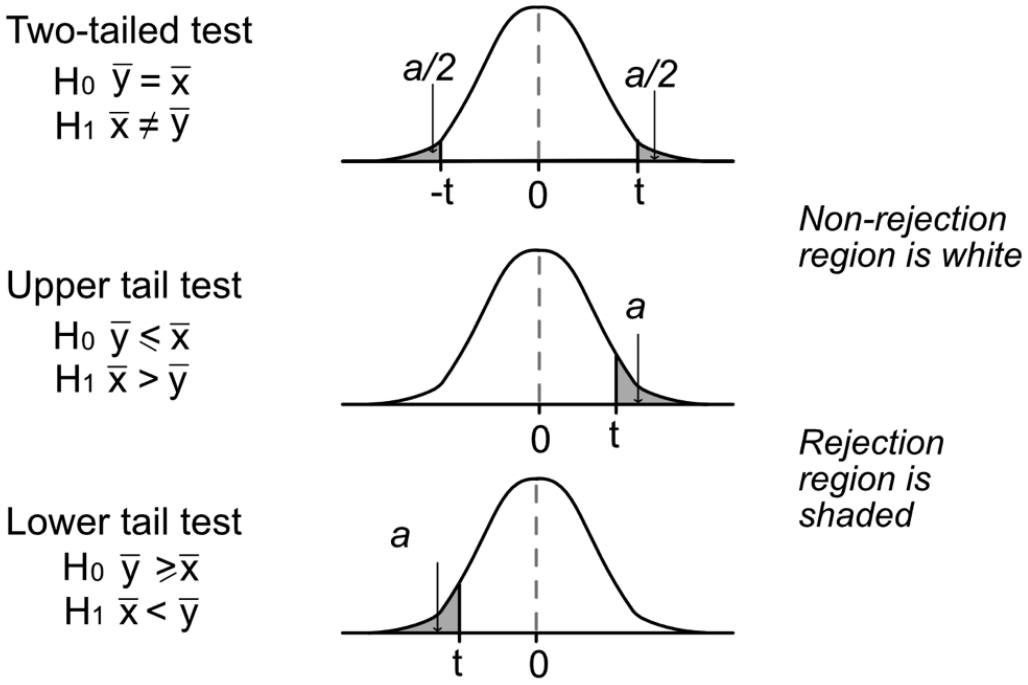


Figure 2.6-14 Hypothesis testing for normal distribution adapted from (Titiunik, 2012)

Other information which can be derived from this type of data is the corelation between one variable and another. Pearson's coefficient is adequate because it computes correlation between each variable. Instead of multiple regression, such as partial least square regression (PLS), which identifies the relationship of one variable with a set of predictor variables which may be strongly correlated between them. This correlation is assumed to be linear; this assumption must be taken into account. The significance of the correlation can be determined, as with hypothesis testing and only correlations with $p < 0.05$ are considered for this work. Moderate ($|0.4| \leq r \leq |0.7|$) correlations and strong ($r \geq |0.7|$) (Dancey, et al., 2014) correlations are considered for this work.

When performing multiple statistical analysis on one set of data it is necessary to account for familywise type I errors. These are the cumulative probability of obtaining a type I error. The Bonferroni procedure accounts for this by decreasing the p for each statistical test by dividing it by the total number of statistical tests carried out on the dataset. This is only applicable if the dataset is relatively small and the statistical tests are planned rather than determined by the experimental results (Hastie, 2009; Huitema, 2011)

2.6.4.2 Multivariate statistical analysis

The challenge faced with untargeted LC-MS data is that there is a large amount of data and it is not straight forward to determine what is relevant (Alygizakis, et al., 2019). After data acquisition, pre-processing is vital in reducing data quantity and complexity. This typically includes peak detection, annotation or subtraction of compounds present in blanks as well as grouping of isotopes, adducts, multicharged ions and in-source fragments to define components (Hollender, et al., 2017). It is now common to develop workflows which can automatically interpret data as it is very time-consuming to manually analyse the large amount of chemical features obtained from untargeted analysis (Richardson, et al., 2018). Other approaches are to develop purpose-built databases which help identify compounds of interest (Schymanski, et al., 2015; Wielens Becker, et al., 2020). Following identification of compounds, prioritization across several samples along gradients of time, space or treatment enables data

reduction and further evaluation. This includes statistical methods such as principal component analysis, clustering and regression analysis (Hollender, et al., 2017). For example computation methods in order to perform peak prioritization based on variation of intensity with time (Alygizakis, et al., 2019). Another approach, which allows for comparisons amongst groups, is metabolomics. The aim of metabolomics is to identify the chemical features against a large and complex background of metabolites that distinguish one group of samples from another (Worley, et al., 2013). Metabolomics has been applied to fields such as the drug discovery process, identification of disease biomarkers, the study of plants and bacteria identification of toxic or nutrition and the environment, among others (Worley, et al., 2013). Another example is for the identification of potentially toxic or carcinogenic chemicals in crumb rubber manufactured from automobile tyres (National Toxicology Program, 2019).

Multivariate analysis is at the core of metabolomics, for multivariate analysis Y and X variables need to be defined. Y-variables are dependent variables, and they depend on the X-variables (Worley, et al., 2013). Y-variables are each of the distinct samples (i.e. not technical replicates) and X-variables are the peak areas of compound identified through untargeted LC-MS/MS (Worley, et al., 2013). During the confirmation processes variables such as retention time, chromatogram shape and mass spectra are used for identification and confidence levels. Once this process is complete a dataset consisting of the magnitude of the peak of each compound in different samples remains (Worley, et al., 2013). The magnitude of the peak area is mainly dependent on the amount present in each sample, however other factors will influence it such as: the analyte itself, matrix effects, the mobile phase and the ionization mode (Worley, et al., 2013). When doing non-targeted analysis, the chromatography and mass spectrometry have not been optimised for each of the analytes, as it has been for targeted analysis. This means that the peak area measured could be deceptively small for a compound which is actually found in relatively high concentrations in the samples. Therefore, it is necessary to normalise the area in order to prevent analytes with large peak areas from dominating the analysis (Worley, et al., 2013). Different types of normalisation can be used depending on the data. The most common are unit variance and Pareto scaling (Umetrics-Sartorious, 2019). Unit variance (or z-scores) completely eliminates the dominance of larger peaks vs smaller peaks for different analytes by making each variable have a mean of 0 and a standard deviation of 1, using Eq. 16. Pareto scaling allows for larger peaks to still be more relevant than smaller peaks by using the following equation (Umetrics-Sartorious, 2019):

$$\frac{X_i - X}{\sqrt{s}} \qquad \textit{Eq. 19}$$

Once the data has been normalised the first step of multivariate analysis is to check whether the samples are grouped as expected, this can be achieved using principal component analysis (PCA). This is an unsupervised method which means that the model is unlikely to be over-fitted. PCA will therefore cluster Y-variables on how similar they are depending on how the X-variables vary within them. The $R^2X[n]$ value refers to the percentage of the variance of the data accounted for by the nth component. PCA is a summary model (Umetrics-Sartorious, 2019), meaning that it does not aim to predict and so it is a good check to do before proceeding to the PLS model as it will reveal whether the grouping of the data is real or due to over-fitting (Umetrics-Sartorious, 2019). The risk of doing PLS straight away is that it is possible to obtain false groups or 'over fitting' (were the model starts to account for the noise instead of for the actual variable changes) (Umetrics-Sartorious, 2019).

PLS is a regression extension analysis of PCA, PLS can differentiate between which X variables predict Y and which do not, identifying variables important for prediction

(Umetrics-Sartorious, 2019). There are two important statistics which are indicators of the quality of the model, X^2 is a measure of how well the model fits the data and Q^2 is a measure of how well Y can be predicted using X (Umetrics-Sartorious, 2019). PCA and PLS have been used to identify important variables in understanding the sorption behaviour of twenty-one BACs to different types of soil (Li, et al., 2020).

PCA and PLS analysis as part of a metabolomics approach, has not been previously applied to comparing influent and effluent samples from WWTP, but there is a lot of potential of using this in this field. It has been used to analyse untargeted. A minimum of n=5 biological replicates (not analytical replicates), are recommended for each group (Sumner, et al., 2007). For this work, a biologically different sample is equivalent to a different sampling time of the same matrix (i.e. Wastewater effluent from the same plant collected at five distinct time points) and a group would be equivalent to either the influent or effluent of a specific WWTP. For this work the relevance of using metabolomics is in being able to compare the presence of BACs identified through untargeted LC-MS/MS analysis in the influent and effluent of a WWTP to gain insights on the removal efficiency of the plant. If two WWTPs are studied it is also possible to compared the removal efficiencies of the two plants for the BACs identified.

2.6.4.3 Environmental risk indicators

Once the concentration of a pollutant in the environment has been determined it is necessary to understand what this means in terms of the effect it has on the ecosystem. Environmental risk can be quantified in terms of hazard and exposure (Peake, et al., 2016b):

$$Risk = Hazard \times Exposure \qquad \textit{Eq. 20}$$

This means an organism needs to be exposed to a hazardous substance for it to be harmed (Braund, et al., 2016b). For BACs the exposure includes all of the chemical's lifecycle and once in the environment it is linked to their persistence, fate, transport and ecotoxicity (Peake, et al., 2016b). Often there is insufficient information to calculate this risk, therefore three concepts are used:

Predicted environmental concertation (PEC): this is a calculated concertation based on modelling and available data on chemical usage or consumption, persistence (ability to be degraded), transport (ability to accumulate in flora and fauna and behaviour in environmental matrices such as soil sorption), fate (ability of organisms to metabolise the chemical) (Peake, et al., 2016b).

Measured environmental concentration (MEC): this is derived from sampling campaigns, detection and quantification of pollutants in environmental matrices (Peake, et al., 2016b).

Predicted no effect concentration (PNEC): this is the concentration of a chemical at which there are no expected adverse effects on the ecosystem at any exposure time. In practical terms this is hard to quantify as an ecosystem includes many different species with multiple interactions. As a simplification, in order to obtain an indicator a minimum set of species are selected for ecotoxicological testing. These species must be practically suitable for testing, i.e., easiness to feed and breed in a laboratory. Most important of all these species must belong to different trophic levels in the environment. For example, for evaluating the PNEC in surface water ecotoxicological tests for algae, daphnids and fish are required. It is important to note that PNEC is an ecotoxicological test and as such it is not about the harm that can occur to an individual organism, but rather about the concentration above which harm to the ecosystem as a whole is likely

to occur (Finizio, et al., 2014). For antibiotics the minimum inhibitory concentration (MIC) is the concentration at which antimicrobial resistance development can occur, and it can be used in a similar way to the PNEC (AMR Industry Alliance, 2018). PNECs and MICs for the BACs studied for this work were obtained from literature for different aquatic organisms and can be seen in Appendix 10.1.

Risk quotients (RQ) are calculated as Eq. 21 below:

$$RQ = \frac{MEC}{PNEC} \qquad \textit{Eq. 21}$$

PEC can be used instead of MEC and MIC can be used instead of PNEC. A ratio < 1 suggests there is no significant risk associated with the environmental presence of the pollutant. A ratio ≥ 1 suggests that further environmental risk characterization or management is required for the chemical in question (Peake, et al., 2016b).

Another indicator, is the total estradiol equivalent (EEQ_{total}) which focuses on potential risks of estrogenic activity to aquatic organisms and is the ratio of the estrogenic effects a chemical has compared to estradiol. As with the PNEC and the MIC it can be used to obtain environmental risk ratios. (Gadd, et al., 2010; Ma, et al., 2016). Potential risks of estrogenic activity to aquatic organisms are calculated based on the total estradiol equivalent (EEQ_{total}) (Gadd, et al., 2010; Ma, et al., 2016). As in Eq. 22 below:

$$EEQ_{totla} ng L^{-1} = \sum EEQ_i = \sum (C_i \times EEF_i) \qquad \textit{Eq. 22}$$

Where EEQ_i is the estrogenic equivalent quotient of a given compound (*i*), C_i is the concentration measured of compound *i* and EEF_i is the estrogenic equivalent factor which is defined as the ratio of the observed half- maximal (EC_{50}) estrogenic activity of estriol to each compound, so it is 1 for estriol (Ma, et al., 2016). Values used for the other steroid pollutants can be found in Appendix 10.1

3 MATERIALS AND METHODS

3.1 Reagents

Ammonium bicarbonate (≥ 99 %), formic acid (≥ 99.5 %) and HPLC grade methanol, water and acetonitrile were acquired from Fisher (Loughborough, UK). Ultrapure water was purified using a Millipore system (Watford, UK).

Four-tert-octylphenol (98.5 %), acetaminophen(≥99 %), atenolol (≥98 %), bezafibrate (98 %), carbamazepine (99%), clofibric acid (98.5 %), codeine (>98 %), diclofenac (≥ 98 %), ethinyl estradiol (98 %), estrone (≥99 %), estradiol (≥98 %), estriol (≥97 %), estriol-3-sulfate sodium salt (>98 %), β-estraiol-3-sulfate sodium salt (≥93 %), erythromycin (≥98 %), gemfibrozil (≥99 %), ketoprofen (≥98 %), levonorgestrel (98 %), metoprolol (≥98 %), morphine (≥98 %), oxytetracycline (96.1 %), N^4-acetylsulfamethoxazole (≥98.5 %), paroxetine (97.1 %), phenazone (≥97.5 %), salicylic acid (≥99 %), simvastatin (≥97 %), sulfadiazine (≥99 %), sulfamethoxazole (>98 %), sulfapyridine (≥99 %), tramadol (≥99 %) and trimethoprim (99 %) were bought from Sigma-Aldrich (Dorset, England). Propranolol (99 %) was bought from Alfa Aesar. Estrone-3-sulfate sodium salt (98 %) was bought from Tornoto Research Chemicals (Upper Heyford, UK). Naproxen was bought from Merck (Bedford, UK).

3.2 Aqueous samples

3.2.1 Wastewater treatment plants sampling in the UK

To ensure sample integrity, samples were collected in the morning and SPE always completed on the same day.

Stk samples were collected regularly from November 2018 to November 2019, plus some samples collected in the summer of 2018. A total of 27 raw influent (WWI) and 30 final effluent (WWE) samples were collected from Stk over this period. A minimum of 5 samples of influent and effluent were analysed for each of the four seasons: Winter (December-February), Spring (March-May), Summer (June-August), Autumn (September- November).

SuB samples were collected from May 2019 to November 2019. Downstream water (WWD), as opposed to pure effluent, was sampled from SuB because pure effluent travels in an underground pipe for approximately 100 m and the point which was sampled is what actually enters the environment, as opposed to Stk where the pure effluent travels for approximately 1 km coming in contact with the environment before joining a river. A total of 15 WWI and WWD samples each were collected.

Samples were collected in an amber glass bottle. In May 2019 glass was forbidden for use in sampling campaigns by the university for safety reasons. Results of the same sample, partly collected in an amber glass bottle and partly collected in the new white plastic bottle were compared using targeted analysis and no significant difference was found between them.

It was not practical to take samples which match the hydraulic retention time, but the large number of samples taken over a long period of time and the mixing taking place inside the WWTPs means the removals calculated are representative (UKWIR (UK Water Industry Reserach), n.d.). For Stk seasonal removals were compared to overall removals using a two-tailed hypothesis test ($p<0.05$), in order to establish if seasonal removals were significantly different to overall yearly removals.This is to determine the

variation of removal seasonally and to assess whether it is an accurate approximation to take the overall removal for a WWTP or whether it is necessary to take evaluate seasonal removalsFor all UK samples, standard water quality parameters were measured on the day of sampling collection. Temperature was measured on site using thermocouple thermometer (Digi-Sense, Cole-Parmer Instrument Ltd., UK), pH was measured using a 3510 pH meter (Jenway 970, Staffordshire, UK). COD was measured photometrically using HACH (UK) DR 2800 Spectrophotometer and ISO 15705 compliant HACH (UK) cuvettes, of the range 0-1000 mgL^{-1} for WWI and 0-150 mgL^{-1} for WWE and WWD. TSS was also measured photometrically using HACH (UK) DR 2800 Spectrophotometer or by weight difference of a filter paper before and after filtering a known volume of water. All wastewater samples were filtered through 1.2 µm pore size Micro glass fibre filter (Fisher, Leicestershire, UK) under vacuum prior to solid phase extraction (SPE).Aqueous sampling in Mexico

Sampling was carried out on the 30th of March 2019 in amber glass bottles. Due to the distance of the sampling site form the laboratory it was not possible to carry out sampling and SPE on the same day. Throughout the day samples were kept in a cool box with ice and once in the lab 1 mL of methanol was added to each 1 L bottle to stop any biological activity. Samples were kept at 4°C until they were processed on the 1st of April 2019. Table 3.2.1 details the location of the samples collected, the pre-treatment and the volume passed through the SPE column.

Sample number	Sample location	Sample pre-treatment	Sample volume (mL)
1	Cerro Colorado spring	None	490
2	Cerro Colorado spring	None	490
3	Atotonilco wastewater effluent	Centrifugation	250
4	Atotonilco wastewater effluent	Centrifugation	250
5	Endho dam	Centrifugation followed by filtration using 8µm particle retention ashless filters (Whatman, UK)	250
6	Endho dam	Centrifugation followed by filtration using 8 µm particle retention ashless filters (Whatman, UK)	250
7	Tula river	Centrifugation	250
8	Tula river	Centrifugation	250
9	Tlamaco irrigation canal	Centrifugation	250
10	Tlamaco irrigation canal	Centrifugation	250
11	Atotonilco wastewater influent	Centrifugation	250
12	Atotonilco wastewater influent	Centrifugation	250

Table 3.2-1 Tula Valley, Mexico sample location, volume and pre-solid phase extraction clean-up

3.2.2 Solid phase extraction of BACs from aqueous matrices

Either HLB or MCX SPE cartridges (500 mg, 6 cc) from (Waters, UK) were used. HLB cartridges were conditioned using 5 mL methanol followed by 5 mL ultrapure water. MCX cartridges were conditioned using 5 mL of methanol followed by 5 mL acidified ultrapure water (pH 2.5 - 3 using sulfuric acid). Sample was loaded (200 mL of influent and 500 mL of effluent or downstream water) at a constant flow rate of 5 mL min^{-1}. MCX cartridges were cleaned using 0.1% formic acid in methanol/ultrapure water (10:90 v/v). Cartridges were then dried under vacuum for 30 min.

Cartridges were stored after drying at -80 °C until analysis (always carried out within a week). Except for the Mexico samples, which were shipped in a cool box to the UK for analysis. Analytes were eluted under atmospheric pressure, with 8 mL of methanol for HLB and with 4 mL of methanol followed by 5% ammonium hydroxide (NH_4OH) in methanol for MCX. Eluents were dried at room temperature using vacuum evaporator Genevac EZ-2 (Biopharma Process Systems, Winchester, UK). Samples were reconstituted in 50:50 v/v (acetonitrile: water), by adding the acetonitrile first and the water second and vortexing for 30 s after each addition.

3.3 Soils

3.3.1 The soils used

A total of 4 soils were used in this thesis. The first was bought from Arthur Bower's and the last three were field samples. Soil 1 was used to develop the soil extraction method and was a John Innes No. 2 (J. Arthur Bower's, Northern Ireland). It was characterized using standard procedures (Flores, et al., 2010). Field soil was sampled from three points at Bunny, South Nottingham (52.2°N, 1.3°W). The soil was collected on the 20th of February 2020. None of the analytes were found to be present in any of these soils above limit of detection. The characteristics of all four soils are reported in Table 3.3.1. All soils were autoclaved at 121 °C for 75 min and homogenized by grinding using a wooden pestle and rubber mortar (to preserve structural integrity) prior to all experiments and sieved through 2mm mesh.

Soil	Source	Soil type	Sand (%)	Clay (%)	Silt (%)	pH	Organic matter (%)	Ref.
1	Bought from John Innes	Silty clay loam	21.6	30.0	48.5	6.20 ± 0.1	28.6	1
2	Bunny soil coordinates	Sand	91.9	7.6	0.5	N.A.	3.4	2
3	Bunny soil coordinates	Loamy sand	83.2	4.7	12.1	6.35	N.A.	3
4	Bunny soil coordinates	Clay loam	31.1	34.5	34.3	6.91	N.A.	4

Table 3.3-1 Soil properties of the soils used for this thesis

Ref. column refers to the following numbered references: **1** Measured following standard procedures (Flores, et al., 2010), **2** (Scott, et al., 2005), **3** (Blunk et al., 2019; Helliwell et al., 2014)), **4** (Atkinson, et al., 2009)

3.3.2 Liquid-solid BAC extraction from soil

1.00 ± 0.005 g of autoclaved and ground soil was placed in a 15 mL polypropylene centrifuge tube. Three consecutive extractions were carried out. First 5 mL of methanol were added, and the samples were vortexed for 30 s. The tubes were then placed in a shaking water bath at 30 °C for half an hour. Samples were then centrifuged for 8 min at 41000 RPM at 4 °C. The supernatant of each sample was collected. The second and third extraction were carried out in the same way, using 5 mL of 85:15 v/v (10 mM ammonium bicarbonate in water: acetonitrile) for the second extraction and finally 5 mL of 50:50 v/v (0.1 % formic acid in water: acetonitrile) for the third extraction. The supernatants were combined.

3.3.3 Solid phase extraction clean-up

One hundred mL ultrapure water was added to the combined extracts to ensure < 10 % solvent content during SPE loading. Oasis HLB cartridges (500 mg, 6 cc) from (Waters, Elstree, UK) were conditioned using 5 mL methanol followed by 5 mL ultrapure water. Sample was loaded at a constant flow rate of 5 $mLmin^{-1}$ and dried under vacuum. Cartridges were stored at -80 °C until analysis (always carried out within a week). Analytes were eluted with 8 mL of methanol under atmospheric pressure. Eluents were dried at room temperature using vacuum evaporator Genevac EZ-2 (Biopharma Process Systems, Winchester, UK). Samples were reconstituted in 50:50 v/v (acetonitrile: water), by adding the acetonitrile first and the water second and vortexing for 30 s after each addition. The samples were then filtered using mini spin filters PTFE, 0.2 µm pore size (VWR, Lutterworth, UK).

3.4 Detection and quantification: LC-MS methods

Once samples have been collected, characterised and BACs have been extracted, they were analysed using LC-MS. Depending on the objective, analysis was either targeted or untargeted, or samples were split to be analysed in both. Both of these analysis methods are described in this section. The development and validation of the methods (from extraction to analysis) is described in Chapter 4.

3.4.1 Targeted methods

3.4.1.1 Instrumental LC-MS parameters

Two chromatographic methods, one basic and one acidic, were used to ensure maximum sensitivity and optimum ionization of all analytes. The gradient of the acidic chromatographic method was made up of 0.1 % formic acid in water (mobile phase A) and acetonitrile (mobile phase B). The basic gradient was made up of 10 mM ammonium bicarbonate in water (mobile phase A) and acetonitrile (mobile phase B). The method was run in two instruments 1290 Series triple quadrupole (Agilent Scientific, UK) and linear ion quadrupole 4000 (AB SCIEX, UK) The details of the conditions for each instrument are presented in Table 3.4-1. The monile phases were the same for both instruments. For the acidic chromatography: *A:* 0.1% formic acid in water (pH~2.6) and *B:* Acetonitrile. For the basic chromatography: *A:* 10mM ammonium bicarbonate in water (pH~8.9) and *B:* Acetonitrile.

All analytes were analysed in multiple reaction monitoring (MRM) with a parent and product ion, except for 4-tert-octylphenol for which no suitable product ion was found for the triple quadrupole (QqQ). The polarity, optimum parent-product ion pair and the fragmentor, collision energy and cell accelerator voltages were optimised individually for each compound and can be found in Table 3.4-2 and Table 3.4-3.

	Linear ion trap (QTrap)		Triple quadrupole (QqQ)	
Instrument details	AB SCIEX Instruments HPLC-MS/MS, 4000 QTRAP (linear ion trap quadrupole), ESI source.		Agilent Scientific 1290 Series UPLC-MS/MS. Triple quadrupole. AJS ESI source	
Column	C18 Gemini Phenomenex (100 x 3mm p.s. 2 µm) and security guard column Phenomenex Security Guard for HPLC Cartridge Kit Product code (KJ0-4282)			
Chromatography gradient	**Acidic chromatography** *0-8 min:* Ramped from 95%A to 5%A *8-9.2 min:* Kept at 5%A *9.2 - 9.6*: Ramped from 5 to 95% A *9.6-10min:* Kept at 95% A	**Basic chromatography** *0-6.6 min:* Ramped from 85% A to 5 %A *6.6-8.0 min:* Kept at 5% A *8.0- 8.6*: Ramped from 5 to 85%A *8.6- 9.0*: Kept at 85% A	**Acidic chromatography** *0-6 min:* Ramped from 95%A to 5 %A *6-7.6 min:* Kept at 5% A *7.6-8.2*: Ramped from 5 to 95% A *9.6-10min:* Kept at 95% A	**Basic chromatography** *0-6.6 min:* Ramped from 85% A to 5 %A *6.6-8.0 min:* Kept at 5% A *8.0- 8.6:* Ramped from 5 to 85%A *8.6- 9.0*: Kept at 85% A
Column temperature (°C)	50	40	50	
Injection volume (µL)	5		10	
Mobile phase flow rate ($mLmin^{-1}$)	0.45	0.30	0.30	
CUR (QTRAP)/ Gas flow ($Lmin^{-1}$) (QqQ)	15	15	7	13
Sheath Gas flow	45	40	12	12
Probe/capillary voltage (V)	5000	4200	3000	5000
Source temperature (°C)	350	300	200	350
Nebulizer (psi)	NA		50	45
Auxiliary gas flow ($Lmin^{-1}$)	25		NA	

Table 3.4-1 Triple quadrupole (QqQ) and linear ion trap (QTrap) Liquid Chromatography-Mass Sepctrometry conditions

Bioactive chemical	Ionisation mode	Precursor ion (*m*/*z*)	Cell exit potential	Fragment ion 1	Delustering potential	Collision energy	Fragment ion 2	Delustering potential	Collision energy
4-Tertoctylphenol	Negative	205.054	9	132.9	80	30	117.8	80	82
Acetaminophen	Positive	152.142	6	135.1	31	9	110.0	31	23
Acetyl-sulfamethoxazole	Positive	296.068	15	87.0	20	30	198.0	20	30
Atenolol	Positive	267.146	14	145.1	81	39	190.1	81	27
Bezafibrate	Negative	361.132	9	275.1	65	24	274.6	65	26
Carbamazepine	Positive	237.153	14	194.1	136	29	193.2	136	47
Clofibric acid	Negative	214.030	7	127.9	30	20	126.9	30	20
Codeine	Positive	300.201	10	152.1	106	91	115.1	106	91
Diclofenac	Negative	294.84	13	251.0	50	16	215.0	50	30
Estrone	Negative	269.095	13	144.9	110	52	142.9	110	76
Estrone-3-sulfate	Negative	371.211	45	267.1	120	38	349.1	120	16
Estradiol	Negative	271.135	13	145.0	175	54	182.9	175	52
Estradiol-3-sulfate	Negative	351.142	15	271.3	110	50	144.8	110	76
Estriol	Negative	287.088	9	170.9	130	52	145.0	130	54
Estriol-3-sulfate	Negative	389.158	6	193.9	30	18	71.0	30	62
Ethinyl estradiol	Negative	295.17	23	142.9	30	74	144.8	30	56
Erythromycin	Positive	734.580	12	158.2	36	43	576.4	36	29
Gemfibrozil	Negative	249.113	1	121.0	60	18	126.8	60	14
Ibuprofen	Negative	204.980	11	158.8	70	10	160.9	70	12
Ketoprofen	Negative	249.113	7	121.0	45	12	126.8	45	10
Levonorgestrel	Positive	313.246	6	109.1	81	41	91.0	81	25
Metoprolol	Positive	268.219	10	116.1	131	27	133.1	131	37
Morphine	Positive	286.1	16	152.1	91	79	201.1	91	37
Naproxen	Negative	229.097	11	169.0	35	42	170.0	35	22
Oxytetracycline	Positive	497.203	0	98.9	71	59	480.1	71	25
Paroxetine	Positive	330.261	14	192.2	101	31	109.2	101	87
Phenazone	Positive	189.188	6	104.1	81	35	106.0	81	39
Propranolol	Positive	260.201	10	116.1	71	27	183.1	71	27
Salicylic acid	Negative	136.935	6	93.0	40	22	65.1	40	38
Simvastatin	Positive	419.300	14	199.1	91	17	285.2	91	17
Sulfadiazine	Positive	251.113	6	156.1	56	23	108.0	56	37
Sulfamethoxazole	Positive	254.102	10	156.1	66	25	108.1	66	35
Sulfapyridine	Positive	250.134	6	156.1	56	25	108.1	56	37
Tramadol	Positive	264.307	18	246.2	66	17	247.3	66	11
Trimethoprim	Positive	291.184	6	231	111	33	261.9	111	33

Table 3.4-2 Mass spectrometry ion transitions and parameters for ion trap instrument (QTrap)

Compound	Mode Chromatography.	Precursor ion and transitions	Fragmentor voltage	Collision Energy	Cell accelerator voltage
4-tert-octylphenol	Negative	205.2 > 161.0	130	2	4
	Basic	205.2 > 135.0	150	30	4
Acetaminophen	Positive	152.0 > 110.0	113	16	4
	Acidic	152.0 > 93.0	120	25	7
Acetyl-sulfamethoxazole	Positive	296.0 > 198.0	100	15	6
	Acidic	296.0 > 134.0	100	15	6
Atenolol	Positive	267.1 > 190.0	120	14	3
	Acidic	267.1 > 145.0	120	22	3
Bezafibrate	Negative	360.0 > 274.0	110	1	7
	Basic	360.0 > 153.9	100	16	1
Carbamazepine	Positive	237.0 > 194.0	130	20	3
	Acidic	237.0 > 165.0	130	65	1
Codeine	Positive	300.1 > 165.0	180	44	5
	Acidic	300.1 > 199.0	170	32	3
Clofibric acid	Negative	213.0>126.9	85	12	2
	Basic	213.0>85.0	70	11	2
Diclofenac	Negative	294.0 >250.0	45	1	1
	Basic	295.2> 143.0	150	60	2
Estrone	Negative	269.2 > 145.0	160	32	2
	Basic	269.2> 143.0	160	32	2
Estrone-3-Sulfate	Negative	349.0 > 269.1	110	22	6
	Basic	349.0 > 145.1	180	55	4
Estradiol	Negative	271.0 > 145.0	180	44	1
	Basic	271.0 > 183.0	180	44	1
Estradiol-3-Sulfate	Negative	351.1 > 271.0	175	24	7
	Basic	351.1 > 80	70	16	3
Estriol	Negative	287.1 > 170.8	70	46	1
	Basic	287.1 > 145.1	190	46	1
Estriol-3-Sulfate	Negative	367.1 > 287.1	130	20	1
	Basic	367.1 > 79.9	80	48	1
Ethynil estradiol	Negative	295.0 > 158.0	100	8	
	Basic	295.0 > 144.0	200	34	

Compound	Mode Chromatography.	Precursor ion and transitions	Fragmentor voltage	Collision Energy	Cell accelerator voltage
Erythromycin	Positive	734.5 > 158.1	165	28	4
	Acidic	734.5 > 576.4	150	12	6
Gemfibrozil	Negative	249.1 > 121.0	105	2	1
	Acidic	249.1 > 127.0	80	2	2
Ketoprofen	Positive	255.0 > 209.0	130	8	7
	Acidic	255.0 > 105.0	125	22	7
Levonorgestrel	Positive	313.2 > 245.0	95	18	4
	Acidic	313.2 > 227.0	135	18	4
Metoprolol	Positive	268.1 > 116.1	140	16	4
	Acidic	268.1 > 72.2	135	20	4
Morphine	Positive	286.1 > 165.0	125	52	5
	Acidic	286.1 > 201.1	125	30	5
Naproxen	Positive	231.1 > 185.0	100	12	4
	Basic	231.1 > 170.0	110	28	1
Oxytetracycline	Positive	461.1 > 426.0	130	10	6
	Acidic	461.1 > 201.0	130	50	6
O-desmethylvenlafaxine	Negative	264.0 > 246.0	100	5	3
	Basic	264.0 > 107.0	135	50	1
Paroxetine	Positive	330.1 > 192.1	110	16	6
	Acidic	330.1 > 70.1	130	28	5
Phenazone	Positive	189.1 > 56.2	135	32	1
	Acidic	189.1 > 77.1	130	44	3
Propranolol	Positive	260.1 > 116.1	125	14	5
	Acidic	260.1 > 183.0	115	14	4
Salicylic Acid	Negative	137.0 > 93.0	150	16	1
	Acidic	137.0 > 65.1	150	32	1
Simvastatin	Positive	419.2 > 199.2	100	16	3
	Acidic	419.2 >225.2	100	10	7
Sulfadiazine	Positive	251.0 > 156.0	100	12	4
	Acidic	251.0 > 92.1	100	20	7
Sulfamethoxazole	Positive	254.0 > 156.0	110	10	5
	Acidic	254.0 > 108.0	100	24	4

Compound	Mode Chromatography.	Precursor ion and transitions	Fragmentor voltage	Collision Energy	Cell accelerator voltage
Sulfapyridine	Positive	250.0 > 156.0	135	12	6
	Acidic	250.0 > 184.0	120	12	7
Tramadol	Positive	264.2 > 58.2	100	28	1
	Acidic	264.2 > 246.0	90	4	7
Trimethoprim	Positive	291.1 > 230.1	150	20	6
	Acidic	291.1 > 261.1	150	20	6

Table 3.4-3 Mass spectrometry ion transitions and parameters for triple quadrupole instrument (QqQ)

3.4.1.2 Quality control and quantification

For each analyte a 1 gL^{-1} stock in methanol was prepared every 6 months. From these individual stocks a mixed stock, containing all analytes, at 0.02 $ngmL^{-1}$ in methanol was prepared and stored in 2 mL aliquots every 3 months. Both, individual and mixed stocks, were stored at -80 °C. Calibration curves were prepared by making two standards in acetonitrile: water (50:50, v/v) at two concentration levels: 300 $ngmL^{-1}$ and 50 $ngmL^{-1}$ and form these the 12 calibration levels (0.5, 1, 2.5, 5, 10, 30, 50, 100, 150 200, 250 and 300 $ngmL^{-1}$) were prepared.

Each run was always started with at least three solvent blanks (acetonitrile: water (50:50, v/v)) for column equilibration, followed by a 50 $ngmL^{-1}$ calibration point to confirm that the signal of all analytes was as expected. The calibration curve was then injected followed by a blank and then the samples. After every six samples a solvent blank (acetonitrile: water (50:50, v/v)) and a quality control (a low (0.5 - 5 $ngmL^{-1}$), mid (10- 50 $ngmL^{-1}$) and high (100 - 300 $ngmL^{-1}$) range concentration prepared separately form the calibration curve) were injected. This is summarised in Figure 3.4.1

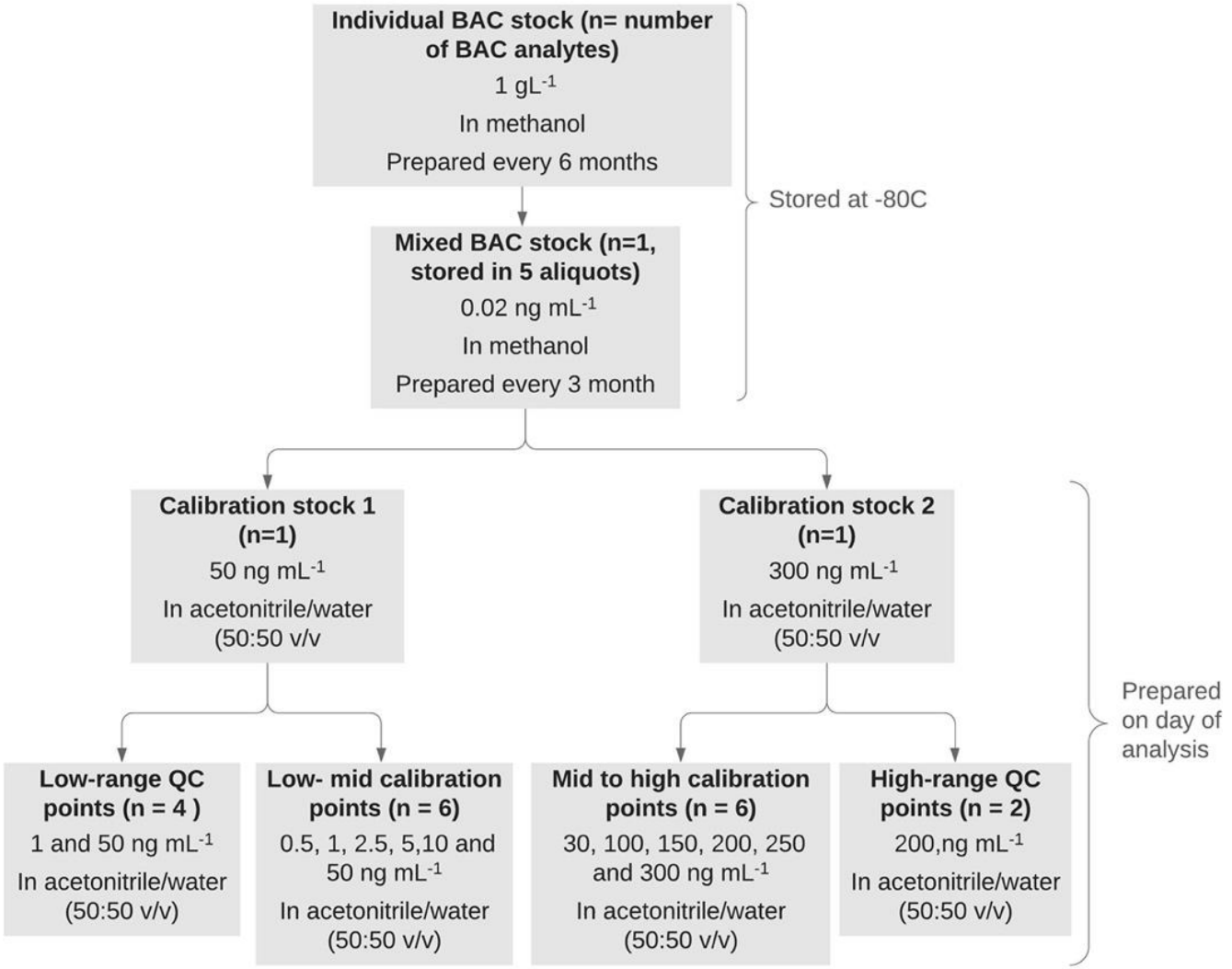


Figure 3.4-1 Stocks and calibration curves

Method recoveries were calculated for each matrix in every sampling event in order to accurately quantify the BACs despite matrix variations. To achieve this, matrix blank (n=3) and pre-spiked samples were prepared (n=3). Matrix blanks were prepared with no spiking, as described in Section 3.2.2 for aqueous matrices and Section 3.3.2- 3.3.3 for solid matrices. Pre-spiked samples were prepared in the same way as matrix blanks, but they were spiked before undergoing extraction. The concentration spiked depended on the matrix and the objective of the experiment. For the regular monitoring of the two WWTPs, 0.1 mL of 2000 $ngmL^{-1}$ of all BACs in methanol was added to n=3 of each matrix (WWE Stk, WWD SuB, WWI Stk and WWI SuB) and mixed thoroughly. Due to the different volumes used for WWI and WWE, this meant influent samples were spiked at 1,000 ngL^{-1} and effluent samples at 400 ngL^{-1} of each analyte. As acetaminophen was found at higher concentrations in WWI, 0.1 mL of 20,000 ngL^{-1} was added, resulting in a total of 2200 ng of acetaminophen in the 200 mL of WWI sample, equating to a concentration of 11 μgL^{-1}. For the soils, 1 g of soil was spiked with 1 mL of 100 $ngmL^{-1}$ of all BACs, the sample was vortexed and then left in a dark place open to atmosphere for 24 h to allow the methanol to evaporate and the BACs to adsorb. After spiking, samples underwent the process described in Section 3.2.2 for aqueous matrices and Sections 3.3.2- 3.3.3 for solid matrices. A third type of samples, referred to as post-spiked samples were prepared. These samples underwent the process as described in Section 3.2.2 for aqueous matrices and Sections 3.3.2- 3.3.3 for solid matrices as if they were blank matrix samples. However, on reconstitution (right before LC-MS injection) they were spiked with a solution in 50/50 v/v water: acetonitrile, as this was the composition of the samples on injection. The concentration spiked depended on the matrix and the experiment, however it had to be the same as one of the concentrations in the calibration in order to be able to calculate Matrix Effects. Figure 3.4.2 illustrates the three types of samples prepared for quantification.

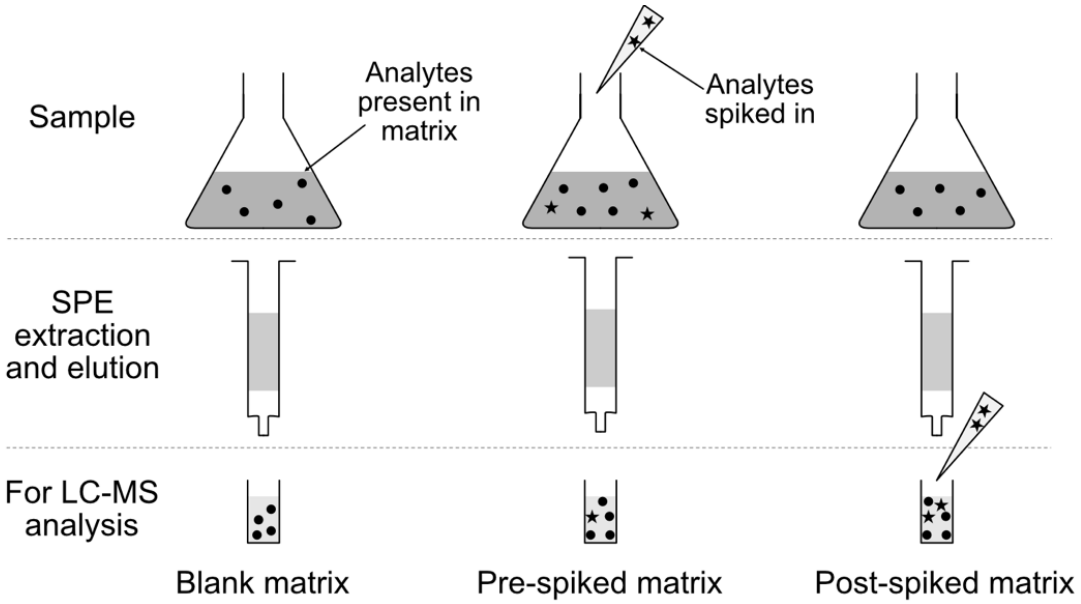


Figure 3.4-2 Schematic of types of samples prepared for targeted analysis

Method recoveries were calculated using the average (n=3) of the concentration found for each analyte in the blank matrix and the pre-spiked matrix, as shown in Eq. 23:

$$Recovery = \frac{[Prespiked\ matrix] - [Blank\ matrix]}{Concentration\ that\ was\ prespiked} \times 100 \qquad \textit{Eq. 23}$$

This recovery is a method recovery, as it accounts for any losses during extraction as well as matrix effects on injection. Therefore, it was then used to calculate the concentration in the matrix blank as follows:

$$Concentration = \frac{Blank\ matrix}{Recovery} \times 100 \qquad \textit{Eq. 24}$$

For the samples collected in Mexico it was not possible to calculate recoveries as explained above, due to the lower volumes of sample that it was possible to collect and to standards not being available. Therefore, the same recoveries were used as those obtained on the day those samples were run along with WWTP Stk samples. WWE Stk samples were used to calculate concentrations of the Cerro Colorado spring, the WWE of Atotonilco WWTP and Endho dam. WWI recoveries from Stk WWTP were used to calculate concentrations of the Tula river and the Tlamaco canal.

Unlike method recoveries, matrix effects were not calculated for every run. They were calculated for method development and validation purposes using the response (peak area) found for each analyte in the post-spiked sample and in the calibration curve point of the same concentration:

$$Matrix\ effects\ (\%) = \frac{(Post - Blank\ matrix) - Calibration\ point}{Calibration\ point} \times 100 \qquad \textit{Eq. 25}$$

Where 0 % would mean total ion suppression and 100% would mean ion total ion enhancement.

Issue	Steps taken to ensure confidence in data obatined
Stability of standards	By comparing the calibration curves from different runs it was determined that preparing the individual BAC standards every 6 months and mixed stocks every 3 months (Figure 3.4.1) ensured consistent peak areas across runs for each BAC and no decrease in sensitivity. Individual BAC solutions were stored in amber glass vials. Mixed stocks were aliquoted also stored in amber glass vials and were aliquoted in order to ensure minimum time outside of the freezer. The same mixed aliquot was used for spiking samples and for the calibration curve. Aliquots were not kept outside of the freezer longer than abuslutly necessary. Before using the mixed BAC aliquot, it was allowed to reach room temperature to avoid volume differences due to temperature.
Instrumental stability	After column equilibration (a minimum of 30 min of running the initial mobile phase gradient at running temperature) three blanks were injected followed by a system suitability sample (SSS). The SSS was the lowest concentration level in the calibration curve which was above LOD for most analytes (5 $ngmL^{-1}$ was used). The peak areas obtained from the SSS were compared to previous results obtained for the same concentration to ensure sensitivity was adequate. If it was not found to be satisfactory the samples were not run until the issue had been addressed. This did not happen often, but when it did it was solved by cleaning the spray needle or autotuning the instrument with the manufacturer's tuning solution. QC samples were run every 6 samples, when a low or mid range calibration level QC was run it was always after a blank. If a high concentration level QC was run it was always followed by a blank. The calibration curve was re-run at the end of the run. When the peak area of QCs varied more than 5% across a run concnetrations were corrected by increasing of decreasing the concentration of the sample using the decrease or increase percentage observed in the QC nearest to the samples.Or by using the end calibration curve for quantitation if this was more adequate, depening on the peak are of the samples and the position in the run. Triplicates were run for every type of sample and quantified connctrations were only considered wen the S/N was >3 for all three.
Carry-over	Apart from the initial blanks run, a blank was run after the highest point in the calibration curve and after every 6 samples. The blank run after every 6 samples was before or after the QC depending on whether it was a low-mid concentration level QC or a high concentration level QC. Analyte signal in the blanks above the limit of detection were not observed for any of the three instruments used. However, if there was even a low signal in the blanks this was subtracted from all the samples. Samples were always run from those with lower concentration of BACs (wastewater effluent before wastewater influent and non-spiked sampes were run before spiked samples).
Linearity	Instrumetal linearity was assessed for every run with a calibration curve prepared as described in Figure 3.4.1. The R^2 was always > 0.99 except for clofibric acid, ethynilestradiol, levonorgestrel. Only the calibration curve points and QCs prepared for that run were used to quantify the samples. For each analyte dynamic ranges of linearity were assessed by changing the lower and upper limits of the calibration curve to identify any concnetratinos levels above or below which linearity was not obsreved. The samples were quantified using only calibration curves within which the peak area was found (no extrapolation).

	Analytes found at very hich contrations, such as acetaminophen were quantified using diluted samples. For compounds found at low concnetrations (<10 $ngmL^{-1}$) the first 5 concnetration levels were used for sample quantification to avoid the high concentration levels of the calibration curve from dominating the calibration equation and giving inaccurate results. Standard addition calibration curves to ensure linearity in matrix were carried out to validate the method and ensure linearity in the matrcies studied, as detailed in Section 4.2.4.
Retention time shifts	QCs were run after every 6 samples, in general no significant retention time shifts were observed between these and the initial calibration curve. However, some rentention time shifts were observed for analytes in the environmental matrix compared to in the mobile phase. In those cases the rention time of the samples spiked post-extraction was taken into account to determine the rentetion time of the analyte in the matrix.
Matrix effects	Matrix effects were quantified using post-spiked samples, for each matrix, as detailed in Eq. 25. Samples were quantified using pre-SPE spiked samples. The recovery calculated form these samples (Eq. 23) accounted for extraction method losses, as well as matrix effects and was used to correct the concnterations measured in the blank matrix samples for accurate quantification. Method validation using standard addition, as presented in Section 4.2.4, was used to validate the method to ensure linearity of the concentration range in the matrcies studied. Furthermore, concentrations calculated from the standard addition calibration curves were shown to be the same was those calculated using the calibration curve in the mobile phase and corrected using the pre-SPE spiked samples (as in Eq 23), which was the approach taken routinely as it was less consumable and time intensive.
LOD	Calibration curve ssesed each time and determined from calibration curve, to determine the conctration which had a S/N>3, based on the S/N of the lowest concentration point. Only samples for which S/N>3 was observed for all three samples of each triplicate, were considered.

Table 3.4-4 Steps taken to ensure robustness of quantification data

3.4.2 Correlations calculated for the UK WWTP sampling campaign

For the correlations, Pearson's coefficient was used, this is adequate because it computes correlation between each variable. Instead of multiple regression, such as partial least square regression (PLS), which identifies the relationship of one variable with a set of predictor variables which may be strongly correlated between them (Dancey, et al., 2014). Q-Q plots were done to confirm the normality of all analytes used for correlations (Ford, 2015). Only analytes with detection frequency >95% were used for the correlations. Only moderate ($|0.4| \leq r \leq |0.7|$) and strong ($r \geq |0.7|$) correlations with $p < 0.05$ were considered. (Dancey, et al., 2014) Three correlation test were run for each data set in MATLAB (MathWorks, USA). There were up to three data points missing for at least one compound in each matrix, so the first correlation was done juts for those ananlytes that had data for all sampling dates. For the second and third runs the sampling day for which there was no data for one analyte was eliminated for all analytes and so on up until 3.

3.4.3 Untargeted method

3.4.3.1 Instrumental LC-MS method

Untargeted LC-MS analysis was performed using Q-Exactive Plus mass spectrometer (MS) equipped with Dionex U3000 UHPLC system (Thermo Fisher Scientific, Hemel Hempstead, UK). The injection volume and temperature was 5 µL and 4 °C. The column used was a C18 Gemini Phenomenex (100 x 3 mm particle diameter) and security guard column Phenomenex Security Guard for HPLC Cartridge Kit Product code (KJ0-4282).

Samples were analysed first using an acidic mobile phase and then using a basic mobile phase. The flow rate was of 300 $\mu L min^{-1}$ at a temperature of 50 °C for 10 min. The gradient for the acidic chromatography started with 95 % A (0.1 % formic acid in water, pH 2.6) and 5 % of B (acetonitrile) and was decreased to 5% A over 6 min, it was kept at this composition for 1.6 minutes and then returned to initial composition over 1.6 minutes, it was re-equilibrated for 0.4 min. For the basic chromatography the gradient started at 85% A (10 mM ammonium bicarbonate in water, pH 8.9) and 15% B (acetonitrile), it was then decreased to 5% A over 6.6 minutes and kept at these conditions for 1.4 min, it was returned to initial conditions over 0.6 min and re-equilibrated for 1.4 min.

The MS settings were the same for both chromatographic injections, it was operated in simultaneous ESI+ and ESI- acquisition modes for LC-MS profiling of the samples and in data-dependent MS/MS (ddMS/MS), with a list including all analytes in from the targeted analysis. The spray voltage was 3.5 kV (ESI+) and 3.5 (ESI-), and the S lens RF level was 50 arbitrary units for both modes. The sheath, auxiliary and sweep gas flow rates were 45, 10 and 2.25 (arbitrary unit) respectively, for both modes. Capillary and heater temperatures were maintained at 375 and 350 °C, respectively. Data was acquired for the LC-MS profiling with a resolution of 70,000 from *m/z* 70-1050.

3.4.3.2 Quality control

Through the method development processes (further explained in Chapter 4), some runs were injected as a targeted run. Therefore, the injection included solvent blanks, calibration curves and analytical replicates (n=3) of the blank matrix, pre-spiked matrix and post-spiked matrix (n=3) as explained in Section 3.4.1.2. However, the calibration curve was at a higher range (1, 5, 10, 50, 100, 400, 800, 1000, 1500, 1800 and 2000 $ngmL^{-1}$).

For runs which were done as a purely untargeted set-up pooled QCs (n=6) were injected at the beginning of the analysis to condition the column and after every 6 samples to check the stability, robustness, repeatability and performance of the analytical system. Pooled QCs are a mixture of all the samples to be injected. For these runs there are no pre-spiked or post-spiked matrix samples and no analytical replicates. For each matrix there are n ≥ 7 for each matrix, referring to the equivalent of biological replicates (samples obtained at different times). This is higher than n=5, which has been cited as the preferable amount for metabolomics analysis (Sumner, et al., 2007).

The targeted runs were mainly used for method development (although new compounds could still be identified). From untargeted runs it was possible to preform multivariate statistical analysis for robust comparison of the matrices studied.

3.4.3.3 Identification and confidence

Trace Finder (Thermo Scientific, UK) was used for the runs done as targeted, to identify and find the parent and product ions of the targeted analytes and confirm the method was working. Calibration curves of the targeted analytes were analysed using this software to assess linearity.

Compound Discoverer 3.1 (Thermo Scientific, UK) was used for data analysis and identification. Figure 3.4-3 is a representation of the custom-made workflow developed. Once the raw files form the Q-Exactive instrument are inputted into Compound Discoverer, the *Select Spectra* node, filters out spectra by mass (100 < Da < 5000). The *Align Retention Times* node compensates for 2 min retention time differences of the compounds in a sequence of sample runs.

After the *Align Retention Times* node comes the *Detect Compounds* node which identifies unknown compounds with the following restrictions: a mass tolerance of 5 ppm, S/N of 3, a minimum peak intensity of 10000 and the following possible adducts: $[M+H]^+$, $[M+H+MeOH]^+$, $[M+K]^+$, $[M+Na]^+$, $[M-H]^-$. The *Group Expected Compounds* node combines chromatographic peaks with the same molecular weight and retention times that the *Detect Compounds* node finds across all the samples.

The unknown compounds found are compared to the MzCould database and give a graphical representation of the similarity between the spectrum obtained and the ones found on the database. This enables the identification of common fragment ions between those experimentally detected and those previously found, providing an important layer of confidence in identification.

The unknown compounds also undergo *Fill Gaps* and *Normalise Areas* nodes, both of which allow for multivariate analysis later on. The *Fill Gaps* node looks for compounds which were found in one sample but not in another. First it calculates the limit of detection of the ion as an area of a simulated Gaussian peak using the expected peak width and the maximum spectrum noise in the expected retention time range multiplied by the S/N threshold. If it does not find the compound it looks for it at a lower intensity threshold than the 100,000 area set. If it still does not find it at a lower intensity threshold or if the peak found is lower, then the limit of detection value is used, and the results table obtained indicates that it has done so. The *Normalize Areas* node compensates for batch effects.

The *Assign Compound Annotations* node assigns the name, formula and structure to the unknown compounds from the mzCloud. If the compound is not found in this database the following databases are used: Predicted Compositions, MassList Search

and ChemSpider Serach. The *Predict Compositions* node populates the formula column of the *Assign Compound Annotations* node. It does so with a 5 ppm mass tolerance, a minimum elemental count of C H and a maximum elemental count of $C_{90}H_{190}Br_3C_{14}O_{18}P_3S_5$.

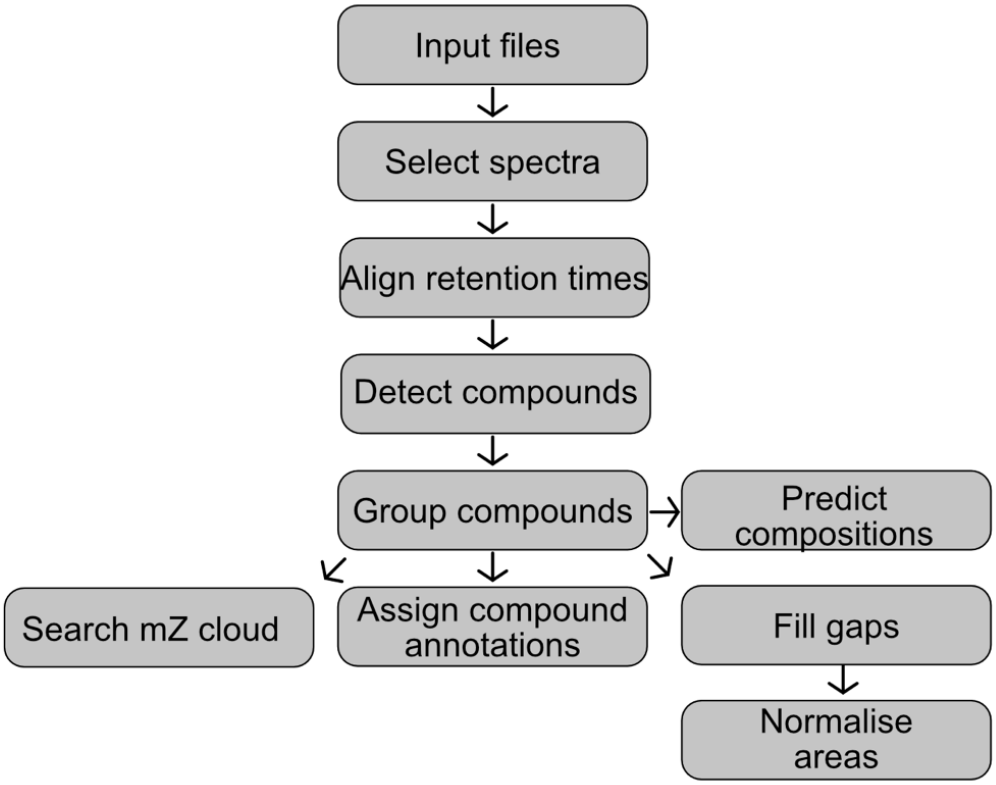


Figure 3.4-3 Compound discoverer workflow

3.4.4 Multivariate analysis

Multivariate analysis was done using SIMCA Multivariate Data Analysis Software tool (Umetrics-Sartorius, Sweden).

4 RESULTS AND DISCUSSION; ANALYTICAL METHOD DEVELOPMENT

4.1 Highlights

- **Three SPE sorbents compared** for the extraction of 36 BACs from four aqueous matrices, with **HLB found to be superior** due to recoveries and reproducability. Steroid hormone glucuronides recovery was better by > 10 % with HLB as compared to MCX. Oxytetracycline was not recovered by Strata-X, but it was successfully recovered using HLB. HLB coefficients of variation (CVs) had an average of 4.1 % for all analytes, while for Strata-X averaged CVs of 12.5 %.
- **Three reconstitution methods trialled** with the best one utilising acetonitrile: water (50/50 v/v) adding the acetonitrile first, vortexing and then adding the water and vortexing again.
- **Two LC-MS instruments (triple quadrupole, QqQ, and linear ion trap, QTrap) compared for targeted analysis of 36 BACs.** Matrix effects and/or iLOD were found to be lower on QTrap for 4-tert-octylphenol, clofibric acid, ethinylestradiol, estrone, estradiol, gemfibrozil, levonorgestrel and simvastatin, while being lower on the QqQ for oxytetracycline, phenazone, salicylic acid and tramadol. Ibuprofen was found to fragment at source on the QqQ.
- **The method was validated across both instruments for four matrices from two WWTPs.** With method linearity > 0.99 for 75% of the cases and >0.95 for 95% of the cases. mLODs were below previously reported concentrations in the respective matrices and below 10 ngL^{-1} for 22-67 % of analytes depending on the matrix. Inter and intra-day precision for over 70 % of the analytes across the four matrices studied was below 15 %.
- **Soil extraction method developed and aging evaluated to determine method recoveries considering real environmental conditions.** Twenty-eight analytes presented a significantly different ($p<0.05$) recovery at one of the five ageing periods (4, 24,72 h and 7 and 14 days) compared to that observed when there was no ageing. Demonstrating, along with literature data, how not considering the decrease in recovery when an ageing period is used for sulfamethoxazole can result in up to a two-fold underestimation of the environmental concentration.
- **Untargeted method developed** for identification of BACs in aqueous matrices'. Built from the targeted method and demonstrated to be adequate for a range of analytes with water solubility ranging from 51 – 51,900 mgL^{-1}, pK_a ranging from 1.4-14.91 and Log_{Kow} ranging from 0.2-2.97. For these analytes linearity was > 0.99 for all analytes across three different injections on three different days. The lower limit of the calibration curve range was 1 $ngmL^{-1}$ except for simvastatin, sulfamethoxazole, salicylic acid and sulphapyridine for which it was 5 $ngmL^{-1}$.

4.2 Rationale for carrying out the targeted analysis first followed by the untargeted analysis

There are arguments in favour of carrying out untargeted analysis first, as this can allow for a scoping approach to identify compounds present in the environmental matrcies studied. However, in terms of practical terms the targeted instruments were more widely available, and it was easier in order to learn to use the LC-MS instrument to start with the more straight forward targeted method. More importantly, this approach allowed for having a validated targeted method including a wide variety of compounds with diverse physicochemical properties as the foundation to build the untargeted method on. This approach allowed an increased confidence in the untargeted compounds identified as the extraction and chromatography had been shown to be effective for a wide variety of BACs. Furthermore, obtaining confirmed

identification of the targeted compounds using the untargeted workflow provided confidence in the identification of unknown compounds. Targeted and untargeted methods are complementary and ideally the compounds identified to be of concern from the untargeted analysis should be then added to the targeted method, there was not enough time to do this but it remains as a future work recommendation.

4.3 Targeted method development: gaps addressed

Multi-residue methods to quantify BACs in environmental matrices such as wastewater influent and effluent as well as surface waters have been published (Aymerich, et al., 2016; Bollmann, et al., 2016; Comtois-Marotte, et al., 2017; Farre, et al., 2008; Gros, et al., 2006; Heath, et al., 2010). However, it is still necessary to optimise mass spectrometry parameters for the analytes studied in the specific LC-MS instrument being used (RSC Analytical Methods Committee, 2013) and to validate the method for the matrices studied (United States Environmental Protection Agency, 1999). The optimisation of mass spectrometry parameters is described for the QqQ in Section 4.2.1, and compared to the literature. Mass spectrometry parameters had been previously optimised for the QTrap instrument (Parthasarathy, 2016), and are those reported in Section 3.4.1. The method validation is detailed in Section 4.2.4 demonstrating the method is fit for purpose according to the parameters described in Section 2.6.3.4. Direct injection, which is becaoming increasingly popular as it presents improved simplicity (Burns, et al., 2017), was not used due to three reasons. Firstly, direct injection is not adequate for sample transportation and given the Mexico-UK sample exchange this was not adequate. Secondly, even with the cleaning aspect of SPE, matrix effects were considerable (Appendix 10.2) and the the guard column filter had to be changed often due to blocking from matrix compomenents. Finally, SPE provides analyte concentration, which is necessary for many of the analytes studied, in particular the hormones.

The SPE method used for this thesis was based on a method which used MCX (Waters, UK) SPE cartridges to analyse BACs in Stoke Bardolph WWTP wastewater influent and effluent (Parthasarathy, 2016). However, two more types of SPE cartridges were trialled, HLB and Strata-X, which as detailed in Section 2.6.2, are common types of sorbents used for BAC environmental analysis.

The soil extraction method developed addresses ageing time, which is an important gap in the literature (Michael, et al., 2017). Ageing time refers to the period between spiking and extraction when calculating method recoveries (Larivière, et al., 2017). The absence of Certified Reference Materials (CRMs) such as those available from bodies such as NIST or IRMM, which are used for other types of analytical work (e.g. specific elements in a specific solid matrix), leads to the common practice of in-house spiking prior to extraction (Michael, et al., 2017). It has been previously recommended that a minimum of 24 h ageing is used for matrices such as soils and slurries (Larivière, et al., 2017) and it has been shown for sulfonamides that ageing time has a significant effect on recovery (Stoob, et al., 2006). However, most published methods do not consider the effect of ageing and often extract analytes immediately after spiking (Goldstein, et al., 2014; Hurtado, et al., 2016; McEachran, et al., 2017; Michael, et al., 2017; Paz, et al., 2016). It has been shown that for certain BACs ageing time can have a considerable impact on method recovery, with recovery decreasing with increasing ageing periods (Michael, et al., 2017). This is of concern as an overestimation of method recoveries can lead to an underestimation of environmental concentrations when these method metrics are used (Michael, et al., 2017). Discrepancies have been found in some studies dealing with the effect of ageing on method recoveries, particularly in relation to sulfonamides (Larivière, et al., 2017). While some have observed decreasing of recoveries by more than half, when ageing time is increased

from 90 h to 17 days, others have only observed a 10% recovery reduction when comparing samples aged for 24 h to those aged for 3 months. These differences have been attributed to soil properties differences (Larivière, et al., 2017). Therefore, during the development of the soil method ageing time was studied in 5 different ageing periods (4, 24, 72, 168 and 336 h). the first three time periods were chosen within the time range of ageing periods studied in the literature for example left overnight (Salvia, et al., 2012), left for 30 min (Martínez-Piernas, et al., 2018b), 90 min, 6 and 17 days (Stoob, et al., 2006). The last two were used to evaluate longer term effects which may take place in the real environment (Martínez-Hernández, et al., 2016). Three field soils one sandy, one loamy sand, and one loam were used in order to study the effect of soil properties on method metrics.

In Chapter 3 the method is presented in the order in which it is used, sampling, followed by extraction and finally LC-MS/MS analysis. However, for method development it is first necessary to have a working detection and quantification method (LC-MS in this case) in order to be able to measure the analytes when the different extraction procedures are trialled (RSC Analytical Methods Committee, 2013). Therefore, this chapter presents the method in the order it was developed, rather than in the order in which it is used.

4.3.1 QqQ LC-MS method development

4.3.1.1 Individual ananlyte optimization

The first step is to optimise the mass spectrometric parameters for optimum fragmentation of each analyte (RSC Analytical Methods Committee, 2013). To achieve this, each analyte was diluted individually at a concentration of 10,000 $ngmL^{-1}$ in acetonitrile/water (50/50 v/v). The acetonitrile is the non-polar mobile phase while the water is the polar mobile phase. For the steroid hormones it was necessary to add a base (10 mM ammonium bicarbonate) to the aqueous fraction in order achieve ionisation. These solutions were directly injected into the mass spectrometer, first in a full scan mode in order to find the parent ion (RSC Analytical Methods Committee, 2013). Once the parent ion was identified, a product ion scan was used to find the possible quantifier and qualifier ions (RSC Analytical Methods Committee, 2013). The collision energy, fragmentor voltage and cell accelerator voltage were changed one by one in equal increments to find the optimum conditions, giving the highest signal for the product ions found. The product ions found were compared to those found in the literature (Table 4.3.1). As can be seen from Table 4.3.1, most of the analytes were successfully fragmented in the QqQ in the same ionisation mode as other methods in the literature. Similarly, most analytes have at least one fragment ion which is the same as those reported in the literature, if not both. Often the fragment ions reported in the literature were also found using the QqQ, but the ones chosen for the final method (Table 3.4.3) had the highest signals.

Bezafibrate was an exception, as can be seen in Table 4.3.1, it could be ionised in positive and negative mode, but when calibrations curves were made in positive mode these were not linear. In negative mode a linearity of > 0.99 could be achieved for this ananlyte, as can be seen in Table 4.3.2. As can be seen in Table 4.3.1, this is in agreement with the literature where it is analysed in negative mode. Similarly, oxytetracycline, sulfamethoxazole and sulfapyridine could be fragmented in both ionisation modes, but in the literature, they were only found to be analysed in positive mode, (Table 4.3.1). When calibration curves were made, these analytes were trialled in both chromatographic modes and both ionisations modes. Lower limits of detection were observed for acidic chromatography and positive ionisation, so this mode was used for the final method (Table 3.4.3).

Clofibric acid, diclofenac, estradiol and naproxen, could be fragmented in both modes, in accordance with the literature, (Table 4.3.1). However, when calibration curves were run for clofibric acid in acidic chromatography and positive ionisation they were not linear, but they were linear (R^2>0.99) in basic chromatography and negative ionisation, so they were analysed in this mode. Naproxen had a lower limit of detection when analysed in basic chromatography and positive ionisation, 0.68 ngL^{-1}, compared to 69.77 ngL^{-1} when analysed in negative ionisation mode. Similarly, diclofenac and estradiol had lower limits of detection in basic chromatography with negative ionisation.

For analytes for which iLODs were above 15 ngL^{-1}, namely ethinyl estradiol, estradiol and estriol, fragment ions from the literature were used instead of those initially found during the optimisation, but this did not yield lower limits of detection, so the ones found originally were kept. As detailed in section 4.3.4.2 lower iLODs were obtained for these analytes using the QTrap instrument. However, due to technical and practical reasons it was not always possible to analyse samples in both instruments.

Ibuprofen was found to be fragmented in-source in the QqQ, which does not allow for control of voltages for fragmentation. It therefore could not be analysed in the QqQ, but it could be analysed in the QTrap. All the data presented in relation to ibuprofen in this thesis was obtained using the QTrap. On an Agilent application note where they used a triple quadrupole to analyse pharmaceuticals in water, ibuprofen was included in the analytes but the collision energy was of 0 V (Werner, et al., 2008). Even though these authors were able to obtain calibration curves for ibuprofen, having a 0 V collision energy indicates the ananlyte needs no energy to fragment (Agilent, 2012), indicating possible in-source fragmentation as was found for this work.

Analyte	Ionisation mode	Parent fragment ions in the QqQ	Fragmentor voltage for the QqQ	Collision energy for the QqQ	Cell accelerator voltage for the QqQ	Parent and fragment ions reported in literature	Reference
4-tert-octylphenol Mr=206.2	Negative	205.2 > 161.0	130	2	4	205.2 > 134.0 205.2 > 133.0	(Henriques, et al., 2010)
		205.2 > 135.0	150	30	4	-	-
Acetaminophen Mr = 151.1626	Positive	152.0 >110.0	113	16	4	152.142>135.1 152.142>110.0	(Parthasarathy, 2016)
		152.0 > 93.0	120	25	7	152.0 > 110.1 152.0 > 65.0	(Mullen, et al., 2017; Zhou, et al., 2017)
Acetyl-sulfamethoxazole Mr = 295.314	Positive	296.0 > 198.0	100	15	6	296.0 > 134.0 296.0 > 198.0	(Le-Minh, et al., 2012)
		296.0 > 134.0	100	15	6	296.068>108.0 296.068>87.0449	(Martínez-Hernández, et al., 2016)
Atenolol Mr = 266.163	Positive	267.1> 190.0	120	14	3	267.1 > 145.1 267.1 > 190.1	(Zhou, et al., 2017)
		267.1> 145.0	120	22	3	267.0 > 145.0 267.0 > 190.0	(Afonso-Olivares, et al., 2017)
Bezafibrate Mr = 361.108	Positive	362.0 > 316.1	100	12	3	No methods were found analysing for this analyte in this mode.	
		362.0 > 138.9	120	18	5		
	Negative	360.0 > 274.0	110	1	7	359.8 > 273.7 359.8 > 153.5	(Afonso-Olivares, et al., 2017)
		360.0 > 153.9	100	16	1	361.132> 275.1 361.132> 274.6	(Parthasarathy, 2016)
Carbamazepine Mr = 236.269	Positive	237.0 > 194.0	130	20	3	237.1 > 194.1 237.1 > 193.1	(Zhou, et al., 2017)
		237.0 > 165.0	130	65	1	237.1 > 194.0 237.1 > 192.0	(Afonso-Olivares, et al., 2017)

Analyte	Ionisation mode	Parent fragment ions in the QqQ	Fragmentor voltage for the QqQ	Collision energy for the QqQ	Cell accelerator voltage for the QqQ	Parent and fragment ions reported in literature	Reference
Clofibric acid Mr = 214.040	Positive	214.8 > 140.8	130	12	6	214 > 128.0 214 > 228	(Öllers, et al., 2001)
		214.8 > 85.1	130	18	1	-	-
	Negative	213.0 > 126.9	85	12	2	213.0 > 127.0 213.0 > 250.1	(Zhou, et al., 2017)
		213.0 > 84.9	70	11	2	214.03 > 127.9 214.03 > 126.9	(Parthasarathy, 2016)
						213.0 > 127.1 213.0 > 85.0	(Martínez-Piernas, et al., 2018b)
Codeine Mr = 299.364	Positive	300.1 > 165.0	180	44	5	300.20> 152.0 300.20> 115.1	(Parthasarathy, 2016)
		300.1 > 199.0	170	32	3	300.0 > 215.0 300.0 > 243.0	(Murphy Huestis Marilyn A., 2005)
						300.0 > 215.0 300.0 >199.0	(Gómez, et al., 2006)
Diclofenac Mr = 296.147	Positive	296.0 > 250.0	70	6	1	295.9 > 214.0 295.9 > 250.0	(Afonso-Olivares, et al., 2017)
		296.0 > 215.0	80	26	1	296.0 > 250.0 296.0 > 214.0	(Mullen, et al., 2017)
	Negative	294.0 > 249.9	45	1	1	294.0 > 250.1 294.0 > 214.1	(Zhou, 2017)
		294.0 > 214.0	45	12	3	294.840>251.0 294.840>215.0	(Parthasarathy, 2016)
Ethinyl Estradiol (EE2) Mr = 296.41	Negative	295.0 > 158.0	100	8	1	295.2 > 144.9 295.2 > 158.8	(Petrie, et al., 2016)
		295.0 > 144.0	200	34	3	295.0 > 145.0 295.0 > 143.0	(Omar, et al., 2017)
Estrone (E1) Mr = 270.366	Negative	269.2 > 145.0	160	32	2	269.0 > 145.0 269.0 > 158.0	(Petrie, et al., 2016)

		269.2> 143.0	160	32	2	269.095>144.9 269.095>142.9	(Parthasarathy, 2016)
Analyte	**Ionisation mode**	**Parent fragment ions in the QqQ**	**Fragmentor voltage for the QqQ**	**Collision energy for the QqQ**	**Cell accelerator voltage for the QqQ**	**Parent and fragment ions reported in literature**	**Reference**
Estrone-3-sulfate (E1-3S) Mr = 350.430	Negative	349.0 > 269.1	110	22	6	371.211>267.1 371.211>349.1	(Parthasarathy, 2016)
		349.0 > 145.1	180	55	4	349.0 > 269.0 349.0 > 145.0	(Zhao, et al., 2014)
Estradiol (E2) Mr = 272.38	Positive	273.1 > 107.0	135	28	3	255.1 > 132.9 255.1 > 159.0	(Galuska, et al., 2013)
		273.1 > 135.0	60	12	1	-	-
	Negative	271.0 > 145.0	180	44	1	271.135>145.0 271.135>182.9	(Parthasarathy, 2016)
		271.0 > 183.0	180	44	1	271.1 > 183.0 271.1 > 144.9	(Petrie, et al., 2016)
Estradiol-3-sulfate (E2-3S) Mr= 352.445	Negative	351.1 > 271.0	175	24	7	351.142>271.3 351.142>144.8	(Parthasarathy, 2016)
		351.1 > 80	70	16	3	351.0 > 271.0 351.0 > 80.0	(Zhao, et al., 2014)
Estriol (E3) Mr = 288.390	Negative	287.1 > 170.8	70	46	1	287.088>170.9 287.088>145.0	(Parthasarathy, 2016)
		287.1 > 145.1	190	46	1	287.4 > 145.4 287.4 > 171.3	(Henriques, et al., 2010)
Estriol-3-sulfate (E3-3S) Mr = 368.44	Negative	367.1 > 287.1	130	20	1	367.0 > 287.0 367.0 > 80.0	(Zhao, et al., 2014)
		367.1 > 79.9	80	48	1	367.0 > 287.0 367.0 > 171.0	(Gorga, 2013)
Erythromycin Mr = 733.93	Positive	734.5 > 158.1	165	28	4	734.5 > 576.3 734.5 > 158.0	(Afonso-Olivares, et al., 2017)
		734.5 > 576.4	150	12	6	734.58 > 158.8 734.58 > 576.4	(Parthasarathy, 2016)

Analyte	Ionisation mode	Parent fragment ions in the QqQ	Fragmentor voltage for the QqQ	Collision energy for the QqQ	Cell accelerator voltage for the QqQ	Parent and fragment ions reported in literature	Reference
Gemfibrozil Mr = 250.33	Negative	249.1 > 121.0	105	2	1	249.113>121.0 249.113>126.8	(Parthasarathy, 2016)
		249.1 > 127.0	80	2	2	249.0 > 121.0 249.0 > 127.1	(Martínez-Piernas, et al., 2018b)
Ketoprofen Mr = 254.281	Positive	255.0 > 209.0	130	8	7	255.0 > 209.0 255.0 > 104.9	(Afonso-Olivares, et al., 2017)
		255.0 > 105.0	125	22	7	255.2 > 105.1 255.2 > 209.2	(Martínez-Piernas, et al., 2018b)
	Negative	253.0 > 209.0	70	1	1	253.0 > 209.0	(Petrie, et al., 2016)
		-	-	-	-	253.0 > 209.2	(Chen, et al., 2010)
Metoprolol Mr = 267.364	Positive	268.1 > 116.1	140	16	4	268.2 > 159.2 268.2 > 116.2	(Martínez-Piernas, 2018b)
		268.1 > 74.1	135	20	7	268.21>116.1 268.219>133.1	(Parthasarathy, 2016)
Morphine Mr = 285.340	Positive	286.1 > 165.0	125	52	5	286.129>152.1 286.129>201.1	(Parthasarathy, 2016)
		286.1 > 181.0	125	36	6	286.2 > 165.1 286.2 > 152.1	(Petrie, et al., 2016)
Naproxen Mr = 230.259	Positive	231.1 > 185.0	100	12	4	231.0 > 185.0 231.0 > 170.0	(Afonso-Olivares, et al., 2017)
		231.1 > 170.0	110	28	1	231.0 > 185.0 231.0 > 116	(Mullen, et al., 2017)
	Negative	228.9 > 185.0	40	3	1	229.097>169.0 229.097>170.0	(Parthasarathy, 2016)
		228.9 > 170.0	40	3	1	229.0 > 185.2 229.0 > 169.1	(Chen, et al., 2010)
Oxytetracycline Mr = 460.434	Positive	461.1 > 426.0	130	10	6	461.3 > 201.1 461.3 > 283.2	(Rocha Guidi, et al., 2018)
		461.1 > 201.0	130	50	6	461.0 > 444.0 461.0 > 426.0	(Lykkeberg, et al., 2004)
	Negative	459.1 > 134.0	10	47	1		

		459.1 > 173.0	10	47	1	No methods were found analysing for this analyte in this mode.	
Analyte	**Ionisation mode**	**Parent fragment ions in the QqQ**	**Fragmentor voltage for the QqQ**	**Collision energy for the QqQ**	**Cell accelerator voltage for the QqQ**	**Parent and fragment ions reported in literature**	**Reference**
Paroxetine Mr = 329.3	Positive	330.1 > 192.1	110	16	6	330.261>192.2 330.261>109.2	(Parthasarathy, 2016)
		330.1 > 70.1	130	28	5	330.3 > 192.2 330.3 > 151.2	(Martínez-Piernas, et al., 2018b)
Phenazone Mr = 188.2258	Positive	189.1 > 56.2	135	32	1	189.188>104.1 189.188>106.0	(Parthasarathy, 2016)
		189.1 > 77.1	130	44	3	189.1 > 77.1 189.1 > 56.0	(Itzel, et al., 2018)
Propranolol Mr = 259.34	Positive	260.1 > 116.1	125	14	5	260.201>116.1 260.201>183.0	(Parthasarathy, 2016)
		260.1 > 183.0	115	14	4	260.0 > 116.2 260.0 > 183.2	(Martínez-Piernas, et al., 2018b)
Salicylic Acid Mr = 138.12	Negative	137.0 > 93.0	150	16	1	136.935 > 93.0 136.935 > 65.1	(Parthasarathy, 2016)
		137.0 > 65.1	150	32	1	137.0 > 93.1 137.0 > 65.2	(Chen, et al., 2010)
Simvastatin Mr = 418.566	Positive	419.2 > 199.2	100	16	3	419.300>199.1 419.300>285.2	(Parthasarathy, 2016)
		419.2 >225.2	100	10	7	419.1 > 285.3 419.1 > 199.1	(Martínez-Piernas, et al., 2018b)
Sulfadiazine Mr = 250.277	Positive	251.0 > 156.0	100	12	4	251.113>156.1 251.113>108.0	(Parthasarathy, 2016)
		251.0 > 92.1	100	20	7	251.2 > 92.1 251.2 > 108.1	(Martínez-Piernas, et al., 2018b)
	Negative	249.0 > 185.0	135	12	5	Have not found a method where this compound is analysed in negative mode.	
		249.0 > 93.9	120	45	4		

Analyte	Ionisation mode	Parent fragment ions in the QqQ	Fragmentor voltage for the QqQ	Collision energy for the QqQ	Cell accelerator voltage for the QqQ	Parent and fragment ions reported in literature	Reference
Sulfamethoxazole Mr = 253.279	Positive	254.0 > 156.0	110	10	5	254.102>156.1 254.102>108.1	(Parthasarathy, 2016)
		254.0 > 108.0	100	24	4	254.06>156.10 254.06 > 91.40	(Baena-Nogueras, et al., 2016)
	Negative	252.0 > 155.9	95	6	4	No methods were found analysing for this analyte in this mode.	
		252.0 > 107.6	95	24	4		
Sulfapyridine Mr = 249.29	Positive	250.0 > 156.0	135	12	6	250.134>156.1 250.134>108.1	(Parthasarathy, 2016)
		250.0 > 184.0	120	12	7	250 > 156 250 > 92	(Le-Minh, et al., 2012)
	Negative	247.9 > 184.0	170	14	6	No methods were found analysing for this analyte in this mode.	
		247.9 > 93.0	170	30	6		
Tramadol Mr = 263.381	Positive	264.2 > 58.25	100	14	6	264.307>246.2 264.307>247.3	(Parthasarathy, 2016)
		264.2 > 246.0	90	4	7	264.0 > 58.0 264.0 > 246.0	(Krizman-Matasic, et al., 2018)
Trimethoprim Mr = 290.32	Positive	291.1 > 230.1	150	20	6	291.184>231.0 291.184>261.9	(Parthasarathy, 2016)
		291.1 > 261.1	150	20	6	291.0 > 230.0 291.0 > 261.0	(Le-Minh, et al., 2012)

Table 4.3-1 Mass spectrometry optimization for QqQ and comparison of precursor and product ions with the literature

4.3.1.2 Chromatography optimization

The gradient was adapted from the two chromatographic methods used in a method developed for the QTrap instrument, as this work had the same analytes (Parthasarathy, 2016). The first method was an acidic chromatography which was paired with positive ionisation. Solvent A was made of 0.1%, v/v, of formic acid in water and solvent B was acetonitrile. The gradient was: 0% B (initial), 100% B (10 min), 100% B (12 min), 0% B (13 min) (Parthasarathy, 2016). The second method had basic chromatography and was paired to negative ionisation mode. For this method solvent A was made of 10mM Ammonium bicarbonate in water and solvent B was acetonitrile. The gradient programme was: 15% B (initial), 100% B (10 min), 100% B (12 min), 15% B (13 min). The mobile phase flow rate was 0.3 $mLmin^{-1}$ (Parthasarathy, 2016). These chromatographic gradients were adapted for the QqQ to increase peak resolution and improve peak shape, as this can have slight variations from instrument to instrument. The gradient was also adapted to ensure there was never 100% organic mobile phase as this can be detrimental to the column. The resulting optimum gradient is the one reported in Section 3.4.1.1 and below Figures 4.3.1 and 4.3.2 depict the two chromatograms for both chromatographic methods. A final improvement was made on the basic chromatography method to improve sensitivity and selectivity by inserting timed MRM acquisition, this means that it is possible to specify which MRM transitions should be monitored at a given time, this increases sensitivity as dwell times per analyte can be increased (Agilent, 2012). The time is based on their retention time ± a tolerance window. This can be visualised in Figure 4.3.2. It was not done for the acidic chromatography as the sensitivity was good enough without it and the peaks were closer to each other.

In the QTrap it is not possible to switch ionisation modes between negative and positive modes for one single chromatographic method. However, for the QqQ it is, once MS conditions had been optimised for each analyte, they were analysed in MRM in both chromatographic methods to assess iLODs and linearity. Some analytes presented a much better signal in either acidic or negative and the steroid hormones were only ionised in the basic chromatography.

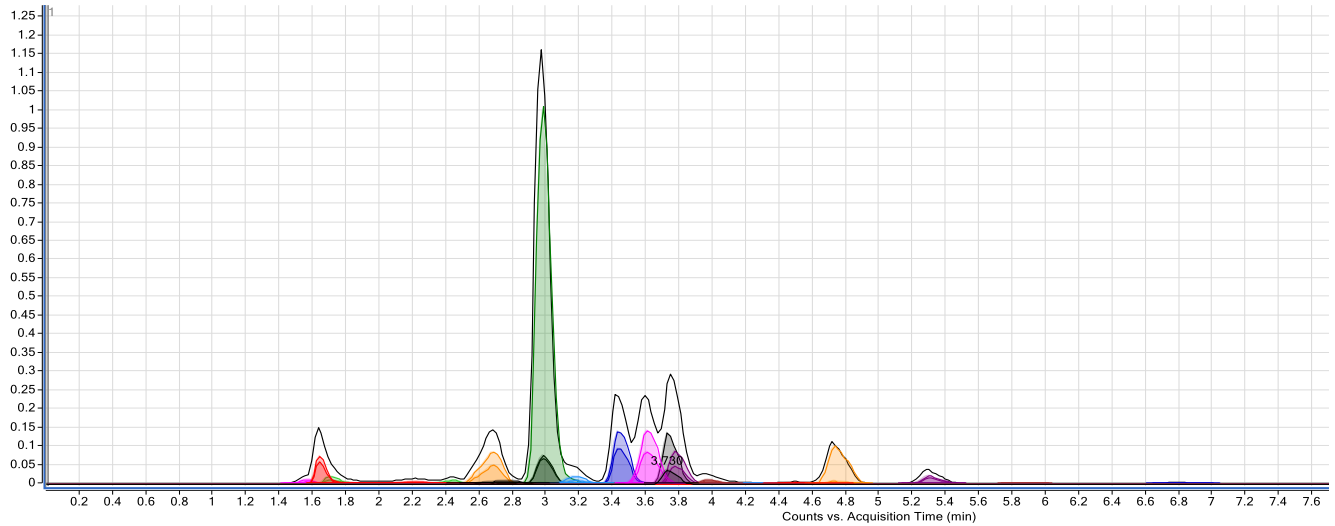


Figure 4.3-1 Acidic chromatography chromatogram for the triple quadruple (QqQ). Y-axis is the intensity in cps x10^6

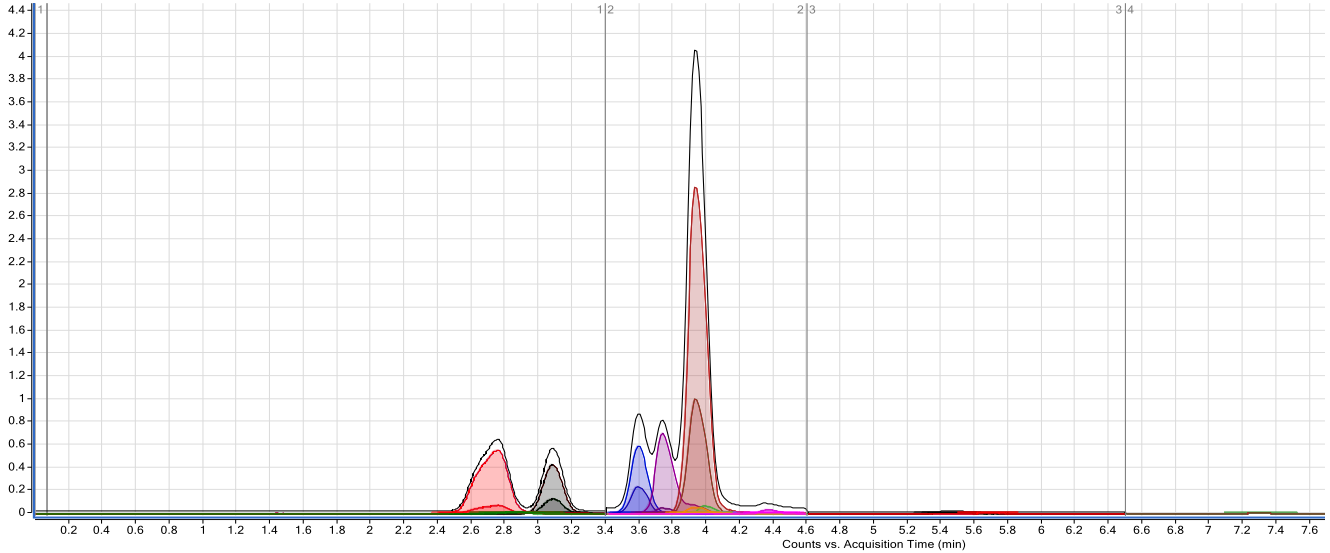


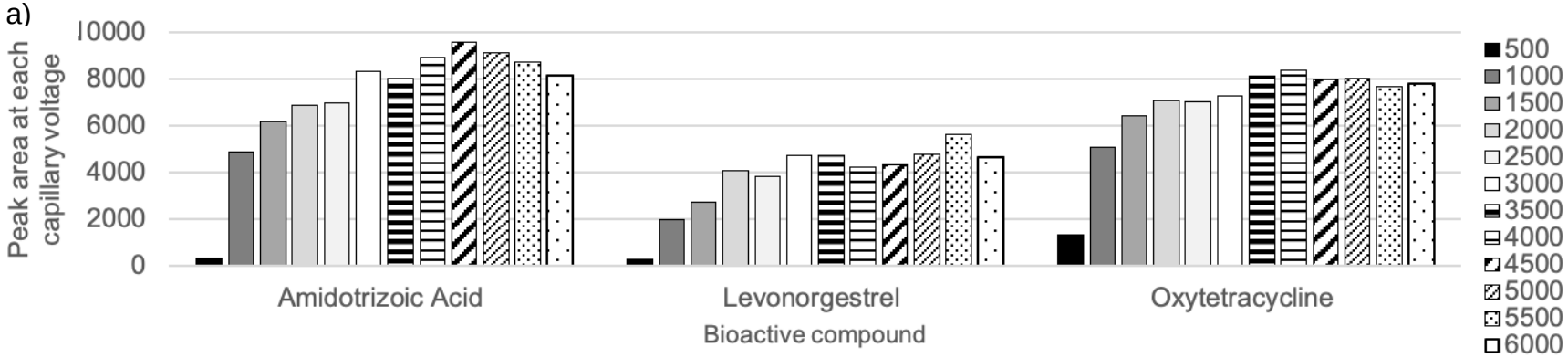
Figure 4.3-2 Basic chromatography chromatogram for the triple quadruple (QqQ) with acquisition windows represented as vertical lines. Y-axis is the intensity in cps x10^4

4.3.1.3 Source optimization

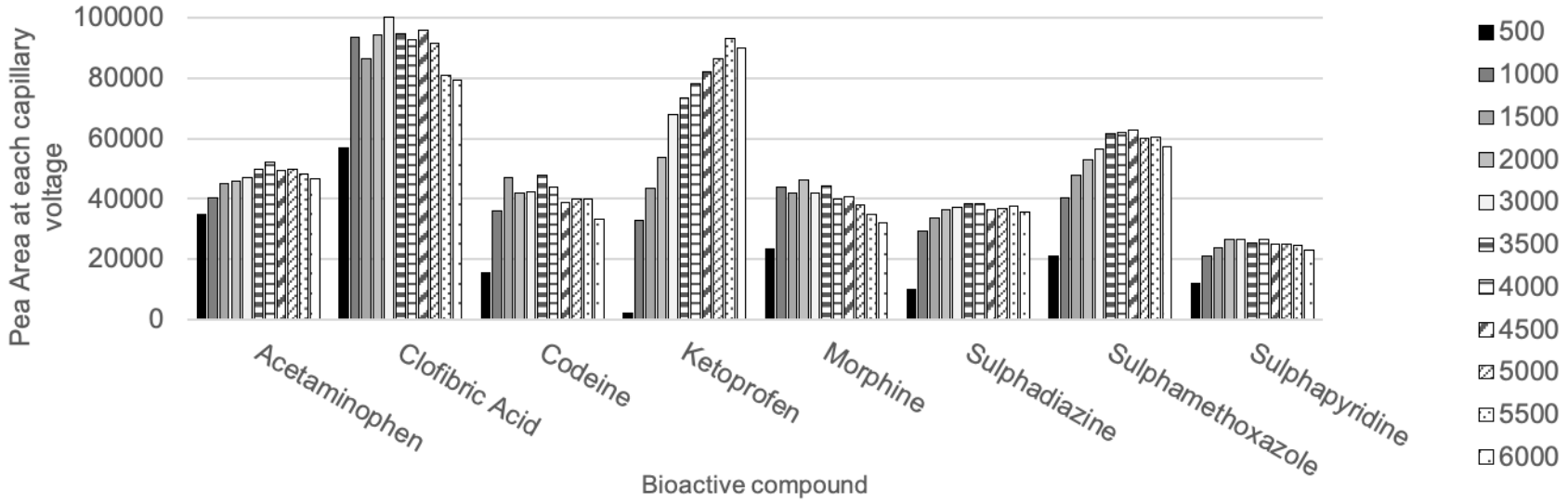
The final optimization step for the LC-MS method were the source parameters (RSC Analytical Methods Committee, 2013). For the QqQ these are: the gas temperature, gas flow, nebuliser pressure, sheath gas heather, sheath gas flow, capillarity and voltage (Agilent, 2012). The analytes were injected in each of the two chromatographic modes through the column with step changes in each of the parameters to determine the optimum value. The peak area obtained for each analyte at each value of each parameter was plotted in a bar graph, along with the other analytes to determine the overall optimum. Below is an example for the capillary voltage in acidic chromatography mode. As the optimum value of each source parameter was different depending on the analyte, those analytes which had shown to have lower sensitivity in the previous stages were prioritised in order to increase their signal.

From Figure 4.3.3 it can be seen that the optimum capillary voltage is highly analyte dependent. Therefore, a compromise had to be made. It is clear that the signal of all analytes decreases with the two most extreme voltages (500 and 6,000) so both of these options were discarded. For the analytes with lower peak area the signal increases from 3,000 V onwards, this is similar for ketoprofen (from the middle figure). Apart from ketoprofen, the analytes in the middle figure have an optimum in the mid-range of capillary between 1,500-5,000, although the exact value varies. For the analytes in the bottom figure the change in signal depending on capillary voltage is less pronounced, however in general the optimum lies again in the mid-range of capillary voltages. Therefore, as a compromise a voltage of 3,000 was chosen. This is similar to the capillary voltage of 3500 used in the Agilent application note using a triple quadrupole for pharmaceuticals analysis, common ones to this method included acetaminophen, codeine, trimethoprim and sulfamethoxazole (Werner, et al., 2008). A similar process was undertaken for the other source parameters and the final values are reported in Section 3.4.1.1.

a)


10000
8000
6000
4000
2000
0
Peak area at each capillary voltage
Amidotrizoic Acid
Levonorgestrel
Oxytetracycline
Bioactive compound
500
1000
1500
2000
2500
3000
3500
4000
4500
5000
5500
6000

b)


100000
80000
60000
40000
20000
0
Pea Area at each capillary voltage
Acetaminophen
Clofibric Acid
Codeine
Ketoprofen
Morphine
Sulphadiazine
Sulphamethoxazole
Sulphapyridine
Bioactive compound
500
1000
1500
2000
3000
3500
4000
4500
5000
5500
6000

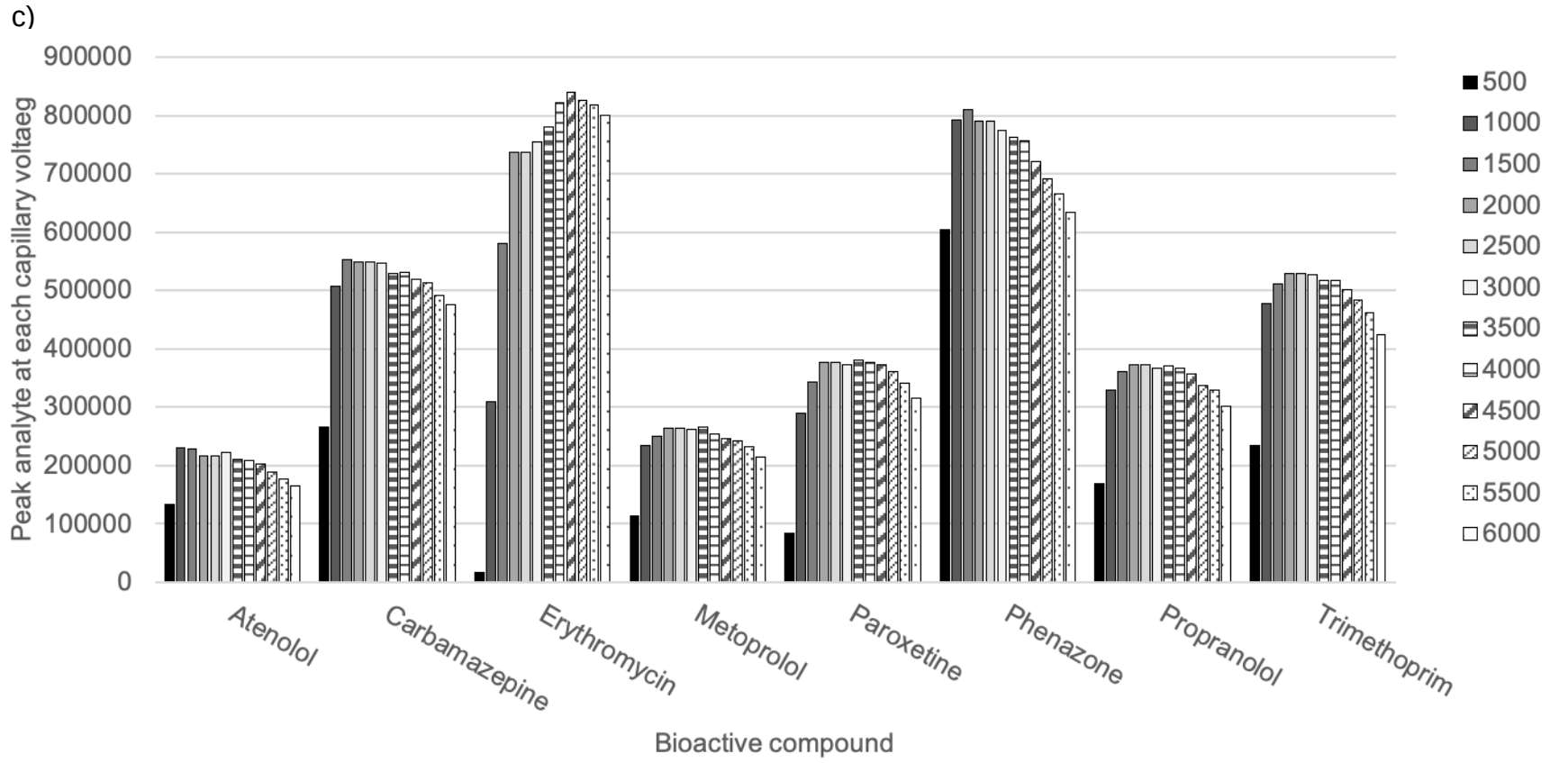


Figure 4.3-3 Peak area per analyte for each capillary voltage in acidic chromatography. a) lowest peak area analytes, b) mid-range peak area analytes,c) highest peak area analytes

4.3.2 QTRAP LC-MS method development

The individual analyte optimisation, source optimisation and chromatography for this instrument were taken from (Parthasarathy, 2016) and are as reported in Section 3.4.1.1. However, a couple of improvements were made in order to enhance repeatability. Scheduled MRM, meaning that each MRM transition is only monitored for at a specific time window in the run, was introduced. The time window for each analyte was the retention time with a tolerance of 20 - 60 s, with 20 s found to be the optimum. A 20 s window for each analyte gave CVs of <15 % for all analytes at each concentration level on repeat (n=5) calibration curve injections. As opposed to when no scheduled MRM was used which gave CVs >15% for most analytes. A shorter time tolerance was not trailed as some peaks had tails longer than 10 s. Similarly, dwell times were optimised, in order to allow for higher sensitivity and repeatability.

4.3.3 SPE optimization

SPE extraction is necessary for both the aqueous and soil matrix extractions. As specified in Section 2.6.2 the most common SPE sorbent types used are MCX (Waters, Herts UK), Strata-X (Phenomenex, Macclesfield UK), and HLB (Waters, Herts UK). These three cartridges were trailed for the analytes selected. The hormone glucuronides recovery was better by more than 10 % with HLB as compared to MCX Better recoveries for pharmaceuticals have been previously observed for HLB compared to MCX (Ngumba, et al., 2016). Oxytetracycline was not recovered by Strata-X, but it was successfully recovered using HLB. The coefficients of variation (CVs) were lower for HLB than for Strat-X. HLB CVs ranged from 0.4 to 9.7 % with an average of 4.1 % for all analytes. While for Strata-X they ranged from 1.5 - 26.0 % with an average of 12.5 %. The conditioning and elution protocol for MCX was taken form (Parthasarathy, 2016) and for HLB and Strata-X the one recommended by the manufacturers was used. The volumes of WWI and WWE were taken from (Parthasarathy, 2016).

Three reconstitution methods were compared:

1) Reconstitution with acetonitrile: water (75/25 v/v)
2) Reconstitution with acetonitrile: water (50/50 v/v)
3) Reconstitution with acetonitrile: water (50/50 v/v) adding the acetonitrile first, vortexing and then adding the water and vortexing again.

The best recoveries were found with option 3, with over 90% of analytes having a recovery above 70%, compared to 82% for option 1 and 76% for option 2. Therefore option 3 was chosen. For the evaporation of the elution solution the Jouan RC10.10 (LabX, Midland, Canada) evaporator was compared to the Genevac EZ-2 (Biopharma Group, Winchester, UK) with no heating and with heating at 30°C. The difference in recovery was not significant between these three methods, with an average of 120.3 ± 34.8 % for Genevac heated, 120.6 ± 35.3 % for Genevac with no heating and 124.0 ± 36.9 % for the Jouan evaporator. However lower CVs were obtained using the Genevac with no heating ranging from 1.8-19.9%, with an average of 6.7 ± 4.5%. Compared to 1.0 - 24.4% and an average of 8.7 ± 6.3 % for Genevac with heating and 1.4 - 26.6% and an average of 6.1 ± 4.9 for Jouan. Evaporation times were about 20 % shorter using the Genevac than Jouan evaporator, therefore the Genevac with no heating method was chosen.

4.3.4 Aqueous method validation for QTrap and QqQ

This work was started using the QTrap instrument, however technical and availability issues with this instrument meant that the method had to be developed in the QqQ too. A comparison was made between both instruments by analysing the same wastewater

effluent (WWE) from Stoke Bardolph WWTP samples on both instruments simultaneously. The samples were exactly the same for both instruments, after reconstitution 500 µL were analysed on each instrument. Most concentrations measured were found to be comparable between both instruments, for example for sulfapyridine 408.3 ± 45.8 ngL^{-1} was measured in WWE using the QTrap and 393.2 ± 3.8 using the QqQ. However, larger CVs were observed using the QTrap, most concentrations CVs are below 10 % for the QqQ and below 15 % for all analytes. For the QTrap they are above 20 % for five analytes. This repeatability issue was one of the main technical issues which lead to most of the analysis being carried out on the QqQ for this work. However, for some analytes, such as 4-tert-octylphenol, and estrone it was possible to measure them in the QTrap but not in the QqQ. Similarly, oxytetracycline, salicylic acid and tramadol could only be measured using the QqQ.

The analytes listed in Table 4.3-2 are those for which either the iLOD, the matrix effects or both had an effect on the quantification of the analyte on one of the instruments. For these analytes the frequency of detection for the sampling campaign in the UK was noticeably lower on the instrument which was not the better one for analysis (Table 4.3.2). The most notable example is ibuprofen, which fragments in source in the QqQ and so it is not possible to quantify it using this instrument, as detailed in Section 4.3.1.1. Other analytes may be better quantified due to lower iLOD or to lower matrix effects in one instrument. Matrix effects are compared in Figure 4.3.4 for Stoke Bardolph and matrix effects for the other matrices studied can be found in Appendix 10.2. 4-tert-octylphenol is better quantified using the QTrap as the iLOD is lower for the QTrap than for the QqQ (10 and 30 $ngmL^{-1}$ respectively, see Table 4.3.2) and matrix effects enhance the signal by about 10% in the QTrap, while matrix effects decrease signal between 10-20% in the QqQ, as can be seen in Figure 4.3.4. For Clofibric acid signal is enhanced in the QTrap and suppressed in the QqQ due to matrix effects (Figure 4.3.4). The same is true for ethinyl estradiol, plus as can be seen in Table 4.3.2, the iLOD is lower on the QTrap than the QqQ. For estradiol even though the iLOD is very similar for both analytes, as can be seen in Figure 4.3.4, matrix effects on the QqQ have a suppression effect of around 80 % on the signal of this analyte, while on the QTrap it is enhanced by around 40 %. Similar effects can be observed for gemfibrozil, levonorgestrel and simvastatin. However, oxytetracycline and salicylic acid could not be quantified using the QTrap due to poor linearity of the calibration curve. Tramadol and phenazone were both better analysed using the QqQ, compared to the QTrap due to a mixture of lower iLODs on the QqQ, (Table 4.3.2) and higher matrix suppression on the QTrap (Figure 4.3.4).

In conclusion the type of instrument can make a significant difference on the analysis, especially for certain analytes. It was possible on several occasions to analyse wastewater samples simultaneously on both instruments in order to get results for as many analytes as possible. However, this was not always possible due to time and resources constraints and so results presented throughput this thesis are for a combination of both instruments. Regardless, the method was validated on both instruments and so all the results presented in this thesis were obtained using a valid method. This also infers how we use data from the literature when multiple instruments have been used, along with differeing extraction approaches and validation. Raising the question of what is comparable and can be translated in time (e.g., seasonal) and/or spatially (e.g., removal efficiencies for a wastewater treatment process). Method metrics are presented in Table 4.3.3 and Table 4.3.4 and discussed below.

Compound	Instrument giving considerably better results	Method metric that was better in the instruments giving better results	Lowest calibration point with a S/N>3 QTrap (ngL^{-1})	Lowest calibration point with a S/N>3 QqQ (ngL^{-1})
4-tert-octylphenol	QTrap	iLOD	10.0	30.0
Clofibric acid	QTrap	Matrix effects	5.0	2.5
Ethinylestradiol (*EE2)*	QTrap	Matrix effects and iLOD	10.0	30.0
Estrone (*E1)*	QTrap	Matrix effects and iLOD	10.0	1.0
Estradiol (E2)	QTrap	Matrix effects	10.0	10.0
Ibuprofen	QTrap	In-source fragmentation in QqQ, this was not the case in QTrap allowing for quantification of ibuprofen with this instrument.	5	-
Gemfibrozil	QTrap	Matrix effects and iLOD	0.5	2.5
Levonorgestrel	QTrap	Matrix effects and iLOD	5.0	10.0
Simvastatin	QTrap	Matrix effects and iLOD	1.0	5.0
Oxytetracycline	QqQ	Fragmentation and linearity	-	10.0
Phenazone	QqQ	iLOD	2.5	0.5
Salicylic acid	QqQ	Fragmentation and linearity	-	5
Tramadol	QqQ	Matrix effects	2.5	0.5

Table 4.3-2 Analyte quantification instrument comparison

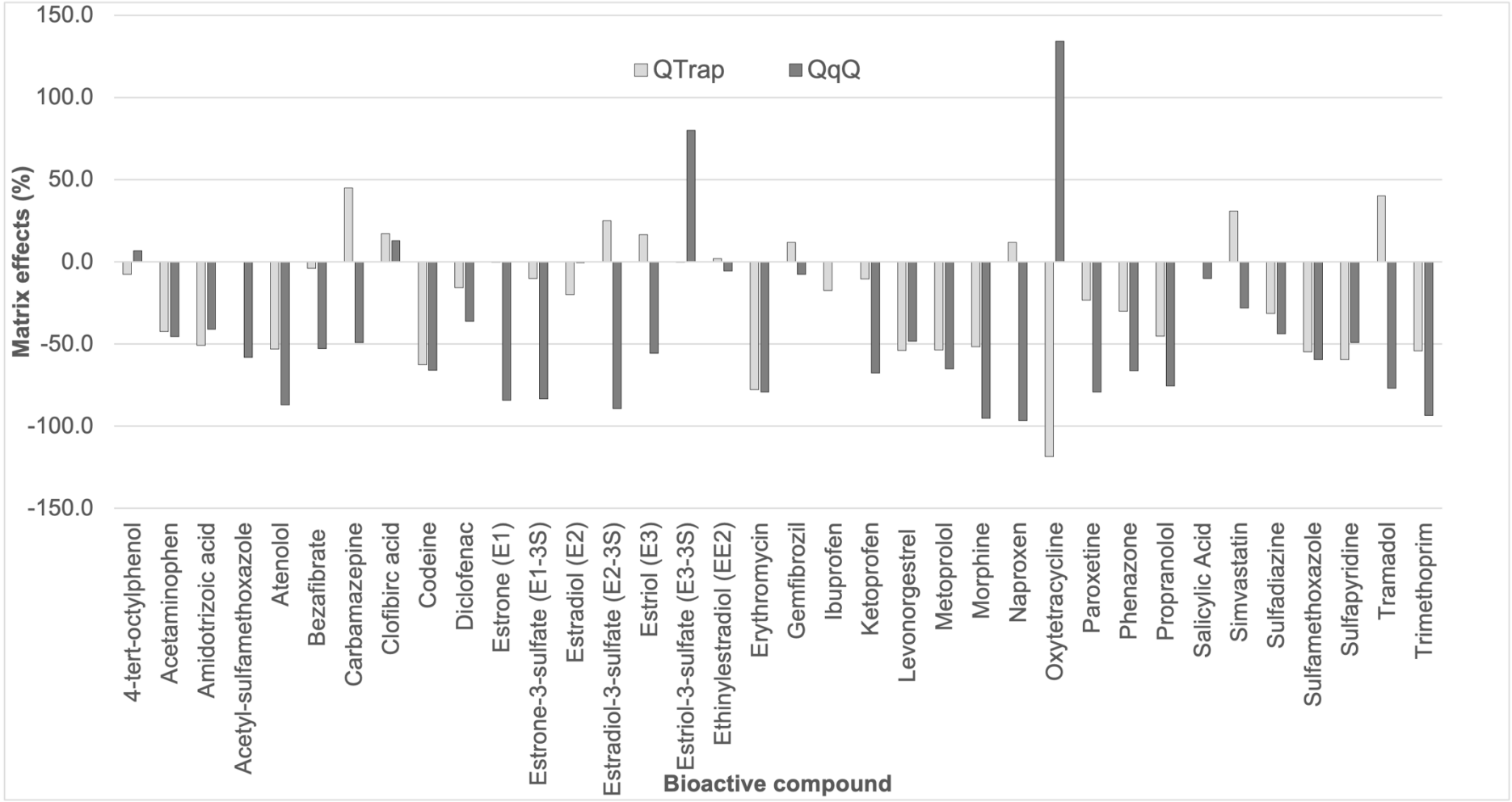


Figure 4.3-4 Matrix effects comparison, exemplar for Stoke Bardolph wastewater effluent presented

As described in Section 3.4.1.2, solvent calibration curve linearity and method recoveries were evaluated on each LC-MS/MS run and used to quantify the samples run on that day. However, for the method validation standard addition curves were made for all four UK matrices studied (Stoke Bardolph wastewater influent and effluent and Sutton Bonington influent and downstream water) and were injected in both QTrap and QqQ instruments. As detailed in Section 2.6.3.4 it has been demonstrated that standard addition calibration curve to be a suitable method to compensate for matrix effects without the use of expensive internal standards (Ngumba, et al., 2016; Rossmann, et al., 2014; Stüber, et al., 2004). Two sets of standard addition calibrations were made on two different days. One day a lower range calibration was made with the following spiked concentrations: 2.5; 10; 20; 50; 100; 200; 500; 1,000; 1,500 ngL^{-1} in both wastewater influents and 0.5; 1; 2.5; 5; 10; 50; 200; 400; 800 ngL^{-1} in Stk wastewater effluent and SuB downstream water. On the other day a higher range with the following concentrations was evaluated: 500; 1,000; 2,000; 4,000; 8,000; 16,000 ngL^{-1} in wastewater influent and 400; 800; 1000; 2,000; 4,000; 6,000 ngL^{-1} in Stk wastewater effluent and SuB downstream water. For each analyte, depending on the concentrations found in wastewater and on the limit of detections of the analyte either the low or high point matrix matched calibration were used for validation. For some analytes there is an overlap, and both are reported. The method limit of detection is the value that has a S/N ratio of 3 and is calculated standard addition matched calibration curve. It is important to note that due to the changing nature of the matrices there is variability in the method limit of detection, given that there is variability in the recovery. This is accounted for when using the recoveries calculated for each sample to calculate the concentrations of the same sample. When two linear ranges are given it is because the calibration had to be split in two ranges as there were two linear dynamic ranges. The intra-day accuracy was evaluated for 1,000 ngL^{-1} in influent wastewater and 400 ngL^{-1} in Stk wastewater effluent and SuB downstream water. It was considered that inter-day accuracy is not relevant as recoveries (i.e. accuracy) were evaluated with each sampling day. Inter-day precision was calculated, as the average coefficient of variation (CV) of pre-spiked samples throughout the whole sampling campaign. Intra-day precision is the coefficient of variation of the pre-spiked recovery samples on one day. Inter-day precision was calculated as the average coefficient of variation for each of the sampling events during the course of the sampling campaign.

Table 4.3.3 and Table 4.3.4 present the combined method metrics for both analytes, with the metrics for the instrument which was better at analysing the analyte presented. However, when samples were analysed just in one instrument the metrics for that instrument were used to evaluate the data and iLODs, method recoveries and repeatability was always calculated for each run. As can be seen in Table 4.3.3, linearity was >0.99 for all analytes in solvent, except for clofibric acid and ethinyl estradiol for which it is >0.95. For most analytes the linearity of the standard addition calibration curve was >0.99 for all or most matrices, (see Table 4.3.3). For 4-tert-octylphenol linearity was below 0.99, but above 0.95 for both influent wastewaters. Lower method metrics have been reported for influents as compared to effluents (Bollmann, et al., 2016; Comtois-Marotte, et al., 2017). Acetyl-sulfamethoxazole had a linearity >0.99 for all matrices except for downstream water of Sutton Bonington WWTP, for which it was 0.9894. However, when the concentration obtained using recovery calculation (Eq. 23 and Eq. 24) and solvent calibrations compared to the standard addition curve the difference between both concentrations was 12.5 %, which is lower than the 20 % considered adequate when comparing matrix-matched calibrations and internal standards (Ngumba, et al., 2016). Similarly, for bezafibrate for which linearity was < 0.99 for both Stk matrices, the difference between the concentration calculations was 7.9 % and 7.5 % for WWI and WWE of Stk respectively. For codeine WWI Stk, the difference was of 0.9 %. For naproxen, which has the lowest

linearities of all analytes, but still >0.9, the percentage difference between the two methods of calculating concentrations was an average of 35.2 % across all matrices. This indicates that results for naproxen should be regarded with caution if this analyte is found to have significant results such as RQs >1. Similarly, for ibuprofen. However other methods in the literature have reported linearity > 0.99 for most of their analytes, with exceptions for a analyte with linearity > 0.92 (Zhe Li, et al., 2018). The matrix linearity for estradiol in Stk WWI was < 0.90, as can be seen in Table 4.2.4, this analyte was therefore not evaluated in this matrix for this thesis. It is difficult to compare the method metrics to other methods as few have done standard addition to wastewater matrices, a study which was found to do standard addition to wastewater influent, effluent and surface water only reported the linearity for surface waters (which was > 0.99 for the antibiotics and antiretroviral drugs studied (Ngumba, et al., 2016). The authors state that comparison between concentrations calculated using internal standard and standard addition curves were > 15 % for both wastewater matrices (Ngumba, et al., 2016).

The mLOD was lower than concentrations reported in wastewater effluents and influents and/or lower than PNECs for most analytes, as can be seen from Table 2.1.1. For some analytes however the mLOD was close to concentrations reported in wastewaters, as is further detailed in Table 5.3.1. In particular 4-tert-octylphenol concentrations have been reported in the range 24-341 ngL^{-1} (Gorga, et al., 2013) and the mLOD for Stoke Bardolph WWI is 22.3 ngL^{-1}, which is close to this range and may account for the lower (<30%) frequencies of detection, as detailed in Table 5.2.1. Similarly, for clofibric acid, estradiol, paroxetine and simvastatin (Table 5.2.1). However, data for these analytes is still reported because it was still measured above the mLOD and this data is valid.

The method recovery accounts for matrix effects, which as shown in Figure 4.3.4 (and Appendix 10.2) can be significant (significant matrix effects are considered > 15% (Ngumba, et al., 2016)). The recovery should allow for an environmentally relevant mLOD, which is mostly the case as previously explained. So as long as recoveries are accounted for the concentration can be accurately calculated (Ngumba, et al., 2016).

In terms of inter and intra-day precision over 70 % of the values are below 15 %, which is within the range reported in the literature (Albero, et al., 2014; Aymerich, et al., 2016; Baena-Nogueras, et al., 2016; Comtois-Marotte, et al., 2017; Gibson, et al., 2007; Chenix Wu, et al., 2012), and the maximum precision value is below the maximum found to be reported in the literature of 48% (Al-Odaini, et al., 2010). Accuracy was in average 103.5 % across all analytes and ranged between 85.8-143 %.

Analyte	iLOD *ngmL^{-1}*	Instrument Linearity (R^2)	Method limit of detection mLOD *ngL^{-1}* (Matrix-matched calibration curve range)				Method linearity (R^2)			
			Stk WWI	***Stk WWE***	***SuB WWI***	***SuB WWD***	***Stk WWI***	***Stk WWE***	***SuB WWI***	***SuB WWD***
4-tert-octylphenol	0.700	0.9982	22.3 (20-1,500)	2.9 (10-1,000)	8.1 (10-40,000)	4.4 (10-2,000)	0.9894	0.9965	0.9536	0.9994
Acetaminophen	2.500	0.9997	0.006 **mgL^{-1}** (1-5 **mgL^{-1}**)	30.5 (50-6,000)	0.006 **mgL^{-1}** (1-5 **mgL^{-1}**)	12.6 (50-400,400-6,000)	0.9922	0.9984	0.9922	0.9974 0.9937
Acetyl-sulfamethoxazole	0.050	0.9974	147.6 (1,000-60,000)	18.5 (10-2,000)	26.4 (500-5,000)	6.5 (50-2,000)	0.9928	0.9960	0.9977	0.9894
Atenolol	0.100	0.9972	3.78 (100-1,500)	11.6 (10-800)	15.8 (100-1,000 , 5,000-40,000)	1.3 (10-2,000)	0.9987	0.9958	0.9803 0.9553	0.9958
Bezafibrate	0.060	0.9912	9.43 (20-1,500)	4.68 (2.5-800)	3.7 (20-1,000, 5,000-40,000)	1.3 (10-2,000)	0.9428	0.9656	0.9994 0.9361	0.9955
Carbamazepine	0.300	0.9976	6.2 (100-5,000, 5,000-60,000)	1.2 (50-400,1,000-2,000)	22.6 (10-1m500)	12.7 (10-800)	0.9996 0.9621	0.9954 0.9992	0.9952	0.9991
Clofibric Acid	7.300	0.9808	21.4 (20-5,000)	12.9 (10-400)	24.0 (20-1,000)	6.0 (10-400)	0.9953	0.9981	0.9943	0.9998
Codeine	0.350	0.9982	103.1 (500-60000)	4.3 (50-2,000)	62.0 (100-5,000)	15.4 (10-2,000)	0.9785	0.9985	0.9996	0.9951
Diclofenac	1.430	0.9960	60.7 (50-5,000)	29.6 (50-2,000)	34.7 (20-5,000)	18.2 (10-2,000)	0.9994	0.9972	0.9997	0.9954
Estrone (*E1)*	0.550	0.9959	44.3 (500-5,000)	10.2 (10-1,500)	11.5 (20-5,000)	7.3 (10-1,500)	0.9988	0.9886	0.9968	0.9985
Estrone-3-sulfate (*E1-3S)*	0.080	0.9968	3.4 (10-1,500)	1.7 (10-2,000)	23.8 (2.5-5,000)	2.6 (40-1,500)	0.9991	0.9982	0.9983	0.9996
Estradiol E2	15.000	0.9982	86.3 (50-1,500)	13.1 (10-400)	100.0 (50-5,000)	8.3 (10-1,500)	0.8812	0.9938	0.9966	0.9962
Estradiol-3-sulfate (*E2-3S)*	0.380	0.9992	5.0 (50-1,500)	3.5 (10-400)	25.8 (20-5,000)	5.0 (10-1,500)	0.9766	0.9863	0.9943	0.9903
Estriol (*E3)*	1.500	0.9906	23.0 (20-1,000)	48.7 (10-2,000)	26.0 (200-16,000)	16.3 (400-6,000)	0.9983	0.9946	0.9947	0.9954
Estriol-3-sulfate (*E3-3S)*	0.500	0.9999	11.6 (20-1,000)	7.5 (2.5-800)	37.3 (50-1,500)	3.2 (0.5-800)	0.9989	0.9489	0.9682	0.9934
Ethynyl estradiol (EE2)	2.300	0.9885	50 (100-15,000)	150 (200-800)	290.3 (100-1,500)	857.1 (400-800)	0.9979	0.9996	0.9998	0.9977
Erythromycin	0.030	0.9999	13.8 (50-1,500)	0.6 (50-6,000)	8.8 (50-16,000)	1.2 (2.5-6,000)	0.9982	0.9990	0.9966	0.9966
Gemfibrozil	1.670	0.9988	10.0 (20-1,000)	3.6 (10-2,000)	23.4 (20-5,000)	7.9 (10-2,000)	0.9995	0.9978	0.9998	0.9972
Ibuprofen	7.300	0.9950	5000 (5000-15,000)	19.7 (10-400,400-2,000)	101.6 (100-40,000)	23.9 (10-1,500)	0.9178	0.9962 0.9486	0.9535	0.9516
Ketoprofen	0.900	0.9964	5.1 (20-500)	4.6 (50-1,500)	8.3 (20-5,000)	1.2 (10-1,500)	0.9983	0.9812	0.9893	0.9960

Analyte	iLOD *ngmL^{-1}*	Instrument Linearity (R^2)	Method limit of detection mLOD *ngL^{-1}* (Matrix-matched calibration curve range)				Method linearity (R^2)			
			Stk WWI	*Stk WWE*	*SuB WWI*	*SuB WWD*	*Stk WWI*	*Stk WWE*	*SuB WWI*	*SuB WWD*
Levonorgestrel	39.130	0.976	300.0 (200-1,500)	44.1 (500-5,000)	103.8 (50-1,000)	150.0 (200-2,000)	0.9906	0.9977	0.9924	0.9982
Metoprolol	0.190	0.9997	7.7 (50-1,500)	3.3 (0.5-800)	5.8 (10-1,500)	1.3 (0.5-800)	0.9999	0.9991	0.9989	0.8141 0.9977
Morphine	0.210	0.9996	220.2 (50- 16,000)	64.1 (10-2,000)	47.5 (50-5,000)	17.1 (10-2,000)	0.9816 0.9971	0.9754	0.9998	0.9944
Naproxen	0.500	0.9971	18.0 (50-2,000)	6.0 (100-5,000)	3.3 (100-40000)	0.6 (10-1,500)	0.9666	0.9477	0.9000	0.9808
O-Desmethylvenlafaxine	0.300	0.9997	16.8 (20-5,000)	11.0 (400-6,000)	15.6 (500-4,000,4,000-16,000)	6.9 (400-1,000 , 2,000-6,000)	0.9991	0.9995 0.9971	0.9993 0.9952	0.9868 0.9953
Oxytetracycline	0.310	0.9442 0.9969	40.8 (500-16,000)	169.6 (400-6,000)	260.9 (200-1,500)	110.0 (10-800)	0.9878	0.9995	0.9676	0.9835
Paroxetine	0.125	0.9971	15.5 (20-200)	15.0 (10-1,500)	13.8 (20-500)	3.3 (10-2,000)	0.9982	0.9913	0.9970	0.9938
Phenazone	0.001	0.9990	9.0 (2.5-1,500)	4.2 (0.5-800)	5.7 (2.5-1,500)	1.4 (0.5-800)	0.9993	0.9989	0.9992	0.9993
Propranolol	0.045	0.9949	16.8 (50-1,500)	2.1 (10-800)	17.0 (10-1,500)	1.5 (10-800)	0.9961	0.9992	0.9993	0.9988
Salicylic Acid	0.375	0.9942	0.001 **mgL^{-1}** (1-5 **mgL^{-1}**)	228.5 (50-800)	0.001 **mgL^{-1}** (1-5 **mgL^{-1}**)	10.2 (10-800)	0.9491	0.9895	0.998	0.9895
Simvastatin	0.109	0.9999	35.9 (20-500)	6.2 (10-2,000)	27.1 (20-1,000)	2.7 (400-2,000)	0.9982	0.9951	0.9665	0.9952
Sulfadiazine	1.500	0.9977	59.3 (20-16,000)	33.3 (50-800)	48.2 (2.5-16,000)	20.8 (10-800)	0.9927	0.9963	0.9842	0.9984
Sulfamethoxazole	1.500	1.0000	10.8 (50-12,000)	5.8 (100-5,000)	39.9 (20-1,000)	8.3 (10-2,000)	0.9993	0.9951	0.9938	0.9972
Sulfapyridine	0.380	0.9998	19.5 (50-500)	28.8 (1000-2000)	19.7 (50-1,000)	6.6 (50-2,000)	0.9992	0.9905	0.9995	0.9899
Tramadol	0.02	0.9972	7.5 (500-16,000)	8.2 (400-6,000)	2.6 (50-1,500)	0.7 (10-800)	0.9939	0.9992	0.9985	0.9989
Trimethoprim	0.04	0.9999	11.8 (50-1,500)	2.68 (10-800)	21.4 (50-1,500)	1.5 (10-800)	0.9913	0.9991	0.9879	0.9978

Table 4.3-3 Method metrics for aqueous method: limits of detection and linearity iLOD: instrumental limit of detection. Stk: Stoke Bardolph wastewater treatment plant, SuB: Sutton Bonington wastewater treatment plant. WWI: wastewater influent. WWE: wastewater effluent. WWD: downstream water

Analyte	Intra-day method precision (%)				Inter-day method precision (%)				Intra-day method accuracy (%)				Recovery (%)			
	Stk WWI	*Stk WWE*	*SuB WWI*	*SuB WWD*	*Stk WWI*	*Stk WWE*	*SuB WWI*	*SuB WWD*	*Stk WWI*	*Stk WWE*	*SuB WWI*	*SuB WWD*	*Stk WWI*	*Stk WWE*	*SuB WWI*	*SuB WWD*
4-tert-octylphenol	5.4	12.5	15.3	13.8	12.1	14.9	28.7	11.8	136.2	99.1	109.8	95.6	70.6 ± 4.9	57.7±4.1	68.0±4.4	50.1±10.0
Acetaminophen	2.9	1.0	1.0	2.2	28.0	9.9	5.8	6.4	104.7	100.5	104.7	100.5	23.3 ± 0.7	2.9 ± 1.2	44.5±2.6	150.4± 3.0
Acetyl-SMX	2.1	7.4	5.9	5.6	9.5	8.0	10.0	9.3	100.1	103.0	100.0	111.5	37.0 ± 2.3	5.7 ± 8.3	34.0±4.4	36.6± 4.3
Atenolol	1.4	6.7	5.4	2.7	2.5	8.1	6.3	6.4	114.7	99.3	96.8	99.6	70.3 ± 6.3	58.2± 6.4	71.4± 5.3	67.6 ± 3.8
Bezafibrate	2.2	0.9	7.8	3.8	9.3	8.3	37.7	24.2	100.8	107.5	90.0	104.3	87.8 ± 5.0	3.3 ± 8.7	3.6± 2.3	99.7 ± 4.9
Carbamazepine	2.4	3.8	8.3	7.9	10.9	9.1	10.6	12.2	99.9	100.5	114.3	113.7	80.6 ± 5.2	7.1 ± 1.6	6.3± 4.3	72.3 ± 16.3
Clofibric acid	1.6	1.1	3.9	10.1	12.2	8.4	15.8	11.7	100.1	92.6	94.3	108.4	89.5 ± 9.3	72.7± 4.2	88.6±9.4	77.6 ± 6.6
Codeine	4.3	11.0	3.3	4.1	17.9	13.6	6.9	9.2	131.9	112.0	100.0	113.2	55.2 ± 4.2	57.8± 4.3	56.9±11.5	67.9 ± 8.5
Diclofenac	2.7	6.7	4.6	5.5	11.2	11.5	6.0	6.0	100.1	106.3	99.9	105.1	51.4 ± 4.6	52.5± 8.6	57.8±9.6	55.4 ± 8.1
Estrone (*E1)*	8.0	10.3	12.7	5.5	16.1	19.2	18.2	4.8	99.8	104.7	85.2	103.1	16.7 ± 0.6	18.0± 0.3	11.1± 0.5	13.0±0.9
Estrone-3-sulfate (*E1-3S)*	3.7	7.6	11.3	8.6	14.8	12.0	11.1	16.6	100.0	103.7	88.4	104.4	86.5±14.2	91.2 ± 6.0	84.7±20.2	4.4 ± 4.9
Estradiol E2	-	-	24.0	-	-	24.0	-	-	112.1	103.4	94.6	100.3	58.0 ± 1.3	84.9 ± 2.1	50.6±2.4	3.8 ± 5.7
Estradiol-3-sulfate (*E2-3S)*	5.2	22.2	21.5	16.9	10.0	22.2	2.6	17.1	104.3	105.2	88.7	110.0	118.3 ± 7.9	13.3±10.1	113.0 ±5.2	126.3±23.0
Estriol (*E3)*	3.9	0.5	4.5	11.2	16.4	11.7	16.5	8.8	99.9	99.6	100.0	105.7	72.3 ± 1.6	109.5±45	92.6±9.5	85.6 ± 6.5
Ethynyl estradiol (EE2)	-	-	-	-	1.7	14.4	3.1	6.5	-	-	-	-	89.7 ± 5.3	78.0±2.9	87.0±3.6	82.2±1.5
Estriol-3-sulfate (*E3-3S)*	4.4	3.1	5.0	4.0	20.2	40.9	8.4	6.4	100.0	106.6	99.8	114.5	94.3 ± 7.6	78.0 ± 5.0	94.7±9.7	83.0±3.9
Erythromycin	2.6	5.4	5.9	9.5	15.5	10.3	5.7	8.8	99.9	101.1	100.1	112.4	27.6±11.6	46.8 ± 3.8	27.1±8.1	12.8±0.8
Gemfibrozil	10.7	3.6	1.8	10.6	10.7	5.4	10.6	14.3	104.7	105.6	99.9	104.5	74.4±2.4	77.1 ± 1.7	8.5 ± 3.6	3.2 ± 2.4
Ibuprofen	18.8	3.2	12.5	7.3	30.0	14.1	15.8	5.7	100.0	100.5	133.8	105.6	15.1± 3.2	86.4± 10.4	2.1 ± 0.4	45.5± 3.7
Ketoprofen	13.3	4.0	12.2	28.6	16.1	17.1	7.0	28.6	100.0	103.4	85.8	113.9	101.1±29.9	97.4 ± 9.4	102.1 ±5.5	95.4±15.3
Levonorgestrel	29.5	20.4	-	-	9.6	41.4	-	-	100.3	107.9	99.6	113.5	41.3 3.6	36.2 ± 1.4	1.6 ± 5.2	40.1±3.2
Metoprolol	3.8	6.0	0.4	7.2	21.1	7.7	5.8	6.4	99.8	106.9	98.3	110.6	53.0±5.9	52.1 ± 4.2	55.9±8.8	63.1±5.1
Morphine	14.2	5.9	5.0	4.0	20.4	12.7	11.9	6.4	100.0	104.7	99.9	105.2	55.0±2.2	3.4 ± 7.1	59.0±8.6	85.6± 3.7
Naproxen	5.1	8.0	11.5	11.3	21.0	10.2	9.4	9.3	104.9	111.2	143.8	116.5	103.7±4.1	137.5± 18.0	70.3±12.3	139.3± 4.2
O-desmethylvenlafaxine	2.3	3.3	1.1	1.1	6.0	3.9	2.0	6.5	99.8	97.9	99.8	112.5	21.9± 1.3	25.2± 1.1	22.8 ± 1.4	28.3± 1.3
Oxytetracycline	3.7	5.1	5.1	2.7	7.0	4.5	10.5	8.5	118.9	99.8	117.5	113.5	15.± 0.9	23.4± 3.0	30.5± 2.6	9.1 ± 2.3
Paroxetine	3.4	11.2	10.0	3.5	17.5	19.7	10.0	7.0	100.0	106.9	100.2	94.4	43.1± 9.6	37.9± 17.8	38.6± 5.2	37.9 ± 9.8

Analyte	Intra-day method precision (%)				Inter-day method precision (%)				Intra-day method accuracy (%)				Recovery (%)			
	Stk WWI	*Stk WWE*	*SuB WWI*	*SuB WWD*	*Stk WWI*	*Stk WWE*	*SuB WWI*	*SuB WWD*	*Stk WWI*	*Stk WWE*	*SuB WWI*	*SuB WWD*	*Stk WWI*	*Stk WWE*	*SuB WWI*	*SuB WWD*
Phenazone	33.1	4.5	3.8	3.2	33.1	10.3	20.7	8.3	100.0	111.8	99.9	113.9	67.9 ± 3.5	71.4 ± 2.1	63.5 ± 8.5	71.9 ± 1.4
Propranolol	2.9	3.7	2.1	2.1	11.3	7.1	13.2	6.5	99.8	100.5	100.2	113.9	55.2 ± 4.2	56.4 ± 2.3	52.7 ± 6.4	63.4 ± 4.4
Salicylic acid	9.0	5.6	1.0	12.7	11.7	26.0	14.7	16.8	101.5	89.9	101.5	111.5	99.1± 0.28	16.8 ± 3.6	21.0± 6.8	37.3 ± 4.7
Simvastatin	2.3	0.9	12.8	1.5	34.0	2.4	20.3	2.1	100.0	111.9	99.9	100.8	26.4 ± 2.9	15.6 ± 3.8	50.9 ± 3.4	72.2 ± 3.0
Sulfadiazine	4.0	4.3	2.2	8.1	17.7	10.2	9.1	7.9	99.9	97.9	99.8	108.4	24.0 ± 6.4	24.3 ± 2.4	23.2 ± 8.1	24.0 ± 6.9
Sulfamethoxazole	1.6	9.3	4.5	3.8	11.4	9.9	6.5	7.7	99.9	105.8	100.0	110.3	32.6 ± 2.2	32.8 ± 1.6	29.6 ± 3.4	31.5 ± 1.8
Sulfapyridine	1.2	9.0	1.9	4.6	12.6	11.2	17.3	26.2	100.0	99.1	99.7	102.9	37.4 ± 4.6	30.3 ± 4.6	33.6 ± 3.1	5.0 ± 1.7
Tramadol	3.0	2.7	0.7	0.3	14.2	5.7	6.4	6.5	99.9	93.1	99.9	109.8	72.3 ± 5.4	60.1 ± 2.9	77.7± 0.6	80.9 ± 7.3
Trimethoprim	2.8	3.6	0.6	1.5	13.1	6.2	0.1	10.2	99.9	115.9	99.8	108.4	46.4 ± 8.5	43.6 ± 4.0	60.6 ± 6.3	57.4 ± 6.4

Table 4.3-4 Aqueous method metrics: precision, accuracy and recoveries

Stk: Stoke Bardolph wastewater treatment plant, SuB: Sutton Bonington wastewater treatment plant. WWI: wastewater influent. WWE: wastewater effluent. WWD: downstream water

4.3.5 Soil extraction method validation

4.3.5.1 Solid-liquid extraction

Even though the largest expense in environmental BAC analysis is the LC-MS instrument, operating costs can be a deterrent once this expense has been addressed. Alternatively, samples can be sent from an area with no access to an LC-MS instrument to a laboratory where there is one. Therefore, a simple solid-liquid extraction was chosen over a more complex sample processing as it is easy to implement in any laboratory with minimum equipment, has low cost and proven efficiency (Chuang, et al., 2015; Martínez-Piernas, et al., 2018b). This is of particular importance when undertaking international collaborations.

Solid-liquid extraction was trialled with solvents identified from the literature including acetone, acetonitrile and methanol (Azanu, et al., 2016; Calderón-Preciado, et al., 2009a; Christou, et al., 2017; Goldstein, et al., 2014; Marsoni, et al., 2014; Martínez-Piernas, et al., 2018b; Petrie, et al., 2017; Riemenschneider, et al., 2017). Methanol was selected because all analytes are stored in methanol and therefore tested to be soluble in methanol even at concentrations of 1 $mgmL^{-1}$. However, as some of the analytes have pK_a <5 or pK_a >9, they would be better extracted with acidic or basic solutions. Therefore, a three-stage extraction to include a solvent (methanol) stage for analytes with a high Log_{Kow} and then a basic and acidic stages for ionizable analytes was designed. The compositions of the second and third extractions were determined from the point at which most pollutants elute from the LC column in the basic and acidic chromatography methods respectively. It was hypothesised and proved by preliminary trials that it was best to start with the solvent extraction, followed by the basic extraction and ending with the acidic extraction. This is because the basic solution is closer to pH 7 than the acidic solution, therefore this order ensures the pH of one extraction does not affect the next. The recovery when performing basic extraction before the acidic extraction was higher for 65.6% of the analytes, so this order was used.

Preliminary work was undertaken to ensure that the spiking volume to soil mass ratio (1 mL: 1 g) ensured a homogeneous mixture by comparing different liquid:solid ratios. These ranged 0.1:1, 0.5:1 and 1:1, based on (OECD, 2000) and visually ensuring the liquid was covering all of the soil.

4.3.5.2 Ageing periods

Twenty-eight analytes presented a significantly different ($p<0.05$) recovery at one of the five ageing periods (4, 24,72 h and 7 and 14 days) compared to that observed when there was no ageing. Analytes showing no significant effect on recovery as an effect of ageing periods were acetaminophen, estradiol-3-sulfate, levonorgestrel, naproxen and oxytetracycline. The results plotted in Figure 4.3.5 consider the recovery obtained at 0 h aging as 100% for each analyte and show the significant ($p<0.05$) percentage differences between this recovery and the recovery obtained at each ageing period. It is clear that for most analytes there is an effect of ageing on method recovery and this effect is analyte dependent. This is very significant in terms of method development and the determination of environmental concentrations.

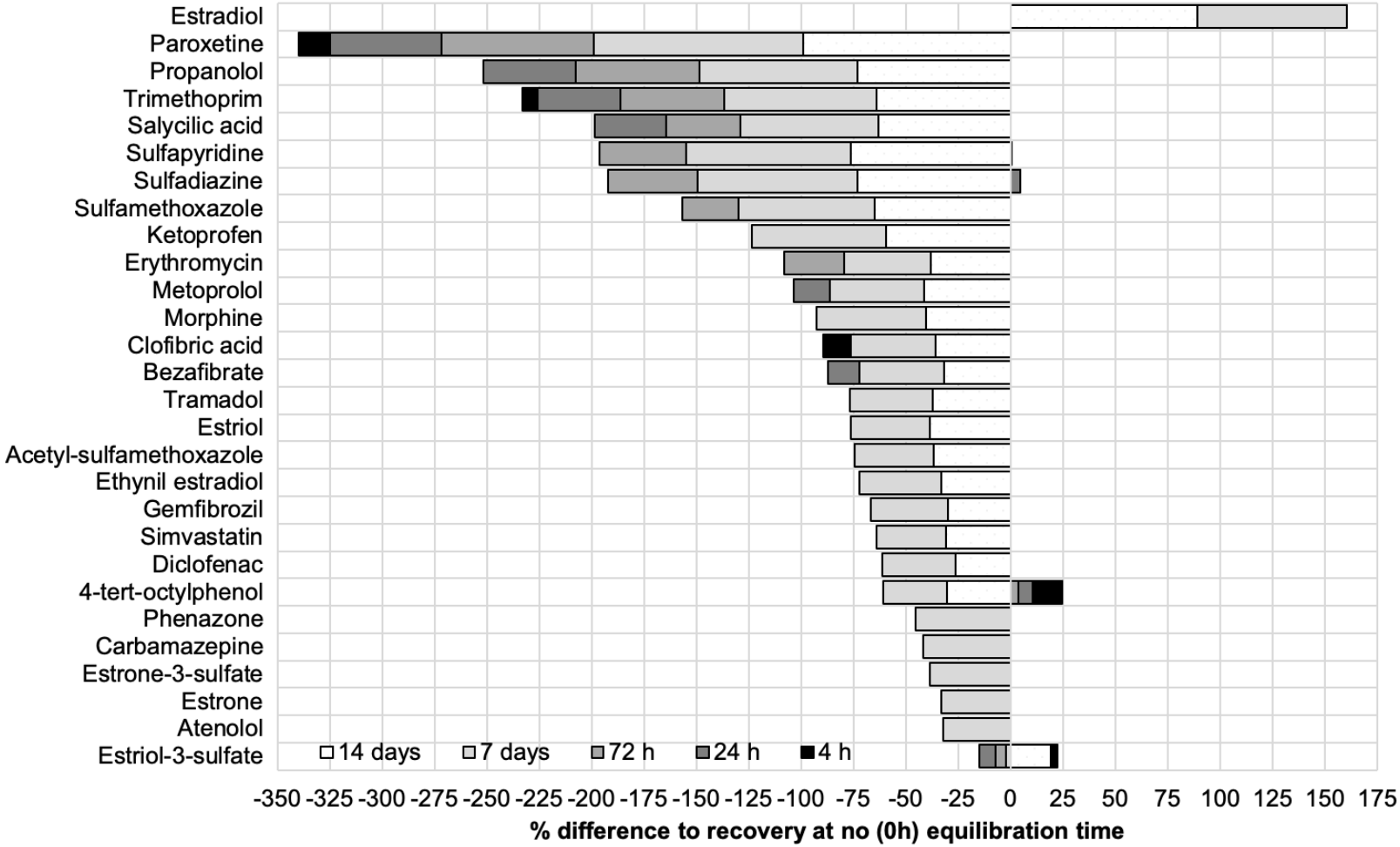


Figure 4.3-5 Effect of ageing time on recovery: percentage difference of recovery obtained at each ageing period compared to the recovery obtained with no ageing period. Only results with ($p<0.05$) significance presented

Using recoveries determined with no ageing period can lead to an over-estimation of recoveries, and therefore an under-estimation of the actual environmental concentrations (Michael, et al., 2017). This is exemplified using sulfamethoxazole, which is the analyte for which the majority of ageing studies have been undertaken in the literature (Hamscher, et al., 2005; Larivière, et al., 2017). The maximum concentration of sulfamethoxazole measured in soil is cited by (Cycoń, et al., 2019) as 54.5 μgkg^{-1} from a study carried out in Chinese agricultural soils (Li, et al., 2011). In this study no ageing period is specified for the recovery calculation and so it is assumed that no ageing period was used. A series of calculations were done in order to calculate approximate values of how different the concentration would be if the recovery had been calculated using an ageing period. In the work done for this thesis no significant difference ($p<0.05$) was found between the aging periods of 4 and 24 h compared to 0 h for sulfamethoxazole. This is similar to the results obtained by (Hamscher, et al., 2005), as can be seen in Table 4.3.5. In this study they compared the recovery of sulfamethoxazole with a 5 min ageing period (42.5 ± 3.9), 10 min (51.6 ± 1.9), 30 min (43.1 ± 6.2), 240 min (33.8 ± 0.8), 1 day (38.2 ± 4.7), 3 day (42.5 ± 3.9), 7 day (22.9 ± 2.7) (Hamscher, et al., 2005). By performing a two tailed hypothesis test ($p<0.05$) on this data, it was found that they (Hamscher, et al., 2005) also obtained a significant difference ($p<0.05$) in recovery after 3 days, compared to their lowest ageing period of 5 min. The other study which looks at ageing periods for sulfamethoxazole compared 90 min (80 ± 0.2), 6 days (50 ± 0.5), 17 days (35 ± 0.1) (Stoob, et al., 2006), these values are approximate because they are provided in a bar chart in the paper and not as numerical values. Given that this second study does not have a recovery using no ageing period, or a very low ageing period like the 5 min (Hamscher, et al., 2005) it could not be used for two-tailed hypothesis test to find which ageing period had a significant difference compared to no ageing period.

Spiked concentration of sulfamethoxazole (μgkg^{-1})	Recovery	Ageing period	Reference
50	42.5 ± 3.9	5 min	(Hamscher, et al., 2005)
50	21.8± 2.1	3 days	(Hamscher, et al., 2005)
100	87.95± 6.0	0 mins	The work for this thesis
100	64.41±7.9	3 days	The work for this thesis
50	71.6±4.3	Not specified, assumed none	(Li, et al., 2011)

Table 4.3-5 Comparison of recoveries obtained for sulfamethoxazole at none or very short ageing periods with those obtained at three day ageing periods in different studies

Taking the 5 min recovery from (Hamscher, et al., 2005) and the 0 min recovery from this work as 100% recovery (in order to be able to compare between all of the different studies), the equivalent percentage for the recovery obtained at the 3 day ageing periods were calculated as 51.3% for (Hamscher, et al., 2005) and 73.2% for my work, as summarised in Table 4.3.6. This was then applied to the concentration presented by (Li, et al., 2011) to obtain a range of concentrations which may be closer to the real environmental concentrations as presented in Table 4.3.6, because of the importance of calculating the recovery using an ageing period (Michael, et al., 2017). It can be seen from Table 4.3.6, that using the recovery obtained with no ageing the concertation reported of 54.5 μgkg^{-1} is obtained (Li, et al., 2011). However, from the data obtained from (Hamscher, et al., 2005), it was calculated that the recovery decrease from no ageing period (42.5 ± 3.9 %) to an ageing period of 3 days (21.8± 2.1 %) (Hamscher, et al., 2005) was 51.3 %. A 51.3 % recovery decrease to the recovery obtained with no ageing period of 71.6 ± 4.3 % (Li, et al., 2011) equates to a recovery of 36.7 ± 5.4

%. The 54.5 μgkg^{-1} (Li, et al., 2011) concentration reported would have been measured as 39.0 μgkg^{-1}, before the recovery correction of 71.6 ± 4.3 % (Li, et al., 2011) was applied. Therefore, using the recovery of 36.7 ± 5.4 % on 39.0 μgkg^{-1} the concentration obtained would be 106.2 ± 15.5 μgkg^{-1} instead of 54.5 μgkg^{-1}. The same process was undertaken for the percentage difference between a no ageing period and an ageing period of 3 days obtained for this work, as can be seen in Table 4.3.6. Similarly, it was done for the average percentage difference between the difference in recovery between 0 h and 3 day recovery for this work and for 5 min and 3 day for (Hamscher, et al., 2005). As can be seen in Table 4.3.6, considering the decrease in recovery when an ageing period is used for sulfamethoxazole can result in up to a two-fold underestimation of the environmental concentration.

Environmental concentration of sulfamethoxazole in soil (μgkg^{-1})	Recovery	Explanation
54.5	71.6 ± 4.3	Original recovery used in study and concentration reported (Li, et al., 2011)
106.2 ± 15.5	36.7 ± 5.4	Using the equivalent 3 day recovery calculated from (Hamscher, et al., 2005), which was 51.3% of the recovery at 5 min. 51.3% of 71.6% is the 36.7% used for this calculation
87.5 ± 17.7	44.6 ± 9.0	44.6% is an average of the recoveries used in the rows above and below.
74.5 ± 11.3	52.4 ± 7.9	Using the equivalent 3 day recovery calculated from my work, which was 73.2% of the recovery at no ageing period. 73.2% of 71.6% is the 52.4% used for this calculation

Table 4.3-6 Approximate environmental concentrations that would be obtained for the (Li, et al., 2011) *study if recoveries were calculated using an ageing period*

The concentrations presented in Table 4.3.6 are only an approximation because other factors such as the soil types and extraction methods are not the same across the three studies compared. However, this is still evidence that the effect of ageing should be considered during method development to avoid large underestimations of environmental concentrations.

Across all analytes the aging periods which have the most effect on recovery were 7 and 14 days (Figure 4.3.5). These periods were added as a comparison with sorption studies and are not in the practical range for ageing. It is possible analytes are being abiotically degraded and biodegraded by external microorganisms colonizing the soil after the initial autoclaving after this period (Martínez-Hernández, et al., 2016; Xu, et al., 2009). However, acetaminophen recovery was not affected by ageing periods and in another study it was shown that its decreasing concentration in soil was mainly due to biodegradation between one and 4 days (Martínez-Hernández, et al., 2016). This indicates that biodegradation, at least for acetaminophen, was minimal in this work. On the other hand 4-tert-octylphenol concentration decrease in soil concertation between 1 and 20 hours was mainly attributed to biodegradation (Ying, et al., 2005). This study observed a decrease only after 7 days ageing, which may indicate some biodegradation after this period. Carbamazepine, which is known to be very persistent and resistant to biodegradation (Paz, et al., 2016), only presented a decrease in recovery at the 7 day ageing period. Given the uncertainty regarding biodegradation with ageing periods above 7 days (Martínez-Hernández, et al., 2016; Xu, et al., 2009) and the practicality, as well as previous recommendations for ageing periods of around 24 h (Larivière, et al., 2017) the 4, 24 and 72 h ageing periods were further considered.

Thirteen analytes presented a significant difference in recovery in at least one of the 4, 24 or 72 h periods. However, for 90% of the analytes the recoveries were not significantly different when extraction was done immediately after spiking and when extraction was done 4 h after, showing that this period is not enough for sorption to occur. The recoveries obtained at 24 and 72 h were compared and no significant difference ($p < 0.05$) between the recoveries obtained at these two periods was found. These findings support the 24 h ageing period as an adequate minimum (Drillia, et al., 2005a; Larivière, et al., 2017; Martínez-Hernández, et al., 2016; Xu, et al., 2009). In an ideal world it would be good to test the recovery of each analyte in the environmental soil where it is being deterimined knowing how long it has been there and performing the ageging study with the same amount of time in a sterile environment (to rule out

biodegrdadtion completley). However, this is at best impractical and often impossible because often it is not known how long pollutants have been in the soil and if it is known it could be periods of months or even years. Therefore, these results show that an ageing period of at least 24 h can give a more realistic recovery, which accounts for sorption time process (Michael, et al., 2017) without the possibility of biodegrdation, however this needs to be evaluated in a case-by-case basis, as previosuly demosntrated a period of 3 days is more adequate for sulfamethoxazole.

Four-tert-octylphenol, estradiol and estriol-3-sulfate presented the opposite trend from that observed for the prior pollutants. Recovery increased with increasing aging periods instead of decreasing. Estrogenic analytes, have been shown to degrade into other estrogenic analytes which could explain this trend for estradiol (Tang, et al., 2019). However, conversion of ethinylestradiol and estradiol to estrone in soils has been wildly reported in literature (Tang, et al., 2019; Ying, et al., 2005) and here the trend observed is an increase in estradiol not in estrone. It is not clear what could have caused it. Desorption of these pollutants found endogenously in the soil prior to spiking is very unlikely as the soils were tested to rule this out.

4.3.5.3 Effect of different soil matrices on recovery

To test the validity of the soil extraction method developed across different soil types and to see the effect of soil properties on recovery, a study was done using real agricultural field soils from Bunny (see Table 4.3.7).

Soil type	Sand (%)	Clay (%)	Silt (%)	pH
SAND	91.9	7.6	0.5	N.A.
LOAMY SAND	83.2	4.7	12.1	6.35
CLAY LOAM	31.1	34.5	34.3	6.91

Table 4.3-7 Soil properties of Bunny field soils (as stated in Table 3.3.1, summarised in this section for ease).

Eighteen out of 33 analytes showed a significant difference ($p<0.05$) in recovery depending on soil type and these are shown in Figure 4.3.6. These differences in method recovery stress the importance of using matrix-specific recovery (United States Environmental Protection Agency, 1999). However, this is not always the case, as sometimes recoveries which are an average of those obtained in different soils are used. For instance recoveries reported as an average of three soils (Li, et al., 2011).

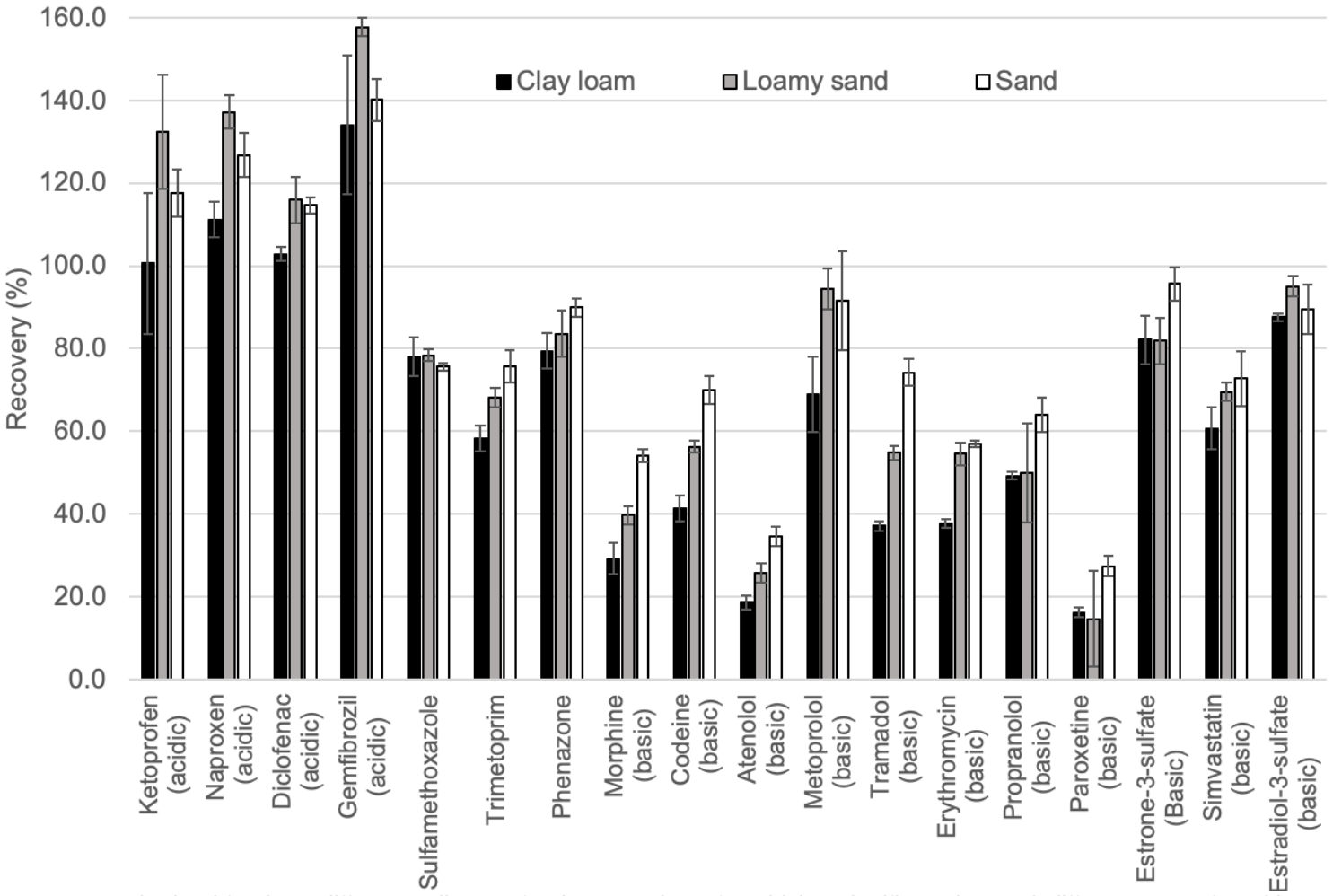


Figure 4.3-6 Recovery obtained for three different soil types for those analytes for which a significant ($p<0.05$) difference was found between at least two soil types

The four acidic analytes presented in Figure 4.3.6, have the highest recovery, out of three soils studied, in loamy sand, followed by sand and the lowest recovery in clay loam. For these pollutants, recoveries increase with decreasing clay content. In soils with pH close to 7, acidic pollutants ($pK_a < 7$) will be positively charged ions and so will have a higher attraction to the negatively charged clay and organic matter particles (Lorphensri, et al., 2007), meaning that recovery of acidic pollutants in soils with higher clay and/or organic matter content, like the clay loam, is expected to be lower. This coincides with previous observations in which sulfamethoxazole recovery was observed to decrease more with aging times in soils with higher clay content (Larivière, et al., 2017).

For basic pollutants ($pK_a > 7$), the log K_{ow} will play a more significant role in determining the affinity of the pollutant to the soil given that charge will not play an important role (Lorphensri, et al., 2007). However, no relationship between the log K_{ow} and the average recovery could be observed. For most of the basic pollutants, it was found that recoveries were highest for the sand, followed by the loamy sand and the clay loam had the lowest recoveries. Sand particles are larger and there are fewer binding places for pollutants (Durán–Álvarez, et al., 2014; Hu, et al., 2019). This exploratory work demonstrates that recovery is dependent on ageing periods and soil types and this factors should be considered from the method development stage.

4.4 Untargeted method development

In order to address the knowledge gaps in relation to metabolites, transformation products and unknown BACs, an untargeted method was developed. Untargeted analysis has two components LC-MS and data analysis. It is not possible to optimise LC-MS parameters in the same way as it is for targeted analysis, as the compounds which will be analysed are unknown. However, it was possible to base the LC-MS method on the targeted method as the BACs selected for targeted analysis have a wide range of physicochemical properties. In terms of data analysis, it is now common to develop workflows which can automatically interpret data as it is very time-consuming to manually analyse the large amount of chemical features obtained from untargeted analysis (Richardson, et al., 2018). Compound Discoverer (Thermo Fisher Scientific, UK) has been used in several environmental studies for peak picking and integration, retention time alignment, isotope and adduct peak grouping, unknown compound grouping, blank subtraction and for comparison with the mzCloud for compound identification (Koba, et al., 2016; Zhe Li, et al., 2020, 2018; National Toxicology Program, 2019; Wang, et al., 2020). mzCloud has been used in an inter-laboratory study in which samples were analysed using different instruments (mainly Orbitraps and time of flight (TOF)) and a wide variety of databases, including other free access databases such as NIST, PubChem, Wiley Registry Mass Spectral Data and vendor databases such as Agilent Pesticide Library LC/Q-TOF MS/MS, Thermo Environmental Food Safety (EFS) with/without retention time (RT) and Waters database with RT, among others (Schymanski, et al., 2015). This study revealed that the main difference between different research group approaches was the data analysis method, with LC-MS methods being fairly harmonised. However, despite the differences, the same most abundant compounds were identified across the different laboratories (Schymanski, et al., 2015).

4.4.1 Liquid Chromatography and mass spectrometry analysis

The liquid chromatography method was taken from the targeted analysis and is the same as was used as in the QqQ or QTrap for the untargeted analysis, as reported in Section 3.4.3.

The MS parameters could not be optimised for specific analytes as with the targeted method, so system defaults as well as source temperatures optimised on the QqQ or QTrap were used and are those reported in Section 3.4.3. The MS was operated in simultaneous ESI+ and ESI- acquisition modes for both acidic and chromatographic methods in order to ensure maximum coverage of possible analytes. Data was acquired for the LC-MS profiling with a resolution of 70,000 from *m/z* 70-1,050. The mass range was chosen within the range of expected masses for BACs, in a study where 18 institutes developed untargeted LC-MS methods to analyse environmental samples for BACs the *m/z* ranges went from *m/z* 50-100 to *m/z* 1000-2000, with resolution ranging from *m/z* 18,000-140,000 (Schymanski, et al., 2015). The instrument was set to perform and record fragmentation patterns for the 10 most abundant compounds in each scan cycle, as well as on analytes in the a custom-made list of analytes including the targeted analytes.

4.4.2 Experimental design for confidence in identification and insights into WWTP removal

A total of five runs were undertaken in the untargeted mode for this thesis. Each with a different objective, this is summarised in Table 4.4-2. Firstly run i was done to ensure the method transfer from the targeted instruments to the Q-Exactive had worked, by injecting a calibration curve of the target analytes in solvent and checking the analytes could be detected and had a linearity > 0.9, using TraceFinder (Thermo Fisher Scinetific, UK). This data was also run using Compound Discoverer workflow to confirm the analytes could be identified with a mzCloud match score > 95 %. This is higher than the match score of 80 % previously reported for standards spiked and identified using Compound Discoverer and mzCloud (Zhe Li, et al., 2018).

Runs a and b comprised of samples injected as a targeted run. The aim of these runs was to determine if the analytes could be successfully detected by the instrument in the wastewater matrices. For these two runs, just one sampling event was injected and calibration curves in acetonitrile/water as well as blanks (just acetonitrile/water), and technical replicates (n=3) were injected. As well as Pre-SPE and Post-SPE extraction spiked samples, to evaluate method recovery and matrix effects respectively (detailed in Section 3.4.1.2). Runs a and b contribute in ruling out false positives, because any compound found in the acetonitrile/water blanks was ruled out for all runs.

Runs i, a, b and d (Table 4.4.2) were used to determine certain method quality parameters for selected compounds as method performance indicators. Untargeted methods do not require the same level of method validation (Sumner, et al., 2007), but these selected parameters give further confidence in the results and are presented in Table 4.4.1. This approach is similar to other published approaches were a targeted LC-MS method is used as the basis for developing an untargeted method (Zhe Li, et al., 2018). The linearity was > 0.99 for all analytes across three different injections on three different days. The lower limit of the calibration curve range was 1 $ngmL^{-1}$ except for simvastatin, sulfamethoxazole, salicylic acid and sulphapyridine for which it was 5 $ngmL^{-1}$. Considering SPE concentration, 1 $ngmL^{-1}$ instrumental is equivalent to 2 and 5 ngL^{-1} in effluent or downstream water and influent wastewater respectively. Similarly, 5 $ngmL^{-1}$ is equivalent to 5 and 10 ngL^{-1} in effluent or downstream water and influent wastewater respectively. Coefficient of variation of repeated (n=3) injections at 800 and 1000 ngL^{-1} was < 15% for all analytes, except for sulfapyridine at 1000 ng mL^{-1} for which it was 16.1%.

These selected analytes have a range of physicochemical properties with water solubility ranging from 51 – 51,900 mgL^{-1}, pK_a ranging from 1.4-14.91 and Log_{Kow} ranging from 0.2-2.97, as can be seen in Table 2.1.1. Even though they are not directly relevant for the untargeted analytes detected, nor necessary as this is not a

quantification method, the valid method metrics presented in Table 4.4.1 provide confidence that the method is in working order.

Analyte	Linearity			LOD $ngmL^{-1}$	Upper linear limit $ngmL^{-1}$	Precision (%)	
	Day 1	*Day 2*	*Day 3*			*800 $ngmL^{-1}$*	*1000 $ngmL^{-1}$*
Atenolol	0.9936	0.9992	0.9970	1	2000	7.2	12.1
Codeine	0.9987	0.9909	0.9950	1	1000	11.7	4.5
Ketoprofen	0.9977	0.9979	0.9946	1	800	1.9	3.4
Metoprolol	0.9982	0.9987	0.9914	1	400	7.1	1.7
Phenazone	0.9961	0.9997	0.9992	1	800	0.7	7.4
Salicylic Acid	0.990	0.9919	0.9992	5	1800	6.1	11.1
Simvastatin	0.9994	0.9971	0.9967	5	1800	6.6	9.3
Sulfamethoxazole	0.9942	0.9921	0.9981	5	2000	8.8	10.6
Sulfapyridine	0.9979	0.9966	0.9974	1	1000	5.4	16.1
Trimethoprim	0.9926	0.9992	0.9958	1	2000	5.8	4.7

Table 4.4-1 Untargeted method metrics

Once it was established that the spiked analytes, plus other untargeted analytes, could be successfully detected in the wastewaters analysed, runs c and d were run using the metabolomics approach (Worley, et al., 2013), previously described in Section 2.6.4.2, in order to be able to compare pollutants found in different matrices and gain insights on the removal efficiency of the WWTPs studied.

Run identifier	Objectives	Type of samples run	Chromatographic methods samples were run in
i.	• Test the method	• Calibration curve with all BACs in targeted method (1-2,000 ngL^{-1} with 11 levels)	• Basic and acidic
a.	• Test the method with real samples • Identify BACs which are not in the targeted method • Rule out false positives present in blanks • Obtain method metrics	• One sampling event at Stk including WWI and WWE • Technical replicates (n=3) • Calibration curve (1-2000 ngL^{-1} with 11 levels) • Blanks (acetonitrile/water)	• Basic and acidic
b.	• Test the method with real samples • Identify BACs which are not in the targeted method • Rule out false positives present in blanks • Obtain method metrics	• WWI and WWE from one sampling event at Stk and WWI and WWD from one sampling event at SuB • Technical replicates (n=3) • Calibration curve (1-2,000 ngL^{-1} with 11 levels) • Blanks (acetonitrile/water)	• Basic and acidic
c.	• Identify BACs which are not in the targeted method • Perform PLS analysis on data from several (n>5) sampling events to find removal trends of untargeted BACs identified	• SuB WWI and WWD samples from seven sampling events • No technical replicates • Pooled QC sample: mixture of all the samples in equal volumes	• Basic and acidic
d.	• Identify BACs which are not in the targeted method • Perform PLS analysis on data from several (n>5) sampling events to find removal trends of untargeted BACs identified	• SuB WWI and WWD and Stk WWI and WWE samples from at least seven sampling events • No technical replicates • Pooled QC sample: mixture of all the samples in equal volumes	• Acidic only due to a technical issue.
e.	• Identify BACs which are not in the targeted method for samples from Mexico. PLS analysis not possible because only one sampling event was undertaken.	• Samples from each of the sampling points in the Tula Valley Mexico • Technical replicates (n=2) • Calibration curve (1-2000 ngL^{-1} with ten levels) • Blanks (acetonitrile/water)	• Basic and acidic

	• Rule out false positives present in blanks • Obtain method metrics		

Table 4.4-2 Summary of runs done for untargeted analysis

For runs c and d, samples were injected with n ≥ 7 (at least 7 different sampling events) making it possible to compare trends between groups in PLS analysis (Sumner, et al., 2007). These runs did not contain any blanks or calibration curve points, as it is necessary for them to be in the same matrix for multivariate analysis (Worley, et al., 2013).

As described in Section 3.4.3.3, compounds were identified by comparison to the mzCloud database. The large amounts of data found meant that it was necessary to have a selection criterion for the compounds to be further studied. The criteria used was:

- Minimum 80% match score to MZ results (calculated by Compound Discoverer software based on the ratio of fragments to parent ion found in the sample compared to those in the database). Previous work has considered a match score of > 70% acceptable (Zhe Li, et al., 2018). For this work 80 % was chosen because targeted analytes were identified with > 95% confidence and below 80 % match score it was observed that compounds had, in many occasions, less than 2 matching fragment ions.
- Similar retention time (± 20 s) across all runs where it was detected in order to decrease the risk of false positives.
- Not present in the blanks in runs i, a and b in order to avoid false positives and peak area in wastewater samples one order of magnitude higher than in solvent blanks (from runs a and b).

Only those compounds which met this confidence in identification criteria were fed into the partial least squares (PLS) models. PLS models allowed to identify similarities and difference between samples which give insights into the WWTP process. This was achieved using multivariate analysis as described in Section 2.6.4.2. SIMCA (Umetrics-Sartorius, Sweden) was used for the multivariate analysis. Data was normalised using univariate scaling. Pareto scaling was trailed but better results were obtained with univariate scaling. Firstly, unsupervised principal component analysis (PCA), including the pooled QCs was done. The PCA score plots was done to confirm the quality of the run (QC samples should be grouped in the middle), and the unsupervised separation of the groups (Worley, et al., 2013). The autofit function in SIMCA was used initially to determine the number of principal components, however these were increased or decreased from 2-5 to find the optimum number of components in relation to the X^2 value and the groupings formed. PCA was also used to determine the most appropriate normalization, Pareto scaling was trialled as it is normally used for LC-MS data given that it retains the magnitude of larger peaks (Worley, et al., 2013), however the univariance (or normalization or z-score), which is the default in SIMCA provided better results. Once the quality and unsupervised separation of the samples was confirmed, as can be seen in Figure 4.4-1 and Figure 4.4-2 below, PLS modelling was undertaken.

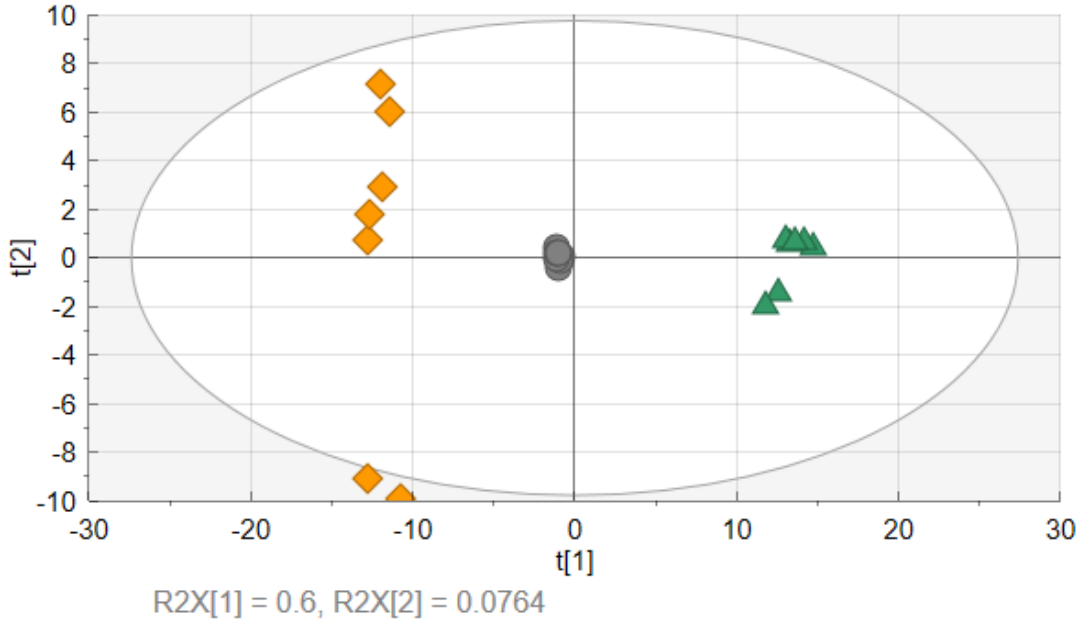


Figure 4.4-1 PCA scores plot with pooled quality controls (gray circles), Sutton Bonington wastewater influent (orange rhombus) and Sutton Bonington downstream water (green triangles). X-axis represents principal component one (t[1]) and Y-axis represents principal component 2 (t[2]). Variance represented by t[1] and t[2] are given by R2[X] and R2X[2] respectively.

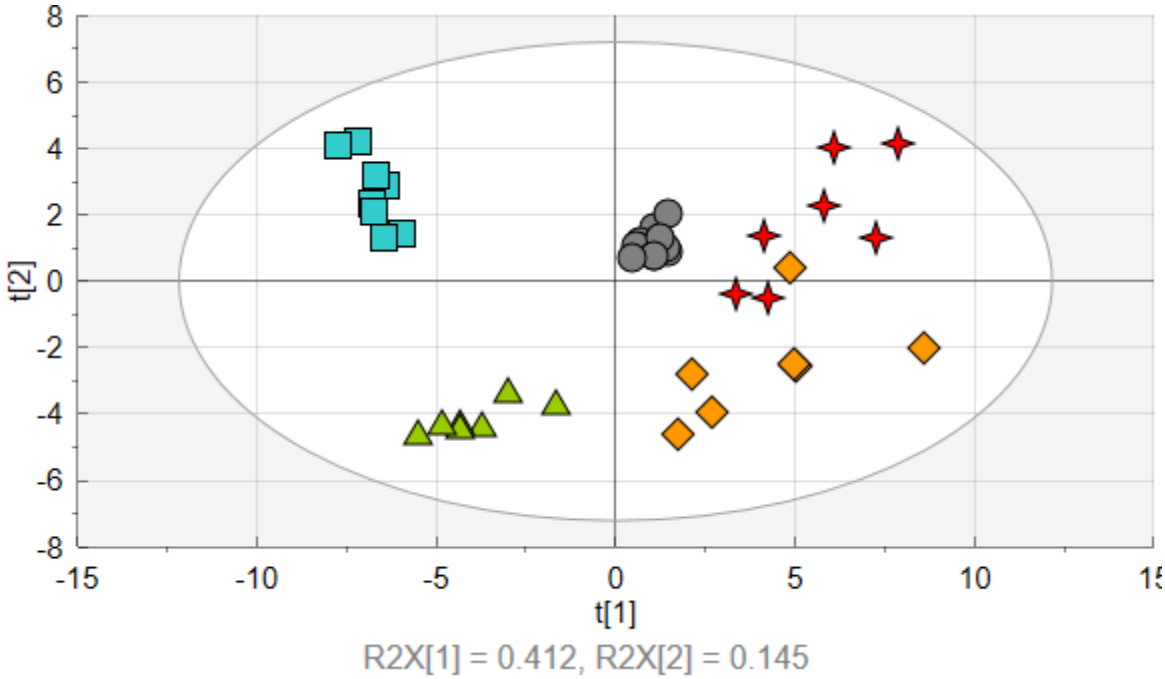


Figure 4.4-2 PCA scores plot with pooled quality controls (Gray circles), Sutton Bonington wastewater influent (orange rhombus), Sutton Bonington downstream water (green triangles), Stoke Bardolph wastewater influent (red 4-point starts) and Stoke Bardolph wastewater effluent (blue squares). X-axis represents principal component one (t[1]) and Y-axis represents principal component 2 (t[2]). Variance represented by t[1] and t[2] are given by R2[X] and R2X[2] respectively.

PLS models determine which X (BAC concentration) variables are predictive of the Y variables (groups based on matrix). In order to validate the PLS model a permutation

was done with 100 permutations to determine that the Q^2 was still higher in the actual model than the random permutations, ruling out the fitting having been by chance (Umetrics, 2016). These validations can be found in Appendices 10.4 and 10.5. The Variable Importance for Prediction (VIP) plot and the VIP scores were used to identify the BACs which had a greater impact on the group separation. VIP scores >1 indicate X-variables (BACs) important for group separation (Umetrics, 2016).

Analyte	Chromatography	Matrix effects on Qexactive (%) evaluated at 100 ngmL^{-1}			
		Wastewater influnet Stoke Bardolph	Wastewater effluent Stoke Bardolph	Wastewater influent Sutton bonington	Downstream wastewater Sutton Bonington
Acetylsulfamethoxazole	Acidic	74.0 ± 24.5	32.5 ± 2.4	29.8 ± 4.5	52.6 ± 12.2
Acetylsulfamethoxazole	Basic	-4.1 ± 1.6	-42.4 ± 8.5	12.9 ± 1.1	-0.9 ± 0.1
Atenolol	Acidic	78.4 ± 3.5	-11.1 ± 1.0	47.2 ± 3.4	1.5 ± 0.1
Atenolol	Basic	-29.6 ± 1.0	-4.9 ± 0.3	-6.7 ±0.3	35.6 ± 3.4
Bezafibrate	Acidic	24.8 ± 2.4	45.1 ± 6.3	53.0 ±10.6	62.2 ± 4.5
Bezafibrate	Basic	-17.4 ± 1.6	-18.7 ± 2.9	7.2 ± 1.5	15.6 ± 5.7
Carbamazepine	Acidic	-45.0 ± 2.5	-40.8 ± 2.6	-42.9 ± 6.0	-14.2 ± 0.5
Carbamazepine	Basic	-39.3 ± 2.9	-31.5 ± 0.9	-0.2 ± 0.02	18.4 ± 1.4
Clofibric acid	Acidic	148.8 ± 50.3	88.3 ± 26.1	73.6 ± 16.8	104.4 ± 28.6
Codeine	Acidic	101.4 ± 6.3	105.0 ± 10.1	85.2 ± 5.9	84.4 ± 3.8
Codeine	Basic	-79.1 ± 9.7	-82.8 ± 8.9	-16.9 ± 1.7	-58.3 ± 11.0
Diclofenac	Basic	-54.4 ± 9.3	-50.0 ± 4.4	-33.0 ± 5.5	-26.7 ± 3.6
Erythromycin	Acidic	-98.7 ± 44.0	-41.4 ± 2.8	-42.2 ± 32.5	-17.9 ± 2.5
Erythromycin	Basic	-15.9 ± 1.6	56.5 ± 5.3	32.7 ± 3.5	74.3 ± 8.6
Estradiol	Basic	18.9 ± 1.1	137.2 ± 42.1	81.1 ± 7.5	125.7 ± 32.8
Ethynylestradiol	Basic	-32.7 ± 13.5	129.7 ± 10.5	46.7 ± 3.4	130.5 ± 8.-
Gemfibrozil	Acidic	-23.9 ± 14.2	-4.5 ± 0.5	15.0 ± 1.9	170.1 ± 42.5
Gemfibrozil	Basic	71.1 ± 5.1	55.6 ± 4.1	181.1 ± 48.1	100.4 ± 10.0
Ketoprofen	Acidic	24.8 ± 5.3	33.6 ± 12.2	55.3 ± 15.9	66.9 ± 10.9
Ketoprofen	Basic	-38.3 ± 3.1	-30.8 ± 5.0	-5.9 ± 1.1	15.7 ± 1.6
Levonorgestrel	Acidic	93.9 ± 53.8	77.5 ± 6.9	81.3 ± 12.4	113.9 ± 62.3
Levonorgestrel	Basic	-92.0 ± 4.0	-71.6 ± 25.6	-75.2 ± 7.5	-46.3 ± 12.6
Metoprolol	Acidic	14.8 ± 3.3	30.8 ± 6.3	31.5 ± 4.6	91.1 ± 5.8
Metoprolol	Basic	6.4 ± 0.9	16.9 ± 1.4	33.3 ± 4.6	75.4 ± 12.7

Analyte	Chromatography	Wastewater influnet Stoke Bardolph	Wastewater effluent Stoke Bardolph	Wastewater influent Sutton bonington	Downstream wastewater Sutton Bonington
Morphine	Acidic	-76.1 ± 12.7	-45.0 ± 5.6	-51.0 ± 14.8	-45.1 ± 8.3
O-Desmethylvenlafaxine	Acidic	-100.0 ± 8.1	-100.3 ± 71.2	-100.0 ± 13.2	-101.2 ± 68.0
Paracetamol	Acidic	-599.6 ± 14.4	83.3 ± 12.1	-836.4 ± 196.4	90.4 ± 23.7
Paracetamol	Basic	-648.0 ± 7.0	-52.3 ± 25.7	-187.5 ± 4.3	-47.8 ± 30.6
Paroxetine	Acidic	-6.7 ± 0.2	5.7 ± 1.9	-9.4 ± 1.6	19.2 ± 12.3
Paroxetine	Basic	-99.2 ± 82.8	0.6 ± 0.4	-98.7 ± 28.1	37.3 ± 21.1
Phenazone	Acidic	-54.6 ± 2.7	-22.5 ± 4.0	-46.2 ± 16.7	7.4 ± 0.5
Phenazone	Basic	-20.6 ± 4.1	-13.2 ± 2.4	7.8 ± 3.6	35.9 ± 8.6
Propranolol	Acidic	-15.7 ± 2.5	6.3 ± 0.4	-4.0 ± 0.7	55.6 ± 6.5
Propranolol	Basic	-73.3 ± 5.7	27.3 ± 1.3	-36.2 ± 4.8	63.7 ± 4.0
Simvastatin	Acidic	-72.5 ± 7.2	-23.1 ± 6.3	-59.2 ± 6.7	16.3 ± 2.1
Sulfamethoxazole	Acidic	33.4 ± 2.0	33.0 ± 7.1	32.3 ± 4.5	68.5 ± 3.4
Sulfapyridine	Acidic	4.7 ± 0.7	15.6 ± 2.9	6.6 ± 2.1	41.6 ± 20.4
Sulfapyridine	Basic	-23.2 ± 1.4	-15.0 ± 3.6	12.2 ± 2.4	47.6 ± 21.9
Tramadol	Acidic	15.9 ± 1.8	41.2 ± 3.3	3.5 ± 1.1	76.0 ± 9.7
Trimethoprim	Acidic	53.9 ± 4.5	27.2 ± 4.3	82.7 ± 5.0	79.6 ± 8.3
Trimethoprim	Basic	-43.5 ± 3.8	-37.7 ± 1.7	-28.7 ± 4.1	-9.1 ± 0.6

Figure 4.4-3 Matrix effects in Qexactive instrument

PLS analysis also provides comparative ratios between the groups identified (Umetrics, 2016). As the peak areas compared are for the same compound it is reasonable to assume that a higher area means a higher concentration. In untargeted analysis peak areas (as opposed to concentrations) have been previously used to compare between like samples and thus calculate WWTP removal efficiency (Zhe Li, et al., 2020). Using the group peak area ratios obtained in the PLS analysis, a WWE/WWI > 1 reveals poor removal and/or conversion of another compound into the one in question. A WWE/WWI < 1 reveals a decrease in concentration across the WWTP. Similarly, for run d it is also possible to compare the WWE of Stk to the WWD of SuB to determine if one plant or the other is releasing higher concentrations of a given pollutant. The same can be done to compare the WWI of both plants using the results from run d.

4.5 Summary

SPE LC-MS/MS targeted and untargeted methods to analyse BACs were developed and validated for aqueous and soil matrices. During targeted method development three SPE sorbents were compared for the extraction of 36 BACs with HLB found to be superior (compared to Strata-X and MCX) due to recoveries and reproducability. Three reconstitution methods were trialled with the best one fount to be utilising acetonitrile: water (50/50 v/v) adding the acetonitrile first, vortexing and then adding the water and vortexing again. Two LC-MS instruments (triple quadrupole, QqQ, and linear ion trap, QTrap) were compared. Matrix effects and/or iLOD were found to be lower on QTrap for 4-tert-octylphenol, clofibric acid, ethinylestradiol, estrone, estradiol, gemfibrozil, levonorgestrel and simvastatin, while being lower on the QqQ for oxytetracycline, phenazone, salicylic acid and tramadol. The targeted method was validated across both instruments for four matrices from two UK WWTPs. With method linearity > 0.99 for 75% of the cases and >0.95 for 95% of the cases. mLODs were below previously reported concentrations in the respective matrices and below 10 ngL^{-1} for 22-67 % of analytes depending on the matrix. Inter and intra-day precision for over 70 % of the analytes across the four matrices studied was below 15 %.

During the soil extraction method development aging was evaluated, for the first time for severalof the analytes, to determine method recoveries considering real environmental conditions. Twenty-eight analytes presented a significantly different ($p<0.05$) recovery at one of the five ageing periods (4, 24,72 h and 7 and 14 days) compared to that observed when there was no ageing. Demonstrating, along with literature data, how not considering the decrease in recovery when an ageing period is used for sulfamethoxazole can result in up to a two-fold underestimation of the environmental concentration.

Untargeted method developed for identification of BACs in aqueous matrices'. Built from the targeted method and demonstrated to be adequate for a range of analytes with water solubility ranging from 51 – 51,900 mgL^{-1}, pK_a ranging from 1.4-14.91 and Log_{Kow} ranging from 0.2-2.97. Linearity was > 0.99 for the analytes evaluated across three different injections on three different days. The lower limit of the calibration curve range was 1 $ngmL^{-1}$ except for simvastatin, sulfamethoxazole, salicylic acid and sulphapyridine for which it was 5 $ngmL^{-1}$.

5 RESULTS AND DISCUSSION; REMOVAL, SEASONALITY AND RISK OF SELECTED BACs IN UK WWTPS

5.1 Highlights:

- **Significant ($p<0.05$) seasonal variations in removal for Stoke Bardolph wastewater treatment plant for 12 pollutants**: diclofenac, carbamazepine, propranolol, trimethoprim, codeine, sulfapyridine, metoprolol, estrone-3-sulfate, morphine, erythromycin, ketoprofen and sulfamethoxazole
- **Correlation between temperatures above 15 °C and increased removal of o-desmethylvenlafaxine** in Sutton Bonington wastewater treatment plant.
- Despite their differences in removal efficiencies, the **discharge from both wastewater treatment plants have environmental risk coefficients consistently above 1 for the same 8 out of 36 pollutants**. Namely, codeine, diclofenac, erythromycin, morphine, oxytetracycline, propranolol, salicylic acid, simvastatin, sulfamethoxazole, tramadol and trimethoprim. Furthermore, the **equivalent estradiol concentrations was found to be at least 8 times above the PNEC** for the effluent or downstream water of both plants.
- Eight strong and significant correlations identified between several emerging pollutants and between pollutants and water quality parameters, these are important for modelling efforts and for pinpointing the effect of process operating conditions on removal efficiencies.

5.2 Summary of samples discussed in this chapter

Table 5.2.1 below presents a summary of the location, frequency and samples colected from the two UK WWTPs and analysed in targeted mode:

WWTP	**Stoke Bardolph WWTP**		**Sutton Bonington WWTP**	
Water type	WWI	WWE	WWI	WWD
Description	Raw influent	Raw effluent sampled at mid point of a 1 km open channel going form the WWTP to the river Trent	Raw influent	Sampled at point where wastewater effluent meets a stream which after 100 m meets the river Soar
Total number of samples taken	27	30	15	15
Frequency of samples taken	A minimum of 5 WWI and WWE samples were analysed for each of the four seasons (November 2018-November 2019): Winter (December-February), Spring (March-May), Summer (June-August), Autumn (September- November). SuB samples were collected from May - November 2019. In addition 3 WWI and WWE samples were taken in the summer of 2018.		Two samples were per month between end of June-November November 2019. Except for August when eight samples were taken.	

Table 5.2-1 Summary of samples taken in the two UK wastewater treatment plants and analysed in targeted mode

5.3 Occurrence

Figures 5.3.1 and 5.3.2 present the concentrations measured throughout the sampling campaign for pollutants detected with >60% frequency for Stk and SuB WWTPs respectively. Table 5.3.1 presents concentrations found for pollutants detected with frequency of detection < 60% and for pollutants found at too high a range for the box plot.

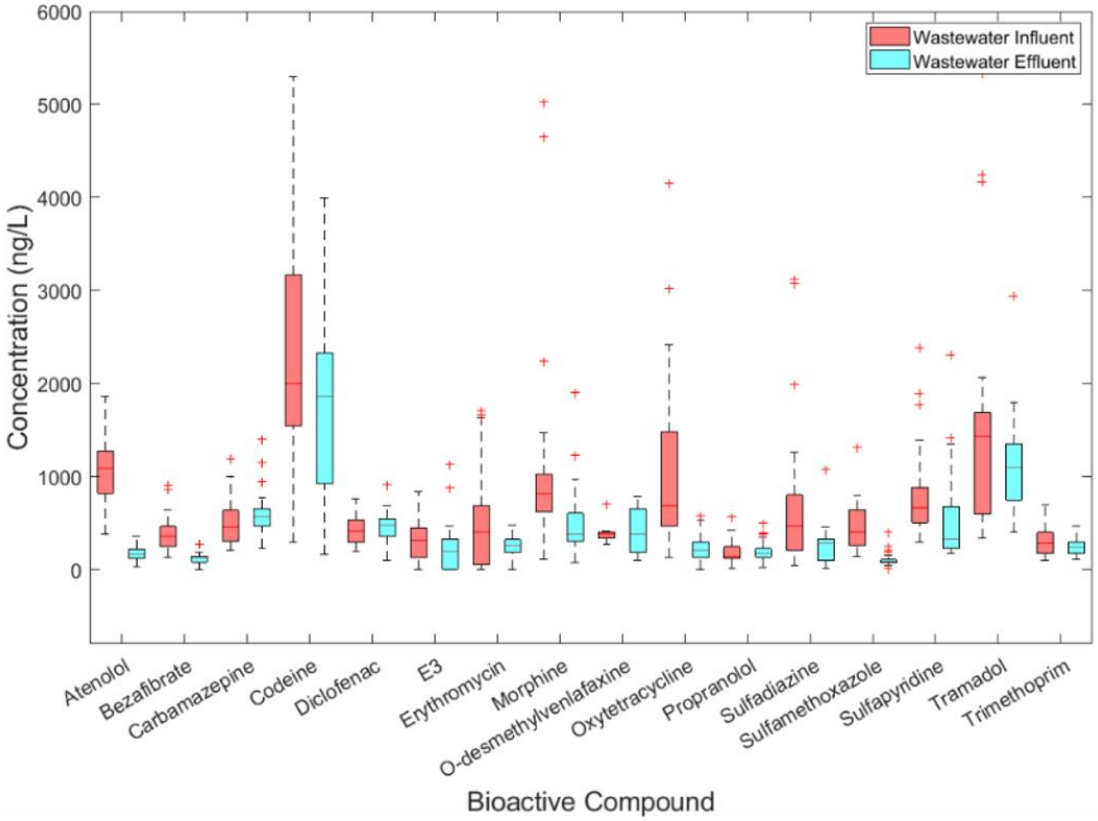


Figure 5.3-1 Wastewater influent and effluent concentrations of pollutants with a detection frequency above 60% in Stoke Bardolph wastewater treatment plant

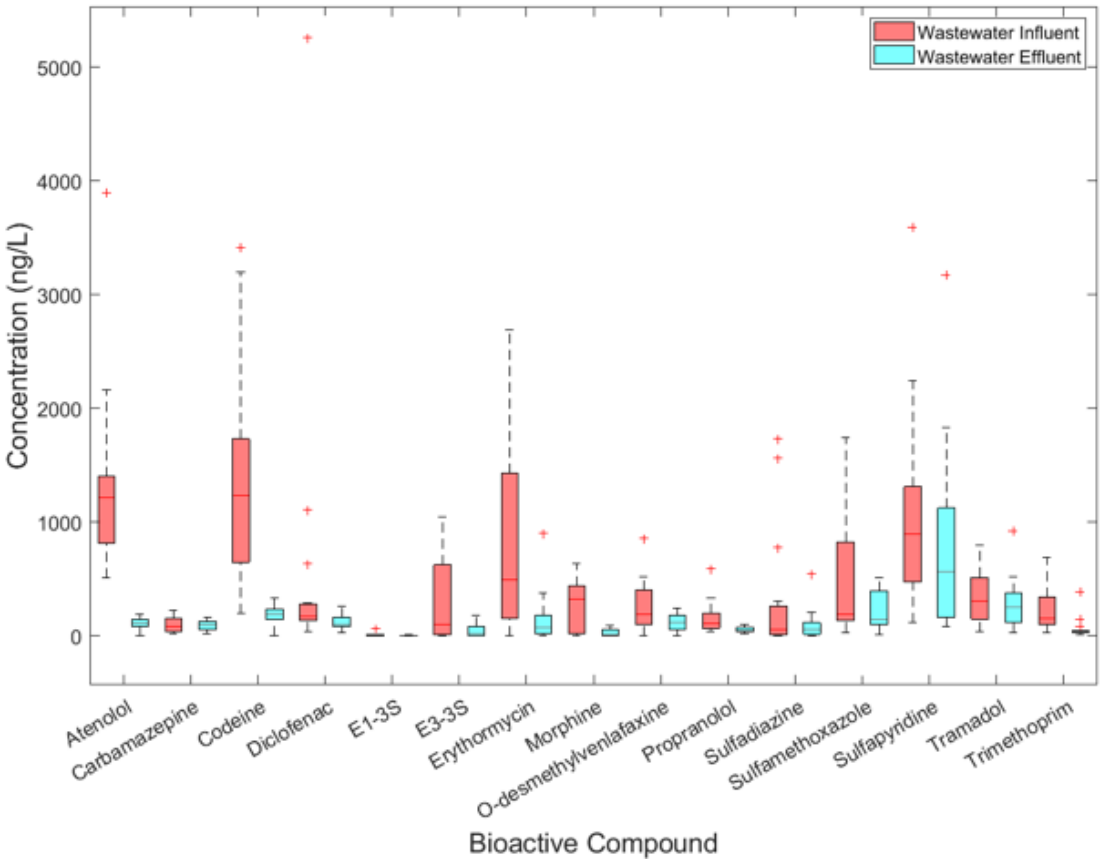


Figure 5.3-2 Wastewater influent and downstream water concentrations of pollutants with >60% frequency of detection for Sutton Bonington wastewater treatment plant

Pollutant	WASTEWATER INFLUNET							WASTEWATER EFFLUENT				WATER DONWSTREAM OF WWTP			
	Stoke Bardolph			Sutton Bonington			Lit.	Stoke Bardolph			Lit.	Sutton Bonington			Lit.
	Freq (%)	*Range (ngL^{-1})*	*mLOD (ngL^{-1})*	*Freq (%)*	*Range (ngL^{-1})*	*mLOD (ngL^{-1})*	*(ngL^{-1})*	*Freq (%)*	*Range (ngL^{-1})*	*mLOD (ngL^{-1})*	*(ngL^{-1})*	*Freq (%)*	*Range (ngL^{-1})*	*mLOD (ngL^{-1})*	*(ngL^{-1})*
4-tert-octylphenol	22.2	25.9-75.4	22.3	26.7	8.1-122.7	8.1	24-351[1]	17.0	9.3-26.2	2.9	10-64 [1]	20.0	5.6-37.7	4.4	<0.14-5.3[1]
Acetaminophen	70.4	13,139.6-498,048.7	5873.1	46.7	8,621-64,243.9	6000	6,924-492,340[8] 100-300,000[26]	90.0	15.6-2670.2		<20-11,733[8]	93.3	45.2-23,554.2		1.5-1,388[8] 0.4-71, 72-571[26]
Acetyl-sulfamethoxazole	85.2	260.0-3552.6	147.6	66.7	37.3-2922.9	26.4	390,445[2];75.2[3]; 67 [4]	3.0	5.0	18.5	690-2,200[5]; 50[3]	53.3	34.4-257.8	6.5	240[5]; 3[4]
Bezafibrate	100	132.1-904.6	9.43	20.0	3.3-19.6	3.7	420-971[8]	90	23.5-270.3	4.5	177-418[8]	20.0	0.8-5.8	1.3	<10-90[8];
Clofibric Acid	18.5	57.4-406.1	21.4	33.3	34.5-409.8	12.9	1-651[8]	7.0	4.9-21.5	12.9	6-44[8]	13.3	58.5-67.4	24.0	<0.3-164 [8]
Estrone (E1)	18.5	7.8-106.8	44.3	20.0	38.4-318.2	11.5	6.54–18.76[10]; 49[8]; 55-77[23]; 42-132[23];0.16-100[24]	10	20.3-53.7	7.5	1.04–4.99[10]; < 9.7[12;] 6-40[23]	13.3	17.5-33.8	7.3	2[4]; <9.7[12]
Estrone-3-sulfate (E1-3S)	66.7	1.4-31.8	3.4	53.3	2.5-68.2	23.8	<76[12]	20	1.7-15.2	1.7	<27[12]	73.3	0.5-7.7	2.6	<27[12]
Estradiol (E2)	0	-	86.3	0	-	100.0	1.02–8[10];9.3-2,200[1]	3	13.0	13.1	0.5–2.2[10] ;<9.5[12]	0	-	8.3	2[4];<9.5[12]
Estradiol-3-sulfate (E2-3S)	25.9	14.7-106.8	5.0	26.7	14.4-53.5	25.8	<13[12]	3	1.7	3.5	<5.3[12]	13.3	4.1-5.1	5.0	<5.3[12]

Pollutant	WASTEWATER INFLUNET							WASTEWATER EFFLUENT				WATER DONWSTREAM OF WWTP			
	Stoke Bardolph			Sutton Bonington			Lit.	Stoke Bardolph			Lit.	Sutton Bonington			Lit.
	Freq (%)	*Range (ngL^{-1})*	*mLOD (ngL^{-1})*	*Freq (%)*	*Range (ngL^{-1})*	*mLOD (ngL^{-1})*	*(ngL^{-1})*	*Freq (%)*	*Range (ngL^{-1})*	*mLOD (ngL^{-1})*	*(ngL^{-1})*	*Freq (%)*	*Range (ngL^{-1})*	*mLOD (ngL^{-1})*	*(ngL^{-1})*
Estriol (E3)	77.8	76.2-841.1	23.0	60	45.1-789.4	26.0	0.35-188.7[24]	63	15.7-1136.3	48.7	<10[12]	26.7	17.8-117.4	16.3	<10[12]
Estriol-3-sulfate (E3-3S)	37.0	20.6-763.1	11.3	66.7	8.2-1048.4	19.2	<41[12]	17	10.77-83.5	7.5	<6.3[12]	66.7	12.9-180.0	5.0	<6.3[12]
Gemfibrozil	3.7	2.8	10.0	6.7	8.5	23.4	137.7[4];116[13]	13	1.9-6.0	3.6	66.9[13]	20.0	0.9-2.8	7.9	5-497[9];0.04-
Ibuprofen	18.5	1355.7-68500.9	5000	13.3	7255.0-12974	101.6	1,681-33,764[8]	23	18.6-110.7	19.7	143-4,230[8]	20.0	801.2-4,062.3	23.9	1-5[8] <42-289[9]
Ketoprofen	44.4	23.6-550.8	5.1	20.0	49.1-118.5	8.3	28-102[8];260-480[11]	73	14.2-93.9	4.6	16-23 [8]	6.7	1.4	1.2	<30-144[9]
Naproxen	85.2	2451.8-15,365.0	2.2	93.3	2,726.1-19,552.0	3.3	838-1,173[8] 2,300-14,000[25]	80.0	8.9-3762.7		70-370[8] 100-1,600[25]	100	328.5-1,192.6	0.6	146[8] 1,687[27]
Metoprolol	44.4	5.9-67.2	7.7	46.7	6.7-45.7	5.8	75-110[8];166 [4]	100	3.0-56.6	4.0	41-69[8]	80.0	1.0-6.4	1.3	<0.5-215[8];20[4]
Oxytetracycline	66.7	129.2-4155.4	194.4	26.7	1035.4-1911.1	260.9	3,600 [8]	63	52.2-584.2	36.5	170[8]	33.3	54.5-922.1	110.0	
Paroxetine	22.2	3.5-124.4	15.5	6.7	12.6	13.8	<6 [9]	20	1.6-54.6	15.0	<7[9]	13.3	6.1-12.9	3.3	<20 [9]
Phenazone	7.4	17.2-38.4	9.0	13.3	4.8-7.8	5.7	91-10[18]	60	0.8 - 12.1	4.2	550-962[18]	33.3	0.9-6.1	1.4	5.9-40.27[19]
Salicylic Acid	33.3	0.012-0.13 mgL^{-1}	0.001mg L^{-1}	40.0	0.0013-	0.001 mgL^{-1}	0.005-0.052 mgL^{-1} [8]	37	84.93-2122.6	228.5	75-209[8];	53.3	11.3-346.2	10.2	201-333[19]

					0.1184 mgL^{-1}						ND-6825 [20]					
Pollutant	**WASTEWATER INFLUNET**							**WASTEWATER EFFLUENT**				**WATER DONWSTREAM OF WWTP**			
	Stoke Bardolph			Sutton Bonington			Lit.	Stoke Bardolph			Lit.	Sutton Bonington			Lit.
	Freq (%)	*Range (ngL^{-1})*	*mLOD (ngL^{-1})*	*Freq (%)*	*Range (ngL^{-1})*	*mLOD (ngL^{-1})*	*(ngL^{-1})*	*Freq (%)*	*Range (ngL^{-1})*	*mLOD (ngL^{-1})*	*(ngL^{-1})*		*Freq (%)*	*Range (ngL^{-1})*	*mLOD (ngL^{-1})*
Simvastatin	22.2	16.3-531.2	35.9	20.0	12.2-83.8	6.2	<7-115[8]; <2.5[21]	17	3.9-17.1	6.2	<3-5[8]; <0.5[21]	13.3	13.0-38.4	2.7	<0.6 [8]

Table 5.3-1 Pollutants detected with a frequency lower than 60% for at least one of the matrices studied in Stoke Bardolph and Stton Bonington wastewater treatment plants

Lit=literature, Freq.= frequency, mLOD=method limit of detection, ND= Not detected;. Superscript numbers refer to the following references: ***1****(Gorga, et al., 2013);* ***2*** *(Le-Minh, et al., 2012);* ***3*** *(Wang et al., 2019);* ***4*** *(Goswami, et al., 2018);* ***5*** *(Hilton, et al., 2003);* ***6*** *(Valls-Cantenys, et al., 2016);* ***7*** *(Loos, et al., 2013);* ***8*** *(Petrie, et al., 2015);* ***9*** *(M. Gros, et al., 2007);* ***10*** *(Mohagheghian, et al., 2014);* ***11*** *(Peña-Guzmán, et al., 2019);* ***12*** *(Naldi, et al., 2016);* ***13*** *(Sui, et al., 2011);* ***14*** *(Han, et al., 2017);* ***15*** *(Besse, et al., 2009);* ***16*** *(Comtois-Marotte, et al., 2017);* ***17*** *(Al-Odaini, et al., 2010);* ***18*** *(Biel-Maeso, et al., 2018);* ***19*** *(Lopez-Serna, et al., 2010);* ***20*** *(Evgenidou, et al., 2015)****;*** ***21*** *(Vanderford, et al., 2006);* ***22*** *(Al-Odaini, et al., 2010)* ***23*** *(Johnson, et al., 2004)* ***24*** *(Kaur, et al., 2018)****25*** (Fernández, et al., 2014) **26** (Phong Vo, et al., 2019) **27** (Sousa, et al., 2018)

For both WWTPs the highest concentrations measured in WWI were salicylic acid>acetaminophen> naproxen, with similar ranges to those previously reported in UK and Europe (Table 5.3.1). Influent BAC concentrations are generally higher for Stk, which is expected given it has a higher PE, receiving wastewater from a city with two universities and two major hospitals. The only exceptions were 4-tert-octylphenol, estrone (E1), estrone-3-sulfate (E1-3S), estriol (E3) and estriol-3-sulfate (E3-3S) which are found at higher concentrations in the influent of SuB. In the UK, 4-tert-octylphenol is used in rubber formulation, insulation varnishes, printing inks, paintings and coatings (DEFRA (Department of environment for and rural affairs), 2008). Therefore, the higher concentrations are likely due to the presence of an industrial estate which includes building restoration, sing-writing and car servicing approximately 3 km from SuB. The amount of steroid hormones, and their corresponding sulfate conjugates, excreted is governed by many factors, such as gender, stage in the menstrual cycle, pregnancy, menopause, hormone replacement treatment and hormone intake (Johnson, et al., 2004). Estriol (E3) has the highest detection frequency and mean concentration of the hormones, this is consistent with results found in other studies (Naldi, et al., 2016). Women excrete more estrone and estriol than men, with pregnant women excreting the most of both hormones (Johnson, et al., 2004). The population of Sutton Bonington is older than that of Nottingham with 50% and 35% of the population being 45 or older respectively (Nottinghamshire County Council, 2020). Despite the percentage of population in reproductive age being higher in Nottingham, 29.7% of those aged 18-29 are full time university students (Nottinghamshire County Council, 2020) and in England only 8% of full time students were found to be parents (Pugh, 2010). The higher estrone and estrone-3-sulfate concentrations found in the influent of SuB compared to Stk and to the literature (Table 5.3.1), could be due to demographic factors such as more pregnant women or more women taking hormone replacement treatment.

Acetyl-sulfamethoxazole was found at about double the concentration of sulfamethoxazole in both influent wastewaters, this is consistent with previous studies indicating that approximately 50% of the ingested sulfamethoxazole dose is excreted as acetyl-sulfamethoxazole and only 15% is excreted unchanged (Dapeng Wang, et al., 2019). This is useful in terms of being able to monitor only the parent antibiotic concentration and calculating the metabolite concentration using this ratio (or vice versa).

5.4 Wastewater treatment plant removal

5.4.1 Pollutants with seasonal variability of removals

Significant difference between the overall removal and at least one of the seasonal removals was observed for 14 pollutants (Table 5.4.1). This is pollutant dependent; no season was found to have worse or better removals for all pollutant. Across all four seasons temperature was found to be significantly different (Figure 5.4.1 a). Total suspended solids were found to be significantly higher in winter and significantly lower in autumn (Figure 5.4.1 d). Erythromycin, ketoprofen and morphine showed significantly different removals for two seasons, compared to the overall removal. Carbamazepine, codeine, diclofenac, propranolol and trimethoprim all had variable removals for three seasons. Estrone-3-sulfate, estriol, sulfadiazine, sulfamethoxazole and tramadol presented significantly different removals for one season, compared to the overall removal.

Bioactive chemical pollutant	STOKE BARDOLPH WASTEWATER TREATMENT PLANT Removals (%)						
	Summer 2018 *n=3*	**Winter 2018-19 *n=6***	**Spring 2019 *n=8***	**Summer 2019 *n=5***	**Autumn 19 *n=5***	**Overall *n=27***	**SUTTON BONINGTON WWTP overall removal *n=15***
4-tert-octylphenol	88.5 ± 141.0	NA	NA	NA	-12.5 ± 17.8	50.7 ± 9.8	NA
Acetaminophen	99.6 ± 136.7	99.0 ± 112.9	99.4 ± 120.1	100.0 ± 100.1	100.0 ± 188.3	99.9 ± 29.6	90.5 ± 194.4
Acetyl-sulfamethoxazole	NA	99.5 ± 221.9	99.9 ±55.8	99.9 ± 114.5	99.5 ± 146.9	99.5 ± 12.3	91.5 ± 186.8
Atenolol	92.3 ± 66.2	85.6 ± 23.6	85.9 ± 31.2	79.8 ± 48.9	71.2 ± 40.7	83.1 ± 12.4	91.8 ± 69.0
Bezafibrate	88.9 ± 72.3	61.6 ± 23.8	62.2 ± 31.2	79.2 ± 65.1	66.7 ± 63.9	66.6± 12.4	NA
Carbamazepine	**-2.1 ± 1.1**	**26.7 ± 12.3**	**-42.4 ± 23.3**	**-71.4 ± 32.5**	-32.2 ± 34.1	-12.9 ± 1.8	2.2 ± 1.8
Clofibric acid	91.0 ± 213.6	NA	NA	NA	93.1 ± 200.1	89.6 ± 13.3	NA
Codeine	59.6 ± 53.3	**4.9 ± 4.2**	67.2 ± 91.7	**16.8 ± 10.7**	**-10.7 ± 8.6**	31.4 ± 7.1	86.1 ± 71.4
Diclofenac	13.9 ± 7.0	-19.9 ± 5.7	**-31.8 ± 12.2**	**28.9 ± 30.6**	**-25.9 ± 9.3**	-16.6 ± 2.7	79.5 ± 180.5
Estrone	NA	NA	NA	NA	37.9 ± 70.0	NA	NA
Estrone-3-sulfate	53.3 ± 31.2	98.0 ± 76.4	98.2 ± 60.4	**-3.2 ± 3.1**	99.7 ± 69.0	41.0 ± 7.8	69.6 ± 123.5
Estriol	94.7 ± 108.9	8.0 ± 13.7	**17.2 ± 4.2**	35.1 ± 54.1	-42.5 ± 61.0	10.1 ± 2.0	92.8 ± 177.2
Estriol-3-sulfate	94.6 ± 123.4	NA	NA	NA	87.2 ± 162.9	83.8 ± 38.2	85.5 ± 147.7
Erythromycin	NA	**21.3 ± 30.8**	67.6 ± 56.5	75.6 ± 85.0	**15.5 ± 13.2**	61.3 ± 11.4	82.1 ± 150.2
Ibuprofen	97.7 ± 63.0	NA	NA	NA	NA	99.6 ± 33.1	80.8 ± 83.1
Ketoprofen	86.0 ± 70.7	32.2 ± 63.9	**-27.5 ± 49.4**	**18.6 ± 26.4**	68.7 ± 141.5	71.0 ± 16.7	NA
Metoprolol	-7.0 ± 6.7	**-26.8 ± 33.3**	**-40.0 ± 65.9**	16.5 ± 24.3	-20.2 ± 141.5	38.2 ± 8.6	86.6 ± 79.4
Morphine	96.5 ± 75.3	**6.7 ± 4.8**	51.0 ± 19.7	44.5 ± 33.8	**8.3 ± 7.5**	55.6 ± 13.4	88.5 ± 117.4
Naproxen	97.3 ± 164.5	84.6 ± 73.8	94.0 ± 116.4	95.7 ± 135.9	98.1 ± 110.4	88.0 ± 20.6	91.7 ± 70.3
O-desmethylvenlafaxine	NA	NA	NA	27.4 ± 31.1	-25.6 ± 14.2	-15.0 ± 1.1	57.0 ± 65.5
Oxytetracycline	NA	74.9 ± 91.8	80.1 ± 59.3	87.5 ± 150.0	86.0 ± 153.2	74.3 ± 6.2	77.5 ± 81.6
Paroxetine	87.9 ± 97.8	NA	NA	33.8 ± 51.3	86.9 ± 164.9	57.1 ± 15.1	NA

Bioactive chemical pollutant	Stoke bardolph wastewater treatment plant Removals (%)						
	Summer 2018 ***n=3***	**Winter 2018-19** ***n=6***	**Spring 2019** ***n=8***	**Summer 2019** ***n=5***	**Autumn 19** ***n=5***	**Overall** ***n=27***	**Sutton Bonington WWTP overall removal** ***N=15***
Phenazone	NA	NA	NA	NA	NA	82.9 ± 28.7	82.9 ± 158.4
Propranolol	**21.9 ± 10.9**	**-39.8 ± 30.0**	**14.7 ± 11.5**	**-38.3 ± 18.3**	-34.5 ± 29.4	-8.0 ± 1.1	65.8 ± 65.9
Salicylic acid	NA	99.7 ± 154.0	99.6 ±257.8	98.3 ± 185.3	NA	97.4 ± 27.8	99.6 ± 256.5
Sulfadiazine	**5.6 ± 9.1**	81.7 ± 67.4	51.6 ± 17.2	59.9 ± 100.2	50.0 ± 39.8	64.7 ± 13.2	70.9 ± 164.5
Sulfamethoxazole	74.8 ± 57.0	87.5 ± 44.2	81.7 ± 44.5	76.1 ± 57.1	**41.2 ± 33.6**	77.1 ± 11.7	52.5 ± 71.8
Sulfapyridine	65.8 ± 58.7	**57.3 ± 14.6**	**45.2 ± 14.8**	**9.5 ± 7.6**	**-9.5 ± 6.8**	34.9 ± 5.9	38.4 ± 56.1
Tramadol	80.9 ± 72.7	24.4 ± 8.5	30.4 ±19.4	37.6 ± 44.8	**-65.6 ± 20.5**	26.4 ± 0.3	16.3 ± 17.5
Trimethoprim	**45.9 ± 20.4**	**28.1 ± 9.4**	14.2 ± 6.1	19.7 ± 13.7	**-21.1 ± 12.3**	17.4 ± 2.5	73.0 ± 117.5

Table 5.4-1 Bioactive chemical pollutant emovals for Stoke Bardolph wastewater treatment plant and Sutton Bonington wastewater treatment plant, bold are those for which tee is a significant ($p<0.05$) difference between the overall and a season removal. NA=not available, for pollutants where the number of detections was n<3.

**Note: removals for Stoke Bardolph WWTP (WWTP 1) and Sutton Bonington WWTP (WWTP 2) are not directly comparable as those of SuB were calculated based on the downstream water, in which BAC concentrations will be more diluted than pure wastewater effluent*

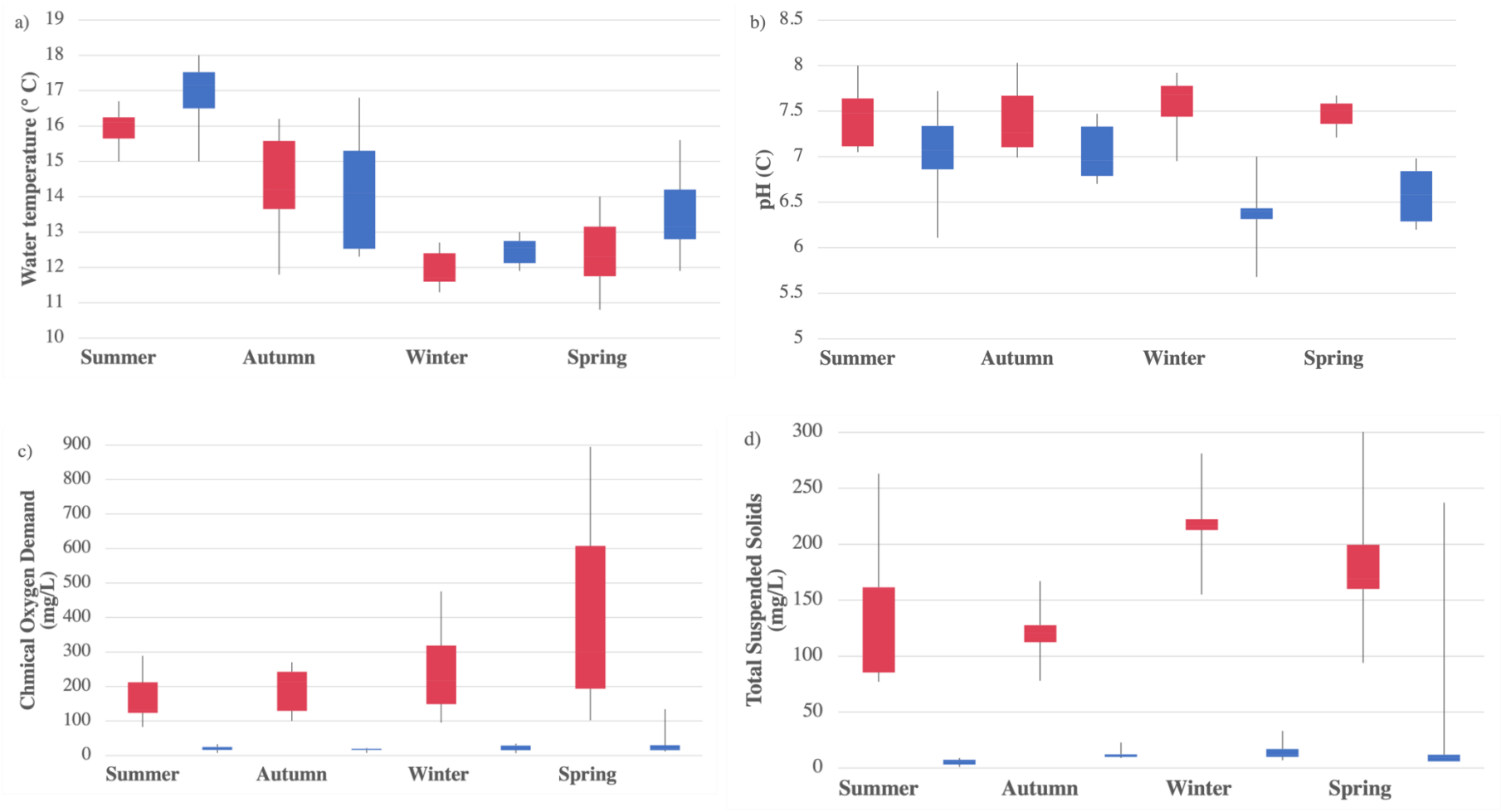


Figure 5.4-1Water quality parameters for Stoke Bardoloph wastewater treatment plant. Red: wastewater influnet. Blue: wastewater efflunet. a) Water temperature, b) pH, c) Chemical oxygen demand, d) Total suspended solids

Sulfapyridine was found to have variable removals across the four seasons (Table 5.4.1). Highly variable removals are reported for sulfapyridine in the literature (<50 - >80 %) (Petrie, et al., 2015), it is possible this variability is partly due to seasonality. E3 has very variable removals for both WWTPs studied, even within one season, standard deviations are high (Table 5.4.1). E3 has a high k_d value, meaning it is mostly removed by sorption (Ting, et al., 2017). Sorption has been shown to be reversible under certain conditions (Ting, et al., 2017), this plus deconjugation (Ting, et al., 2017) could explain the variability in E3 removal.

Metoprolol presents low and negative removals in WWTP 1, particularly during winter and spring (Table 5.4.1). No significant difference between influent loads and effluent concentrations of metoprolol have been previously reported (Robert Gurke, et al., 2015a). This pollutant was detected at lower concentrations in the WWI of both plants than those reported in the literature (Robert Gurke, et al., 2015a; Petrie, et al., 2015). Metoprolol was detected with less frequency in the influent than the WWE or WWD of both plants. Metoprolol is metabolised in the body with 10-30% excreted unchanged (Robert Gurke, et al., 2015a; Petrie, et al., 2015) and approximately 85% excreted as metabolites α-hydroxymetoprolol and O-demethylmetoprolol(Brocker, et al., 2020). There could be some conversion of metabolites back to the parent compound, which would explain the negative removals.

Carbamazepine had higher removals in winter (Table 5.4.1), compared to mostly negative removals for the rest of the year. There was no increase in influent concentration which could explain the higher winter removals, although previously higher winter influent carbamazepine concentrations have been reported(Fernández, et al., 2014). Negative carbamazepine removals have been previously reported and attributed to its low biodegradability, low affinity for sludge(Zhang, et al., 2008) and transformation of the free forms by microorganisms (Petrie, et al., 2015; Sui, et al., 2011).

Codeine presented significantly ($p<0.05$) lower removals in autumn and significantly higher in Spring (Table 5.4.1). This is different to previously reported higher codeine summer removals, related to warmer temperatures and more sunlight (Yadav, et al., 2019). Significantly lower, and negative, autumn removals were also observed for tramadol, trimethoprim and sulfapyridine. Negative removals of BACs are associated to hydrolysis of conjugated metabolites or desorption from suspended solids or sludge(Petrie, et al., 2015). Codeine is partially excreted as codeine-6-glucuronide(Petrie, et al., 2015). Trimethoprim is excreted primarily unchanged, with known metabolites being 1,3-oxides and 3'4-hydroxyderivatives(Petrie, et al., 2015). Tramadol metabolites are mainly O-demethylation, N-demethylation and conjugation (Gong, et al., 2014). For sulfapyridine metabolites are mainly hydroxyl and acetyl metabolites(Petrie, et al., 2015). Some conversion of these metabolites to the parent compounds may explain the negative removals, however it is not clear why this would be more prominent in the autumn. TSS and COD were lower and less variable in the autumn than the spring (Figure 5.4.1). All four of these pollutants, except tramadol, are soluble in water (Log_{Kow}< 1.5 (dos Santos, et al., 2019; Grossberger, et al., 2014; Wishart, et al., 2017)). For tramadol less TSS and COD could mean less partitioning to the solid phase, or lower available sorption sites resulting in desorption and therefore negative removals. Tramadol was found to have a significantly better removal in Stk, despite the dilution effect that would be observed for SuB downstream water. Low tramadol removals have been previously reported (Petrie, et al., 2015). For trimethoprim the average wastewater influent concentration during the autumn was the lowest of all the seasons (183 ± 87 ngL^{-1} compared to the overall average of 301 ± 39 ngL^{-1}). The wastewater effluent concentrations were not very variable across the

seasons, with an average of 249 ± 15 ngL^{-1}. This may indicate a certain amount always passes unchanged, however this does not explain the negative removals. Negative trimethoprim removals have been previously observed in a plant using activated sludge (Wang, et al., 2016), however it has also been reported to be between in 36-70% in other studies (Wang, et al., 2016). It is possible that some trimethoprim is absorbed to the sludge, but as trimethoprim is not strongly bonded to the sludge (Göbel, et al., 2005) it could be released into the water after being absorbed.
Diclofenac has a significant difference between summer removal and the rest of the year. The reduced removal for diclofenac during winter in a biological treatment plant has been previously reported (Sui, et al., 2011). Diclofenac presents low biodegradability and low sorption to sludge (Zhang, et al., 2008) and its low removal has been previously reported. Similarly, significantly lower removals were observed for erythromycin in the winter and autumn (Table 2), this may be related to lower temperatures not favouring biological process and lower sun irradiation. The lower removals observed for morphine in autumn and winter are in accordance with those previously reported (Yadav, et al., 2019).

5.4.2 Pollutants with variability in removals across treatment type

The water quality parameters measured fo SuB are reported in Appendix 10.3, it is important to report these along WWTP removals so that other studies can be compared. Trimethoprim and o-demsethylvenlafaxine were found to have medium removal in SuB and low in Stk, with significant ($p<0.05$) difference between the two plants. This could be due to the dilution effect of the downstream water used for the calculation for SuB. However, trimethoprim has been previously reported to be removed by slow growing nitrifying bacteria (Radjenović, et al., 2009), which may explain the better removal in SuB as the microorganism populations would be more stable in a trickling filter than in activated sludge. Sorption has been identified as a possible removal pathway (Radjenović, et al., 2009) so the sand filtration step at SuB may be the reason for the improved trimethoprim removal of SuB. Previous trimethoprim removals reported have been -53.1% in a plant with conventional activated sludge (Wang, et al., 2016) and 38-55% for a plant with anaerobic and aerobic activated sludge (Rodriguez-Narvaez, et al., 2017). O-desmethylvenlafaxine removal has been reported to vary between -20 – 100% in a plant with biological and chemical phosphorus removal and a sand filtration polishing step (Kosma, et al., 2019). If not due simply to dilution, the results in this study could indicate that the sand filtration step is more efficient in o-desmethylvenlafaxine removal than the nitrogen or phosphorus removal stages.

Propranolol presents a significantly better removal in SuB compared to Stk, this cannot be simply attributed to dilution as the overall removal for STK is negative. For Stk, propranolol presented significantly lower (and negative) removals in the winter and summer and a significantly higher removal in the spring. Twenty percent of propranolol is excreted as glucuronide conjugates(Petrie, et al., 2015), and their deconjugation in the WWTP is likely the reason negative removals are observed. In the literature the removals have been reported to be highly variable (<50 - >80%)(Petrie, et al., 2015). This variability is likely due to seasonality as well as treatment type. In a study assessing the removal of beta-blockers from WWTP biodegradation was identified as a removal mechanisms for all the compounds studied and sorption only for propranolol (Radjenović, et al., 2009), this may explain the better removals in SuB due to sorption in the sand filtration step. It is possible the activated sludge organisms are better at deconjugation of propranolol, which may explain the negative removals observed for this plant.

5.4.3 Pollutants with consistent removal

Acetaminophen, ibuprofen, salicylic acid and naproxen were found to have high overall removal (>80%) for both plants and were not found to have a significant ($p<0.05$) seasonal removal variation for Stk. The constant seasonal removals for acetaminophen are in contrast to a previous study were higher removal was observed with increasing seasonal temperatures (~70% in autumn (9 °C) and ~95% (19 °C) in summer) (Fernández, et al., 2014). Better summer removal has been previously observed for ibuprofen and acetaminophen in Greek WWTPs with activated sludge (Kosma, et al., 2014), however others report consistent high removal (Petrie, et al., 2015). Naproxen has been reported to have removals >75% for a range of different plants, mainly using activated sludge (Rodriguez-Narvaez, et al., 2017; Wang, et al., 2016). All three of these pollutants have high standard deviations in the removal, due to the wide range of concentrations measured in the wastewater (Table 5.4.1). For acetaminophen in SuB, concentrations 2-3 orders of magnitude higher were observed on the last three sampling dates in October and November (12,525-23,554 ngL^{-1}), compared to the rest of the concentrations measured (45-317 ngL^{-1}). This spike did not correlate to any water quality parameters or concentrations of other BACs. Atenolol (Table 5.4.1) also presented a high overall removal (>80%) for both plants with no significant seasonal difference for Stk. Atenolol has been reported to have a wider range of removals in different WWTPs, for example in a plant using a bioreactor it was found to have a removal of 47.1% (Rodriguez-Narvaez, et al., 2017), while in other plants it has been reported to have removals from 50->80%(Petrie, et al., 2015). All three steroid sulphate conjugates present high and consistent (>80%) removal for both plants, but this could mean they are just being deconjugated.

Oxytetracycline and sulfadiazine were found to have overall medium (50-80%) removal for both plants, with no significant seasonal variation. For oxytetracycline this is lower than the high removal (>80%) reported elsewhere (Petrie, et al., 2015), but different conditions such as temperature, pH and type of biological set up have been shown to affect removal (Shao, et al., 2019). Sulfadiazine has a negative week removal for SuB, with a high standard deviation. Sulfadiazine has been previously shown to present high removal in the aerobic carbon and nitrification stage (compared to very low removal in the anaerobic denitrification stage)(Dapeng Wang, et al., 2019). Ketoprofen and bezafibrate were found to have medium removals for Stk with no significant seasonal variation. This is similar to what was found in literature (Kosma, et al., 2019; Petrie, et al., 2015), due to their low frequency it was not possible to determine accurate removals for SuB.

The overall removal of acetyl-sulfamethoxazole for Stk was > 90%, with no significant seasonal variation. A similar high removal was observed for SUB but with a high standard deviation. These removals are higher than a previous study which found removals ranging from -141 to 33.6 % in the summer and -51.2 to 30.3 % in the winter over 5 WWTPs treating domestic wastewater in China, three of which utilised anaerobic-anoxic-oxic biodegradation, one which used adsorption biodegradation and one utilising an oxidation ditch as secondary treatment (Zhang, et al., 2015). The influent concentrations were lower (82.7-299 ngL^{-1}) (Zhang, et al., 2015) than those observed in the present study, so the lower removals cannot be explained by higher influent concentrations and are likely due to better removal. Sulfamethoxazole presented medium (50-80%) removal for both plants, lower than the high (>80%) removal observed for acetyl-sulfamethoxazole. A study found that most sulfamethoxazole removal was in the nitrification unit (Dapeng Wang, et al., 2019), which may explain why removal was slightly higher for Stk than SuB. The overall removals for both plants are within previously reported ranges (Petrie, et al., 2015) and higher than the negative removal observed for a plant with activated sludge(Wang, et

al., 2016). Sulfamethoxazole had a significantly ($p<0.05$) worse removal in autumn (41.2 ± 34.0), compared to the overall removal (77.1 ± 7.4) for Stk, similar to sulfapyridine.

Most 4-tert-octylphenol removal has been reported to be due to adsorption to sludge and high removal rates have been observed (Höhne, et al., 2008), which is lower than those measured for Stk (Table 5.4.1). However, 4-tert-octylphenol concentrations in Stk WWE are within the range found in literature, while SuB WWD concentrations are higher(Gorga, et al., 2013), likely due to the higher influent concentrations.

5.5 Quantifying the risk of bioactive pollutants in wastewater effluents

The estradiol predicted no effect concentration (PNEC) for freshwater life is 1 ngL^{-1} (Ma, et al., 2016). The estradiol equivalent value was calculated using Eq. 22 and the values in Appendix 10.1 and it was between 8-16.9 and 22.6-47 ngL^{-1} in SuB WWD and Stk WWE, respectively. The mean is 12.5 and 31 times the accepted value respectively in SuB WWD and Stk WWE. The free hormones are the main contributors to these vales. However, sulfate conjugates have been shown to deconjugate (Gomes, et al., 2009) and could potentially make these values even higher. This values are higher than those found in streams and groundwater (Tremblay, et al., 2018) but similar to those found in other wastewater effluents in Europe (Comtois-Marotte, et al., 2017).

Pollutants with RQ > 1 (calculated as described in Section 2.6.4.3) are codeine, diclofenac, erythromycin, morphine, oxytetracycline, propranolol, salicylic acid, simvastatin, sulfamethoxazole, tramadol and trimethoprim as can be seen from Figure 5.5-1. Some of these pollutants have been previously found to be a risk quotient above 1 (diclofenac (Daouk, et al., 2016; Hernando, et al., 2006), erythromycin, sulfamethoxazole, trimethoprim (Johnson, et al., 2015), oxytetracycline and propranolol (Hernando, et al., 2006)). Carbamazepine, naproxen, ketoprofen, metoprolol and ibuprofen have been previously found to have higher RQ values than 1 in other studies (Daouk, et al., 2016; Hernando, et al., 2006) and they did not in this study. RQ variability within literature is due to many factors. Unknown PNEC data for some pollutants, plus the different sensitivities of different organisms means there are variable PNECs available in the literature (Qu, et al., 2019). Conversion of metabolites to parent bioactive drugs or metabolites with more bioactive effects than the parent drug may mean the actual risk is higher (Han, et al., 2017). For example, although acetyl-sulfamethoxazole has an RQ < 1, it can undergo deacetylation in the environment, reforming sulfamethoxazole (Aurore Andriamalala, et al., 2018), which in this study was found to have an RQ > 1 for both sites.

Other factors can add to the environmental toxicity of BACs in effluents such as one enantiomer being more potent than another (Petrie, et al., 2015) and the largely unknown mixture effects (Petrie, et al., 2015). However, for this study it appears that both plants need improvement in removal for the same pollutants. Ibuprofen removal has been previously reported to occur mainly in the aeration stage of biological removal, which would explain the lower concentrations entering the environment from Stk compared to SuB (Smook, et al., 2008).

In terms of minimum inhibitory concentration for antimicrobial resistance, only oxytetracycline was found to have an RQ value above 1. However, this was not observed for the mean concentrations (when the RQ values were 0.52 and 0.68 respectively for Stk and SuB) but rather for the maximum concentrations measured at each site. The RQ values at maximum concentrations were 1.17 and 1.84 for Stk and SuB respectively. Despite oxytetracycline being the only antibiotic with an RQ>1 for the minimum inhibitory concentration for antimicrobial resistance, it is important to note

that knowledge of the consequences of long-term exposure of antimicrobials towards living organisms is still negligible (Felis, et al., 2019).

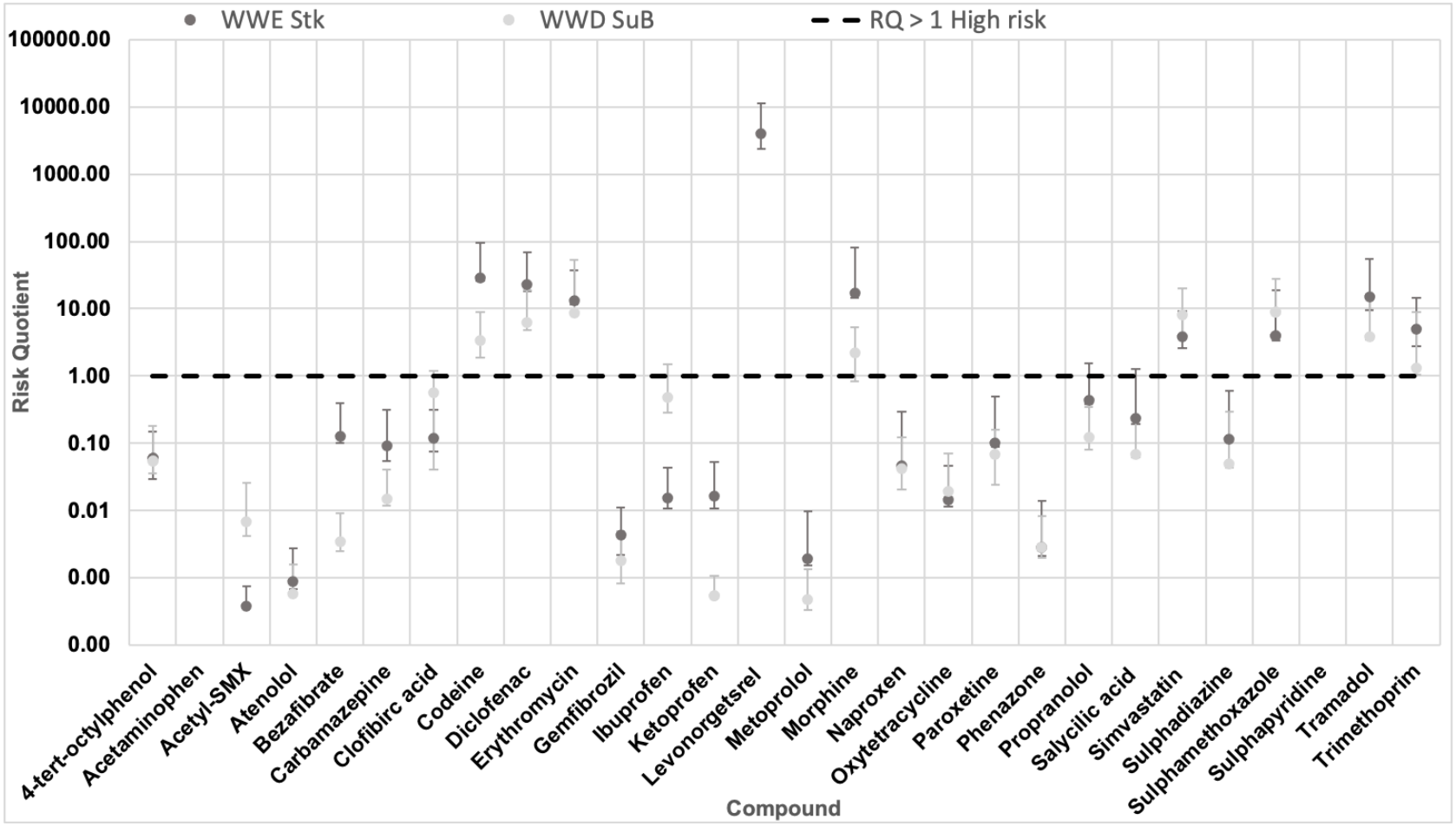


Figure 5.5-1 Risk quotients of Stoke Bardolph wastewater effluent (WWE Stk) and Sutton Bonington downstream water (WWD SuB)

These risk quotients above 1 are of concern for local aquatic wildlife living in the wastewater effluent open channel of Stoke Bardolph and in the stream immediately receiving the effluent from Sutton Bonington. However, there are uncertainties associated to this calculation, given that PNECs are estimated for key species and vary according to the literature consulted, which is why several publications were consulted (Appendix 10.1) and the lowest PNEC found (or the PNEC which accounted for aquatioc toxicity as a whole, as opposed to one specific species was used). In order for a pollutant to be toxic to aquatic life bioaccumulation (how much of the pollutant dissolved in water is actually uptaken by aquatic organisms) must occur (Arnot, et al., 2006). Methods to measure bioaccumation are varied and there are uncertainties associated to it, given factors such as the assumption of a steady state between the uptake through dermal, respiratory and food routes and the elimination of the pollutant through routes such as excretion and metabolitc transformation and the dilution due to growth (Arnot, et al., 2006). However, the approach taken for this thesis is a standard approach for identifying BACs of concern from wastewater effluents previously published (Phonsiri, et al., 2019).

The dilution that would take place once the pure effluent from Stoke Bardolph reaches the river was taken into consideration to obtain a quick estimate of the pollutants which would remain at RQs>1 in the river.The wastewater flowrate from Stk WWTP was estimated as $1.3 m^3 s^{-1}$ based on the PE of 650,000 and the standard 200 L^{-1} day^{-1} $habitant^{-1}$. The minimum, average and maximum flow rates for the River Trent in 2019 were obtained from the National River Flow Archive, at Trent at Colwick guage station (6 km upstream from the point where the effluent from Stk WWTP is discharged. The values were 28 $m^3 s^{-1}$, 85 $m^3 s^{-1}$, 330 $m^3 s^{-1}$ resulting in dilution rations ranging from 0.005-0.054 depending on the river flowrate. Therefore, the RQs which would remain above 1 in the river would be for levonorgestrel at all river flowrates, for codeine, total estrogenicity and morphine (at minimum and average Trent River flow rates) and for diclofenac, tramadol, and erythromycin (only at minimum River Trent flowrates). These RQs are likely to be higher when considering pollutants which would likely be present in the river from upstream discharges.

In the current situation in the UK in which mucnicipal wastewater is not directly reused for agricultural irrigation thses finidngs are significant in terms of the environmental pollution presented by WWTP effluents to the aquatric environment. The data collected is important in terms of site-specific information which will inform treatment process improvents neceseray as more stringetnt wastewater effluent discharge regulations are put in place(DEFRA, 2018; UK Water Industry Research, 2017). It is also of importance in terms of future-planning, with increasing pressure on freshwater resources and increasing food demand (Lazarte, 2017; UN-WWAP, 2015; WWAP, 2017), direct reuse of wastewater effluent for agricultural irrigation in the UK has the potential to be a part of the solution. This solution could potentially protect aquatic life from direct contact with BACs at RQs>1. However, further work is necessary in terms of the behaviour of BACs once in the soil environment and their potential to accumulate in crops and enter the animal and human food chains. In this sense international collaborations with countries, such as Mexico, where wastewater irrigation is a common practice are relevant as examples and as learning points for different water management strategies.

5.6 Correlations between prevalence of different pollutants and between pollutants and water quality parameters

Statistical relationships do not prove causation; however correlations are valuable in modelling efforts and determining better treatment methods and operating conditions. Overall, more significant correlations were found for Stk WWE and SuB WWD than the

WWI of either plant. Eight strong correlations were found, all for SuB. Taking into account that several statistical tests were carried out, Bonferroni correction was applied to avoid family-wise type I error. A $p< 0.05$ was considered and 8 strong correlations were found, therefore that a significant p-value should be below 0.00625 for each of them. Two of the strong correlations were found in influent wastewater for SuB, the first one was a positive correlation between sulfamethoxazole and trimethoprim (R=0.743, p=0.001506), this positive correlation was also found in WWI Stk (R=0.6589, p=0.000252). The reason is most probably that trimethoprim and sulfamethoxazole are often prescribed as a joint medicine to treat a wide variety of bacterial infections (Falagas, et al., 2015). The second one was between Chemical Oxygen Demand and Total Suspended solids (R=0.7918, p=0.000435), for well understood reasons.

The remaining six strong correlations were found in downstream water of SuB. The strongest one was between o-desmethylvenlafaxine and estriol-3-sulfate (E3-3S) (R=0.8472, p=0.02375), however this is not statistically significant using the Bonferroni correction. O-desmethylvenlafaxine was also correlated to temperature (R=0.7461, p=0.0005330). O-desmethylvenlafaxine seems to have lower effluent concentrations when temperature is lower (approximately below 15 °C). Most of O-desmethylvenlafaxine removal is most likely occurring in the sand filtration step, given it is very resistant to degradation in conventional biological units (García-Galán, et al., 2019; Llorca, et al., 2019), so the lower temperatures could favour sorption in the sand filtration step.

The four remining strong correlations, were between tramadol and trimethoprim (R=0.7177, p=0.002589), tramadol and diclofenac (R=0.7404, p=0.001597), sulfamethoxazole and diclofenac (R=0.7503, p=0.001272) and propranolol and sulfamethoxazole (R=0.7595, p=0.001021). By plotting all five of these pollutants (Figure 5.4.1) it was apparent that they all have a spike on the 22/08/2019, which is less pronounced for propranolol. Their concentrations all converge in October, the influent concentrations of all of these pollutants also reduced in October, except that of tramadol. Tramadol has consistent low recovery and similar effluent concentration throughout the sampling period. A similar thing occurs with sulfamethoxazole, although the removals are higher. Trimethoprim has consistent medium to high removals and is most likely eliminated mostly in the sand filtration step, as previously explained. All five of these pollutants, except diclofenac, have an amide group, however it would be expected for other pollutants like atenolol, carbamazepine, sulfadiazine and sulfapyridine to behave in the same way if this were the only reason for their correlation. All five of these pollutants are relatively insoluble in water (except tramadol), all have log koc < 3. It is possible all are mostly eliminated in the sand filtration step and so their correlation may be due to this unit operator. Further experiments would be necessary to determine whether this is the case, looking into competitive sorption, sorption saturation, etc.
Even though it is not a strong correlation, the negative correlation between bezafibrate and temperature (R=-0.5889, p=0.001232) found in the effluent of Stoke Bardolph, supports previous studies which have found that biodegradation is the main removal mechanism of bezafibrate and can be described as pseudo-first-order reaction (Sui, et al., 2016). The reaction rate constant reduced by 86 % when the temperature dropped from 20 °C to 10 °C (Sui, et al., 2016).

Future work, including obtaining more results to increase reliability of the moderate correlations found and determining and testing their usefulness in modelling and predictive work would be necessary. This information is useful in determining optimum conditions for unit operators and maximum removal of BACs.

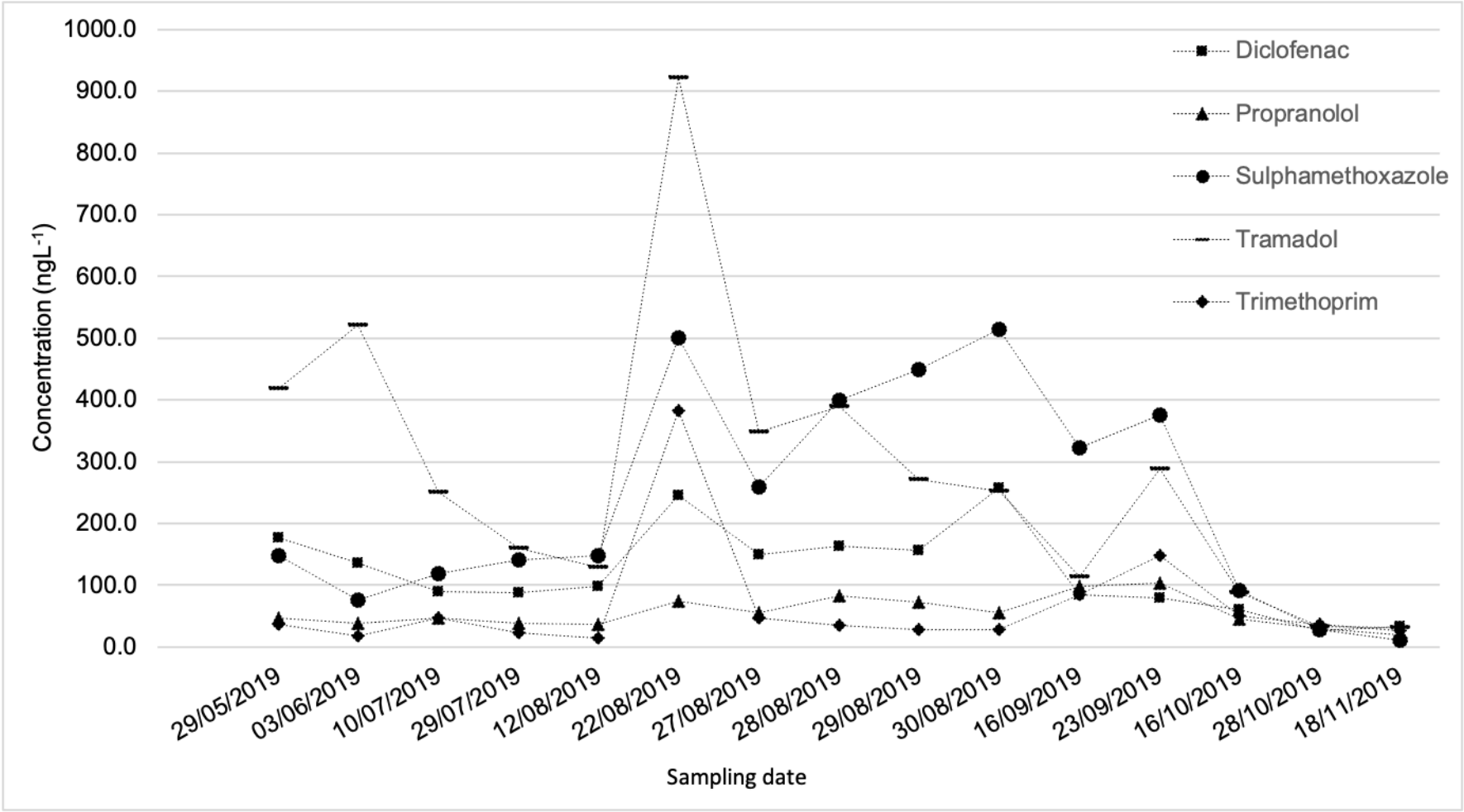


Figure 5.6-1 Sutton Bonington wastewater treatment plant concentrations in downstream water of pollutants with strong correlations

5.7 Summary

Occurrence of pollutants were found to be similar to those found in literature, with some exceptions which could be explained due to the demographics and types of establishments in the area. Significantly better overall removal was found for tramadol in Stoke Bardolph WWTP (PE approx. 650,000; advanced secondary treatment), compared to Sutton Bonington (PE approx. 2,000; trickling filter and sand filtration). Twelve pollutants were found to have significant seasonal variation in removals, namely diclofenac, carbamazepine, propranolol, trimethoprim, codeine, sulfapyridine, metoprolol, estrone-3-sulfate, morphine, erythromycin, ketoprofen and sulfamethoxazole. Eight strong and significant correlations were found between the temporal variation in concentration for different pollutants. These are significant as they can help in modelling efforts, reducing time and resources in sampling and analysis as well as in unveiling insights into the removal process. For example, temperatures above 15°C were found to increase removals of estriol and o-desmethylvenlafaxine in Sutton Bonington treatment plant. This is important in designing and operating plants with better removals for bioactive pollutants. Despite their differences in removal efficiencies, both plants have risk coefficients consistently above 1 for the same 8 out of 36 pollutants. Namely, codeine, diclofenac, erythromycin, morphine, oxytetracycline, propranolol, salicylic acid, simvastatin, sulfamethoxazole, tramadol and trimethoprim. Furthermore, the equivalent estradiol concentrations was found to be at least 8 times above the PNEC for both plants.

The removal of these pollutants in the literature is very variable, this variability is due to different treatment process, seasonality, sampling strategies, among other factors. As more stringent legislation is approved it is important to have site-specific data to identify pollutants of concern (low removal and high environmental risk). This work identifies 8 pollutants both of concern for two UK WWTPs, as well as identifying 12 pollutants which have seasonally variable removal. These findings are relevant in terms of comparing with other UK WWTPs, modelling efforts to understand necessary improvements to treatment process at a national scale or potential direct uses of wastewater effluent for agricultural irrigation.

6 RESULTS AND DISCUSSION; IDENTIFICATION OF OTHER BACS, IN UK WWTPS

6.1 Highlights

- **25 compounds not previously identified** in relation to wastewater pollution, including: 2-Amino-6-methylmercaptopurine, active drug metabolite of 6-Thioguanine, a drug used to treat leukaemia; 4-Hydroxyindole a pollutant banned by banned by ASEAN (Association of Southwast Asian Nations) Harmonized Cosmetic Regulation Scheme and, 6-methylquinoline, a Heterocyclic aromatic hydrocarbon with estrogenic effects.
- **10 biomarkers identified** including caffeine and four of its metabolites, nicotine and its metabolite cotinine, acesulfame, saccharin and creatinine. Their identification using an untargeted method not designed specifically to identify them, evidences their stability and amenability for analysis (key requirements of biomarkers).
- **2 illicit drugs** rarely reported in wastewater were identified: cannabinoid A-836339 and designer drug ADBICA.
- Wastewater-based epidemiology: **3 pollutants identified in relation to potential disease monitoring**. Adenine and its metabolite 1-methyl-adenine which have been found in higher concentrations in the urine of cancer patients. GABA, of which higher amounts in human urine are associated with more advanced dementia and piperine, which has been found to have a co-variance with SARS-CoV-2 RNA, but this relationship has not been studied in detail.
- Several sampling days allowing for **PLS analysis which gives information on removal of untargeted pollutants identified across two different plants**, for example better removal was observed for 16-hydroxyhexadecanoic acid, 2-amino-6-methylmercaptopurine, dihydrocodeine, lidocaine, PPG n5 and PPG n6 in a wastewater treatment plant comprised of trickling filter and sand filtration than a plant with advanced activated sludge removal process.
- Complementing the **fate of several of the analytes in the targeted monitoring** by transformation product and metabolite identification. For example, 2 metabolites of carbamazepine, one of which is pharmacologically active, as well as 2 active metabolites and one active transformation product of tramadol were found in the influent and effluent of both plants. As well as 4-aminophenol, a toxic biodegradation product of paracetamol was identified in the effluent or downstream water of both treatment plants.

6.2 Summary of samples discussed in this chapter

LC-MS run identifier (run set-up detailed in section 4.3)	Samples analysed			
	Stoke Bardolph WWTP		Sutton Bonington WWTP	
	WWI	WWI	WWI	WWD
	Raw influent	Raw effluent sampled at mid ppoint of a 1 km open channel going form the WWTP to the river Trent	Raw influent	Raw effluent sampled at mid ppoint of a 1 km open channel going form the WWTP to the river Trent
a.	One sample taken on 9th August 2018	One sample taken on 9th August 2018	-	-
b.	One sample taken on 10th July 2019	One sample taken on 10th July 2019	One sample taken on 10th July 2019	One sample taken on 10th July 2019
c.	-	-	Seven samples taken on one taken at the start of August 2020 and the other on 6 consecutive days at the end of August 2019	Seven samples taken on one taken at the start of August 2020 and the other on 6 consecutive days at the end of August 2019
d.	Seven samples taken from August to October 2019, with five taken in October, one in September and one in August	Eight samples taken from August to October 2019, with five taken in October, two in September and one in August	Seven samples taken from Septmber to October 2019, with five taken in October and two in September	Seven samples taken from Septmber to October 2019, with five taken in October and two in September
Total number of samples	**9**	**10**	**15**	**15**

Table 6.2-1 Summary of location and frequency of samples collected from the UK wastewater treatment plants for untaregted analysis

6.3 Pollutants identified

As explained in Sections 2.6.3.4 analytes which were in the targeted method, were identified with confidence level 1 as standards were used (Sumner, et al., 2007). The rest of the compounds were identified with confidence level 2 as they were compared to the mzCloud database (Sumner, et al., 2007).

The compounds identified have been categorised into three main groups. Firstly, *Unveiling environmental toxicity risk beyond the targeted monitoring).* This section encompasses metabolites and transformation products of the targeted analytes, completing the fate and prevalence of several of the compounds monitored in the previous chapter. This section also includes other pharmaceutical and personal care products which were not in the targeted analysis as well as BACs related to industrial processes and products. These are divided into those which have been widely reported in the literature and those that haven't. Several of these compounds have known toxic effects, which even though cannot be quantified as those from the targeted method, add to the environmental risk of the effluents from Stk and SuB WWTPs.

Secondly, the section *Compounds not previously identified or* with unknown effects*,* deals with the identification of compounds which have not been previously identified in relation to wastewater or in the aquatic environment. Even though these compounds have not been characterised in terms of environmental toxicity they are likely to have some effect as most of them are known to be bioactive.

Thirdly, pollutants in the *Public Health: pollutants relevant to wastewater-based epidemiology*, these pollutants have been previously associated to quantifying habits such as caffeine and nicotine intake or illicit drug use, used as markers of human pollution or are indicators of disease in the population.

Compounds classified in one category may well fit into another, as the topics are interlinked, for example a compound which may provide insights into public health may also pose a risk to the environment. However, the categories are useful in understating the different insights which untargeted monitoring can provide.

Finally, the section *Insights into treatment process removal efficiency*, discusses pollutants which presents significant ($p<0.05$) reductions or increases in signal from influent to effluent in either plant. This is quantified using the WWE/WWI ratio of the average of the areas in runs c or d, (where $n>6$, for different sampling days). As the areas compared are for the same compound it is reasonable to assume that a higher area means a higher concentration. Therefore, a WWE/WWI > 1 reveals poor removal and/or conversion of other compounds into the one in question. A WWE/WWI < 1 reveals a decrease in concentration across the WWTP. Similarly, for run d it is also possible to compare the WWE of Stk to the WWD SuB to determine if one plant or the other is releasing a higher concentration of a certain pollutant to the environment. The same can be done to compare the WWI of both plants using the results from run d. This section also discusses the grouping obtained using PLS from runs c and d. As expected, samples were grouped according to WWI and WWE, as well as according to plant (Stk or SuB). The VIP (variable of importance prediction) score assigned to each compound during PLS, indicates which compounds are more responsible for the grouping of the samples. This is of importance as it reveals the main differences between WWI and WWE as well as between the plants, giving insights into the removal process. The compounds discussed in this section had to be detected in runs c and d with a frequency of detection >40% in order to make PLS analysis valid.

6.3.1 Unveiling environmental toxicity risk beyond the targeted monitoring

6.3.1.1 Metabolites and transformation of compounds in the targeted method

The targeted analytes along with their identified metabolites and transformation products are reported in Table 6.3.1. Compounds which were in the targeted method were identified with level 1 confidence in the untargeted study, as standards were used, the rest with level 2 confidence (Sumner, et al., 2007). Expected metabolites were identified, behaving in similar ways as those found in literature, with regard to frequency and removal, as detailed below. Although these results are not new, they provide a more complete picture of the fate of the targeted pollutants in the WWTPs studied as well as giving further confidence in the methodology used and results obtained.

Atenolol and its biotransformation product atenolol acid were identified. Between 50 to over 85% of the atenolol dose is excreted unchanged in urine within 24 hours (Karaman, 2015) and it easily undergoes biodegradation into atenolol acid in treatment plants with different kinds of biological treatments (Xu, et al., 2017). In the targeted monitoring, atenolol was found to have a high removal (>80%) in both plants and for this removal to be independent of seasonal variation for Stk WWT. The untargeted results for SuB are in agreement with this as the peak area of atenolol is seen to significantly ($p<0.01$) decrease by one order of magnitude form influent to effluent, as shown in Table 6.3.1. On the other hand, the peak area of atenolol acid significantly ($p<0.01$) increases by one to two orders of magnitude between influent and downstreamwater. This is in accordance with the literature, in terms of atenolol being biodegraded to atenolol acid (Xu, et al., 2017). Atenolol acid has been mostly predicted not to be carcinogenic (Wielens Becker, et al., 2020).

Carbamazepine (CBZ) was detected with 100% frequency in the targeted analysis and in this untargeted study it was detected in all but the last run. The two and a half higher carbamazepine signal found in WWD compared to WWI for WWTP SuB is consistent with the low and even negative removals found previously for this plant in the targeted study as well as in literature (Petrie, et al., 2015; Sui, et al., 2011). Of the ingested carbamazepine dose approximately 25-30% is excreted unchanged from the human body and the rest is metabolised. The most abundant CBZ metabolite is 10-11-dihydro-10,11-trans-dihydro-carbamazepine (DiOH-CBZ), it is found in human urine as 30% of the ingested dose and it is often detected in wastewater in concentrations equal or higher to the parent compound. DiOH-CBZ is not therapeutically active (Paz, et al., 2016). Approximately 2% of the ingested dose is excreted as the therapeutically active metabolite 10,11-epoxy carbamazepine (CBZ-Ep). CBZ-Ep has been detected in reclaimed wastewater in the range of 50-120 ngL^{-1} (Leclercq, et al., 2009). CBZ-Ep is expected to be unstable in aquatic solutions and to be converted to DiOH-Cbz in the treatment process (Leclercq, et al., 2009). However, in SuB CBZ-Ep was found to have a signal 4.5 times significantly ($p<0.01$) larger in WWD than WWI, contradicting the results predicted by (Leclercq, et al., 2009). However, an increase in CBZ-Ep (a negative removal of -17%) was also observed in a targeted study of a WWTP in South-West England with PE of around 105,000 and comprised of a trickling filter (Petrie, et al., 2016). These authors also reported a -457% removal for DiOH-CBZ (Petrie, et al., 2016), the results presented in this untargeted study do not present this increase of DiOH-CBZ within the treatment process. It is possible this due to the reduced selectivity and sensitivity of the untargeted method as compared to a targeted method. The negative removal observed for CBZ-Ep in this study and in other studies is of concern as this is a pharmaceutically active metabolite of a parent compound which is prevalent in the environment and could have synergistic negative effects (Leclercq, et al., 2009).

Acetylsalicylic acid is known as a prodrug as its metabolites are pharmacologically active (Han, et al., 2017). Acetylsalicylic acid is hydrolysed to salicylic acid which undergoes transformation to gentisic acid, salicyluric acid and glucuronide conjugates (Celiz, et al., 2009). Salicylic acid and gentisic acid have been found to affect reproduction and growth of *Cladocera*, although the effect concertation was lower than the concentrations observed in the aquatic environment (Han, et al., 2017). Acetylsalicylic acid is mainly expected to be found in the environment as salicyluric acid, followed by salicylic acid and to a lesser extent gentisic acid (Han, et al., 2017). Previously acetylsalicylic acid has been found in WWI in concentrations in the range 1,500-5,000 ngL^{-1} while salicylic acid was found between 49,000- 68,000 ngL^{-1}, up to 12,000 ngL^{-1} for salicyluric acid and 3,600-5,500 ngL^{-1} for gentisic acid (Wiegel, et al., 2004).

In the targeted study salicylic acid was found at very high (mgL^{-1}) concentrations in the influent of both WWTPs. In WWE Stk concentrations were found to be 84.93-2122.6 ngL^{-1} and 11.3-346.2 ngL^{-1} in WWD SuB. This reduction in concertation of salicylic acid form influent to effluent or downstream water, is confirmed in the untargeted results, with the significant ($p<0.01$) 2-3 order of magnitude reduction in peak area between WWI and WWD for SuB. It was not possible to obtain the WWE/WWI ratio for Stk as it was not found in run d, most likely because samples were only run in the acidic mobile phase.

Salicyluric acid, seems to have high removals in both plants, with the frequency of detection being 100% in both influents and 20 % and 0 in WWE Stk and WWD SuB respectively. Furthermore, there is a significant ($p<0.01$) reduction of 2-3 orders of magnitude in signal area from WWI to WWE or WWD for both pants, as shown in Table 6.3.1.

Even though there was a reduction in peak area for influent and effluent concentration of gentisic acid for both plants, this was only significant for Stk indicating possible better removal for this pollutant in this plant. This is confirmed by the similar influent concentrations, but significantly lower for WWE Stk than WWD SuB as can be seen in Table 6.4-1. Similar results can be observed for salicyluric acid (2-hydroxyhippuric acid), indicating better removal in Stk for both of these metabolites. Gentisic acid has been previously detected in wastewater effluent and river effluent in the μgL^{-1} range (Celiz, et al., 2009; Wiegel, et al., 2004). It has been reported to have high toxicity to aquatic life (Celiz, et al., 2009; Han, et al., 2017). The identification of toxic metabolites in effluents highlights the missing quantification in toxicity when doing targeted analysis.

Tramadol and 3 of its metabolites or transformation products were identified, namely O-Desmethyltramadol (ODT), N-Desmethyltramadol (NDT) and Tramadol-N oxide (TNO). The most frequently detected one was ODT, which is the main metabolite of tramadol and it is biologically active (Gong, et al., 2014). NDT is also a tramadol metabolite and it is biologically active but to a lesser extent (Gong, et al., 2014). TNO has been investigated as an analgesic in its own right (Juzwin, et al., 2000) and it has been identified as a transformation product of tramadol during ozonation (Kharel, et al., 2020). ODT has been previously measured at 122 ngL^{-1} in surface waters and 261 ngL^{-1} in wastewater effluents (Rúa-Gómez, et al., 2012). In this study, neither NDT or TNO present a significant difference between influent and effluent concentrations for SuB WWTP, this points towards poor removal and/or formation of ODT within the WWTP. Further work would be necessary to determine if there is a significant increase or reduction between influent and effluent in Stk WWT of this same pollutant. The identification and prevalence of these tramadol metabolites in both effluents is of particular concern as the concentrations of tramadol on its own entering the

environment had already been identified as RQ > 1 for both of these plants in the targeted study. NDT has been previously observed to increase throughout the WWTP process, it has been measured in UK wastewater influent at 209 ± 4.2 ngL^{-1} and effluent at 341 ± 4.9 ngL^{-1} (Petrie, et al., 2016) . In this study neither NDT nor TNO were detected in the samples ran in runs c and d, so it is not possible to determine the statistical significance of the influent and effluent signal across different sample dates.

Acetaminophen has been widely characterised in wastewater and was also previously detected in these two plants in the targeted study. In this study its main transformation product, 4-aminophenol, which has been previously reported to be more toxic than the parent product, was also detected (Gómez, et al., 2010). It has been shown that acetaminophen is converted by microbes into 4-aminophenol in a reaction catalysed by amidohydrolase and is then converted into hydroquinone by the replacement of the amino group with a hydroxyl group. Strains capable of performing this transformation have been identified in activated sludge samples (Shijin Wu, et al., 2012). This biodegradation pathway may explain the significant ($p<0.05$) 3 order of magnitude decrease in signal of this pollutants from WWI to WWD in SuB. However, if the removal of acetaminophen is leading to another toxic product in the environment the high (>80%) acetaminophen removals determined in the targeted study, should be regarded with caution. In the targeted study acetaminophen RQ was < 1, however if another toxic product is entering the environment as a result of its biotransformation in the WWTP this should be studied in parallel in the future.

Both, the anti-depressant venlafaxine and its metabolite O-desmethylvenlafaxine were found to have a significant ($p<0.05$) near doubling of the signal in WWD compared to WWI in SuB. O-desmethylvenlafaxine has been previously reported to have very low biodegradability in conventional WWTPs (García-Galán, et al., 2019). Only 5 % of the ingested venlafaxine dose is excreted unchanged, its reported concentration range is between 120-249 ngL^{-1} in WWI and 95-199 ngL^{-1} in WWE and its removal is reported to be <50% (Petrie, et al., 2015).

Codeine and metoprolol were also identified. Codeine with similar removal trends as those found in the targeted study, with significant reduction in signal from WWI to WWD in SuB for the untargeted study matching with high removals measured for this pollutant in the targeted study for SuB. This gives further confidence in the untargeted results. Metoprolol was found at too low frequencies (22.2% in WWI Stk, 20.0% in WWE Stk, 6.66 % in WWI and WWD SuB), to determine statistical trends form the untargeted results.

Parent compound *Metabolite or transformation product*	**Level of confidence in identification**	**Formula Monoisotopic mass**	**RT range among runs (min)**	**Detection (%)**				**Ratio for peak area WWE/WWI or WWD/WWI** ***p value of ratio***	
				WWI Stk n=9	*WWE Stk n=10*	*WWI SuB n=15*	*WWD SuB n=15*	*Stk n=9*	*SuB n=15*
Atenolol	1	$C_{14}H_{22}N_2O_3$ 266.16307	0.81-1.21[a] 2.15-2.31[b]	100	90.0	100	66.6	0.2 *p>0.05*	0.4-0.7 *p>0.05*
Atenolol Acid	2	$C_{14}H_{21}NO_4$ 267.1471	1.24- 1.81[a] 1.18-1.73[b]	22.2	20.0	53.3	53.3	-	4.4-12.5 *p<0.01*
Codeine	1	$C_{18}H_{21}NO_3$ 299.1516	1.49-1.54[a] 4.10-4.12[b]	22.2	20.0	53.3	53.3	-	0.33 *p<0.01*
Carbamazepine (CBZ)	1	$C_{15}H_{12}N_2O$ 236.0949	3.77-3.78[a] 4.41-4.49[b]	22.2	20.0	53.3	53.3	-	2.6 *p<0.01*
Carbamazepine10,11-epoxide (CBZ-Ep)	2	$C_{15}H_{12}N_2O_2$ 252.0899	2.88-2.94[a] 3.81-3.85[b]	22.2	20.0	53.3	53.3	-	4.5 *p<0.01*
10,11-dihydro-10,11-dihydroxycarbamazepine(DiOH-CBZ)	2	$C_{15}H_{14}N_2O_3$ 270.1005	2.88-2.94[a] 3.19[b]	22.2	20.0	6.66	6.66	-	-
Tramadol	1	$C_{16}H_{25}NO_2$ 263.1882	2.11-3.30[a] 1.35[b]	11.1	10.0	46.6	46.6	-	3.62 *p<0.01*
O-Desmethyltramadol (ODT)	2	$C_{15}H_{23}N\ O_2$ 249.1729	1.75-1.79[a] 3.40-3.53[b]	22.2	20.0	53.3	53.3	-	2.3 *p<0.01*
N-Desmethyltramadol (NDT)	2	$C_{15}H_{23}NO_2$ 249.1728	2.03-2.07[a]	22.2	20.0	6.66	6.66	-	-
Tramadol-N oxide (TNO)	2	$C_{16}H_{25}NO_3$ 247.1573	2.27-2.35[a] 3.40-3.02[b]	22.2	20.0	6.66	6.66	-	-
Salicylic acid	1	$C_7H_6O_3$ 138.03176	3.18-3.48[a] 1.17-1.23[b]	22.2	0	100	40	-	0.009-0.01, *p<0.01*
Gentisic acid	2	$C_7H_6O_4$ 154.0267	9.53[a]	77.7	0	40.0	26.6	0.005 *p<0.01*	0.045, *p<0.05*
2-Hydroxyhippuric acid (Salicyluric acid)	2	$C_9H_9NO_4$ 195.05324	2.50-2.76[a] 1.09-1.39[b]	100	20.0	100	0	0.007 *p<0.01*	0.046-0.008 *p<0.01*
Venlafaxine	2	$C_{17}H_{27}NO_2$	2.54-2.55[a]	22.2	20.0	53.3	53.3	-	1.9

		277.2042	5.17-5.39[b]						*p<0.01*
Parent compound *Metabolite or transformation product*	**Level of confidence in identification**	**Formula Monoisotopic mass**	**RT range among runs (min)**	**Detection (%)**				**Ratio for peak area WWE/WWI or WWD/WWI *p value of ratio***	
				WWI Stk n=9	*WWE Stk n=10*	*WWI SuB n=15*	*WWD SuB n=15*	*Stk n=9*	*SuB n=15*
O-desmethylvenlafaxine	1	$C_{16}H_{25}NO_2$ 263.1887	1.57[a] 3.80-3.95[b]	11.1	20.0	53.3	53.3	-	2.1 *p<0.05*
Paracetamol (Acetaminophen)	1	$C_8H_9O_2$ 151.0633	1.67-1.93[a] 1.60-1.68[b]	100	40	100	100	0.001 *p<0.01*	0.001-0.003, *p<0.01*
4-aminophenol (p-aminophenol)	2	C_6H_7NO 109.05274	1.66-1.81[a]	22.2	20.0	46.6	0.06	-	0.0056 *p<0.01*

Table 6.3-1 Targeted analytes and their metabolites and transformation products identified through untargeted analysis

WWI: wastewater influent; WWE: wastewater effluent; Stk: Stoke Bardolph wastewater treatment plant; SuB: Sutton Bonington wastewater treatment plant. Superscripts a and b above the retention times refer to either the acidic (a) or basic (b) run.

6.3.1.2 BACs widely reported in the literature

Several pharmaceutical and personal care products as well as pollutants related to industrial process and products, which have been widely reported in the literature were identified in this study. Those which were identified with >40% frequency are reported in Table 6.3-2, along with their known toxicity, legal status (if they have it) and environmental fate and prevalence. When the toxicity or prevalence of the identified BACs were not reported in the literature, they were categorised in Section 6.3.1.3. When a significant ratio was observed which could provide insights into removal efficiency of Stk and Sub, this is discussed in Section 6.3

Compound	Formula Monoisotopic mass *RT range among runs (min)*	Detection (%) *WWI Stk n=9*	 *WWE Stk n=10*	 *WWI SuB n=15*	 *WWD SuB n=15*	Ratio for peak area WWE/WWI or WWD/WWI *p value of ratio* *Stk n=9*	 *SuB n=15*	Literature
2,4,5-trimethylaniline	$C_9H_{13}N$ 135.10477 *8.81*[a]	0	0	46.7	46.7	-	0.945, *p>0.01*	• Found in the 1994 EU list of hazardous by-products which must not be formed by azo-dyes (Ekici, et al., 2001). • Found in sludge from Chinese industrial textile WWTPs (Ning, et al., 2015). • Higher affinity for solids than water (Ning, et al., 2015).
4-nitrophenol	$C_6H_5NO_3$ 139.0271 *3.06-3.29*[a]	22.2	20.0	53.3	53.3	-	0.088-0.163 *p>0.01*	• Used to manufacture plasticisers, pesticides, pharmaceuticals, explosives and dyes (Balasubramanian, et al., 2019) • US limit in natural water: 10 ngL^{-1}, due to its high toxicity (Huang, et al., 2018) • Biodegradation is possible with acclimatized biomass (Tomei, et al., 2004)
5-nitro-o-toludine	$C_7H_8N_2O_2$ 152.05856 *0.89-1.21*[a] *1.17-1.27*[b]	100	80	100	100	0.007, *p<0.01*	0.115-0.160, *p<0.01*	• Used in synthetic dyes manufacturing process (Ning, et al., 2015). • Found more frequently than 2,4,5-trimethylaniline in sludges (Ning, et al., 2015). • Higher affinity for solids (Ning, et al., 2015).
Benzotriazole	$C_6H_5N_3$ 119.04845 *2.33-2.41*[a] *2.19-2.35*[b]	22.2	20.0	53.3	53.3	-	1.23-2.108 *p<0.01*	• Corrosion inhibitor for example in dishwashing detergents(Rhodes-Dicker, et al., 2019). • Anti-estrogenic and suspected human carcinogen (Rhodes-Dicker, et al., 2019). • Found in WWE up to 227 ngL^{-1} and in groundwater up to 1,032 ngL^{-1} (Rhodes-Dicker, et al., 2019).

								• Removal of 36-46 % using activated sludge has been observed (Rodriguez-Narvaez, et al., 2017).
Compound	**Formula Monoisotopic mass** ***RT range among runs (min)***	**Detection (%)**				**Ratio for peak area WWE/WWI or WWD/WWI** ***p value of ratio***		**Literature**
		WWI Stk n=9	*WWE Stk n=10*	*WWI SuB n=15*	*WWD SuB n=15*	*Stk n=9*	*SuB n=15*	
Benzoic acid	$C_7H_6O_2$ 122.03698 2.36-2.69[a] 1.11-1.16[b]	100	100	100	100	0.197 *p<0.01*	0.21-0.54 *p<0.01*	• Fungistatic, widely used as a food preservative (S. Kim, et al., 2019). • Previously identified in sewage sludge samples (Black, et al., 2019). • Participates in photochemical reactions and can affect the climate and atmospheric reactions. (Santos, et al., 2019).
Bisphenol A	$C_{15}H_{16}O_2$ 228.11522 *4.86-4.91[b]*	10.0	11.1	53.3	53.3	-	1.545 *p<0.05*	• Plasticiser (Lapworth, et al., 2015). • Can cause developmental and reproductive dysfunctions, banned in the EU (Ryeo Ok Kim, et al., 2019). • WWI: 416- 2050 ngL^{-1} , WWE: 35-86 ngL^{-1} (Petrie, et al., 2015). • High (>80%) removal using both trickling filter and activated sludge. Trickling filter slightly better likely due to stable microorganism populations (Kasprzyk-Hordern, et al., 2009).
Caprolactam	$C_6H_{11}NO$ 113.08409 *1.88-2.04[a]* *1.92[b]*	100	100	100	100	0.625 *p<0.05*	0.829-1.387 *p>0.05*	• Used to manufacture nylon 6 filament, fibre and plastic (Gedam, et al., 2014). • Has caused widespread environmental pollution (Rajoo, et al., 2013), it has high COD and toxicity (Wang, et al., 2007) • Readily biodegradable (Gedam, et al., 2014)

Compound	Formula Monoisotopic mass *RT range among runs (min)*	Detection (%)				Ratio for peak area WWE/WWI or WWD/WWI *p value of ratio*		Literature
		WWI Stk n=9	*WWE Stk n=10*	*WWI SuB n=15*	*WWD SuB n=15*	*Stk n=9*	*SuB n=15*	
6-aminocaproic acid	$C_6H_{13}NO_2$ 131.09474 *1.32-1.35*[b]	22.2	20.0	0.07	0.07	-	-	• Biodegradation product of caprolactam
Crotamiton	$C_{13}H_{17}NO$ 203.13107 4.36-4.48[a] 5.29-5.44[b]	22.2	20.0	33.3	53.3	-	6.035 *p<0.01*	• Antipruritic (McCance, et al., 2018) • Persistent pollutant, considered as a tracer for wastewater pollution in groundwater due to its persistence (McCance, et al., 2018). • Has been detected in WWE form ND- 4500 ngL^{-1} (McCance, et al., 2018).
Catechol	$C_6H_6O_2$ 110.0369 *1.89-2.09*[a]	22.2	10.0	53.5	0	-	0.004 *p<0.01*	• Used in rubber, medicine, dyestuff, etc. Also an important metabolite in aromatic pollutant biodegradation (Zhuang Liu, et al., 2019). • Can cause lympho- hematopoietic cancer, toxicity to the central nervous system and DNA replication inhibition (Zhuang Liu, et al., 2019). • Fatally toxic to fish at 5- **ga** 25 mgL^{-1} and inhibits biological growth in microorganisms (Aghapour, et al., 2013) • Good aerobic biodegradation (Aghapour, et al., 2013).
DEET	$C_{12}H_{17}NO$ 191.13095 *4.06-4.18*[a] *4.89-5.05*[b]	22.2	20.0	53.3	53.3	-	0.538-0.641 *p>0.05*	• Common active ingredient in topical insect repellent (Ying Li, et al., 2018). • Causes reproductive disorders after prolonged environmental exposure (Lakshminarasimman, et al., 2018) and inhibits activity of a central nervous system enzyme in insects and mammals (Stuart, et al., 2012) .

								• Incomplete WWTP removal. One of 30 most commonly detected pollutants in GW in UK and EU. (Stuart, et al., 2012) • Rapid biodegradation in aerobic activated sludge but not in anaerobic or anoxic conditions (Lakshminarasimman, et al., 2018).
Compound	**Formula Monoisotopic mass *RT range among runs (min)***	**Detection (%)**				**Ratio for peak area WWE/WWI or WWD/WWI *p value of ratio***		**Literature**
		WWI Stk n=9	*WWE Stk n=10*	*WWI SuB n=15*	*WWD SuB n=15*	*Stk n=9*	*SuB n=15*	
Fexofenadine	$C_{32}H_{39}NO_4$ 501.2878 *3.18*[a] *4.21-4.27*[b]	22.2	20.0	53.3	53.3	-	0.5698 *p>0.05*	• Antihistamine (Loos, et al., 2013) • Previously found in WWE 165-1287 ngL^{-1}(Loos, et al., 2013). • Low WWTP removal (Assress, et al., 2019)
Gabapentin	$C_9H_{17}NO_2$ 171.12588 *1.37-1.53*[a]	100	100	53.3	46.6	0.11 *p<0.01*	0.114 *p<0.01*	• Anticonvulsant (R. Gurke, et al., 2015), excreted unchanged, no known metabolites (Petrie, et al., 2015) • WWI (15 034-18 474 ngL^{-1}), WWE (2592- 21 417 ngL^{-1}) (Petrie, et al., 2015). • Variable removal (<50-80%), depending on the WWTP (Petrie, et al., 2015). Better (average of 84%) removal during activated sludge compared to trickling filter (Kasprzyk-Hordern, et al., 2009)
Galaxolidone	$C_{18}H_{24}O_2$ 272.17747 *5.57-5.84*[a] *6.88*[b]	33.3	100	40.0	53.3	9.442 *p<0.01*	2.106 *p<0.01*	• Biotransformation product of synthetic fragrance galaxolide. This transformation has been observed in WWTPs with subsequent release of galaxolidone to the environment (Tasselli, et al., 2020; Vallecillos, et al., 2017). • 7,000 ngL^{-1} and 5,000 ngL^{-1} mean concentrations of galaxolide and galaxolidone in WWE from a Milan municipal WWTP serving

								between 10,000-50,000 inhabitants (Tasselli, et al., 2020).
Compound	**Formula Monoisotopic mass** ***RT range among runs (min)***	**Detection (%)**				**Ratio for peak area WWE/WWI or WWD/WWI** ***p value of ratio***		**Literature**
		WWI Stk n=9	*WWE Stk n=10*	*WWI SuB n=15*	*WWD SuB n=15*	*Stk n=9*	*SuB n=15*	
Lidocaine	$C_{14}H_{22}N_2O$ 234.17324 *1.84-1.97*[a] *5.53-5.69*[b]	100	100	100	100	3.798*p >0.05*	8.452-11.523, *p<0.01* 1.287, *p>0.05*	• Anaesthetic (Rúa-Gómez, et al., 2012) • Found in municipal and hospital WWE (Rúa-Gómez, et al., 2012). Found in 2010 in the WWE of 10 different German WWTPs with a mean concertation of 107 ngL^{-1}, in surface water where effluents were discharged, concentrations did not pose an acute threat, however chronic effects on aquatic organisms were not evaluated (Rúa-Gómez, et al., 2012) • Persistent in surface water due to slow biodegradation. Main degradation pathways are indirect photodegradation and hydrolysis (Rúa-Gómez, et al., 2013). • Degradation half-life > 100 days in surface water (Zhe Li, et al., 2020) .
Norlidocaine	$C_{12}H_{18}N_2O$ 206.14192 *1.51*[a]*,3.85*[b]	11.1	10.0	0	6.7	-	-	• Main lidocaine metabolite, not often detected (Rúa-Gómez, et al., 2012)
Methyl salicylate	$C_8H_8O_3$ 152.04744 *3.24-3.32*[a] *3.40-3.57*[b]	100	0	100	53.3	0.034 *p<0.01*	0.025-0.094 *p<0.01*	• Derivative of salicylic acid naturally produced by many plants and produced industrially too. Acts as a rubefacient, analgesic and anti-inflammatory (Real, et al., 2012). • Readily biodegradable (Saxe, et al., 2018)

Compound	Formula Monoisotopic mass *RT range among runs (min)*	Detection (%)				Ratio for peak area WWE/WWI or WWD/WWI *p value of ratio*		Literature
		WWI Stk n=9	*WWE Stk n=10*	*WWI SuB n=15*	*WWD SuB n=15*	*Stk n=9*	*SuB n=15*	
Ranitidine	$C_{13}H_{22}N_4O_3S$ 314.14113 *1.19-1.30*[a] *3.39-3.43*[b]	88.8	90.0	73.3	60.0	0.947 *p>0.05*	0.523, *p<0.01* 0.73, *p>0.05*	• Antihistamine (T. H. Miller, et al., 2016). • RQ<1 in surface water, but only considering one trophic level and not accounting for genotoxicity (Bergheim, et al., 2012). • WWI: <12- 5,050; WWE <9-425 ngL^{-1}. 30% excreted unchanged (Petrie, et al., 2015). • Retransformation of conjugates to parent compound resulting in higher WWE concnetrations (Meritxell Gros, et al., 2007).Biodegradation only occurs with high microbial diversity, optimal nutrients and aerobic conditions. (Bergheim, et al., 2012).
Sulpiridie	$C_{15}H_{23}N_3O_4S$ 341.14107 *1.33-1.45*[a] *3.36-3.35*[b]	100	100	0	0	2.835 *p<0.05*	-	• Antipsychotic, measured in German WWTP, in WWI: 113-1,100 ngL^{-1} , WWE: 110-294 ngL^{-1} (Bollmann, et al., 2016). • Low biodegradability, so poor biological removals (Rodriguez-Narvaez, et al., 2017). • One of most used pharmaceuticals targeting G-protein coupled receptors in England and Japan (Han Zhang, et al., 2020).
Undecanoic acid	$C_{11}H_{22}O_2$ 186.16199 4.18-4.37[b]	11.1	10.0	53.3	53.3	-	0.543 *p>0.05*	• EU approved flavouring (European Commission, 2012) • Has been previously identified as a wastewater pollution marker (Sirivedhin, et al., 2005).

Table 6.3-2 BACs identified with > 40% frequency in at least once matrix which have been widely reported in the literature

WWI: wastewater influent; WWE: wastewater effluent; Stk: Stoke Bardolph wastewater treatment plant; SuB: Sutton Bonington wastewater treatment plant. Superscripts a and b above the retention times refer to either the acidic (a) or basic (b) run

Several BACs, which have been previously detected in other studies, were detected with <33.3% for all matrices in this untargeted study and so are not included in the Table 6.3.2 above. However, they are briefly summarised in the Table 6.3.3 below as they exemplify the wide range of bioactive compounds found in wastewaters.

Pollutant identified	Literature
Amisulpride	Antipsychotic predominantly excreted unchanged and previously detected in wastewaters (1.1-1.3 μgl^{-1}) and in groundwater (0.07 μgl^{-1}). Not efficiently removed in WWTPs (Bollmann, et al., 2016).
Butylparaben	Food preservative, measured in WWI (50-723 ngL^{-1}) and in WWE (<1 ngL^{-1}), it has been reported to have high (>80%) removal in the WWTP process (Petrie, et al., 2015).
Cinnamoyl glycine	Human urinary metabolite produced either from plant consumption containing cinnamate or is a plant product excreted unchanged (Wishart, et al., 2018). This pollutant was found in WWI of Stk and SuB but only in one SuB WWE sample.
Clopidogrel	An antiplatelet (National Health Service, 2018) found in muscle of fish in Uruguay rivers (Rojo, et al., 2019) its metabolite clopidogrel carboxylic acid was found up to 7 times higher than the parent compound (Subedi, et al., 2017), but this metabolite was not detected in this study.
Clozapine	Antipsychotic detected in hospital wastewaters (n.d.-105.7 ngL^{-1}), hospital wastewater effluent (n.d.-78.2 ngL^{-1}) and municipal wastewater effluent (n.d.-9.3 ngL^{-1}). (Robert Gurke, et al., 2015b).
Ephedrine	Drug with multiple uses, has been previously detected in wastewater influent (465 ± 17.7 ngL^{-1}) and wastewater effluent (125 ± 4.2 ngL^{-1}), it has been reported to have high (>80%) removal in WWTPs (Petrie, et al., 2015). It was detected at least once in each matrix in this study.
Ferulic acid	Identified at least once in all matrices, it is found naturally in apples (Lee, et al., 2017) and in soils (Olofsson, et al., 2014). It had a WWD/WWI ratio of 0.491 ($p<0.01$) for SuB which indicates some removal this WWTP.
Furosemide	Diuretic, detected in wastewater influent between 1,476-2,789 ngL^{-1} and in wastewater effluent: 629-1,161 ngL^{-1} (Petrie, et al., 2015).
Ketamine	Measured in wastewater influent (52-235 ngL^{-1}) and effluent (83-130 ngL^{-1}) (Petrie, et al., 2015).
Metformin	Antidiabetic, one of the most abundant pharmaceuticals found in effluents and wastewaters. Metformin has been shown to act as an endocrine disruptor at environmentally relevant concentrations (Niemuth, et al., 2015).
Methadone	Synthetic opioid, previously identified in surface wastewaters with wastewater effluent influence (Campos-Mañas, et al., 2019).
Meptazinol	Identified in a list of 200 most common opioids, but not found in surface water and wastewater effluent samples (Campos-Mañas, et al., 2019),
Lamotrigine	Concentrations have been found to increase from 1.1 to 1.6 μgL^{-1} from influent to effluent in wastewater treatment plants (Bollmann, et al., 2016). This is consistent with the significant ($p<0.01$) WWD/WWI ratio of 5.091 in SuB.
Norephedrine	Excreted 80-90% unchanged, it has been previously detected in WWI in the 40-86 ngL^{-1} range with high (>80%) removal and in final effluent with a concertation of 59 ngL^{-1}. In this study it was detected in 5 samples only in WWI SuB and in no WWE SuB samples, in accordance with the previous high removals reported (Petrie, et al., 2015)

Pollutant identified	Literature
Nortriptyline	Antidepressant, measured in the aquatic environment and ranked in number 35 out of 39 in a study to find toxicological levels for 10 end points of several pharmaceuticals (dos Santos, et al., 2019).
Pulegone	Minty tasting naturally occurring organic compound found in corn mint, orange mint, peppermint, and spearmint. Potential biomarker for the consumption of these food products (S. Kim, et al., 2019). It was only detected in the influents of both plants, 10 and 40% respectively for Stk and SuB.
Sertraline	Widely detected in aquatic environments influenced by wastewater (Silva, et al., 2014).
Triphenyl phosphate	Identified as top 7 substance of concern due to concentration and toxicity in arctic region study (Choi, et al., 2020). Has been found at 3.98 ngL^{-1} in Chinese surface waters and 1.13 ngL^{-1} in Chinese tap water, has also been measured in wastewater in Sweden and Australia (Ying Li, et al., 2020).
Tris(2-butoxyethyl) phosphate	Flame retardant and environmental pollutant (S. Kim, et al., 2019). Identified as high production chemical and previously identified in wastewater effluent, has lower toxicity index than other organophosphate flame retardants (Choi, et al., 2020).

Table 6.3-3 Pollutants identified with < 40% frequency in all matrices, which have been previously identified in wastewaters

6.3.1.3 BACs reported with lesser frequency in the literature

Compounds which have only been found in one or two previous studies, or for which toxicity and environmental fate are not widely characterised are reported in Table 6.3.4.

Four-methylbenzotriazole has been previously detected as one of the most relevant compounds identified in the aquatic environment of the Danube (Loos, et al., 2017). 16-hydroxyhexadecanoic acid has been previously measured in a river in India with wastewater influence at a concentration of 325 ngL^{-1} (Khalid, et al., 2018). This compound is a key monomer of cutin in the plant cuticle. It has a role as a plant metabolite (S. Kim, et al., 2019).

Boldione is a growth promotor which is banned for meat producing animals in the EU (Destrez, et al., 2009), but is available for human bodybuilders (Oda, et al., 2012). It has been previously reported in Californian sewage sludge with level 2 confidence (Black, et al., 2019).

In the literature escitalopram has not been as widely studied and its removal in WWTP as well as toxicity in the environment is not so well defined. In a study based on Spanish data escitalopram and its metabolites have been classified as hazard level 3 (out of 4 levels where 4 is the most hazardous) based on persistence, bioaccumulating, toxicity and occurrence in Spanish waters (Ortiz de García, et al., 2013). In a study of five Brazilian WWTPs there seems to be a reduction in escitalopram concentration from WWI to WWE and in Brazilian rivers with WWE influence the risk quotient values were found to be below 1 (Pivetta, et al., 2020).

Choline is an essential micronutrient for humans and can be found in urine (Wei, et al., 2017). Choline has been previously identified in sewage sludge and has been found to have a half -life > 100 days in surface water (Zhe Li, et al., 2020). In this untargeted study it was found to have a significant ($p<0.01$) one order of magnitude reduction in signal form WWI to WWE in SuB, however frequency remains above 50% for WWD SuB, meaning it is still entering the environment.

Daidzein is a phytoestrogen, these group of pollutants has generally been overlooked as compared to endocrine disrupting pollutants, despite evidence of the developmental and sexual abnormalities they can cause in aquatic organisms. They are found in wastewater mainly due to human and animal consumption of plants and excretion in urine and faeces of these compounds as metabolites of their precursors found in plants (Kang, et al., 2009).

Erucic acid, is an omega-9 fatty acid found mainly in the brassica family of plants such as canola, rapeseed, wallflower seed, mustard seed as well as brussels sprouts and broccoli (S. Kim, et al., 2019). Its degradation in WWTP has been previously studied in relation to treatment of effluents from mustard oil refineries (Chakraborty, et al., 2011). This pollutant has been reported to be biodegradable and high (>80%) removal has been observed using an upflow anaerobic filter (Chakraborty, et al., 2011). In this untargeted study it was only detected in the basic chromatography, so it is likely why it was not detected in run d. In SuB there is a significant ($p<0.01$) reduction of one order of magnitude in signal from WWI wo WWD.

Mycophenolic acid is an immunosuppressant (Pitt, 2009), it has been identified as potentially inducing chronic effects on aquatic organisms and has been previously detected in Portuguese WWE in the range 395-874 ngL^{-1} and in river samples in concentrations ranging from 117- 541 ngL^{-1}(Santos, et al., 2018).

Testosterone sulfate is a human metabolite of the androgen testosterone steroid excreted in urine (Forsdahl, et al., 2020). Androgen hormones have recently become of interest as they have been found to pose a risk to aquatic organisms and have been widely measured in the aquatic environment (Zhao, et al., 2019). Testosterone has been previously reported to have high frequency of detection (>75%) in four municipal Swiss WWTPs with a maximum concentration of 390 ngL^{-1} in WWI, lower concentrations were observed in the effluent (Kun Zhang, et al., 2017)

Compound	Formula Monoisotopic mass	Monoisotopic mass	Retetion time range among runs (min)	Detection (%)				Ratio for peak area WWE/WWI or WWD/WWI *p value of ratio*	
				WWI Stk n=9	*WWE Stk n=10*	*WWI SuB n=15*	*WWD SuB n=15*	*Stk n=9*	*SuB n=15*
4-Methylbenzotriazole	$C_7H_7N_3$	133.06402	2.85-3.02[a] 3.12-3.25[b]	22.2	20.0	53.3	53.3		1.97-2.842, *p<0.01*
16-hydroxyhexadecanoic	$C_{16}H_{32}O_3$	272.23497	5.19-5.46[a]	100	100	93.3	40	0.358, *p<0.01*	0.179-0.23, *p<0.01*
Boldione	$C_{19}H_{24}O_2$	284.17743	5.52-5.67[a] 5.10-5.14[b]	22.2	20.0	53.3	46.7	-	0.015, *p<0.01*
Escitalopram	$C_{20}H_{21}FN_2O$	324.16374	2.87[a] 5.72-6.07[b]	22.2	20.0	53.3	53.3	-	0.9, *p>0.05*
Choline	$C_5H_{13}NO$	103.09965	0.58-0.64[a] 1.55-1.68[b]	22.2	20.0	53.3	53.3	-	0.184, *p<0.01*
Daidezin	$C_{15}H_{10}O_4$	254.05786	3.37-3.42[a] 3.23[b]	22.2	20.0	53.3	20.0	-	0.025, *p<0.01*
Dodecyl trimethylammonium	$C_{15}H_{33}N$	227.26145	3.86-3.99[a]	100	10.0	100	60.0	0.015 *p<0.01*	0.27, *p<0.01* 0.382, *p>0.05*
Erucic acid	$C_{22}H_{42}O_2$	338.31849	7.54-7.76[b]	22.2	10.0	53.3	40	-	0.238, *p<0.01*
Mycophenolic acid	$C_{17}H_{20}O_6$	320.12583	4.11-4.22[a] 3.10-3.30[b]	22.2	20.0	46.6	0	-	0.057, *p<0.01*
PEG n6	$C_{12}H_{26}O$	282.1678	2.03-2.12[a]	100	20.0	53.3	46.6	0.016, *p<0.01*	0.152, *p<0.01*
PEG n7	$C_{14}H_{30}O_8$	326.1939	2.25-2.28[a]	88.8	11.	6.7	6.7	0.009, *p<0.01*	0.154, *p<0.01*
PPG n5	$C_{15}H_{32}O_6$	308.21979	3.19-3.30[a] 3.68-3.77[b]	100	90.0	53.3	53.3	2.514, *p<0.01*	0.508, *p<0.01*
Testosterone sulfate	$C_{19}H_{28}O_5S$	368.16579	8.95[a] 3.87-4.10[b]	22.2	20.0	53.3	40.0	-	1.566, *p>0.05* 0.024, *p<0.01*

Table 6.3-4 Pollutants lesser reported in the literature. Superscripts a and b above the retention times refer to either the acidic (a) or basic (b) ru

6.3.2 Compounds not previously identified or with unknown effects

A total of 25 pollutants, which have not been previously identified in relation to wastewater or the aquatic environment were identified. They are detailed in Table 6.3-5. Exemplar spectrum and peak is given for one of the pollutants identified in Figure 6.3.1, spectrums for all analytes reported in this section can be found in Appendix 10.4.

Parent compound *Metabolite or transformation product*	**Formula Monoisotopic mass**	**Retention time range among runs (min)**	**Detection (%)**				**Ratio for peak area WWE/WWI or WWD/WWI** ***p value of ratio***		**LIterature**
			WWI Stk n=9	*WWE Stk n=10*	*WWI SuB n=15*	*WWD SuB n=15*	*Stk n=9*	*SuB n=15*	
2-Amino-6-methylmercaptopurine	$C_6H_7N_5S$ 181.04239	1.70-1.86[a] 1.93-2.05[b]	100	100	100	100	12.404 *p<0.01*	4.966-5.254 *p<0.01*	Active metabolite of 6-Thioguanine, a drug used to treat leukaemia (Huynh, et al., 2015).
2-Aminonicotinic acid	$C_6H_6N_2O_2$ 138.04301	1.04-1.9[b]	22.2	20.0	53.3	53.3	-	0.072 *p<0.01*	Human metabolite deriving from nicotinic acid (S. Kim, et al., 2019). Nicotinic acid is used in food industry, agriculture and cosmetic production, with a worldwide output around 35,000 ton (Karabacak, et al., 2012). It is also found in coffee, chicken, beef, fish, peanuts and legumes. Nicotinic acid has not been widely studied in the environment (Martínez Bueno, et al., 2011).
2,5-Dimethylpyrazine	$C_6H_8N_2$ 108.0688	1.86-1.92[b]	10.0	11.1	53.3	53.3	-	4.35, *p<0.01*	EU approved food additive (S. Kim, et al., 2019)
2,6-Dimethylpyrazine	$C_6H_8N_2$ 108.06874	0.62-0.66[a]	88.8	80.0	46.7	46.7	3.26, *p<0.01*	1.179, *p>0.05*	EU approved food additive (S. Kim, et al., 2019)
2-Hydroxymyristic acid	$C_{14}H_{28}O_3$ 244.20366	5.85-6.07[a] 4.81-5.01[b]	22.2	10.0	46.6	6.7	-	0.11, *p<0.01*	Long chain fatty acid, deriving from the fatty acid myristic acid (S. Kim, et al., 2019).
4-Hydroxybenzoic acid	$C_7H_6O_3$ 138.03178	1.99-2.18[a]	11.1	10.0	46.6	40.0	-	0.339, *p<0.01*	Found in apples (Lee, et al., 2017) and human metabolite of flavonoid naringenin (Hernández-Aquino, et al., 2017). Parabens, esters of 4-hydroxybenzoic acid, are used as preservatives in personal care products, pharmaceuticals and canned foods. They have shown

									estrogenic activity in the environment (Evgenidou, et al., 2015).
Parent compound *Metabolite or transformation product*	**Formula Monoisotopic mass**	**Retention time range among runs (min)**	**Detection (%)**				**Ratio for peak area WWE/WWI or WWD/WWI** ***p value of ratio***		**LIterature**
			WWI Stk n=9	*WWE Stk n=10*	*WWI SuB n=15*	*WWD SuB n=15*	*Stk n=9*	*SuB n=15*	
4-Hydroxyindole	C_8H_7NO 133.05273	2.65-2.75[a] 2.93-3.05[b]	11.1	10.0	53.3	6.7	-	0.014, *p<0.01*	Used in drug manufacturing, component of some hair dyes and banned by ASEAN (Association of Southwast Asian Nations) Harmonized Cosmetic Regulation Scheme (Sukakul, et al., 2018).
6-methylquinoline	$C_{10}H_9N$ 143.07357	*1.60[a] 3.18-3.20[b]*	88.8	40.0	100	40.0	0.014, *p<0.01*	0.017-0.272, *p<0.01*	Heterocyclic aromatic hydrocarbon (hetero-PAHs). Hetero-PAHs have been used in several industrial processes such as wood preservation, production of manufactured gas, dyestuffs, among others and have been found in soils of contaminated sites as well a in groundwater (Brinkmann, et al., 2014). Has estrogenic activity with an estradiol equivalence factor of just under $1x10^{-6}$ (Brinkmann, et al., 2014).
Acetophenone	C_8H_8O 120.0575	*0.7-0.703[a] 1.77-1.81[b]*	22.2	20.0	53.3	53.3	-	0.03, *p<0.01*	Used for fragrance in soaps and perfumes, as flavouring in foods and as a solvent for plastics and resins. Has accute toxcity in humans but no chronic exposure

									data has been found (Environmental Protection Agency (US), 2000)
Parent compound *Metabolite or transformation product*	**Formula Monoisotopic mass**	**Retention time range among runs (min)**	**Detection (%)**				**Ratio for peak area WWE/WWI or WWD/WWI** ***p value of ratio***		**LIterature**
			WWI Stk n=9	*WWE Stk n=10*	*WWI SuB n=15*	*WWD SuB n=15*	*Stk n=9*	*SuB n=15*	
Acedoben (4-acetamidobenzoic acid)	$C_9H_9NO_3$ 179.05837	2.21-2.40[a] 1.19[b]	22.2	20.0	53.3	53.3	-	0.01-0.101, *p<0.01*	Contained in a number of pharmaceutical products as antiviral drug Isoprinosine, Deanol. In the human body it has a role as a metabolite (Velcheva, et al., 2017).
Bis(2-ethylhexyl) amine	$C_{16}H_{35}N$ 241.27658	4.01-4.02[a]	88.8	20.0	60.0	53.3	*0.005, p<0.01*	0.059-*0.183, p<0.01*	Used in hair conditioners and for several industrial processes (S. Kim, et al., 2019)
Docosahexaenoic acid ethyl ester	$C_{24}H_{36}O_2$ 356.27123	*4.96-5.14[a] 4.27-4.41[b]*	100	30.0	100	60.0	0.001, *p<0.01*	0.002-0.072, *p<0.01*	Found in fish oil products (Fan, et al., 2020)
Lauric acid	$C_{12}H_{24}O_2$ 200.17764	4.69[a] 6.17-6.28[b]	11.1	10.0	46.6	53.3	-	0.47, *p<0.01*	Main fatty acid in coconut and palm kernel oil (S. Kim, et al., 2019). Has antibacterial properties and is routinely used to treat acne (Kozan, et al., 2020). It is also used in commercial detergents for dishwashers or soaps (Otsuka, et al., 2020)
Nifenazone	$C_{17}H_{16}N_4O_2$ 308.12601	3.99[a] 2.99[b]	11.1	10.0	53.3	20.0	-	0.07, *p<0.01*	A drug which around 1964 replaced phenylbutazone to treat rheumatic disorders and was found to be 10 times less toxic (Colley, 1964).
PEG n12	$C_{24}H_{50}O_{13}$ 563.35146	2.74-2.97[a]	22.2	20.0	53.3	53.3	-	0.009, *p<0.01*	Previously tentatively identified in synthetic turf/ recycled tyre crumb

PPG n6	$C_{18}H_{38}O_7$ 366.26148	3.52-3.65[a]	100	100	53.3	53.3	*1.578, p<0.05*	*0.381, p<0.01*	rubber biotoxicity assays which also used mzCloud databased in Compound Discoverer (National Toxicology Program, 2019).
PPG n7	$C_{21}H_{44}O_8$ 441.33	3.92-3.95[a]	88.8	90.0	46.6	46.6	*0.637, p>0.05*	*0.331, p<0.01*	
PPG n9	$C_{27}H_{56}O_{10}$ 557.41354	4.49-5.23[a]	88.8	90.0	46.6	46.6	*0.591, p<0.05*	*0.218, p<0.01*	
Parent compound *Metabolite or transformation product*	**Formula Monoisotopic mass**	**Retention time range among runs (min)**	**Detection (%)**				**Ratio for peak area WWE/WWI or WWD/WWI** ***p value of ratio***		**LIterature**
			WWI Stk n=9	*WWE Stk n=10*	*WWI SuB n=15*	*WWD SuB n=15*	*Stk n=9*	*SuB n=15*	
Senkyunolide H	$C_{12}H_{16}O_4$ 206.09431	4.27-4.38[a] 5.14-5.18[b]	22.2	20.0	53.3	53.3	-	0.371, *p<0.01*	One of the phthalides in *Ligusticum chuanxiong*, one of the most common traditional Chinese medicine herbs used for pain treatment, inflammation and menstrual pains (Zhao, et al., 2015), it is sold in the UK (Bristol Botanicals Limited, 2021; G Baldwin & Co, 2021).
Triethanolamine	$C_6H_{15}NO_3$ 149.10518	*0.57-0.644[a] 1.20-1.32[b]*	22.2	10.0	53.3	40.0	-	0.008 *p<0.01*	Used in dry cleaning and wood scouring and found in cosmetics, households detergents, polishes and others (S. Kim, et al., 2019).
Zeatin	$C_{10}H_{13}N_5O$ 219.11204	1.31-1.35[b]	11.1	10.0	53.3	53.3	-	0.68 *p>0.05*	Plant hormone, cytokinin (Smith, et al., 2017), no information could be found in relation to human excretion of this compound through urine or faeces.
α-Eleostearic acid	$C_{18}H_{30}O_2$ 278.22446	6.04[a] 5.37-5.57[b]	33.3	20.0	86.7	66.6	0.095 *p<0.01*	0.035-0.101 *p<0.01*	An essential fatty acid for mammals, found in seed oils such as tung and rapeseed (Cheng, et al., 2020).
Y-aminobutyric acid (GABA)	$C_4H_9NO_2$ 103.06335	1.04-1.11[b]	22.2	20.0	13.3	46.6	-	1.746 *p<0.05*	Naturally occurring neurotransmitter (S. Kim, et al., 2019), it is also a plant-signalling molecule (Carter, et al.,

									2018). Higher amounts of GABA in human urine are associated with more advanced dementia (Zhou, et al., 2020).
Parent compound *Metabolite or transformation product*	**Formula Monoisotopic mass**	**Retention time range among runs (min)**	**Detection (%)**				**Ratio for peak area WWE/WWI or WWD/WWI** ***p value of ratio***		**LIterature**
			WWI Stk n=9	*WWE Stk n=10*	*WWI SuB n=15*	*WWD SuB n=15*	*Stk n=9*	*SuB n=15*	
δ-Valerolactam	C_5H_9NO 99.06838	1.27-1.46[a] 1.39-1.43[b]	100	100	100	93.3	0.191 *p<0.01*	0.227-0.373 *p<0.01*	Previously identified in Swedish lake through untargeted analytes (Zhe Li, et al., 2020). Identified as a fish metabolite (Maha, et al., 2019).

Table 6.3-5 Pharmaceutical and personal care products not previously identified

WWI: wastewater influent; WWE: wastewater effluent; Stk: Stoke Bardolph wastewater treatment plant; SuB: Sutton Bonington wastewater treatment plant. Superscripts a and b above the retention times refer to either the acidic (a) or basic (b) run.

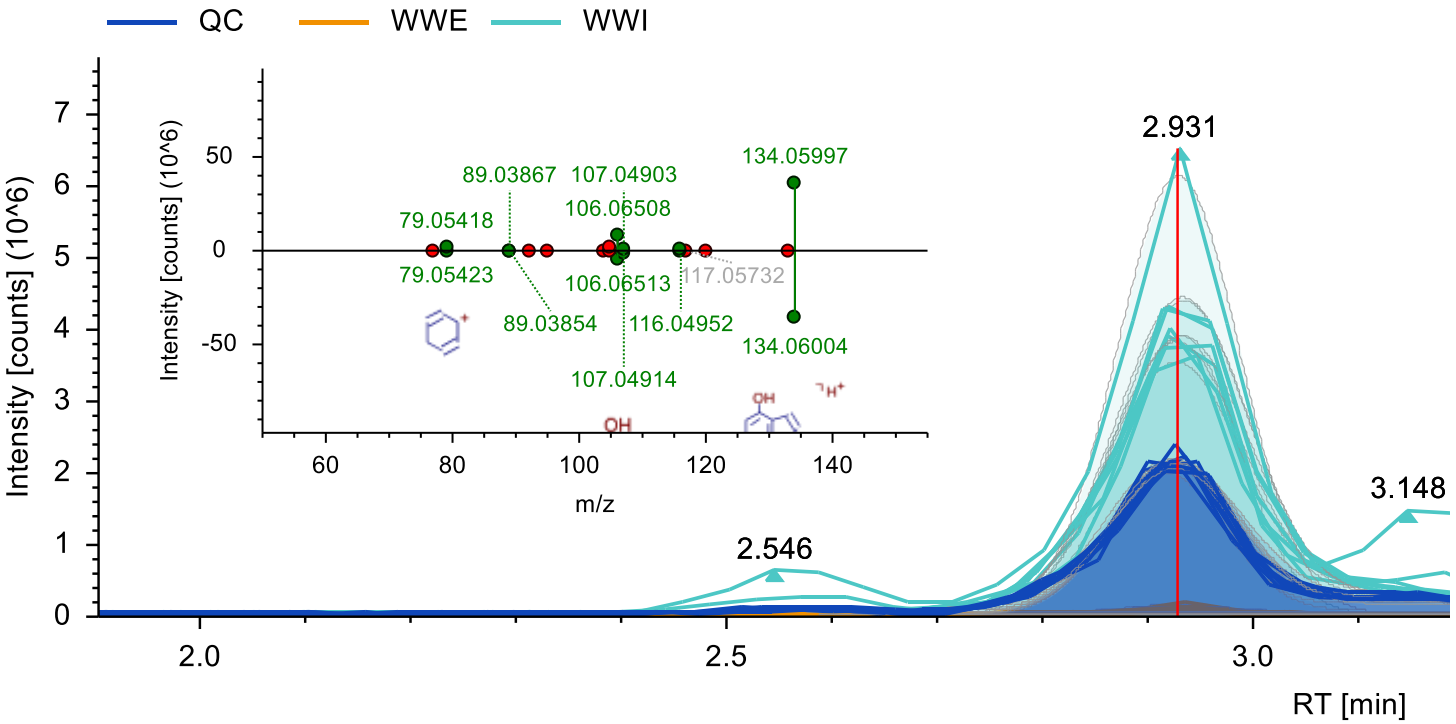


Figure 6.3-1 4-Hydroxindole chromatogram and mass spectrum for run c in acidic chromatography. Top spectrum is from the sample and bottom spectrum from mzCloud

Several PEG and PPG compounds were detected, those with the most frequency are reported in Table 6.3.5. Those detected with <25% frequency in all matrices were PEG n5, PEG n8, PEG n10, PEG n11, PEG n13, PEG n14, PEG n15a and PEG n16. These are not widely reported in the literature with relation to wastewater. However PEG n6, PEG n7and PPG n5 have been previously detected in a Swedish lake with wastewater effluent influence (Zhe Li, et al., 2020). Both, PEG n6 and PPG n5, have been shown to be persistent with a degradation half-life in surface waters of over 100 days (Zhe Li, et al., 2020).

6.3.3 Public health: compounds relevant to wastewater-based epidemiology

Wastewater-based epidemiology is a new approach to obtain information on the health of a population in near-real time. Compared to other techniques such as questionaries or surveys, mortality and morbidity rates, prescription rates, among others, it presents several advantages. It is unbiased, as opposed to questionnaires or sentinel and clinical-based surveillance, it can focus on a broad range of diseases and biomarkers (Sims, et al., 2020). Compared to prescription data, it reveals what the population has actually consumed, including over-the-counter drugs and illicit drugs (Archer, et al., 2018). However some challenges remain such as the selection of biomarkers which need to be stable in water, time-lag between data collection and analysis and uncertainties related to the contribution population and wastewater flows (Sims, et al., 2020). This last one, is being addressed with the use of biomarkers. For example, by allowing for the determination of an illegal drug consumption per capita without the need of an accurate estimate of the population (Chen, et al., 2014):

$$Drug\ consumption\ per\ capita = \frac{Drug\ concentration}{Biomarker\ concentration} \times \frac{Biomarker\ excretion\ rate}{Drug\ excretion\ rate} \qquad \textit{Eq. 26}$$

Biomarkers must be quantifiable, stable in wastewater, constantly excreted and total excretion should correlate to census population, so humans must be the only source of the biomarker in wastewater (Chen, et al., 2014). Some of the pollutants identified in this study are of interest because even though the analysis was not designed specifically for them, they were detected, and this indicates they are stable, amenable to analysis and prevalent in wastewaters. Pollutants identified related to wastewater-based epidemiology are summarised in Table 6.3.6 and discussed in Seactions 6.3.3.1-6.3.3.3.

Parent compound *Metabolie/transfotmation product*	**Formula Monoisotopic mass**	**Retention time range among runs (min)**	**Detection (%)**				**Ratio for peak area WWE/WWI or WWD/WWI** ***p value of ratio***		**Relevance to public health and/or wastewater-based epidemiology**
			WWI Stk n=9	*WWE Stk n=10*	*WWI SuB n=15*	*WWD SuB n=15*	*Stk n=9*	*SuB n=15*	
A-836339	$C_{16}H_{26}N_2O_2S$ 310.16776	4.55[b]	0	0	46.6	46.6	-	0.28, *p<0.01*	Illicit drug
ADBICA	$C_{20}H_{29}N_3O_2$ 343.22047	2.13-2.25[a]	2.2	0.0	53.3	53.3	-	0.022, *p<0.01*	Illicit drug
Acesulfame	$C_4H_5NO_4S$ 162.9943	2.07-2.79[a] 1.13[b]	77.7	70.0	100	100	0.094, *p<0.01*	0.275-0.053, *p<0.01*	Biomarker and population habit indicator
Adenine	$C_5H_5N_5$ 135.05468	1.23-1.27[b]	11.1	10.0	53.3	53.3	-	2.199, *p<0.01*	Disease monitoring
1-methyl adenine	$C_6H_7N_5$ 149.07016	0.59-0.66[a] 1.17[b]	22.2	20.0	46.6	6.7	-	0.11, *p<0.01*	Disease monitoring
Caffeine	$C_8H_{10}N_4O_2$ 194.08028	2.18-2.34[a] 2.00-2.12[b]	100	10.0	100	60.0	0.126, *p<0.01*	0.126-0.005, *p<0.01*	Biomarker and population habit indicator
Paraxanthine	$C_7H_8N_4O_2$ 180.0647	1.62-2.03[a]1.36[b]	100	70.0	100	86.6	0.003, *p<0.01*	0.008-0.025, *p<0.01*	Biomarker and population habit indicator
1,7-dimethyluric acid	$C_7H_8N_4O_3$ 196.0598	1.75-1.82[a] 1.02[b]	11.1	10.0	53.3	6.6	-	0.036, *p<0.01*	Biomarker and population habit indicator
3-methylxanthine	$C_6H_6N_4O_2$ 166.0492	1.26-1.34[a]1.14[b]	11.1	10.0	53.3	6.6	-	0.006, *p<0.01*	Biomarker and population habit indicator
7-methylxanthine	$C_6H_6N_4O_2$ 166.0491	1.13-1.18[b]	11.1	0	53.3	33.3	-	0.005, *p<0.01*	Biomarker and population habit indicator

Parent compound *Metabolie/transfotmation product*	**Formula Monoisotopic mass**	**Retention time range among runs (min)**	**Detection (%)**				**Ratio for peak area WWE/WWI or WWD/WWI** ***p value of ratio***		**Relevance to public health and/or wastewater-based epidemiology**
			WWI Stk n=9	*WWE Stk n=10*	*WWI SuB n=15*	*WWD SuB n=15*	*Stk n=9*	*SuB n=15*	
Creatinine	$C_4H_7N_3O$ 113.0589	0.63-0.65[a] 1.11-1.18[b]	100	100	100	100	0.008, *p<0.01*	0.016-0.111, *p<0.01*	Biomarker and population habit indicator
MDMA	$C_{11}H_{15}NO_2$ 193.11032	1.81[a]	77.7	80.0	46.7	46.7	2.597, *p<0.01*	2.471, *p<0.05*	Illicit drug
Nicotine	$C_{10}H_{14}N_2$ 162.11577	3.68-3.87[b]	11.1	0	11.1	0	-	0.070, *p<0.01*	Biomarker and population habit indicator
Cotinine	$C_{10}H_{12}N_2O$ 176.09506	2.08-2.17[b]	100	100	100	100	0.217, *p<0.01*	0.226, *p<0.01* 0.475, p>0.05	Biomarker and population habit indicator
Saccharin	$C_7H_5NO_3S$ 182.99962	2.51-3.22[a] 2.75[b]	100.0	66.7	100.0	53.3	0.012, *p<0.01*	0.105, *p<0.01*	Biomarker and population habit indicator
Piperine	$C_{17}H_{19}NO_3$ 285.13641	4.60-4.77[a] 5.71-5.83[b]	100.0	22.2	100.0	53.3	-	0.049-0.091, *p<0.01*	Potential relationship to disease monitoring
Y-aminobutyric acid (GABA)	$C_4H_9NO_2$ 103.06335	1.04-1.11[b]	22.2	20.0	13.3	46.6	-	1.746, *p<0.05*	Potential for disease monitoring

Table 6.3-6 Pollutants with public health relevance

WWI: wastewater influent; WWE: wastewater effluent; Stk: Stoke Bardolph wastewater treatment plant; SuB: Sutton Bonington wastewater treatment plant. Superscripts a and b above the retention times refer to either the acidic (a) or basic (b) run.

6.3.3.1 Population habit indicators and anthropogenic biomarkers

The most prevalent compound and its metabolites found in relation to food was caffeine. An intake of 300 $mgday^{-1}$ is not associated with adverse health effects; however, it is difficult to estimate how much a population is consuming from dietary surveys. One limitation is that caffeine is often underestimated as some products do not specify the amount of caffeine contained. Furthermore, surveys may focus on products such as coffee and tea, but caffeine is also contained in products such as analgesics. Therefore, methods have been developed in order to obtain an accurate approximation of a population's caffeine intake from wastewater (Gracia-Lor, et al., 2017). Caffeine has been extensively measured in wastewaters and has been suggested as a biomarker of human wastewater pollution (Chen, et al., 2014).

Nicotine and its main metabolite cotinine have been widely detected in wastewaters and the aquatic environment (Bisceglia, et al., 2010) and were also detected in this untargeted study. Nicotine is metabolised quickly and 70-80% is turned to cotinine in the liver and excreted in urine (Evgenidou, et al., 2015), this explains the higher frequency of detection of cotinine compared to nicotine in this study. Cotinine has been detected in WWI at concentrations reaching the μgL^{-1} level (Evgenidou, et al., 2015). Both nicotine and cotinine have been suggested as potential biomarkers (Chen, et al., 2014).

Two sweeteners, previously detected in wastewaters and the aquatic environment acesulfame and saccharin (McCance, et al., 2018) were identified. Both of these have been previously suggested as biomarkers (Chen, et al., 2014). Acesulfame has been identified as persistent throughout the WWTP process. Saccharin has been reported to be greatly reduced through the WWTP process with around 90% removal in activated sludge (McCance, et al., 2018). In this study both show a significant ($p<0.01$) one to two order of magnitude reduction in signal from WWI to WWE in both plants, indicating some removal. However, acesulfame does not have a reduction in frequency form WWI to WWE in either plant in this study, indicating it is still entering the aquatic environment, as can be seen in Table 6.3.6. Saccharin, as well as the significant ($p<0.01$) reduction in signal for both plants form WWI to WWE had a reduction in frequency of detection, as can be seen in Table 6.3.6. Both acesulfame and saccharin have been considered as tracers for wastewater pollution in groundwaters (McCance, et al., 2018).

Dihydrocodeine is an alternative or adjunct to codeine, 54% of the ingested dose is excreted unchanged (Petrie, et al., 2015). However dihydrocodeine has also been identified as a transformation product of codeine in aerobic batch experiments seeded with activated sludge (Bletsou, et al., 2015). Dihydrocodeine has been found to be a pollutant where NHS (National Health Service for the UK) prescriptions statistics have good agreement with loadings measured in WWTPs (Baker, et al., 2014). This is important as it can be a good indicator of public health. The WWE/WWI ratio was not always significant ($p>0.05$), indicating possible variable removal. This pollutant has been reported to have low WWTP removal (< 20 %) with concentrations ranging between 227-386 and 118-146 ngL^{-1} in WWI and WWE respectively (Petrie, et al., 2015).

6.3.3.2 Disease monitoring

Piperine has been previously identified in sewage sludge (Black, et al., 2019), but only in a few studies (Wang, et al., 2020). Piperine is a natural alkaloid occurring in black pepper and has therapeutic properties. It has been semi-quantified in New York wastewaters ranging from 22- 2,020 ngL^{-1}. Piperine has been found to have a co-

variance with SARS-CoV-2 RNA, but this relationship has not been studied in detail (Wang, et al., 2020), however it points in an interesting direction on the important of non-targeted screening of wastewater to find potential biomarkers or trends related to public health.

Some compounds which are naturally excreted in human urine or faeces were also identified. These compounds may be indicators of public health. Adenine and methyl-adenine and methyl-guanine nucleosides exist in urine, mainly methylated (Raćkowska, et al., 2019). They are polar (elute at the beginning of the chromatography) (Raćkowska, et al., 2019), which is consistent with the elution times found in this study as can be seen in Table 6.3.6. They have been found in higher concentrations in urine of patients with cancer (Raćkowska, et al., 2019). Adenine signal was seen to increase in one order of magnitude (2.199 ratio, $p<0.01$) in SuB, this may be due to demethylation of 1-methyl-adenine, which was found in 53% SuB WWI but only in 6.7% WWE, as can be seen in Table 6.236. 1-methylguanine was also found at least once in all matrices, but with frequencies under 25% for all and not with enough detections to evaluate removal on either plant.

Creatinine, is a waste product from the normal breakdown of muscle tissue. It has been widely detected in wastewater as in endogenous in human urine and therefore proposed as biomarker for wastewater epidemiology studies, however it was found not be stable after 24 hours in wastewater samples (Chen, et al., 2014). The 100% frequency of detection in all matrices in this study, as can be seen in Table 6.3.6, are consistent with literature(Chen, et al., 2014). Despite the 2-3 order of magnitude significant ($p<0.01$) reduction in signal from WWI to WWE/WWD for both plants, the removal is incomplete as creatinine can still be found in effluents at the same frequency, as can be seen in Table 6.3.6.

Naturally occurring neurotransmitter, GABA (S. Kim, et al., 2019), it is also a plant-signalling molecule (Carter, et al., 2018). Higher amounts of GABA in human urine are associated with more advanced dementia (Zhou, et al., 2020). In this study it was found to have a significant ($p<0.01$) one order of magnitude increases in signal from WWI to WWD in SuB, as well as an increase in frequency of detection, as can be seen in Table 6.3.6. This may mean that another compound is being converted into this compound. This compound is also reported in Section 6.2.2, given that apart from being relevant to disease monitoring it has not been previously identified in samples related to wastewater.

6.3.3.3 Illegal drugs

Wastewater monitoring has been used as a way to monitor the use of known illicit drugs as well to identify new drugs being developed and used recreationally (Baker, et al., 2014). Apart from the public health implications, there are also environmental toxicity aspects associated to illicit drugs in the environment. Three illicit drugs were identified with frequencies above 40% in this study, as can be seen in Table 6.3.6. A-836339 is a cannabinoid which was first characterised in 2009 and has since been identified in dubious drug products (Uemura, et al., 2014). In this study it was only detected in SuB and showed a significant ($p<0.01$) reduction in signal of one order of magnitude between WWI and WWD, as can be seen in Table 6.3.6. This drug has not been previously identified in wastewater and its removal in WWTPs is unknown.

ADBICA was identified in 2015 as part of an effort to characterize synthetic cannabinoid designer drugs which are often first found by forensic researchers and law enforcement agencies. It was found that ADBICA, along with other synthetic cannabinoids have parallel effects to those of Δ^9-THC, but with greater potency

(Banister, et al., 2015). This is of concern as the environmental toxicity, behaviour and fate of synthetic cannabinoids is not well studied and they may be adding to the negative effects of emerging pollutants. In this study it seems that ADBICA has some removal in SuB, with a significant ($p<0.01$) decrease in signal from WWI to WWD.

MDMA, or ecstasy was also found with high frequency in this study, as can be seen in Table 6.3.6. This drug has been previously studied in wastewaters and patterns such as its increased concentration during weekends have been reported (Petrie, et al., 2015). MDMA mean consumption in the UK has been estimated from wastewater measurements, sewage epidemiology, as 148 $mgday^{-1}$ per 1000 people (Baker, et al., 2014). MDMA methylene homolog was also detected in this study, in only one sample in WWI and WWE Stk. This is a potential designer drug and its biological and toxicological activities have not been evaluated (Cayman Chemicals, 2015). MDMA had a VIP score of 1.3, meaning it was an important variable for the groupings, as detailed in Section 6.4

Other drugs, which were found with under 40% frequency and therefore not reported in Table 6.3.6 were identified. Ab-chminaca 2'-indazole, is a cannabinoid used for forensic and research applications and not human or veterinary use (Cayman Chemicals, 2019) was identified only once in each matrix. As well as N-methyl-2-ai, a psychoactive drug and research chemical sold online as a designer drug (S. Kim, et al., 2019), this drug was detected twice in WWI and WWE each of Stk and once in WWI SuB.

6.4 Insights into treatment process removal efficiency

Validated PLS models provided insights into the removal efficiency of the identified compounds in both plants. As can be seen in the PLS plot scores for each run, in Figure 6.4.1, the samples were grouped as expected according to whether they were WWE/WWD or WWI or belonged to Stk or SuB. This means that each of these matrices have significantly different distributions of the BACs identified. As explained previously this can give us insights into the removal efficacies of several BACs in both WWTPs.As has been previously stated, peak areas were conly compared against those of the same analyte, as it is not a valid comparison to compare peak areas across different analytes. As can be seen from Table 6.4-1, run c is particularly useful in comparing the removal efficiency of both of the WWTPs studied for the compounds identified in this run. When better removal is identified for Stk, there is a chance it is due to dilution effects as WWE of Stk is compared to WWD of SuB. Further study would be necessary to confirm these removals as some of the difference in peak area for a pollutant across the different matrices could be due to matrix effects. However, as can be seen in Appendix 10.2 (Table 10.2.3) for most analytes the matrix effects are similar across all four matrcies. However, this is compound dependent and no standard was used for the pollutants identified in this section (as its untargeted work) and so it is not possible to quantify the matrix effects. This work is still indicative of pollutants which should be further studied and which likely have poor WWTP removals.

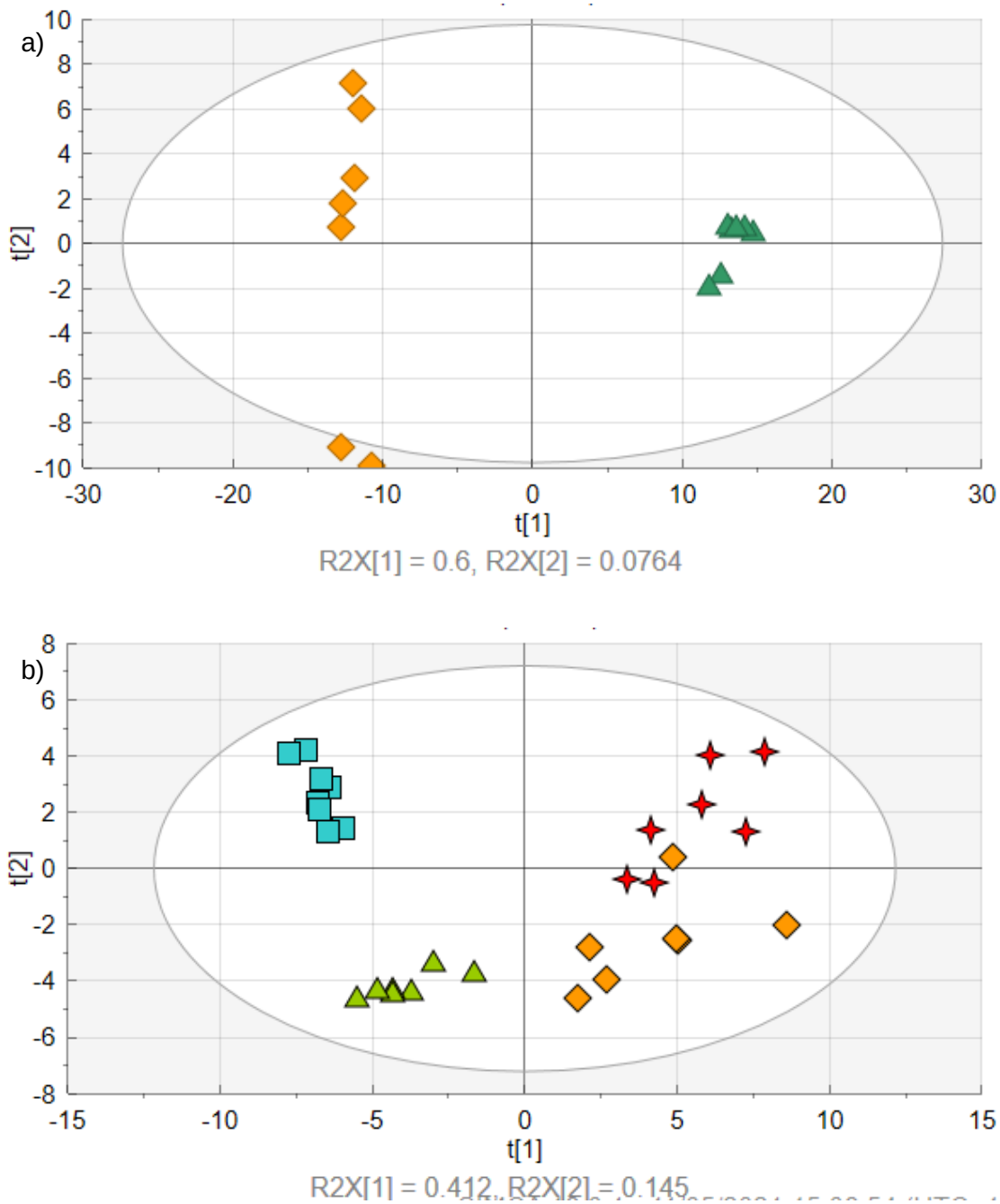


Figure 6.4-1 PLS plot score a) for run c and b) for run d

Sutton Bonington wastewater influent (orange rhombus), Sutton Bonington downstream water (green triangles), Stoke Bardolph wastewater influent (red 4-point starts) and Stoke Bardolph wastewater effluent (blue squares). X-axis represents component one (t[1]) and Y-axis represents principal component 2 (t[2]). Variance represented by t[1] and t[2] are given by R2[X] and R2X[2] respectively.

Pollutant	VIP	Ratio: WWI Stk / WWI SuB *n=7*	p-value: WWI Stk/ WWI SuB *n=7*	Ratio: WWE Stk/ WWD SuB *n≥7*	p-value: WWE Stk / WWD SuB *n≥7*	Better removal	
						Stk	*SuB*
16-Hydroxyhexadecanoic acid	0.38	1.26	p>0.05	2.45	p<0.01		X
2-Amino-6-methylmercaptopurine	1.19	0.96	p>0.05	2.27	p<0.01		X
2-Hydroxyhippuric acid	0.89	0.65	p>0.05	0.10	p<0.01	X	
2,6-Dimethylpyrazine	1.48	1.74	p<0.01	4.81	p<0.01	-	-
5-Nitro-o-toluidine	1.09	1.04	p>0.05	0.06	p<0.01	X	
6-Methylquinoline	0.92	0.54	p>0.05	0.03	p<0.01	X	
Benzoic acid	0.99	1.81	p>0.05	0.66	p>0.05	-	-
Bis(2-ethylhexyl) amine	0.98	2.29	p<0.05	0.06	p<0.01	X	
Caffeine	1.03	0.93	p>0.05	0.02	p<0.01	X	
Creatinine	0.63	1.13	p>0.05	0.08	p<0.01	X	
Dihydrocodeine	1.63	1.53	p>0.05	16.45	p<0.01		X
Docosahexaenoic acid ethyl ester	0.98	0.98	p>0.05	0.02	p<0.01	X	
Gabapentin	0.86	1.36	p>0.05	1.45	p>0.05	-	-
Galaxolidone	1.34	0.89	p>0.05[1]	3.98	p<0.01		X
Gentisic acid	0.92	1.00	p>0.05	0.10	p<0.01	X	
Lidocaine	0.53	3.61	p<0.05	10.65	p<0.01		X
MDMA	1.28	2.99	p<0.01	3.15	p<0.01	-	-
Paracetamol	1.10	1.22	p>0.05	0.01	p<0.01	X	
Paraxanthine	1.12	1.26	p>0.05	0.03	p<0.01	X	
Piperine	0.94	1.132	p>0.05	0.133	p<0.01	X	
PEG n6	1.08	1.04	p>0.05	0.11	p<0.01	X	
PEG n7	1.03	1.27	p>0.05	0.08	p<0.01	X	

Pollutant	VIP	Ratio: WWI Stk / WWI SuB *n=7*	p-value: WWI Stk/ WWI SuB *n=7*	Ratio: WWE Stk/ WWD SuB *n≥7*	p-value: WWE Stk / WWD SuB *n≥7*	Better removal	
						Stk	*SuB*
Piperine	0.94	1.13	p>0.05	0.13	p<0.01	X	
PPG n5	1.18	0.67	p>0.05	3.31	p<0.01		X
PPG n6	0.85	0.70	p>0.05	2.90	p<0.01		X
Ranitidine HCl	1.43	4.62	p<0.01	5.99	p<0.01		
Saccharin	1.00	1.61	p>0.05	0.18	p<0.01	X	
Sulpiride	1.69	-	-	-	-		
Xylenesulfonate	0.99	6.72	p<0.01	0.74	p>0.05	X	
α-Linolenic acid	0.94	7.29	p<0.01	0.61	p>0.05	X	
δ-Valerolactam	0.45	1.43	p>0.05	0.83	p>0.05	-	-

Table 6.4-1 PLS results to compare removal in Stoke Bardolph wastewater treatment plant (Stk) and Sutton Bonington wastewater treatment plant (Sub)

VIP: variable importance for prediction; WWI: wastewater influent; WWE: wastewater effluent; WWD: water downstream of WWTP.

6.4.1 Compounds for which WWTP fate has not been widely studied

Eight of the compounds which have not been previously detected in relation to wastewter were found to have a significnat ($p<0.01$) reduction in signal of between one and two orders of magnitude between WWI and WWD for SuB, as can be seen in Table 6.3.5. They were 2-Hydroxymyristic acid, 4-Hydroxyindole, acedoben, acetophenone, bis(2-ethylhexyl) amine, lauric acid, senkyunolide and nifenazone. Bis(2-ethylhexyl) amine has three order of magnitude reduction in signal between WWI and WWE for Stk, as can be seen in Table 6.3.5, indicating good removal for this plant too. Comparing the removal of bis(2-ethylhexyl) amine from both plants, a better removal for Stk than SuB can be inferred, as can be seen in Table 6.4.1. This is due to the not significantly different WWI signals between Stk and SuB, but the significantly lower WWE Stk signal compared to WWD SuB, as can be seen in Table 6.4.1. However, it is possible this is due to dilution effects as WWE of Stk is compared to WWD of SuB. Further study would be necessary to confirm this.

For some compounds it was only possible to assess the removal in SuB WWTP. Those which showed a significant ($p<0.01$) reduction in signal for this plant were 2-Aminonicotinic, 4-Hydroxybenzoic Y-aminobutyric acid (GABA), daidezin, triethanolamine, mycophenolic acid, choline, boldione, as can be seen in Tables 6.3.5 and 6.3.4 adenine and the two illicit drugs -836339 and ADBICA, as can be seen in Table 6.3.6. However, for choline frequency remains above 50% for the effluent of SuB, as can be seen in Table 6.3.4, meaning it is still entering the environment from WWTPs. Boldione has not been extensively studied and in the literature has been identified (level 2 confidence) in sludge (Black, et al., 2019). The significant ($p<0.01$) two order of magnitude reduction in signal from WWI to WWD observed for SuB WWTP, which can be seen in Table 6.3.4, may be partly due to accumulation in sludge. For adenine the signal decrease, as can be seen in Table 6.3.6, may be due to demethylation of 1-methyl-adenine, which was found in 53% SuB WWI but only in 6.7% WWE. 1-methylguanine was also found at least once in all matrices, but with frequencies under 25% for all and not with enough detections to evaluate removal on either plant. 2-Aminonicotinic acid and 4-Hydroxybenzoic have a significant ($p<0.01$) reduction in signal of two orders of magnitude between WWI and WWE/WWD, as can be seen in Table 6.3.5, signalling some removal. However, the frequency of detection was virtually the same for WWI and WWE, meaning the compound is still entering the aquatic environment.

5-nitro-o-toludine was found to have significant ($p<0.01$) removal in both plants, with better removal in Stk (as can be seen in Table 6.4-1). This potentially indicates biodegradation as a better removal method than adsorption and that activated sludge is more effective than trickling filter, but would need to be confirmed with further studies. Similar results were obtained for 6-methylquinoline, 16-hydroxyhexadecanoic, piperine and α-Eleostearic acid, as can be seen in Table 6.4.1, the removal for none of these pollutants in WWTPs has been extensively studied, as can be seen in Tables 6.3.4 and 6.3.5. The removal of piperine and Docosahexaenoic acid ethyl in Stk also seems superior to that of SuB as it was detected with less frequency in the effluent of this plant, as can be seen in Table 6.3.5.

A few compounds which showed no removal, or a significant increase in signal from WWI to WWE or WWD in both plants. δ-Valerolactam showed a significant ($p<0.01$) reduction in signal from WWI to WWE or WWD in both plants, without either plant having better removal. Similarly, both 2,5-Dimethylpyrazine and 2,6-Dimethylpyrazine, show a significant ($p<0.01$) increase in signal of one order of magnitude in SuB and Stk respectively, as can be seen in Table 6.4.1. 2,6-Dimethylpyrazine does not have

a significantly different removal in both plants, so it is likely incompletely removed in both plants. 2-Amino-6-methylmercaptopurine was detected in all samples in this study, as can be seen in Table 6.3.5, and it has a significant ($p<0.01$) increase in signal between WWI and WWE or WWD, for both plants (as can be seen in Table 6.4.1). This compound also had a VIP>1 for both run c and d, as can be seen in Table 6.4.1. The ratio between the influents of both plants was not significant, indicating that similar amounts are entering both plants, However, the ratio (WWE Stk/WWD SuB) was 2.268, $p<0.01$ indicating better removal in SuB WWTP for this pollutant.

The removal of PEG and PPG compounds has not been widely studied. In this study most of these compounds were found to have a significant ($p<0.01$-0.05) reduction in signal from WWI to WWE or WWD of at least one order of magnitude if not more, showing at least some removal in the WWTP process. However, PPG n5 and PPG n6 had a significant ($p<0.05$) increase in signal of one order of magnitude in Stk. Given the known persistence of PPG n5 (Zhe Li, et al., 2020), it would be advisable to further study the concentrations entering the environment as well as the toxicity of these pollutants. The two PEG compounds (n6 and n7) detected in run d were found to have better removal in Stk compared to SuB and the opposite was true for the two PPG compounds detected (n5 and n6).

6.4.2 Pollutants for which removal has been widely studied

6.4.2.1 Very persistent pollutants

Pollutants which are known to be persistent and showed significant increase in signal from WWI to WWE in SuB are: crotamiton (McCance, et al., 2018), carbamazepine and its metabolite carbamazepine 10,11-epoxide (Petrie, et al., 2015), bisphenol A (Petrie, et al., 2015) and benzotriazole .

Ranitidine shows variable treatment with no significant ($p>0.05$) ratios for either plant as well as a significant ($p<0.01$) reduction for SuB for run c. This may be due to re-transformation of conjugates to parent compounds (Meritxell Gros, et al., 2007). Also likely to the specific conditions necessary for biodegradation to occur: aerobic environments with high microbial diversity and optimal nutrient conditions (Bergheim, et al., 2012).

Benzoic acid has significant ($p<0.01$) reduction in signal form WWI to WWE or WWD in both plants, as can be seen in Table 6.4.1, however it was still found with 100% frequency in both plants, as can be seen in Table 6.3.3, with means there is incomplete removal. The removal seems similar for both plants, as can be seen in Table 6.4.1. In the past it has been detected in sludge samples, which may be its main removal mechanism (Black, et al., 2019).

The 100% frequency of detection in all matrices of creatinine are consistent with literature (Chen, et al., 2014). Despite the 2-3 significant ($p<0.01$) reduction in signal from WWI to WWE or WWD for both plants, the removal is incomplete as creatinine can still be found in effluents at the same frequency, as can be seen in Table 6.3.6. This pollutant was found to have potentially better removal in Stk WWTP compared to SuB WWTP.

Sulpiridie was not detected in the WWI or WWD of SuB, however in Stk it showed a significant ($p<0.05$) WWE/WWI ratio of 2.835, as can be seen in table 6.4.1, which indicates an increase in concentration through the WWTP. This pollutant has been reported to have low biodegradability and thus poor removals in secondary biological

treatment (Rodriguez-Narvaez, et al., 2017). Sulpiridie has been previously found to have similar WWI and WWE concentrations (Bollmann, et al., 2016).

MDMA (ecstasy) showed a significant ($p<0.01$) increase from WWI to WWE for both plants, as can be seen in Table 6.4.1. Comparing both plants, it is possible to see that they are not significantly different in their influents or effluents, meaning they have likely similar removal, as can be seen in Table 6.4.1. MDMA has been previously reported to have low (<50%) WWTP removal (Petrie, et al., 2015), which is consistent with the results obtained for this work.

Galaxolidone is seen to significantly ($p<0.01$) increase from WWI to WWE or WWD in both plants, as can be seen in Table 6.4.1, this is expected as it is the biotransformation product of synthetic fragrance galaxolide (Tasselli, et al., 2020; Vallecillos, et al., 2017). In order to obtain a complete picture, it would be necessary to assess the concentration of galaxolide too, but this pollutant was not detected in this study. Galaxolidone had a VIP > 1 in run d, as can be seen in Table 6.4.1, meaning that it is significantly different in all or some of the groups. The signal between both plants in the WWI is not significantly different, however the effluent from Stk has a significantly higher area than the WWD of SuB, indicating better removal in SuB WWTP. Galaxolidone has a high (5.90) $\log_{kow}$ (S. Kim, et al., 2019), which indicates a higher affinity for solids and may mean most of the removal occurs in the sand filters in SuB and is the reason this plant presents better removal for this pollutant.

4-methylbenzotriazole has been previously detected as one of the most relevant pollutants identified in the aquatic environment of the Danube (Loos, et al., 2017), in this study a significant ($p<0.01$) one order of magnitude increase in signal from WWI to WWD in SuB was found, as can be seen in Table 6.4.1. This is a study which shows that its half-life in wastewater is of 8.5 days and that WWTP residence times are insufficient for biodegradation to be a considerable removal factor (Janssen, et al., 2015). As SuB also has a sand filtration step, this indicates this pollutant is recalcitrant to sand adsorption, as well as biodegradation. Other removal methods identified for it have been photodegradation in surface waters, however this is dependent on several hours of sunlight (Janssen, et al., 2015).

6.4.2.2 Pollutants with better removal

Methyl salicylate has been reported to be readily biodegradable (Saxe, et al., 2018) which is likely the reason that for both plants there is a significant ($p<0.01$) reduction in signal from WWI to WWE or WWD of two orders of magnitude, as can be seen in Table 6.4.1. It seems that Stk removal is better than SuB as this pollutant was not detected in WWE Stk, but it was in WWD SuB. Similarly, catechol has been previously reported to have good aerobic biodegradation (Aghapour, et al., 2013), which is consistent with the significant ($p<0.01$) reduction in SuB, where it was not detected in the effluent, as can be seen in Table 6.3.2.

Caffeine has been reported to have high (>80%) removal in the WWTP process, WWI concentrations in the range: 9,902-25,138 ngL^{-1} and in effluents 25-200 ngL^{-1}. This is consistent with the 100% frequency found in both WWIs and only 10-60% in WWE and WWD, as can be seen in Table 6.3.6. As well as the significant ($p<0.01$) at least one order of magnitude reduction in signal from WWI to WWE or WWD in both plants, as can be seen in Table 6.4.1. Caffeine is extensively metabolised to form three major metabolites: 3,7-dimethylxanthine (known as theobromine), 1,7-dimethylxanthine (paraxanthine) and 1,3-dimethylxanthine (theophylline) (Gracia-Lor, et al., 2017). Paraxanthine has been previously found at higher mass loadings than caffeine in WWI (Gracia-Lor, et al., 2017). In this study it was found with more frequency in the effluents

than caffeine, however removal seems better with significant ($p<0.01$) 2-3 order of magnitude decrease in signal from WWI to WWE or WWD, as can be seen in Table 6.3.6. The other three metabolites detected, 1-7 dimethyluric acid, 3-methylxanthine and 7-methylxanthine were all found at lower frequencies than the other two, as can be seen in Table 6.3.6. All three seem to have good removal in SuB with 2-3 order of magnitude significant ($p<0.01$) reduction in signal from WWI to WWD, as can be seen in Table 6.4.1. Caffeine and paraxanthine both show better removal in Stk, with significantly lower in WWE Stk than WWD SuB, despite there not being a significant difference between WWI SuB and WWI Stk, as can be seen in Table 6.4.1.

Nicotine has been previously reported to have high (>80%) removal in WWTPs (Petrie, et al., 2015), which is consistent with the 2 order of magnitude significant ($p<0.01$) reduction in signal between in WWI and WWD for SuB, as can be seen in Table 6.4.1. Cotinine has been similarly reported to have good removal, or at least a reduction from concentrations in WWI to WWE (Evgenidou, et al., 2015), which is mostly consistent with the results found here, as can be seen in Table 6.3.6. However the frequency of detection remains 100% for all effluents in this study, as can be seen in Table 6.3.6, which may be more an indication of the high influent concentrations rather than poor removal, as this pollutants has been considered as a marker of human wastewater pollution due to its high concentrations in influent leading it to still being found in effluents, despite relatively good removals reported (Evgenidou, et al., 2015).

Gabapentin has been previously reported to have variable removal (<50-80%), depending on the WWTP (Petrie, et al., 2015). With better (average of 84%) removal during activated sludge compared to trickling filter (Kasprzyk-Hordern, et al., 2009). In this study the WWI and WWE or WWD of both plants were not significantly different between them, however they both presented a significant ($p<0.01$) order of magnitude decrease in Gabapentin signal, as can be seen in Table 6.4.1, indicating similar removals in both plants despite the different process. It is possible that the better removal in activated sludge in Stk is compensated by the sand filters in SuB, but further studies would be necessary to confirm this.

The two sweeteners identified have been reported to have different fates in the WWTP removal process (McCance, et al., 2018). Acesulfame has been identified as persistent throughout the WWTP process. Saccharin has been reported to be greatly reduced through the WWTP process with around 90% removal in activated sludge (McCance, et al., 2018). This is in agreement with the better removal of saccharin in Stk compared to SuB found in this study, as can be seen in Table 6.4.1. Both sweeteners show a significant ($p<0.01$) one to two order of magnitude reduction in signal from WWI to WWE in both plants, as can be seen in Table 6.4.1, indicating some removal. However, acesulfame does not have a reduction in frequency form WWI to WWE in both plants in this study, as can be seen in Table 6.3.6, indicating it is still entering the aquatic environment. Saccharin, as well as the significant ($p<0.01$) reduction in signal for both plants form WWI to WWE or WWD had a reduction in frequency of detection, as can be seen in Table 6.3.6. Both acesulfame and saccharin have been considered as tracers for wastewater pollution in groundwaters (McCance, et al., 2018).

Lidocaine showed variable and often not significant ($p>0.05$) ratios, so it is not possible to reach a conclusion regarding its removal in these two plants without further study. Lidocaine was found in 2010 in the WWE of 10 different German WWTPs with a mean concertation of 107 ngL^{-1} and an ecotoxicity evaluation determined that it did not pose an acute threat at the concentrations found in surface waters receiving WWE from the WWTPs, however constant entering into the enviornment of this pollutant could still cause chronic effects on aquatic organisms (Rúa-Gómez, et al., 2012). Lidocaine has been reported to be persistent in surface waters, with slow biotic degradation and direct

photodegradation. The main degradation pathway has been found to be indirect photodegradation as well as hydrolysis, to some extent (Rúa-Gómez, et al., 2013). It has been reported to have a degradation half-life < 100 days in surface water (Zhe Li, et al., 2020). The main metabolite of lidocaine is norlidocaine (monoethylglycinexylidide), which was identified in once in all four matrices in this study. This metabolite has not been detected often (Rúa-Gómez, et al., 2012).

Caprolactam was detected in all samples in this study. Caprolactam has caused widespread environmental pollution (Rajoo, et al., 2013), it has high COD and toxicity (Wang, et al., 2007). It is mainly used in the manufacturing of Nylon-6 fibres. It has been reported to be readily biodegradable (Gedam, et al., 2014). This is in accordance to the significant ($p<0.05$) one order of magnitude reduction in signal from WWI to WWE in Stk, as can be seen in Table 6.4.1, using activated sludge process. However, reduction was not significant for SuB, as can be seen in Table 6.4.1, signalling probable better removal using activated sludge thank trickling filter. Caprolactam biodegradation has been described to produce 6-aminocaproic acid (Rajoo, et al., 2013), which was also identified in this study. However, the frequency of detection was not high enough to get an understanding of the removal on either plant.

Fatty acid removal has been studied in WWTPs and in general they are biodegradable, however they can inhibit microbial action and therefore reduce effluent quality if found in large concentrations. Long chain fatty acids have been found to not be full degraded in the WWTP and have been measured in effluents (Chipasa, et al., 2006). Linoleic acid**,** is a long chain fatty acid generated from the hydrolysis of vegetable oils, it has been found at high concentrations in food processing and kitchen wastewaters (Zhang, et al., 2011). It is an essential fatty acid for humans, mainly found in plant oils (S. Kim, et al., 2019). Similar to linoleic acid 2-hydromyristic acid is a long chain fatty acid, deriving from the fatty acid myristic acid (S. Kim, et al., 2019). In this study this pollutant was found to have a significant ($p<0.01$) reduction of one order of magnitude in signal from WWI to WWD in SuB and a reduction in frequency of detection for both plants in the WWE or WWD compared to WWI, as can be seen in Table 6.3.5. Similarly, α-Eleostearic acid is an essential fatty acid for mammals, found in seed oils such as tung and rapeseed (Cheng, et al., 2020). In this study it seems to have some removal from both plants with significant ($p<0.01$) one to two order reduction in signal from WWI to WWE in both plants a well as reduction in frequency, as can be seen in table 6.3.5.

Erucic acid, is an omega-9 fatty acid found mainly in the brassica family of plants such as canola, rapeseed, wallflower seed, mustard seed as well as brussels sprouts and broccoli (S. Kim, et al., 2019). Its degradation in WWTPs has been previously studied in relation to treatment of effluents from mustard oil refineries (Chakraborty, et al., 2011). This pollutant has been reported to be biodegradable and high (>80%) removal has been observed using an upflow anaerobic filter(Chakraborty, et al., 2011). In this study it was only detected in the basic chromatography, so it is likely why it was not detected in run d. In SuB there is a significant ($p<0.01$) reduction of one order of magnitude in signal from WWI wo WWD, as can be seen in Table 6.4.1.

6.5 Summary

The untargeted analytical method developed and applied allows for the identification of a wide variety of emerging pollutants in wastewater which are relevant for environmental toxicology and public health monitoring fields. In terms of environmental toxicology, 25 compounds not previously identified in relation to wastewater monitoring were identified. These compounds are either potentially or certainly toxic, for example 4-Hydroxyindole which is used in the hair dyes and banned by ASEAN. In complementing the fate of several of the analytes in the targeted monitoring by

transformation product and metabolite identification, more information is gained on environmental toxicology and WWTP removal efficiency. For example, 2 metabolites of carbamazepine, one of which is pharmacologically active, as well as 2 active metabolites and one active transformation product of tramadol were found in the influent and effluent of both plants. As well as 4-aminophenol, a toxic biodegradation product of paracetamol was identified in the effluent or downstream water of both treatment plants.

In terms of public health two illicit designer drugs not often identified (A-836339 and ADBICA) were found with >40% frequency for at least one site. Furthermore biomarkers (cotinine and creatinine) necessary for wastewater-based epidemiology were identified with 100% frequency in all matrices. Their identification using an untargeted method not designed to identify them in particular, evidences their stability and amenability for analysis (key requirements of biomarkers). Three pollutants with potential disease monitoring attributes for wastewater epidemiology were identified (namely adenine, GABA and piperine). Finally, multivariate analysis allowed for insights into the removal efficiency of two common wastewater treatment process (activated sludge compared to trickling filter + sand filtration). The valuable information gained using LC-MS/MS and multivariate PLS analysis approach indicates further work can be carried out using this same approach on other treatment methods.

The pollutants identified in this section are of importance in terms of ensuring that future plans for direct resue of wastewater effluent agricultural irrigation consider pollutants which are likely of concern but which are not identified when only targeted analysis is undertaken.

7 RESULTS AND DISCUSSION; BAC DISTRIBUTION IN AGRIGULTURAL REGION: TULA VALLEY, MEXICO

7.1 Highlights

- **Seven pollutants quantified which had not been previously studied in the Tula Valley**, of which 4-tert-octylphenol, levonorgestrel, simvastatin and tramadol were found to have environmental risk quotients above one.
- **Five pollutants found with measurable concentrations in spring groundwater**, with sulfamethoxazole and levonorgestrel found at a concentration with an environmental risk quotient above one.
- Acetaminophen, diclofenac, erythromycin, trimethoprim, bezafibrate and simvastatin also found to have **environmental risk quotients above one at several sampling points**.
- **Total estradiol equivalent estrogenicity found to have environmental risk quotient above one**, with influence form estriol and its sulfate conjugate which have not been previously measured in the Tula Valley.
- A total of **eighteen pollutants which have not been previously detected** in the aquatic environment and which were not found in the two UK sites studied were detected. For example, 1-Adamantanamine, a compound used in textiles and some food packaging products and with estrogenic effects and 10-Hydroxycarbazepine, active metabolite of oxcarbazepine were found at all sampling points except for the spring
- A total of **31 compounds not previously detected in the aquatic environment but found in the UK sites studied** for this thesis were detected in the Tula Valley samples. Some examples include 1-Adamantanamine, which is used to treat the influenza virus and boldenone, which is banned in the EU and is used as a growth promoter for meat-growing animals. Both were detected in all sampling sites, except for the spring.
- **More than 20 pollutants known for their toxicity and bioactivity, but not previously detected in the Tula Valley** were identified using untargeted analysis. For example, the benzotriazole 5-methylbenzotriazole, cocaine metabolite benzoylecgonine and ciprofloxacin.
- **Two illicit drugs** were found: cocaine and ADBICA
- Caffeine, paraxanthine, nicotine and cotinine: **potential biomarkers, which were also found in the UK samples** were detected. This is important in terms of internationally relevant biomarkers.

7.2 Targeted pollutants: occurrence, distribution and environmental risk

Part of the aim of this thesis was to address the issue of bioactive pollutants in the environment from an international perspective. An important part of achieving this is developing international collaborations which allow data from two different countries with different wastewater practices, to be analysed in one single piece of work. Therefore, the collaboration developed allowed for sampling in the Tula Valley in Mexico, a particularly interesting site as it has been irrigated using wastewater for over 100 years, as detailed in Section 2.5.3. The five points sampled are marked on Figure 7.2.1. Ths were canal water (blue cross), which is mainly raw wastewater coming from Mexico City, followed by Endho dam water (blue dot) which is raw wastewater combined with treated wastewater effluent from the Atotonilco WWTP (red dot in Figure 7.2.1). The effluent from Atotonilco WWTP was also sampled. River water is from the Tula river downstream of the dam (green cross) and finally spring water from

the Cerro Colorado spring (yellow triangle) is water which, over long periods of time, has percolated through the soils of the Tula Valley and recharged an artificial aquifer.

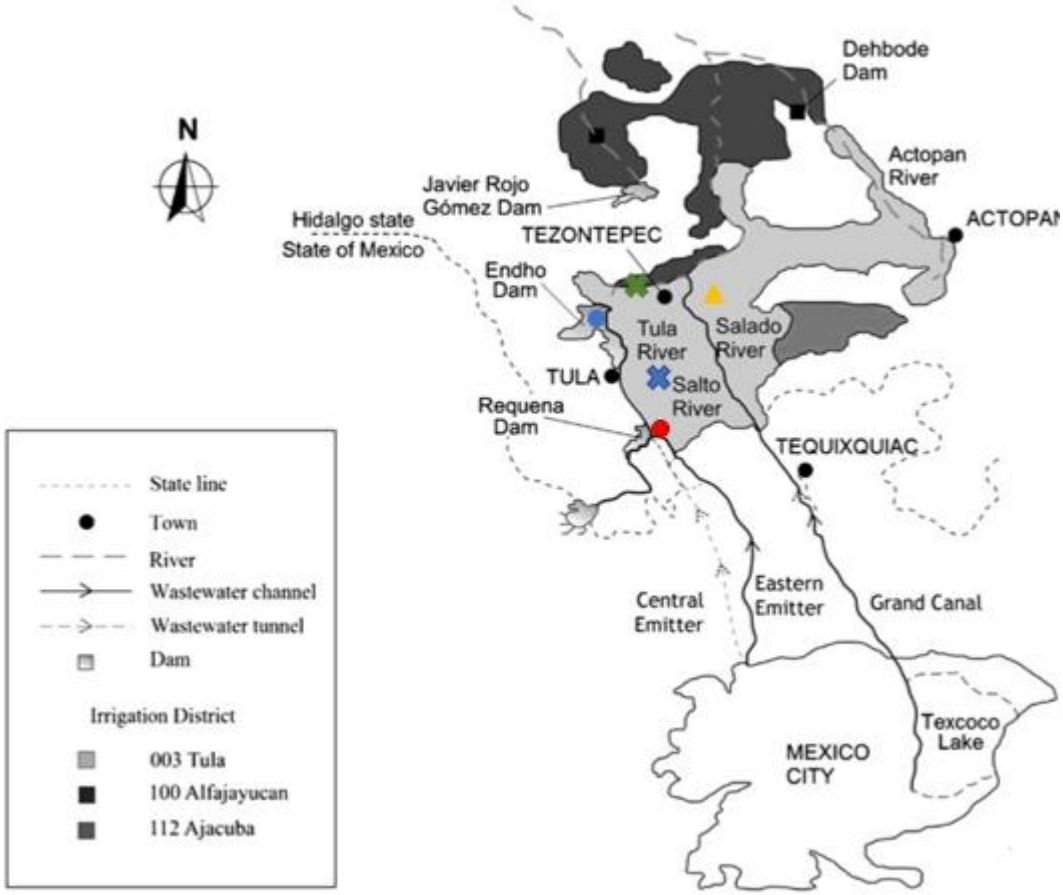


Figure 7.2-1 Tula Valley map (Chavez et., al 2011) with sampling locations marked. Blue cross: agricultural canal sampling point, Blue dot: Endho dam sampling point, Red dot: Atotonilco WWTP, Green cross: Tula River sampling point, Yellow triangle: Cerro Colorado sampling point.

The values of the concentrations measured are summarised in Appendix 10.8, and further analysed and presented throughout this chapter. As described in Section 3.2.2, given the distance from the Tula Valley to the laboratory facilities it was only possible to collect a maximum of 1 L of sample at each location, this is only enough for duplicate technical replicates (as opposed to n=3), so standard deviations could not be calculated. Furthermore the low sample volume and the fact that the standards were not available in the laboratory in Mexico where sample processing took place, meant it was not possible to pre-spike the samples in order to calculate recoveries for each matrices or to undertake matrix-specific method validation. However, as detailed in Section 4.2.4 the method was validated using a range of wastewaters. In order to be able to quantify this samples, the recoveries obtained for UK WWTP samples run on the day the Mexico samples were used. Wastewater influent recoveries were used for the dam and the canal, while recoveries from Stoke Bardolph WWTP effluent were used for Atotonilco WWTP effluent and recoveries from downstream water from Sutton Bonington samples were used for the spring samples. Even though it is not ideal, the practicalities of the situation meant it was necessary.

The non-steroidal anti-inflammatory drug acetaminophen was the pollutant detected with the highest concentrations in all matrices, except in the spring water (Figure 7.2.1) , where it was below the limit of detection. The concentration range was 20,960.0 ngL^{-}

[1] (river water) – 29,912.6 ngL^{-1} (canal water). The Mexican national system of medicinal products and packaging residue management (SINGERM) is an organization which collects medicines (mainly from Mexico City) past their use-by-date from homes and disposes them in a safe way. According to them the most commonly disposed pharmaceutical was acetaminophen (CANIFARMA, 2017), which is in accordance with acetaminophen being the pollutant found at the highest concentrations in this study. Acetaminophen has not been widely measured in the Tula valley, however a multi-residue study conducted in 2018 measured this pollutant in the range of 612 to 67,200 ngL^{-1} in water from several irrigation canals. The lowest concentration was found in a site downstream of the Endho dam, where wastewater will have travelled for longer after leaving Mexico City, therby allowing further sorption and degradation to take place. The highest concentration was found in a canal in the southern area of the valley, which is closer to the main canals transporting wastewater from Mexico City (Lesser, et al., 2018). The results of this thesis show a similar trend, with the water from the canal having the highest concentration and the concentration then decreasing from wastewater effluent to dam and finally to river. The acetaminophen concentrations measured are within the ranges previously reported in Latin America for wastewaters (16,630- 46,600 ngL^{-1}), with an average concentration in surface water of 10,283.51 ngL^{-1} (Peña-Guzmán, et al., 2019). Acetaminophen has not been detected in spring water in the Tula Valley previously and this is likely due to its high solubility in water (14,000 mgL^{-1} at 25 °C (S. Kim, et al., 2019)) and therefore preferential partitioning to water rather than soil sorption, which would then lead to leaching into groundwater. Acetaminophen has also been reported to be very biodegradable in soil (Martínez-Hernández, et al., 2016) so if partitioning onto soil occurs then it is likely biodegraded before reaching the aquifer.

The concentrations for acetaminophen obtained in the Tula Valley were lower than the range of concentrations measured in the wastewater influent of Stoke Bardolph WWTP (100,000 – 85,000,000 ngL^{-1} with a mean of 750,715 ngL^{-1}), but considerably higher than the concentrations in the effluent (15.6 – 2,670.2 with an average of 749.2 ngL^{-1}) demonstrating the role of wastewater treatment in reducing pollutant load before release to the wider water environment. The range of concentrations found in the Tula Valley were near the maximum measured in the effluent of Sutton Bonington WWTP (23,554.2 ngL^{-1}). The influent concentrations of Sutton Bonington plant were still higher (8,621.0 – 64,243.9, average 38,493.2 ngL^{-1}) than those measured for acetaminophen in the sampling points studied in the Tula Valley. The higher wastewater influent concentrations in the UK compared to those measured in Mexico were expected as the water sampled in Mexico had either been treated in the Atotonilco WWTP or undergone a degree of natural attenuation. However, higher concentrations of acetaminophen are directly entering the environment in the Tula Valley than through the effluents of the two UK WWTPs studied. This is of concern as the concentrations found in Mexico equate to a risk quotient above one for aquatic life (as can be seen in Figure 7.2.3). As a comparison, in the two UK WWTPs effluents the RQs for acetaminophen were below one. This means the Atotonilco WWTP is not removing enough of this pollutant. Acetaminophen has been shown to have high removal using secondary biological treatment due to its biodegradability (>98 %) (Peake, et al., 2016a; Petrie, et al., 2015), however it has been reported to have very low (<3%) removal using coagulation (Teh, et al., 2016). Therefore, it is likely the aerobic activated sludge part of the treatment in Atotonilco WWTP is doing most of the removal of this pollutant while the physicochemical side is not. In order to confirm this, it would be necessary to sample the effluent of each parallel treatment to compare them.

After acetaminophen the pollutants found with the highest concentrations were erythromycin, naproxen, sulfamethoxazole and its metabolite acetyl-sulfamethoxazole, bezafibrate, diclofenac, 4-tert-ocylphenol and carbamazepine with

concentrations in the range of thousands of ngL^{-1} for at least a couple of the sampling sites. These can be seen in Figure 7.2.1 and Figure 7.2.2, as well as Appendix 10.8. Out of the 36 pollutants measured only 5 were detected in spring water (Figure 7.2.2), with three of these: sulfamethoxazole, carbamazepine and bezafibrate were also found with concentrations in the range of thousands of ngL^{-1} at sampling points other than the spring. As expected, the lowest concentrations were found for the spring water due to sorption and degradation. Even though this sampling campaign was undertaken for one temporal point, this demonstrates the natural attenuation capacity of the area. However, the concentrations for sulfamethoxazole and levonorgestrel the concentrations in the spring equate to an RQ above one, as can be seen in Figure 7.2.3. For sulfamethoxazole the PNEC used for the calculation was for aquatic toxicity, while for levonorgestrel it was for fish larvae, as can be seen in Appendix 10.1, these were the lowest values reported in the literature for these pollutants.

After acetaminophen, erythromycin was found with highest concentrations. The highest level was measured in the dam (9,199.4 ngL^{-1}) and canal (9,059.8 ngL^{-1}), compared to the river (5,506.9 ngL^{-1}) and wastewater effluent where it was at its lowest with 206.6 ngL^{-1}. These erythromycin concentrations translated to RQs > 1 for all sampling points in terms of aquatic toxicity. In terms of antibiotic resistance, (minimum inhibitory concentration), the RQs were above one for all except wastewater effluent, as can be seen Figure 7.2.3. Erythromycin has often been found to be of concern, with RQs above one in the environment (Petrie, et al., 2015). In this thesis, erythromycin RQs >1 were found in the effluent or downstream water of both UK WWTPs studied. The samples during the Tula Valley sampling campaign were taken at a single time point and therefore do not reflect the overall situation, which would have to account for the Atotonilco WWTP hydraulic retention time as well as for the travelling time of the water from Mexico City and through the valley. However, these results still indicate some removal of erythromycin because wastewater effluent concentration is hundreds of nanograms per litre lower than the concentrations at the other sampling points, which are influenced by raw wastewater. Erythromycin has shown variable removal in activated sludge from 19-72 % (Peake, et al., 2016a), the results from this study indicate that Atotonilco WWTP likely has high removal capability for erythromycin. In the Tula Valley concentrations previously reported range from 25.9 – 1,140 ngL^{-1} with lower concentrations being found further from Mexico City and higher concentrations found closer to the main raw wastewater channels (Lesser, et al., 2018). Concentrations decreasing in the course of wastewater transportation along the Tula Valley have been previously observed for bezafibrate, diclofenac, erythromycin, gemfibrozil, metoprolol, naproxen and trimethoprim (Chávez-Mejía, et al., 2019).

Compared to previous studies in the Tula Valley where naproxen has been measured in canals in the range 96.4-18,600 ngL^{-1} (Lesser, et al., 2018), it was detected at lower concentrations in this work. In raw wastewater from Mexico City it has been measured at 5,861 ± 190 ngL^{-1} in the wet season and 16,336 ± 1,484 ngL^{-1} in the dry season (Chávez, et al., 2011). The samples studied for this thesis were taken towards the end of the dry season, and they show a reduction in concentration from canal water (7,216.6 ngL^{-1}) to dam (2,851.4 ngL^{-1}) and river (3,544.5 ngL^{-1}). This reduction is consistent with previous studies showing that naproxen can be photo-catalytically degraded in water and is also attenuated by soil sorption (Chávez-Mejía, et al., 2019). In this study naproxen was not detected in spring water above the LOD (0.6 ngL^{-1} for downstream Sutton Bonington WWTP and 6.0 ngL^{-1} for WWE of Stoke Bardolph). However it has been previously detected in well water from the Tula Valley at 11.5 and 11.1 ngL^{-1} (Lesser, et al., 2018). Other studies have shown that soil sorption is the main mechanism of attenuation for naproxen, due to the high organic content of the Tula Valley soil, while carbamazepine has been shown to be highly recalcitrant and having high likelihood of reaching groundwater (Chávez-Mejía, et al., 2019). This is

consistent with the results found in this study where carbamazepine was one of the five pollutants detected in spring water, while naproxen was not.

Both naproxen and carbamazepine presented their lowest concentration in wastewater effluent, which indicates potentially good removal in Atotonilco WWTP. Carbamazepine concentration in wastewater effluent of Atotonilco WWTP (407.1 ngL^{-1}) was lower than the average of 576.8 ngL^{-1} (with a range from 235.52- 1,407.3 ngL^{-1}) measured for effluent wastewater from Stoke Bardolph WWTP, but higher than the range measured in downstream water from Sutton Bonington WWTP (20.1 to 167.0 ngL^{-1}). Carbamazepine is known to be very recalcitrant and not prone to biodegradation (Petrie, et al., 2015) as well as showing poor removal in coagulation processes (Teh, et al., 2016). The relatively low carbamazepine concentration in Atotonilco WWTP wastewater effluent could be due to lower influent concentrations at the corresponding time or to good removal in this plant. This would have to be further studied with longer sampling campaigns including influent, effluent from the biological treatment, effluent form the physicochemical treatment and combined effluent. Both for naproxen and carbamazepine the removal of the treatment process of Atotonilco WWTP and natural attenuation translated to RQs below one for this sampling campaign, as can be seen in Figure 7.2.3.

In previous studies undertaken in the Tula Valley sulfamethoxazole and carbamazepine have been amongst the most frequently detected pollutants in groundwater, along with the pesticide DEET (Lesser, et al., 2018). This is consistent with both of these pollutants being detected in spring water in this study. Carbamazepine has been previously detected in the same Cerro Colorado spring sampled for this thesis, at a concertation of 7.7 ± 1.3 ngL^{-1} in 2012 (Ruíz, et al., 2012), it would be interesting to do more sampling campaigns in this spring to see if the concentration is increasing or if it is due to seasonal variation. Sulfamethoxazole was the pollutant with the highest concentration of 74.8 ngL^{-1} in spring water. This concentration is concerning as it is high enough to cause aquatic toxicity, with an RQ above one, as can be seen in Figure 7.2.3. Sulfamethoxazole concentrations were high enough in all the sampling points to have an RQ above one. Sulfamethoxazole also had RQs above one for both effluents of the WWTPs studied in the UK. Sulfamethoxazole metabolite, acetyl-sulfamethoxazole concentrations were not equivalent to an RQ > 1, however as this pollutant can undergo deacetylation in the environment, reforming sulfamethoxazole (Aurore Andriamalala, et al., 2018), its presence at relatively high concentrations (703.6 ngL^{-1} in the dam up to 2,166.6 ngL^{-1} in the river) are also of concern. Sulfamethoxazole concentrations are within the range previously measured in canal water in the Tula Valley (279 - 6,570 ngL^{-1}) (Lesser, et al., 2018). Sulfamethoxazole concentration in Atotonilco wastewater effluent (3,462 ngL^{-1}) is higher than those measured in both Stk WWE: (17.5 – 406.8 ngL^{-1}) and SuB WWD (10.0 -513.6 ngL^{-1}). Sulfamethoxazole removal has been previously reported to be highest in nutrient removal units (Dapeng Wang, et al., 2019), Atotonilco WWTP does not have this kind of removal due to the nature of the use of its effluent water for irrigation (CONAGUA, 2007) and this is likely why poor sulfamethoxazole removal is achieved. This is interesting because it highlights the complexity of maintaining water with the nutrient levels that farmers in the area have come to expect as well as protecting the environment.

Similar to sulfamethoxazole and carbamazepine, bezafibrate was detected in spring water (3.9 ngL^{-1}). Bezafibrate has been previously found to be less retained in Tula Valley soils and therefore measured in groundwater, compared to other pollutants with water solubilities higher than 10 gL^{-1} (Siemens, et al., 2008). Bezafibrate has a very low water solubility of 1.55 mgL^{-1} (Wishart, et al., 2018). The highest concentration for bezafibrate was measured in the wastewater effluent (1,610.1 ngL^{-1}). This could

indicate poor removal of this pollutant in the Atotonilco WWTP. The concentration measured was higher than the ranges measured in the two UK WWTP (Stk WWE: 23.5- 270.3 ngL^{-1}, SuB WWE: 0.9-5.8 ngL^{-1}). Bezafibrate removal has been observed to be >90% in a WWTP comprising of aerobic activated sludge (Rodriguez-Narvaez, et al., 2017), which is the treatment received by part of the wastewater in Atotonilco WWTP. This indicates that the parallel physicochemical treatment likely does not have high bezafibrate removal. Bezafibrate concentrations are of environmental concern for all sampling points except the spring water, as can be seen in Figure 7.2.3. Bezafibrate has been previously reported to often fall within the toxic environmental range (Petrie, et al., 2015), however this is in contrast to the results obtained for the two UK WWTPs studied were the effluent concentrations of bezafibrate were equivalent to RQs below one.

Diclofenac was also detected with concentrations often above 1,000 ngL^{-1}, as can be seen in Figure 7.2.1, this is within previously reported ranges in the region. With concentrations ranging from 2,129 ± 79 ngL^{-1} in the wet season and 3,424 ± 391 ngL^{-1} in the dry season (Chávez, et al., 2011). These concentrations are slightly higher than those measured in the UK for this thesis, as reported in Chapter 5, and in some European countries (60-1500 ngL^{-1}) (Petrie, et al., 2015). Despite the higher concentrations found in the Tula Valley in Mexico compared to the UK in both locations diclofenac has RQs above one, making it an important pollutant of concern in both countries.

Four-tert-octylphenol was found in the range 695.2 ngL^{-1} (canal water) – 1,041.8 ngL^{-1} (river water), it has not been previously studied in the Tula Valley. These concentrations equate to environmental toxicity RQs below one, however they are very close to 1 in relation to antimicrobial resistance development (minimum inhibitory concentration), as can be seen in Figure 7.2.3, making this pollutant of potential concern. This is in contrast to the RQs obtained for Stk WWE and Sub WWD which were below one for this pollutant.

Levonorgestrel and oxytetracycline were both found at concentrations below 1,000 ngL^{-1}, in all sampling points but they were also found in spring water like sulfamethoxazole, bezafibrate and carbamazepine (see Figure 7.2.2). Levonorgestrel has not been previously measured in the Tula Valley. In Latin America concentrations in surface water have been measured in the range of 19-2,600 ngL^{-1} (Peña-Guzmán, et al., 2019), the range of concentrations measured in this study were lower than this. With a maximum of 88.3 ngL^{-1} in wastewater effluent and a minimum of 41.8 ngL^{-1} in spring water and it was not detected in the dam. Levonorgestrel is very toxic even at concentrations below 1 ngL^{-1} (Besse, et al., 2009). Even though the concentrations measured in this study are lower than for other pollutants the RQ is above one for all the sampling points were it was measured, as can be seen in Figure 7.2.3. Sorption studies have revealed that progestins (including levonorgestrel) have no significant desorption hysteresis and thus can be readily desorbed under rainfall or irrigation events (Yang, et al., 2020), which is likely the reason levonorgestrel can be found in the groundwater of the Tula Valley. Levonorgestrel has been previously reported to have low removal (<50%) in several different WWTPs (Yu, et al., 2019b) and is likely the reason this pollutant has a relatively high concertation in the wastewater effluent of Atotonilco WWTP (88.3 ngL^{-1}). Oxytetracycline has been found in Latin America in the range of 10.6-24.5 ngL^{-1} in wastewater effluent (Peña-Guzmán, et al., 2019), which is lower than the 228.1 ngL^{-1} measured in this study for the Atotonilco WWTP effluent. However, the oxytetracycline concentrations measured in the canal and wastewater effluent are within the range previously measured in irrigation canals of the Tula Valley of 23.5-225 ngL^{-1} (Lesser, et al., 2018). Oxytetracycline has not been previously measured in Tula Valley groundwater.

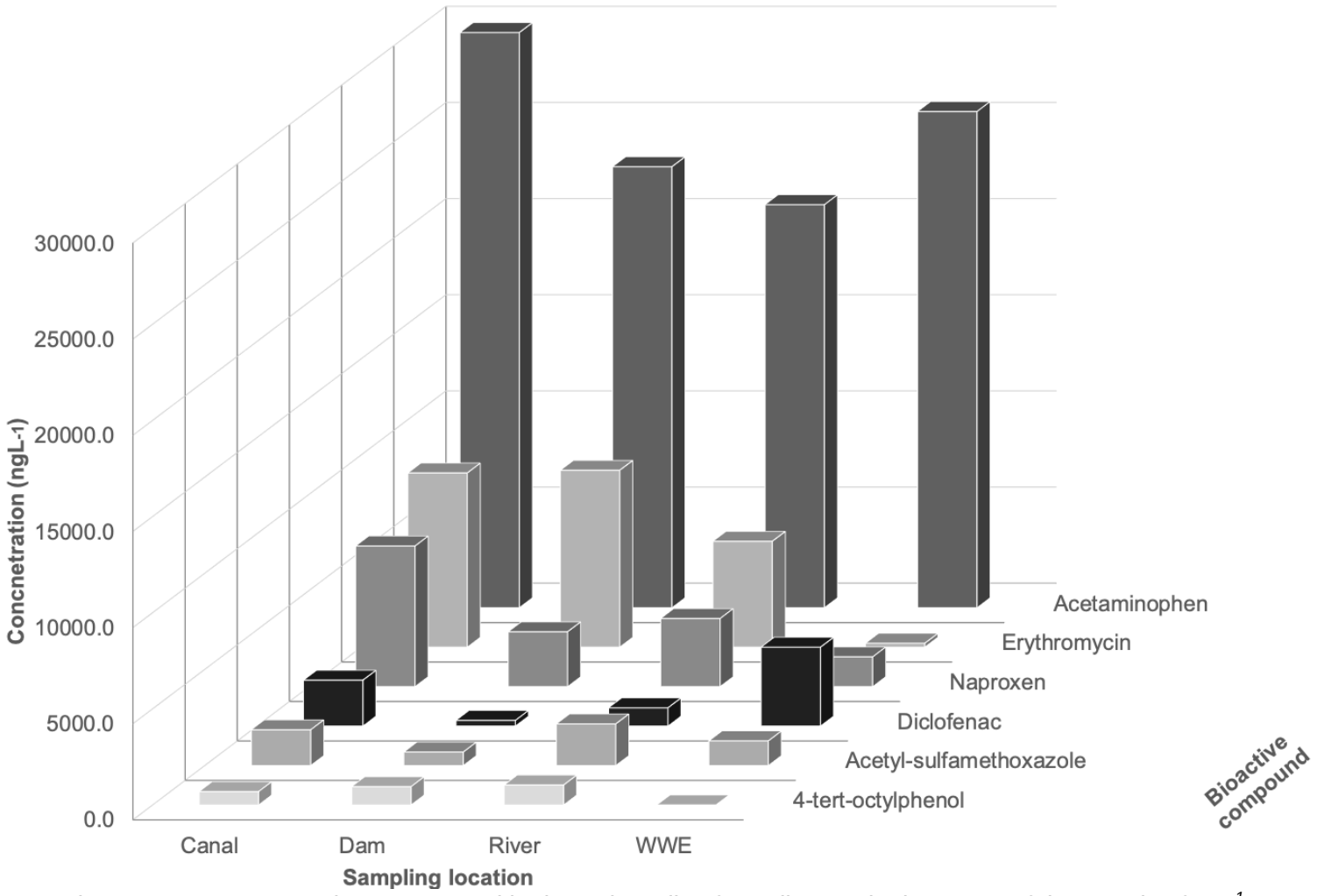


Figure 7.2-2 Concentrations measured in the Tula Valley for pollutants in the range of thousands of ngL^{1-}

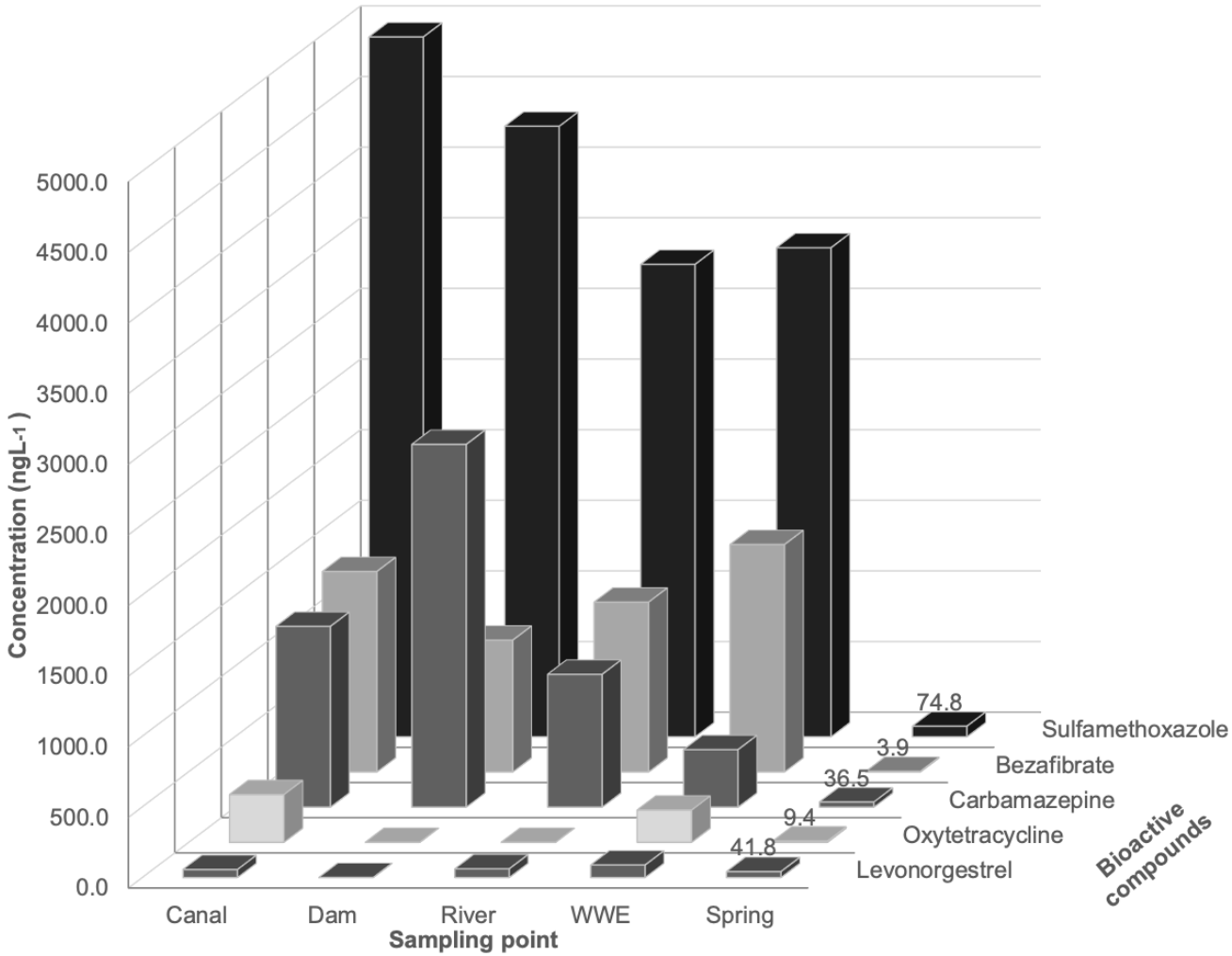


Figure 7.2-3 Concentrations measured in the Tula Valley for pollutants detected in spring water

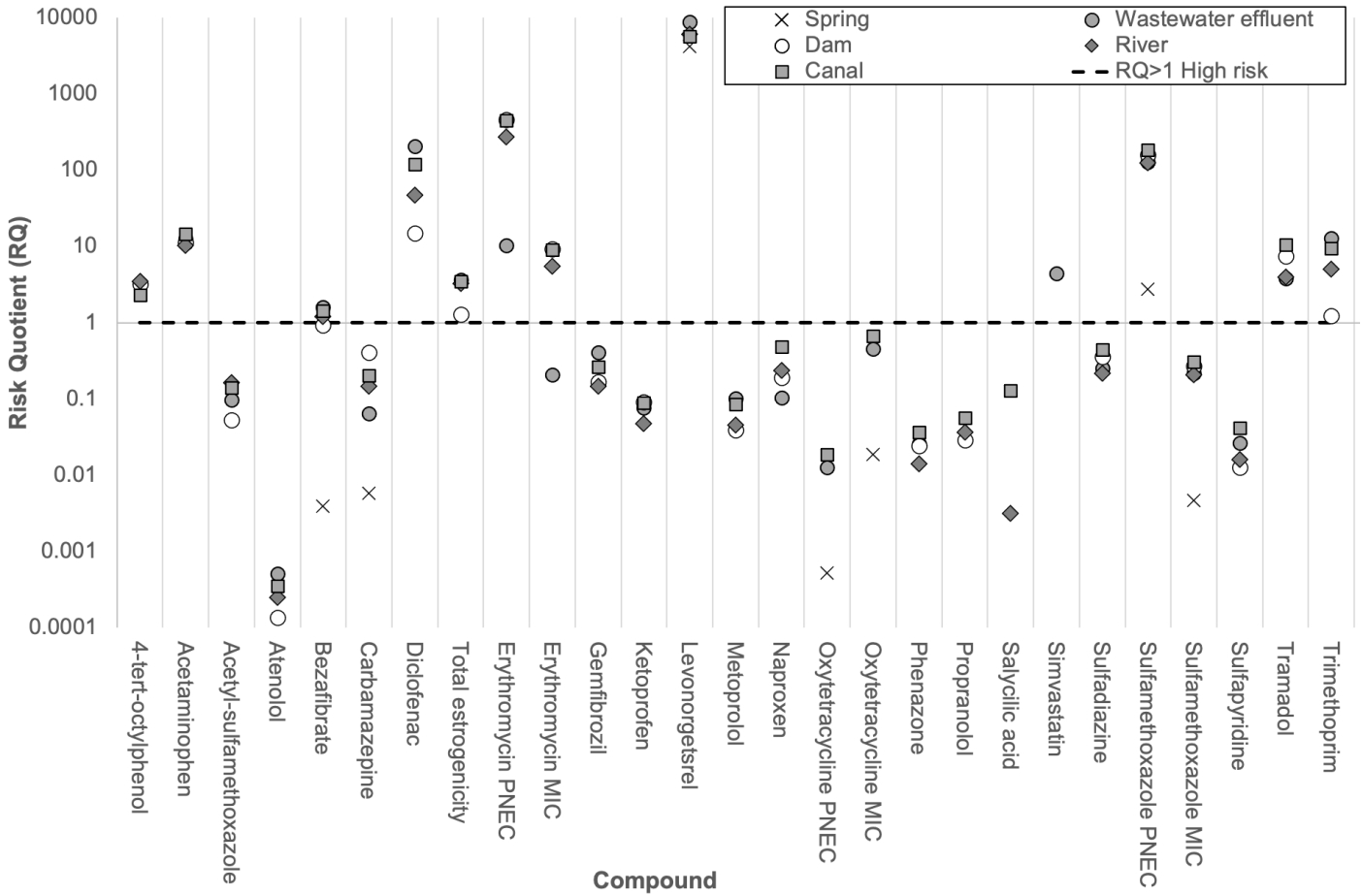


Figure 7.2-4 Risk quotients (RQs) for bioactive pollutants measured in the Tula Valley, Mexico Total estrogenicity accounts for Estrone-3-Sulfate (E1-3S), Estriol (E3) and Estriol-3-Sulfate (E3-3S), which were the three natural estrogens found at concentrations above the limit of detection. For some antibiotics RQs were calculated for PNEC (predicted no effect concentration in terms of aquatic toxicity) and for MIC (Minimum inhibitory concentration in terms of antibiotic resistance development)

Fifteen pollutants were found with concentrations below 1,000 ngL^{-1} and were not detected in the spring water (see Figure 7.2.4 and Figure 7.2.6). However, for simvastatin, tramadol and trimethoprim the concentrations detected are still of concern as they equate to RQs above one, (see Figure 7.2.3). Tramadol and trimethoprim were also identified to have RQs > 1 in the effluents of the two UK WWTPs studied for Stk WWE and Sub WWD. Simvastatin has not been previously measured in the Tula Valley and in this study, it was only found in wastewater effluent, so a more thorough sampling camping would be necessary to establish its prevalence in the region. Similarly, tramadol has not been previously identified in this region, however in this study it was found in all the sampling points except for the spring. The concentrations measured were lower (14.2 ngL^{-1} in WWE of Atotonilco WWTP) than those detected for Stoke Bardolph influent (340.5-5,341, average 1,483 ngL^{-1}) and effluent (400.4-2942.9, average 1,090.8 ngL^{-1}), but they remain of concern given the RQ above one, as can be seen in Figure 7.2.3. Trimethoprim has been previously identified as a pollutant in a concentration range which causes environmental toxicity (Petrie, et al., 2015), concentrations detected are within ranges previously reported for the Tula Valley in the irrigation canals (Lesser, et al., 2018). The concentration found in the dam was lower (62.1 ngL^{-1}) than at the rest of the sampling points. This could be due to removal due to slow growing nitrifying bacteria or sorption (Radjenović, et al., 2009), which could be part of long-term processes taking place at the dam.

Atenolol, gemfibrozil, metoprolol, propranolol and sulfadiazine were found in a similar range to that previously reported in this region (Lesser, et al., 2018). Ketoprofen, paroxetine, phenazone and sulfadiazine have not been previously measured in the Tula Valley, however the concentrations found in this study were not of environmental concern, based on the PNECs. Salicylic acid was found at the lower concentrations (6.5 ngL^{-1} in the river and 264.4 ngL^{-1} in the canal) compared previously reported concentrations of this pollutant in the Tula Valley which were above 10,000 ngL^{-1} in raw wastewater and irrigation canals (Chávez-Mejía, et al., 2019). The main attenuation pathway for salicylic acid has been found to be photolysis (Chávez-Mejía, et al., 2019), which is likely the reason why at the river, which is further from Mexico City there is less concentration of this pollutant, than at the canal.

The total estrogenic effect of the pollutants measured is above one for all sampling points (except for the spring), as can be seen in Figure 7.2.3. The RQ for estrogenicity is above one, but it would likely be higher if pollutants like ethinyl estradiol and estrone, which have been previously quantified in irrigation canals of the Tula Valley in the range of 32.3-70.3 ngL^{-1} and 46.8-166 ngL^{-1} respectively (Lesser, et al., 2018) were included in this calculation. The limit of detection was likely not low enough in the LC-MS QqQ to identify these pollutants. Estriol, as well as its sulfate conjugate have not been previously measured in the Tula Valley and its concentrations were higher than those previously reported for estrone and ethynyl estradiol (Chávez, et al., 2011; Lesser, et al., 2018), this indicates these pollutants merit further study.

Clofibric acid was not detected in any of the studied sampling locations, the method limits of detection ranged from 6.0 ngL^{-1} to 24.0 ngL^{-1} for this pollutant. Clofibric acid has been previously reported to be below limit of detection (0.5 ngL^{-1} in spring water and 100 ngL^{-1} in wastewater) (Chávez, et al., 2011; Gibson, et al., 2007). It is possible more sensitive methods are necessary to quantify this pollutant. However, the lowest PNEC found in the literature was 110 ngL^{-1} for *Daphnia magna* (Appendix 10.1) and given the method limits of detection used to study this pollutant in Tula Valley are higher than this it is likely this pollutant is not of concern for the region.

The opioids codeine and morphine were not detected in any of the samples and neither of these pollutants has been monitored in the Tula Valley previously. This is likely

because opioids are not produced in Mexico and in general it is difficult to obtain them as medicines (Transform Drug Policy Foundation, et al., n.d.). Codeine and morphine have both been previously detected in Latin American wastewaters in the range 143-538 and 16 -14,130 ngL^{-1} respectively and in surface waters in the range of 10-252 and 36 ngL^{-1} respectively (Peña-Guzmán, et al., 2019)

An important consideration for the Tula Valley is the difference between the wet and the dry season. The sampling undertaken for this thesis was done during the dry season and so the concnetrationsof BACs in the irrigation channels would be reduced during the wet season. Furthermore, less irrigation water will be used during the wet season. This means that the data presented here is for the worse period during the year. Therefore the assement done in the dry season is of importance as its when higher concnetrations of BACs are likely to be reaching agirucltural soil and potentially uptaken by crops. A recomentation would be to further study crop uptake of the pollutants found with higher concnetrations and which present higher toxicity and recommend that only those crops presenting an uptake below concnetrations which could affect animals and humans consuming crops be grown during the dry season.

Another consideration in terms of the risk quotients calculated is that these were obtained from the literature, mostly focusing on European species. It was not possible to find equivalent values for Mexican species, a recomedation would be to study PNECs for Mexican species the most toxic and prevalent pollutants (e.g. 4-tert-octylphenol, acetaminophen, diclofenac, steroid hormones, erythromycin, levonorgestrel, sulfamethoxazole, tramadol and trimethoprim).

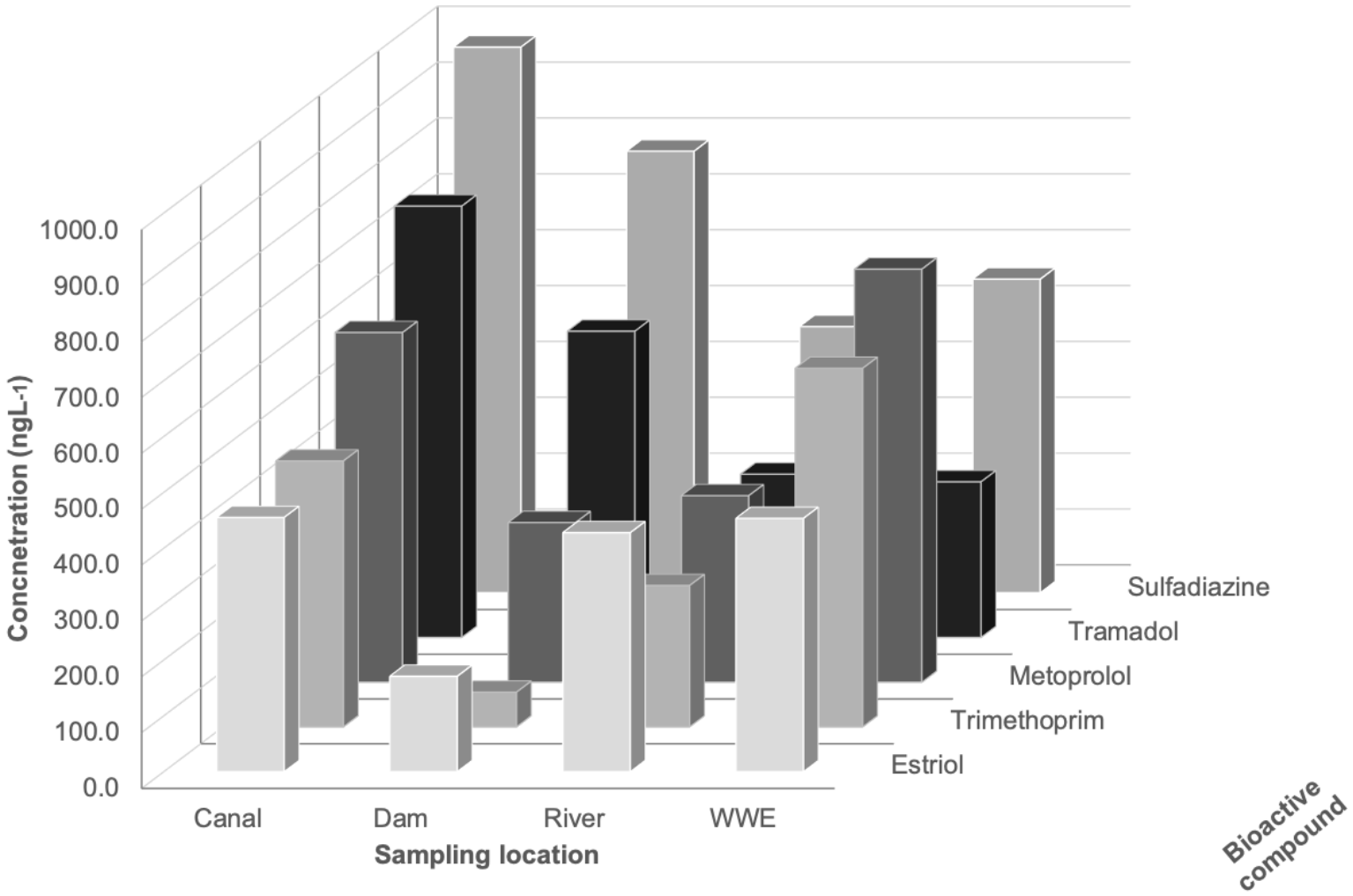


Figure 7.2-5 Concentrations of bioactive pollutants measured in the range of hundreds of ngL^{-1}

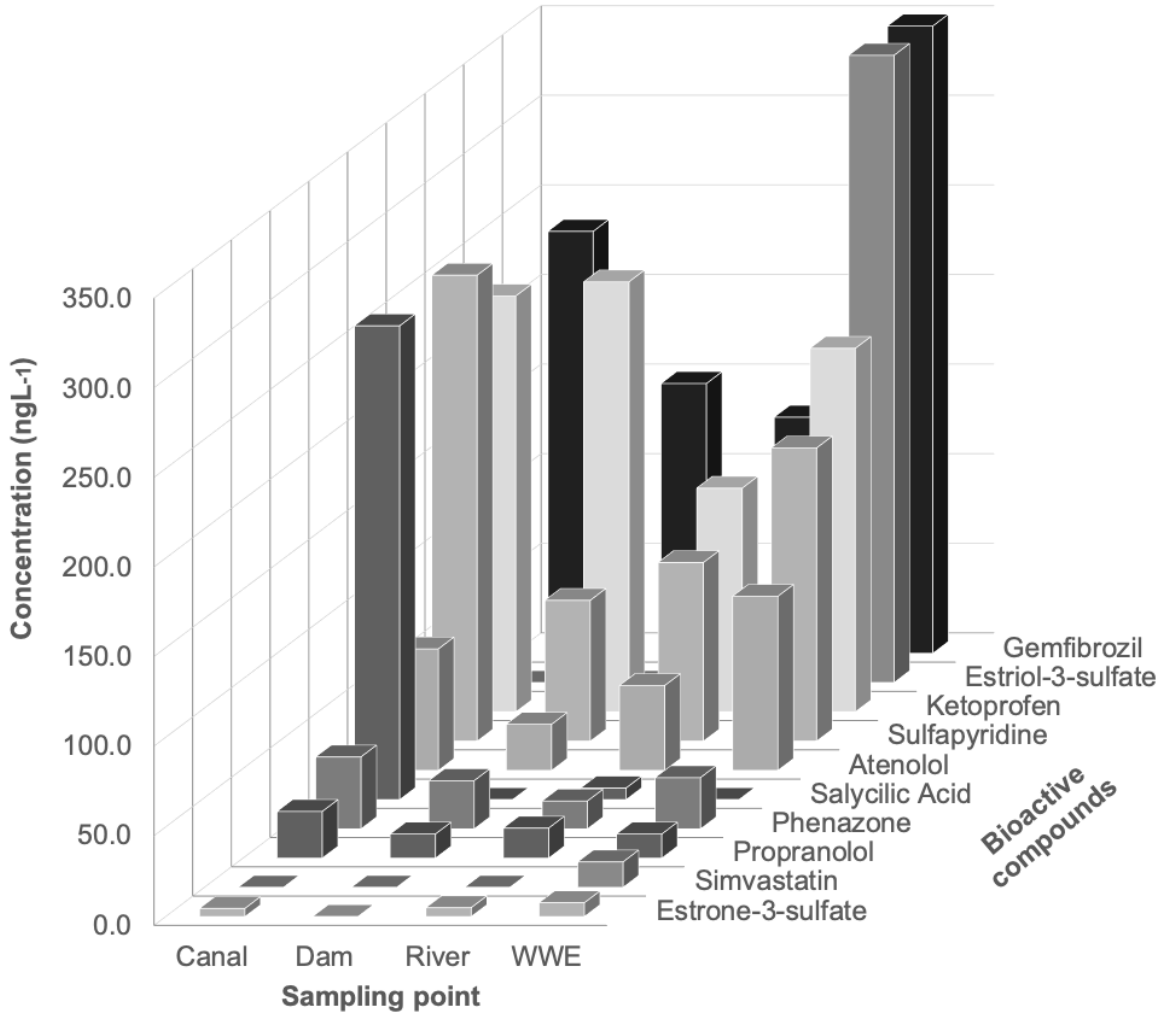


Figure 7.2-6 Concentrations of bioactive pollutants measured in the range of tens to hundreds of ngL^{-1}

7.3 Untargeted results

Untargeted analysis has not been previously undertaken in the Tula Valley. Untargeted analysis can complement targeted analysis in understanding the fate of targeted analytes (transformation products and metabolites) as well as identifying other potentially toxic pollutants not previously considered in targeted analysis (Alygizakis, et al., 2019; Di Carro, et al., 2018; Richardson, et al., 2018). All compounds reported in this section were identified with level of confidence in identification 2, according to the Metabolic Standards Initiative (Sumner, et al., 2007). This is because they were identified based on their mass spectrum compared to the mzCloud library and not against a standard.

7.3.1 Compounds not previously identified in the aquatic environment (and not detected in the UK samples)

A total of eighteen compounds which have not been previously detected in the aquatic environment and which were not found in the two UK sites studied were detected. The spectrums used to identify them are found in Appendix 10.5. Three of these compounds are related to industrial processes or products, namely: 4,4'-Dihydroxydiphenylsulfone, 4-Coumaric acid and Tetrakis(2-hydroxypropyl)ethylenediamine, as can be seen in Table 7.3.1. These industrially related compounds have their highest peak area for the wastewater influent and the canal samples, indicating there is likely some removal at the Atotonilco WWTP as well as some natural attenuation. However, this would have to be confirmed with a long-term sampling campaign or with samples accounting for the hydraulic retention time of the WWTP and for the time it takes for the water to travel through the valley. Since very little is known about the toxicity of these compounds it is difficult to assess their significance.

Four of the compounds found are naturally occurring in foods consumed in Mexico such as avocados and tomatoes, as can be seen in Table 7.3.1, demonstrating the importance of wastewater-based epidemiology to inform on profiles specific to time or space e.g. for distinguishing diet, along with relationships with medication intake, diseases, etc (Archer, et al., 2018; He, et al., 2020). Two flavouring additives were also found, (see Table 7.3.1). One of these, decanoic acid, has been previously identified as potentially toxic (Wishart, et al., 2018). Further work determining their environmental fate and toxicity would help to identify which compounds should be prioritised for further studies. Several compounds which are naturally occurring in plants and in humans were also found, some of which are potentially bioactive such as ricin and rubiadin, (see Table 7.3.1). A toxin related to cyanobacteria, Homo-anatoxin, was also detected, (Table 7.3.1). Only three compounds were detected in the spring water two of which are naturally produced compounds: formononetin and rubiadin. However, one compound: α-Piperidinobutiophenone was found with relatively high peak intensity in the spring, as can be seen in Table 7.3.1, but no information related to its use could be found.

Analyte	Formula Monoisotopic mas *Retention Time*	Peak area (n=2)						Literature
		WWI	*Canal*	*River*	*Dam*	*WWE*	*Spring*	
16-Heptadecyne-1,2,4-triol	$C_{27}H_{32}O_3$ 266.2245 *5.86*[a]	1.16 x10^8	3.56 x10^7	6.51 x10^7	4.75 x10^6	2.64 x10^6	-	Also known as avocadyne, found in avocado (S. Kim, et al., 2019). Not detected in the UK but *1,2-dihydroxyheptadec-16-yn-4-yl acetate*, which is also found in avocados (S. Kim, et al., 2019), was found at least once in all UK matrices studied, not presented in Chapter 6 as the frequency was below 40%.
2-Oxindole	C_8 H_7NO 133.05275 *2.83*[a]	9.98 x10^8	7.60 x10^8	4.08 x10^8	-	1.40 x10^7	-	Natural metabolite found in faeces and saliva (S. Kim, et al., 2019)
4,4'-Dihydroxydiphenylsulfone	$C_{12}H_{10}O_4S$ 250.02999 *3.18*[b]	2.66 x10^7	1.74 x10^7	5.58 x10^7	1.53 x10^7	1.02 x10^7	-	Role as a metabolite and endocrine disruptor (S. Kim, et al., 2019). Used as a textile dye and in some food packaging products (S. Kim, et al., 2019).
4-Coumaric acid	C_9 H_8 O_3 164.04764 *2.32*[a]	2.49 x10^7	1.13 x10^7	8.91 x10^6	2.89 x10^6	8.56 x10^6	-	Used as components in flavours, perfumes, synthetic indigo and pharmaceuticals (S. Kim, et al., 2019).
4'-Methoxyacetophenone	$C_9H_{10}O_2$ 150.06548 *1.10*[a]	1.33 x10^8	4.94 x10^7	2.58 x10^7	3.67 x10^6	2.99 x10^7	-	Found in alcoholic beverages and some fruits (pepper, tomato, cranberries, anise). Flavouring agent for vanilla, nut, tobacco and butter flavours (Wishart, et al., 2018).
4'-Methoxyacetophenone	$C_9H_{10}O_2$ 150.06517 *1.63*[b]	1.57 x10^8	1.16 x10^8	6.34 x10^7	5.37 x10^7	2.57 x10^8	-	Approved EU flavouring agent (European Commission, 2012).
4-Methyl-5-thiazoleethanol	C_6H_9NOS 143.04047 *1.24*[a]	3.01 x10^8	6.81 x10^7	7.50 x10^7	1.66 x10^7	1.92 x10^7	-	Found in alcoholic beverages, cooked beef, cocoa, porcini and roasted peanuts (Wishart, et al., 2018).
10-Hydroxycarbazepine	$C_{15}H_{14}N_2O_2$ 254.10526 *3.60*[b]	2.93 x10^7	3.92 x10^7	2.61 x10^7	3.81 x10^7	6.46 x10^7	-	Major pharmacologically active metabolite of oxcarbazepine (widely used antiepileptic drug) (Chen, et al., 2019). Not detected in wastewater, although oxcarbazepine has been detected in

								surface waters with wastewater influence in a concentration of around 300 ngL^{-1} (Scott, et al., 2018).
Analyte	**Formula** **Monoisotopic mass** ***Retention Time***	**Peak area (n=2)**						**Literature**
		WWI	*Canal*	*River*	*Dam*	*WWE*	*Spring*	
Decanoic acid	$C_{10}H_{20}O_2$ 172.14648 *5.01*[a]	9.02 x10^7	8.45 x10^7	1.43 x10^7	1.89 x10^7	4.33 x10^7	-	Naturally occurring metabolite in humans, found in some foods such as radish and tomatoes, it is potentially toxic (Wishart, et al., 2018). Not detected in the UK samples, however decanamide, which derives from decanoic acid (S. Kim, et al., 2019), was found at least once in each matrix, not presented in Chapter 6 as the frequency was below 40%.
Dodecanedioic acid	$C_{12}H_{22}O_4$ 230.15189 *1.20*[b]	1.68 x10^8	1.43 x10^8	2.73 x10^7	2.45 x10^7	2.31 x10^7	-	Human metabolite and chemical used for anti-freezing and industrial manufacturing (S. Kim, et al., 2019).
Formononetin	$C_{16}H_{12}O_4$ 268.07319 *4.67*[b]	-	-	2.06 x10^7	1.64 x10^8	1.22 x10^7	6.74 x10^8	Plant metabolite present in clover grass (Gadd, et al., 2010).
Homo-anatoxin	$C_{11}H_{17}NO$ 179.13098 *0.80*[a]	2.43 x10^7	3.38 x10^7	2.06 x10^8	2.88 x10^7	4.84 x10^7	-	Cyanobacteria related toxin (Milena, et al., 2017).
Maltol	$C_6H_6O_3$ 126.03167 *1.94*[a]	1.09 x10^8	3.33 x10^7	1.04 x10^7	-	1.82 x10^7	-	Flavouring agent (European Commission, 2012).
Myristic acid	$C_{14}H_{28}O_2$ 228.209 *7.56*[b]	1.97 x10^8	4.30 x10^7	2.98 x10^7	1.27 x10^7	-	-	Free fatty acid in animal fatty wastewater sludges (Wallis, et al., 2017).
PPG n8	$C_{24}H_{50}O_9$ 499.37148 *5.11*[b]	5.51 x10^8	6.00 x10^8	1.09 x10^9	1.13 x10^9	2.75 x10^8	-	-
Ricinine	$C_8H_8N_2O_2$ 164.05857 *2.12*[a]	1.02 x10^8	1.06 x10^8	1.72 x10^8	1.27 x10^7	5.98 x10^7	-	Alkaloid previously identified in two Mexican *Ricinus communis* leaves. The seeds of this plant are usually used for the extraction of castor bean oil. The leaves have been shown to have antinociceptive, antioxidant and anti-inflammatory properties and

								are being investigated for use instead of a as waste as a by-product from caster-oil production (Vasco-Leal, et al., 2020)**.**
Analyte	**Formula** **Monoisotopic mass** ***Retention Time***	**Peak area (n=2)**						**Literature**
		WWI	*Canal*	*River*	*Dam*	*WWE*	*Spring*	
Rubiadin	$C_{15}H_{10}O_4$ 254.06128 *8.97*[a]	4.53 x10^6	3.99 x10^7	4.53 x10^6	3.28 x10^7	3.49 x10^7	3.91 x10^6	Isolated from *Rubia yunnanensis,* has antibacterial, antioxidant and hepatoprotective properties (S. Kim, et al., 2019).
Tetrakis(2-hydroxypropyl) ethylenediamine	$C_{14}H_{32}N_2O_4$ 292.23594 *2.88*[b]	1.29 x10^9	5.17 x10^8	1.58 x10^8	1.40 x10^8	1.06 x10^9	-	Used as adhesive, agricultural husbandry, crops and air cleaners (S. Kim, et al., 2019).
α-Piperidinobutiophenone * *Acidic areas*	$C_{15}H2_1NO$ 231.16212 *4.73, 5.76*[b]	2.11 x10^8	3.76 x10^8	3.66 x10^8	9.24 x10^7	2.21 x10^8	1.43 x10^8	Not in literature

Table 7.3-1 Compounds not previously detected in the aquatic environment. WWI: wastewater influent, WWE: wastewater effluent.

Note: Peak area does not equate to concentration and they are not comparable from one compound to another, but within the same compound a larger peak area very likely relates to a higher concentration. Superscripts a and b above the retention times refer to either the acidic (a) or basic (b) run.

7.3.2 Compounds not previously reported in the literature in aquatic environments, detected in the UK and Mexico samples

The compounds in this section have not been widely reported in the literature in relation to the aquatic environment, therefore their occurrence and fate is not well known. The spectrums used to identify them are found in Appendix 10.5. They were detected in the samples from Mexico as well as in the samples from the UK, further details about several of them are given in section 6.3.2, and this information is not repeated here. Although little is known about the toxicity of these compounds, several of them like bolidone, testosterone sulfate, nonanoic acid, 2-anisic acid, 2-Amino-6-methylmercaptopurine and 1-Adamantanamine have known bioactive properties, as can be seen in Table 7.3.2, and therefore are likely of concern in terms of environmental toxicity. Further studies would be necessary to determine their PNECs and environmental concentrations in order to quantify the risk they pose to the environment. All the compounds in this section were either not detected in groundwater or detected at the lowest peak area of all the samples indicating some natural attenuation.

Analyte	Formula Monoisotopic mass *Retention Time*	Peak area (n=2)						Literature	Frequency (%) of detection in UK WWTPs			
		WWI	*Canal*	*River*	*Dam*	*WWE*	*Spring*		*WWI Stk n=9*	*WWE Stk n=10*	*WWI SuB n=15*	*WWD SuB n=15*
1-Adamantanamine	$C_{10}H_{17}N$ 151.13599 *3.10*[b]	1.22 x10^8	2.95 x10^8	1.86 x10^8	1.36 x10^8	2.50 x10^8	-	1-Adamantanamine used to treat influenza virus (Scott, et al., 2018)	10.0	11.1	6.7	6.7
2,6-Dimethylpyrazine	$C_6H_8N_2$ 108.06874 *2.81*[b]	3.55 x10^6	6.17 x10^6	7.69 x10^8	8.22 x10^7	5.34 x10^6	-	Food additive (S. Kim, et al., 2019).	88.8	90.0	46.6	46.6
2-Amino-6-methylmercaptopurine	$C_6H_7N_5S$ 181.04216 *1.88*[a]	1.07 x10^7	1.91 x10^7	1.30 x10^7	3.15 x10^6	2.36 x10^7	-	Metabolite of thioguanine (S. Kim, et al., 2019).	100	100	100	100
8-(4-Sulfophenyl) octanoic acid	$C_{14}H_{20}O_5S$ 300.10318 *1.22*[b]	1.70 x10^8	8.44 x10^8	2.63 x10^8	6.22 x10^8	1.41 x10^9	-	Not in literature	20.0	22.2	53.3	53.3
10-(4-Sulfophenyl) decanoic acid	$C_{16}H_{24}O_5S$ 328.13451 *1.22*[b]	1.07 x10^8	3.16 x10^8	1.17 x10^8	2.75 x10^8	5.16 x10^8	7.12 x10^6	No literature	10.0	10.0	26.6	13.3
4-Methylphenol	C_7H_8O 108.05765 *4.21*[b]	3.78 x10^9	1.07 x10^9	2.51 x10^7	1.65 x10^7	7.82 x10^6	1.21 x10^7	EU approved flavouring agent (European Commission, 2012)	22.2	10.0	6.7	0.0
Acetophenone	C_8H_8O 120.05743 *6.02*[a]	1.07 x10^8	7.53 x10^7	5.44 x10^7	3.61 x10^7	1.11 x10^8	2.57 x10^6	Used as a flavouring, solvent, and polymerization catalyst (S. Kim, et al., 2019).	22.2	20.0	53.3	53.3
Boldenone	$C_{19}H_{26}O_2$ 286.19303 *5.13*[b]	1.17 x10^7	4.53 x10^6	5.67 x10^7	6.85 x10^6	5.19 x10^6	-	Growth promoter for meat producing animals, banned in the EU (Destrez, et al., 2009), available for human bodybuilders use or illegally on racing horses (Oda, et al., 2012).	22.2	20.0	53.3	46.6

Analyte	Formula Monoisotopic mass *Retention Time*	Peak area (n=2)						Literature	Frequency (%) of detection in UK WWTPs			
		WWI	*Canal*	*River*	*Dam*	*WWE*	*Spring*		*WWI Stk n=9*	*WWE Stk n=10*	*WWI SuB n=15*	*WWD SuB n=15*
Docosahexaenoic acid ethyl ester	$C_{24}H_{36}O_2$ 356.27101 *4.47*[b]	2.51 x10^8	1.30 x10^8	-	-	-	-	Found in fish oil products (Fan, et al., 2020).	100	30.0	100	60.0
Ferulic acid	$C_{10}H_{10}O_4$ 194.05796 *3.00*[a]	7.46 x10^7	2.55 x10^7	5.56 x10^7	4.45 x10^6	1.45 x10^7	-	Found naturally in applies (Lee, et al., 2017) and in soils (Olofsson, et al., 2014).	11.1	10.0	40.0	26.6
Imino(methyl)phenyl-lambda~4~-sulfane 2,4,6-trimethylbenzenesulfonic acid	$C_9H_{12}O_3S$ 200.05079 *3.12*[b]	3.41 x10^8	2.36 x10^8	1.95 x10^8	1.76 x10^8	1.76 x10^8	-	Not in literature.	22.2	20.0	53.3	53.3
Lauric acid	$C_{12}H_{24}O_2$ 200.1776 *4.89*[b]	1.39 x10^{10}	1.50 x10^9	5.58 x10^7	2.69 x10^8	2.58 x10^8	4.69 x10^7	Main fatty acid in coconut and palm kernel oil (S. Kim, et al., 2019). Has antibacterial properties and is routinely used to treat acne (Kozan, et al., 2020).	11.1	10.0	46.6	53.3
Linoleic acid	$C_{18}H_{32}O_2$ 280.24008 *6.18*[b]	6.28 x10^9	9.37 x10^8	4.96 x10^8	-	1.61 x10^9	-	Essential fatty acid for humans, mainly found in plant oils (S. Kim, et al., 2019).	22.2	10.0	53.3	53.3
Sodium [dodecanoyl(methyl)amino]acetate	$C_{15}H_{29}NO_3$ 271.21456 *4.79*[b]	2.56 x10^8	1.01 x10^8	9.65 x10^7	1.02 x10^7	2.89 x10^6	1.80 x10^6	Anionic surfactant not extensively studied. Surfactants have wide applications for example detergency, drug delivery, personal care products, among others (Das, et al., 2013).	22.2	0	53.3	46.6
Tetranor-12(S)-HETE	$C_{16}H_{26}O_3$ 248.17744 *4.71*[a], *5.75*[b]	2.11 x10^8	3.38 x10^8	1.46 x10^8	3.49 x10^8	4.40 x10^8	2.31 x10^6	Hydroxy fatty acid product of arachidonic acid metabolism through the 12-lipoxygenase pathway (S. Kim, et al., 2019).	20.0	22.2	53.3	53.3

Analyte	Formula Monoisotopic mass *Retention Time*	Peak area (n=2)						Literature	Frequency (%) of detection in UK WWTPs			
		WWI	*Canal*	*River*	*Dam*	*WWE*	*Spring*		*WWI Stk n=9*	*WWE Stk n=10*	*WWI SuB n=15*	*WWD SuB n=15*
Tropine	$C_8H_{15}NO$ 141.11528 *3.31[a], 3.86[b]*	3.61 x10^7	6.86 x10^7	5.16 x10^7	5.33 x10^7	7.33 x10^7	-	Tropane alkaloids can be found in several plants from the Solanaceae and other related families and in tea and pasta as an impurity. Many of these tropane alkaloids are degraded into tropinone and tropine during the cooking process (Marín-Sáez, et al., 2019).	10.0	11.1	0	6.6
Tropinone	$C_8H_{13}NO$ 139.09969 *3.04[a], 3.47[b]*	4.73 x10^7	3.69 x10^8	9.80 x10^7	-	4.85 x10^7	-		-	-	46.6	46.6
Undecanoic acid	$C_{11}H_{22}O_2$ 186.16196 *4.36[b]*	8.28 x10^8	5.54 x10^8	2.16 x10^8	1.61 x10^8	1.52 x10^8	1.53 x10^7	Approved EU flavouring agent (European Commission, 2012).	11.1	10.0	53.3	53.3
Palmitoleic acid	$C_{16}H_{30}O_2$ 254.22436 *5.46[a]*	3.75 x10^8	8.93 x10^7	-	2.21 x10^7	-	-	Metabolite of several organisms such as: *Daphnia galeata*, humans, algae and *Escherichia coli* (S. Kim, et al., 2019).	10.0	0.0	53.3	0.0
PEG n6	$C_{12}H_{26}O_7$ 282.16761 *1.73[b]*	2.57 x10^8	8.47 x10^7	1.82 x10^8	7.28 x10^7	1.61 x10^7	-	Previously detected in Swedish lake with a degradation half-life of <100 days (Zhe Li, et al., 2020)**.**	100	20.0	53.3	46.6
PPG n5	$C_{15}H_{32}O_6$ 308.21961 *3.82[b]*	1.94 x10^8	2.68 x10^8	4.62 x10^8	4.57 x10^8	2.27 x10^8	-	Previously detected in Swedish lake with a degradation half-life of <100 days (Zhe Li, et al., 2020)	100	90.0	53.3	53.3
PPG n6	$C_{18}H_{38}O_7$ 366.26144 *4.29[b]*	7.10 x10^7	9.36 x10^7	1.39 x10^8	1.53 x10^8	8.11 x10^7	-	Previously tentatively identified in synthetic turf/ recycled tyre crumb rubber biotoxicity assays which also used mzCloud databased in Compound Discoverer (National Toxicology Program, 2019).	100	100	53.3	53.3
PPG n7	$C_{21}H_{44}O_8$ 441.32973 *4.71[b]*	8.26 x10^8	6.40 x10^8	1.11 x10^9	1.08 x10^9	3.57 x10^8	-		88.8	90.0	46.6	46.6
PPG n9	$C_{27}H_{56}O_{10}$	1.81 x10^8	2.58 x10^8	4.66 x10^8	9.71 x10^8	2.37 x10^8	-		88.8	90.0	46.6	46.6

	557.41336 *5.47*[b]											
Analyte	**Formula** **Monoisotopic mass** ***Retention Time***	**Peak area (n=2)**						**Literature**	**Frequency (%) of detection in UK WWTPs**			
		WWI	*Canal*	*River*	*Dam*	*WWE*	*Spring*		*WWI Stk n=9*	*WWE Stk n=10*	*WWI SuB n=15*	*WWD SuB n=15*
PEG n13	$C_{26}H_{54}O_{14}$ 272.18444 *3.10*[b]	1.23 x10^9	4.21 x10^8	1.38 x10^9	3.70 x10^8	8.96 x10^7	4.89 x10^6	Not in literature.	11.1	0	0	0
PEG n15	$C_{30}H_{62}O_{16}$ 695.42967 *3.27*[b]	8.44 x10^8	2.20 x10^8	4.92 x10^8	1.28 x10^8	5.55 x10^7	5.60 x10^6	Tentatively identified in synthetic turf (National Toxicology Program, 2019).	22.2	0	6.7	0
Pentadecanoic acid	$C_{15}H_{30}O_2$ 242.22461 *5.94*[b]	8.49 x10^8	5.05 x10^8	1.22 x10^8	3.89 x10^8	1.09 x10^9	9.30 x10^7	Found in trace amounts in some EU-approved flavouring (European Commission, 2012).	11.1	0	6.7	0
Resorcinol monoacetate	$C_8H_8O_3$ 152.04754 *2.43*[a]	7.27 x10^7	1.77 x10^7	1.63 x10^7	2.20 x10^6	4.21 x10^6	-	Antibacterial agent used against acne (S. Kim, et al., 2019).	10.0	11.1	33.3	20.0
Tridecylic acid	$C_{13}H_{26}O_2$ 214.19331 *5.16*[b]	3.30 x10^9	1.08 x10^9	4.63 x10^7	1.13 x10^8	1.05 x10^8	1.82 x10^7	Plant metabolite (S. Kim, et al., 2019).	10.0	11.1	6.6	6.6
α-Eleostearic acid	$C_{18}H_{30}O_2$ 278.22436 *5.80*[b]	4.30 x10^8	1.13 x10^8	7.79 x10^7	7.65 x10^7	1.51 x10^9	5.54 x10^7	Essential fatty acid for mammals, found in seed oils such as tung and rapeseed (Cheng, et al., 2020)	33.3	20.0	86.6	66.6
δ-Valerolactam * *basic areas shown*	C_5H_9NO 99.06837 *0.83*[a]*, 1.49*[b]	2.95 x10^8	1.58 x10^8	-	-	-	-	Identified as a fish metabolite (Maha, et al., 2019)**.**	100	100	100	93.3

Table 7.3-2 Compounds which were also detected in the two UK sites, but which are not widely reported in the literature.

Note: Peak area does not equate to concentration and they are not comparable from one compound to another, but within the same compound a larger peak area very likely relates to a higher concentration. Superscripts a and b above the retention times refer to either the acidic (a) or basic (b) run. WWI: wastewater influent, WWE: wastewater effluent.

7.3.3 Agriculturally relevant pollutants

The Tula Valley is an agricultural zone and so it is expected that agriculturally related pollutants would be present in the aquatic environment. As shown in Table 7.3.3, two herbicides, one pesticide and one pesticide ingredient were identified. All of these pollutants, except diuron, were detected in spring water, as can be seen in Table 7.3.3. These four pollutants have not been previously detected in the Tula Valley. However, in another study of the area, pollutants used for the fabrication of pesticides, were detected in wastewaters; namely the chlorinated hydrocarbons chloroform and 1,2-dichloroethane and the phthalates bis-2-(ethylhexyl) phthalate, diethyl phthalate, dimethyl phthalate, and dibutyl phthalate (Lesser, et al., 2018). In a previous study of the area targeting different classes of micropollutants, two pesticides (2,4-dichlorophenoxyacetic acid and pentachlorophenol) were not found in groundwater or dam water, however 2,4-dichlorophenoxyacetic acid was found in tanks with mixed rain water and wastewater (Félix–Cañedo, et al., 2013).

Fomesafen has its highest peak area in the spring sample, as can be seen in Table 7.3.3, which is of concern as it indicates possible accumulation in groundwater. Both diuron and 2,6-di-tert-Butyl-4-methoxyphenol have high peak areas in the wastewater effluent, as can be seen in Table 7.3.3, this study was done for only one time point so the wastewater effluent cannot be directly related to the wastewater influent. However high peak areas in the effluent are of concern as they indicate possible poor removal of the pollutant which is then entering the environment. For all four agriculturally relevant pollutants detected (except for desethylatrazine), the concentration is higher in the irrigation canal than in the wastewater influent. This indicates these pollutants are likely being used in the area for agriculture rather than being brought in through the wastewater from Mexico City.

Analyte	Formula Monoisotopic mass *Retention Time*	Peak area (n=2)						Literature
		WWI	*Canal*	*River*	*Dam*	*WWE*	*Spring*	
2,6-di-tert-Butyl-4-methoxyphenol	$C_{15}H_{24}O_2$ 236.17768 *6.75*[b]	9.32 x10^7	1.13 x10^8	5.11 x10^7	1.16 x10^8	1.16 x10^8	1.10 x10^7	Inert ingredient in pesticide(S. Kim, et al., 2019). Not previously detected in wastewater.
Desethylatrazine	$C_6H_{10}ClN_5$ 187.06227 *3.51*[a]	2.05 x10^6	1.85 x10^6	6.76 x10^6	3.55 x10^6	1.80 x10^6	9.13 x10^7	Pesticide, found in Swedish artificial recharge plants <1-2 ngL^{-1} (Kuster, et al., 2010), found in European groundwaters as one of 19 emerging pollutants most frequently detected or with highest concentrations (Stuart, et al., 2012). In Latin American wastewater effluent it has been detected at 35 ngL^{-1} (Peña-Guzmán, et al., 2019).
Diuron	$C_9H_{10}Cl_2N_2O$ 232.0168 *5.21*[b]	7.24 x10^6	5.32 x10^7	6.41 x10^7	1.09 x10^8	1.04 x10^8	-	Herbicide identified as an environmental pollutant (S. Kim, et al., 2019). Found in Latin American surface waters at 123.5 ngL^{-1} and in surface waters 95.80-75,000 ngL^{-1} (Peña-Guzmán, et al., 2019).
Fomesafen	$C_{15}H_{10}ClF_3N_2O_6S$ 437.98958 *4.72*[b]	3.29 x10^5	1.20 x10^6	2.79 x10^6	-	4.07 x10^5	1.79 x10^7	Herbicide (S. Kim, et al., 2019) sold in Mexico (Syngenta Mexico, n.d.).

Table 7.3-3 Agriculturally relevant pollutants detected in the Tula Valley

WWI: wastewater influent, WWE: wastewater effluent. Note: Peak area does not equate to concentration and they are not comparable from one analyte to another, but within the same analyte a larger peak area very likely relates to a higher concentration. Superscript b on retention time means basic chromatography, superscript a means acidic chromatography.

7.3.4 Pollutants widely reported in the literature in samples with wastewater influence

Most of the pollutants in this section have known toxic or bioactive effects, as can be seen in Table 7.3.4, as this study is qualitative and not quantitative it is not possible to determine if the concentrations present an environmental risk. However, concentrations and environmental risk quotients determined elsewhere indicate they are likely of significance. These results indicate that the quantified environmental risk in section 0 is incomplete. Untargeted analysis had not been previously carried out in the Tula Valley and these results are valuable because they highlight the range of bioactive pollutants found in the aquatic environment and provide indications on which pollutants should be studied further. Four pollutants were detected in spring groundwater, namely laurolactam, methocarbamol, tributyl phosphate, PFOA and PFOS, as can be seen in Table 7.3.4. Their presence in groundwater highlights their prevalence and resistance to natural attenuation and indicates they merit further study. PFOS shows its highest peak area in groundwater, as can be seen in Table 7.3.4, which is of concern as it could indicate accumulation in groundwater. However, this would have to be explored further with long-term environmental monitoring and adequate quantification.

7.3.4.1 Not detected in UK Samples

Analyte	Formula Monoisotopic mass *Retention Time*	Peak area (n=2)						Literature
		WWI	*Canal*	*River*	*Dam*	*WWE*	*Spring*	
4'-Hydroxydiclofenac	$C_{14}H_{11}Cl_2NO_3$ 311.01172 *3.56*[b]	6.59 x10^7	6.20 x10^7	8.28 x10^6	2.04 x10^6	8.13 x10^7	-	Human phase I metabolite of diclofenac which degraded slowly but more than diclofenac in an activated sludge study. Diclofenac and its metabolites have been found to accumulate in aquatic organisms (Peake, et al., 2016b).
4-Acetamidoantipyrine	C_6 H_7 NO 245.11635 *2.36*[a]	3.38 x10^8	3.94 x10^8	2.33 x10^8	2.51 x10^8	5.25 x10^8	-	Metabolite of metamizole (S. Kim, et al., 2019), which is prohibited in the US but used in Mexico and other countries (Bonkowsky, et al., 2002). Previously measured in German wastewaters (Feldmann, et al., 2008).
4-Formylaminoantipyrine	$C_{12}H_{13}N_3O_2$ 231.1007 *2.35*	1.20 x10^8	1.36 x10^8	7.80 x10^7	7.61 x10^7	1.70 x10^8	-	Metabolite of aminophenazone. Previously detected in Hungary (Gyenge-Szabó, et al., 2014).
5-Methylbenzotriazole	$C_7H_7N_3$ 133.06388 *1.79*[b]	5.03 x10^6	5.35 x10^7	8.50 x10^6	1.10 x10^7	1.35 x10^8	-	Benzotriazole identified as an environmental contaminant (S. Kim, et al., 2019). Previously identified in USA urban estuary (0.4-27 ngL^{-1}), likely from wastewater sources or atmospheric deposition (Tian, et al., 2020).
5α-Androstan-3,6,17-trione	$C_{19}H_{26}$ O_3 302.18807 *3.767*[a]*,4.212*[b] *(acidic peak areas presented)*	5.05 x10^7	4.22 x10^7	1.96 x10^8	3.31 x10^7	2.90 x10^7	-	DEA Schedule III controlled substance (S. Kim, et al., 2019)
7-Methylxanthine	$C_6H_6N_4O_2$ 166.04932 *1.13*[b]	3.94 x10^7	1.44 x10^7	8.45 x10^6	-	2.04 x10^6	-	Caffeine metabolite, previously measured in ten European city's wastewater influent (Gracia-Lor, et al., 2017).

Analyte	Formula Monoisotopic mass *Retention Time*	Peak area (n=2)						Literature
		WWI	*Canal*	*River*	*Dam*	*WWE*	*Spring*	
Androsterone	$C_{19}H_{30}O_2$ 272.21735 *7.44*[b]	5.16 x10^6	1.51 x10^6	1.78 x10^6	1.90 x10^7	2.02 x10^6	2.48 x10^6	Steroid metabolite derived from sex hormones (S. Kim, et al., 2019). Widely reported in wastewater influent and effluent (Pomiès, et al., 2013). In Mexican wastewater influent in the State of Mexico (north of Mexico City it was found at 1,980 ngL^{-1} in the wet season and 3,020 ngL^{-1} in the dry season (Estrada-Arriaga, et al., 2016).
Atazanavir	$C_{38}H_{52}N_6O_7$ 704.3889 *6.15*[b]	5.64 x10^6	1.73 x10^7	7.42 x10^6	2.15 x10^7	4.13 x10^7	-	Antiretroviral. Detected in South African wastewater effluent (78- 740 ngL^{-1}) and influent (64-1,400 ngL^{-1}), incomplete removal observed, for three different WWTPs (Abafe, et al., 2018).
Benzoylecgonine	$C_{16}H_{19}NO_4$ 289.13123 *2.46*[a]*, 2.7*[b] *(acidic peak areas presented)*	1.62 x10^8	1.15 x10^8	6.48 x10^7	1.37 x10^8	4.60 x10^7	-	Metabolite of cocaine, previously measured in a UK WWTP at 1,247 ± 70.7 ngL^{-1} in wastewater influent and 389 ± 35.3 ngL^{-1} in effluent (Petrie, et al., 2016).
Carbofuran	$C_{12}H_{15}NO_3$ 221.10501 *4.82*[b]	8.83 x10^8	5.98 x10^8	2.51 x10^7	3.92 x10^7	1.53 x10^9	-	Found in Latin American drinking water in the range 8.9 – 50,000 ngL^{-1} (Peña-Guzmán, et al., 2019).
Carbendazim	$C_9H_9N_3O_2$ 191.06933 *1.80*[a]	6.15 x10^7	4.59 x10^7	2.12 x10^7	2.45 x10^7	3.30 x10^7	-	Fungicide, found in an agricultural soil in Spain (1.27 ngg^{-1}) and lettuce crops (2.24-19.9 ngg^{-1}) (Margenat, et al., 2018).
Ciprofloxacin	$C_{17}H_{18}FN_3O_3$ 331.13323 *2.15*[a]	5.66 x10^6	2.09 x10^7	2.27 x10^6	1.93 x10^6	1.24 x10^6	-	Has been widely detected in wastewater influent and effluent and in the aquatic environment, found with concentrations up to 6.5 mgL^{-1} (Petrie, et al., 2015). Was detected in a Brazilian study of hospital wastewater with

								environmental risk quotients above one (Wielens Becker, et al., 2020).
Analyte	**Formula** **Monoisotopic mass** ***Retention Time***	**Peak area (n=2)**						**Literature**
		WWI	*Canal*	*River*	*Dam*	*WWE*	*Spring*	
Dodecyl sulfate	$C_{12}H_{26}O_4S$ 266.15497 *5.25*[b]	7.35 x10^9	1.80 x10^9	2.13 x10^9	3.30 x10^7	-	-	Anionic surfactant commonly used in detergents, soaps, shampoos and shower gels (Nguyen, et al., 2016). Extensively detected in wastewater and aquatic environments, found to have negative effects such as depression, laboured breath, diarrhoea and carbon metabolism disruption in animals (Nguyen, et al., 2016).
Isoquinoline	C_9H_7N 129.05788 *1.22*[a]	1.66 x10^8	6.21 x10^7	3.84 x10^7	-	1.20 x10^7	-	Fragrance (Meyer, et al., 2019), EU approved flavouring substance (European Commission, 2012). Aromatic compound frequently found in coking wastewater (Ren, et al., 2019). Isoquinoline has been found to be mainly removed by aerobic secondary treatment (Ren, et al., 2019).
Laurolactam	$C_{12}H_{23}NO$ 197.17775 *5.28*[b]	-	-	-	6.62 x10^9	-	1.40 x10^{10}	Used in engineering plastics such as nylon-12 and copolyamides (Oenbrink, et al., 2009).
Lincomycin	$C_{18}H_{34}N_2O_6S$ 406.2134 *4.06*[b]	6.65 x10^7	5.37 x10^7	3.77 x10^7	4.08 x10^7	4.98 x10^7	-	Found in Latin American wastewater influent (33.5-1,450 ngL^{-1}), wastewater effluent (5.8-125 ngL^{-1}) in surface water detected at 1 ngL^{-1} (Peña-Guzmán, et al., 2019). In a nutrient-removal wastewater treatment plant it was found in wastewater influent at 204 ngL^{-1} and 1,450 ngL^{-1}, in the wet and dry season respectively, in the effluent in the dry season it was found at a maximum of 183 ngL^{-1}. Removal process at this WWTP, were grit

								removal, micro screen, oxidation ditch and clarifier and removal of this pollutant ranged from 57 % in the rainy season to 87 % in the dry season (Estrada-Arriaga, et al., 2016).
Analyte	**Formula** **Monoisotopic mass** ***Retention Time***	**Peak area (n=2)**						**Literature**
		WWI	*Canal*	*River*	*Dam*	*WWE*	*Spring*	
Methocarbamol	$C_{11}H_{15}NO_5$ 241.09477 *3.34*[b]	7.60 x10^7	1.08 x10^8	6.73 x10^7	6.49 x10^7	1.43 x10^8	7.60 x10^7	Muscle relaxant, confirmed detection with level 1 confidence in a German river (Beckers, et al., 2020). Quantified in wastewater effluent with concentration of 300 ngL^{-1} for sites with no pharmaceutical manufacturing facilities (PMF) discharge and with around 400 ngL^{-1} for sites with PMF discharge in the USA. In other locations with no PMF the concentration measured was in the range of 10-1,560 ngL^{-1} (Scott, et al., 2018). In a UK study of two rivers, this pollutant was found with 25 % detection frequency and was measured ~ 2ngL^{-1} above the worst case predicted environmental concentration (PEC) and an order of magnitude above the expected PEC range (Burns, et al., 2017).
Metoclopramide	$C_{14}H_{22}ClN_3O_2$ 299.13674 *3.72*[b]	4.07 x10^6	1.67 x10^7	9.84 x10^6	6.28 x10^7	2.61 x10^7	-	Dopamine antagonist, can be used to increase milk production in breast-feeding women(Hale, et al., 2017).Often used to treat gastroesophageal reflux disease, gastroparesis and nausea (S. Kim, et al., 2019). Identified in untargeted study in raw hospital wastewater in Brazil, with environmental risk quotient of 0.3 for green algae (Wielens Becker, et al., 2020). Found in greenhouse soils irrigated with reclaimed water

								at a maximum of 1.4 ngL^{-1} (Martínez-Piernas, et al., 2018a)**.**
Analyte	**Formula** **Monoisotopic mass** ***Retention Time***	**Peak area (n=2)**						**Literature**
		WWI	*Canal*	*River*	*Dam*	*WWE*	*Spring*	
Nalidixic acid	$C_{12}H_{12}N_2O_3$ 232.08468 *2.70*[b]	1.34 x10^8	1.79 x10^8	9.09 x10^7	5.91 x10^7	2.19 x10^8	-	Quinolone, maximum residue levels measured in fish in Brazil was 20 μgkg^{-1} (Rocha Guidi, et al., 2018). In a UK river catchment receiving treated wastewater it was found as an ubiquitous pollutant with wastewater influent loadings of: 0.8-4.9 $gday^{-1}$, removal efficiency was WWTP dependent and ranged between 38 and 82% with better removal in activated sludge compared to trickling filter with wastewater effluent loads of 1.8-3.1 $gday^{-1}$ (Castrignanò, et al., 2020).
Ofloxacin	$C_{18}H_{20}FN_3O_4$ 361.14371 *2.53*[a]	3.80 x10^7	1.44 x10^6	1.17 x10^6	2.55 x10^6	-	-	Antibiotic, previously measured in Latin American surface water at an average of 335 ngL^{-1} and in wastewater effluent at 231.63 ngL^{-1} (Peña-Guzmán, et al., 2019).
Pentoxifylline	$C_{13}H_{18}N_4O_3$ 278.13774 *3.36*[b]	3.40 x10^7	6.34 x10^7	3.51 x10^7	7.00 x10^7	9.44 x10^7	-	Vasodilating drug, tested for but not found in a 10 consecutive day sampling campaign in a WWTP in Germany during the winter (Robert Gurke, et al., 2015a). Predicted environmental concentration in wastewater effluent ranges between 5.1-6.9 μgL^{-1}, and in river between 0.20-0.27 μgL^{-1} in Spain (Gómez-Canela, et al., 2019).
Perfluoro-1-octanesulfonic acid (PFOS)	$C_8HF_{17}O_3S$ 499.93722 *5.67*[b]	8.57 x10^6	6.91 x10^6	7.75 x10^6	9.88 x10^6	3.53 x10^6	1.71 x10^8	Perfluorinated compound, toxic, used in stain repellents and fire-fighting foams commonly detected in the aquatic environment (Wilkinson, et al., 2017).

Analyte	Formula Monoisotopic mass ***Retention Time***	Peak area (n=2)						Literature
		WWI	*Canal*	*River*	*Dam*	*WWE*	*Spring*	
Perfluorooctanoic acid (PFOA)	$C_8HF_{15}O_2$ 413.97331 *4.94*[b]	5.32 x10^5	1.02 x10^6	6.29 x10^6	1.09 x10^7	1.07 x10^7	3.85 x10^7	Perfluorinated compound, toxic, used in Teflon commonly detected in the aquatic environment (Wilkinson, et al., 2017)**.**
Theobromine	$C_7H_8N_4O_2$ 180.06461 *1.86*[a]	1.55 x10^8	6.54 x10^7	7.38 x10^7	5.56 x10^6	2.07 x10^7	-	Identified as an emerging pollutant with high concentrations in WWTP effluents (Egea-Corbacho, et al., 2019). Found in chocolate and is a caffeine metabolite, found in arctic circle water samples (Choi, et al., 2020).
Tributyl phosphate	$C_{12}H_{27}O_4P$ 266.16457 *5.47*[a]	4.69 x10^8	4.09 x10^8	6.41 x10^8	8.04 x10^8	1.04 x10^9	3.78 x10^7	Identified as an emerging pollutant and in arctic circle water samples (Choi, et al., 2020).

Table 7.3-4 Pollutants which have been reported in the literature and detected in the Tula Valley and were not found in the UK samples

WWI: wastewater influent, WWE: wastewater effluent. Note: Peak area does not equate to concentration and they are not comparable from one analyte to another, but within the same analyte a larger peak area very likely relates to a higher concentration. Superscript b on retention time means basic chromatography, superscript a means acidic chromatography

7.3.4.2 Also detected in UK samples: pollutants with wastewater-based epidemiology relevance

As described in section 6.2.3, wastewater-based epidemiology is a powerful technique which can reveal what a population has actually consumed, including over-the-counter drugs and illicit drugs (Archer, et al., 2018). Biomarkers are pollutants necessary for adequate quantification of the per capita consumption of substances such as illegal drugs. As can be seen in Eq. 26, biomarkers must be quantifiable, stable in wastewater, constantly excreted and total excretion should correlate to census population, so humans must be the only source of the biomarker in wastewater (Chen, et al., 2014). It is significant that four pollutants identified as potential biomarkers (caffeine, paraxanthine, nicotine and cotinine), as can be seen in Table 7.3.5 and Table 6.2.6, were found both in Mexico and UK samples as it means they are very prevalent in two different contexts and can be used for wastewater-based epidemiology in the future. Of particular interest is nicotine as it is the only one detected in spring water, as can be seen in Table 7.3.5, indicating it is very persistent

Analyte	Formula Monoisotopic mass *RT*	Peak area concentration (n=2)						Literature	Frequency (%) of detection in UK WWTPs			
		WWI	*Canal*	*River*	*Dam*	*WWE*	*Spring*		*WWI Stk n=9*	*WWE Stk n=10*	*WWI SuB n=15*	*WWD SuB n=15*
ADBICA	$C_{20}H_{29}N_3O_2$ 343.22049 *2.28*[a]	5.68 x10^8	2.45 x10^8	6.26 x10^8	3.69 x10^8	7.92 x10^7	2.12 x10^6	Designer drug (Banister, et al., 2015).	2.2	0.0	53.3	53.3
Caffeine	$C_8H_{10}N_4O_2$ 194.08032 *2.33*[a], *2.23*[b] *(Acidic peak areas presented)*	8.66 x10^8	4.84 x10^8	4.29 x10^8	5.56 x10^7	2.57 x10^8	-	Naturally occurring stimulant, found in coffee beans, tea leaves, kola nuts, cocao beans, yerbamate and guarana berries. Important marker for wastewater pollution (Archer, et al., 2018; Castiglioni, et al., 2018; Gorga, et al., 2013). Previously detected in the Tula Valley irrigation canals (115-42,000 ngL^{-1}), but not in groundwater, likely due to its high affinity for organic matter in soil and high biodegradability (Lesser, et al., 2018).	100	10.0	100	60.0
Cocaine	$C_{17}H_{21}NO_4$ 303.14675 *5.29*[b]	1.19 x10^8	1.13 x10^8	6.09 x10^7	6.37 x10^6	5.61 x10^7	5.03 x10^6	Illicit drug, measured in Latin American wastewater influent in the 9.54- 2,710 ngL^{-1} range, in wastewater effluent in the range 0.29-62 ngL^{-1}, in surface wate at 6-537 ngL^{-1}, in drinking waters at 6-22 ngL^{-1} and in groundwater at 1.87 ngL^{-1} (Peña-Guzmán, et al., 2019). Maximum concentration measured in Mexico of 271 ngL^{-1} (Peña-Guzmán, et al., 2019).	22.2	10.0	6.7	6.7

Analyte	Formula Monoisotopic mass *RT*	Peak area concentration (n=2)						Literature	Frequency (%) of detection in UK WWTPs			
		WWI	*Canal*	*River*	*Dam*	*WWE*	*Spring*		*WWI Stk n=9*	*WWE Stk n=10*	*WWI SuB n=15*	*WWD SuB n=15*
Cotinine	$C_{10}H_{12}N_2O$ 144.06543 *0.79, 2.26[b](basic areas shown)*	1.96 x10^8	9.98 x10^7	1.25 x10^8	3.45 x10^7	6.99 x10^7	-	Main nicotine metabolite (Chen, et al., 2014). Widely detected in wastewaters, human-use marker (Bisceglia, et al., 2010). Previously measured in the Tula Valley irrigation canals (27.4-1,670 ngL^{-1}), but not found in groundwater (Lesser, et al., 2018), in agreement with this study.	100	100	100	100
Nicotine	$C_{10}H_{14}N_2$ 162.11553 3.52[b]	3.18 x10^8	1.52 x10^8	2.19 x10^8	1.19 x10^7	1.28 x10^7	3.62 x10^6	Widely detected in wastewaters, human-use marker (Bisceglia, et al., 2010). Detected in Uruguay raw wastewater (79 ngL^{-1}) and Latin American wastewater efflunets (20ngL^{-1})(Peña-Guzmán, et al., 2019)**.**	22.2	10.0	53.3	53.3
Paraxanthine	$C_7H_8N_4O_2$ 180.06466 2.05	2.35 x10^8	1.32 x10^8	9.64 x10^7	7.96 x10^6	5.17 x10^7	-	Caffeine metabolite, found at higher mass loadings than caffeine in WWI (Gracia-Lor, et al., 2017). Previously detected in the Tula Valley irrigation canals, but not in groundwater likely due to its high affinity for organic matter in soil and biodegradability (Lesser, et al., 2018), in agreement with this study.	100	70.0	100	86.6

Table 7.3-5 Pollutants with wastewater-based epidemiology relevance detected in the Tula Valley, and also in the UK samples.

WWI: wastewater influent, WWE: wastewater effluent. Note: Peak area does not equate to concentration and they are not comparable from one analyte to another, but within the same analyte a larger peak area very likely relates to a higher concentration. Superscript b on retention time means basic chromatography, superscript a means acidic chromatography.

1.1.1.1 Also detected in UK samples: metabolites/transformation products of targeted analytes

The parent compound carbamazepine was not found with environmental risk quotients above one in any of the sampling points studied in either country using the targeted method, as can be seen in Figure 5.3.1 and Figure 7.2.3. However, 70-75% of the ingested dose of carbamazepine is excreted as 10,11-epoxy carbamazepine which is therapeutically active (Paz, et al., 2016). This compound was identified in the Tula Valley through untargeted analysis and it reveals unaccounted for toxicity of carbamazepine when only the parent compound is monitored. Neither of the carbamazepine metabolites have been previously measured in the Tula Valley. Compared to the number of metabolites and transformation products found for the targeted analytes in the UK samples, very few were found in the samples from the Tula Valley. Further untargeted sampling campaigns are likely needed to find more and unveil further information on the fate and transformations of bioactive pollutants in the agricultural environment.

Analyte	Formula Monoisotopic mass *RT*	Peak area (n=2)						Literature	Frequency (%) of detection in UK WWTPs			
		WWI	*Canal*	*River*	*Dam*	*WWE*	*Spring*		*WWI Stk n=9*	*WWE Stk n=10*	*WWI SuB n=15*	*WWD SuB n=15*
10,11-Dihydro-10,11-dihydroxycarbamazepine	$C_{15}H_{14}N_2O_3$ 270.1002 *2.94[a], 3.28[b] (Acidic peak areas presented)*	9.57 x10^6	3.08 x10^7	1.95 x10^7	2.47 x10^7	3.96 x10^7	1.66 x10^6	Carbamazepine metabolite previously detected in wastewater influent and wastewater effluent 47.2 ngL^{-1} (Miao, et al., 2003).	22.2	20.0	6.66	6.66
Carbamazepine 10,11-epoxide	$C_{15}H_{12}N_2O_2$ 252.08957 *2.94, 3.28[b] (Acidic peak areas presented)*	7.49 x10^6	2.81 x10^7	2.14 x10^7	2.74 x10^7	4.03 x10^7	1.42 x10^6	Previously detected in a constructed wetland plants treating secondary (trickling filter) effluent in the UK (Petrie, et al., 2017).	22.2	20.0	53.3	53.3

Table 7.3-6 Metabolites or transformation products of the targeted analytes detected in the Tula Valley, and also in the UK samples,

WWI: wastewater influent, WWE: wastewater effluent. Note: Peak area does not equate to concentration and they are not comparable from one compound to another, but within the same compound a larger peak area very likely relates to a higher concentration. Superscript b on retention time means basic chromatography, superscript a means acidic chromatography

7.3.4.3 Also detected in UK samples: widely reported BACs

The pollutants reported in Table 7.3-7 have been widely characterised in the environment and often have known bioactivity and toxicity in the environment. Despite their prevalence in the environment and in the literature many of them have not been previously measured or detected in the Tula Valley. Nine of these pollutants were detected in spring ground water, namely: 2-Hydroxybenzothiazole, 4-Methylbenzotriazole, 4-Nitrophenol, fumaric acid, galaxolidone, benzoic acid, caprolactam, methyl salicylate, nefopam and PPG n4. As is detailed in the Table 7.3.7, these pollutants are known to be persistent and toxic, therefore their presence in a drinking water source merits further attention to determine if they are at levels which could cause harm to the environment and humans using this water as a drinking water source, at the concentrations at which they are found.

Analyte	Formula Monoisotopic mass *RT*	Peak area does not equate to concentration (n=2)						Literature	Frequency (%) of detection in UK WWTPs			
		WWI	*Canal*	*River*	*Dam*	*WWE*	*Spring*		*WWI Stk n=9*	*WWE Stk n=10*	*WWI SuB n=15*	*WWD SuB n=15*
2-Hydroxybenzothiazole	C_7H_5NOS 151.00926 *4.00*[a]	1.49 x10^7	6.89 x10^6	4.59 x10^6	3.85 x10^8	1.50 x10^6	3.45 x10^6	Benzotriazole micropollutant detected previously in wastewaters (Scott, et al., 2018). Not previously detected in the Tula Valley.	11.1	10.0	13.3	20.0
4-Methylbenzotriazole	$C_7H_7N_3$ 133.06394 *3.53*[b]	9.40 x10^7	1.94 x10^8	1.12 x10^8	1.82 x10^9	1.81 x10^8	9.18 x10^7	Methylbenzotriazoles combined found at a maximum concentration of 290 ngL $^{-1}$ in river water (Loos, et al., 2017). Not previously detected in the Tula Valley.	22.2	20.0	53.3	53.3
4-Nitrophenol	$C_6H_5NO_3$ 139.02709 *2.35*[b]	6.07 x10^6	4.98 x10^6	6.08 x10^6	3.69 x10^7	8.27 x10^6	6.07 x10^6	Previously measured in surface waters between 1.6-18 ngL^{-1} in urban estuary in the United States (Tian, et al., 2020). The limit set by the United States Environmental Agency in natural water is 10 ngL^{-1} due to its high toxicity (Huang, et al., 2018). Not previously detected in the Tula Valley.	22.2	20.0	53.3	20.0
6-Methylquinoline	$C_{10}H_9N$ 143.07338 *4.66*[b]	3.95 x10^7	8.16 x10^7	3.48 x10^7	3.07 x10^7	9.72 x10^7	-	Quinolone antibiotic (S. Kim, et al., 2019), found to have estrogenic activity (Brinkmann, et al., 2014). Not previously detected in the Tula Valley.	88.8	40.0	100	40.0

Analyte	Formula Monoisotopic mass *RT*	Peak area does not equate to concentration (n=2)						Literature	Frequency (%) of detection in UK WWTPs			
		WWI	*Canal*	*River*	*Dam*	*WWE*	*Spring*		*WWI Stk n=9*	*WWE Stk n=10*	*WWI SuB n=15*	*WWD SuB n=15*
Benzoic acid	$C_7H_6O_2$ 122.03705 *2.66[a], 1.21[b] (Basic peak areas presented)*	8.13 x10^7	4.26 x10^7	9.20 x10^6	3.86 x10^7	1.52 x10^8	3.21 x10^6	Fungistatic, widely used as a food preservative (S. Kim, et al., 2019). Has been previously measured in surface waters in the Tula Valley at concentrations of up to 286 µgL^{-1} (Downs, et al., 2000). Found as atmospheric smoke particles, it dissolves in rain and reaches surface waters and is a tracer for biomass burning (Santos, et al., 2019). In water it participates in photochemical reactions and can affect the climate and atmospheric reactions. Previously identified in sewage sludge samples (Black, et al., 2019).	100	100	100	100
Benzotriazole	$C_6H_5N_3$ 119.0484 *2.54[b]*	2.98 x10^7	2.34 x10^7	1.85 x10^7	8.24 x10^7	1.98 x10^7	-	Corrosion inhibitor used in engine coolants, antifreeze liquids, dishwashing detergents, silver protection and more (Rhodes-Dicker, et al., 2019). Has been found in tap water samples at concentrations up to 227 ngL^{-1} and in groundwater at concentrations up to 1,032 ngL^{-1}. Its toxicity has been determined as having anti-estrogenic effects and is suspected as a human	22.2	20.0	53.3	53.3

								carcinogen (Rhodes-Dicker, et al., 2019). Not previously detected in the Tula Valley.				
Analyte	**Formula** **Monoisotopic mass** ***RT***	**Peak area does not equate to concentration (n=2)**						**Literature**	**Frequency (%) of detection in UK WWTPs**			
		WWI	*Canal*	*River*	*Dam*	*WWE*	*Spring*		*WWI Stk n=9*	*WWE Stk n=10*	*WWI SuB n=15*	*WWD SuB n=15*
Bisphenol A	$C_{15}H_{16}O_2$ 228.11502 *5.05*[b]	7.24 x10^7	6.49 x10^7	8.20 x10^7	6.57 x10^7	5.00 x10^7	-	Widely measure in wastewaters, found , in Mexico City wastewater influent found in the range of 432-3,177 ngL^{-1}, this was reported to be within the range found in other countries (Gibson, et al., 2010). Has been detected in groundwater in the Tula Valley, but evidence shows it is not likely to be found in crops (Chávez-Mejía, et al., 2019).	11.1	10.0	53.3	53.3
Butylparaben	$C_{11}H_{14}O_3$ 194.09762 *3.77*[b]	1.04 x10^8	4.83 x10^7	1.56 x10^8	8.73 x10^6	1.51 x10^6	-	Preservative and flavouring agent (S. Kim, et al., 2019). In UK wastewater influent has been found in the range of 50-723 ngL^{-1}, and in wastewater effluents at <1 ngL^{-1} (Petrie, et al., 2015). In Latin American surface waters it has been reported in the range 13-55 ngL^{-1} (Peña-Guzmán, et al., 2019). Not previously detected in the Tula Valley.	22.2	0	-	-

Analyte	Formula Monoisotopic mass *RT*	Peak area does not equate to concentration (n=2)						Literature	Frequency (%) of detection in UK WWTPs			
		WWI	*Canal*	*River*	*Dam*	*WWE*	*Spring*		*WWI Stk n=9*	*WWE Stk n=10*	*WWI SuB n=15*	*WWD SuB n=15*
Caprolactam	$C_6H_{11}NO$ 113.08405 *2.08[a], 1.93[b](acidic areas shown)*	2.82 x10^9	1.96 x10^9	3.48 x10^9	8.16 x10^9	1.30 x10^9	1.31 x10^9	Mainly used to manufacture nylon 6 filament, fibre and plastic. Also used in textile stiffeners, film coatings, synthetic leather, paint vehicles and more (S. Kim, et al., 2019). Global demand is 5 million tons per year and it has a high COD and toxicity (Wang, et al., 2007). Has caused widespread environmental pollution (Rajoo, et al., 2013). Not previously detected in the Tula Valley.	100	100	100	100
Catechol	$C_6H_6O_2$ 110.03700 *2.22[a]*	4.78 x10^7	1.24 x10^7	-	-	-	-	Vital industrial reagent used in petroleum, chemicals, rubber, medicine and dyes. Also an important metabolite in aromatic pollutant biodegradation. It is water-soluble and widespread in environmental soil and water. It is biotoxic and can cause lympho-hematopoietic cancer and DNA replication inhibition (Zhuang Liu, et al., 2019). Not previously detected in the Tula Valley.	22.2	10.0	53.3	0.0
Climbazole	$C_{15}H_{17}ClN_2O_2$ 292.09746 *6.03[b]*	5.10 x10^7	8.68 x10^7	5.34 x10^7	1.68 x10^8	2.53 x10^8	-	Active ingredient in antidandruff shampoo and has been widely detected in wastewaters (Brienza, et al., 2017). Not previously detected in the Tula Valley.	11.1	10.0	6.7	6.7

Analyte	Formula Monoisotopic mass *RT*	Peak area does not equate to concentration (n=2)						Literature	Frequency (%) of detection in UK WWTPs			
		WWI	*Canal*	*River*	*Dam*	*WWE*	*Spring*		*WWI Stk n=9*	*WWE Stk n=10*	*WWI SuB n=15*	*WWD SuB n=15*
Dextrorphan	$C_{17}H_{23}NO$ 257.17783 *3.87*[b]	1.45 x10^7	7.06 x10^7	4.94 x10^7	8.65 x10^7	7.49 x10^7	-	Metabolite of the opioid dextromethorphan. One of the most frequently detected opioids in surface water and wastewater in Minnesota (Campos-Manas, et al., 2017). Has been previously measured in activated sludge (Richardson, et al., 2018). Not previously detected in the Tula Valley.	11.1	10.0	-	-
Fumaric acid	$C_4H_4O_4$ 116.01124 *0.94*[b]	5.68 x10^6	7.44 x10^6	6.10 x10^6	9.97 x10^6	1.20 x10^7	2.12 x10^7	EU approved food additive (European Commission, 2012). Identified as a pollutant in industrial and agrochemical production plant (Yabalak, et al., 2020). Not previously detected in the Tula Valley.	11.1	10.0	6.7	6.7
Galaxolidone	$C_{18}H_{24}O_2$ 272.17736 *5.84*[a]	4.40 x10^8	5.32 x10^8	3.27 x10^8	9.47 x10^8	6.68 x10^8	1.51 x10^7	Synthetic fragrance, often found as pollutant in environments with wastewater influence (Vallecillos, et al., 2017). Not previously detected in the Tula Valley.	33.3	100	40.0	53.3
Losartan	$C_{22}H_{23}ClN_6O$ 422.16181 *4.07*[b]	1.77 x10^8	2.26 x10^8	1.51 x10^8	1.67 x10^8	3.19 x10^8	-	G-protein receptor inhibitor, previously detected in the aquatic environment (Ihara, et al., 2015). In Latin American wastewater influent it has been measured in the range 580-4,540 ngL^{-1}, in wastewater effluent: 1,000-1,970	11.1	10.0	6.7	6.7

								ngL^{-1} and in surface water 32 ngL^{-1} (Peña-Guzmán, et al., 2019). Not previously detected in the Tula Valley.				
Analyte	**Formula** **Monoisotopic mass** ***RT***	**Peak area does not equate to concentration (n=2)**						**Literature**	**Frequency (%) of detection in UK WWTPs**			
		WWI	*Canal*	*River*	*Dam*	*WWE*	*Spring*		*WWI Stk n=9*	*WWE Stk n=10*	*WWI SuB n=15*	*WWD SuB n=15*
Lidocaine	$C_{14}H_{22}N_2O$ 234.17311 *1.91*[a]	5.13 x10^7	1.16 x10^8	5.38 x10^7	2.77 x10^7	6.78 x10^7	-	Anaesthetic, found at an average of 107 ngL^{-1} in effluent samples of a WWTP from households and hospitals (Rúa-Gómez, et al., 2012). Not previously detected in the Tula Valley.	100	100	100	100
Methyl salicylate	$C_8H_8O_3$ 152.04747 *3.77*[b]	4.60 x10^7	-	4.64 x10^6	4.91 x10^7	1.98 x10^6	3.72 x10^6	Rubefacient, naturally produced by many plants. However, it is also commercially produced as a rubefacient, anti-inflammatory and analgesic. Classified as an emerging contaminant (Real, et al., 2012). Not previously detected in the Tula Valley.	100	0	100	53.3
Nefopam	$C_{17}H_{19}NO$ 253.14644 *5.25*[a]*, 6.47*[b] *(Acidic peak areas reported)*	2.56 x10^7	3.81 x10^7	4.32 x10^7	3.13 x10^7	3.83 x10^7	5.33 x10^7	Non-opioid analgesic drug (Aymard, et al., 2003) Part of the Contaminant Candidate List 4, a list from the Environmental Protection Agencey of pollutants known or anticipated to exist in drinking water (Richardson, et al., 2018). Not previously detected in the Tula Valley.	10.0	11.1	-	-

Analyte	Formula Monoisotopic mass *RT*	Peak area does not equate to concentration (n=2)						Literature	Frequency (%) of detection in UK WWTPs			
		WWI	*Canal*	*River*	*Dam*	*WWE*	*Spring*		*WWI Stk n=9*	*WWE Stk n=10*	*WWI SuB n=15*	*WWD SuB n=15*
Palmitoylethanolamide	$C_{18}H_{37}NO_2$ 299.28221 *7.94*[b]	3.37 x10^8	7.64 x10^6	3.56 x10^6	2.65 x10^6	9.27 x10^6	-	Used to prevent viral infections, available for human use as supplements (Wishart, et al., 2018). Previously identified in sewage sludge (Black, et al., 2019). Not previously detected in the Tula Valley.	10.0	0	-	-
PPG n4	$C_{12}H_{26}O_5$ 250.17782 *3.26*[b]	2.44 x10^8	3.84 x10^8	5.61 x10^8	6.06 x10^8	3.14 x10^8	3.54 x10^7	Previously detected in Swedish lake with a degradation half-life of <100 days (Zhe Li, et al., 2020). Not previously detected in the Tula Valley.	11.1	10.0	6.7	6.7
Tetramethylpyrazine	$C_8H_{12}N_2$ 136.10005 *0.70*[a]	2.08 x10^7	3.07 x10^7	2.19 x10^7	1.27 x10^7	4.84 x10^7	-	Widely detected in water matrices and related to agricultural and industrial activities (Guo, et al., 2020). Not previously detected in the Tula Valley.	20.0	22.2	6.7	6.7
Testosterone sulfate	$C_{19}H_{28}O_5S$ 368.16546 *4.27*[b]	1.36 x10^8	4.58 x10^7	4.90 x10^7	-	3.33 x10^5	-	Human urinary metabolite (Forsdahl, et al., 2020). Has been widely detected in the aquatic environment (Zhao, et al., 2019). Not previously detected in the Tula Valley.	22.2	20.0	53.3	40.0
Venlafaxine	$C_{17}H_{27}NO_2$ 277.2041 *2.31*[a], *4.86*[b]	-	4.38 x10^7	3.99 x10^6	9.71 x10^6	3.82 x10^7	-	Frequently detected antidepressant (Rúa-Gómez, et al., 2012) found at in a range of 20- 400,000 ngL^{-1} in wastewater influent and 30-56 ngL^{-1} in	20.0	22.2	53.3	53.3

								wastewater effluent in Latin America (Peña-Guzmán, et al., 2019). Not previously detected in the Tula Valley.				
Analyte	**Formula** **Monoisotopic mass** ***RT***	**Peak area does not equate to concentration (n=2)**						**Literature**	**Frequency (%) of detection in UK WWTPs**			
		WWI	*Canal*	*River*	*Dam*	*WWE*	*Spring*		*WWI Stk n=9*	*WWE Stk n=10*	*WWI SuB n=15*	*WWD SuB n=15*
Xylenesulfonate	$C_8H_{10}O_3S$ 186.0352 *2.32*[b]	4.58 x10^8	4.02 x10^8	4.48 x10^8	1.60 x10^8	1.91 x10^8	-	Normally in the form sodium xylenesulfonate, has been tested for toxicity in rodents and it was found that only some isomers were toxic and so the mixture might not reach toxic level (Author Y. P. Zhang, et al., 2020) . Not previously detected in the Tula Valley.	77.7	50.0	53.3	53.3

Table 7.3-7 Pollutants widely reported in the literature and detected in the Tula Valley, and also in the UK samples

WWI: wastewater influent, WWE: wastewater effluent. Note: Peak area does not equate to concentration and they are not comparable from one analyte to another, but within the same analyte a larger peak area very likely relates to a higher concentration. Superscript b on retention time means basic chromatography, superscript a means acidic chromatograph

7.4 Summary

The work undertaken for this chapter contributes to the knowledge of bioactive pollution in the Tula Valley in three broad categories. Firstly, in terms of pollutants not previously measured or detected in this area. From the targeted analysis, a particularly important contribution was the quantification of seven pollutants not previously quantified, of which 4-tert-octylphenol, levonorgestrel, simvastatin and tramadol were found to have environmental risk quotients above one. Total estradiol equivalent estrogenicity was found to have an environmental risk quotient above one, with influence form estriol and its sulfate conjugate which have not been previously measured in the Tula Valley. Similarly, the untargeted results contribute in terms of pollutants never detected in the Tula Valley. A total of eighteen pollutants which have not been previously detected in the aquatic environment and which were not found in the two UK sites studied were detected. For example, 1-Adamantanamine, a pollutant used in textiles and some food packaging products and with estrogenic effects and 10-Hydroxycarbazepine, active metabolite of oxcarbazepine were found at all sampling points except for the spring. A total of 31 compounds not previously detected in the aquatic environment but found in the UK sites studied for this thesis were detected in the Tula Valley samples. Some examples include 1-Adamantanamine, which is used to treat the influenza virus and boldenone, which is banned in the EU and is used as a growth promoter for meat-growing animals. Both were detected in all sampling sites, except for the spring. More than 20 pollutants known for their toxicity and bioactivity, but not previously detected in the Tula Valley were identified using untargeted analysis. For example, the benzotriazole 5-methylbenzotriazole, cocaine metabolite benzoylecgonine and the antibiotic ciprofloxacin.

Secondly in terms of identifying persistent pollutants, as they were measured or detected in spring groundwater.. Five pollutants were found with measurable concentrations in spring groundwater (bezafibrate, carbamazepine, oxytetracycline, sulfamethoxazole and levonorgestrel). Of these five pollutants, sulfamethoxazole and levonorgestrel were found at a concentration with an environmental risk quotient above one in this matrix. From the untargeted analysis pollutants of particular concern identified were pesticide desethylatrazine and herbicide fomesafen, as the highest peak area was found in the spring sample for both pollutants. Other pollutants of concern, due to the peak area being similar or higher than at the other sampling points were: androsterone, laurolactam, methocarbamol, PFOS, PFOA, tributyl phosphate, 4-Methylbenzotriazole, 2-Hydroxybenzothiazole, 4-Nitrophenol, benzoic acid, caprolactam, fumaric acid and nefopam.

Thirdly, in terms of comparable results from those obtained in the UK sampling campaign, pointing out pollutants of international environmental importance. Despite the differences between the Tula Valley and the two UK sites studied, four pollutants were found to have environmental risk quotients above one in both sites, namely diclofenac, erythromycin, trimethoprim and tramadol. From the untargeted analysis potential biomarkers, which were also found in the UK samples were detected, namely: caffeine, paraxanthine, nicotine and cotinine. This is important in terms of internationally relevant biomarkers.

There is a risk to the ecosystem, with concentrations found in aquatic environments found to be higher than the PNECs for aquatic life (section 7.2) and this could potentially be worse as evidenced by the pollutants identified using the untargeted approach. However, the PNECs used are for European species (as they could not be found for species particular to Mexico) and so some uncertainty remains. I believe in the Tula Valley the benefits of wastewater reuse currently outweigh risks to the environment and to human health. However, I do strongly believe this situation must

continue to be monitored and studied because many unknowns remain (mixture effects, antimicrobial resistance, etc). However, these potential negative effects need to be constantly balanced out against the benefits of wastewater reuse and the cost of additional treatment. Currently the benefits outweigh known and unknown negative effects, but the situation should continue to be monitored. In terms of future work, a study with distinct date samples ($n \geq 5$) for each location in order to undertake partial least squares (PLS) analysis, as the one done in Chapter 6, would be very valuable. This would help to further pinpoint specific bioactive pollutants for further study in terms of their removal at the Atotonilco WWTP and their fate throughout the valley. This could be followed with targeted work to quantify the environmental risk of the pollutants identified.

8 RESULTS AND DISCUSSION; MODELLING OF SORPTION OF BIOACTIVE POLLUTANTS IN SOIL

8.1 Highlights

- **First model work of BAC sorption in soil using data from across different papers** to identify literature gaps and unveil experimental biases.
- Literature gaps identified which require further study:
  - More **data is still needed for understudied pollutants such as tramadol** and for pollutants which present inconsistencies across the literature like sulfamethoxazole.
  - Soil parameters, such as pH should be reported using standard methods to **ensure data is comparable across literature.**
  - Sorption studies are needed with **environmentally relevant concentrations.**
  - The **biodegradation quenching method was found to influence sorption**, with NaN_3 having a greater influence than autoclaving. This needs to be further studied.
  - Sorption **studies at different temperatures** are needed to account for variations which will occur seasonally and geographically.
  - Sorption studies using **treated and untreated liquid phase** (realistic irrigation water) are needed.
  - **Untargeted analysis** of soil and liquid phases in sorption studies could elucidate competitive sorption effects as well as transformation process which may account for discrepancies in the literature.

8.2 Pollutants of interest identified

So far in this thesis, accurate and reliable methods have been developed to measure BACs in soil and water, as detailed in Chapter 4. Then in Chapters 5 to 7 literature gaps in relation to waters which will influence irrigation water or water which will or could be used directly for irrigation were addressed. In this chapter the aim is to use the information obtained from Chapters 5-7 and the literature to address knowledge gaps in relation to the behaviour of BACs once they enter the soil compartment from irrigation water. The aim is not to develop mechanistic models, as these have been done in the literature, but rather to identify experimental biases which may be hindering the environmental applicability of lab-scale sorption studies. The parameters, and the ranges, currently studied are analysed and recommendations are made for future sorption experiments in order to obtain more environmentally relevant data. This is of high importance because sorption behaviour of pollutants will determine their bioavailability for plant uptake or their likelihood to leach to groundwater (as discussed in Section 2.3.2). Sorption parameters are of great importance in modelling efforts to understand the fate and prevalence of BACs in the agricultural enviornment and therefore the suitability of different water sources (raw wastewater/treated wastewater/ diluted treated wastewater) for use in agricultural irrigation.

The approach taken was to collect and model data available in the literature on BAC sorption to soil. Firstly, to determine the parameters which affect soil sorption and the ranges in which they are studied in the literature. Secondly to build models to find trends across different papers and gaps which need addressing. In order to make the task manageable, three pollutants were selected to focus on. The criteria to select these pollutants had three aspects. The first was that the pollutants should be of environmental relevance for both Mexico and the UK. The second that there should be enough data in the literature to make some models. The third was that the pollutants should have limits of detection low enough to measure environmentally relevant

concentrations in both LC-MS instruments available (QqQ and Qtrap) and that they could be analysed in the same chromatographic mode to save time and resources in terms of analysis. No three pollutants met all the criteria, but a satisfactory compromise was found.

In terms of environmental relevance for both countries, three criteria were taken into account, the:

i. detection in all locations studied,
ii. the frequency of detection, and
iii. environmental risk quotients.

Pollutants detected in all sampling points studied were: bezafibrate, carbamazepine, diclofenac, oxytetracycline, salicylic acid, sulfamethoxazole and tramadol. Of these, those quantified in acidic chromatography are carbamazepine, sulfamethoxazole and tramadol. The rest are quantified in basic chromatography. The analytes quantified in acidic chromatography have lower limits of detection and can be measured using either instrument with satisfactory results. Therefore carbamazepine, sulfamethoxazole and tramadol were selected. In both countries both sulfamethoxazole and tramadol have RQs > 1. Carbamazepine and sulfamethoxazole were both found with >90% frequency in both WWI and WWE/WWD of both UK WWTPs studied. Tramadol was found with 100% frequency in the WWE/WWD of both UK WWTPs studied. In this thesis carbamazepine and sulfamethoxazole were the two pollutants with the highest concentrations in Tula Valley groundwater, demonstrating persistence. Sulfamethoxazole concentration was even found to be of environmental concern in the spring water of the Tula Valley with and RQ >1.

Carbamazepine and sulfamethoxazole are the most commonly studied BACs in soil sorption studies (Ahmed, et al., 2015; Aurore Andriamalala, et al., 2018; Carter, et al., 2019, 2014; Chefetz, et al., 2008; Corada-Fernández, et al., 2015; Cycoń, et al., 2019; de Santiago-Martín, et al., 2020; Drillia, et al., 2005a; Durán-Alvarez, et al., 2009; Kodesova, et al., 2019; Kodešová, et al., 2019, 2015; Koroša, et al., 2020; Martínez-Hernández, et al., 2016; Prosser, et al., 2014; Revitt, et al., 2015; Thelusmond, et al., 2016; Wojsławski, et al., 2019) and so it would be possible to find enough data in the literature to model for these two pollutants, at least. Carbamazepine is known to be very persistent in the environment (Durán-Alvarez, et al., 2009; Martínez-Hernández, et al., 2016). Sulfamethoxazole has variable biodegradability and sorption in the literature (Aurore Andriamalala, et al., 2018; Martínez-Hernández, et al., 2016).

8.3 Modelling of literature data to identify gaps in the literature

8.3.1 Types of experimental setups

The two main types of experiments studying bioactive pollutants sorption behaviour in soil are batch sorption-desorption tests and column percolation tests (Casey, et al., 2005; Chefetz, et al., 2008; M. Conde-Cid, et al., 2020; Conde-Cid, et al., 2019; Drillia, et al., 2005a; Durán–Álvarez, et al., 2014; Kiecak, et al., 2019; Naka, et al., 2016; Paz, et al., 2016; Revitt, et al., 2015; Wojsławski, et al., 2019; Yu, et al., 2020; Y. Zhang, et al., 2017). Batch sorption and desorption experiments are mainly based on the OECD guideline 106 (OECD, 2000). The main objective of this type of study is to obtain a sorption value for the pollutant of interest as a function of different soil properties. Therefore, the sorption behaviour of the pollutant must be studied in several types of soils (OECD, 2000). These experiments consist of contacting a liquid phase, containing the pollutant of interest, with the soil and after an equilibration period measuring the amount of pollutant in the solid and liquid phase to determine the soil

partition coefficient (K_d), as in Eq. 2 in Chapter 2 (OECD, 2000). As a reminder, Eq. 2 is:

$$K_d = \frac{m}{(1-m)}\frac{v}{s} \qquad \textit{Eq. 27}$$

Where m is the fraction of BAC sorbed onto soil particles. For batch tests: v is the volume of liquid phase and s the amount of soil (Durán–Álvarez, et al., 2014). K_d is a linear approximation of the sorption behaviour (Kiecak, et al., 2019).

For the desorption experiments the soil containing the pollutant of interest is contacted with a liquid phase (not containing the pollutant). K_d for the sorption and desorption experiments can be calculated as a function of the initial concentration of the pollutant (OECD, 2000). Apart from the K_d data is also fitted to the Langmuir (Eq. 3) and Freundlich (Eq.4) model (OECD, 2000). Column percolation tests study the leaching potential of pollutants through a soil column (Durán–Álvarez, et al., 2014; Naka, et al., 2016; Wojsławski, et al., 2019). Compared to batch tests they have been shown to be more reproducible and more comparable to real environmental conditions (Wojsławski, et al., 2019). Both of these types of experiments can be done using either a single pollutant or a mixture of pollutants in order to study competitive sorption (Conde-Cid, et al., 2019; Durán-Álvarez, et al., 2012; Paz, et al., 2016).

Initially, data from both types of experiments was collected, however it soon became apparent that batch experiments tend to be more similar between them and therefore more comparable than column tests. There are different types of column experiments. The setup can be different, one option is to extract undisturbed soil columns to study the transport of pollutants through them with water (Durán–Álvarez, et al., 2014) or using lysimeters to control other variables like evapotranspiration (Paz, et al., 2016), or using a pot with vegetables grown or without them (Koroša, et al., 2020). Another difference between them is the way in which water is contacted with the soil, like drip irrigation (Paz, et al., 2016), sprinkle irrigation (Koroša, et al., 2020) or saturation of soil column (Uddin, et al., 2020; Yu, et al., 2020). Therefore, it was difficult to find enough comparable column experiments in the literature to model. Furthermore, for every column study found in the literature approximately two batch studies were found. The wider availability of data for batch studies, as well as the more uniform experimental set-up meant that these studies were a more adequate starting point for the modelling. Modelling of BACs sorption behaviour in soil has been done, but only using the results obtained by the same group (Conde-Cid, et al., 2020; Li, et al., 2020). This is very valuable work, firstly because in both studies they produce a large amount of data which is amenable to modelling and secondly because modelling work can allow to make predictions for different situations (Conde-Cid, et al., 2020; Li, et al., 2020). However, using data from across different studies can give insights into wider trends, identify literature gaps and unveil experimental biases. This has been done previously for the sorption of per and polyfluoroalkyl (PFASs) in soil (Yasong Li, et al., 2018b). The aim of this chapter is to undertake a first step in comparing pollutant sorption in literature across different studies and use the information to design specific experiments that address literature gaps.

8.3.2 Parameters and ranges reported and studied in the literature

In modelling terms, for batch experiments the dependent or y variable is the partition coefficient K_d (or other sorption coefficients), as explained in section 2.3.2.1. For the purposes of the model K_d was used, as opposed to Freundlich or Langmuir coefficients, because it is not a fitted curve but rather a number describing the partition at a given concentration, as explained in section 2.3.2.1. This was more amenable for the model

and K_d was either given in the literature studied (Table 8.3.1) or could be calculated from the data reported. In some of the papers evaluated, data was presented in graphs as a function of the initial concentration (Conde-Cid, et al., 2019; Hu, et al., 2019; Liang, et al., 2016; Paz, et al., 2016). In these cases, a MATLAB code (can be seen in Appendix 10.9) was used to extract the actual concentrations of BAC in the soil and liquid phase at equilibrium and used them to calculate K_d using Eq.1. In one case reported K_{oc} (Chefetz, et al., 2008) was converted to K_d using Eq.2.

The parameters studied which can influence K_d can be divided into soil properties, pollutant or pollutants studied and experimental conditions (type of liquid phase, volumes and quantities of liquid and soil phases, temperature, initial concentration of pollutant, etc.). One hundred and ninety-five data points were collected from eleven different papers (Table 8.3.1). Each data point refers to a different K_d value.

8.3.2.1 Soil properties

Different papers report different soil characteristics, for example some report cation exchange capacity (CEC) (Aurore Andriamalala, et al., 2018; Chefetz, et al., 2008; Gibson, et al., 2010; Hu, et al., 2019; Kiecak, et al., 2019; Martínez-Hernández, et al., 2016; Wojsławski, et al., 2019) or the specific ions present in the soil (Durán-Álvarez, et al., 2012; Li, et al., 2020; Uddin, et al., 2020), but others do not report either (Dalkmann, et al., 2014; Paz, et al., 2016). Only a few papers reported calcium carbonate percentage (Avetta, et al., 2016; Chefetz, et al., 2008; Durán-Álvarez, et al., 2012), soil conductivity (Martínez-Hernández, et al., 2016; Paz, et al., 2016) and soil bulk density (Durán-Álvarez, et al., 2012). Therefore, these parameters could not be included in the model. Only the soil characteristics reported across all the papers studied could be included in the model. These were soil pH, fraction of organic carbon content and soil texture (percentage of sand, clay and silt). According to the OECD guidelines these are the most important parameters determining sorption (OECD, 2000). However, other paremeters which may affect sorption are CEC, content of amorphous iron and aluminium oxides and specific surface areas (OECD, 2000). Iron and aluminium oxides content were only given in a few papers (Durán-Álvarez, et al., 2012; Li, et al., 2020; Uddin, et al., 2020) and specific surface area of the soil was not given in any of the references studied.

Soil texture was reported uniformly across all papers, using standard methods for determining the percentage of each texture. The range found in the literature for the soil properties reported across all papers has a wide range, as can be seen in Figure 8.3.1 below. Most of the soils were taken from real agricultural areas. Two studies used soil from the Tula Valley(Dalkmann, et al., 2014; Durán-Álvarez, et al., 2012). In one study they used soil which had been irrigated with untreated water for approximately 85 years (Durán-Álvarez, et al., 2012), in the other soils which had been irrigated with untreated wastewater for 0, 14, 35 or 100 years (Dalkmann, et al., 2014). Two studies used agricultural soil form Iran (Chefetz, et al., 2008; Paz, et al., 2016) in one of them the soil was from a field irrigated with treated wastewater for over 25 years (Chefetz, et al., 2008). Two studies used Chinese agricultural soil (Hu, et al., 2019; Uddin, et al., 2020). One study used soil from an experimental field in France to compare soil never amended with organic waste, soil amended with compost and sewage sludge and soil amended with manure (Aurore Andriamalala, et al., 2018). Soils were also taken from unsaturated zones of groundwater in Spain in two studies (Kiecak, et al., 2019; Martínez-Hernández, et al., 2016). Finally reference soils were also used (Wojsławski, et al., 2019) and technical sand with low clay and organic content as a comparison to aquifer soils was used in one study (Kiecak, et al., 2019). When field soil was collected it was mostly collected from the top layer of the soil (0-30cm) (Dalkmann, et al., 2014; Hu, et al., 2019; Martínez-Hernández, et al., 2016; Uddin, et al., 2020). However some

studies compared sorption using different soil depths: 10 cm was compared to 40 cm in (Durán-Álvarez, et al., 2012). Some aquifer soils were collected from deeper layers (80-300 cm) (Kiecak, et al., 2019). The depth was not always specified (Paz, et al., 2016).

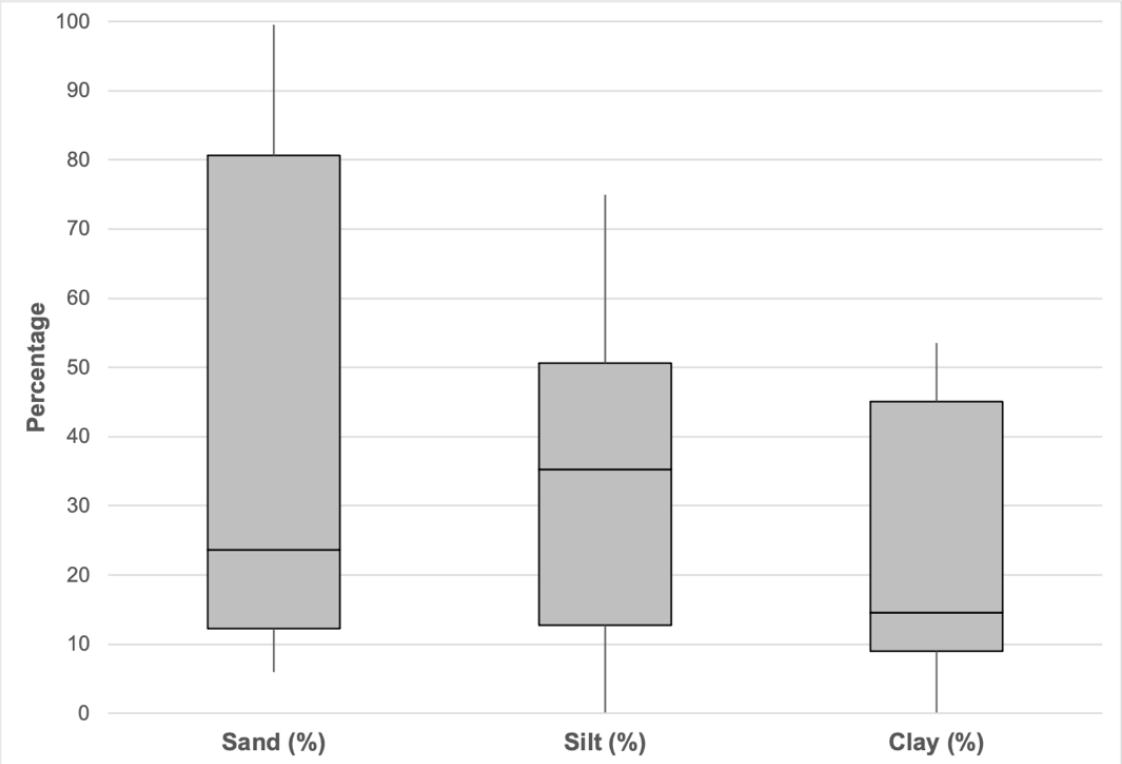


Figure 8.3-1 Range of soil textures in the soils studied

Soil pH, along with the pollutant's pk_a, is a very important factor determining sorption of bioactive pollutants to soil (Kodešová, et al., 2015; Lorphensri, et al., 2007), as detailed in section 2.3.2.1. However it was challenging to compare soil pH values in the literature as they are often obtained in different ways, mainly by using different solutions (Minasny, 2011). The two solutions used for measuring it in the literature studied for this work were either in water (pH_{H_2O}) or in 0.01 M of $CaCl_2$ (pH_{CaCl_2}). Measurements in $CaCl_2$ solution have been shown to be less affected by soil electrolyte concentration and therefore more stable (Minasny, 2011). Models have been developed to convert from pH_{H_2O} to pH_{CaCl_2} (Minasny, 2011) and they have been shown to be more accurate when the electrical conductivity of the soil is known (Minasny, 2011). However this parameter is not often reported, only two out of the eleven references used to extract data reported it (Martínez-Hernández, et al., 2016; Paz, et al., 2016). Therefore, an older conversion was used, one which was built from comparing 7844 pH measurements obtained in pure water to the same soil measured using a solution of 0.01 M $CaCl_2$ (Henderson, et al., 2002). This study found that below a pH of 4.2 the difference between pH_{H_2O} and pH_{CaCl_2} is very small, between 5-7 the difference is linear and above a pH of 7 a large increase in pH_{H_2O} results in a small pH_{CaCl_2} difference (Henderson, et al., 2002). Most of the data used in this study was in the pH range 4.5-9.0 and the authors state that their model is more reliable within this range (Henderson, et al., 2002). Using this data they built a conversion table (Henderson, et al., 2002), which was used for the work presented in this chapter. When the solution used to measure the pH was not specified (Dalkmann, 2014; Martínez-Hernández, 2016a; Aurore Andriamalala, 2018b; Uddin, 2020) it was assumed that it was measured in water.

Three models were built, in one the pH values were not converted, in the second all pH_{H_2O} values were converted to their equivalent pH_{CaCl_2} and a third one the pH_{CaCl_2} values were converted to pH_{H_2O}. The difference between these three models was not big, as can be seen form the R^2Y and R^2X values in Table 8.3.1.

Model	RX^2	RY^2	Q^2 cumulative
pH no conversion (as reported)	0.640	0.380	0.36
pH_{CaCl_2} converted to pH_{H2O}	0.59	0.368	0.355
pH_{H2O} converted to pH_{CaCl}	0.651	0.384	0.364

Table 8.3-1 Comparison of models with different pH values

The range of soil pH studied in the papers used to build this model is shown in Figure 8.3.2 below. The values shown are for pH_{CaCl_2}. It can be seen that most of the data was obtained in soil within pH 7-8. The optimum range for most agricultural crops is between 5.5 and 7.5 (Odutola Oshunsanya, 2018) so the range in the majority of the literature is at the higher end of this range. The pH during the experiment was not monitored in most papers, however in one paper it was controlled (Martínez-Hernández, 2016b). This could not be accounted for the model as most studies did not consider pH variations during the experiment. Any changes of pH during the experiment could affect sorption and so it would be good if this vriable was kept constant.

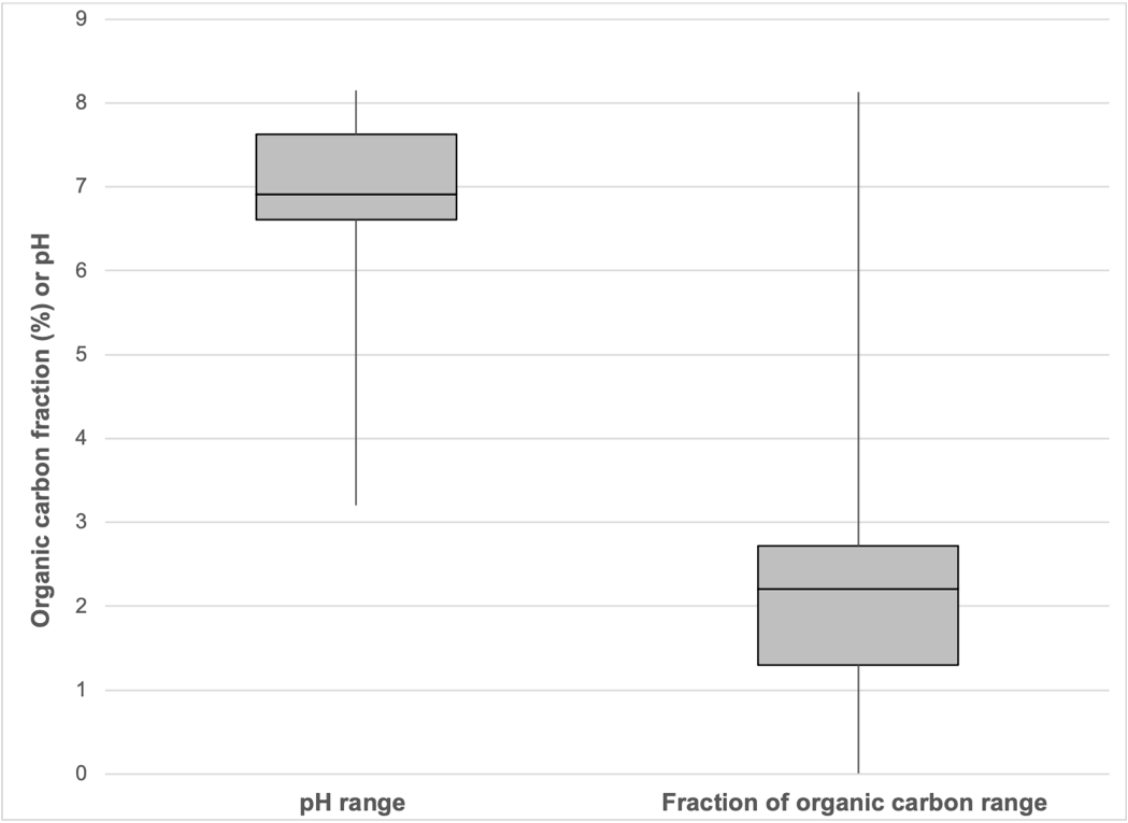


Figure 8.3-2 Range of pH_{CaCl_2} values (all pH_{H2O} converted to pH_{CaCl_2}) and Fraction of organic carbon studied in the literature

As explained in section 2.3.2, organic carbon is the measurable fraction of organic matter. Organic matter contains approximately 58% of organic carbon and so a factor

of 1.72 is used to convert between total organic carbon and organic matter (Fenton, 2008). This conversion was used for one reference which reported organic matter (Uddin, et al., 2020), instead of organic carbon as all the others. On the other hand, all carbamazepine references reported fraction of organic carbon (Chefetz, et al., 2008; Durán-Álvarez, et al., 2012; Kiecak, et al., 2019; Li, et al., 2020; Martínez-Hernández, et al., 2016; Paz, et al., 2016; Wojsławski, et al., 2019), so no conversion was needed for the model built using only carbamazepine. The range of fraction of organic carbon content in the literature studied is reported in Figure 8.3.2.

8.3.2.2 Experimental conditions

Important parameters in batch experiments are the amount of soil and the volume of liquid used, as well as the initial concentrations of the pollutant used (OECD, 2000). These were not always specified, or were given as a range and it was not possible to determine which results corresponded to which initial conditions (Li, et al., 2020). Liquid volume and soil amount were always specified and ranged from 0.03-3.0 L and 0.5- 300 mg respectively (Aurore Andriamalala, et al., 2018; Chefetz, et al., 2008;Dalkmann, et al., 2014; Durán-Álvarez, et al., 2012; Gibson, et al., 2010; Hu, et al., 2019; Kiecak, et al., 2019; Li, et al., 2020; Martínez-Hernández, et al., 2016; Paz, et al., 2016; Uddin, et al., 2020; Wojsławski, et al., 2019). Carbamazepine initial concentrations in the liquid phase ranged from 0.05-3,000,000 μgkg^{-1} (Chefetz, et al., 2008; Durán-Álvarez, et al., 2012; Kiecak, et al., 2019; Li, et al., 2020; Martínez-Hernández, et al., 2016; Paz, et al., 2016; Wojsławski, et al., 2019) while for sulfamethoxazole it ranged from 20-22,083.3 μgkg^{-1} (Aurore Andriamalala, et al., 2018; Dalkmann, et al., 2014; Hu, et al., 2019; Martínez-Hernández, et al., 2016; Uddin, et al., 2020). Environmental concentrations in agricultural soils for sulfamethoxazole have been reported as: 0.2-54.5 μgkg^{-1} (Christou, et al., 2019; Cycoń, et al., 2019) and for carbamazepine 1.2-98.3 μgkg^{-1} (Christou, 2019). Therefore, the concentrations studied in sorption studies are mostly higher than those that have been measured in the environment. This is a significant gap in the literature because K_d has been shown to be concentration dependent (Kiecak, et al., 2019; Singh, 2016).

The type of liquid phase used was always specified. The most common was a $CaCl_2$ solution, with concentrations of either 0.005 or 0.01 M $CaCl_2$ (Chefetz, 2008; Durán-Álvarez, Juan-Carlos Prado-Pano, 2012; Dalkmann, 2014; Paz, 2016; A Andriamalala, 2018; Hu, 2019; Wojslawski, 2019; J. Li, 2020; Uddin, 2020). Other liquid phases used were synthetic reclaimed water (Martínez-Hernández, 2016b), aquifer water (Kiecak, et al., 2019) and secondary treated wastewater (Chefetz, 2008). It has been shown that the K_d changes when the same sorption experiment is done with freshwater compared to when it is done with secondary treated water (Chefetz, 2008). This is a literature gap as most studies are done with $CaCl_2$ solution and in reality, soil is irrigated with either treated or untreated wastewater or surface water.

The method used to sterilise the soil, in order to rule out biodegradation, was not always specified (Andriamalala, et al., 2018; Dalkmann, et al., 2014) and this can have a significant effect on soil sorption, as it can change soil properties (Lotrario, 1995). The methods reported were autoclaving (Hu, et al., 2019; Li, et al., 2020; Martínez-Hernández, et al., 2016; Uddin, et al., 2020) and NaN_3 (Chefetz, et al., 2008; Kiecak, et al., 2019; Paz, et al., 2016; Wojsławski, et al., 2019). Methods to prevent photodegradation were mostly stated and included amber glass (Durán-Álvarez, et al., 2012; Li, et al., 2020) and aluminium (Hu, et al., 2019; Kiecak, et al., 2019; Martínez-Hernández, et al., 2016; Uddin, et al., 2020), however it was not always specified (Chefetz, et al., 2008;Dalkmann, et al., 2014; Wojsławski, et al., 2019). The temperature at which the experiment was conducted has an effect on sorption (Yasong

Li, et al., 2018a; OECD, 2000) and this was not always specified (Uddin, et al., 2020). When it was specified, most experiments were conducted at 20 or 25 ºC (A. Andriamalala, et al., 2018; Chefetz, et al., 2008; Dalkmann, et al., 2014; Durán-Álvarez, et al., 2012; Hu, et al., 2019; Kiecak, et al., 2019; Martínez-Hernández, et al., 2016; Paz, et al., 2016; Wojsławski, et al., 2019) except for an experiment done at 4 ºC for carbamazepine (J. Li, 2020). The OECD guideline specifies that experiments should be conducted between 20- 25 ºC (OECD, 2000), this provides comparable results. However, it does not account for temperature variations which will occur seasonally and geographically.

Another parameter which is not always specified is the test vessel used (A. Andriamalala, et al., 2018; Uddin, et al., 2020). The OECD guideline states that checks should be carried out to rule out adsorption on the vessel material (OECD, 2000) and studies have shown it does make a difference in relation to BAC sorption (Li, et al., 2020). Most common materials used are glass (Dalkmann, et al., 2014; Durán-Álvarez, et al., 2012; Hu, et al., 2019; Kiecak, et al., 2019; Martínez-Hernández, et al., 2016), teflon (Chefetz, et al., 2008; Paz, et al., 2016) and polypropylene tubes (Li, et al., 2020; Wojsławski, et al., 2019).

The linear soil partitioning coefficient (K_d) is measured at the equilibrium time which is the time at which the concentration in either phase has stopped changing (OECD, 2000). This needs to be determined before the experiment and depends on the pollutant and soil studied and on the initial BAC concentration, liquid volume and soil volume (OECD, 2000). In the papers studied equilibrium times ranges from 12 hours to 7 days (Chefetz, et al., 2008; Dalkmann, et al., 2014; Durán-Álvarez, et al., 2012; Hu, et al., 2019; Kiecak, et al., 2019; Li, et al., 2020; Martínez-Hernández, et al., 2016; Paz, et al., 2016; Wojsławski, et al., 2019). Only two papers included an equilibration period between the liquid and solid phase (before adding the pollutant), one was for 12 hours (Kiecak, et al., 2019) and the other for 24 hours (Dalkmann, et al., 2014)

8.3.2.3 Pollutants studied

The papers studied for the purpose of the models built in this section were initially filtered in order to find references which studied carbamazepine, sulfamethoxazole and tramadol. However, when papers studied other pollutants along these, those values were also collected. When no more references, containing data adequate for modelling, for carbamazepine, sulfamethoxazole and tramadol could be found then other pollutants were also added. This would allow for comparison of K_d trends across different pollutants in a model for multiple pollutants. The references used are given in Table 8.3.1, along with the numbering system used for the models. These references include those which were not used in the model in the end as their studies are about column sorption or about competitive sorption with multiple pollutants. The simplest case was modelled (single pollutant batch experiments) as a starting point. Although in the future it would be valuable to do the same with more experimental setups and more datapoints.

The pollutant's physicochemical properties were accounted for by calculating the $LogD_{ow}$ of eacha anlyte at the pH_{CaCl2} of the soil in which the reference carried out the study. The sulfamethoxazole model was found not be valid, as explained in section 8.4.2, however the multi-component and the carbamazepine models were valid and showed interesting trends.

#	Reference
1	V. Martínez-Hernández, R. Meffe, S. Herrera López, I. de Bustamante, The role of sorption and biodegradation in the removal of acetaminophen, carbamazepine, caffeine, naproxen and sulfamethoxazole during soil contact: A kinetics study, Sci. Total Environ. 559 (2016) 232–241.
2	B. Durán-Álvarez, Juan-Carlos Prado-Pano, B. Jiménez-Cisneros, Sorption and desorption of carbamazepine, naproxen and triclosan in a soil irrigated with raw wastewater: Estimation of the sorption parameters by considering the initial mass of the compounds in the soil, Chemosphere. 88 (2012) 84–90.
3	Liang, C. *et al.* (2016) 'Mechanism for the primary transformation of acetaminophen in a soil/water system', *Water Research*. 98, pp. 215–224.
4	Paz, A. et al. (2016) 'Fate of carbamazepine, its metabolites, and lamotrigine in soils irrigated with reclaimed wastewater: Sorption, leaching and plant uptake', Chemosphere. Pergamon, 160, pp. 22–29.
5	Li, J., Carter, L. J. and Boxall, A. B. A. (2020) 'Evaluation and development of models for estimating the sorption behaviour of pharmaceuticals in soils', Journal of Hazardous Materials. Elsevier, 392(March), p. 122469.
6	Conde-Cid, M. *et al.* (2019) 'Competitive adsorption of tetracycline, oxytetracycline and chlortetracycline on soils with different pH value and organic matter content', *Environmental Research*. Elsevier Inc., 178(August), p. 108669.
7	Uddin, M. et al. (2020) 'Insight into dynamics and bioavailability of antibiotics in paddy soils by in situ soil moisture sampler', Science of the Total Environment. Elsevier B.V., 703, p. 135562. doi: 10.1016/j.scitotenv.2019.135562.
8	Hu, S. et al. (2019) 'Adsorption/desorption behavior and mechanisms of sulfadiazine and sulfamethoxazole in agricultural soil systems', Soil and Tillage Research.
9	Andriamalala, A. et al. (2018) 'Fate of sulfamethoxazole, its main metabolite N-ac-sulfamethoxazole and ciprofloxacin in agricultural soils amended or not by organic waste products', Chemosphere. Pergamon, 191, pp. 607–615.
10	Wojslawski, J. *et al.* (2019) 'Leaching behavior of pharmaceuticals and their metabolites in the soil environment', *Chemosphere*, 231, pp. 269–275.
11	Chefetz, B., Mualem, T. and Ben-Ari, J. (2008) 'Sorption and mobility of pharmaceutical compounds in soil irrigated with reclaimed wastewater', Chemosphere, 73, pp. 1335–1343.
12	Kiecaka, A. et al. (2019) 'Sorption properties and behaviour at laboratory scale of selected pharmaceuticals using batch experiments', Journal of Contaminant Hydrology, 225(103500).
13	Dalkmann, et al. (2014) 'Long-term Wastewater Irrigation Reduces Sulfamethoxazole Sorption, but Not Ciprofloxacin Binding, in Mexican Soils', Journal of Environmental Quality, 43(3), pp. 964–970.

Table 8.3-2 References studied for the modelling of bioactive pollutants sorption to soil

8.4 Models and gaps in the literature they revealed

This aim of this work was to identify the main variables affecting soil sorption across different papers. Partial least squares (PLS) models are a powerful tool to identify the most important independent variables influencing a dependent variable (Li, et al., 2020; Syms, 2018). Furthermore the visualization of the score plot is very useful in identifying underlying similarities and differences between different studies (Li, et al., 2020; Syms, 2018) so PLS was used for this task. However, PLS is an supervised model (meaning it is more likely to be over-fitted). Therefore principal component analysis (an unsupervised model) was undertaken before all PLS models in order to confirm the groupings shown in PLS models were not due to overfitting (Umetrics-Sartorious, 2019). SIMCA (Umetrics-Sartorius, Sweden) was used for the modelling.

8.4.1 Multiple pollutants models

The first model developed utilised all 195 data points, the proportion of pollutants are summarised in Figure 8.4.1. The majority of data points for each pollutant were obtained from only one reference, with the exceptions of carbamazepine (seven references), sulfamethoxazole (six refences) and two references each for ciprofloxacin, diclofenac and naproxen. It is important to be aware of this proportion, as the conclusions drawn from the multiple pollutants models may be more relevant to the pollutants which had a larger proportion of datapoints, rather than to all of them. Which is why models for individual compounds were then evaluated (Section 8.4.2 and Section 8.4.3) .

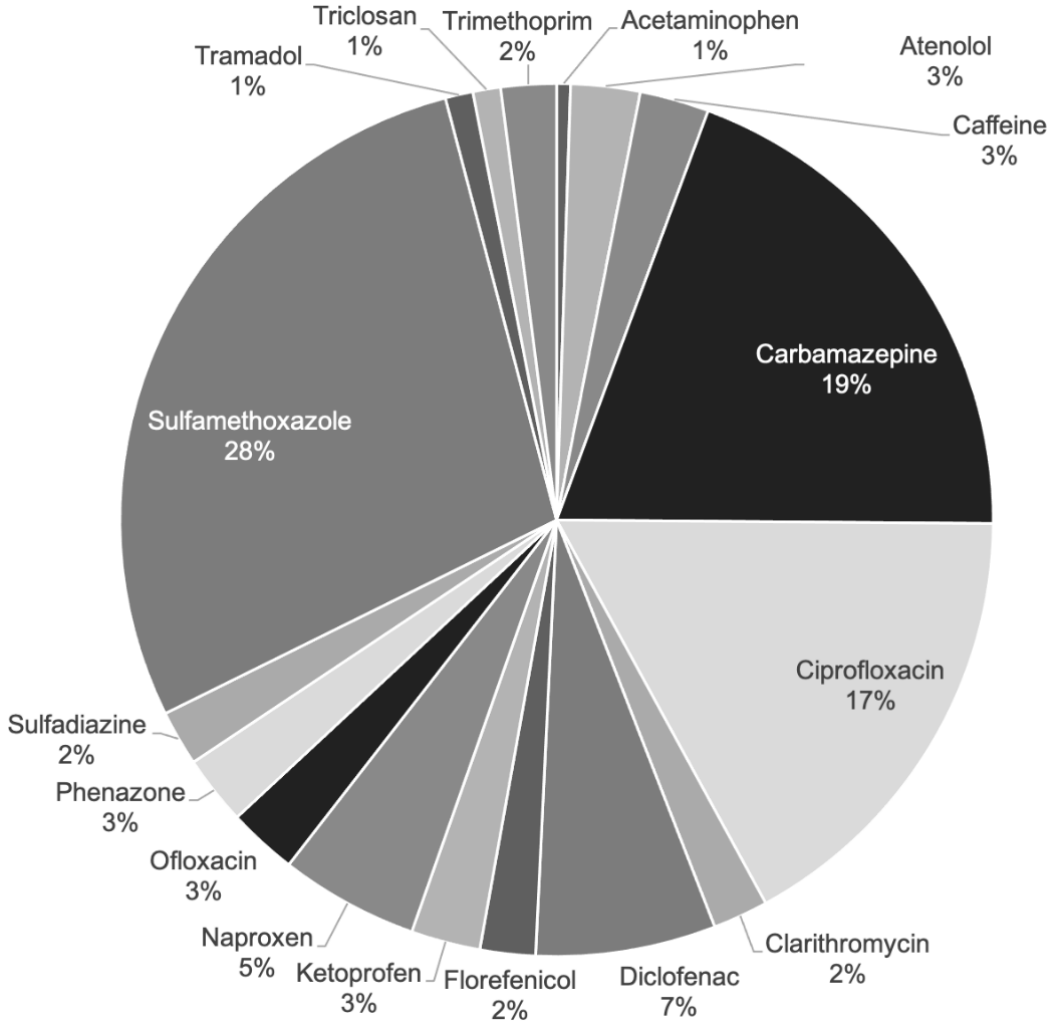


Figure 8.4-1 Proportion of pollutants in the multicomponent model

The y-variable of the model was the K_d and the x-variables were, percentage of sand, silt and clay, soil fraction of organic content, type of liquid phase, method for biodegradation quenching, time to reach equilibrium and the pollutant's $LogD_{ow}$ at the pH_{CaCl2} of the soil studied for each data point. The qualitative variables were turned into quantitative variables using the quantification matrix method (Agudelo-Jaramillo, et al., 2016). The type of liquid phase was entered by having four variables called *Liquid phase: $CaCl_2$ solution*, *$CaCl_2$ plus other salts solution*, *secondary treated water and well/aquifer water*; for each of these variables 1 or 0 was entered depending on whether that liquid phase was used. This is in accordance with (Agudelo-Jaramillo, et al., 2016) on how to model qualitative data. The same was done for the biodegradation quenching using two variables: *autoclaving* or *NaN_3*, when it was not specified zero was entered for both variables.

Pollutant name and soil type were both entered as secondary identifiers in SIMCA, in order to see if the data points were grouped according to these variables. The quality parameters of the three models run, with the different pH conversions, is shown in Table 8.4.1.

All of the models were valid, based on the permutation test (n=100), as can be seen in Figure 8.4.2, because the random R^2 and Q^2 values were lower than the real model R^2 and Q^2 values.

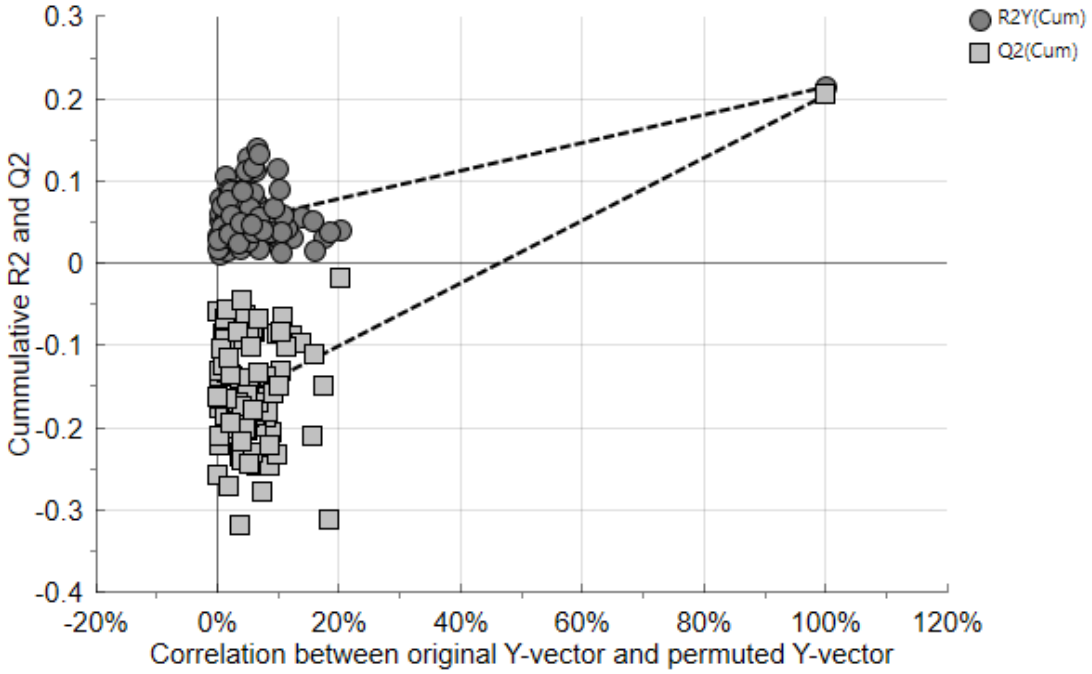


Figure 8.4-2 Validation of the multicomponent model through permutation (n=100)

From Figure 8.4.3 there is no clear grouping according to pollutant across all the refences. However, within each reference it is possible to see different clusters corresponding to different pollutants. The clearest ones are the two clusters, one for sulfamethoxazole and one for ciprofloxacin both for reference 13 (Dalkmann, et al., 2014). This study has a total of 54 data points, with 27 each for sulfamethoxazole and ciprofloxacin. Four different soils were used each with either 0,14,35 or 100 years under irrigation using untreated wastewater. Seven different initial concentrations of the pollutant (100, 250,500,1250,2500,5000,10 000 $\mu g kg^{-1}$) were used (Dalkmann, et al., 2014).

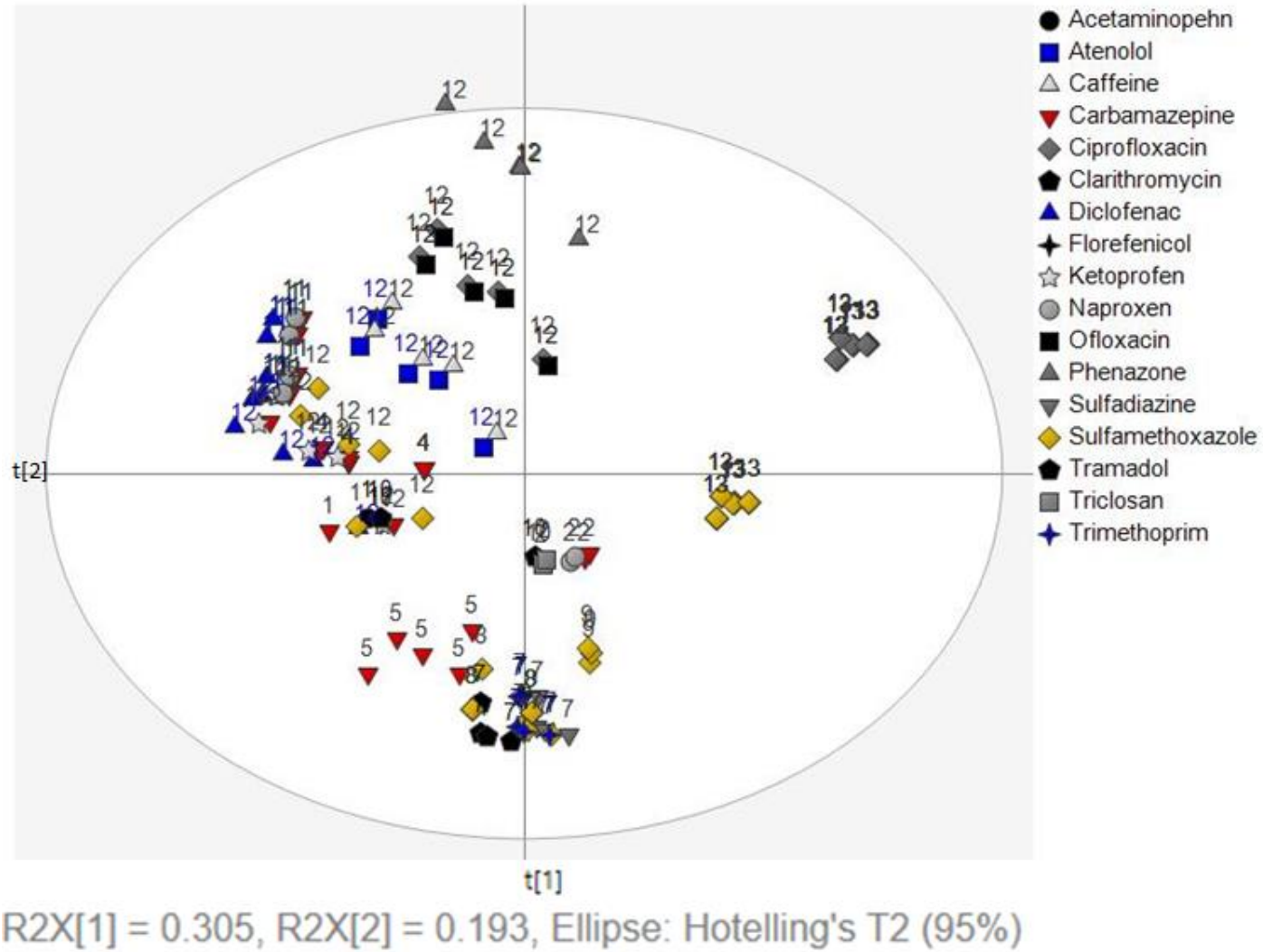


Figure 8.4-3 Scores plot: visualization of principal component 1 t[1] and principal component 2 t[2], variance represented by each principal component innthe figure is given by R2X[1] and R2X[2] respectively. Datapoints shaped and coloured according to pollutant and labelled according to reference

In Figure 8.4.3 there are four different groups with a similar pattern for both pollutants. The authors of this study stated that sorption of ciprofloxacin was stronger and mostly irreversible, irrespective of the amount of time the soil had been under untreated wastewater irrigation and the organic matter content (Dalkmann, et al., 2014). They observed a decrease in sorption with increasing soil pH due to a decreasing fraction of cationic species. For sulfamethoxazole they observed stronger sorption in the non-irrigated soil. pH was not expected to cause great differentiation in sorption for sulfamethoxazole because it has pk_a (1.83 and 5.57) lower than the pH range of the soils studied (7.1-7.4 in pH_{H2O}) meaning this compound would be found predominantly in its anionic from (Dalkmann, et al., 2014). From Figure 8.4.4 it is possible to see that the main four clusters formed for both pollutants correspond to the 4 periods under irrigation of the soils and within each are the seven concentrations studied for each pollutant. This separation and the fact it has the same pattern for both pollutants might mean there is a greater influence, which acts on both pollutants, from the irrigation time than the authors highlighted. This also shows a greater dependence on irrigation under wastewater length than on initial concentration in the range studied.

For reference 12 there are seven linear shapes, mostly on the top left quadrant, corresponding to the seven pollutants studied in this paper (atenolol, caffeine, carbamazepine, ciprofloxacin, diclofenac, ketoprofen, ofloxacin, phenazone and sulfamethoxazole) (Kiecak, et al., 2019). Each of the seven lines are spaced out in a similar pattern corresponding to soil type in the following order: medium sand and coarse sand, sandy loam and clay with some overlap between sandy loam and clay. In the paper the authors state that no clear pattern across all pollutants was found depending on soil type and that sorption is more pollutant dependent (Kiecak, et al., 2019). However, using PLS analysis in the context of other literature it is possible to see a pattern where for all pollutants medium sand and coarse sand were grouped and sandy loam and clay were grouped. In this study the authors perform a correlation analysis between K_d and several soil properties, namely total carbon, total organic carbon, pH, cation exchange capacity (CEC) and specific surface area (SSA) (Kiecak, et al., 2019). They found that for phenazone there was a high correlation between K_d and soil pH. For caffeine, sulfamethoxazole and ciprofloxacin CEC and SSA were the most crucial factors. For ciprofloxacin, K_d was correlated to clay minerals. Sorption of carbamazepine presented moderate correlation to pH, and atenolol to CEC and SSA. For diclofenac and ketoprofen they found no correlation (Kiecak, et al., 2019). For the PLS multicomponent model performed for this thesis the variable with the most importance for prediction was the pollutant's solubility in water, as can be seen in Figure 8.4.5, this is consistent with reference 12 (Kiecak, et al., 2019). as it shows the pollutant is one of the most important variables. This means it is important to do sorption studies for pollutants which have not been largely studied in the literature. Clay and sand content were also found as important variables in the PLS model, as can be seen in Figure 8.4.5. It is possible that the large number of datapoints for ciprofloxacin has some dominance over the model, making clay an important variable. CEC and SSA could not be include in the PLS model as they were not provided by all of the references studied. For diclofenac and ketoprofen the authors found no correlation (Kiecak, et al., 2019), however in the PLS model they were found to be the least separated by soil type as the other pollutants.

Reference 11 is divided by compound (diclofenac, naproxen carbamazepine) and the sand values are close in the score plot to the sand values of reference 12. All three of these refences used silt loam (Aurore Andriamalala, et al., 2018; Hu, et al., 2019; Uddin, et al., 2020), which may indicate higher dependence on soil type than on experimental conditions or pollutant (for these pollutants).

Silt loam is clustered at the bottom across references 7,8 and 9 for sulfamethoxazole, clarithromycin, trimethoprim, florfenicol and sulfadiazine, as can be seen from Figure 8.4.4. The one thing these group of datapoints have in common, that is not shared with any other datapoint, is the soil type. The soil pH_{CaCl_2} range among these datapoints is 4.8-7.0, the sand percentage is 6.0-37.0%, silt percentage 55.9-73.6, clay percentage 4.28-20.53 and organic carbon 0.47-4.71% (A. Andriamalala, et al., 2018; Hu, et al., 2019; Uddin, et al., 2020). All of the datapoints were carried out in liquid phase of a solution of $CaCl_2$ (A. Andriamalala, et al., 2018; Hu, et al., 2019; Uddin, et al., 2020) and all were sterilized by autoclaving, except for reference 9 which did not specify a biodegradation quenching method (Aurore Andriamalala, et al., 2018). None of the datapoints had an equilibration time between the solid and liquid phase before adding the pollutant and all had 24 h time to equilibrium (A. Andriamalala, et al., 2018; Hu, et al., 2019; Uddin, et al., 2020). All the compounds in these references have a log_{kow} below 3.16 and a water solubility between 77-610 mgL^{-1}. The pollutants studied all have two pk_a points. Clarithromycin: 8.4 and 12.5, florfenicol 1.8 and 5.5, sulfadiazine 2.0 and 6.5, sulfamethoxazole 1.8 and 5.5, trimethoprim 7.7 and 17.3 (A. Andriamalala, et al., 2018; Hu, et al., 2019; Uddin, et al., 2020). This means that even across different pollutants and experimental conditions soil type remains an important variable, this is supported by clay and sand percentage being found as variables important for prediction (Figure 8.4.5)

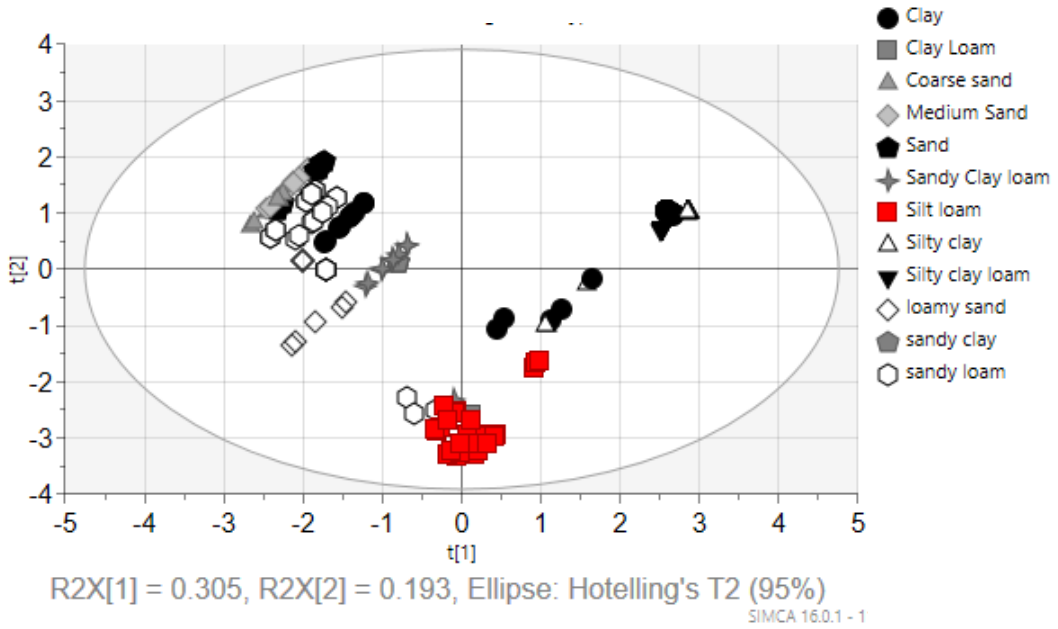
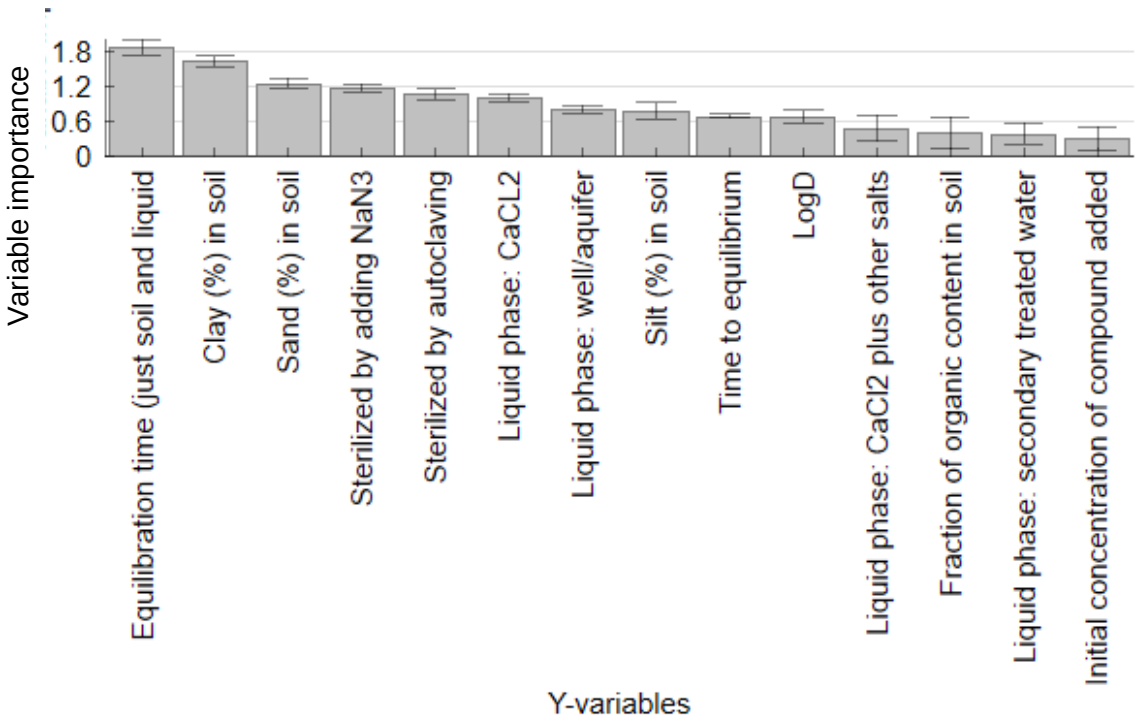


Figure 8.4-4 Scores plot: visualization of principal component 1 (t[1]) and principal component 2 (t[2]) with datapoints shaped and coloured according to soil type for multicomponent PLS. The variance repsented by each component is given by R2X[1] and R2X[2].

The variables for importance (Figure 8.4.5) are in agreement with the references (Table 8.3.1) in that soil type is very important. The equilibration time of the soil with the liquid phase was found to be an important variable, which is not considered in most of the refences studied The sterilization method was also a variable of importance, as can be seen in Figure 8.4.5, this requires further attention as it may affect conclusions drawn from experiments suing different types of sterilization.. According to the models study using NaN_3 has a greater influence than autoclaving. The Q^2 cumulative for the models was below 1, which shows a low ability to make predictions based on the data in the model (Umetrics-Sartorious, 2019). This could be because more datapoints are needed or the models.

Figure 8.4-5 Variables of importance for the multicomponent PLS model



8.4.2 Sulfamethoxazole models

Two more models were run, one only for the datapoints related to sulfamethoxazole and one only for carbamazepine. Even though there were also relatively large amounts of datapoints for ciprofloxacin, as can be seen in Figure 8.4.1, these were only from 2 different references and so a model for this pollutant was not made.

The sulfamethoxazole model however was not valid. It failed on the permutation test and the R^2Y and Q^2 values were both below 0.5. In the PCA and PLS score plot reference 12 was outside the model area, so this reference was removed but the model was still not valid. It is not clear why the model was invalid, K_d values were between 0.12 – 25.78 with a mean of 3.46 and a standard deviation of 4.45 (Andriamalala, et al., 2018; Dalkmann, et al., 2014; Hu, et al., 2019; Kiecak, et al., 2019; Uddin, et al., 2020), so they were not too widely spread which could have affected the data granularity. Correlations between K_d values and percentage of clay, silt and soil, $pH_{CaCl_2,}$ fraction of organic carbon and initial concentration were explored but nothing significant was found. Sulfamethoxazole has been reported to have variable behaviour in soil (Aurore Andriamalala, et al., 2018; Martínez-Hernández, et al., 2016) and inconsistencies between experimental and field observations have been reported (Kiecak, et al., 2019). All the references studied for this pollutant used a liquid phase of $CaCl_2$ solution or $CaCl_2$ plus other salts solution. It would be interesting to add sorption experiments using liquid phases closer to environmental conditions such as treated or untreated wastewater. It is also necessary to compare sorption values for initial concentrations closer to environmental values), as detailed in section 8.3.2.2. The biodegradation quenching method could also be tested as it has shown in the multicomponent model to have an effect.

8.4.3 Carbamazepine models

This PLS model was built, same as the multicomponent model, with the K_d as the y-variable and soil pH_{CaCl_2}, percentage of sand, percentage of silt, percentage of clay and fraction of organic content as x-variables. LogDow was not used for these models because it was not significantly different across the datapoints, given this model was for the same pollutant. The fit of the model was very similar irrespective of which pH conversion was used and it was better than for the multicomponent model as can be seen in Table 8.4.2. The model was valid as can be seen in Figure 8.4.6.

Model	RX^2	RY^2	Q^2 cumulative
pH_{CaCl_2} converted to pH_{H2O}	0.390	0.767	0.554
pH_{H2O} converted to pH_{CaCl}	0.394	0.766	0.554

Table 8.4-1 Carbamazepine PLS model parameters

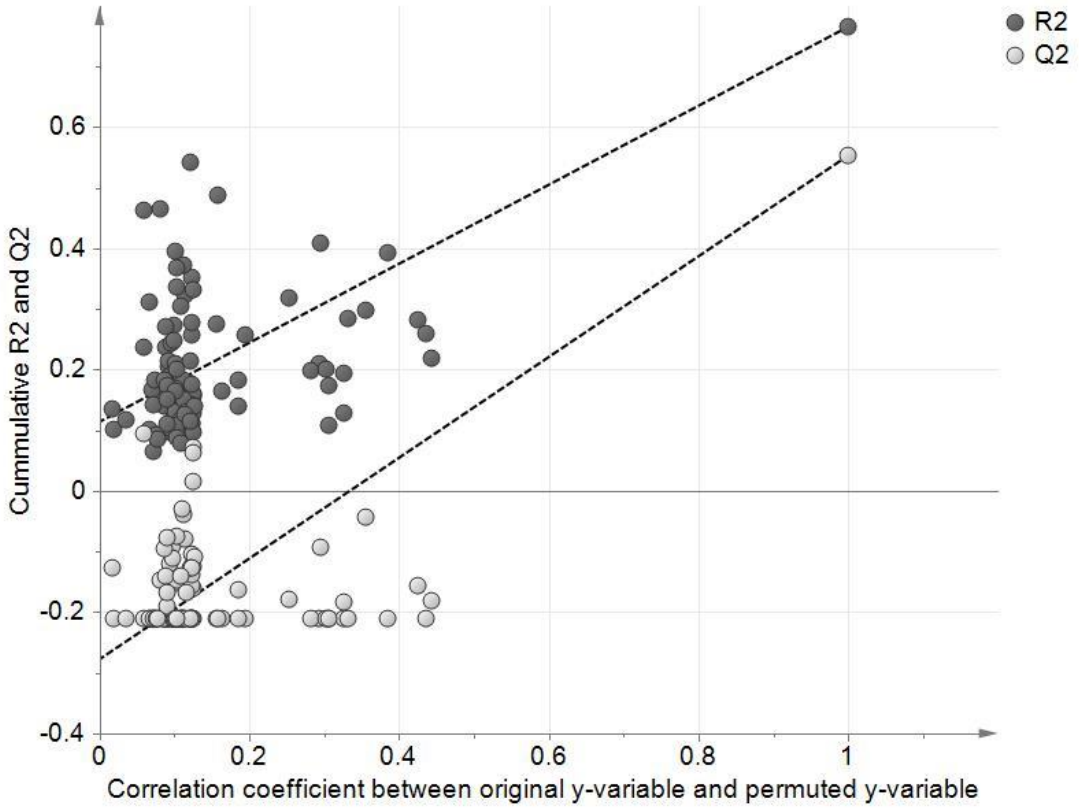


Figure 8.4-6 Carbamazepine PLS model validation by permutation (n=100)

From Figure 8.4.7 it can be seen that the most relevant variable, of those in the model, was the fraction of organic content. This is consistent with literature, which has shown increased carbamazepine sorption with increasing organic matter and previously found strong positive correlations between both (Evyatar, et al., 2018; Gibson, et al., 2010; Paz, et al., 2016).From Figure 8.4.8, there is no clear grouping depending on soil type across all the references. However, clay and sand percentage were also found to be important variables in using the PLS models, however this was not found in previous studies, but authors have speculated that clay will certainly have an effect due to the hydrophobic interactions with clay particles (Gibson, et al., 2010).

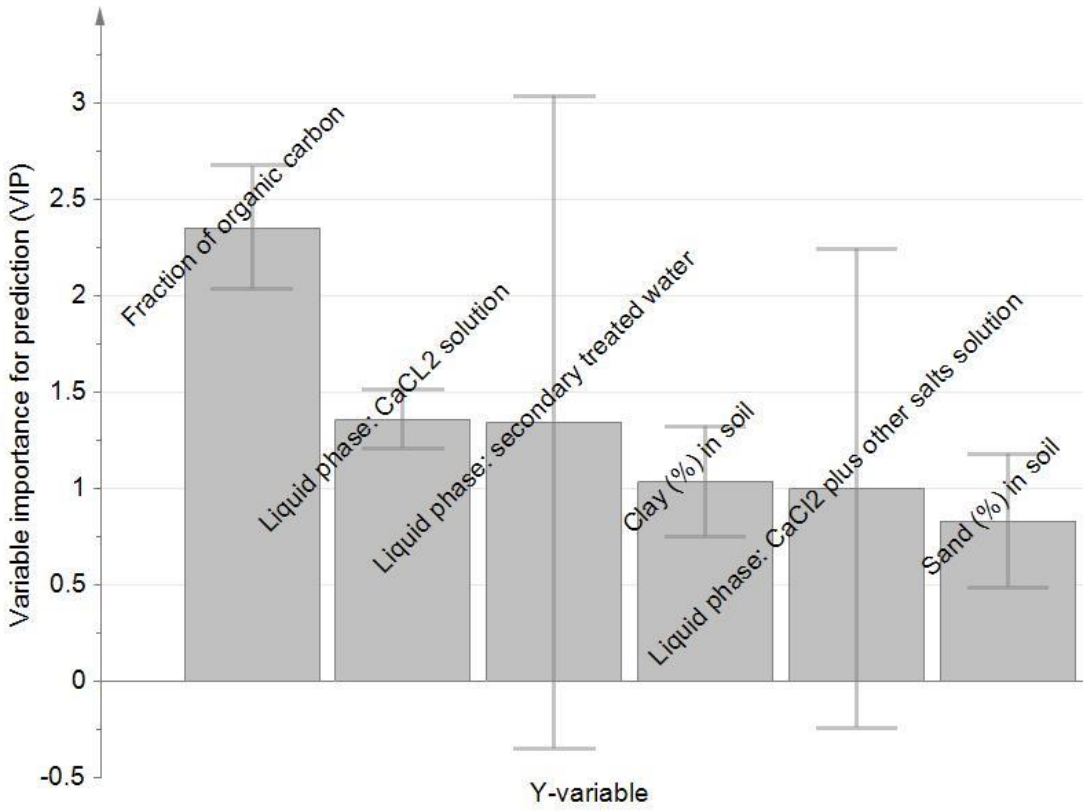


Figure 8.4-7 Variables for importance of prediction for carbamazepine PLS model

It seems that the liquid phase also has an effect on the model, however this is only valid for $CaCl_2$ solution. There are more datapoints using this solution (24 out of 38) while for $CaCl_2$ solution with other salts it is only 5 datapoints and for secondary treated water it is only 4. It is possible that more datapoints are required with these last two liquid phases to discern how important this variable is for prediction.

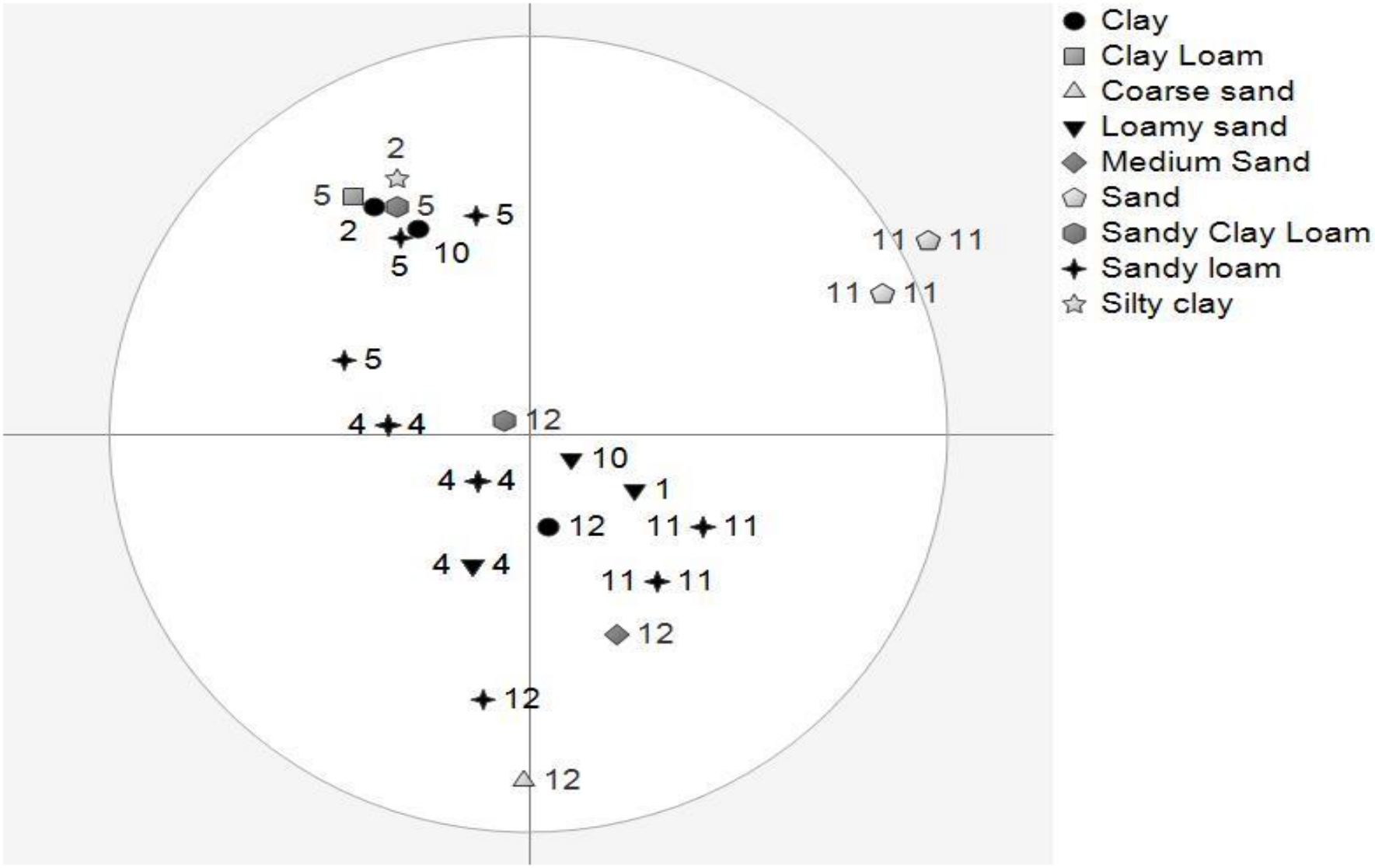


Figure 8.4-8 Scores plot: visualization of principal component 1 (x-axis) and principal component 2 (y-axis) for Carbamazepine kd soil sorption PLS model, datapoint marks based on soil type and labels based on reference

8.5 Summary and future work

In this section variables which were found to be of importance through literature review and modelling are discussed and the ranges which should be studied are stated and justified. These are specifically for batch sorption studies. After these have been addressed the next step would be to find relationships between batch and column and real field experiments in order to be able to adequately model real-life and draw conclusions with less experiments needed. Steps have been taken towards this in the literature (Manuel Conde-Cid, et al., 2020; Li, et al., 2020), but as identified throughout this chapter more work is still needed. Other variables to consider beyond those explored in this chapter would be biotic vs abiotic degradation (Lin, et al., 2010; Martínez-Hernández, et al., 2016; Mazioti, et al., 2015), contact times beyond equilibrium times, for example exploring 4 week and 6 week contact periods as these are the time periods used for flood irrigation in the Tula Valley (Chávez-Mejía, et al., 2019).

8.5.1 Pollutants

The main gap identified through this modelling exercise was that more data is needed for pollutants which are understudied, such as tramadol. Although pollutants which are extensively studied like carbamazepine and sulfamethoxazole still have unknowns. In particular for sulfamethoxazole the inconsistencies between laboratory and field studies (Kiecak, et al., 2019) and the fact a valid model was not obtained shows the need for more data. Tramadol has not been widely studied, but it has been shown that there are discrepancies between its behaviour in batch test and column tests. In batch tests it was found to be highly mobile in soils, however in column studies it was found to strongly bind to the soil (Wojsławski, et al., 2019).

8.5.2 Equilibrium time between the soil and liquid phase (prior to adding the pollutant)

As shown from the variable importance plot for the multiple component model this variable has a significant effect on $K_{d.}$ The OECD guidelines recommend that an equilibrium period is used (OECD, 2000), but not all studies specify whether it was done(A. Andriamalala, et al., 2018; Chefetz, et al., 2008; Durán-Álvarez, et al., 2012; Hu, et al., 2019; Uddin, et al., 2020; Wojsławski, et al., 2019). My suggestion would be to compare soprtion with and without an equilibration water between the liquid and soil phase. However it is also important to question the extent of the environmental relevance of carrying out an equilibration phase with a buffer liuquid, which is not what will be found in the environment. It will likely be more relevant in terms of an equilibration period between the soil and the actual liquid phase used for irrigation.

8.5.3 Environmentally relevant concentrations

As detailed in section 8.3.2.2 sorption experiments with environmentally relevant concentrations are still needed. In practical terms LODs must be considered. For the method developed for this thesis, the highest iLOD of the pollutants used with either instrument was: 0.18 ngg^{-1} for tramadol, 0.14 ngg^{-1} for carbamazepine: 0.14 and 1.43 ngg^{-1}. for sulfamethoxazole. Considering both, the environmentally relevant concentrations (specified in section 8.3.2.2) and the iLODs for the pollutants, the concentrations to be studied for these pollutants would be for sulfamethoxazole: 1, 2.5, 5, 10, 15 ngg^{-1} and for tramadol and carbamazepine 0.1, 0.5, 2.5, 5, 10 ngg^{-1}.

8.5.4 Biodegradation quenching method

Given that the two biodegradation quenching methods were found to be variables which influence K_d in the multicomponent PLS model, these would require further study. A comparison between sorption in soil sterilized by autoclaving vs soil sterilised using NaN_3 would be carried out for the three pollutants of interest.

8.5.5 Temperature

As a variable not often considered in batch tests a range of 1, 5, 10, 15, 20 and 25, 30 and 35 °C would be studied. The range was chosen to include the range of temperatures in Nottingham (1 - 22°C , with an average of 9.8°C) (Clima-Data.org, n.d.; Met Office, 2016) and range in the Tula Valley 4 - 34 °C (CONAGUA, n.d.) with an average of 16.7°C (Jimenez-Cisneros, et al., 2008).

8.5.6 Liquid phase and untargeted analysis

Some inconsistencies in the literature may be due to metabolites and transformation products. Such as for sulfamethoxazole, which is partly excreted as acetyl-sulfamethoxazole and this pollutant has the potential to retransform to sulfamethoxazole (Martínez-Hernández, et al., 2016). Untargeted analysis would be undertaken in order to compare variables, as done in the PLS analysis in Chapter 6. In this case sorption with buffer liquid phase would be compared to wastewater effluent and influent as liquid phases. This would allow the elucidation of competitive sorption using more realistic liquid phases as well as transformations taking place.

8.5.7 Soil type

Soil type is not a literature gap, however it has a great impact on soil sorption and so a range needs to be included in sorption experiments (OECD, 2000). 3- 5 soils would be used and comparisons between standard soils such as those from LUFA-Speyer (Germany) and field studies, both from the UK, like Bunny filed soils(Atkinson, et al., 2009), and Mexico from the Tula valley would be compared.

9 CONCLUSIONS AND FUTURE WORK

9.1 Conclusions

This thesis contributes to the knowledge in the area of BACs in the agricultural environment in five specific areas, corresponding to the five results chapters, by addressing specific gaps identified from the literature review. This research contributes to knowledge of the presence, fate, and prevalence of BACs in wastewater applied to the agricultural environment and gives indications of specific pollutants requiring further attention in terms of improving treatment technologies to prevent environmental toxicity and/or antimicrobial resistance development in Mexico and the UK.

1. **Environmentally-relevant and practical methods for accurate quantification and flexible detection of BACs in the environment were developed.** These methods address issues of accurate environmental reporting, expense and practicality such as accurate quantification without isotopically labelled standards, as well as simple solid-liquid extraction approaches. This is of particular importance when addressing geographical knowledge gaps, ensuring methods are practical and applicable in rural areas (where most agricultural areas are found) as well as areas with lower economic resources. A particularly important contribution in terms of method development, was studying the effect of ageing on method recoveries used to calculate environmental concentrations of pollutants present in soil matrices following irrigation. For the majority of the pollutants for which ageing was investigated, this aspect had not been previously considered. The importance of considering ageing in method development was demonstrated using sulfamethoxazole to show that the previously reported maximum concentration in an agricultural soil may be underestimated by around 50%, which can have environmental risk consequences and impact to policy and regulation.

2. **Long-term monitoring of two WWTPs has generated valuable data in terms of treatment process removals, risk quotients and correlations.** Significant seasonal variations in removal for Stoke Bardolph WWTP for 12 pollutants were identified, highlighting the importance of considering seasonal variations during wastewater treatment design and implementation. Despite their differences in removal efficiencies, the discharge from both WWTPs have environmental risk coefficients consistently above 1 for the same 8 out of 36 pollutants. Pinpointing specific pollutants which should be further considered in future efforts to improve wastewater treatment as well as legislation for WWTP discharge of BACs. The eight correlations found between pollutants and between pollutants and water quality parameters aid in modelling efforts to predict and understand the fate and prevalence of BACs in the environment and the needs in terms of wastewater treatment improvements.

3. **Untargeted analysis carried out to complement targeted analysis of pollutants.** Identification of potential precursors or transformation products linked to targeted pollutants allow a better understanding of the environmental risk of the targeted pollutants and wastewater treatment efficiency. For example, 4-aminophenol, a toxic biodegradation product of paracetamol was identified in the effluent or downstream water of both treatment plants. Untargeted analysis allowed for the identification of 25 compounds not previously identified in relation to wastewater pollution, including: 2-Amino-6-methylmercaptopurine, active drug metabolite of 6-Thioguanine, a drug used to treat leukaemia, 4-Hydroxyindole a pollutant banned by ASEAN (Association of Southwest Asian Nations) and, 6-methylquinoline, a heterocyclic aromatic hydrocarbon with estrogenic effects. This work also made valuable contributions in terms of public health by identifying two illicit designer drugs not often identified (A-836339 and ADBICA). Also, the identification of biomarkers (cotinine and creatinine) using an untargeted method not designed to identify them in particular, evidences their

stability and amenability for analysis (key requirements of biomarkers). Furthermore, three pollutants with potential disease monitoring attributes were identified, namely adenine, GABA and piperine. Using the PLS metabolomics approach it was possible to compare wastewater treatment methods providing insights into the removal efficiency of two common wastewater treatment process (activated sludge compared to trickling filter + sand filtration) for many BACs which are understudied or have never been studied before.

4. **The sampling campaign undertaken in the Tula Valley, an agricultural region which has been under wastewater irrigation for over 100 years, contributes the bridging of geographical knowledge gaps. Specifically, in terms of pollutants not previously measured or detected in this area.** Four-tert-octylphenol, levonorgestrel, simvastatin and tramadol were quantified for the first time and were found to have environmental risk quotients above one. Similarly, never before quantified in this region, estriol and its sulfate conjugate, were found to contribute to the total estrogenicity which was found to have an environmental risk quotient above one. The detection of pollutants not previously detected in this area included, 1-adamantanamine, a compound used in textiles and some food packaging products and with estrogenic effects, 10-hydroxycarbazepine, active metabolite of oxcarbazepine the benzotriazole 5-methylbenzotriazole, cocaine metabolite benzoylecgonine and the antibiotic ciprofloxacin. Pollutants of particular concern were those quantified or detected in spring groundwater, due to their persistence and because this spring is used as a drinking water source. Five pollutants were quantified in spring water, of which sulfamethoxazole and levonorgestrel were found to have an environmental risk quotient above one. Thirteen pollutants were identified in spring water, including pesticide desethylatrazine, herbicide fomesafen and toxic perfluorinated hydrocarbons PFOS and PFOA. Despite the differences between the Tula Valley and the two UK sites studied, four pollutants were found to have environmental risk quotients above one in both sites, namely diclofenac, erythromycin, trimethoprim and tramadol, pinpointing pollutants of international environmental relevance. Furthermore, four common potential biomarkers were identified in both countries (caffeine, paraxanthine, nicotine and cotinine). This is important in terms of internationally relevant biomarkers.

5. The environmental monitoring and evaluating process efficiency on pollutant remediation (Chapters 5-7, using the methods developed in Chapter 4), informed the identification of three environmentally-relevant and analytically practical pollutants for study following the application of irrigated water to soils. **First model work of BAC sorption in soil using data from across different papers to identify literature gaps and unveil experimental biases**. Results from this modelling show that more data is still needed for understudied pollutants such as tramadol and for pollutants which present inconsistencies across the literature like sulfamethoxazole. Sorption studies are needed that evaluate pollutant fate using environmentally-relevant concentrations. The biodegradation quenching method was found to influence sorption, with sodium azide having a greater influence than autoclaving. This needs to be further studied to avoid affecting BAC soil sorption when aiming to control biodegradation. Soil parameters, such as pH should be reported using standard methods to ensure data is comparable across literature. A balance between ensuring the data is amenable to modelling (comparable with existing literature, obtained using standard methods, etc) and avoiding guideline parameters from preventing wider range data from being obtained must be considered. For example, the effect of temperature is rarely studied in BAC soil sorption studies as guidelines recommend it is studied at room temperature. However, temperature is known to affect BAC soil sorption so it must be taken into account to understand the effect of seasonal and geographical temperature variations. BAC soil sorption studies using treated and untreated liquid phase (realistic

irrigation water) are needed in order to consider realistic environmental conditions. Untargeted analysis of soil and liquid phases in sorption studies could elucidate competitive sorption effects as well as transformation process which take place in the real environment and may account for discrepancies in the literature.

Throughout the results chapters, three important considerations can be highlighted. Firstly, the necessity of environmentally-relevant and practical analytical methods which are applicable worldwide. For instance, it was not possible to undertake full matrix validation for the Mexico samples, but the collaboration allowed for the use of a method which had been thoroughly validated in four different aqueous matrices in the UK. This leads onto the second consideration, the importance of addressing pollution issues with a global perspective. This is largely due to the fact that pollution is not restricted by political borders. Finally, the importance of producing data which is amenable to modelling, as well as modelling existing data to ensure experimental and field work have maximum impact on policy and practice, with minimum resources expenditure (e.g. time, and consumables).

9.2 Future work and Recommendations

Given the findings from the thesis, as well as the literature review, I would recommend that in the Tula Valley water management (including resue of wastewater and treated wastewater for agriculture) continues as the economic and social benefits of this practice currently outweigh the risks from BACs. The expense of removing BACs from the water used for irrigation in the Tula Valley would far outweigh the benefits, which include the presence of nutrients in the wastewater, nutriens would be removed along with BACs if further treatment were to be implemented. However, I strongly believe it is necessary to continue to monitor the situation closely, as it is undeniable that there are negative effects (e.g. antimicrobial resistance and toxicity to aquatic and terrestrial life) given the presence of BACs in the aquatic and agricultural environment. Many unkowns regarding metabolites, new pharmaceuticals being developed, and toxicity remain. For the UK I think there is the potential to consider utilising wastewater effluent for direct irrigation (forgoing river dilution). This is based on long-term raw wastewater irrigation in other areas, including Mexico, proving highliy beneficial and on concentrations of BACs in effluents in the UK being generally lower than those found in irrigation water elsewhere. This practice could protect aquatic life in rivers from BACs and help alleviate water stress in areas of the UK, especially with upcoming weather pattern changes due to climate change. However, I strongly believe more research in terms of sorption to soil and therefore biovaialability of these pollutants to soil biota as well as their leaching potential to groundwater and uptake by crops needs to be further studied.

Further work is required in terms of untargeted analysis to identify metabolites, transformation products and new pollutants not included in current targeted methods. Specifically related to the sites studied in this thesis, more untargeted campaigns with ($n>5$) for each of the two UK WWTPs and PLS analysis to understand the process removal effcicieny for the pollutants identified. Four untargeted campaigns for each of the four seasons would be valuable. Also, it would be valuable to test the effluent of Sutton Bonington WWTP as opposed to downstream water in order to evalute only the removal efficieny of the plant, without the additional dilution effects. Similarily, it would be valuable to add Trent river water, the river onto which Stoke Bardolph WWTP is discharged in order to evalute what is entering the wider environment. In the Tula Valley, a study with distinct date samples ($n \geq 5$) for each location in order to undertake partial least squares (PLS) analysis, would be very valuable. This would help to further pinpoint specific bioactive pollutants for further study in terms of their removal at the Atotonilco WWTP and their fate and persistence throughout the valley. This could be

followed with targeted work to quantify the environmental risk of the pollutants identified in order to establish which BACs need more attention in terms of their presence in the agricultural enviornment. It would be very interesting to compare UK and Mexico samples in one single untargeted run using this method. This data can then be used to continue improving existing wastewater treatment methods as well as identifying pollutants which could present a risk to the agricultural environment, given current and potential future irrigation practices.

In terms of wastewater reuse for irrigation many unknowns remain, and several can be addressed by studying the sorption variables identified in Chapter 8. Experiements targeting pollutants found to be persistent throughout conventional wastewater treatment and environmentally persistent (resistance to biodegrdadation and other natural attenuation processes). Other understudied variables affecting sorption which require study are the effect of temperature, sorption at environmentally-relevant concentrations, using environmentally-sourced liquid phases (e.g. surface water, wastewater effluent and/or untreated as opposed to buffers), and analysing solid and liquid phases using untargeted methods to identify transformation products, metabolites and other pollutants which may be causing competitive sorption. Similar studies can then be carried out using column or lysimeter approaches. The results from this type of sorption experiments, as well as field studies will then inform on the availability of BACs for leaching to groundwater, affecting soil fauna and being uptaken by plants. Many unkowns remain in terms of BACs uptake and translocation in plants, the effects they have in plants and the effect their presence in plants that are then consumed by animals and humans can have.

9.3 Personal reflection

The problem of BACs in the urban water environment can at times seem daunting. There are hundreds of different pollutants entering the environment due to anthropogenic use. Once they enter WWTPs and/or direct to the wider environment they can undergo multiple transformations and there are many ways in which these pollutants can interact and affect living organisms. Throughout this PhD I have learnt the importance of critically evaluating the available information and finding specific ways in which my work can contribute to gaps in our understanding of this issue. The learning process I went through of developing and validating analytical methods which are matrix specific is very valuable in terms of my future career, as it means I can use these skills to address knowledge gaps in other geographical areas and other types of matrices. The collaboration with Mexico allowed for measuring and detecting pollutants not measured in the Tula Valley before and to compare data from two different countries, but more importantly it formed international ties between like-minded scientists, and this has long-term beneficial implications for the field. In a personal level from my PhD what I will take into my future career is the ability to question what has been done, ensuring I can justify all of my decisions and not lose sight of where this research fits into the world and the societal value.

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10 APPENDICES

10.1 Predicted no effect concetrations form literature

Compound	Estrogenic equivalent factor (ngL^{-1})	Reproductive toxicity to aquatic life (ngL^{-1})	PNEC for fish (ngL^{-1})	PNEC for *Daphnia magna* (ngL^{-1})	PNEC for algae (ngL^{-1})	PNEC for invertebrates (ngL^{-1})	Minimum inhibitory concentration[11] (ngL^{-1})
4-tert-octylphenol		330 [3]					
Acetaminophen		9,580[4]	>160,000[13] 378,000[14] 378,000[15]	9,200 [14] 2040[15]	134,000[13]	30,100[14]	
Acetyl-SMX		13,300[4]		,			
Atenolol			1096,000[13]		190,000[13]	205,000[13]	
Bezafibrate			5,300[13] 5,300[14] 26,435[15]	30,000[14] 23,00[15]	18,000[13] 1,352[15]	1,000[13]	
Carbamazepine			35,400 [13]	13,800[14]	31,600[13] 33,600[14]	6,360[13]	
Clofibric acid			53,000[14]	110[14]	86,000[14]		
Codeine		60 [5]					
Diclofenac		1,000[6] 20 [5]	532,000[13] 5,300[14] 50[15]	22,000[14] 20,000[15]	14,500[13] 1,000,000[15]	20,000[13]	
Estrone (E1)	0.47[1]	3 [7] 3.6 [8] 6 [9]					
Estrone-3-sulfate (E1-3S)	0.000012[2]						
Estradiol (E2)	1 [1]	1.52 [10] 0.4 [8]					

Compound	Estrogenic equivalent factor (ngL^{-1})	Reproductive toxicity to aquatic life (ngL^{-1})	PNEC for fish (ngL^{-1})	PNEC for *Daphnia magna* (ngL^{-1})	PNEC for algae (ngL^{-1})	PNEC for invertebrates (ngL^{-1})	Minimum inhibitory concentration[11] (ngL^{-1})
Estradiol-3-sulfate (E2-3S)	0.000017[2]						
Estriol (E3)	0.0076 [1]	60 [9]					
Estriol-3-sulfate (E3-3S)	0.00055 [2]						
Erythromycin		500 [11] 20 [12] 200 [6]	>1,000,000[13]		36.6[13]	22,450[13]	1,000[11]
Gemfibrozil		17,800 [4]	1,180[13] 900[14]	10,400[14] 9,020[14]	4,000[13]	6,000[13]	
Ibuprofen		8,610[4]	5,000[13] 42,000[14] 41,561[15]	102 000[15]	31,5000[13] 4,010[15]	4,000[13]	
Ketoprofen			32,000[13] 264,080[14]		164,000[13] 64,000[14]	2,620[13] 179,455[14]	
Levonorgestrel			0.01(larvae)[16]				
Metoprolol			81,557[13] 31,000[14]	63,900[14]	7,300[13]	9,383[13]	
Morphine		30 [5]					
Naproxen		1,570,000 [4]	193,337[13]	166,300[14]	137944[13] 625,500[14]	15,000[13]	
O-desmethylvenlafaxine							
Oxytetracycline		18,000[11]					500[11]
Paroxetine			3,864[14]	580[14]	140[14]		
Phenazone			3,000[13] 5,781[14]	36,797[14]	1100[13] 1346[14]	6,700[13]	
Propranolol			29,500[13] 1,210[14]	460[14]	550[13] 6680[14]	2,700[13]	
Salicylic acid		82,500 [4]	37,000[13]	118,000[14]	>100000[13]	2,040[13]	

					233760[14]		
Compound	**Estrogenic equivalent factor (ngL^{-1})**	**Reproductive toxicity to aquatic life (ngL^{-1})**	**PNEC for fish (ngL^{-1})**	**PNEC for *Daphnia magna* (ngL^{-1})**	**PNEC for algae (ngL^{-1})**	**PNEC for invertebrates (ngL^{-1})**	**Minimum inhibitory concentration[11] (ngL^{-1})**
Simvastatin			765,000[15]	3.2[15]	22 800[15]		
Sulfadiazine		720,000 **[11]**		10,000[17]	2200[13]	221,000[13]	N/A**[11]**
Sulfamethoxazole		600 [11] 27 [12]	>750,000[13] 5,62,500[17] 1,516,102[14]	25,200[14]	1540[13] 30[14]	189,200[13]	16,000[11]
Sulfapyridine							
Tramadol			130[14]	73[14]	959[14]		
Trimethoprim		100,000 [11] 50 [12]	255,000[17] >100,000 317,910[14]	8,210 [14]	110000[13] 16000[14]	120,700[13]	500**[11]**

Figure 10.1-1 Predicted no effect concentrations from the literature

Empty cells mean values could not be found in the literature. N/A means not available, as specified in the literature.
Superscripts refer to the following references: **1**(Ma, et al., 2016), **2** (Tremblay, et al., 2018), **3** (Quednow, et al., 2008), **4** (Han, et al., 2017), **5** (Daouk, et al., 2016), **6** (Kötke, et al., 2019), **7** (Burkhardt-Holm, 2010), **8** (Loos, et al., 2018), **9** (Fekadu, et al., 2019), **10** (Huang, et al., 2019), **11** (AMR Industry Alliance, 2018), **12** (Phonsiri, et al., 2019), **13** (Biel-Maeso, et al., 2017), **14** (Thomaidi, et al., 2015), **15** (Pereira, et al., 2017), **16** (Yu, et al., 2019b), **17** (Nguyen Dang Giang, et al., 2015)

10.2 Matrix effects

As detailed in section 3.4.1.2, matrix effects were quantified using the following equation:

$$Matrix\ effects\ (\%) = \frac{(Post\ spiked - Blank\ matrix) - Calibration\ point}{Calibration\ point} \times 100$$

Where the post spiked sample is spiked on reconstitution (immidetaly prior to LC-MS injection) and at the same concnetration as the calibration point used in the equation. This concnetration is specified for each instrument in the Tables 10.2.1- 10.2.3. Blank matrix is the non-spiked sample, it is subtracted from the post spiked sample in order to remove the signal for the ocmpound inherently present in the matrix.

Analyte	Matrix effects on triple quadrupole (QqQ) (%) evaluated at 200 ngmL^{-1}			
	Wastewater influnet Stoke Bardolph	Wastewater effluent Stoke Bardolph	Wastewater influent Sutton bonington	Downstream wastewater Sutton Bonington
4-Tertoctylphenol	-14.4 ± 0.8	6.5 ± 0.1	-6.4 ± 0.1	1.9 ± 0.02
Acetaminophen	-740.9 ± 126.5	-45.4 ± 10.1	597.4 ± 12.7	-17.6 ± 4.2
Acetyl-sulfamethoxazole	-62.3 ± 22.2	-58.3 ± 3.7	-52.8 ± 2.4	-38.7 ± 1.5
Amidotrizoic acid	-70.3 ± 22.2	-41.0 ± 0.2	-28.9 ± 1.0	-19.9 ± 0.9
Atenolol	-82.5 ± 2.9	-87.2 ± 4.6	-78.9 ±2.4	-76.1 ± 3.8
Bezafibrate	-109.8 ± 1.7	-52.9 ± 1.2	-44.1 ± 10.1	-53.4 ± 1.5
Carbamazepine	-80.8 ± 13.1	-49.2 ± 1.3	-56.7 ± 4.2	-33.2 ± 1.7
Clofibric acid	13.4 ± 0.6	12.8 ± 0.04	19.8 ± 0.3	7.6 ± 0.1
Codeine	-96.2 ± 28.6	-66.0 ± 3.5	-74.1 ± 4.8	-70.3 ± 7.3
Diclofenac	-41.6 ± 0.8	-36.3 ±1.2	-29.4 ± 1.9	-31.2 ± 1.5
Ethinyl estradiol	-57.5 ± 5.7	-5.8 ± 0.2	-41.7 ± 4.0	-12.3 ± 0.3
Erythromycin	-99.7 ± 18.9	-79.3 ± 3.9	-99.0 ± 5.1	-73.2 ± 12.2
Estradiol	-49.6 ± 2.7	-0.6 ± 0.01	-38.4 ± 0.9	-3.8 ± 0.1
Estraiol-3-sulfate	-94.2 ± 7.0	-89.5 ± 4.5	-87.5 ± 8.3	-75.7 ± 2.6
Estriol	-61.9 ± 7.1	-55.7 ± 4.8	-60.4 ± 6.3	-40.0 ± 2.5
Estriol-3-sulfate	-96.1 ± 1.5	-94.2 ± 3.1	-45.2 ± 47.1	-101.5 ± 77.8
Estrone	-88.0 ± 6.6	-84.3 ± 1.6	-77.1 ± 6.3	-64.6 ± 1.5
Estrone-3-sulfate	-87.1 ± 38.2	-83.6 ±3.4	-75.5 ± 7.9	-63.5 ± 14.7
Gemfibrozil	-12.9 ± 0.6	-7.6 ± 8.3	-12.3 ± 0.3	-16.8 ± 0.2
Ketprofen	-73.6 ± 25.8	-67.7 ± 12.2	-55.8 ± 0.6	-43.0 ± 1.3
Levonorgestrel	-50.7 ± 9.1	-48.2 ± 0.9	-21.6 ± 2.8	-30.0± 2.1
Metoprolol	-91.0 ± 29.2	-65.2 ± 3.8	-72.5 ± 7.2	-46.0 ± 4.0
Morphine	-95.0 ± 3.8	-95.2 ± 3.9	-93.5 ± 18.1	-93.3 ± 12.0
Naproxen	-97.4 ± 3.2	-96.7 ± 14.3	-96.2 ± 9.4	-89.7 ± 11.2
Oxytetracycline	29.1 ± 6.8	134.2 ± 3.8	142.5 ± 8.9	184.2 ± 3.0
Paroxetine	-91.6 ± 18.7	-79.4 ± 5.5	-82.1 ± 1.2	-71.2 ± 11.0
Phenazone	-83.5 ± 9.3	-66.2 ± 12.0	-70.6 ± 17.3	-45.5 ± 1.4
Propranolol	-90.0 ± 10.8	-75.6 ± 1.8	-82.2 ± 2.0	-56.1 ± 2.9
Salicylic Acid	-183.6 ± 47.8	-10.2 ± 2.8	-34.2 ± 9.1	37.6 ± 11.9
Simvastatin	-16.3 ± 8.1	-28.2 ± 2.1	-22.5 ± 1.8	-20.0 ± 0.8

Analyte	Wastewater influnet Stoke Bardolph	Wastewater effluent Stoke Bardolph	Wastewater influent Sutton bonington	Downstream wastewater Sutton Bonington
Sulfadiazine	-74.3 ± 9.0	-43.8 ± 2.4	-45.0 ± 9.1	-10.7 ± 0.2
Sulfamethoxazole	-77.1 ± 8.7	-59.5 ± 4.7	-53.7 ± 2.3	-37.1 ± 1.9
Sulfapyridine	-69.2 ± 14.4	-49.3 ± 10.7	-43.9 ± 12.2	-25.4 ± 12.3
Tramadol	-99.9 ± 21.8	-76.9 ± 3.6	-81.9 ± 0.6	-56.2 ± 4.3
Trimethoprim	-92.0 ± 17.8	-93.6 ± 3.9	-86.1 ± 1.9	-66.5 ± 2.6

Table 10.2-1 Matrix effects on triple quadrupole (QqQ) for UK matrcies stuided

Analyte	Matrix effects on linear ion trap (QTrap) (%) evaluated at 200 ngmL^{-1}	
	Wastewater influnet Stoke Bardolph	Wastewater effluent Stoke Bardolph
4-Tertoctylphenol	-17.8 ± 3.9	-7.5 ± 2.1
Acetaminophen	-290.2 ± 8.0	-42.4 ± 5.5
Amidotrizoic acid	-51.4 ± 29.3	-50.9 ± 22.9
Atenolol	-70.8 ± 4.4	-53.2 ± 2.3
Bezafibrate	5.3 ± 0.4	-3.9 ± 0.4
Carbamazepine	27.0 ± 1.0	45.0 ± 1.8
Clofibric Acid	37.4 ± 6.3	16.9 ± 4.5
Codeine	-55.8 ± 5.7	-62.6 ± 1.7
Diclofenac	7.2 ± 0.9	-15.9 ± 2.4
E1	-2.1 ± 0.3	-0.2 ±0.1
E1-3S	-1.5 ± 0.2	-10.1 ±2.0
E2	-17.1 ± 3.7	-20.0 ± 15.8
E2-3S	46.1 ± 16.7	24.8 ± 5.7
E3	0.7 ± 0.04	16.4 ± 4.3
E3-3S	14.0 ± 6.5	-0.4 ± 0.3
EE2	-26.7 ± 11.5	1.1 ± 0.3
Erythromycin	-50.1 ± 14.5	-77.8 ± 5.9
Gemfibrozil	23.0 ± 2.4	11.7 ± 6.2
Ibuprofen	NA	-17.6 ± 1.3
ketoprofen	14.9 ± 3.5	-10.4 ± 1.3
Levonorgestral	-83.4 ± 31.2	-54.1 ± 33.8
Metoprolol	-57.3 ± 15.9	-53.7 ± 27.9
Morphine	-80.1 ± 9.8	-51.7 ± 4.5
Naproxen	-169.8 ± 3.0	11.8 ± 1.4
Oxytetracycline	-544.8 ± 135.7	-118.5 ± 51.3
Paroxetine	-24.6 ± 5.2	-23.4 ±10.4
Phenazone	-53.9 ± 1.8	-30.2 ± 9.1
Propranolol	-44.5± 5.0	-45.3 ± 1.6
Simvastatin	3.2 ± 0.6	31.0 ± 7.6
Sulfadiazine	-34.0 ± 6.0	-31.6 ± 6.2
Sulfamethoxazole	-55.3 ± 2.9	-54.9 ± 4.6
Sulfapyridine	-41.4 ± 9.2	-59.7 ± 15.5

Analyte	Wastewater influnet Stoke Bardolph	Wastewater effluent Stoke Bardolph
Tramadol	-432.8 ± 41.7	40.0 ± 1.1
Trimethoprim	-54.5 ± 6.3	-54.3 ± 3.0

Table 10.2-2 Matrix effects on quadrupole ion trap (QTrap) for UK matrcies stuided

Analyte	Matrix effects (%) on QExactive instrument				Ratios			
	Stoke Bardolph wastewater treatment plant		Sutton Bonington wastewater treatment plant					
	Wastewater influent (WWI Stk)	*Wastewater effluent (WWE Stk)*	*Wastewater influent (WWI Stk)*	*Dowstream water (WWD SuB)*	*WWE Stk/WWI Stk*	*WWE Stk/ WWD SuB*	*WWD SuB/WWI SuB*	*WWI Stk/ WWISuB*
Acetylsulfamethoxazole	74.0±24.5	32.5±2.4	29.8±4.5	52.6±12.2	0.4	0.6	1.8	2.5
Atenolol	78.4±3.5	-11.1±1.0	47.2±3.4	1.5±0.1	-0.1	-7.6	0.0	1.7
Bezafibrate	24.8±2.4	45.1±6.3	53.0±10.6	62.2±4.5	1.8	0.7	1.2	0.5
Carbamazepine	-45.0±2.5	-40.8±2.6	-42.9±6.0	-14.2±0.5	0.9	2.9	0.3	1.0
Clofibric acid	148.8±50.3	88.3±26.1	73.6±16.8	104.4±28.6	0.6	0.8	1.4	2.0
Codeine	101.4±6.3	105.0±10.1	85.2±5.9	84.4±3.8	1.0	1.2	1.0	1.2
Diclofenac	-54.4±9.3	-50.0±4.4	-33.0±5.5	-26.7±3.6	0.9	1.9	0.8	1.6
Erythromycin	-15.9±1.6	56.5±5.3	32.7±3.5	74.3±8.6	-3.5	0.8	2.3	-0.5
Estradiol	18.9±1.1	137.2±42.1	81.1±7.5	125.7±32.8	7.3	1.1	1.6	0.2
Ethinylestradiol	-32.7±13.5	129.7±10.5	46.7±3.4	130.5±8.0	-4.0	1.0	2.8	-0.7
Gemfibrozil	71.1±5.1	55.6±4.1	181.1±48.1	100.4±10.0	0.8	0.6	0.6	0.4
Ketoprofen	-38.3±3.1	-30.8±5.0	-5.9±1.1	15.7±1.6	0.8	-2.0	-2.6	6.5
Levonorgestrel	93.9±53.8	77.5±6.9	81.3±12.4	113.9±62.3	0.8	0.7	1.4	1.2
Metoprolol	6.4±0.9	16.9±1.4	33.3±4.6	75.4±12.7	2.6	0.2	2.3	0.2
Morphine	-76.1±12.7	-45.0±5.6	-51.0±14.8	-45.1±8.3	0.6	1.0	0.9	1.5

Analyte	Matrix effects (%)on Qexactive instrument				Ratios			
	Stoke Bardolph wastewater treatment plant		Sutton Bonington wastewater treatment plant					
	Wastewater influent (WWI Stk)	*Wastewater effluent (WWE Stk)*	*Wastewater influent (WWI Stk)*	*Dowstream water (WWD SuB)*	*WWE Stk/WWI Stk*	*WWE Stk/ WWD SuB*	*WWD SuB/WWI SuB*	*WWI Stk/ WWISuB*
O-Desmethylvenlafaxine	-100.0±8.1	-100.3±71.2	-100.0±13.2	-101.2±68.0	1.0	1.0	1.0	1.0
Paracetamol	-599.6±14.4	83.3±12.1	-836.4±196.4	90.4±23.7	-0.1	0.9	-0.1	0.7
Paroxetine	-6.7±0.2	5.7±1.9	-9.4±1.6	19.2±12.3	-0.9	0.3	-2.0	0.7
Phenazone	-54.6±2.7	-22.5±4.0	-46.2±16.7	7.4±0.5	0.4	-3.0	-0.2	1.2
Propranolol	73.3±5.2	27.3±1.3	-36.2±4.8	63.7±4.0	-0.4	0.4	-1.8	2.0
Simvastatin	72.5±7.2	-23.1±6.3	-59.2±6.7	16.3±2.1	0.3	-1.4	-0.3	1.2
Sulfamethoxazole	33.4±2.0	33.0±7.1	32.3±4.5	68.5±3.4	1.0	0.5	2.1	1.0
Sulfapyridine	4.7±0.7	15.6±2.9	6.6±2.1	41.6±20.4	3.3	0.4	6.3	0.7
Sulfapyridine	-23.2±1.4	-15.0±3.6	12.2±2.4	47.6±21.9	0.6	-0.3	3.9	-1.9
Tramadol	15.9±*1.8*	41.2±3.3	3.5±1.1	76.0±9.7	2.6	0.5	21.6	4.5
Trimethoprim	53.9±*4.5*	27.2±4.3	82.7±5.0	79.6±8.3	0.5	0.3	1.0	0.7

Table 10.2-3 Matrix effects on Qexactive instrument

10.3 Water quality parameters during the sampling campaign at Sutton Bonington wastewater treatment plant

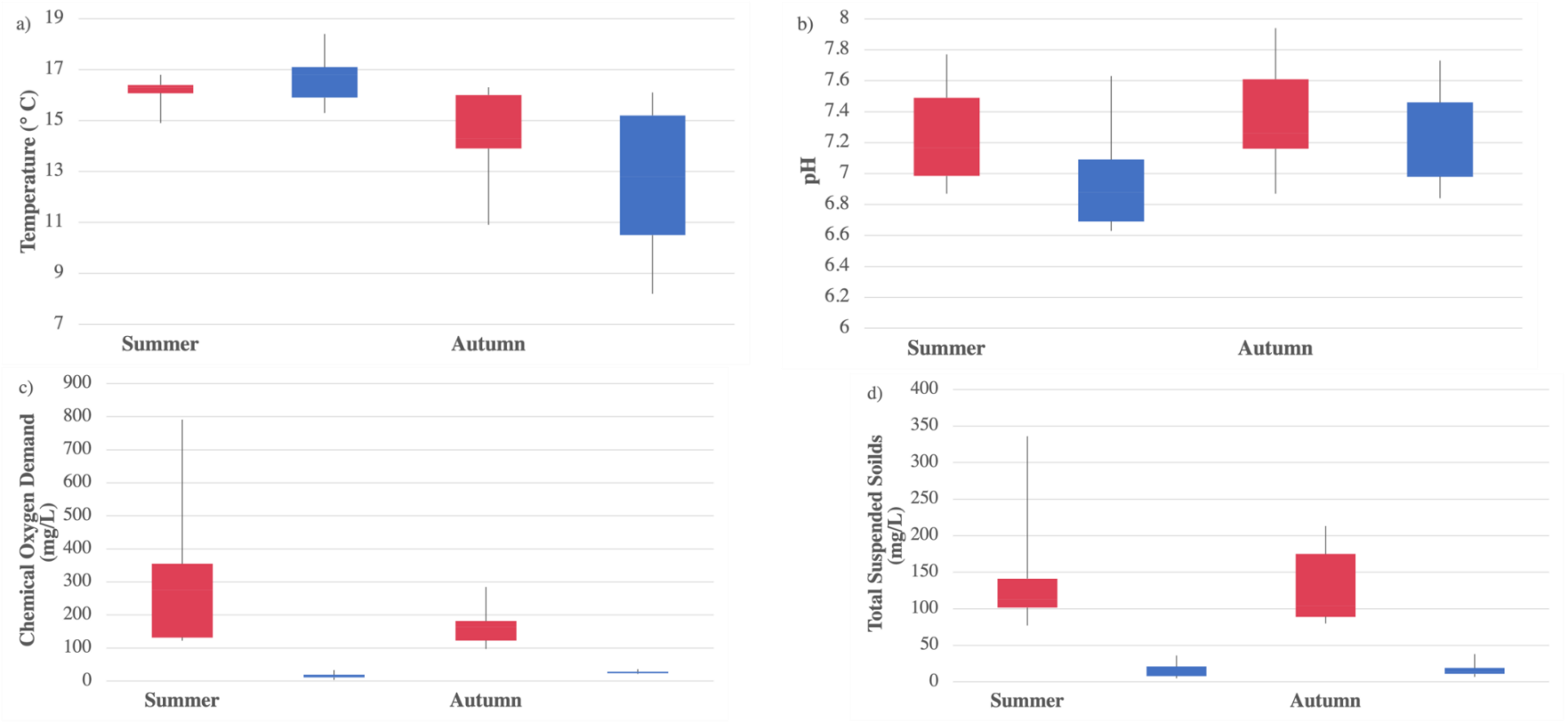


Figure 10.3-1 Water quality paremeters measured during the sampling campaing at Sutton Bonington wastewater treatment plant

10.4 UK samples mass spectrums obtained for compounds not previously identified in the aquatic environment

In all figures in this appendix, the part above is the sample spectra and below is the refrence from the *mzCloud*. All the spectrums are from run c, unless otherwise specified. Green spectra refer to fragments (or the parent ion) which matched with the refrence standard and red ones are those which did not match.

10.4.1 Spectrums for compounds not previously identified in relation to wastewater pollution

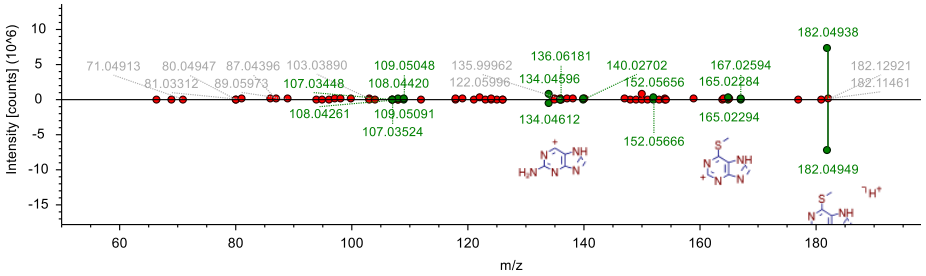


Figure 10.4-1 Obtained (top) and reference MzCloud (bottom) spectrum for 2-Amino-6-methylmercaptopurine in basic chromatography mode

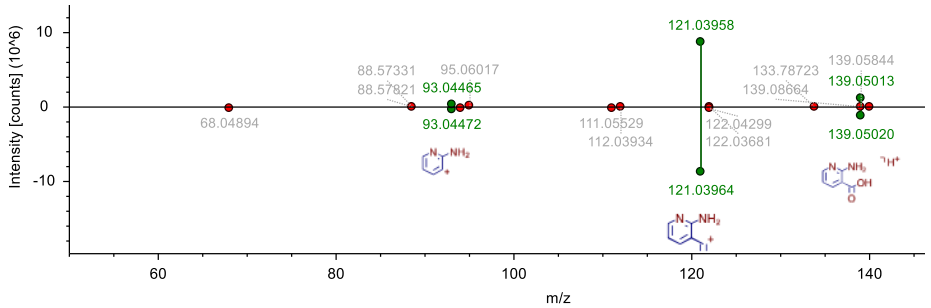


Figure 10.4-2 Obtained (top) and reference MzCloud (bottom) spectrum for 2-aminonicotinic acid in basic chromatography

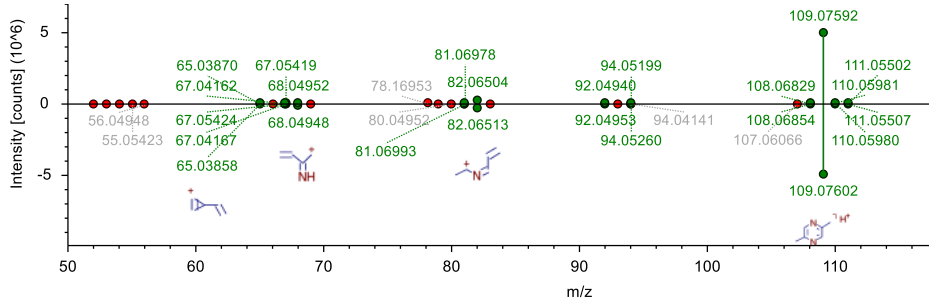


Figure 10.4-3 Obtained (top) and reference MzCloud (bottom) spectrum for 2,5-Dimethylpyrazine in basic chromatography

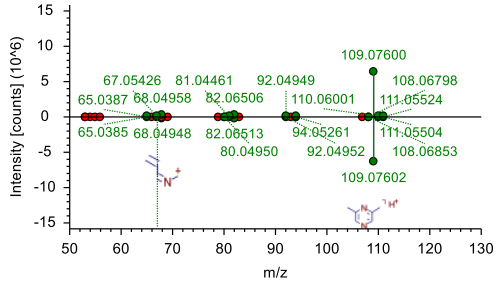


Figure 10.4-4 Obtained (top) and reference MzCloud (bottom) spectrum for 2,6-Dimethylpyrazine in acidic chromatography for run a

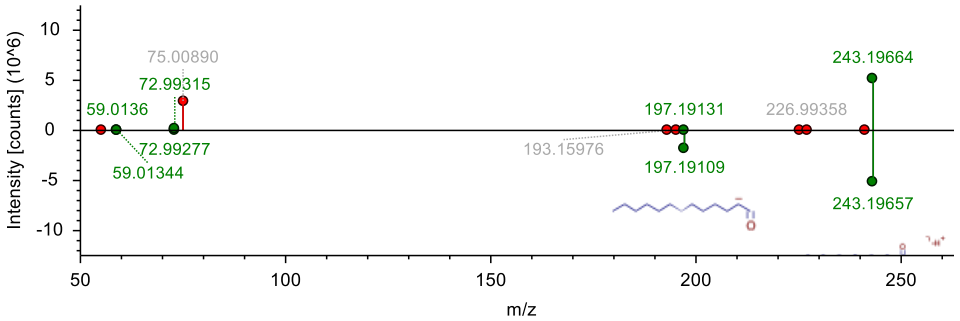


Figure 10.4-5 Obtained (top) and reference MzCloud (bottom) spectrum for 2-Hydromyristic in acidic chromatography

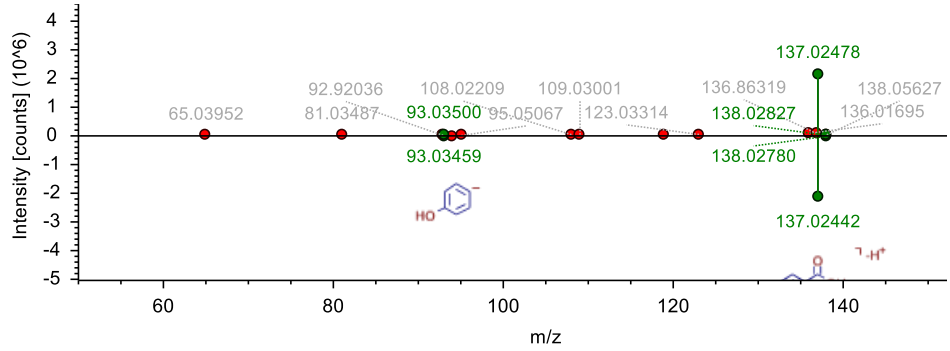


Figure 10.4-6 Obtained (top) and reference MzCloud (bottom) spectrum for 4-Hydroxybenzoic acid in acidic chromatography

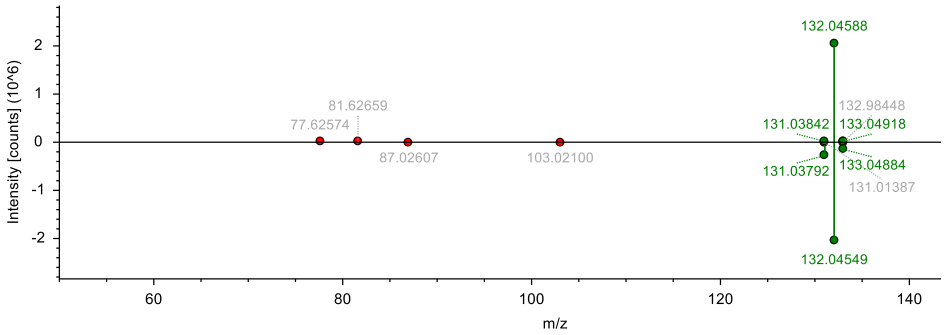


Figure 10.4-7 Obtained (top) and reference MzCloud (bottom) spectrum for 4-hydroxyindole in basic chromatography

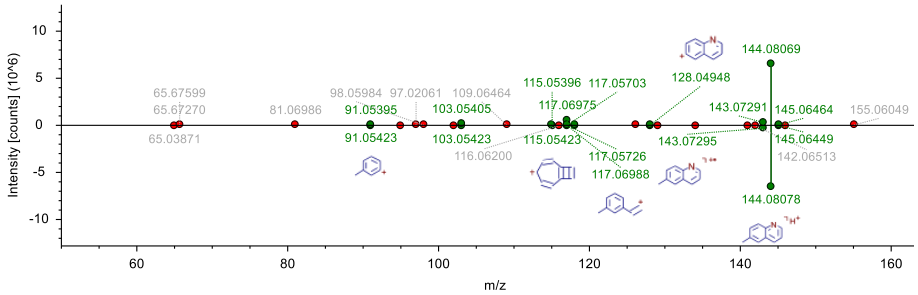


Figure 10.4-8 Obtained (top) and reference MzCloud (bottom) spectrum for 6-methylquinoline in basic chromatography

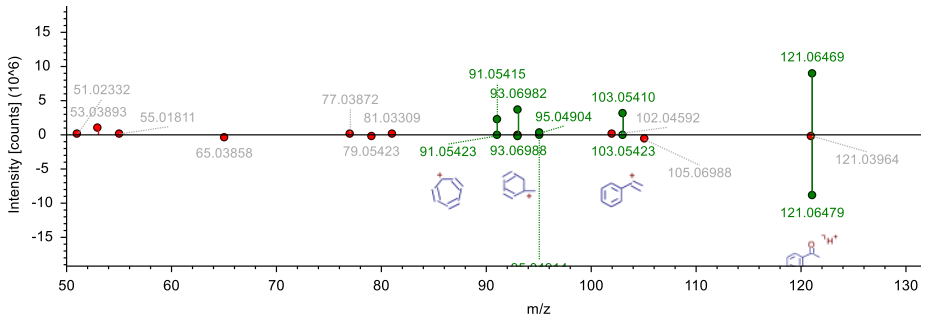


Figure 10.4-9 Obtained (top) and reference MzCloud (bottom) spectrum for acetophenone in basic chromatography

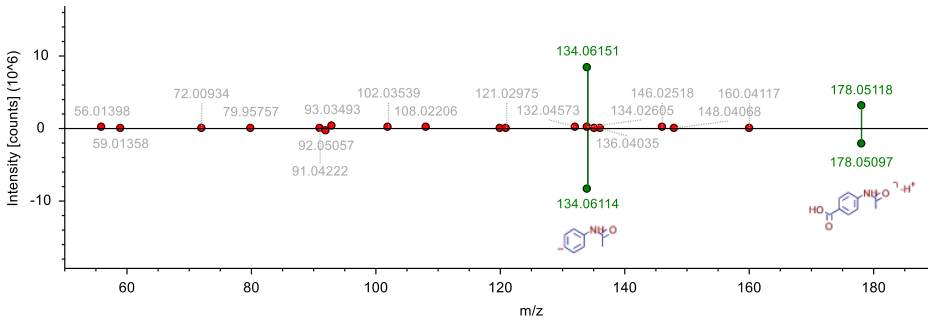


Figure 10.4-10 Obtained (top) and reference MzCloud (bottom) spectrum for acedoben (4-acetamidobenzoic acid) for basic chromatography

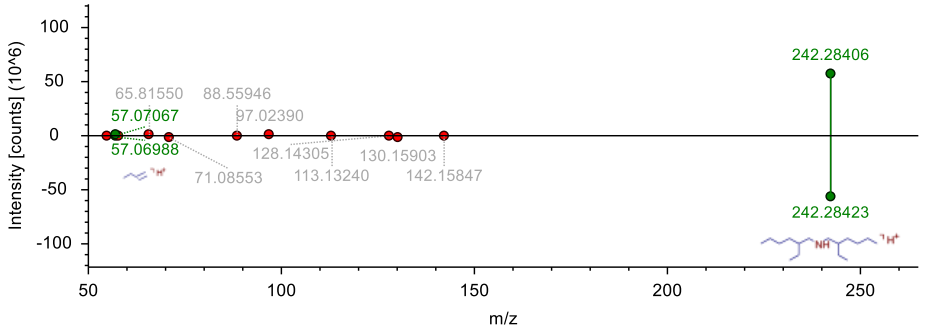


Figure 10.4-11 Obtained (top) and reference MzCloud (bottom) spectrum for bis(2-ethylhexyl) amine for acidic chromatography

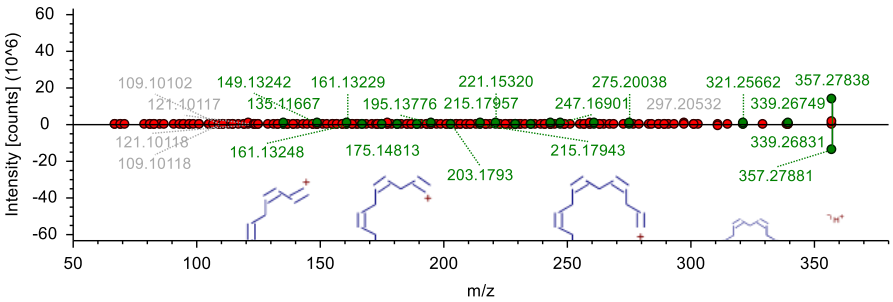


Figure 10.4-12 Obtained (top) and reference MzCloud (bottom) spectrum for docosahexaenoic acid ethyl ester for acidic chromatography

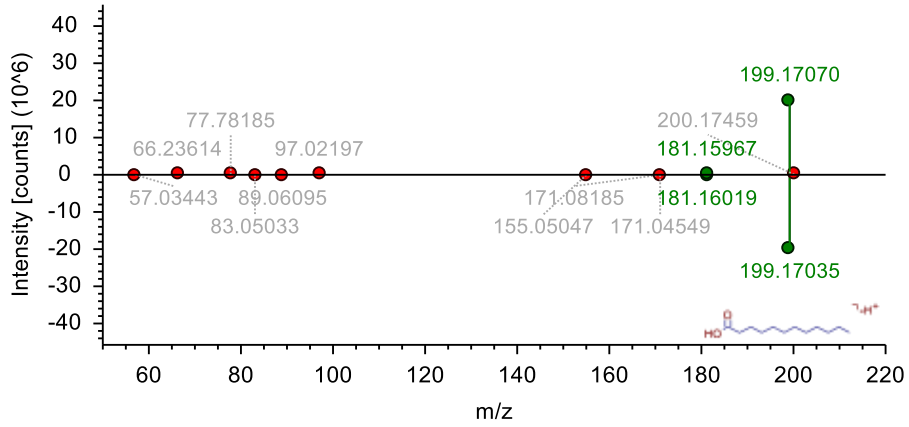


Figure 10.4-13 Obtained (top) and reference MzCloud (bottom) spectrum for lauric acid for basic chromatography

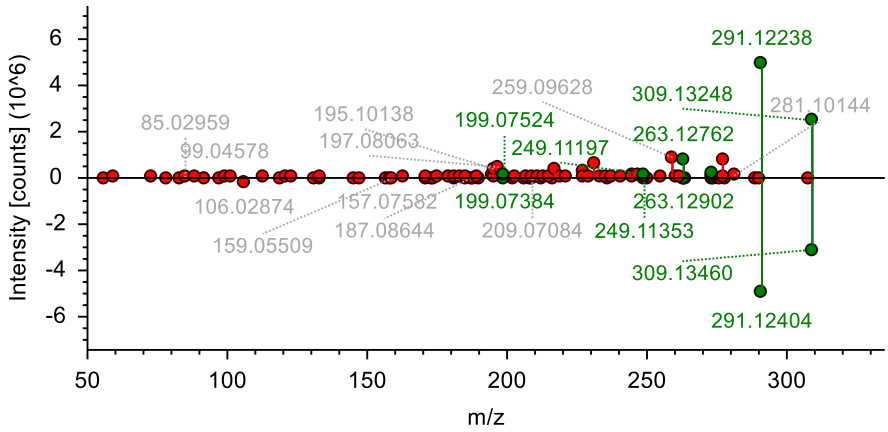


Figure 10.4-14 Obtained (top) and reference MzCloud (bottom) spectrum for nifenazone for basic chromatography

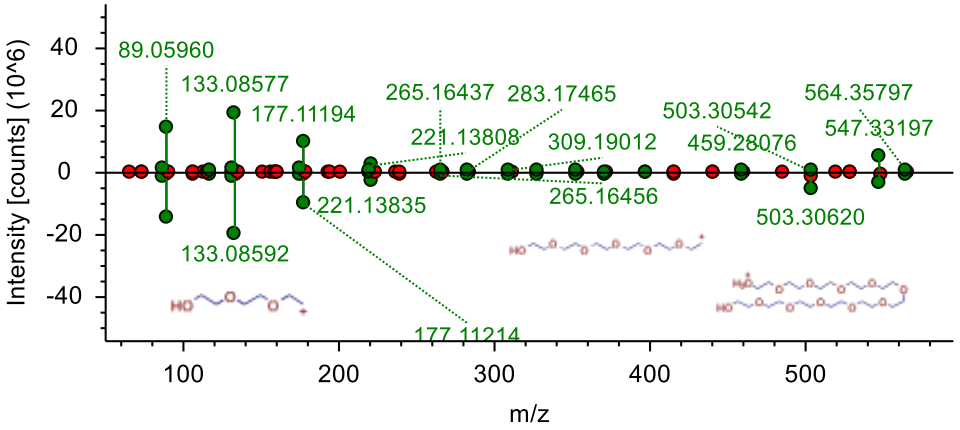


Figure 10.4-15 Obtained (top) and reference MzCloud (bottom) spectrum for PEG n12 for basic chromatography

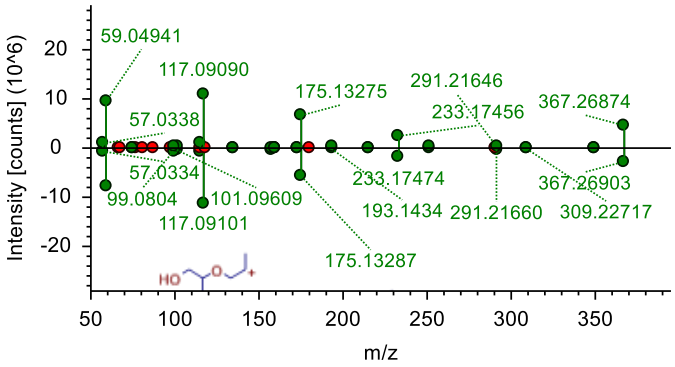


Figure 10.4-16 Obtained (top) and reference MzCloud (bottom) spectrum for PPG n6 for acidic chromatography in run a

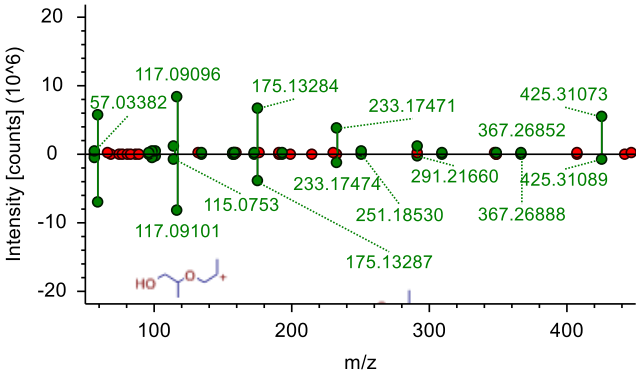


Figure 10.4-17 Obtained (top) and reference MzCloud (bottom) spectrum for PPG n7 for acidic chromatography in run a

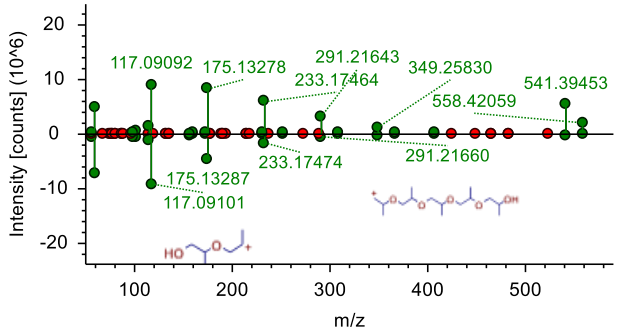


Figure 10.4-18 Obtained (top) and reference MzCloud (bottom) spectrum for PPG n9 for acidic chromatography in run a

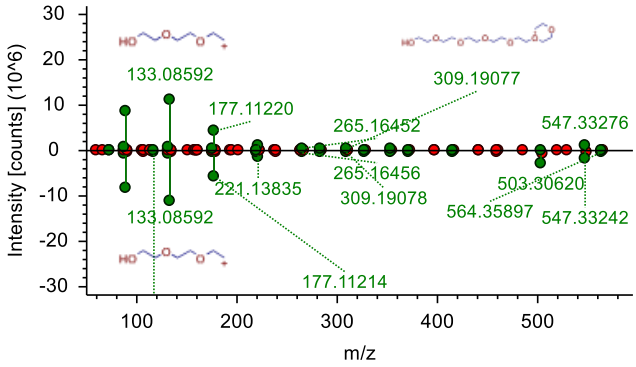


Figure 10.4-19 Obtained (top) and reference MzCloud (bottom) spectrum for PPG n12 for basic chromatography in run a

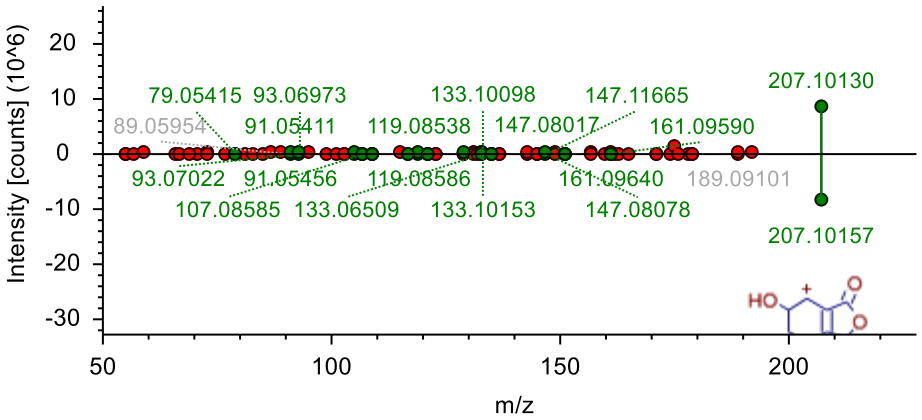


Figure 10.4-20 Obtained (top) and reference MzCloud (bottom) spectrum for Senkyunolide H for basic chromatography

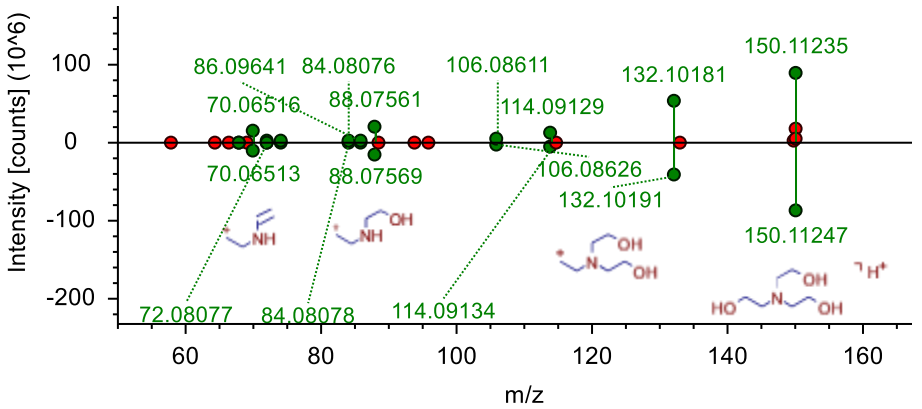


Figure 10.4-21 Obtained (top) and reference MzCloud (bottom) spectrum for triethanolamine for basic chromatography

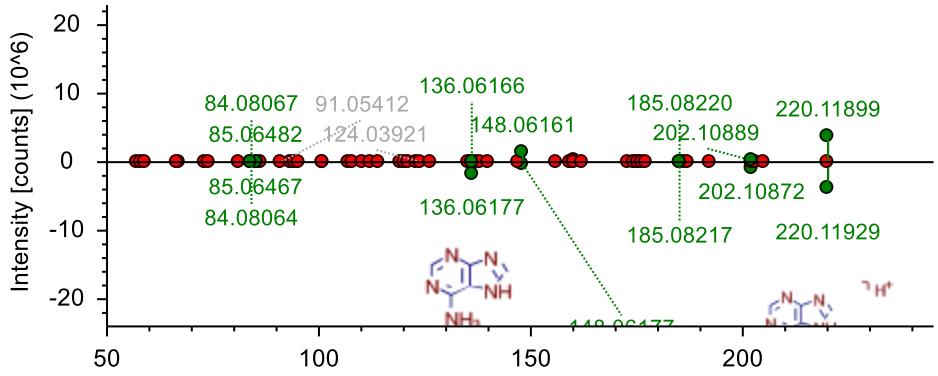


Figure 10.4-22 Obtained (top) and reference MzCloud (bottom) spectrum for zeatin for basic chromatography

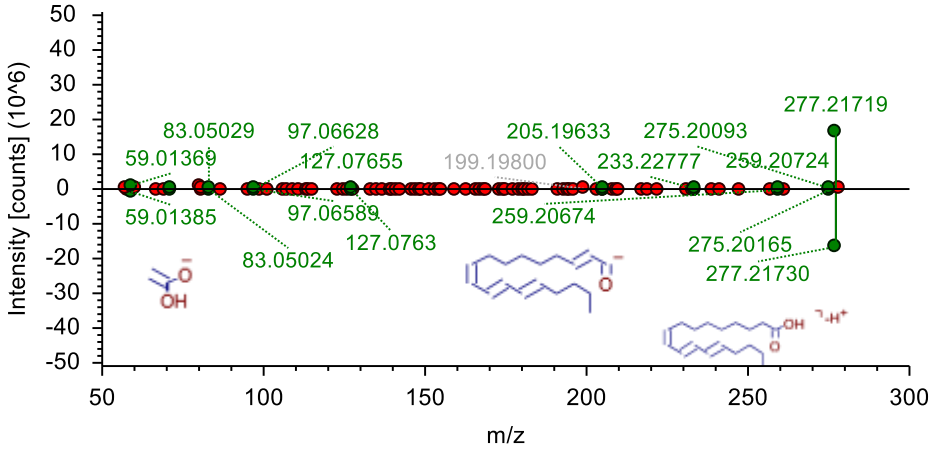


Figure 10.4-23 Obtained (top) and reference MzCloud (bottom) spectrum for α-Eleostearic acid for basic chromatography

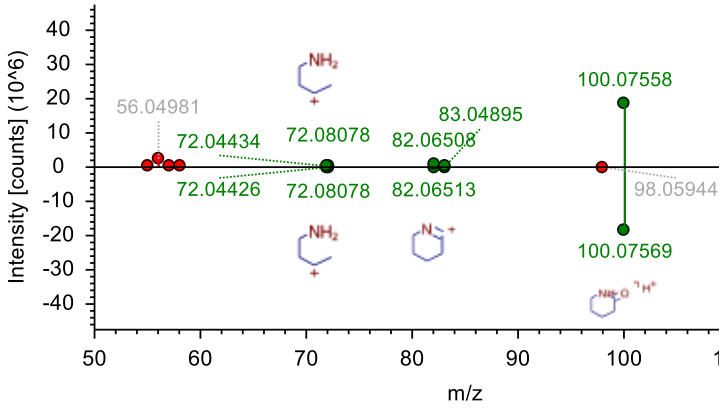


Figure 10.4-24 Obtained (top) and reference MzCloud (bottom) spectrum for δ-valerolactam for basic chromatography

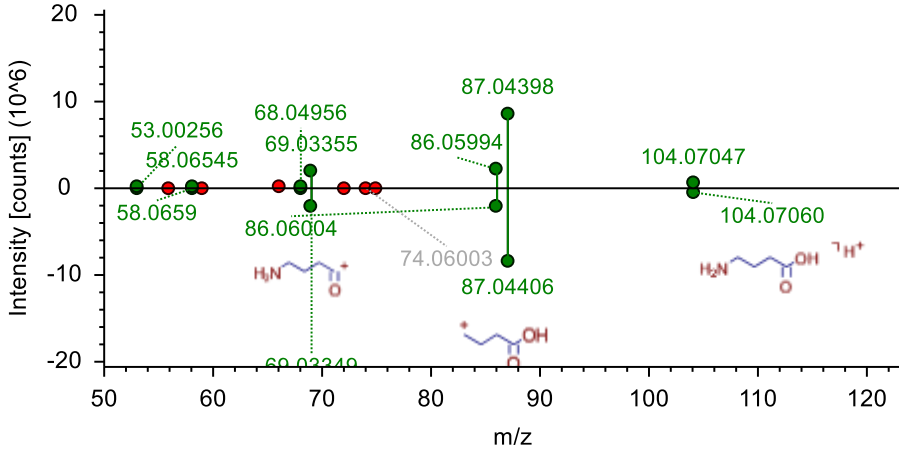


Figure 10.4-25 Obtained (top) and reference MzCloud (bottom) spectrum for Gamma-aminobutyric acid (GABA) for basic chromatography

10.4.2 Spectrums for compounds identified which have been rarely identified in relation to wastewater

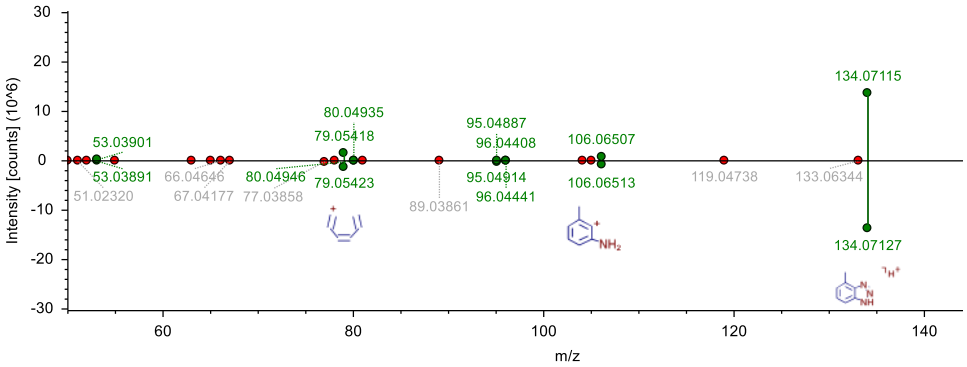


Figure 10.4-26 Obtained (top) and reference MzCloud (bottom) spectrum for4-methylbennzotriazole in basic chromatography

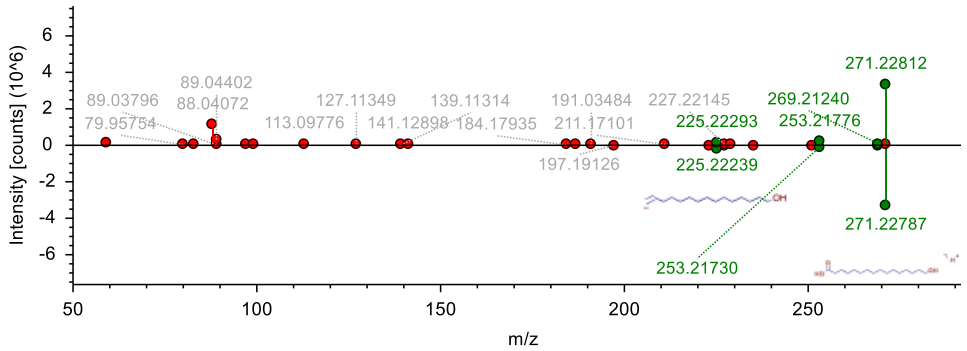


Figure 10.4-27 Obtained (top) and reference MzCloud (bottom) spectrum for 16-hydroxydecanoic aicd in acidic chromatography run a

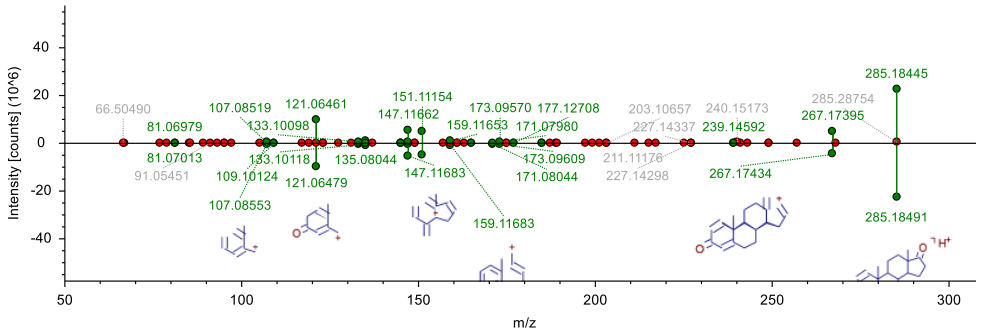


Figure 10.4-28 Obtained (top) and reference MzCloud (bottom) spectrum for boldione in basic chromatography

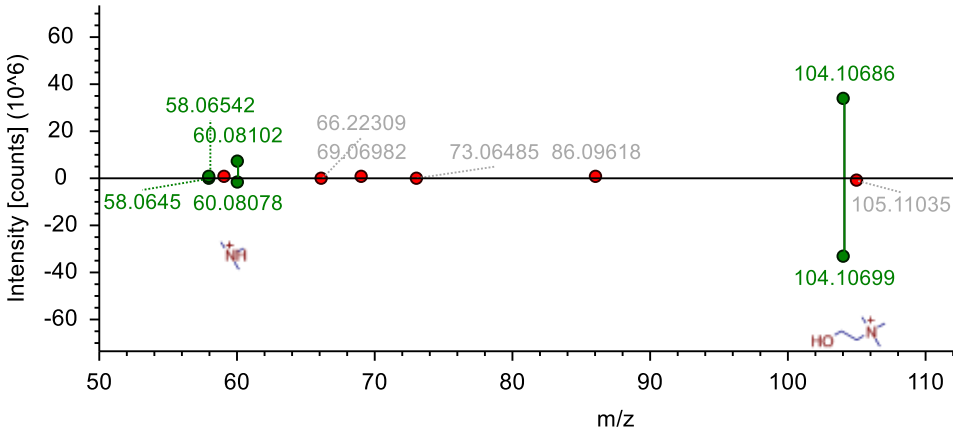


Figure 10.4-29 Obtained (top) and reference MzCloud (bottom) spectrum for choline in basic chromatography

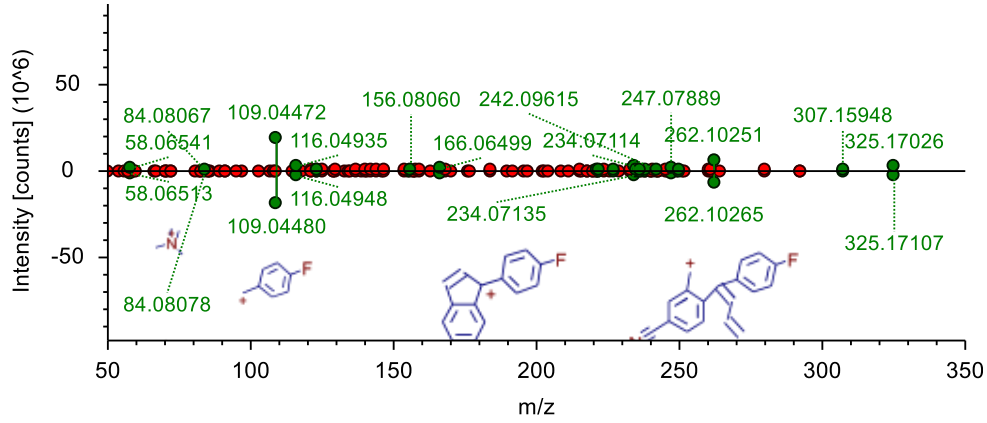


Figure 10.4-30 Obtained (top) and reference MzCloud (bottom) spectrum for escitalopram in basic chromatography

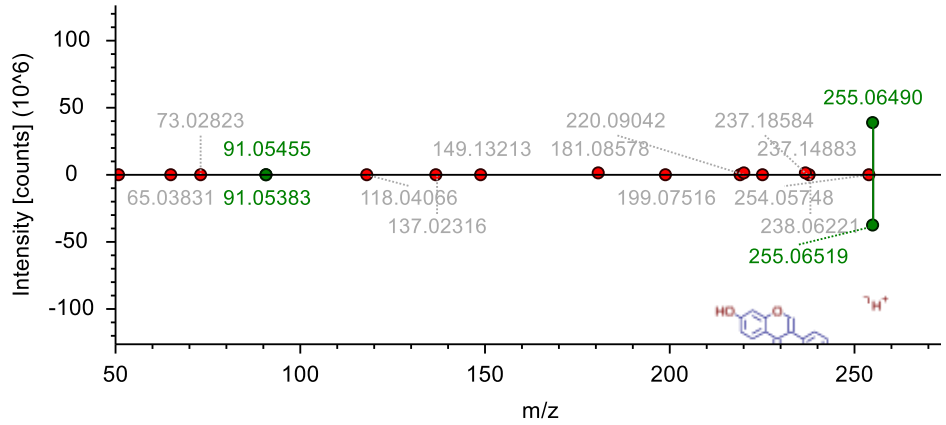


Figure 10.4-31 Obtained (top) and reference MzCloud (bottom) spectrum for daidezin in basic chromatography

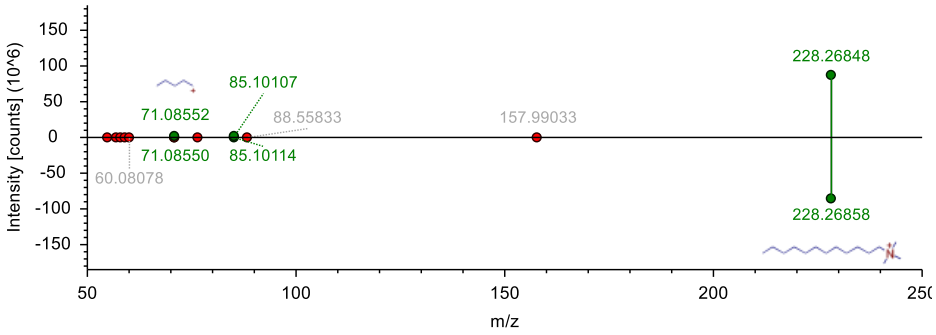


Figure 10.4-32 Obtained (top) and reference MzCloud (bottom) spectrum for dodecyl triethylammonium in acidic chromatography

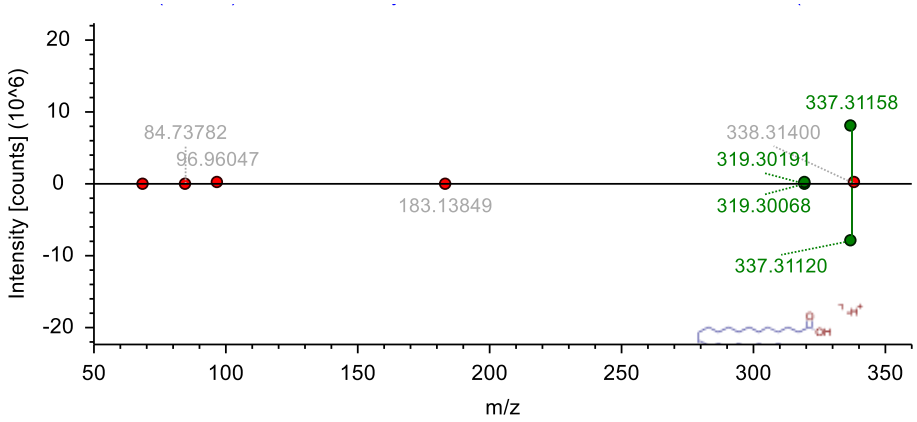


Figure 10.4-33 Obtained (top) and reference MzCloud (bottom) spectrum for erucic acid in basic chromatography

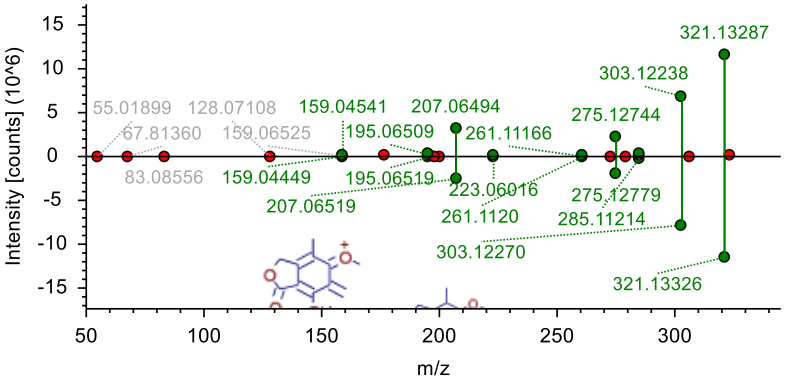


Figure 10.4-34 Obtained (top) and reference MzCloud (bottom) spectrum for mycophenolic acid in basic chromatography

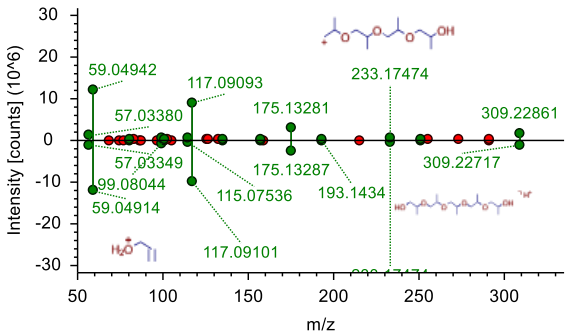


Figure 10.4-35 Obtained (top) and reference MzCloud (bottom) spectrum for PPG n5 in acidic chromatography in run a

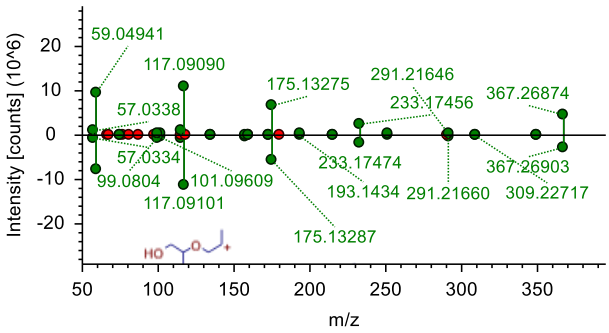


Figure 10.4-36 Obtained (top) and reference MzCloud (bottom) spectrum for PPG n6 in acidic chromatography in run a

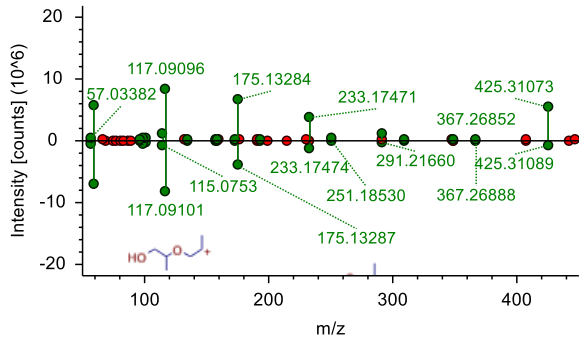


Figure 10.4-37 Obtained (top) and reference MzCloud (bottom) spectrum for PPG n7 in acidic chromatography in run a

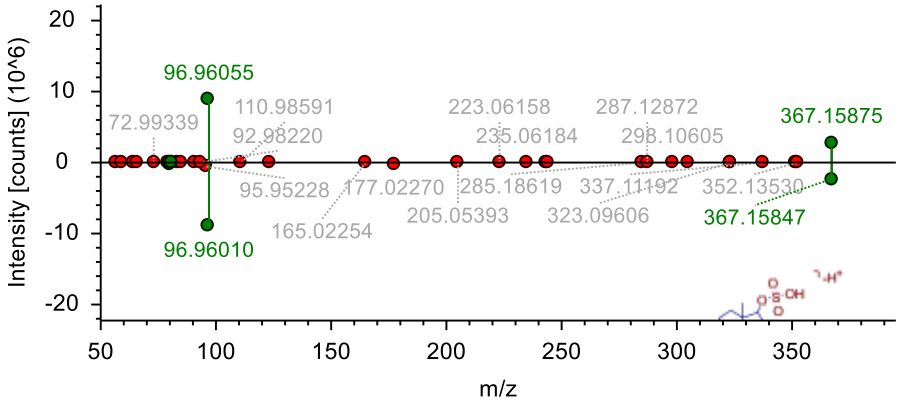


Figure 10.4-38 Obtained (top) and reference MzCloud (bottom) spectrum for testosterone sulfate in basic chromatography

10.5 Spectrums for Mexico samples not previously detected in aquatic samples

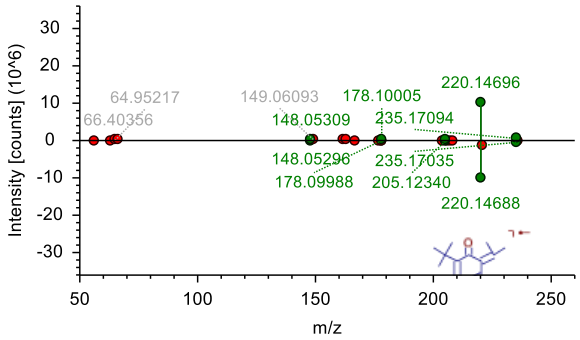


Figure 10.5-1 Spectrum obtained (TOP) and reference MzCloud spectrum (TOP) for 2,6-di-tert-Butyl-4-methoxypenonol in basic chromatography

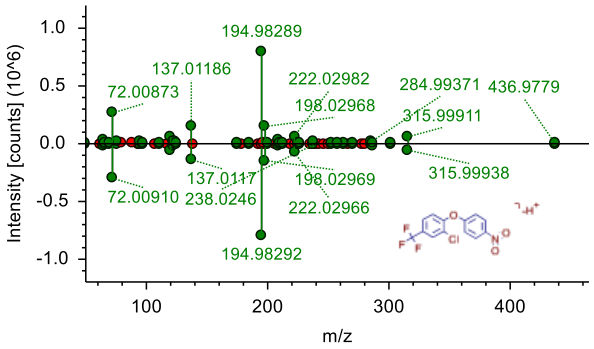


Figure 10.5-2 Spectrum obtained (TOP) and reference MzCloud spectrum (TOP) for fomesafen in basic chromatography

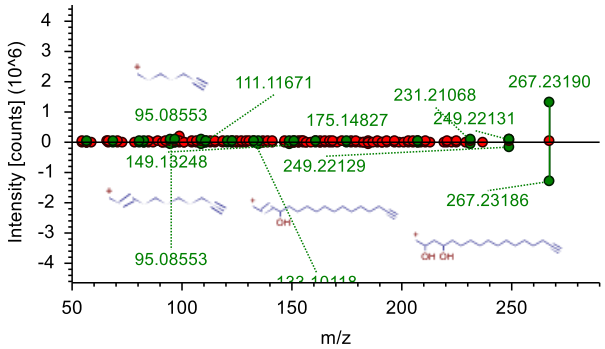


Figure 10.5-3 Spectrum obtained (TOP) and reference MzCloud spectrum (TOP) for 16-Heptadecyne-1,2,4-triol in acidic chromatography

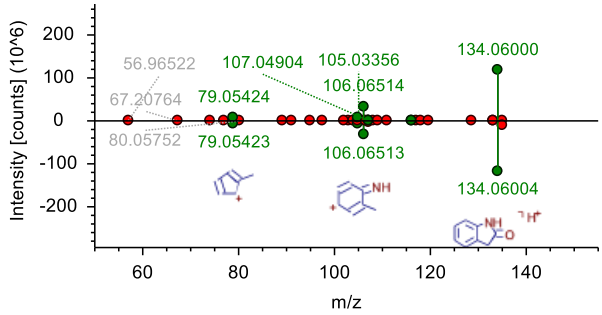


Figure 10.5-4 Spectrum obtained (TOP) and reference MzCloud spectrum (TOP) for 2-oxindole in acidic chromatography

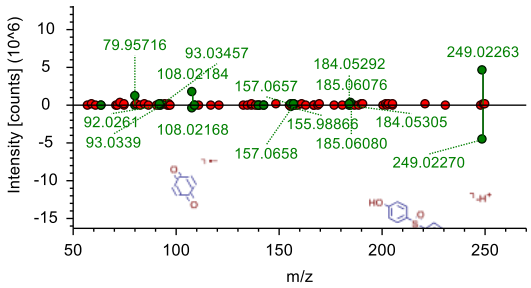


Figure 10.5-5 Spectrum obtained (TOP) and reference MzCloud spectrum (TOP) for 4,4'-Dihydroxydiphenylsulfone in basic chromatography

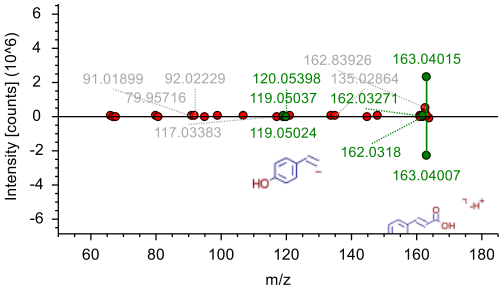


Figure 10.5-6 Spectrum obtained (TOP) and reference MzCloud spectrum (TOP) for 4-coumaric acid in acidic chromatography

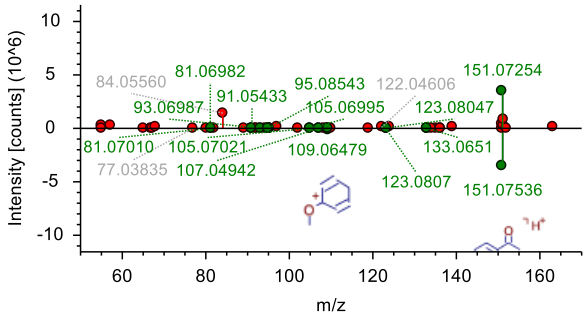


Figure 10.5-7 Spectrum obtained (TOP) and reference MzCloud spectrum (TOP) for 4'-Methoxyacetophenone acid in acidic chromatography

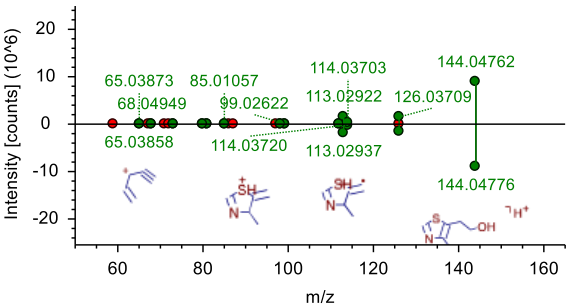


Figure 10.5-8 Spectrum obtained (TOP) and reference MzCloud spectrum (TOP) for 4-Methyl-5-thiazoleethanol acid in acidic chromatography

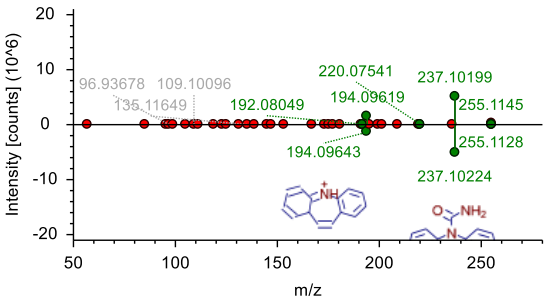


Figure 10.5-9 Spectrum obtained (TOP) and reference MzCloud spectrum (TOP) for 10-Hydroxycarbazepine acid in acidic chromatography

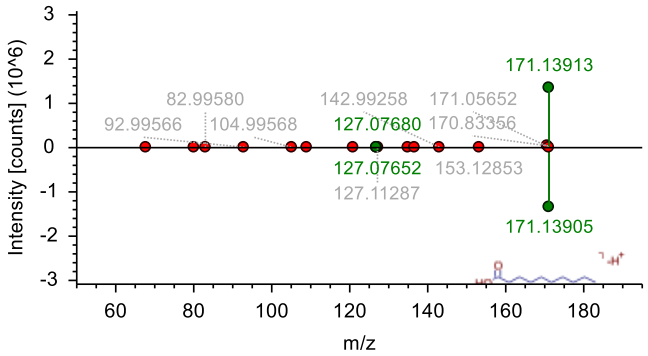


Figure 10.5-10 Spectrum obtained (TOP) and reference MzCloud spectrum (TOP) for decanoic acid in acidic chromatography

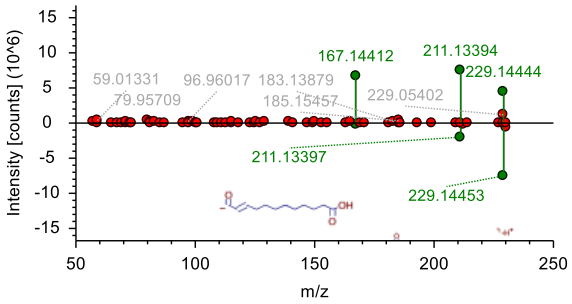


Figure 10.5-11 Spectrum obtained (TOP) and reference MzCloud spectrum (TOP) for dodecanedioic acid in basic chromatography

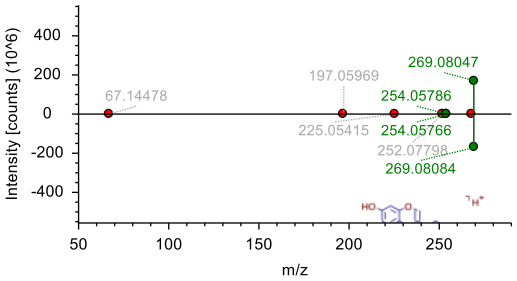


Figure 10.5-12 Spectrum obtained (TOP) and reference MzCloud spectrum (TOP) for formononetin in basic chromatography

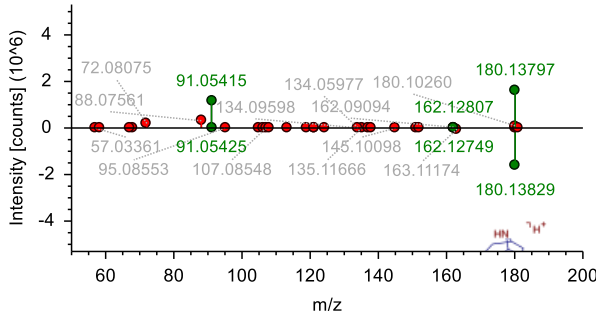


Figure 10.5-13 Spectrum obtained (TOP) and reference MzCloud spectrum (TOP) for homo-anatoxin in acidic chromatography

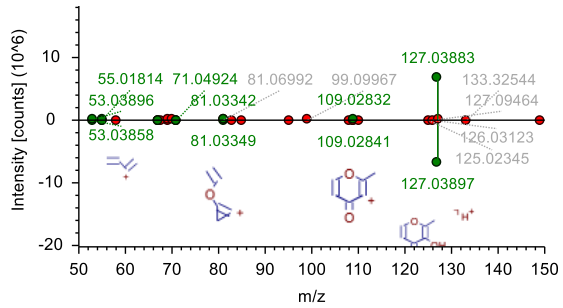


Figure 10.5-14 Spectrum obtained (TOP) and reference MzCloud spectrum (TOP) for maltol in acidic chromatography

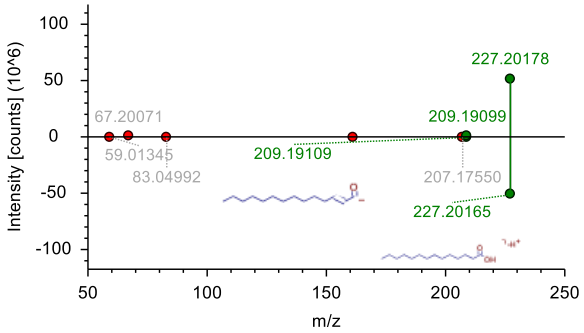


Figure 10.5-15 Spectrum obtained (TOP) and reference MzCloud spectrum (TOP) for myristic acid in basic chromatography

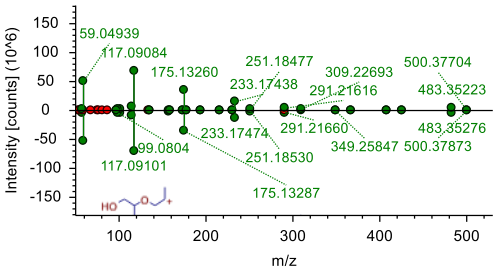


Figure 10.5-16 Spectrum obtained (TOP) and reference MzCloud spectrum (TOP) for PPG n8 in basic chromatography

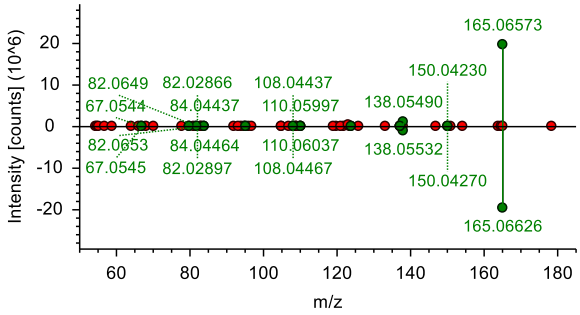


Figure 10.5-17 Spectrum obtained (TOP) and reference MzCloud spectrum (TOP) for ricinine in acidic chromatography

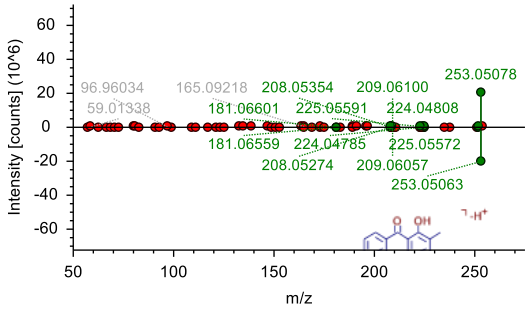


Figure 10.5-18 Spectrum obtained (TOP) and reference MzCloud spectrum (TOP) for rubiadin in acidic chromatography

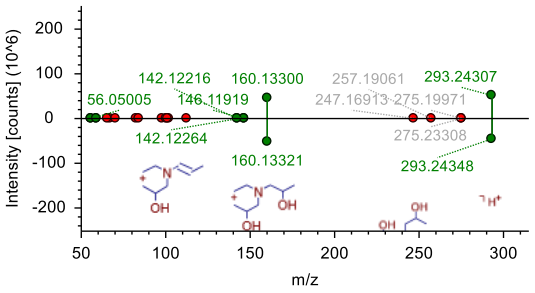


Figure 10.5-19 Spectrum obtained (TOP) and reference MzCloud spectrum (TOP) for Tetrakis(2-hydroxypropyl)ethylenediamine in basic chromatography

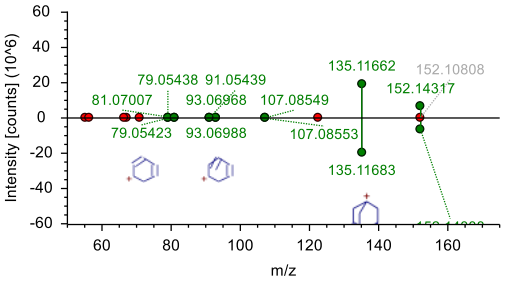


Figure 10.5-20 Spectrum obtained (TOP) and reference MzCloud spectrum (TOP) for 1-adamantanamine in basic chromatography

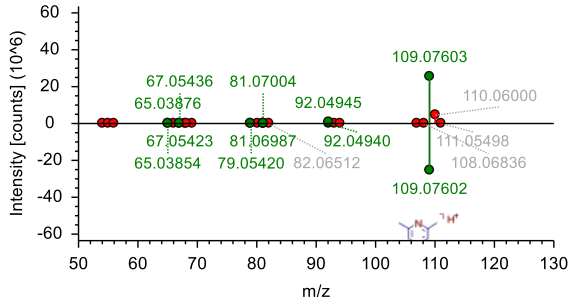


Figure 10.5-21 Spectrum obtained (TOP) and reference MzCloud spectrum (TOP) for 2,6-Dimethylpyrazine in basic chromatography

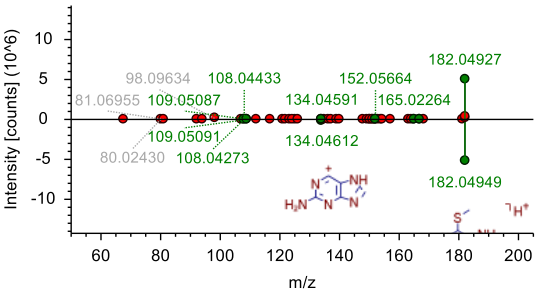


Figure 10.5-22 Spectrum obtained (TOP) and reference MzCloud spectrum (TOP) for 2-Amino-6-methylmercaptopurine in basic chromatography

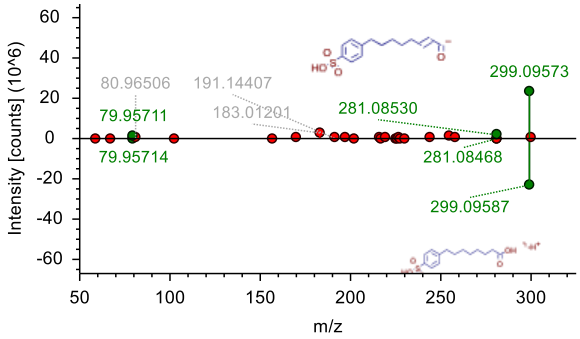


Figure 10.5-23 Spectrum obtained (TOP) and reference MzCloud spectrum (TOP) for 8-(4-Sulfophenyl) octanoic acid in basic chromatography

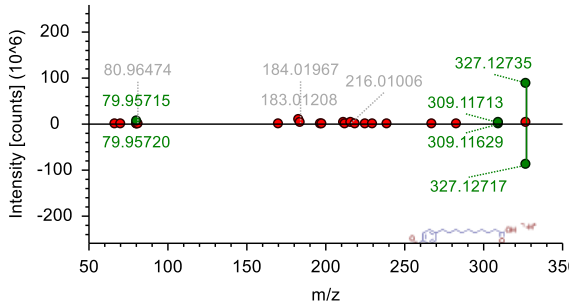


Figure 10.5-24 Spectrum obtained (TOP) and reference MzCloud spectrum (TOP) for 10-(4-Sulfophenyl) decanoic acid in acidic chromatography

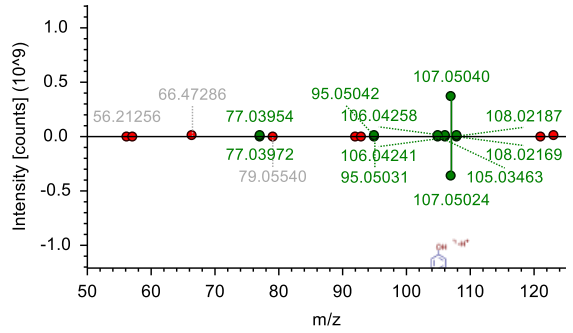


Figure 10.5-25 Spectrum obtained (TOP) and reference MzCloud spectrum (TOP) for 4-methylphenol in basic chromatography

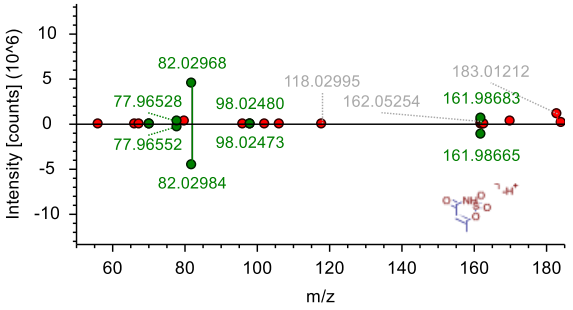


Figure 10.5-26 Spectrum obtained (TOP) and reference MzCloud spectrum (TOP) for acetophenone in basic chromatography

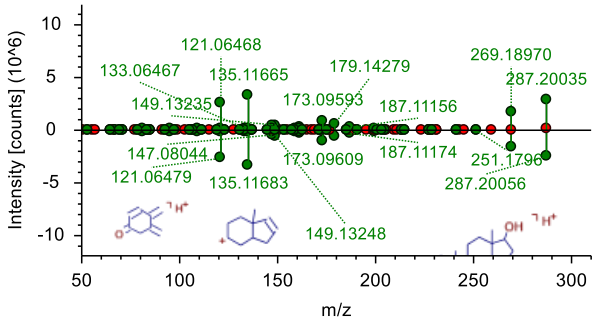


Figure 10.5-27 Spectrum obtained (TOP) and reference MzCloud spectrum (TOP) for boldenone in basic chromatography

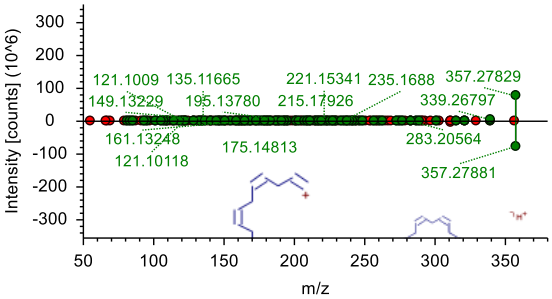


Figure 10.5-28 Spectrum obtained (TOP) and reference MzCloud spectrum (TOP) for docosahexaenoic acid ethyl ester in basic chromatography

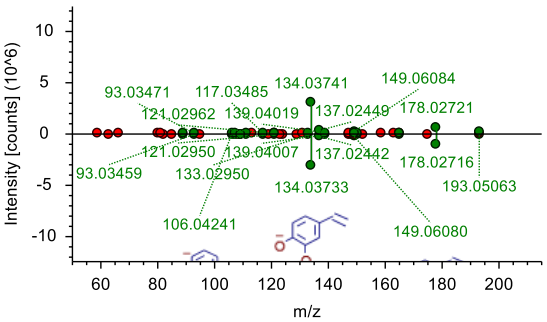


Figure 10.5-29 Spectrum obtained (TOP) and reference MzCloud spectrum (TOP) for ferulic acid in acidic chromatography

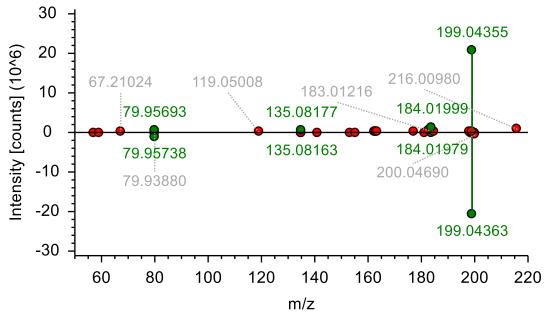


Figure 10.5-30 Spectrum obtained (TOP) and reference MzCloud spectrum (TOP) for Imino(methyl)phenyl-lambda~4~-sulfane 2,4,6-trimethylbenzenesulfonic acid in basic chromatography

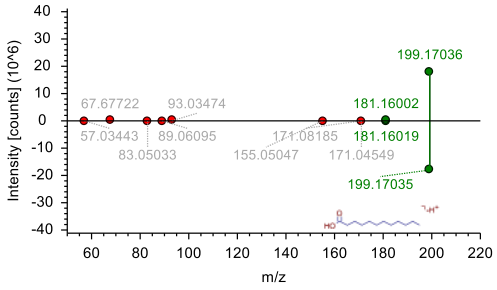


Figure 10.5-31 Spectrum obtained (TOP) and reference MzCloud spectrum (TOP) for lauric acid in basic chromatography

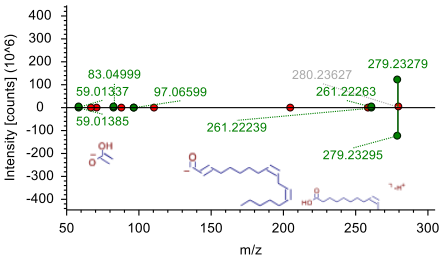


Figure 10.5-32 Spectrum obtained (TOP) and reference MzCloud spectrum (TOP) for linoleic acid in basic chromatography

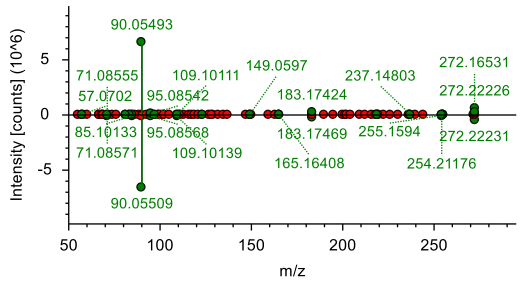


Figure 10.5-33 Spectrum obtained (TOP) and reference MzCloud spectrum (TOP) for Sodium [dodecanoyl(methyl)amino]acetate in basic chromatography

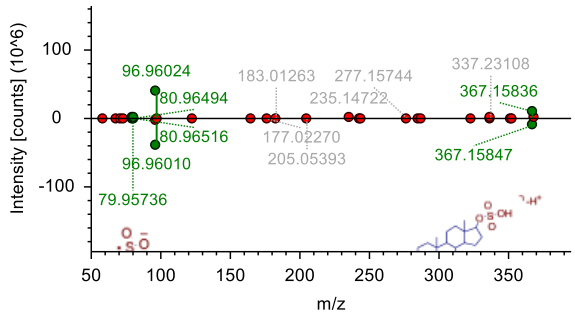


Figure 10.5-34 Spectrum obtained (TOP) and reference MzCloud spectrum (TOP) for testosterone sulfate in basic chromatography

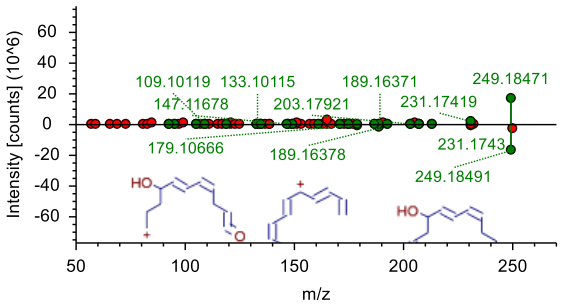


Figure 10.5-35 Spectrum obtained (TOP) and reference MzCloud spectrum (TOP) for tetranor-12(S)-HETE in basic chromatography

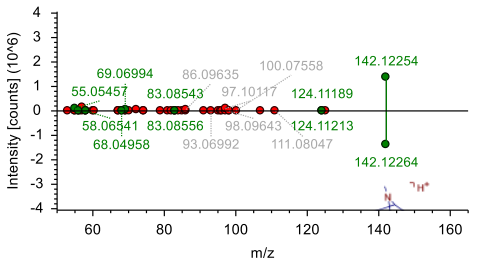


Figure 10.5-36 Spectrum obtained (TOP) and reference MzCloud spectrum (TOP) for tropine in basic chromatography

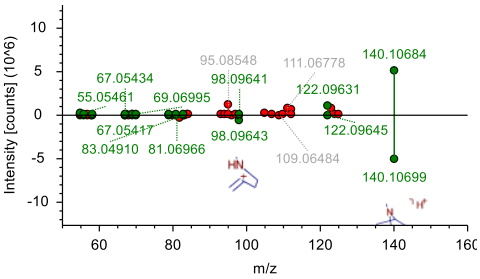


Figure 10.5-37 Spectrum obtained (TOP) and reference MzCloud spectrum (TOP) for tropinone in basic chromatography

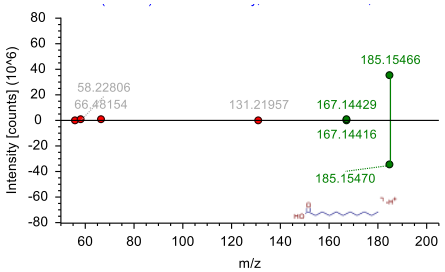


Figure 10.5-38 Spectrum obtained (TOP) and reference MzCloud spectrum (TOP) for undecanoic acid in basic chromatography

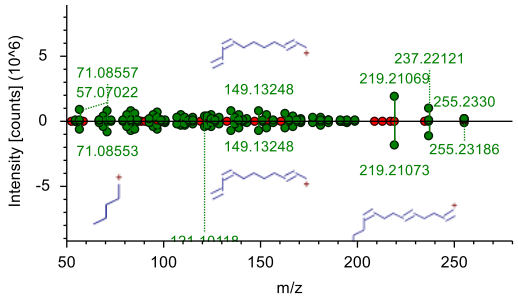


Figure 10.5-39 Spectrum obtained (TOP) and reference MzCloud spectrum (TOP) for palmitoleic acid in acidic chromatography

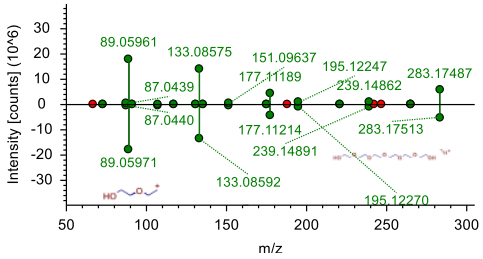


Figure 10.5-40 Spectrum obtained (TOP) and reference MzCloud spectrum (TOP) for PEG n6 in basic chromatography

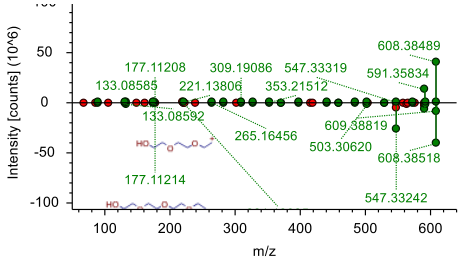


Figure 10.5-41 Spectrum obtained (TOP) and reference MzCloud spectrum (TOP) for PEG n13 in basic chromatography

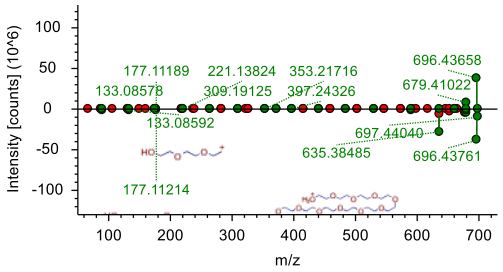


Figure 10.5-42 Spectrum obtained (TOP) and reference MzCloud spectrum (TOP) for PEG n15 in basic chromatography

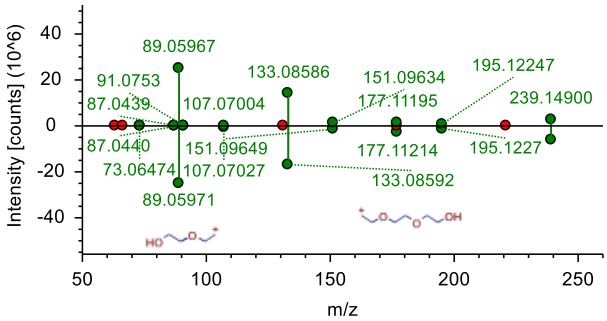


Figure 10.5-43 Spectrum obtained (TOP) and reference MzCloud spectrum (TOP) for PPG n5 in acidic chromatography

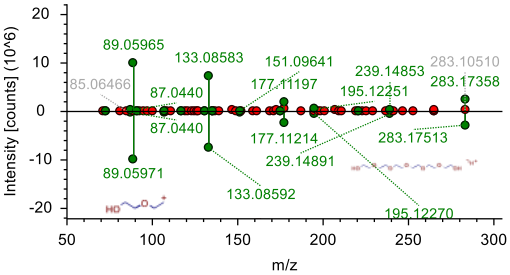


Figure 10.5-44 Spectrum obtained (TOP) and reference MzCloud spectrum (TOP) for PPG n6 in acidic chromatography

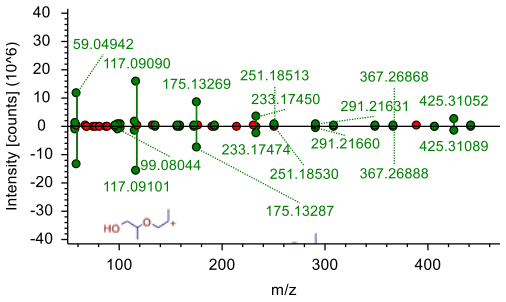


Figure 10.5-45 Spectrum obtained (TOP) and reference MzCloud spectrum (TOP) for PPG n7 in basic chromatography

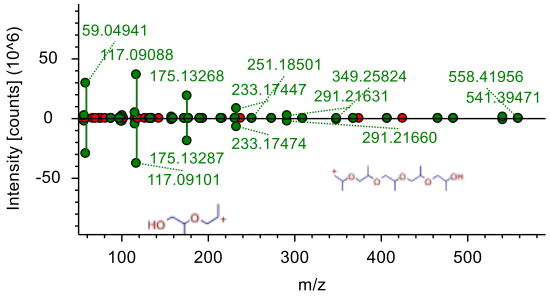


Figure 10.5-46 Spectrum obtained (TOP) and reference MzCloud spectrum (TOP) for PPG n9 in basic chromatography

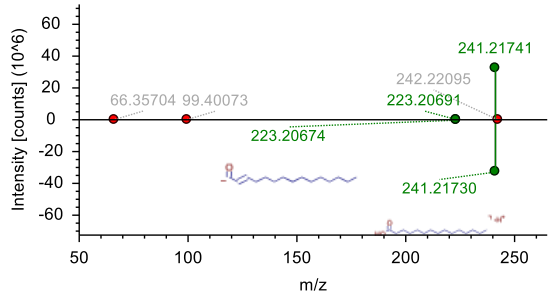


Figure 10.5-47 Spectrum obtained (TOP) and reference MzCloud spectrum (TOP) for pentadecanoic acid in basic chromatography

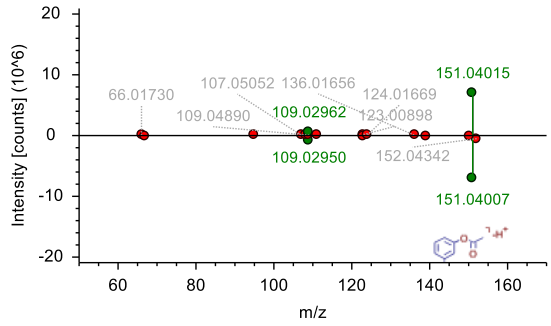


Figure 10.5-48 Spectrum obtained (TOP) and reference MzCloud spectrum (TOP) for resorcinol monoacetatein acidic chromatography

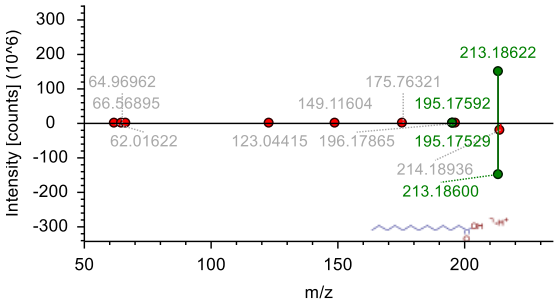


Figure 10.5-49 Spectrum obtained (TOP) and reference MzCloud spectrum (TOP) for tridecylic acid in basic chromatography

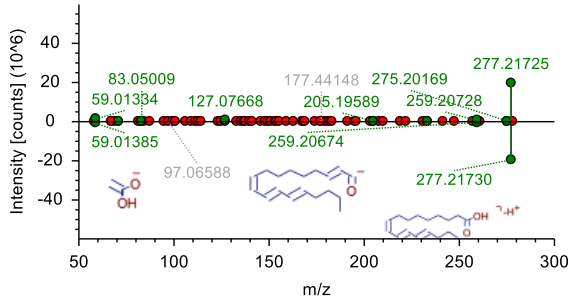


Figure 10.5-50 Spectrum obtained (TOP) and reference MzCloud spectrum (TOP) for α-Eleostearic acid in basic chromatography

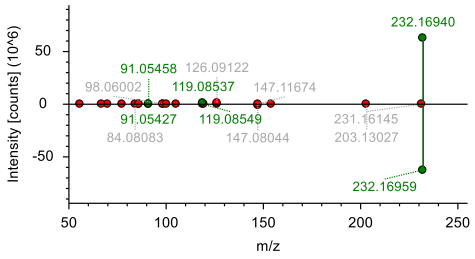


Figure 10.5-51 Spectrum obtained (TOP) and reference MzCloud spectrum (TOP) for α-piperidinobutiophenone acid in basic chromatography

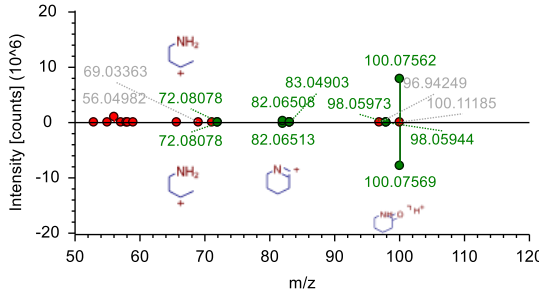


Figure 10.5-52 Spectrum obtained (TOP) and reference MzCloud spectrum (TOP) for δ-Valerolactam acid in basic chromatography

10.6 Model validation through permutations for run c

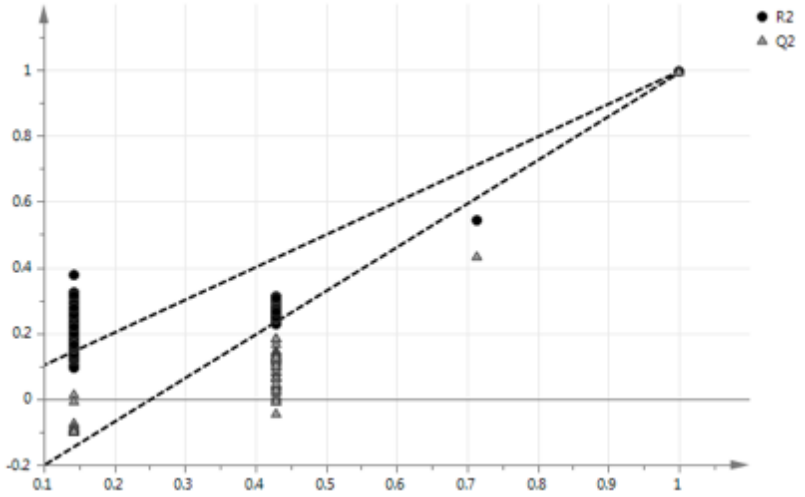


Figure 11.6.1 Run c PLS-DA permutations for Sutton Bonington wastewater influent

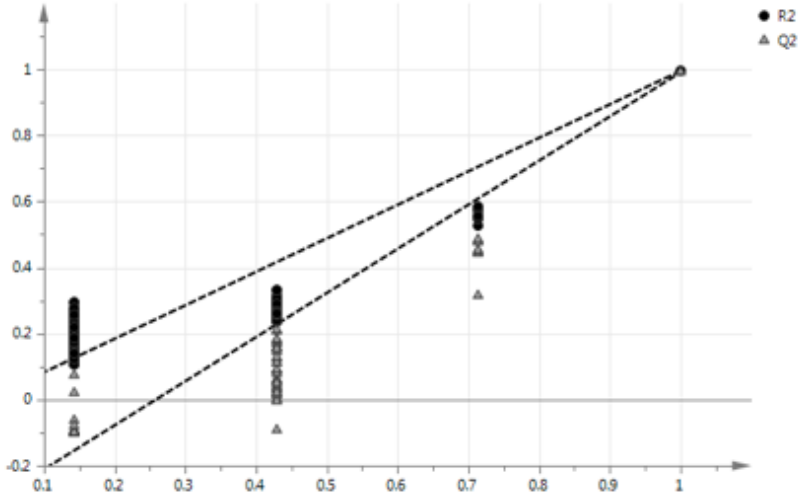


Figure 11.6.2 Run c PLS-DA permutations for Sutton Bonington downstream water

10.7 Model validation through permutations for untargeted run d

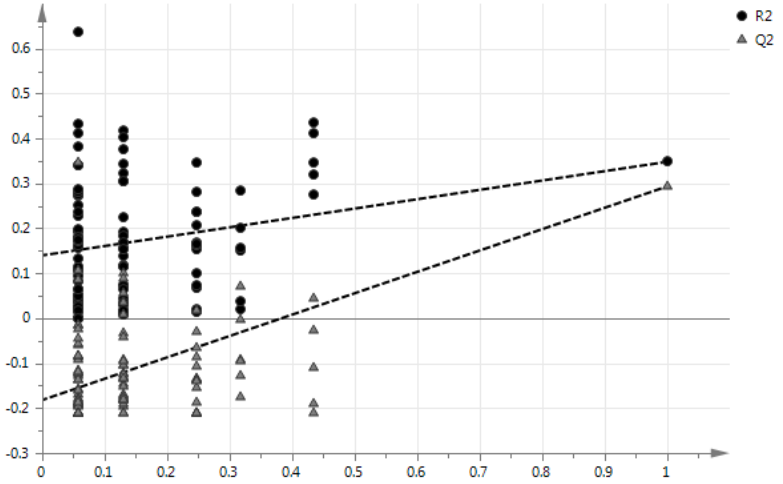


Figure 11.7.1 Run d PLS permutations for Sutton Bonington wastewater influent

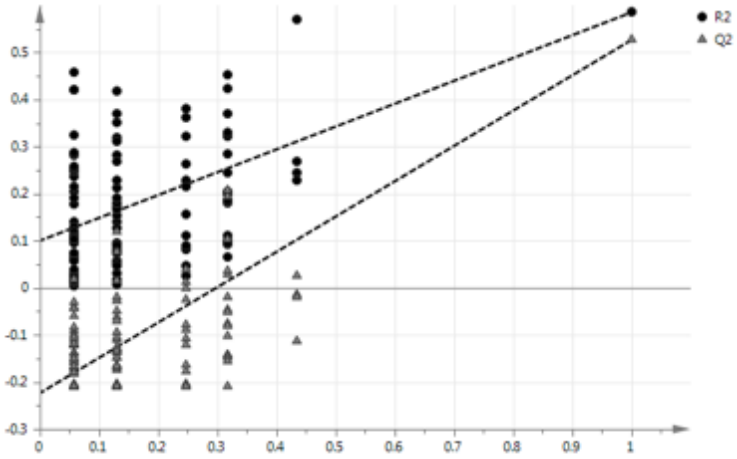


Figure 11.7.2S Run d PLS permutations for Stoke Bardolph wastewater influent

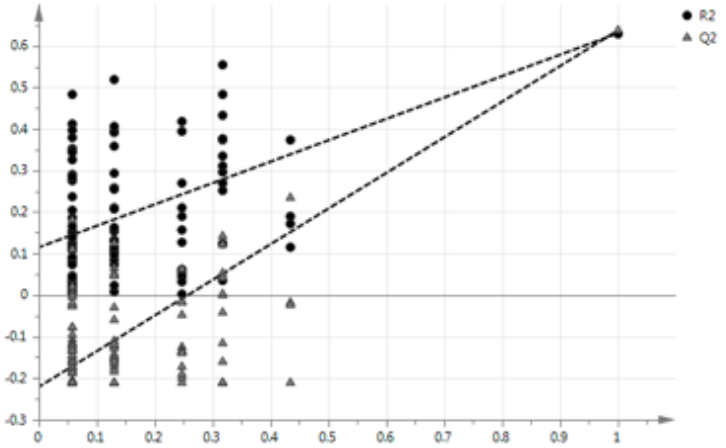


Figure 11.7.3S Run d PLS permutations for Sutton Bonington downstream water

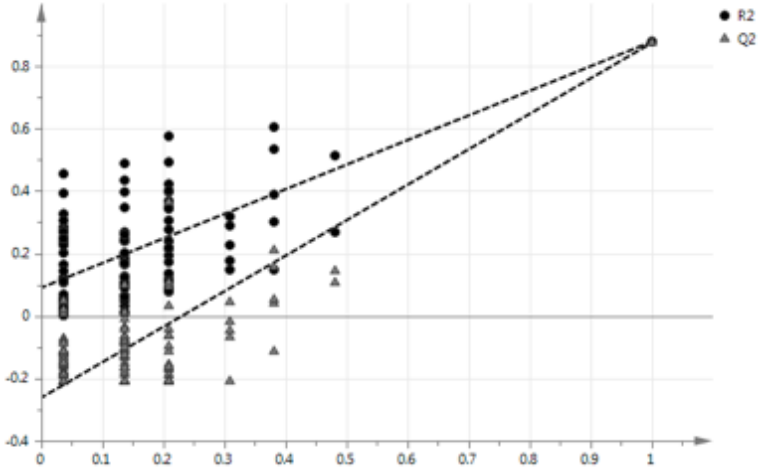


Figure 11.7.4S Run d PLS permutations for Stoke Bardolph wastewater effluent

10.8 Concentrations from targeted analysis in the Tula Valley

BAC	Spring ngL^{-1}	WWE ngL^{-1}	Dam ngL^{-1}	River ngL^{-1}	Canal ngL^{-1}
4-tert-octylphenol	BLD	ND	954.0	1041.8	695.8
Acetaminophen	ND	25808.4	22934.77	20960.9	29912.6
Acetyl-SMX	ND	1282.1	703.6	2166.6	1860.2
Atenolol	ND	97.1	25.7	47.3	67.7
Bezafibrate	3.9	1610.1	933.1	1202.5	1419.2
Carbamazepine	36.5	407.1	2568.0	940.6	1279.7
Clofibric Acid	ND	ND	ND	ND	ND
Codeine	ND	ND	ND	ND	ND
Diclofenac	BLD	4101.6	297.9	949.6	2387.9
Ethinyl estradiol (EE2)	ND	ND	ND	ND	ND
Estrone (E1)	ND	ND	ND	ND	ND
Estrone-3-sulfate (E1-3S)	ND	7.7	ND	5.0	4.5
Estradiol (E2)	ND	ND	ND	ND	ND
Estradiol-3-sulfate (E2-3S)	ND	ND	ND	ND	ND
Estriol (E3)	ND	453.3	170.7	427.8	454.9
Estriol-3-sulfate (E3-3S)	ND	385.7	ND	ND	ND
Erythromycin	ND	206.6	9199.4	5506.9	9059.8
Gemfibrozil	ND	369.5	150.6	131.8	235.5
Ketoprofen	ND	202.8	239.9	124.8	232.0
Levonorgestrel	41.8	88.3	ND	61.2	56.4
Metoprolol	ND	739.3	285.8	333.8	625.9
Morphine	ND	ND	ND	ND	ND
Naproxen	ND	1548.9	2852.4	3544.5	7316.6
Oxytetracycline	9.4	228.1	BLD	BLD	337.9
Paroxetine	ND	ND	ND	BLD	BLD
Phenazone	BLD	28.6	26.8	15.5	40.1
Propranolol	ND	13.4	13.4	16.7	26.0
Salicylic Acid	BLD	BLD	BLD	6.5	264.4
Simvastatin	ND	14.2	ND	ND	ND
Sulfadiazine	ND	561.2	790.3	476.3	976.7
Sulfamethoxazole	74.8	3462.1	4321.7	3344.5	4954.2
Sulfapyridine	ND	163.5	78.5	99.7	260.0
Tramadol	BLD	278.6	548.2	292.2	772.1
Trimethoprim	ND	641.8	62.1	253.0	476.0

Table 10.8-1 Concentrations measured in the Tula Valley

10.9 MATLAB code used for obtaining values from graphs

```
function grabitvnew(fname)

%GRABIT Extracts data points from an image file.
%
% GRABIT starts a GUI program for extracting data from an image file.
% It is capable of reading in BMP, JPG, TIF, GIF, and PNG files (anything
% that is readable by IMREAD).  Multiple data sets can be extracted from a
% single image file, and the data is saved as an n-by-2 matrix variable in
% the workspace.  It can also be renamed and saved as a MAT file.
%
% Following steps should be taken:
%   1. Load the image file.
%   2. Calibrate axes dimensions.  You will be prompted to select 4 points
%      on the image. Zoom and pan enabled.
%   3. Grab points by clicking on points.  Right-click to delete a point.
%      Image can be zoomed  and panned.
%   4. Multiple data sets will remain in memory so long as the GUI is open.
%      Variables can be renamed, saved to file, or edited in Array Editor.
%
% Panning is achieved by clicking and dragging on the image. Double-click
% to center view. Right click and drag to zoom in and out. In addition,
% there are keyboard shortcuts for zooming:
%   <a>     - zoom in
%   <b>     - zoom out
%   <space> - reset view
%
% This code will also work for extracting data points from a tilted or a
% skewed image (even upside-down or mirrored).  The calibration stage
% ensures that the imperfect orientation or quality of the image is
% accounted for.
%
% The types of files that will most likely work are BMP, JPG, TIF, GIF (up
% to 8-bit), and PNG files.  Basically,  any format supported by the IMREAD
% is accepted.
%
% GRABIT(FILENAME) will start the GUI program and open the image file
% FILENAME.
%
% Type GRABIT('-sample') to load a sample image.
%
%
% VERSIONS:
%   v1.0 - first version
%   v1.1 - use imshow instead of image (takes care of colormap)
%   v1.5 - convert to a GUI version
%   v1.6 - added functionality to open a file from the command window and
%          embedded a sample image file to the function
%   v1.6.1 - changed cross cursor to crosshair
%   v1.6.2 - brought back 'image' in case the user doesn't have Image Toolbox
%   v1.6.5 - fixed zoom problem in R14
%   v2.0 - major code change. added zoom feature during calibration. added
%          panning feature. (March 3, 2006)
%   v2.1 - store sample image as HEX to reduce file size. (March 6, 2006)
```

```
%   v2.1.1 - animate view change and zooming for a better visual perception
%          (March 11, 2006)
%   v2.1.5 - added features: double-click to center view. right-click and
%          drag to zoom. other minor code changes. (March 16, 2006)
%   v2.2 - fixed loadImageFcn bug. (May 4, 2006)
%   v2.3 - fixed bug to work with grayscale image (Jan, 2007)
%

% Created in Matlab R13. Tested up to R2006b
%
% Copyright 2003
% Jiro Doke
%
% To Do: Capability to deal with logarithmic axes
%

%--------------------------------------------------------------------------
% Initialize
%--------------------------------------------------------------------------
sh = get(0, 'ShowHiddenHandles');
set(0, 'ShowHiddenHandles', 'on');

% close existing windows
im  =  findobj('type',  'figure',  'tag',  'GrabitGUI');
if ishandle(im)
  close(im);
end

set(0, 'ShowHiddenHandles', sh);

% background colors
bgcolor1 = [.8, .8, .8];
bgcolor2 = [ 1,  1,  1];
bgcolor3 = [.7, .7, .7];
bgcolor4 = [ 1, .5, .5];

%--------------------------------------------------------------------------
% Custom cursor pointers
%--------------------------------------------------------------------------
zoomPointer = [
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    2    1    1    1    1    1    2  NaN  NaN  NaN  NaN  NaN  NaN  NaN  NaN
NaN
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NaN
    2    1    2  NaN  NaN  NaN  NaN    2    2    2    2  NaN  NaN  NaN  NaN
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  NaN  NaN  NaN    2    1    2    1    1    1    1    1    1    2    1    2  NaN
  NaN  NaN  NaN    2    1    2    2    2    1    1    2    2    2    1    2  NaN
```

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  NaN  NaN  NaN  NaN  NaN  NaN  NaN   2   2   2   2  NaN   2   1   1   1
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2   1   2
 ];

zoomInOutPointer = [
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NaN  NaN
 ];

% closed hand pointer (from Jrme Briot)
closedHandPointer = [
  NaN  NaN  NaN  NaN  NaN  NaN  NaN  NaN  NaN  NaN  NaN  NaN  NaN  NaN
NaN  NaN  NaN
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 ];
```

```
% X-axis Origin pointer
xoPointer = [
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   NaN
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   NaN
    2     1     1     2     2     2     2   NaN   NaN   NaN   NaN   NaN   NaN   NaN   NaN   NaN
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    2     1     2     2     1     2   NaN   NaN   NaN   NaN   NaN   NaN   NaN   NaN   NaN   NaN
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  NaN   NaN   NaN   NaN   NaN   NaN   NaN   NaN   NaN   NaN   NaN   NaN   NaN   NaN   NaN   NaN
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   1   NaN
 ];

% X-axis Max pointer
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    2     1     1     1     1     1     2   NaN   NaN   NaN   NaN   NaN   NaN   NaN   NaN   NaN
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    2     1     1     2     2     2     2   NaN   NaN   NaN   NaN   NaN   NaN   NaN   NaN   NaN
   NaN
    2     1     2     1     2   NaN   NaN   NaN   NaN   NaN   NaN   NaN   NaN   NaN   NaN   NaN
   NaN
    2     1     2     2     1     2   NaN   NaN   NaN   NaN   NaN   NaN   NaN   NaN   NaN   NaN
   NaN
    2     1     2   NaN     2     1     2   NaN   NaN   NaN   NaN   NaN   NaN   NaN   NaN   NaN
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  NaN   NaN   NaN     1     1   NaN   NaN   NaN     1     1   NaN   NaN   NaN   NaN   NaN   NaN
  NaN   NaN
```

```
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NaN   1
  NaN  NaN  NaN  NaN   1   1   1   1   1  NaN  NaN   1   1  NaN   1   1
  NaN  NaN  NaN   1   1   1  NaN   1   1   1  NaN   1  NaN   1  NaN   1
  NaN  NaN  NaN   1   1  NaN  NaN  NaN   1   1  NaN   1  NaN  NaN  NaN
1
  NaN  NaN  NaN  NaN  NaN  NaN  NaN  NaN  NaN  NaN  NaN   1  NaN
NaN  NaN   1
 ];

% Y-axis Origin pointer
yoPointer = [
    2    2    2    2    2    2    2  NaN  NaN  NaN  NaN  NaN  NaN  NaN  NaN
NaN
    2    1    1    1    1    1    2  NaN  NaN  NaN  NaN  NaN  NaN  NaN  NaN
NaN
    2    1    1    2    2    2    2  NaN  NaN  NaN  NaN  NaN  NaN  NaN  NaN
NaN
    2    1    2    1    2  NaN  NaN  NaN  NaN  NaN  NaN  NaN  NaN  NaN  NaN
NaN
    2    1    2    2    1    2  NaN  NaN  NaN  NaN  NaN  NaN  NaN  NaN  NaN
NaN
    2    1    2  NaN    2    1    2  NaN  NaN  NaN  NaN  NaN  NaN  NaN  NaN
NaN
    2    2    2  NaN  NaN    2    2  NaN  NaN  NaN  NaN  NaN  NaN  NaN  NaN
NaN
  NaN  NaN  NaN  NaN  NaN  NaN  NaN  NaN  NaN  NaN  NaN  NaN  NaN
NaN  NaN  NaN
  NaN  NaN  NaN   1   1  NaN  NaN  NaN   1   1  NaN  NaN  NaN  NaN
NaN  NaN
  NaN  NaN  NaN   1   1   1  NaN  NaN   1   1  NaN  NaN  NaN  NaN  NaN
NaN
  NaN  NaN  NaN  NaN   1   1   1   1   1   1  NaN  NaN  NaN  NaN  NaN
NaN
  NaN  NaN  NaN  NaN  NaN   1   1   1   1  NaN  NaN  NaN   1   1   1
NaN
  NaN  NaN  NaN  NaN  NaN   1   1   1  NaN  NaN  NaN   1  NaN  NaN
NaN   1
  NaN  NaN  NaN   1   1   1   1   1  NaN  NaN  NaN   1  NaN  NaN  NaN
1
  NaN  NaN  NaN   1   1   1  NaN  NaN  NaN  NaN  NaN   1  NaN  NaN
NaN   1
  NaN  NaN  NaN  NaN  NaN  NaN  NaN  NaN  NaN  NaN  NaN  NaN   1   1
1  NaN
 ];

% Y-axis Max pointer
ymPointer = [
    2    2    2    2    2    2    2  NaN  NaN  NaN  NaN  NaN  NaN  NaN  NaN
NaN
```

```
   2   1   1   1   1   1   2  NaN  NaN  NaN  NaN  NaN  NaN  NaN  NaN
NaN
   2   1   1   2   2   2   2  NaN  NaN  NaN  NaN  NaN  NaN  NaN  NaN
NaN
   2   1   2   1   2 NaN  NaN  NaN  NaN  NaN  NaN  NaN  NaN  NaN  NaN
NaN
   2   1   2   2   1   2 NaN  NaN  NaN  NaN  NaN  NaN  NaN  NaN  NaN
NaN
   2   1   2 NaN   2   1   2 NaN  NaN  NaN  NaN  NaN  NaN  NaN  NaN
NaN
   2   2   2 NaN NaN   2   2 NaN  NaN  NaN  NaN  NaN  NaN  NaN  NaN
NaN
 NaN NaN NaN NaN NaN NaN NaN NaN NaN NaN NaN NaN NaN NaN
NaN NaN NaN
 NaN NaN NaN   1   1 NaN NaN NaN   1   1 NaN NaN NaN NaN
NaN NaN
 NaN NaN NaN   1   1   1 NaN NaN   1   1 NaN NaN NaN NaN NaN
NaN
 NaN NaN NaN NaN   1   1   1   1   1   1 NaN NaN NaN NaN NaN
NaN
 NaN NaN NaN NaN NaN   1   1   1   1 NaN NaN   1 NaN NaN NaN
1
 NaN NaN NaN NaN NaN   1   1   1 NaN NaN NaN   1   1 NaN   1
1
 NaN NaN NaN   1   1   1   1   1 NaN NaN NaN   1 NaN   1 NaN   1
 NaN NaN NaN   1   1   1 NaN NaN NaN NaN NaN   1 NaN NaN
NaN   1
 NaN NaN NaN NaN NaN NaN NaN NaN NaN NaN NaN   1 NaN
NaN NaN   1
 ];

% zoom button icon
zoomIcon = [
   1   1   1   1   1   0   0   0   0   1   1   1   1   1   1   1
   1   1   1   0   0   0   0   0   0   0   0   1   1   1   1   1
   1   1   0   0   0   1   1   1   1   0   0   0   1   1   1   1
   1   0   0   1   1   1   0   0   1   1   1   0   0   1   1   1
   1   0   0   1   1   1   0   0   1   1   1   0   0   1   1   1
   0   0   1   1   1   1   0   0   1   1   1   1   0   0   1   1
   0   0   1   0   0   0   0   0   0   0   0   1   0   0   1   1
   0   0   1   0   0   0   0   0   0   0   0   1   0   0   1   1
   0   0   1   1   1   1   0   0   1   1   1   1   0   0   1   1
   1   0   0   1   1   1   0   0   1   1   1   0   0   1   1   1
   1   0   0   1   1   1   0   0   1   1   1   0   0   1   1   1
   1   1   0   0   0   1   1   1   1   0   0   0   0   0   1   1
   1   1   1   0   0   0   0   0   0   0   0   0   0   0   0   1
   1   1   1   1   1   0   0   0   0   1   1   0   0   0   0   0
   1   1   1   1   1   1   1   1   1   1   1   1   0   0   0   0
   1   1   1   1   1   1   1   1   1   1   1   1   1   0   0   0
 ];

% create zoom icon (RGB matrix)
fgID = zoomIcon==0;
bgID = ~fgID;
zI1  = zeros([size(zoomIcon), 3]);
```

```
zI2  = zeros([size(zoomIcon), 3]);
for id = 1:3
  tmp        = zoomIcon;
  tmp(fgID)   = 0;
  tmp(bgID)   = bgcolor3(id);
  zI1(:,:,id) = tmp;
  tmp(bgID)   = bgcolor4(id);
  zI2(:,:,id) = tmp;
end

% get screen size in pixels
un         = get(0, 'units');
set(0, 'units', 'pixels');
screenSize  = get(0, 'ScreenSize');
sW          = screenSize(3);
sH         = screenSize(4);
set(0, 'units', un);

% figure width and height (in pixels)
fW         = sW-200;
fH         = sH-100;

im = figure(...
  'units'                       , 'pixels', ...
  'position'                     , [100, 50, fW, fH], ...
  'backingstore'                    , 'off', ...
  'doublebuffer'                    , 'on', ...
  'name'                         , 'Grabit', ...
  'numbertitle'                    , 'off', ...
  'menubar'                        , 'none', ...
  'color'                       , bgcolor1, ...
  'pointer'                      , 'arrow', ...
  'visible'                     , 'off', ...
  'interruptible'                  , 'off', ...
  'busyaction'                     , 'cancel', ...
  'resizefcn'                     , @figResizeFcn, ...
  'windowbuttonupfcn'                , @winBtnUpFcn, ...
  'keypressfcn'                    , @keyPressFcn, ...
  'deletefcn'                     , 'delete(timerfind(''name'', ''BtnUpTimer''));', ...
  'tag'                         , 'GrabitGUI', ...
  'defaultUicontrolUnits'           , 'pixels', ...
  'defaultUicontrolBackgroundColor' , bgcolor3, ...
  'defaultUicontrolFontname'         , 'Verdana', ...
  'defaultUicontrolFontUnits'       , 'pixels', ...
  'defaultUicontrolFontsize'         , 10, ...
  'defaultUicontrolInterruptible'   , 'off', ...
  'defaultUicontrolBusyAction'      , 'cancel', ...
  'defaultAxesFontName'              , 'Verdana', ...
  'defaultAxesUnits'               , 'pixels', ...
  'defaultAxesFontSize'             , 8);

panelW = 0.8*fW;
uicontrol(...
  'style'                       , 'frame', ...
  'position'                     , [10, fH-110, panelW, 100], ...
```

```
  'backgroundcolor'               , bgcolor2);
uicontrol(...
  'style'                    , 'pushbutton', ...
  'string'                   , 'Load Image...', ...
  'callback'                   , {@loadImageFcn, []}, ...
  'position'                   , [15, fH-40, 200, 25], ...
  'tag'                      , 'LoadImageBtn');
uicontrol(...
  'style'                    , 'edit', ...
  'backgroundcolor'                , bgcolor2, ...
  'string'                   , '', ...
  'position'                   , [220, fH-40, panelW-215, 25], ...
  'horizontalalignment'             , 'left', ...
  'enable'                   , 'inactive', ...
  'tag'                      , 'ImageFileLoc');

uicontrol(...
  'style'                    , 'frame', ...
  'position'                   , [15, fH-75, 3*(panelW-20)/4-5, 30], ...
  'backgroundcolor'                , bgcolor2);
uicontrol(...
  'style'                    , 'togglebutton', ...
  'string'                   , 'Calibrate', ...
  'buttondownfcn'                  , @calibrateImageFcn, ...
  'position'                   , [20, fH-72, panelW/4-25, 25], ...
  'enable'                   , 'off', ...
  'tag'                      , 'CalibrateImageBtn');
calibFrameX = panelW/4-5;
calibFrameW = panelW/2-10;
uicontrol(...
  'style'                    , 'text', ...
  'position'                   , [calibFrameX+5, fH-70, calibFrameW/8, 15], ...
  'string'                   , 'Xo:', ...
  'fontweight'                   , 'bold', ...
  'horizontalalignment'             , 'right', ...
  'tooltipstring'                , sprintf('Calibration:\nX-Axis Origin'), ...
  'backgroundcolor'                , bgcolor2);
uicontrol(...
  'style'                    , 'text', ...
  'position'                       , [calibFrameX+5+calibFrameW/8, fH-70, calibFrameW/8,
15], ...
  'string'                   , ' NaN', ...
  'horizontalalignment'             , 'left', ...
  'tag'                      , 'hXoValue', ...
  'tooltipstring'                , sprintf('Calibration:\nX-Axis Origin'), ...
  'backgroundcolor'                , bgcolor2);
uicontrol(...
  'style'                    , 'text', ...
  'position'                       , [calibFrameX+5+calibFrameW/4, fH-70, calibFrameW/8,
15], ...
  'string'                   , 'Xm:', ...
  'fontweight'                   , 'bold', ...
  'horizontalalignment'             , 'right', ...
  'tooltipstring'                , sprintf('Calibration:\nX-Axis Max'), ...
  'backgroundcolor'                , bgcolor2);
```

```
uicontrol(...
  'style'                , 'text', ...
  'position'               , [calibFrameX+5+3*calibFrameW/8, fH-70, calibFrameW/8,
15], ...
  'string'               , ' NaN', ...
  'horizontalalignment'          , 'left', ...
  'tag'                  , 'hXmValue', ...
  'tooltipstring'          , sprintf('Calibration:\nX-Axis Max'), ...
  'backgroundcolor'            , bgcolor2);
uicontrol(...
  'style'                , 'text', ...
  'position'                 , [calibFrameX+5+calibFrameW/2, fH-70, calibFrameW/8,
15], ...
  'string'               , 'Yo:', ...
  'fontweight'             , 'bold', ...
  'horizontalalignment'          , 'right', ...
  'tooltipstring'          , sprintf('Calibration:\nY-Axis Origin'), ...
  'backgroundcolor'            , bgcolor2);
uicontrol(...
  'style'                , 'text', ...
  'position'               , [calibFrameX+5+5*calibFrameW/8, fH-70, calibFrameW/8,
15], ...
  'string'               , ' NaN', ...
  'horizontalalignment'          , 'left', ...
  'tag'                  , 'hYoValue', ...
  'tooltipstring'          , sprintf('Calibration:\nY-Axis Origin'), ...
  'backgroundcolor'            , bgcolor2);
uicontrol(...
  'style'                , 'text', ...
  'position'               , [calibFrameX+5+3*calibFrameW/4, fH-70, calibFrameW/8,
15], ...
  'string'               , 'Ym:', ...
  'fontweight'             , 'bold', ...
  'horizontalalignment'          , 'right', ...
  'tooltipstring'          , sprintf('Calibration:\nY-Axis Max'), ...
  'backgroundcolor'            , bgcolor2);
uicontrol(...
  'style'                , 'text', ...
  'position'               , [calibFrameX+5+7*calibFrameW/8, fH-70, calibFrameW/8,
15], ...
  'string'               , ' NaN', ...
  'horizontalalignment'          , 'left', ...
  'tag'                  , 'hYmValue', ...
  'tooltipstring'          , sprintf('Calibration:\nY-Axis Max'), ...
  'backgroundcolor'            , bgcolor2);

uicontrol(...
  'style'                , 'togglebutton', ...
  'string'               , 'Grab Points', ...
  'buttondownfcn'              , @grabPointsFcn, ...
  'position'               , [3*(panelW)/4, fH-72, panelW/4+5, 25], ...
  'enable'                 , 'off', ...
  'tag'                  , 'GrabPointsBtn');
uicontrol(...
  'style'                , 'togglebutton', ...
```

```
  'cdata'                 , zI1, ...
  'buttondownfcn'              , @zoomBtnFcn, ...
  'position'              , [15, fH-105, 25, 25], ...
  'enable'                , 'inactive', ...
  'tag'                  , 'ZoomStateBtn');
uicontrol(...
  'style'                , 'pushbutton', ...
  'string'               , 'Reset View ', ...
  'callback'              , @resetViewFcn, ...
  'position'              , [45, fH-105, 100, 25], ...
  'tag'                  , 'ResetViewBtn');
uicontrol(...
  'style'                , 'text', ...
  'position'              , [150, fH-108, panelW-145, 31], ...
  'horizontalalignment'        , 'center', ...
  'foregroundcolor'           , [0 0 .5], ...
  'backgroundcolor'           , bgcolor2, ...
  'fontsize'              , 10, ...
  'string'                , {'Click and drag to pan. Double-click to center. Right-click
and drag to zoom.', ...
    'Keyboard Shortcuts: <a> - zoom in, <z> - zoom out, <space> - reset view'});

rPanelX = 0.82 * fW;
rPanelW = fW - rPanelX - 10;
uicontrol(...
  'style'                , 'listbox', ...
  'callback'              , @selectVariableFcn, ...
  'position'              , [rPanelX+10, 100, rPanelW-20, 0.6*fH], ...
  'backgroundcolor'           , bgcolor2, ...
  'tooltipstring'          , sprintf('Double-click to edit\nvariable in Array Editor'), ...
  'tag'                  , 'VariableList');
uicontrol(...
  'style'                , 'text', ...
  'string'               , 'Data in Memory', ...
  'position'              , [rPanelX+10, 0.6*fH+100, rPanelW-20, 20], ...
  'backgroundcolor'           , bgcolor1, ...
  'fontweight'             , 'bold');
uicontrol(...
  'style'                , 'pushbutton', ...
  'string'               , 'Save to file as...', ...
  'position'              , [rPanelX+10, 70, rPanelW-20, 25], ...
  'callback'              , @variableManipulationFcn, ...
  'tooltipstring'           , sprintf('Save variable as a MAT file or\nDouble-precision,
tab-delimited TXT file'), ...
  'tag'                  , 'SaveAs');
uicontrol(...
  'style'                , 'pushbutton', ...
  'string'               , 'Rename...', ...
  'position'              , [rPanelX+10, 45, rPanelW-20, 25], ...
  'callback'              , @variableManipulationFcn, ...
  'tooltipstring'          , 'Rename variable in workspace', ...
  'tag'                  , 'Rename');
uicontrol(...
  'style'                , 'pushbutton', ...
  'string'               , 'Delete...', ...
```

```
  'position'                  , [rPanelX+10, 20, rPanelW-20, 25], ...
  'callback'                  , @variableManipulationFcn, ...
  'tooltipstring'             , 'Delete variable from workspace', ...
  'tag'                       , 'Delete');

axes(...
  'position'                  , [10, 10, 0.8*fW, fH-200], ...
  'visible'                   , 'on', ...
  'handlevisibility'          , 'on', ...
  'box'                       , 'on', ...
  'SortMethod'                , 'depth', ...
  'xtick'                     , [], ...
  'ytick'                     , [], ...
  'interruptible'             , 'off', ...
  'buttondownfcn'             , @winBtnDownFcn, ...
  'tag'                       , 'ImageAxis');
imAxRatio = (fH - 200) / (0.8 * fW);
title('', 'fontunits', 'pixels', 'fontsize', 24, 'color', 'red');

axes(...
  'position'                  , [rPanelX+20, 0.6*fH+150, rPanelW-30, 0.4*fH-190], ...
  'box'                       , 'on', ...
  'tag'                       , 'PreviewAxis');

uicontrol(...
  'style'                     , 'text', ...
  'string'                    , 'Preview Box', ...
  'fontweight'                , 'bold', ...
  'position'                  , [rPanelX+20, fH-40, rPanelW-30, 25], ...
  'backgroundcolor'           , bgcolor1);

uicontrol(...
  'style'                     , 'edit', ...
  'backgroundcolor'           , bgcolor4, ...
  'string'                    , 'Enter Value', ...
  'position'                  , [0 0 100 25], ...
  'horizontalalignment'       , 'left', ...
  'visible'                   , 'off', ...
  'callback'                  , @coordinateEditFcn, ...
  'tag'                       , 'CoordinateEdit');

set(findobj(im, 'type', 'uicontrol'), 'units', 'normalized');
set(findobj(im, 'type', 'axes'), 'units', 'normalized');

% create handles structure
handles                       = guihandles(im);
handles.zoomPointer           = zoomPointer;
handles.zoomPointerHotSpot    = [2 2];
handles.zoomInOutPointer      = zoomInOutPointer;
handles.zoomInOutPointerHotSpot  = [9 9];
handles.closedHandPointer     = closedHandPointer;
handles.closedHandPointerHotSpot  = [9 9];
handles.xoPointer             = xoPointer;
handles.xoPointerHotSpot      = [2 2];
handles.xmPointer             = xmPointer;
```

```
handles.xmPointerHotSpot          = [2 2];
handles.yoPointer                 = yoPointer;
handles.yoPointerHotSpot          = [2 2];
handles.ymPointer                 = ymPointer;
handles.ymPointerHotSpot          = [2 2];
handles.zoomIconUp                = zI1;
handles.zoomIconDown              = zI2;
handles.curPointer                = 'arrow';
handles.curPointerData.CData      = zoomPointer;
handles.curPointerData.HotSpot    = [1 1];
handles.state                     = 'normal';
handles.bgcolor1                  = bgcolor1;
handles.bgcolor2                  = bgcolor2;
handles.bgcolor3                  = bgcolor3;
handles.bgcolor4                  = bgcolor4;
handles.imAxRatio                 = imAxRatio;
handles.I                         = [];
handles.map                       = [];
handles.savedVars                 = struct;
handles.isPanning                 = false;
handles.curTitle                  = '';
handles.CurrentPointAxes          = [];
handles.CurrentPointFig           = [];
handles.timer                     = timer(...
                                  'Name', 'BtnUpTimer', ...
                                  'StartDelay', 0.2, ...
                                  'TimerFcn', {@btnUpTimerFcn, im});

guidata(im, handles);

set(im, 'handlevisibility', 'callback');

if nargin == 1 && ischar(fname)
  switch lower(fname)
    case '-sample'
      fname = createSampleImageFcn;
      if ~isempty(fname)
        loadImageFcn(handles.LoadImageBtn, [], fname);
        try
          pause(0.5);
          delete(fname);
        catch
          errordlg(lasterr);
        end
      end

    otherwise
      filename = which(fname);
      if ~isempty(filename)
        loadImageFcn(handles.LoadImageBtn, [], filename);
      else
        errordlg(sprintf('%s\nnot found.', fname));
        return;
      end
  end
```

```
end

set(im, 'visible', 'on');


%---------------------------------------------------------------------
%---------------------------------------------------------------------
% variableManipulationFcn
%---------------------------------------------------------------------
%---------------------------------------------------------------------
function variableManipulationFcn(varargin)
% This callback is called when one of the three buttons under the variable
% listbox is pressed.

obj = varargin{1};

handles = guidata(obj);

vars = fieldnames(handles.savedVars);
if isempty(vars)
  return;
end
listboxVal = get(handles.VariableList, 'value');
varName = vars{listboxVal};
switch get(obj, 'Tag')

  case 'SaveAs' % save the variable as a .mat file
    [fname, pname] = uiputfile(...
      {'*.mat', 'MAT files (*.mat)'; ...
      '*.txt', 'TXT files (*.txt)'}, ...
      sprintf('Save "%s" as:', varName), ...
      varName);
    if ~isequal(fname, 0) && ~isequal(pname, 0)
      saveDatFcn(pname, fname, handles.savedVars.(varName))
    end

  case 'Rename'  % rename the variable in the base workspace
    answer = inputdlg({sprintf('Rename "%s" as:', varName)}, ...
      'Rename...', 1, {varName});
    if ~isempty(answer) && ~strcmp(varName, answer{1})
      if ~(evalin('base', sprintf('exist("%s", "var")', answer{1}))) && ...
          isempty(strmatch(answer{1}, vars, 'exact'))
        newVarNames = vars;
        newVarNames{listboxVal} = answer{1};
        for id = 1:length(vars)
          tmp.(newVarNames{id}) = handles.savedVars.(vars{id});
        end
        handles.savedVars = tmp;
        showAllVarsFcn(handles);
        set(handles.VariableList, 'value', listboxVal);
        assignin('base', answer{1}, handles.savedVars.(answer{1}));
        evalin('base', sprintf('clear %s;', varName));
      else
        errordlg('That name is already in use.');
        return;
```

```
      end
    end

  case 'Delete' % delete the variable from the base workspace
    btn = questdlg(sprintf('Delete "%s" from the workspace?', varName), ...
      'Delete from workspace...', 'Yes', 'No', 'No');
    switch btn
      case 'Yes'
        handles.savedVars = rmfield(handles.savedVars, varName);
        showAllVarsFcn(handles);
        evalin('base', sprintf('clear %s;', varName));
      case 'No'
    end

end

guidata(obj, handles);


%--------------------------------------------------------------------------
%--------------------------------------------------------------------------
% saveDatFcn
%--------------------------------------------------------------------------
%--------------------------------------------------------------------------
function saveDatFcn(pname, fname, var)
% this function saves the variable to file

[p, fname, ext] = fileparts(fname);
switch lower(ext)
  case '.mat'
    eval(sprintf('%s = var;', fname));
    save(fullfile(pname, [fname, ext]), fname, '-v6');
  case '.txt'
    eval(sprintf('%s = var;', fname));
    save(fullfile(pname, [fname, ext]), fname, '-ascii', '-double', '-tabs');
  otherwise
    errordlg('Unknown extension.');
end


%--------------------------------------------------------------------------
%--------------------------------------------------------------------------
% selectVariableFcn
%--------------------------------------------------------------------------
%--------------------------------------------------------------------------
function selectVariableFcn(varargin)
% this callback is called when a variable is selected from the variable
% listbox.  When the variable is clicked, it will plot the data in the
% Preview Box above.  If the variable is double-clicked, it will be opened
% in the Array Editor.  It also ensures that the variable in the base
% workspace is the same copy as the variable stored in the GRABIT
% workspace.  This means that if you change the contents of a variable in
% the base workspace (via other functions), then it will allow you to
% update the variable in the GRABIT workspace.
```

```
obj = varargin{1};

handles = guidata(obj);

sType = get(handles.GrabitGUI, 'SelectionType');
switch sType
  case {'normal', 'open'} % single or double click
    vars = fieldnames(handles.savedVars);
    if isempty(vars)
      return;
    end
    listVal = get(obj, 'value');
    varName = vars{listVal};

    % check to see if the stored var is the same as the var in the
    % base workspace
    if evalin('base', sprintf('exist(''%s'', ''var'')', varName))

      % the copy in the base workspace must be a n-by-2 DOUBLE array
      if strcmp(class(evalin('base', varName)), 'double') && ...
         ndims(evalin('base', varName)) == 2 && ...
         size(evalin('base', varName), 2) == 2

        if ~isequal(evalin('base', varName), handles.savedVars.(varName))
          % may have been modified in the base workspace
          btn = questdlg(sprintf('''%s'' may have been modified in the base workspace (e.g.
Array Editor).\nUpdate the variable?', varName), ...
            'Modified Variable', 'Update Base Workspace', 'Update Grabit', 'Neither',
'Update Grabit');

          switch btn
            case 'Update Base Workspace'
              assignin('base', varName, handles.savedVars.(varName));

            case 'Update Grabit'
              handles.savedVars.(varName) = evalin('base', varName);
              showAllVarsFcn(handles);
              set(obj, 'value', listVal);

          end

        end

      else
        btn = questdlg(sprintf('''%s'' in the base workspace is different from the one in
Grabit.\nUpdate the base workspace variable?', varName), ...
          'Wrong Variable', 'Update Base Workspace', 'Leave Untouched', 'Update Base
Workspace');

        switch btn
          case 'Update Base Workspace'
            assignin('base', varName, handles.savedVars.(varName));

        end
```

```
      end

    else % the variable does not exist in base workspace, so make a copy
      assignin('base', varName, handles.savedVars.(varName));
    end

    switch sType
      case 'normal' % single click
        axes(handles.PreviewAxis);
        set(handles.PreviewLine, ...
          'xdata', handles.savedVars.(varName)(:, 1), ...
          'ydata', handles.savedVars.(varName)(:, 2));
        axis auto;

      case 'open' % double click
        try
          openvar(varName);
        catch
          errordlg(lasterr);
        end

    end
end

guidata(obj, handles);


%---------------------------------------------------------------------------
%---------------------------------------------------------------------------
% loadImageFcn
%---------------------------------------------------------------------------
%---------------------------------------------------------------------------
function loadImageFcn(varargin)
% this function loads an image file

[obj, filename] = splitvar(varargin([1, 3]));

handles = guidata(obj);

if isempty(filename)
  [fname, pathname] = uigetfile(...
    {'*.bmp;*.jpg;*.jpeg;*.tif;*.tiff;*.gif;*.png', ...
    'Image Files (*.bmp,  *.jpg,  *.jpeg,  *.tif,  *.tiff,  *.gif,  *.png)';
    '*.bpm', 'BPM files (*.bpm)';
    '*.jpg;*jpeg', 'JPG files (*.jpg,  *.jpeg)';
    '*.tif;*tiff', 'TIFF files (*.tif,  *.tiff)';
    '*.gif', 'GIF files (*.gif)';
    '*.png', 'PNG files (*.png)';
    '*.*', 'All files (*.*)'}, 'Select an image file');
  if ischar(fname)
    filename = fullfile(pathname, fname);
  else
    return;
  end
end
```

```
set(handles.ImageFileLoc, 'string', filename);

try
  %warning off;
  [A, map] = imread(filename);
  %warning on;
catch
  errordlg(lasterr);
  return;
end

if ndims(A) == 3   %some TIFF files have wrong size matrices.
  if size(A, 3)>3
    A = A(:, :, 1:3);
  elseif size(A, 3)<3
    errordlg('This is a weird image file...possibly a bad TIFF file...');
    return;
  end
end

% Need this so that image shows up when not called by a CALLBACK
set(0, 'ShowHiddenHandles', 'on');

cla(handles.PreviewAxis);
handles.PreviewLine = line(NaN, NaN, ...
  'color'     , 'blue', ...
  'linestyle' , '-', ...
  'marker'    , '.', ...
  'tag'       , 'PreviewLine', ...
  'parent'    , handles.PreviewAxis);
axes(handles.ImageAxis);
cla(handles.ImageAxis);
NP = get(handles.GrabitGUI, 'nextplot');  % for compatibility with R14

if isempty(map)
  imageInfo = imfinfo(filename);
  if strcmpi(imageInfo.ColorType, 'grayscale')
    colormap(gray(2^imageInfo.BitDepth));
  end
else
  colormap(map);
end
iH = image(A);
set(iH, 'HitTest', 'off');
set(handles.ImageAxis, 'xtick', [], 'ytick', []);
axis equal;

set(handles.GrabitGUI, 'nextplot', NP);
set(handles.ImageAxis, ...
  'SortMethod'      , 'depth', ...
  'tag'             , 'ImageAxis', ...
  'handlevisibility', 'callback', ...
  'buttondownfcn'   , @winBtnDownFcn);
set(get(handles.ImageAxis, 'title'), ...
```

```
  'string'          , '', ...
  'fontunits'       , 'pixels', ...
  'fontsize'        , 24, ...
  'color'           , 'red');
handles.ImageLine = line(NaN, NaN, ...
  'color'           , 'red', ...
  'linestyle'       , 'none', ...
  'marker'          , '.', ...
  'tag'             , 'ImageLine', ...
  'hittest'         , 'off');
handles.CalibPtsH(:,1) = line(repmat(NaN, 2, 4), repmat(NaN, 2, 4));
handles.CalibPtsH(:,2) = line(repmat(NaN, 2, 4), repmat(NaN, 2, 4));
set(handles.CalibPtsH(:,1), ...
  {'marker','color'}, {'o','r';'o','r';'o','b';'o','b'}, ...
  'markersize'      , 10, ...
  'hittest'         , 'off');
set(handles.CalibPtsH(:,2), ...
  {'marker','color'}, {'+','r';'x','r';'+','b';'x','b'}, ...
  'markersize'      , 20, ...
  'hittest'         , 'off');

% initialize image data
handles.I           = A;
handles.map         = map;
handles.CalibVals   = [];
handles.CalibPts    = [NaN, NaN, NaN, NaN];
handles.CalibPtsIm  = repmat(NaN, 4, 2);
handles.ImLimits    = [get(handles.ImageAxis, 'xlim'); ...
                       get(handles.ImageAxis, 'ylim')];

set(handles.CalibrateImageBtn, ...
  'string', 'Calibrate', ...
  'enable', 'inactive', ...
  'value' , 0);
set(handles.GrabPointsBtn, ...
  'enable', 'off', ...
  'value' , 0);
set(handles.ZoomStateBtn, ...
  'enable', 'inactive', ...
  'value' , 0);
zoom off;

set(0, 'ShowHiddenHandles', 'off');

guidata(obj, handles);


%--------------------------------------------------------------------------
%--------------------------------------------------------------------------
% calibrateImageFcn
%--------------------------------------------------------------------------
%--------------------------------------------------------------------------
function calibrateImageFcn(varargin)
% this function performs calibration of the image by prompting the user to
% select 4 points on the image as reference points.
```

```
obj = varargin{1};

handles = guidata(obj);

if isempty(handles.I)
  set(obj, 'enable', 'off');
else
  switch get(obj, 'value')
    case 0  % start calibration
      set(obj, 'value', 1, 'backgroundcolor', handles.bgcolor4);

      handles.curPointer              = 'custom';
      handles.curPointerData.CData    = handles.xoPointer;
      handles.curPointerData.HotSpot  = handles.xoPointerHotSpot;
      set(handles.GrabitGUI, ...
        'PointerShapeCData'     , handles.curPointerData.CData, ...
        'PointerShapeHotSpot'   , handles.curPointerData.HotSpot, ...
        'WindowButtonMotionFcn' , {@pointerFcn, handles, handles.curPointer});

      set([handles.LoadImageBtn, ...
          handles.SaveAs, ...
          handles.Rename, ...
          handles.Delete], ...
        'enable', 'off');

      set(handles.CalibPtsH, 'xdata', NaN, 'ydata', NaN);
      handles.CalibVals = [];
      handles.CalibPts = [NaN, NaN, NaN, NaN];
      handles.CalibPtsIm = repmat(NaN, 4, 2);
      set([handles.hXoValue, handles.hXmValue, ...
          handles.hYoValue, handles.hYmValue], ...
        'string', ' NaN');

      % this is the first calibration point: X-Axis Origin
      handles.curTitle = 'Click on the ORIGIN of x-axis';
      set(get(handles.ImageAxis, 'title'), ...
        'string', handles.curTitle);

      % change state to CALIBRATION
      handles.state = 'calibration';

    case 1  % stop (prematurely) calibration
      set(obj, ...
        'value'           , 0, ...
        'backgroundcolor' , handles.bgcolor3, ...
        'string'          , 'Calibrate');
      handles.curTitle = '';
      set(get(handles.ImageAxis, 'title'), 'string', '');

      handles.curPointer          = 'arrow';
      set(handles.GrabitGUI, ...
        'WindowButtonMotionFcn' , {@pointerFcn, handles, handles.curPointer});

      set([handles.LoadImageBtn, ...
```

```
        handles.SaveAs, ...
        handles.Rename, ...
        handles.Delete], ...
      'enable', 'on');

    % calibration was prematurely stopped, so reset all values
    set(handles.CalibPtsH, 'xdata', NaN, 'ydata', NaN);
    handles.CalibVals = [];
    handles.CalibPts = [NaN, NaN, NaN, NaN];
    handles.CalibPtsIm = repmat(NaN, 4, 2);
    set([handles.hXoValue, handles.hXmValue, ...
       handles.hYoValue, handles.hYmValue], ...
     'string', ' NaN');

    set(handles.GrabPointsBtn, 'enable', 'off');
    set(handles.CoordinateEdit, 'visible', 'off');

    % change state to NORMAL
    handles.state = 'normal';
  end
end

guidata(obj, handles);


%-----------------------------------------------------------------------
%-----------------------------------------------------------------------
% grabPointsFcn
%-----------------------------------------------------------------------
%-----------------------------------------------------------------------
function grabPointsFcn(varargin)
% this function is used to extract data points by prompting the user to
% select points on the image.

obj = varargin{1};

handles = guidata(obj);

switch get(obj, 'value')
  case 0  % initiate point grabbing
    calib = handles.CalibVals;

    axes(handles.ImageAxis);
    set(handles.ImageAxis, ...
      'xlim', handles.ImLimits(1,:), ...
      'ylim', handles.ImLimits(2,:));
    set(handles.ImageLine, 'xdata', NaN, 'ydata', NaN);
    handles.curTitle = {'Grab points by clicking on data points.', ...
    '<BACKSPACE> or <DEL> to delete previous point. <ENTER> to finish.'};
    set(get(handles.ImageAxis, 'title'), ...
      'string', handles.curTitle);

    set(handles.PreviewLine, 'xdata', NaN, 'ydata', NaN);
    set(handles.PreviewAxis, ...
      'xlim', [min([calib.Xo calib.Xm]) max([calib.Xo calib.Xm])], ...
```

```
    'ylim', [min([calib.Yo calib.Ym]) max([calib.Yo calib.Ym])]);

  handles.ImDat   = [];
  handles.TrueDat = [];

  handles.curPointer          = 'crosshair';
  set(handles.GrabitGUI, ...
    'WindowButtonMotionFcn', {@pointerFcn, handles, handles.curPointer});

  set(obj, ...
    'value'           , 1, ...
    'string'          , 'Grabbing Points (0)', ...
    'backgroundcolor' , handles.bgcolor4);
  set([handles.CalibrateImageBtn, ...
      handles.LoadImageBtn, ...
      handles.SaveAs, ...
      handles.Rename, ...
      handles.Delete], ...
    'enable', 'off');

  % change state to GRAB
  handles.state = 'grab';

 case 1  % finish point grabbing
  set(obj, ...
    'value'           , 0, ...
    'string'          , 'Grab Points', ...
    'backgroundcolor' , handles.bgcolor1);

  handles.curTitle = '';
  set(get(handles.ImageAxis, 'title'), 'string', '');
  handles.curPointer            = 'arrow';
  set(handles.GrabitGUI,     'WindowButtonMotionFcn',     {@pointerFcn,     handles,
handles.curPointer});
  set(handles.CalibrateImageBtn, 'enable', 'inactive');
  set([handles.LoadImageBtn, ...
      handles.SaveAs, ...
      handles.Rename, ...
      handles.Delete], ...
    'enable', 'on');
  if ~isempty(handles.TrueDat) % some points were grabbed
    varNames                        = fieldnames(handles.savedVars);
    varNames{end + 1}               = findNextVarNameFcn(varNames);
    handles.savedVars.(varNames{end}) = handles.TrueDat;
    assignin('base', varNames{end}, handles.savedVars.(varNames{end}));
    showAllVarsFcn(handles);
  end

  % change to NORMAL state
  handles.state = 'normal';
end

guidata(obj, handles);
```

```
%--------------------------------------------------------------------------
%--------------------------------------------------------------------------
% showAllVarsFcn
%--------------------------------------------------------------------------
%--------------------------------------------------------------------------
function showAllVarsFcn(handles)
% this function shows all data set variables that exist in the GRABIT
% workspace in the variable listbox.

varNames = fieldnames(handles.savedVars);

if isempty(varNames)
  listboxStr = {''};
else
  for id = 1:length(varNames)
    [m, n] = size(handles.savedVars.(varNames{id}));
    listboxStr{id} = sprintf('%s [%dx%d]', varNames{id}, m, n);
  end
end

set(handles.VariableList, 'string', listboxStr, 'value', length(listboxStr));


%--------------------------------------------------------------------------
%--------------------------------------------------------------------------
% findNextVarNameFcn
%--------------------------------------------------------------------------
%--------------------------------------------------------------------------
function newVarName = findNextVarNameFcn(varNames)
% this helper function determines the next available variable name by
% checking the existing variable names in the base workspace and GRABIT
% workspace.

wsVarNames = evalin('base', 'who');
vars = unique([wsVarNames(:); varNames(:)]);
idx = 1;
while 1
  if isempty(strmatch(sprintf('Data%03d', idx), vars, 'exact'))
    newVarName = sprintf('Data%03d', idx);
    return;
  else
    idx = idx + 1;
  end
end


%--------------------------------------------------------------------------
%--------------------------------------------------------------------------
% zoomBtnFcn
%--------------------------------------------------------------------------
%--------------------------------------------------------------------------
function zoomBtnFcn(varargin)
% this function toggles the zoom state

obj = varargin{1};
```

```
handles = guidata(obj);

switch get(obj, 'value')
  case 0
    set(obj, ...
      'value'         , 1, ...
      'backgroundcolor', handles.bgcolor4, ...
      'cdata'         , handles.zoomIconDown);
    udata.titlestring = get(get(handles.ImageAxis, 'Title'), 'string');
    udata.btnstate = get([handles.LoadImageBtn, ...
        handles.CalibrateImageBtn, ...
        handles.GrabPointsBtn, ...
        handles.VariableList, ...
        handles.SaveAs, ...
        handles.Rename, ...
        handles.Delete],'enable');
    udata.imgstate = get(handles.ImageAxis, 'ButtonDownFcn');
    udata.handlevisibility = get(handles.ImageAxis, 'handlevisibility');
    set(obj, 'userdata', udata);

    set(get(handles.ImageAxis, 'Title'), 'string', 'Zoom ON');
    set([handles.LoadImageBtn, ...
        handles.CalibrateImageBtn, ...
        handles.GrabPointsBtn, ...
        handles.VariableList, ...
        handles.SaveAs, ...
        handles.Rename, ...
        handles.Delete], 'enable', 'off');
    set(handles.ImageAxis, 'ButtonDownFcn', '');
    zoom('on');
    handles.curPointerData.CData   = get(handles.GrabitGUI, 'PointerShapeCData');
    handles.curPointerData.HotSpot = get(handles.GrabitGUI, 'PointerShapeHotSpot');
    set(handles.GrabitGUI, ...
      'PointerShapeCData'     , handles.zoomPointer, ...
      'PointerShapeHotSpot'   , handles.zoomPointerHotSpot, ...
      'WindowButtonMotionFcn' , {@pointerFcn, handles, 'custom'}, ...
      'keypressfcn'           , ';'); % this prevents switching to command window

    % this seems necessary in some versions of Matlab
    set(handles.ImageAxis, 'handlevisibility', 'on');

  case 1
    set(obj, ...
      'value', 0, ...
      'backgroundcolor', handles.bgcolor3, ...
      'cdata', handles.zoomIconUp);
    zoom('off');

    %--------------------------------------------------------------------
    % If zoom created a compact view window, expand it to fill the whole
    % axes.
    %--------------------------------------------------------------------
    xl = get(handles.ImageAxis, 'xlim'); xrng = diff(xl);
    yl = get(handles.ImageAxis, 'ylim'); yrng = diff(yl);
```

```
    if abs(yrng/xrng - handles.imAxRatio) > .01 % wrong axes ratio
      if yrng/xrng > handles.imAxRatio
        xrng = yrng / handles.imAxRatio;
        xl   = mean(xl) + [-0.5, 0.5] * xrng;
      else
        yrng = xrng * handles.imAxRatio;
        yl   = mean(yl) + [-0.5, 0.5] * yrng;
      end
      set(handles.ImageAxis, 'xlim', xl, 'ylim', yl);
    end
    %-----------------------------------------------------------------------

    udata = get(obj, 'userdata');
    set(get(handles.ImageAxis, 'Title'), 'string', udata.titlestring);
    set(handles.ImageAxis, 'handlevisibility', udata.handlevisibility);
    set([handles.LoadImageBtn, ...
        handles.CalibrateImageBtn, ...
        handles.GrabPointsBtn, ...
        handles.VariableList, ...
        handles.SaveAs, ...
        handles.Rename, ...
        handles.Delete], {'enable'}, udata.btnstate);
    set(handles.ImageAxis, 'ButtonDownFcn', udata.imgstate);
    set(handles.GrabitGUI, ...
      'PointerShapeCData'     , handles.curPointerData.CData, ...
      'PointerShapeHotSpot'   , handles.curPointerData.HotSpot, ...
      'WindowButtonMotionFcn' , {@pointerFcn, handles, handles.curPointer}, ...
      'keypressfcn'           , @keyPressFcn);
end

guidata(obj, handles);


%-----------------------------------------------------------------------
%-----------------------------------------------------------------------
% pointerFcn
%-----------------------------------------------------------------------
%-----------------------------------------------------------------------
function pointerFcn(varargin)
% this changes the pointer based on whether the cursor is in the image
% axes.

[handles, ptr] = splitvar(varargin(3:4));

pt = get(handles.ImageAxis, 'CurrentPoint');
xl = get(handles.ImageAxis, 'xlim');
yl = get(handles.ImageAxis, 'ylim');
if pt(1,1) > xl(1) && pt(1,1) < xl(2) && pt(1,2) > yl(1) && pt(1,2) < yl(2)
  set(handles.GrabitGUI, 'pointer', ptr);
else
  set(handles.GrabitGUI, 'pointer', 'arrow');
end


%-----------------------------------------------------------------------
```

```
%--------------------------------------------------------------------------
% figResize
%--------------------------------------------------------------------------
%--------------------------------------------------------------------------
function figResizeFcn(varargin)
% this function makes sure the axis fills the whole axes extent

obj = varargin{1};

handles = guidata(obj);

axis(handles.ImageAxis, 'equal');
handles.imAxRatio = diff(get(handles.ImageAxis, 'ylim')) / ...
  diff(get(handles.ImageAxis, 'xlim'));
handles.ImLimits = [get(handles.ImageAxis, 'xlim'); ...
    get(handles.ImageAxis, 'ylim')];

guidata(obj, handles);


%--------------------------------------------------------------------------
%--------------------------------------------------------------------------
% keyPressFcn
%--------------------------------------------------------------------------
%--------------------------------------------------------------------------
function keyPressFcn(varargin)
% this is for the keyboard shortcuts. During 'grab' mode, <backspace>
% deletes the last point clicked and <return> ends the 'grab' mode.

obj = varargin{1};

handles = guidata(obj);

if ~isempty(handles.I)
  k = lower(get(obj, 'CurrentKey'));

  switch k
    case 'a'        % zoom in
      xl = get(handles.ImageAxis, 'xlim'); xrng = diff(xl);
      yl = get(handles.ImageAxis, 'ylim'); yrng = diff(yl);

      % prevent zooming in too much.
      % set the limit to 64x zoom.
      if xrng >= size(handles.I, 2)/64*2
        % animate zoom
        for id = 0:0.2:1
          set(handles.ImageAxis, ...
            'xlim', xl + id * xrng / 4 * [1, -1], ...
            'ylim', yl + id * yrng / 4 * [1, -1]);
          drawnow;
        end
      end

    case 'z'        % zoom out
      xl = get(handles.ImageAxis, 'xlim'); xrng = diff(xl);
```

```
    yl = get(handles.ImageAxis, 'ylim'); yrng = diff(yl);
    % animate zoom
    for id = 0:0.2:1
      set(handles.ImageAxis, ...
        'xlim', xl + id * xrng / 2 * [-1, 1], ...
        'ylim', yl + id * yrng / 2 * [-1, 1]);
      drawnow;
    end

  case 'space'    % reset view
    resetViewFcn(handles.ResetViewBtn);

  case {'backspace', 'delete'}

    switch handles.state
      case 'grab'
        if ~handles.isPanning

          if isempty(handles.ImDat)
            return;
          else
            handles.ImDat(end, :)   = [];
            handles.TrueDat(end, :) = [];
          end

          set(handles.PreviewLine, ...
            'xdata', handles.TrueDat(:, 1), ...
            'ydata', handles.TrueDat(:, 2));
          set(handles.ImageLine, ...
            'xdata', handles.ImDat(:, 1), ...
            'ydata', handles.ImDat(:, 2));

          set(handles.GrabPointsBtn,    'string',    sprintf('Grabbing    Points    (%d)',
size(handles.ImDat, 1)));

          guidata(obj, handles);
        end

    end

  case {'return', 'enter'}

    switch handles.state
      case 'grab'
        grabPointsFcn(handles.GrabPointsBtn);

    end

  end
end


%---------------------------------------------------------------------
%---------------------------------------------------------------------
% resetViewFcn
```

```
%--------------------------------------------------------------------------
%--------------------------------------------------------------------------
function resetViewFcn(varargin)
% this function resets the view

obj = varargin{1};

handles = guidata(obj);

if ~isempty(handles.I)
  xl = get(handles.ImageAxis, 'xlim');
  yl = get(handles.ImageAxis, 'ylim');
  xd = (handles.ImLimits(1, :) - xl) / 10;
  yd = (handles.ImLimits(2, :) - yl) / 10;
  % animate zoom
  for id = 0:10
    set(handles.ImageAxis, ...
      'xlim', xl + id * xd, ...
      'ylim', yl + id * yd);
    drawnow;
  end
end

% take focus away
loseFocusFcn(handles)


%--------------------------------------------------------------------------
%--------------------------------------------------------------------------
% loseFocusFcn
%--------------------------------------------------------------------------
%--------------------------------------------------------------------------
function loseFocusFcn(handles)
% attempt to take focus away by setting the ENABLE property to off and then
% back to the original setting

settings = get([handles.LoadImageBtn, ...
    handles.ResetViewBtn, ...
    handles.VariableList, ...
    handles.SaveAs, ...
    handles.Rename, ...
    handles.Delete], ...
  'enable');
set([handles.LoadImageBtn, ...
    handles.ResetViewBtn, ...
    handles.VariableList, ...
    handles.SaveAs, ...
    handles.Rename, ...
    handles.Delete], ...
  'enable', 'off');
drawnow;
set([handles.LoadImageBtn, ...
    handles.ResetViewBtn, ...
    handles.VariableList, ...
    handles.SaveAs, ...
```

```
    handles.Rename, ...
    handles.Delete], ...
 {'enable'}, settings);


%---------------------------------------------------------------------
%---------------------------------------------------------------------
% winBtnDownFcn
%---------------------------------------------------------------------
%---------------------------------------------------------------------
function winBtnDownFcn(varargin)
% this function is called when the mouse click initiates

obj = varargin{1};

handles = guidata(obj);

if strcmpi(get(handles.timer, 'Running'), 'on') || isempty(handles.I)
  return;
end

handles.CurrentPointAxes = get(handles.ImageAxis, 'CurrentPoint');
handles.CurrentPointFig  = get(handles.GrabitGUI, 'CurrentPoint');

switch get(handles.GrabitGUI, 'SelectionType')
  case 'normal'

    set(handles.CoordinateEdit, 'visible', 'off');
    id = find(isnan(handles.CalibPts));
    if ~isempty(id)
      set(handles.CalibPtsH(id(1),:), 'xdata', NaN, 'ydata', NaN);
    end

    % first call winBtnMotionPauseFcn to prevent immediate click-n-drag
    set(handles.GrabitGUI, ...
      'WindowButtonMotionFcn', ...
      {@winBtnMotionPauseFcn, handles, handles.CurrentPointAxes(1,1:2), clock});

  case 'alt'

    set(handles.CoordinateEdit, 'visible', 'off');
    id = find(isnan(handles.CalibPts));
    if ~isempty(id)
      set(handles.CalibPtsH(id(1),:), 'xdata', NaN, 'ydata', NaN);
    end

    xl = get(handles.ImageAxis, 'XLim');midX = (xl(1)+xl(2))/2;
    yl = get(handles.ImageAxis, 'YLim');midY = (yl(1)+yl(2))/2;
    figPos = get(handles.GrabitGUI, 'Position');
    handles.curTitle = get(get(handles.ImageAxis, 'title'), 'string');
    set(handles.GrabitGUI, ...
      'Pointer', 'custom', ...
      'PointerShapeCData'     , handles.zoomInOutPointer, ...
      'PointerShapeHotSpot'   , handles.zoomInOutPointerHotSpot, ...
      'WindowButtonMotionFcn' , {@zoomMotionFcn, handles, ...
```

```
                        get(handles.GrabitGUI, 'CurrentPoint'), ...
                        figPos(4), ...
                        size(handles.I, 2), ...
                        midX, ...
                        midY, ...
                        diff(xl)/2, ...
                        diff(yl)/2});

   set(get(handles.ImageAxis, 'title'), 'string', 'Zooming...');

end

guidata(obj, handles);


%---------------------------------------------------------------------
%---------------------------------------------------------------------
% zoomMotionFcn
%---------------------------------------------------------------------
%---------------------------------------------------------------------
function zoomMotionFcn(varargin)
% this performs the click-n-drag zooming function. The pointer location
% relative to the initial point determines the amount of zoom (in or out).

[obj, handles, initPt, figHt, horizPx, midX, ...
  midY, rngXhalf, rngYhalf] = splitvar(varargin([1, 3:end]));

pt = get(obj, 'CurrentPoint');

% get relative pointer location (y-coord only).
% power law allows for the inverse to work:
%   C^(x) * C^(-x) = 1
% Choose C to get "appropriate" zoom factor
r = 30 ^ ((initPt(2) - pt(2)) / figHt);

% make sure it doesn't zoom in too much.
% the limit is based on the size of the original image.
% set limit to 64x zoom.
if r < horizPx/64/rngXhalf/2  % stop zoom
  set(get(handles.ImageAxis, 'title'), 'string', 'Max Zoom Reached');
  set(obj, ...
    'Pointer'              , 'arrow', ...
    'WindowButtonMotionFcn' , '');
else
  set(handles.ImageAxis, ...
    'XLim', [midX - r * rngXhalf, midX + r * rngXhalf], ...
    'YLim', [midY - r * rngYhalf, midY + r * rngYhalf]);
end


%---------------------------------------------------------------------
%---------------------------------------------------------------------
% winBtnMotionPauseFcn
%---------------------------------------------------------------------
%---------------------------------------------------------------------
```

```
function winBtnMotionPauseFcn(varargin)
% this prevents click-n-drag from happening for X seconds. This is useful
% because users may move the mouse as they are clicking.

[obj, handles, xy, c] = splitvar(varargin([1, 3:end]));

if etime(clock, c) > .15  % waits .15 seconds before dragging occurs
  set(obj, ...
    'Pointer'            , 'custom', ...
    'PointerShapeCData'   , handles.closedHandPointer, ...
    'PointerShapeHotSpot' , handles.closedHandPointerHotSpot);
  set(obj, 'WindowButtonMotionFcn', {@winBtnMotionFcn, handles.ImageAxis, xy});
  handles.curTitle = get(get(handles.ImageAxis, 'title'), 'string');
  set(get(handles.ImageAxis, 'title'), 'string', 'Panning...');
  handles.isPanning = true;
  guidata(obj, handles);
end


%----------------------------------------------------------------------
%----------------------------------------------------------------------
% winBtnMotionFcn
%----------------------------------------------------------------------
%----------------------------------------------------------------------
function winBtnMotionFcn(varargin)
% this function is called when click-n-drag (panning) is happening

[axH, xy] = splitvar(varargin(3:4));

pt = get(axH, 'CurrentPoint');
set(axH, ...
  'xlim', get(axH, 'xlim')+(xy(1)-pt(1,1)), ...
  'ylim', get(axH, 'ylim')+(xy(2)-pt(1,2)));


%----------------------------------------------------------------------
%----------------------------------------------------------------------
% winBtnUpFcn
%----------------------------------------------------------------------
%----------------------------------------------------------------------
function winBtnUpFcn(varargin)
% this is called when the mouse button is released. It initiates the button
% down timer object (see btnUpTimerFcn). This timer waits for 0.2 seconds
% before any action is taken. This is in order to detect a double click. If
% a double click is detected, the single click action is NOT executed.

obj = varargin{1};

handles = guidata(obj);
if ~isempty(handles.I)  % there is an image displayed
  switch get(obj, 'SelectionType')
    case 'normal'
      if strcmpi(get(handles.timer, 'Running'), 'off')

        % start the timer which waits some time to see if double-clicking
```

```
      % occurs
      start(handles.timer);

      set(obj, ...
        'Pointer'              , handles.curPointer, ...
        'PointerShapeCData'    , handles.curPointerData.CData, ...
        'PointerShapeHotSpot'  , handles.curPointerData.HotSpot, ...
        'WindowButtonMotionFcn' , {@pointerFcn, handles, handles.curPointer});

      set(get(handles.ImageAxis, 'title'), 'string', handles.curTitle);
    end

  case 'alt'
    set(obj, ...
      'Pointer'              , handles.curPointer, ...
      'PointerShapeCData'    , handles.curPointerData.CData, ...
      'PointerShapeHotSpot'  , handles.curPointerData.HotSpot, ...
      'WindowButtonMotionFcn' , {@pointerFcn, handles, handles.curPointer});

    set(get(handles.ImageAxis, 'title'), 'string', handles.curTitle);
 end
end


%--------------------------------------------------------------------------
%--------------------------------------------------------------------------
% btnUpTimerFcn
%--------------------------------------------------------------------------
%--------------------------------------------------------------------------
function btnUpTimerFcn(varargin)

figH = varargin{3};

handles = guidata(figH);

switch get(handles.GrabitGUI, 'SelectionType')
 case 'open' % double click
   % this will center the view

   % get current units
   un = get([0, handles.GrabitGUI, handles.ImageAxis], 'units');

   set([0, handles.GrabitGUI, handles.ImageAxis], 'units', 'pixels');
   pt    = get(0, 'PointerLocation');
   figPos = get(handles.GrabitGUI, 'Position');
   pt2   = pt - figPos(1:2);
   axPos  = get(handles.ImageAxis, 'position');

   % check to see if pointer is inside the image axes
   if pt2(1) > axPos(1) && pt2(1) < axPos(1)+axPos(3) && ...
      pt2(2) > axPos(2) && pt2(2) < axPos(2)+axPos(4)
     xl = get(handles.ImageAxis, 'xlim'); xrng = diff(xl);
     yl = get(handles.ImageAxis, 'ylim'); yrng = diff(yl);
     x  = (pt2(1)-axPos(1))/axPos(3)*xrng+xl(1);
     y  = (axPos(2)+axPos(4)-pt2(2))/axPos(4)*yrng+yl(1);
```

```
    % animate (slide) to the new location. this may give a better
    % visual perception of the view change

    % determine how fast to animate, depending on the location of the
    % pointer
    interval = ceil(norm((figPos(1:2)+axPos(1:2)+axPos(3:4)/2) - pt)/30);

    if interval
      ld = (([x, y] - [xrng, yrng]/2) - [xl(1), yl(1)])/interval;
      pd = ((figPos(1:2)+axPos(1:2)+axPos(3:4)/2) - pt)/interval;
    else
      % interval == 0, meaning it's the same point
      ld = [0, 0];
      pd = [0, 0];
    end

    for id = 0:interval
      set(handles.ImageAxis, ...
        'xlim', xl + id * ld(1), ...
        'ylim', yl + id * ld(2));

      % center the pointer location
      set(0, ...
        'PointerLocation', pt + id * pd);
      drawnow;
    end
  end

  % reset UNITS
  set([0, handles.GrabitGUI, handles.ImageAxis], {'units'}, un);

case 'normal'  % single click

  switch handles.state
    case 'grab'
      if ~handles.isPanning && ~isempty(handles.CurrentPointAxes)
        calib = handles.CalibVals;

        X = handles.CurrentPointAxes(1, 1);
        Y = handles.CurrentPointAxes(1, 2);

        % the point on the image
        handles.ImDat(end + 1, :) = [X, Y];

        % the true data point coordinates
        handles.TrueDat(end + 1, :) = ([calib.Xo; calib.Yo] + ...
          [calib.e1 calib.e2] \ [X - calib.Xxo; Y - calib.Yyo])';
        set(handles.PreviewLine, ...
          'xdata', handles.TrueDat(:, 1), ...
          'ydata', handles.TrueDat(:, 2));
        set(handles.ImageLine, ...
          'xdata', handles.ImDat(:, 1), ...
          'ydata', handles.ImDat(:, 2));
```

```
        set(handles.GrabPointsBtn, 'string', sprintf('Grabbing Points (%d)',
size(handles.ImDat, 1)));

      end

    case 'calibration'
      if ~handles.isPanning && ~isempty(handles.CurrentPointAxes)
        id = find(isnan(handles.CalibPts));
        pt = handles.CurrentPointAxes;
        set(handles.CalibPtsH(id(1),:), 'xdata', pt(1,1), 'ydata', pt(1,2));
        handles.CalibPtsIm(id(1),:) = pt(1,1:2);
        set(handles.CoordinateEdit, ...
          'units'   , 'pixels', ...
          'position', [handles.CurrentPointFig+[5, 5], 100, 25], ...
          'visible' , 'on', ...
          'string'  , 'Enter Value');
      end

  end
end

handles.CurrentPointAxes = [];
handles.CurrentPointFig  = [];
handles.isPanning        = false;

guidata(handles.GrabitGUI, handles);


%--------------------------------------------------------------------------
%--------------------------------------------------------------------------
% coordinateEditFcn
%--------------------------------------------------------------------------
%--------------------------------------------------------------------------
function coordinateEditFcn(varargin)
% this function is called when a value is entered in the edit box for
% calibration values

obj = varargin{1};

handles = guidata(obj);

strs = {'Click on the MAXIMUM value of x-axis', ...
   'Click on the ORIGIN of y-axis', ...
   'Click on the MAXIMUM value of y-axis', ...
   ''};
ptrs = {'xmPointer', 'yoPointer', 'ymPointer'};
hs = {'hXoValue', 'hXmValue', 'hYoValue', 'hYmValue'};

val = str2double(get(obj, 'string'));
if ~isnan(val)
  id = find(isnan(handles.CalibPts));
  if ~isempty(id)
    handles.CalibPts(id(1)) = val;
    set(handles.(hs{id(1)}), 'string', sprintf(' %g', val));
    set(obj, 'visible', 'off');
```

```
    handles.curTitle = strs{id(1)};
    set(get(handles.ImageAxis, 'title'), 'string', strs{id(1)});
    if id(1) == 4 % last calibration point
      handles = calculateCalibrationFcn(handles);
    else
      handles.curPointerData.CData    = handles.(ptrs{id(1)});
      handles.curPointerData.HotSpot  = handles.([ptrs{id(1)} 'HotSpot']);
      set(handles.GrabitGUI, ...
        'PointerShapeCData'   , handles.curPointerData.CData, ...
        'PointerShapeHotSpot' , handles.curPointerData.HotSpot);
    end
  else
    set(obj, 'string', '');
  end
else
  set(obj, 'string', '');
end

guidata(handles.GrabitGUI, handles);


%--------------------------------------------------------------------------
%--------------------------------------------------------------------------
% calculateCalibrationFcn
%--------------------------------------------------------------------------
%--------------------------------------------------------------------------
function handles = calculateCalibrationFcn(handles)
% this function calculates the calibration values

Xxo = handles.CalibPtsIm(1,1);
Yxo = handles.CalibPtsIm(1,2);
Xxm = handles.CalibPtsIm(2,1);
Yxm = handles.CalibPtsIm(2,2);
Xyo = handles.CalibPtsIm(3,1);
Yyo = handles.CalibPtsIm(3,2);
Xym = handles.CalibPtsIm(4,1);
Yym = handles.CalibPtsIm(4,2);

v1  = [Xxm - Xxo; Yxm - Yxo];
v2  = [Xym - Xyo; Yym - Yyo];

Xo  = handles.CalibPts(1);
Xm  = handles.CalibPts(2);
Yo  = handles.CalibPts(3);
Ym  = handles.CalibPts(4);

Xlength = Xm - Xo;
Ylength = Ym - Yo;

% the basis vectors in the MATLAB domain
e1 = v1 / Xlength;
e2 = v2 / Ylength;

% rearrage axes
C     = [e1 e2] \ [Xyo - Xxo; Yyo - Yxo];
```

```
blahX = [Xxo; Yxo] + C(2) * e2; Xxo = blahX(1); Yxo = blahX(2);
blahY = [Xyo; Yyo] - C(1) * e1; Xyo = blahY(1); Yyo = blahY(2);

calib.Xo  = Xo;
calib.Xm  = Xm;
calib.Yo  = Yo;
calib.Ym  = Ym;
calib.e1  = e1;
calib.e2  = e2;
calib.Xxo = Xxo;
calib.Yyo = Yyo;

handles.CalibVals = calib;
set(handles.GrabPointsBtn, ...
  'enable', 'inactive');
set(handles.CalibrateImageBtn, ...
  'value'           , 0, ...
  'backgroundcolor' , handles.bgcolor3, ...
  'enable'          , 'inactive', ...
  'string'          , 'Re-Calibrate');

handles.state = 'normal';

handles.curPointer = 'arrow';
set(handles.GrabitGUI     , ...
  'WindowButtonMotionFcn' , {@pointerFcn, handles, handles.curPointer});

set([handles.LoadImageBtn, ...
    handles.SaveAs, ...
    handles.Rename, ...
    handles.Delete], ...
  'enable', 'on');


%--------------------------------------------------------------------------
%--------------------------------------------------------------------------
% splitvar
%--------------------------------------------------------------------------
%--------------------------------------------------------------------------
function varargout = splitvar(varargout)
% this function splits input arguments into individual variables.


%--------------------------------------------------------------------------
%--------------------------------------------------------------------------
% createSampleImageFcn
%--------------------------------------------------------------------------
%--------------------------------------------------------------------------
function fname = createSampleImageFcn
% this function creates a temporary image file in the temp directory by
% loading in a sample binary image data.
```