ENZYME FOCUSED PROCESS ENGINEERING: EVALUATING THE INFLUENCE OF COMPLEX PROCESS ENVIRONMENTS ON ENZYME ACTIVITY

Kristoffer Still

BSc, MSc

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ABSTRACT

Complex process environments such as the treatment of wastewater are susceptible to a high amount of variability. Industrial processes that incorporate a biological component, either microorganisms or enzymes, are especially prone to this variability from multiple external influences. Other examples apart from wastewater treatment include the production of biologically derived chemicals and biopharmaceuticals. Wastewater is highly dynamic as both its characteristics and composition vary over time substantially increasing the level of complexity. Therefore understanding the variability associated with wastewater treatment through enzyme activity provides a foundation for studying the other mentioned biotechnology processes.

Enzymes are critical in the treatment of wastewater as they are responsible for remediating the majority of organic pollutants. The incoming wastewater feedstock is converted to a final product that is safe for release into the receiving environment. The main aim of this research was to demonstrate enzyme variability, and identify the environmental and process factors that contribute to this for enzymes both exogenous and inherent to the wastewater system. Exogenous enzymes proposed as endocrine disrupting compound (EDC) treatment technologies and enzymes inherent to the wastewater system were studied.

Laccase and tyrosinase were the chosen exogenous enzymes and their ability to remove EDCs investigated. Estrone (E1) was the EDC primarily studied with diclofenac (DCF) included later to analyse the influence mixed substrate solutions have on enzyme performance. E1 and DCF are both on the European Union Watch List due to their adverse effects on aquatic wildlife. An enzymatic water quality parameter was developed to study the temporal variability of E1 removal by laccase and tyrosinase. Previous literature had mostly shown laccase to degrade EDCs in wastewater from a single sample snapshot. The experimental conditions for the developed enzymatic parameter remained constant with the tested effluent varying between sample days.

The enzymatic parameter with laccase and tyrosinase was performed over a sampling period of four months. E1 removal (%) was variable for the two enzymes as E1 removal ranged between 54% and 92%. There was little correlation between laccase and tyrosinase performance over this sampling period, even though the two enzymes

are oxidoreductases. Average E1 removal (%) for laccase during this sampling period was 74.8% (± 9.6%) and for tyrosinase the average was 83.0% (±. 8.3%). This variability in removal was due to the dynamic nature of the related process and environmental factors such as pH and dissolved oxygen.

Constituents in the wastewater can also inhibit laccase and tyrosinase activity. In this research chloride (Cl^-) and copper (Cu^{2+}) were the studied inhibitors. DCF was also used to analyse EDC mixture effects with E1. The inhibitory effects were examined in a wastewater matrix unlike the literature that has mainly investigated clean buffer solutions. The addition of Cu^{2+} at 0.05 mg/L to the enzymatic water quality parameters greatly inhibited tyrosinase performance reducing E1 removal. Compared to the control that contained no added Cu^{2+} E1 removal was 20% lower. Laccase unlike tyrosinase was also able to remove DCF and the addition of E1 increased DCF removal between 11% and 27% in the effluent, depending on DCF concentration.

Inherent enzymes in particular hydrolases are essential in the breakdown of the majority of organic pollutants and the subsequent assimilation by microorganisms within the WWTP activated sludge. Five inherent hydrolases were analysed in this research and they were α-glucosidase, β-glucosidase, alkaline phosphatase, esterase and sulfatase. The activities for these enzymes in the literature had shown to vary considerably between WWTPs and the same WWTP. This understanding was expanded on by measuring inherent activity across a WWTP and correlating the recorded activities with measured standard water quality parameters using Pearson's correlation. The WWTP sampled from was unique in the UK as it incorporated a membrane bioreactor (MBR) system.

Not previously discussed in the literature has been inherent activity associated with returned activated sludge (RAS). The MBR system unlike conventional WWTPs is far more efficient at recycling RAS and retaining enzymes in the system. Inherent activity was reported to be higher in the RAS than the mixed liquor for all hydrolases apart from esterase even when activity was corrected against solid concentration. This shows other influences apart from biomass concentration influencing enzyme activity. The reduction in esterase activity between the RAS and the mixed liquor ranged between 4.7% and 22.1%. Analysing inherent activity throughout the WWTP demonstrated

temporal and spatial variability with the contributory environmental and process factors highlighted.

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PRESENTATIONS

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The University of Nottingham BBSRC Doctoral Training Partnership (DTP) is undertaken in partnership with Rothamsted Research. Its aim is to develop an innovative, integrated, student-led programme of individualised research training in the Biosciences through an effective cohort-based programme. Students are not recruited to an individual project but, through a series of lab rotations, identify a PhD programme that they wish to pursue in one of three BBSRC priority research areas (Food Security; Industrial Biotechnology and Bioenergy; World Class Biosciences)

Research Training Structure

The research training structure is framed by individual development needs analysis and has the following core components:

- Generic and research skills development
- High-level modular training
- Laboratory rotations
- Experiential learning through public engagement and outreach activity

Generic and research skills development comprises the following: induction, introductory training programme, annual 'Spring School' conference-style training event, participation in Sysmic (online systems and mathematical biology course), specialist training days at partner organisations, twilight (after-lab) training which is arranged on an *ad hoc* basis to meet identified needs, and seminar series.

In Year One there is an initial period of intensive training which takes place over the course of the first six months. This comprises a two-week introductory training programme, 24 weeks of laboratory rotations, followed by a week-long 'Spring School' (conference-style training event for all DTP cohorts) and ending in a science outreach event. Each lab rotation is assessed focussing on a different form of scientific communication (Report, Poster, Presentation) and students receive feedback from supervisors. The PhD project begins in Month 7. Students are also encouraged to choose from a portfolio of relevant Master Courses which they can attend alongside their lab rotation projects.

In Years Two to Four training is mainly concentrated in the week long annual Spring School, although additional training and seminars are delivered where required throughout the year. A three month Professional Internship for PhD Students (PIPS) placement is usually taken in Year Three and, as BBSRC specifies, this is not in an area related to the students' PhD research programme.

The integrated programme of training is mapped to BBSRC priority areas, BBSRC Skills Statement: Scientific and Core Skills for UK Bioscience and the Researcher Development Framework (RDF) to ensure breadth and depth of provision, and to encompass vulnerable skills and strategic competencies. It builds on the best practice principles outlined in the Statement of Expectations for Doctoral Training, RCUK (2013) and the 'Good Practice Elements in Doctoral Training', LERU (2014).

It offers a wide range of skills development opportunities to prepare students for a successful bioscience-related career and supports the exchange of ideas and experiences within and across cohorts. It also generates a strong community of postgraduates and supervisors to stimulate the formation of cross-disciplinary links through cohort development, which prepares students for a career in an increasingly multidisciplinary environment.

Reflection Form

Name of Organization

Thames Water Ltd.

Details of Placement

The placement was carried out at the operational and research facility, Old Ford Water Recycling Plant operated by Thames Water. Thames Water is the largest water and wastewater services company within the UK so provided a great insight into the industry. Furthermore, the Old Ford treatment process is unique in the UK as wastewater is treated and fed straight into a non-potable water distribution network around the Olympic Park site in London. Activities from the placement included learning more about wastewater reuse and drivers for perusing this treatment approach such as current drinking water supplies being unsustainable. Other activities included establishing the benefits of the treatment process employed at Old Ford as well as understanding the governing legislations and regulations. Provided help in the monitoring of the treatment plant by collecting wastewater samples and performing data analysis.

Placement Achievements

The main outcomes of the placement were the increased exposure to the water industry and the governing legislations. The Old Ford Water Recycling Plant is also a research facility and so the treatment process was tested under different modifications. Achieved greater understanding on how water companies prioritize research and translate to full scale operations. Worked in a team with different specialties so was able to interact with both the operational and research sides as well as share any gained knowledge with them.

Skill development

Communication skills improved due to the daily interaction with a wide range of people. Acquired a greater understanding on the analysis performed to measure water quality and the tests undertaken to check operational parameters, set by Thames Water and the governing legislations. The water quality analysis tests were demonstrated and performed developing on previous technical skills. Attended a creative thinking workshop that provided strategies to enhance innovation.

Future Work

The placement provided a great opportunity to experience working in the water industry and confirming a viable career pathway in the future.

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LIST OF ABBREVIATIONS

α-GLU	α-Glucosidase
β-GLU	β-Glucosidase
ABTS	2,2′-Azino-Bis(3-Ethylbenzothiazoline-6-Sulfonic Acid)
AC	Ammonium Acetate
ACN	Acetonitrile
ALP	Alkaline Phosphatase
BOD	Biochemical Oxygen Demand
BPA	Bisphenol A
CIP	Chemical Investigation Programme
COD	Chemical Oxygen Demand
DCF	Diclofenac
DO	Dissolved Oxygen
E1	Estrone
E2	17α-Estradiol
E3	Estriol
EC	Electrical Conductivity
EDCs	Endocrine Disrupting Compounds
EE2	17α-Ethinylestradiol
EST	Esterase
HCl	Hydrochloric Acid
HPLC	High Performance Liquid Chromatography
HRT	Hydraulic Retention Time
KH_2PO_4	Potassium Phosphate Monobasic
K_2HPO_4	Potassium Phosphate Dibasic
LOD	Limit of Detection
LOQ	Limit of Quantification
MeOH	Methanol
MWD	Multiple Wavelength Detector
NaCl	Sodium Chloride
NaOH	Sodium hydroxide
RAS	Returned Activated Sludge
SA	Specific Activity
SMX	Sulfamethoxazole
SRT	Solids Retention Time
SUL	Sulfatase
TSS	Total Suspended Solids
TDS	Total Dissolved Solids
UV	Ultraviolet
WWTP	Wastewater Treatment Plant

CHAPTER 1

Introduction

1.1 Thesis Overview

This work investigates the complex nature of wastewater and how the inherent variability influences enzyme behaviour. Enzymes inherent to the wastewater system and the application of external enzymes to remove endocrine disrupting compounds (EDCs) were studied as described in Chapter 2, Literature Review. Wastewater treatment is a complex process environment that can be considered to be a biotechnological processes as a biological component converts the raw feedstock into a final product. The findings from this work can be used to inform on enzyme response for other biotechnological processes. Materials and methods used within this research are described in Chapter 3. The following Chapter 4 provides the justification and reasoning for the selected enzymes, substrates and experimental designs to study enzyme variability relating to the wastewater environment.

Chapter 5 focuses on external enzymes, specifically laccase and tyrosinase, and their performance in removing estrone (E1) from the wastewater matrix. E1 removal is a concern to the water industry as the estrogen is an EDC and on the European Watch List. The temporal variability of the wastewater matrix was studied by performing standard characterisation experiments and the influence of this variability was studied on laccase and tyrosinase performance through E1 removal. In addition to this inhibitors of laccase and tyrosinase were investigated and their impact on E1 elimination assayed at concentrations reflecting the wastewater environment. The source of enzymes switched focus in Chapter 6 as inherent enzymes were instead studied. The activities of selected hydrolases, responsible for the degradation of the majority of organic pollutants within wastewater, were analysed. For the first time inherent activity was analysed throughout a wastewater treatment process and correlated with the standard measured characteristics of each sampled wastewater matrix. Relationships between inherent activity and wastewater influences were identified to understand the temporal and spatial variability observed. Chapter 7 contains the overall conclusions and recommendations of the thesis.

1.2 Research Background

The literature on this research commonly overlooks the variability of wastewater and fails to highlight the inherent complexity of wastewater. The complexity arises from the dynamic nature of the feedstock and multiple process and environmental factors such as temperature that have an impact on treatment efficiency. For example the performance of laccase to degrade various EDCs in a wastewater matrix has been investigated. However, most of these studies have analysed EDC removal from one wastewater sample and not considered temporal variability. This research developed enzymatic parameters to demonstrate this temporal variability and influence on exogenous enzyme performance. Furthermore the majority of studies investigating laccase and tyrosinase as potential treatment technologies have done so in buffer solutions that do not accurately represent the wastewater matrix. Inherent enzymes have been extensively studied in activated sludge for benchscale processes that optimise pollutant removal. The activity for inherent enzymes indicates biological efficiency and so treatment potential. Few papers have analysed how inherent enzyme activity varies throughout a wastewater treatment plant and correlated to wastewater characteristics.

1.3 Aim and Objectives

The aim of this research is to understand the degree of variability of enzyme activity in the face of process complexity. Understanding the response of enzymes to multiple process and environmental factors will allow processes to be more resilient to variability. The project objectives are to evaluate and address utilising enzymes in process industries with a focus on wastewater as a case study:

1. Development of methodologies for inherent and external enzyme activity in the wastewater matrix. Justification for the selected enzymes and substrates.
2. Demonstrate the temporal variability of the complex wastewater matrix by standardised characteristic tests.
3. Analyse the temporal response of commercial enzymes, laccase and tyrosinase to wastewater variability through the removal of a case study EDC. Compare the trend in EDC removal between the enzymes.
4. Evaluate the variability of inherent enzyme activity throughout a wastewater treatment process and identify factors that result in activity to increase or decrease.

CHAPTER 2

Literature Review

2.1 Enzymes in Industrial Biotechnological Processes

Biotechnological processes are heavily reliant on enzymes and are used widely in many industries including food, detergents, water, pulp and paper, therapeutics and diagnostics. Recombinant DNA technology greatly advanced the application of enzymes, as production levels could be improved from transferring the genes from native species into industrial strains. Over 50% of the enzyme market in 2011 was from recombinant sources (Sanchez and Demain, 2011). Specific examples of enzymes incorporated into biotechnological processes include the production of biodiesel from *Jatropha curcas* seeds, the use of oxidising enzymes to provide alternative routes for chemical synthesis and production of food products (Burton 2003) (Deeba et al., 2012) (Gurung et al., 2013). Wastewater from the textile and pharmaceutical industry is also being treated by enzymes at an early developmental stage (Demarche et al., 2012).

Enzymes are biological catalysts that lower the activation energy of a reaction favouring the conversion of a substrate into a product. They are proteins or glycoproteins that have a region that directly participates in the catalytic process referred to as the active site (Karigar and Rao, 2011). There are two types of mechanisms in which the enzyme can form an enzyme-substrate complex [ES] as shown in Figure 2.1. The two mechanisms are the 'Lock and Key' or 'Induced Fit' model. In the 'Lock and Key' model the shape of the substrate and active site are complementary to one another. For the 'Induced Fit' model there is an initial weak interaction between the substrate and enzyme that results in a rapid conformational change strengthening the binding between the substrate and active site bonds. The formation of the [ES] complex then undergoes a transitional state before the enzyme and product dissociate into free components (Whiteley and Lee, 2006).

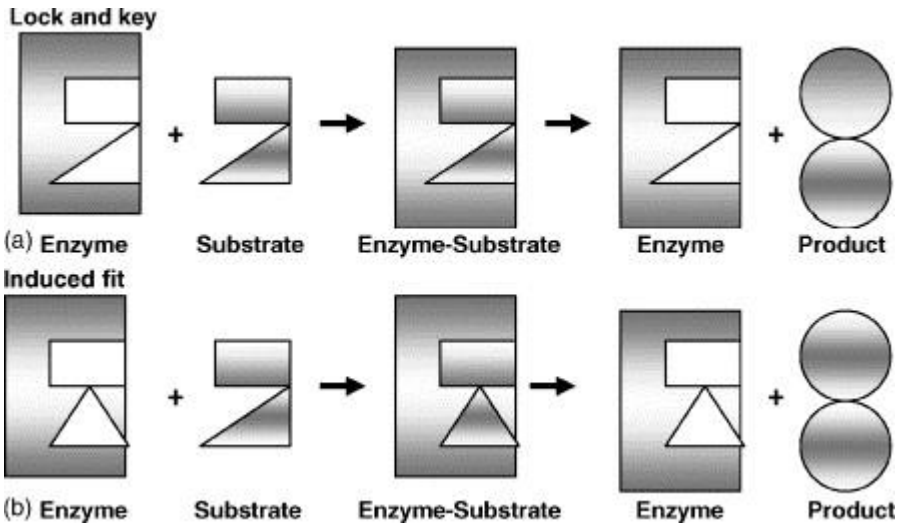


Figure 2.1 (a) "Lock and Key" (b) "Induced Fit" enzymatic mechanisms (Whiteley and Lee, 2006).

Enzymes can be influenced by a wide range of environmental and process factors resulting in variable performance and inconsistent product. Temperature and pH are two factors widely investigated for their impact on enzyme activity. Greater understanding on enzyme variability is required for improved process adaptions or performance. Wastewater is a dynamic and highly variable matrix that was used in this research as an indicator for process complexity to provide a foundation for other industrial processes. Furthermore, enzymes are vital biological components in wastewater treatment, responsible for degrading a wide range of organic pollutants. Enzyme behaviour was studied in the wastewater matrix for inherent enzymes, as well as enzymes external to the system able to degrade more recalcitrant pollutants. Wastewater treatment is a biotechnological process as the wastewater entering a wastewater treatment plant (WWTP) is the raw feedstock, with enzymes as the biological component that transform this raw feedstock into a final product being water that is fit for purpose and safe for release.

2.2 Wastewater Treatment and Role of Enzymes

Wastewater from household and industrial sources requires treatment before re-entering the water cycle. The sewage system transports discharged wastewater to a wastewater treatment plant (WWTP) where it is treated to a quality acceptable for release into a river. The primary role of a WWTP is to remove organic matter and eliminate pathogens (Cadoret et al., 2002). The WWTP effluent that enters the river system is required to have no negative impact on the receiving environment.

Consequently, there are multiple legislations that enforce this water quality. They stipulate the acceptable concentrations of ammonia, nitrates and pesticides, for example (Gardner et al., 2013).

Treatment of wastewater relies heavily on a biological process, activated sludge, to remove the majority of organic pollutants. Activated sludge is a mixed population of heterotrophic organisms that degrade organic matter through biological oxidation (Burgess and Pletschke, 2008). Figure 2.2 details the overall treatment process for a conventional WWTP. Wastewater entering a WWTP is commonly referred to as the influent and is subjected first to a screening process where large debris are removed. The screened wastewater then enters a primary settling tank before entering the biological process stage that contains the activated sludge. There are a few WWTPs with a sand filtration unit at the end of their treatment processes to provide a final polishing step for the effluent, commonly referred to as tertiary treatment (de Mes et al., 2005).

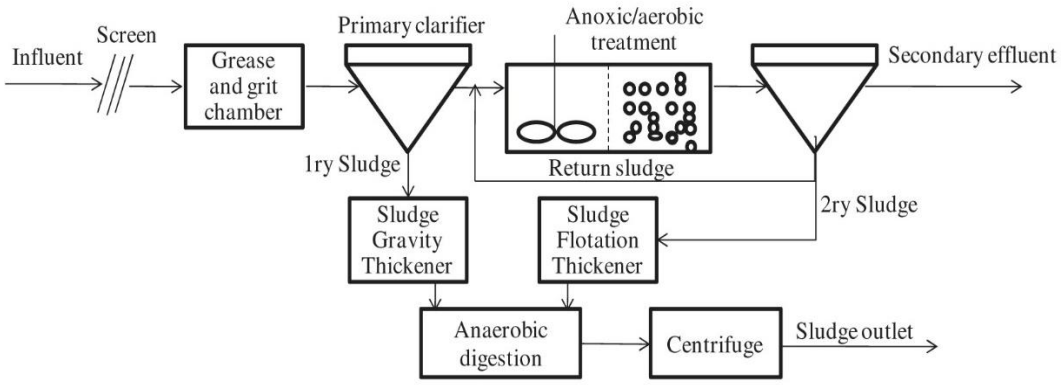


Figure 2.2 Schematic of a conventional municipal wastewater treatment plant (Jelic et al., 2011).

A recent advancement to the conventional wastewater treatment plant has been the incorporation of a membrane bioreactor (MBR). This technology has been trialled within the UK and Thames Water are currently operating an MBR-based water recycling system in London. Other countries where direct wastewater reuse is more common and encouraged have adopted this recent technology more widely. The treatment process with an MBR is combined with membrane filtration. Unlike conventional WWTPs the distinction between sludge retention time (SRT) and hydraulic retention time (HRT) is far greater leading to high sludge age. Benefits include greater bacterial diversification and high biomass concentrations improving

pollutant removal. However, issues such as fouling of the filtration membranes and high biomass concentration limiting oxygen transfer have restricted MBR processes replacing conventional WWTPs (Das et al., 2017).

Wastewater treatment is a biological process where the production of enzymes from the microbial activated sludge plays a large part in breaking down the organic constituents in wastewater. Analysing these enzymes can provide feedback on the treatment efficiency for a given WWTP (Fischer et al., 2013). Carbohydrates, lipids and proteins are broken down by a group of enzymes classified as hydrolases. The reduced molecular weights of these pollutants are then able to be assimilated by the activated sludge cells used for energy and carbon (Nybroe et al., 1992). Bioactive chemicals are a recent class of pollutants that have received worldwide attention regarding their occurrence in wastewater. This has arisen from their reported poor removal by conventional WWTPs and harmful effects on wildlife. Consequently, enzymes not belonging to the wastewater system have been investigated instead as potential treatment options. Examples of enzymes studied to remove such organic pollutants are given in Table 2.1.

Table 2.1 Examples of enzymes with the ability to remove organic pollutants (modified from Eibes et al., 2015).

Enzymes	Coenzyme/required substrates	Pollutant
Cellobiose dehydrogenase (EC. 1.1.99.18)	$-/O_2$ or Fe^{+3}	TNT, RDX (explosives), dyes
Azo reductase (EC 1.7.1.6)	NAD(P)H/–	Azo dyes
Laccases (EC 1.10.3.2)	$-/O_2$	Phenolic compounds (including halogenated phenols), PAH, synthetic dyes, pesticides
HRP (EC 1.11.1.7)	$-/H_2O_2$	Phenolic compounds (including chlorinated phenols), PAHs, dyes
Chloroperoxidase (EC 1.11.1.10)	$-/Cl^-$, H_2O_2	Phenolic compounds (including chlorinated phenols), PAHs, chlorinated anilines
Manganese peroxidase (EC 1.11.1.13)	$-/Mn^{+2}$, H_2O_2	Phenolic compounds, PAHs, synthetic dyes, TNT (explosive), pesticides
Lignin peroxidase (EC 1.11.1.14)	$-/H_2O_2$	Non-phenolic (veratryl alcohol) and phenolic aromatic compounds (including chlorinated phenols), PAHs, dyes, nitroaromatics and explosives
Versatile peroxidase (EC 1.11.1.16)	$-/H_2O_2$	Phenolic compounds (including chlorinated phenols), PAHs, dyes, pesticides
Dyoxygenase (EC 1.14.12)	NAD(P)H/O_2	BTEX, PAHs
Cytochrome P450 monoxygenase (EC 1.14.14.1)	NAD(P)H/O_2	PAHs, aliphatic hydrocarbons, pesticides
Tyrosinase (EC 1.14.18.1)	$-/O_2$	Phenolic compounds (including halogenated phenols) dyes, phthalates derivatives
Carboxylesterases (EC 3.1.1.1)	$-/H_2O$	Pesticides
Lipase (ec 3.1.1.3)	$-/H_2O$	Waste cooking oil, phthalates derivatives
Cutinase (EC 3.1.1.74)	$-/H_2O$	Malathion (pesticide), phthalates derivatives
Phosphotriesterase (EC 3.1.8)	$-/H_2O$	Organophosphorous pesticides
Haloalkane dehalogenases (EC 3.8.1.5) LinB/AtzA, TrzN	$-/H_2O$	β-Hexachlorocyclohexane/chloro-s-triazine (pesticides), chlorinated solvents
Amidase (EC 3.5.1.4)	$-/H_2O$	Nitrile compounds
Nitrilase (EC 3.5.5.1)	$-/H_2O$	Nitrile compounds
Nitrile hydratase (EC 4.2.1.84)	–/–	Nitrile compounds
Haloalkane dehydrochlorinase (EC 4.5.1) LinA	$-/H_2O$	α-, δ-, γ-Hexachlorocyclohexane (pesticides)

2.3 Entry of Bioactive Chemicals into the Wastewater Environment

Bioactive chemicals or substances are endocrine disrupting compounds (EDCs) or pharmaceuticals that invoke a negative response in aquatic organisms (Cirja et al., 2008). Figure 2.3 shows the possible removal pathways estrogens, strong EDCs, can undergo in a WWTP and is representative of most bioactive chemicals in general. However, effluent released by a WWTP into surrounding surface water is the main route of entry for EDCs into the environment (de Mes et al., 2005). Conventional WWTPs were never designed to remove EDCs so most of them pass through the process unaltered (Abejón et al., 2015). Biodegradation is the favoured route of

bioactive chemical removal as the compound is forever eliminated from the water cycle.

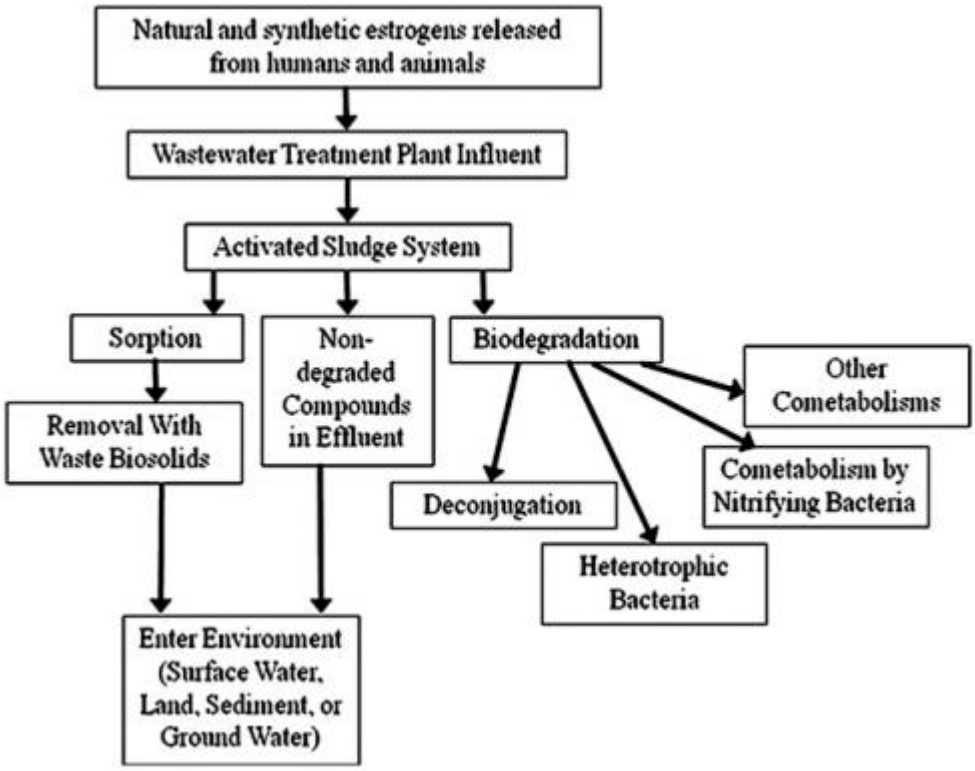


Figure 2.3 Fate of estrogens on a wastewater treatment plant and the environment (Racz and Goel, 2010).

As shown in Figure 2.3, the EDCs and pharmaceuticals that enter the environment all derive from human or animal excretion. Hormones and pharmaceuticals usually exit the body as conjugated metabolites in the urine or feces. The occurrence of these conjugated metabolites has to be considered alongside the parent compounds. Pharmaceuticals and steroids in the body are almost entirely metabolised within the liver by cytochrome P450 enzymes. There are two metabolism phases, as compounds in Phase I first undergo modification to unmask or introduce functional groups accessible to conjugation through oxidation, reduction and hydrolysis reactions. Conjugation in Phase II can happen at the following functional groups; sulfhydryl, amino, carboxyl and hydroxyl groups. The compounds once in Phase II can then undergo sulfation, methylation, acetylation, glucuronidation, glycine and glutathione conjugation. Conjugation generally results in less active compounds and increases their solubility to ease excretion from the body (Ternes et al., 1999). Deconjugation

occurs in the wastewater system leading to high environmental concentrations of the parent compound.

2.3.1 Drivers for Eliminating Bioactive Chemicals

The detection of bioactive chemicals especially EDCs within the environment is of huge concern to scientists and policymakers alike (Anku et al., 2017). Sources of EDCs include excreted hormones, household products, pharmaceuticals and industrial waste. Controlling and reducing the effects of this pollution is high on the agenda for the Environmental Agency (EA), European Union (EU) and World Health Organisation (WHO) (Ahmed et al., 2017). The release of EDCs into the environments needs to be addressed, as it has been shown that estrogens discharged into the environment has led to male fish having reduced reproductive capabilities (Anderson et al., 2012).

Since the 1970s the EU has been progressively implementing directives that have focused on improving surface water quality. The Water Framework Directive 200/60/EC (Directive 200/60/EC) controls EU members with regards to the chemical quality of surface water (Bueno et al., 2012). The EU has since created a Watch List that requires the monitoring of selected bioactive chemicals in the wastewater environment. In total there are 17 compounds on the list ranging from macrolide antibiotics to natural estrogens. These bioactive chemicals would not be such a problem if they were well removed by WWTPs. Table 2.2 shows that for diclofenac (DCF), a compound included in the EU Watch List, removal was below 35% and so the compound is classed as highly recalcitrant (Deblonde et al., 2011). Current removal strategies for bioactive chemicals are unfeasible at a large scale, therefore sustainable alternatives are being sought after, such as enzymatic technologies.

Table 2.2 Concentrations of emerging contaminants (µg/L) in influent and effluent samples collected from multiple WWTPs (Deblonde et al., 2011).

Pharmaceuticals compounds	Molecules	Means	RSD	Median	Min	Max	*n*	Means	RSD	Median	Min	Max	*n*	
	Clarithromycin	0.344					2	0.15					2	56.4
	Ciprofloxacin	0.62	1.48	0.157	0.09	5.524	13	0.234	0.649	0.021	0.007	2.378	13	62.3
	Doxycyclin	0.65	0.94	0.098	0.067	2.48	10	0.420	0.426	0.227	0.038	1.09	9	35.4
	Erythromycin	0.58	0.242	0.56	0.346	0.83	3	0.297	0.237	0.2305	0.109	0.62	4	48.8
	Erythromycin–H_2O	2.025					2	0.59					2	70.9
	Methronidazole	0.09					1	0.055					1	38.9
Antibiotics	Norfloxacin	0.115	0.056	0.0905	0.066	0.25	12	0.0526	0.0985	0.0195	0.007	0.33	10	54.3
	Ofloxacin	0.482	0.884	0.156	0.007	2.275	6	0.171	0.317	0.0485	0.007	0.816	6	64.5
	Roxithromycin	0.78	0.737	0.81	0.0272	1.5	3	0.472	0.435	0.54	0.008	0.87	3	39.5
	Sulfamethoxazole	0.32	0.248	0.2905	0.02	0.674	10	0.264	0.150	0.243	0.07	0.62	11	17.5
	Sulfapyridin	0.492					1	0.081					1	83.5
	Tetracyclin	48					1	2.375					2	95.1
	Trimethoprim	0.43	0.401	0.251	0.0535	1.3	15	0.424	0.363	0.32	0.04	1.34	17	1.4
Antiepileptics	Carbamazepine	0.732	0.869	0.25	0.0819	1.68	6	0.774	0.789	0.37	0.042	2.1	13	−5.7
	4-aminoantipyrine	1.517					1	0.676					1	55.4
	Antipyrin	0.04					1	0.027					1	32.5
	Codein	2.8605					2	1.93					2	32.5
	Diclofenac	1.039	1.283	0.232	0.16	3.1	6	0.679	0.701	0.55	0.04	2.448	11	34.6
Analgesics and anti-inflammatories	Ibuprofen	13.482	25.639	3.495	0.0143	22.7	10	3.480	1.489	0.56	0.03	12.6	17	74.2
	Indomethacine	0.136					2	0.166	0.118	0.19	0.037	0.27	3	−22.1
	Ketoprofen	0.483	0.286	0.441	0.146	0.94	5	0.333	0.148	0.34	0.125	0.63	9	31.1
	Ketorolac	0.407					1	0.228					1	44.0
	Naproxen	5.077	8.251	2.363	0.206	23.21	7	0.934	0.873	0.452	0.017	2.62	13	81.6
	Clofibric acid	0.215	0.251	0.12	0.026	0.5	3	0.131	0.136	0.12	0.012	0.36	5	39.1
	Fenofibric acid	0.079					1	0.196	0.161	0.13	0.078	0.38	3	−148.1
Lipid regulators	Bezafibrate	1.948	2.320	1.4205	0.05	4.9	4	0.763	0.963	0.13	0.035	2.2	5	60.8
	Gemfibrozil	1.562	1.704	0.71	0.453	3.525	3	0.757	1.068	0.323	0.0112	2.86	6	51.5
	Acebutolol	0.335					1	0.140					1	58.2
	Atenolol	1.080	0.946	0.996	0.03	1.197	4	0.468	0.381	0.345	0.16	1.025	4	56.7
	Celiprolol	0.44					1	0.28					1	36.4
Betablockers	Metoprolol	1.535	2.290	0.61	0.02	4.9	4	0.679	0.657	0.73	0.019	1.7	5	55.8
	Propanolol	0.198	0.269	0.005	0.036	0.51	3	0.102	0.0712	0.093	0.03	0.18	5	48.5
	Sotalol	1.667					2	0.79					2	52.6

2.3.2 Bioactive Chemicals of Interest

The bioactive chemicals of most interest are EDCs due to their adverse impact on aquatic wildlife such as male fish feminisation (de Mes et al., 2005). From an environmental and regulatory standpoint the chosen EDCs in this research were E1 and DCF. Further justification is given in Chapter 4 for their selection. The parent compounds were the sole focus of the research even though both EDCs enter the sewage system as conjugates. There is no legislation that covers the removal or monitoring of conjugated EDCs. Possible reasons for the exclusion of conjugated EDCs include their reduced estrogenicity and transformation back into the parent compound within the sewage system. Therefore their environmental impact is less of a concern.

Glucuronide conjugation is the dominant form for excreted estrone in the urine. Natural hormones are not excreted in the feces to the same high amounts as seen for urine. Estrone excreted in the feces is typically in its unconjugated form due to the presence of the enzyme β-glucuronidase that cleaves off the conjugate group. The excretion of this enzyme results in the high conversion of estrone-3-glucuronide to estrone. However, the other potential conjugate group for estrone is sulphate and is vastly more recalcitrant (de Mes et al., 2005).

DCF a widely administered anti-inflammatory drug is also excreted as either a glucuronide or sulphate conjugate (Vieno and Sillanpää, 2014). A high amount of β-glucuronidase is released by Escherichia coli (*E.coli*) in faecal matter and sewage system (Kumar et al., 2012). Glucuronide conjugate metabolites revert back to the parent compound rapidly before arriving at a WWTP. Sulfatase is mainly produced by bacteria in the activated sludge and therefore most EDCs become completely deconjugated by the time they exit a WWTP (Liu et al., 2009). A tertiary treatment step is best adapted to prevent the release of EDCs into the receiving aquatic environment.

2.4 Current Strategies for Removing Bioactive Chemicals

Tertiary treatments, mainly advanced oxidation processes, have been developed to combat the issue of EDCs in the environment. Examples include ozonation, photodegradation and introducing hypochlorite or chlorite oxides. Associated

disadvantages with these methods as seen in Table 2.3 has led to greener alternatives being sought such as biological technologies including enzymatic removal. The drive to utilise enzymes instead comes from their desired characteristics of reducing operational costs and the formation of less toxic by-products. Enzymes produced by white rot fungi have been shown to degrade a wide range of EDCs and are the most promising as a result (Taboada-Puig et al., 2016).

Table 2.3 Advantages and disadvantages of advanced oxidation and biological treatment of organic pollutants (Mohapatra et al., 2014).

Advanced oxidation technologies	Biological treatment technologies
Advantages	Advantages
1. Complete oxidation possible	1. Partial mineralization
2. Can handle large recalcitrant molecules	2. Eco-friendly
3. Easy process operability	3. Low capital and operating costs
4. Ability to handle fluctuating flow rates and compositions	4. True destruction of organics versus mere phase separation
5. Absence of secondary wastes	5. Simultaneous removal of reduced inorganic compounds, such as sulfides and ammonia, and total nitrogen removal possible through denitrification
6. Inconvenient to produce undesired products more toxic than the initial ones	6. Operational flexibility to handle a wide range of flows and wastewater characteristics
	7. No use of chemicals
	8. Requires less energy inputs
Disadvantages	Disadvantages
1. Higher capital and operating costs	1. Digestion rates are slow, in general days or weeks, requiring large storage tanks.
2. Difficult to manage utilities in wastewater treatment plants	2. Microorganisms must be fed constantly, or they will die off. If they die off, re-acclimating the microorganisms to the waste stream may take several days
3. Radical scavenging which may lead to lowered degradation efficiency	3. Changing from one organic removal compartment to another can greatly reduce or eliminate effective treatment.
4. Simultaneous removal of inorganics not reported	
5. Operational flexibility is not feasible	
6. Often uses harsh chemicals	
7. Energy input is higher	

Oxidative enzymes secreted by white rot fungi laccase, manganese peroxidase and versatile peroxidase have all been shown to degrade EDCs. A group of intracellular enzymes cytochrome P450 monooxygenases additionally contribute to EDC degradation (Asif et al., 2017). Laccase has been explored the most for EDC degradation because the only required substrate is atmospheric oxygen. As shown in Table 2.1 the other white rot fungi enzymes require other substrates not present in the wastewater system increasing costs substantially. Tyrosinase is another oxidative enzyme isolated from fungi that has also been shown to degrade EDCs with molecular

oxygen as a substrate (Shesterenko et al., 2012). These two enzymes have been put forward as the enzymes to solve the EDC wastewater issue (Ba and Vinoth Kumar, 2017). Current disadvantages with enzymatic treatment are with isolating and purifying the proteins, as both are expensive. Nevertheless, researchers are starting to reduce costs by extending the active life of both enzymes through more effective immobilisation methods (Durán and Esposito, 2000).

Laccase and tyrosinase were the two exogenous enzymes selected to investigate the influence of WWTP conditions on enzymatic EDC removal. The two enzymes are similar as both are copper containing phenoloxidases (Durán and Esposito, 2000). Furthermore the enzymes are being widely explored as final tertiary treatments for WWTPs (Demarche et al., 2012). The integration of the enzymes at the end of the treatment process provides many advantages. As already discussed the majority of EDC conjugated metabolites are transformed back to the parent compound in the WWTP effluent. Furthermore, the effluent is the least polluted wastewater matrix resulting in less constituents that can interfere with enzyme performance and hinder EDC removal.

Most papers within the literature use commercially available laccase and tyrosinase that has been purified from a fungal origin. Other treatment adaptions include using whole white rot fungi to treat wastewater. However, biomass growth needs to be sustained and so requires an additional energy source that increases costs (Yu et al., 2013). Researchers have begun to investigate different immobilisation methods for laccase and tyrosinase to make their industrial application more economically appealing. Immobilisation improves feasibility as enzyme stability is increased and enzyme recovery is more efficient (Donato et al., 2014). Before evaluating the behaviour of the immobilised enzymes there is still a gap in knowledge for understanding the performance of the free enzymes in the wastewater matrix, and characterisation at relevant wastewater conditions.

2.4.1 Laccase Characteristics

Laccase has been investigated for the biodegradation of a wide range of EDCs as demonstrated by the previous literature studies summarised in Table 2.4. The most common source of laccase was from *Trametes versicolor*.

Table 2.4 Published studies on laccase to remove EDC substrates.

Laccase Source	Substrates	Inhibitors/ Mediators	Analytical Method	Matrix	Experimental Conditions	Inactivation	Reference
Trametes versicolor	BPA	Inhibitors: Cu^{2+}, Fe^{3+}, Cl^-, F^-	Substrate concentration (HPLC-UV)	Synthetic Water (Buffer Solution)	pH → 3-8 Temperature → 25 to 55ºC Contact time → 10 hours	—	(Kim and Nicell., 2006)
Trametes versicolor	E1 E2 E3 EE2	—	Substrate removal (LC-MS/MS)	Synthetic Water (Phosphate buffer) Wastewater (AS Settling Reactor)	pH → 7 (synthetic water), 8±0.5 (wastewater) Temperature → 25ºC Contact time → 1 hour	HCl	(Auriol et al., 2007a)
Trametes versicolor	E1 E2 E3 EE2	—	Residual estrogen % (LC-MS/MS)	Synthetic Water (Phosphate buffer) Wastewater (AS Settling Reactor)	pH → 7 Temperature → 25 ± 1ºC Contact time → 1 hour Enzyme → 0, 2, 5, 10, 15 & 20 U/ml	HCl 0.45µm filter	(Auriol et al., 2008)
Trametes versicolor	Phenol	—	Substrate concentration (HPLC-UV)	Buffer Solution (Britton-Robinson buffer)	pH → 3 to 9 (at 25ºC) Temperature → 10 to 60ºC (at pH6)	Sampled diluted to be 10% acetic acid	(Kurniawati and Nicell, 2008)
Trametes sp. Ha1	E1 E2 EE2	—	Substrate concentration (HPLC-UV)	Buffer Solution (Mcllvaine buffer)	pH → 5 Temperature → 30ºC Shaker → 45 stroke/min	Reaction tube quenched in an ice water bath and then ACN added (70% v/v)	(Tanaka et al., 2009)

Coriolus versicolor	2,4-DCP	—	Substrate removal % (UV/Vis spectrophotometer)	Buffer Solution	Chitosan-immobilised pH → 3, 4, 5, 5.5, 6, 7, 8 & 9 Temperature → Ambient Contact time → 6 hours	—	(Zhang et al., 2009)
Myceliophthora thermophila	E1 E2 EE2 DCF NPX	Mediators: HBT, VA, SA, FA, p-CA, V	Substrate removal % (HPLC-UV)	Buffer Solution Sodium acetate	pH → 4 to 7 Temperature → 22 ±2ºC Contact time → 4, 8 & 24 hours	0.25M HCl	(Lloret et al., 2010)
Trametes versicolor	Oxybenzone	Mediators: ABTS, ACE, FA, HBA, HBT, SIN, SYR, PCA	Substrate remaining (LC-MS/MS)	Buffer Solution (Sodium phosphate) Wastewater (WWTP primary effluent)	pH → 7 Temperature → 23ºC Contact time → 2, 6 & 24 hours	HCl	(Garcia et al., 2011)
Myceliophthora thermophila	E1 E2	—	Substrate removal % (HPLC-UV)	Buffer Solution (Sodium phosphate)	Enzymatic membrane reactor O2 supply → 2 to 8 pulses/HRT Temperature → 26ºC Contact time → 1 to 4 hours Stir speed → 250 rpm	—	(Lloret et al., 2012)
Myceliophthora thermophila	E1 E2 EE2	Inhibitors: NaN_3, $ZnCl_2$, $CoCl_2$, $CaCl_2$	Substrate removal % (HPLC-UV)	Buffer Solution (Phosphate buffer)	Packed bed reactor pH → 7 Temperature → 24 ±2ºC Contact time → 8 hours	—	(Lloret et al., 2012)

Myceliophthora thermophila	E1 E2 EE2	—	Substrate removal % (HPLC-UV)	Buffer Solution (Sodium phosphate)	Enzymatic membrane reactor O_2 → 15, 30, 60 $mgO_2/(L.h)$ Contact time → 1, 2 & 4 hours Stir speed → 250 rpm	—	(Lloret et al., 2013)
Myceliophthora thermophila	E1 E2 EE2	—	Degradation rate (HPLC-UV)	Buffer Solution (Phosphate buffer) Wastewater (filtered secondary effluent)	Enzymatic membrane reactor Temperature → 26ºC Contact time → 4 hours Stir speed → 250 rpm	HCl 0.45µm	(Lloret et al., 2013)
Myceliophthora thermophila	DCF NPX	Mediators: HBT & SA	Substrate concentration (HPLC-UV)	Buffer Solution (Acetate & phosphate buffer)	pH → 4 & 7 Temperature → 25ºC	HCl	(Lloret et al., 2013)
Trametes versicolor	BPA DCF MFA TCN	Substrate mixture	Substrate removal % (HPLC-UV)	Buffer Solution (Citric acid, dibasic sodium phosphate & tris-HCl)	pH → 3 to 9 Temperature → 10, 25 & 40ºC Contact time → 1 to 7 hours	Samples directly injected into HPLC	(Margot et al., 2013)
Trametes versicolor Bacterial genus: *Streptomyces* (*S.cyaneus, S.ipomoea, S.griseus, S.psammoticus*)	BPA DCF MFA	Inhibitor: NaCl	Substrate concentration (HPLC-UV)	Buffer Solution (Citrate phosphate buffer)	pH → 5, 6 & 7 Temperature → 25ºC Contact time → 0 to 12 days	Samples directly injected into HPLC	(Margot et al., 2013)

Trametes versicolor	Analgesics Antibiotics Psychiatric drugs	—	Removal rate % (UPLC-TQMS)	Urban Wastewater (Sterile & non-sterile samples)	Fluidised bed reactor (WRF) pH → 4.5 Temperature → 25°C.	0.45µm	(Cruz-Morató et al., 2013)
Paraconiothyrium variable	BPA Phenol	—	Removal % (Colorimetric assay at 560nm)	Buffer solution (Citrate & phosphate buffer)	pH → 3 to 7 Temperature → 25ºC Enzyme → 24 hours	—	(Asadgol et al., 2014)
Trametes versicolor	Trace organic contaminants (14 phenolic + 16 non-phenolic)	Mediators: HBT & SA	Substrate removal % (GS/MS system)	Buffer Solution (Sodium citrate buffer)	pH → 4.5 Temperature → 25ºC Contact time → 20 hours	2M H_2SO_4 0.45µm	(Nguyen et al., 2014)
Aspergillus oryzae	BPA DCF	Mediator: SA	Substrate removal % (HPLC-UV)	Buffer Solution (Sodium citrate buffer)	Enzymatic membrane reactor pH → 3, 4.5, 6 ,7 & 9 Temperature → 28ºC Contact time → 2, 4, 8 & 22 hours	—	(Nguyen et al., 2014)
Trametes versicolor	E2	—	Substrate removal % (HPLC-Fluorescence)	Buffer Solution (Phosphate buffer)	pH → 4 to 8 TOC → 0, 2, 5 & 10 mg/L Contact time → 2,4,8 & 24 hours	1:1 MeOH	(Xia et al., 2014)
Phlebia radiata	m-Cresol	—	Substrate removal % (HPLC-UV)	Culture media	Temperature → 28ºC	Sterilised (20 minutes at 121ºC)	(Cobas et al., 2015)
Trametes versicolor	SMX IPN	Mediators: ABTS SA AS	Substrates removal % (HPLC-UV)	Buffer Solution (Citrate & phosphate buffer)	pH → 3 to 9 Temperature → 25ºC Contact time → 20 hours	Samples directly injected into HPLC	(Margot et al., 2015)
Trametes versicolor	BPA EE2	—	Substrate removal % (HPLC-UV)	Ultrapure Water	Temperature → 37ºC	1:2 MeOH	(Maryšková et al., 2016)

Trametes versicolor *Myceliophthora thermophila*	BPA DCF	—	Substrate removal % (Decrease in radioactivity for the tagged compounds)	Wastewater (filtered secondary effluent)	Nano-immobilsation pH → 8.7 ±0.7 Temperature → 25 ±2ºC Contact time → 24 hours	—	(Arca-Ramos et al., 2016)
Pleurotus ostreatus	Oxybenzone Pentachlorophenol NPX Atrazine	Mediators: HBT, HPI, TEMPO, VA, SA, VA, ABTS	Substrate removal % (HPLC-UV)	Ultrapure water	Temperature → 25ºC Contact time → 24 hours	—	(Ashe et al., 2016)
Trametes versicolor	38 antibiotics	Mediator: SYR	Substrate concentration (UPLC-MS/MS)	Buffer Solution (Phosphate buffer)	Enzymatic membrane reactor pH → 7 Temperature → 25ºC Contact time → 24 hours	—	(Becker et al., 2016)
Trametes versicolor *Coriolopsis gallica* *Bjerkandera adusta*	BPA	Mediator: HBT	Substrate removal % (GC-MS)	Buffer Solution (Sodium acetate buffer)	pH → 5 Temperature → 45ºC Contact time → 24 hours	NaOH	(Daâssi et al., 2016)
Trametes versicolor	Triclosan	—	Substrate conversion % (HPLC-UV)	Buffer Solution (Citrate-phosphate buffer)	pH → 4.5 Temperature → 25ºC	Phosphoric acid	(Melo et al., 2016)
Pycnoporus sanguineus	DCF DHQ β-NP 2,4-DCP	—	Substrate removal % (HPLC-UV)	Buffer Solution (Mcllvaine buffer) Groundwater	Temperature → Ambient Contact time → 1 to 9 hours	—	(Rodríguez-Delgado et al., 2016)
Cerrena unicolor	TC, OTC, SMX, Ampicillin, Erythromycin	Mediator: ABTS	Substrate removal % (UPLC-MS/MS)	Buffer Solution	Magnetic cross-linked enzyme aggregate (M-CLEAs) pH → 7 Temperature → 25ºC Contact time → 48 hours	—	(Yang et al., 2017)

Trametes versicolor *Myceliophthora thermophila*	E1, E2, E3, EE2, BPA, NP, PrPb, BBP	—	Substrate removal % (Triple quadrupole LC-MS)	Buffer Solution Wastewater (Raw wastewater)	Temperature → 22 ±1°C Contact time → 2, 6 & 24 hours	4M HCl	(Becker et al., 2017)

Fungal laccases compared to other laccases typically exhibit higher redox potentials, resulting in a far greater amount of EDCs that can be degraded. For example the redox potential for fungal laccase is between 0.7V and 0.8V whereas plant laccases are lower at 0.4V to 0.5V (Gasser et al., 2014). All laccases are characterised by a copper centre within their catalytic site. Four copper ions are contained in the catalytic site where the type -1 copper (T1) first oxidises the substrate. The generated electrons are then transferred through a tripeptide sequence to a type-2 (T2) and type-3 (T3) copper site where oxygen is reduced to water (Catherine et al., 2016). Figure 2.4 shows the removal enzymatic mechanism that the EDC substrate BPA undergoes when in contact with laccase.

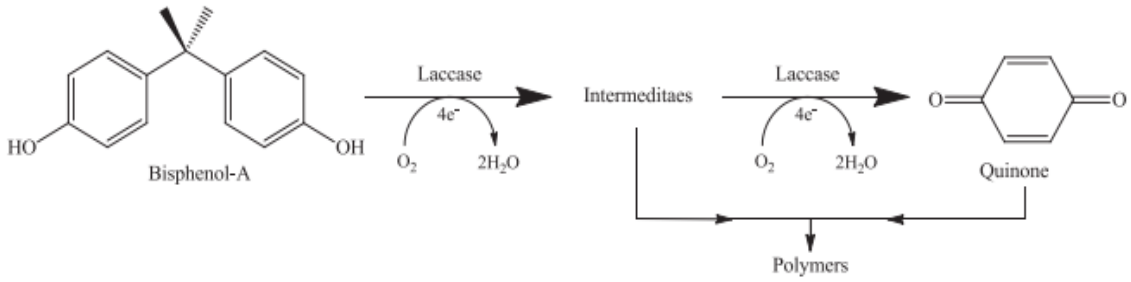


Figure 2.4 Schematic degradation pathway of bisphenol-A (BPA) in the presence of laccase (Hou et al., 2014).

Radical-radical coupling of the substrate occurs rather than degradation, in turn forming polymers (Xia et al., 2014). Laccase first oxidises the substrate to form a free radical. In this process one electron is lost and molecular oxygen is reduced to water. Free radicals polymerise through non-enzymatic reactions to produce insoluble products that precipitate out and then can be removed either by filtration or sedimentation (Steevensz et al., 2009). Dimers still exhibiting phenolic functionality can be oxidised again by laccase, resulting in formation of trimers, tetramers, and oligomers until no longer soluble (Xia et al., 2014).

For the first time (Becker et al., 2017) showed laccase immobilisation to match the costs of conventional advanced oxidation processes highlighting the potential WWTP application in the near future. The removal of bisphenol A (BPA), benzylbutylphthalate (BBP), nonylphenol (NP) and propylparaben (PrPb) was investigated in synthetic and wastewater matrices. Complete removal of the four substrates was reported after 24 hours contact with the laccase immobilised on polymeric beads. The cost for adopting both laccase and the carrier material was

between 0.018 and 0.130 €/m^3 whereas the traditional advanced oxidation methods were calculated to between 0.04 and 0.20 €/m^3 (Becker et al., 2017). The industrial application of laccase is becoming ever more feasible. However, in the meantime further work needs to be performed investigating the performance of laccase in wastewater over a sustained period of time.

A few papers within Table 2.4 explored the addition of mediators to improve EDC removal by laccase. Mediators were not included in this research as the associated costs are inhibitive to large scale applications (Ashe et al., 2016). Feasibility of the laccase studies in Table 2.4 are a concern as they do not always reflect the wastewater environment. Recent papers are beginning to investigate laccase performance at WWTP conditions rather than manipulating WWTP conditions to achieve optimal laccase performance. For example Lloret et al. (2010) in their study concluded the optimal pH for laccase to be pH 4 and Zhang et al. (2009) showed an optimal temperature range between 35 ^oC - 45 ^oC. These reported optimal conditions are not reflective of a WWTP. Enzyme variability at conditions representing the WWTP environment has not been examined in detail and the impact on EDC removal not considered.

Temperature and pH are the most commonly analysed factors when analysing EDC removal by laccase as seen in Table 2.4. Another factor that can impact laccase performance is salinity (Kim and Nicell, 2006). The composition of wastewater is highly complex, containing numerous different chemicals depending on the source being either municipal or industrial. Constituents in the wastewater can inhibit or enhance laccase activity (Margot et al., 2013). Metals, ions and organic chemicals can all have an impact on laccase activity. A clean water matrix will not provide information on this influence unless the pollutants are added into the sample matrix. The research carried out in Chapter 5 studied the impact of a common metal Cu^{2+} and Cl^- anion on laccase performance including a combination of EDCs added to the matrix. A comparison between a clean buffer and wastewater matrix was made to determine if a clean water model could similarly predict laccase behaviour in wastewater.

2.4.2 Tyrosinase Characteristics

Laccase and tyrosinase share similar characteristics with the first being no other oxidant is required apart from atmospheric oxygen. Tyrosinase also has two copper ions in the active site. Lastly, the generated by-products, as shown in Figure 2.5, both undergo polymerisation to generate insoluble compounds that can be removed by either precipitation or filtration (Ba and Vinoth Kumar, 2017). Tyrosinase was characterised from the edible mushroom Ag*aricus bisporus* when investigating the cause for their rapid browning once harvested. Tyrosinase has been proposed in numerous biotechnological and environmental applications (Halaouli et al., 2006).

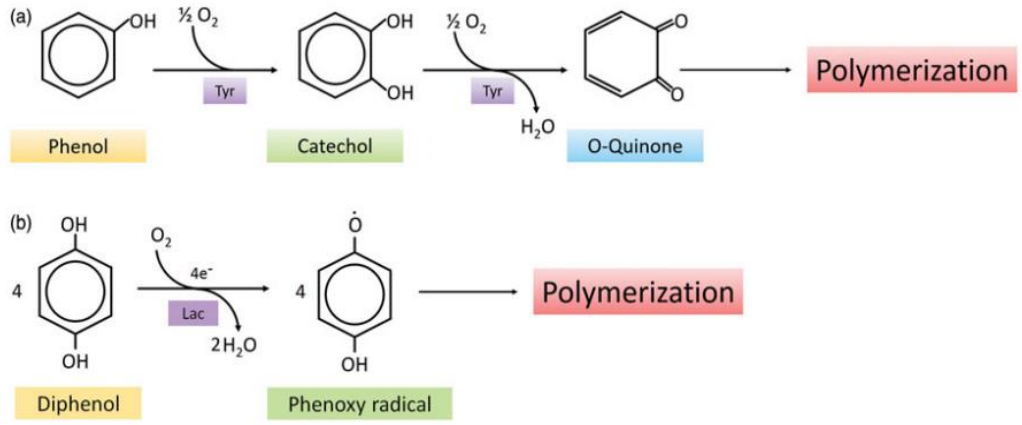


Figure 2.5 (a) Catalytic reaction of tyrosinase (Tyr) with monophenol and *o*-diphenol to *o*-quinine formation (b) catalytic reaction of laccase (Lac) with diphenols to phenoxy radical formation (Ba and Vinoth Kumar, 2017).

Tyrosinase forms no free radicals as shown in Figure 2.5 instead the phenolic compounds are instead oxidised to *o*-quinones which then undergo further oligomerization generating insoluble macromolecules that precipitate out of solution (Shesterenko et al., 2012). The transformation of a monophenols by tyrosinase first generates o-diphenol intermediates and then o-quinones that polymerise leading to high molecular weight polyphenolics that can be removed by solid-liquid separation (Pigatto et al., 2013). As well as degrading phenols Muñoz-Muñoz et al. (2011) demonstrated that tyrosinase could also oxidise aromatic amines in Table 2.5. The difference in kinetic constants are reported for the oxidation of both aromatic monoamines and monophenols. From Table 2.5 it can be concluded that aromatic monoamine oxidation is far slower and less favourable than the oxidation of monophenols.

Table 2.5 Values of the kinetic constants characterising the oxidation of aromatic monoamines and monophenols by tyrosinase (Muñoz-Muñoz et al., 2011).

Aromatic monoamines	k_{cat} (s^{-1})	K_m (mM)
Aniline	0.45 ± 0.03	0.64 ± 0.09
2-aminobenzoic acid	$(0.13 \pm 0.01) \times 10^{-3}$	2.36 ± 0.34
3-aminobenzoic acid	$(0.33 \pm 0.01) \times 10^{-3}$	0.28 ± 0.02
4-aminobenzoic acid	$(5.04 \pm 0.32) \times 10^{-3}$	0.14 ± 0.01
2-aminotoluene	0.33 ± 0.02	2.93 ± 0.38
3-aminotoluene	0.46 ± 0.04	1.26 ± 0.07
4-aminotoluene	1.01 ± 0.03	1.04 ± 0.12
2-aminoanisole	0.09 ± 0.01	0.26 ± 0.03
3-aminoanisole	0.12 ± 0.01	0.15 ± 0.02
4-aminoanisole	1.24 ± 0.21	0.09 ± 0.01
4-aminophenylalanine	0.01 ± 0.01	0.42 ± 0.05

Phenols	k_{cat} (s^{-1})	K_m (mM)
Phenol	10.81 ± 1.51	0.61 ± 0.04
2-hydroxybenzoic acid	0.046 ± 0.002	2.62 ± 0.18
3-hydroxybenzoic acid	0.64 ± 0.03	2.35 ± 0.32
4-hydroxybenzoic acid	1.70 ± 0.07	0.22 ± 0.03
2-methylphenol	7.37 ± 0.40	1.48 ± 0.18
3-methylphenol	7.51 ± 0.65	0.62 ± 0.09
4-methylphenol	16.12 ± 1.53	0.38 ± 0.05
2-hydroxyanisole	5.47 ± 0.21	5.40 ± 0.60
3-hydroxyanisole	46.87 ± 2.06	2.53 ± 0.26
4-hydroxyanisole	184.20 ± 6.10	0.08 ± 0.01
4-hydroxyphenylalanine	8.12 ± 1.44	0.27 ± 0.03

Palma et al. (2013) studied the removal of phenols that are widely present in both industrial and municipal waste streams. Crude extracts of vegetable products containing tyrosinase were added to a bioreactor to remove the following harmful pollutants phenol, o-cresol and p-nitrophenol. Yet again the determined optimal conditions do not reflect an operational WWTP similar to the laccase studies. The expense of heating wastewater to an optimal temperature of 45 ^{o}C is not feasible but the optimal pH of 6.6 is more environmentally relevant. A greater understanding on tyrosinase and laccase performance at conditions that relate to a typical WWTP is needed.

The research with the chosen EDC substrates and testing sampled wastewater matrices aims to inform on this missing knowledge. Inherent limits of a WWTP need to be considered when investigating the application of exogenous enzyme treatment technologies. Table 2.6 shows that the use of tyrosinase has so far been centred on simple phenol degradation. Unlike laccase there is a gap in knowledge regarding the potential of tyrosinase to degrade EDC substrates and this gap offers novel findings to discuss.

Table 2.6 Published studies on tyrosinase to remove chemical pollutants.

Tyrosinase Source	Substrates	Inhibitors/ Mediators	Analytical Method	Matrix	Experimental Conditions	Sample Inactivation	Reference
Mushroom	L-DOPA Phenol Chlorinated phenols	—	Substrate oxidation (UV/Vis spectrophotometer)	Buffer Solution (Citrate, phosphate, borate & NaOH/Borax buffers)	pH → 3 to 10 Temperature → 10 to 50ºC Contact time → 3 hours	—	(Ikehata and Nicell, 2000)
Mushroom	4-*tert*-butylcatechol	Inhibitors: Tropolone, Salicylhydroxamic acid Mediators: SOD, Mannitol	Oxygen production	Buffer Solution (Sodium acetate & sodium phosphate)	pH → 3.5 to 8 Temperature → 37ºC	—	(García-Molina et al., 2005)
Bacillus thuringiensis	2,4-dichlorophenol	Mediators: PEG 2000, Tween 20, SDS, Triton X-100	—	Buffer Solution (Phosphate buffer)	pH → 7 Temperature → 10 to 50ºC Contact time → 3 hours	0.1ml of H_3PO_4 8.5% (w/v) added to 3ml samples	(El-Shora and Metwally, 2008)
Mushroom	Phenol Para-cresol Phenyl acetate	—	Substrate degradation (UV/Vis spectrophotometer)	Buffer Solution (Phosphate buffer)	pH → 7 Temperature → 35ºC Contact time → 12 hours Shaker → 100rpm	—	(Bayramoglu et al., 2013)
Mushroom	*o*-Diphenols *o*-Aminophenols Aromatic *o*-diamines	—	Spectrophotometric and oxymetric assays	—	—	—	(Munoz-Munoz, *et. al.*, 2013)

Mushroom	Phenol *p*-Nitrophenol *o*-Cresol	—	Substrate absorbance (UV/Vis Spectrophotometer)	Buffer Solution (Phosphate buffer)	Stirred vessel pH → 5.6 to 7.6 Temperature → 15 to 55ºC Stirrer → 200 to 800 rpm Contact time → 30 to 500 minutes	—	(Palma et al., 2013)
Mushroom (A. bisporus)	Phenol	—	Substrate concentration (HPLC-UV)	Olive Mill Wastewater (1:20 diluted)	pH → 4.5 to 8.5	240µL of H_3PO_4 8.5% (w/v) added to 3ml samples	(Pigatto et al., 2013)
Mushroom	BPA Phenol *p*-Cresol *p*-Chlorophenol	—	Substrate conversion (HPLC-UV)	Buffer Solution (Phosphate buffer)	Immobilised (cross-linked) pH → 5.6 to 8.6 Contact time → 0 to 11 hours	0.22µM filter	(Xu and Yang, 2013)
Mushroom	Phenol	Inhibitor: Cyanide	Substrate concentration (HPLC-UV)	Buffer Solution (Phosphate buffer) Synthetic WW (Sodium acetate + multiple salts) Coking Wastewater	pH → 8 Temperature → 25 & 35ºC Contact time → 2 to 20 hours	—	(Martínková and Chmátal, 2016)
Streptomyces pharetrae *Streptomyces polyantibioticus*	3-HAA 4-Methylcatechol 4-TBC Caffeic acid Catechol L-DOPA L-Tyrosinase Aminophenol *p*-Cresol Pyrogallol	Inhibitors: EDTA, l-Ascorbic acid, 1-Cysteine, NaCl, SDS, Sodium metabisulphite, Cu^{2+}, Zn^{2+} Mediators: Ca^{2+}, Co^{2+}, Fe^{2+}, Mg^{2+}, Mn^{2+}, Zn^{2+}	Substrate oxidation (UV/Vis spectrophotometer)	Buffer Solution (Sodium acetate, potassium phosphate & Tris-HCl buffers)	pH → 3 to 10 Temperature → 5 to 60ºC Contact time → 0 to 24 hours	—	(Le Roes-Hill et al., 2015)

Similar to laccase, the presence of inhibitors and mediators in the wastewater matrix can influence tyrosinase performance. (Han et al., 2007) identified Zn^{2+} to be an inhibitor of tyrosinase. The binding of Zn^{2+} to tyrosinase resulted in conformational changes. The analysis of trace contaminants in UK wastewaters by Gardner et al. (2013) showed Zn^{2+} to be a persistent metal in wastewater. Consideration has to be taken into account for any difference in Zn^{2+} concentration between the papers resulting in a reduced effect. Phenol was shown to enhance tyrosinase activity (Wada et al., 1995) and is a common pollutant in wastewater released from the following coal conversion, plastics, textiles and pulp industries (El-Shora and Metwally, 2008). The same approach for laccase was taken for tyrosinase with the common metal ion Cu^{2+} and Cl^{-} anion in wastewater added to a buffer and sampled wastewater matrix to compare any varying influences. Additionally the combination of EDCs was tested that has not been previously observed or discussed within the literature for tyrosinase.

2.4.3 Detection of Bioactive Chemicals to Assess Laccase and Tyrosinase Activity

From Tables 2.4 and 2.6 it can be seen that high performance liquid chromatography (HPLC) was the most popular analytical method for analysing EDC removal and in turn enzyme performance. Substrate removal in this research project was also determined by HPLC-UV analysis. HPLC is a powerful separation method that provides users with both qualitative and quantitative information for the separated analytes. Compounds analysed by HPLC in a mixture are distinguished from each other by their own unique elution time. A detector attached to a HPLC instrument registers a signal for each eluted compound with a UV detector being the most commonly used. The popularity of a UV detector arises from its wide linear range, high sensitivity and not being affected by temperature changes. There are different chromatographic separation methods available but reverse-phase HPLC is the most applicable to the detection of small organic compounds such as EDCs and widely adopted because of its reproducibility.

For reverse-phase HPLC there is a non-polar stationary phase and a polar mobile phase. This leads to non-polar compounds taking longer to elute than more polar compounds. The stationary phase provides a high specific surface area for the adsorption of compounds that is often chemically bonded octadecylsilane (ODS) in reverse-phase HPLC. The mobile phase, typically a mixture a two solvents, dictates

the retention time of the compound adsorbed to the stationary phase. Water and acetonitrile are common solvents with water being far more polar than acetonitrile. Polar compounds are weakly retained by the stationery phase and therefore elute off first as similar polarities attract. Composition of the stationary and mobile phases greatly influences retention times as well as the temperature of the used stationary phase column and the compound properties (Meyer, 2013).

2.5 Wastewater Conditions and Impact on Enzyme Activity

Comparison between Tables 2.4 and 2.6 show that laccase within the literature has been investigated more so than tyrosinase for EDC removal. Table 2.7 highlights recent studies that have evaluated the combined removal of chemical pollutants by laccase and tyrosinase treatment. (Ba et al., 2014). Combined crosslinked enzyme aggregates of laccase and tyrosinase showed removal of acetaminophen exceeded 80% in municipal and hospital wastewater. Furthermore this paper reflected the recent trend in the literature with more experiments involving laccase and tyrosinase being performed in sampled wastewater matrices. The majority of the previous work has been carried out in clean buffer solutions and the repeatability of those results in wastewater are often unknown. Publications by Garcia et al. (2011) and Lloret et al. (2013) have shown laccase to behave differently in a clean water matrix to wastewater. As previously noted, a range of different wastewater constituents such as metals and chloride can influence enzyme activity. The temporal variability of wastewater arising from its dynamic nature is not often taken into account with removal not analysed over a sustained period.

Table 2.7 Published studies on combined laccase and tyrosinase treatments to remove chemical pollutants.

Laccase/ Tyrosinase Source	Substrates	Inhibitors/ Mediators	Analytical Method	Matrix	Experimental Conditions	Sample Inactivation	Reference
Laccase: *Pyricularia oryzae* Tyrosinase: *Mushroom*	α-Naphtanol, 2-4 Dichlorophenol, 4-Methoxyphenol, β-Naphtanol, 4-Chloro-3-methylphenol, Catehin	—	Substrate removal (%) (HPLC-UV)	Deionised Water Buffer Solution (Phosphate buffer)	Tubular reactor pH → 6.5 Temperature → 25ºC Contact time → 15 minutes	—	(Krastanov, 2000)
Laccase: *Trametes versicolor* Tyrosinase: *Mushroom*	Acetaminophen	Inhibitors: $CaCl_2$, $CuCl_2$, $ZnCl_2$, SDS, NaN_3, EDTA	Substrate concentration (LC-MS/MS)	Buffer Solution (Citrate phosphate, sodium phosphate, boric acid hydroxide) Municipal and Hospital Wastewater	pH → 3 to 9 Temperature → 5 to 60ºC Contact time → 8 hours	—	(Ba et al., 2014)

Other factors apart from temperature and pH impact on enzyme performance with salinity being an example (Kim and Nicell, 2006). Wastewater constituents can also inhibit or enhance enzyme performance. Margot et al. (2013) investigated the degradation of BPA, DCF and mefenamic acid (MFA). The majority of papers within the literature tend to only analyse the removal of a single bioactive chemical each time. However, Margot et al. (2013) evaluated the combined degradation of the substrates together. DCF removal significantly improved in the presence of BPA and MFA. Wastewater in the real world contains a mixture of different bioactive chemicals. Cyanide in coking wastewater has shown to inhibit laccase and tyrosinase activity, resulting in poor phenol removal (Steevensz et al., 2009) (Martínková and Chmátal, 2016). Cyanide in both cases had to be removed before enzymatic treatment to produce acceptable phenol elimination, once again highlighting how essential it is to study the wastewater matrix and its inherent complexity.

Ikehata and Nicell (2000) explored the use of tyrosinase to degrade phenols instead of previously utilised peroxidases as they required hydrogen peroxide as a cofactor, increasing costs. Tyrosinase was able to degrade phenol over a wide range of concentrations at an optimal pH range between 5 and 8 (Ikehata and Nicell, 2000). Compared to laccase, the optimal conditions exhibited by tyrosinase appear to be more reflective of a WWTP. The application of tyrosinase to remove EDCs in wastewater might be an improved alternative to laccase and additionally provide more stable activity.

Optimal conditions determined in the literature are often based on relative enzyme activity using an analytical substrate such as L-tyrosine for tyrosinase for example. EDC substrate removal may not mirror the optimal conditions observed for the analytical substrate especially in the complex wastewater matrix. Margot et al. (2013) investigated the pH profile for laccase from *T.versicolor* by utilising four different analytical substrates; ABTS, 2,6-dimethoxyphenol, guaiacol and syringaldazine. The pH profiles varied for each analytical substrate but activity was only observed under acidic conditions. First principles help to inform on enzyme activity and in turn removal but the optimal conditions for the treatment technology should relate to the EDCs being tested.

First principles regarding enzyme kinetics can be used to inform on the behaviour of the analysed enzymes. The potential response of enzymes at conditions reflecting a WWTP can be calculated. As touched on before, many of the studies performed in Tables 2.4 and 2.6 have tested temperature and pH outside the scope of a WWTP. For example, the typical temperature range in the UK for a WWTP is between 10 ^{o}C and 25 ^{o}C. The pH range, between pH 6.5 and 8.5, is tightly controlled to minimise any adverse effects on the aquatic environment that receives the discharged effluent (Tchobanoglous et al., 2003). Oxygen can be a limiting step for the laccase and tyrosinase experiments so the introduction of additional oxygen would be expected to increase activity leading to greater EDC removal. The publications by Lloret et al. (2012) and Lloret et al. (2013) investigated the effect of aerating with pure oxygen on laccase performance. The expense associated with pure oxygen from handling and storing the flammable gas restricts its large scale use at a WWTP.

Temperature and pH are the two factors most associated with influencing enzyme activity. The first principles are not only relevant to laccase and tyrosinase but also inherent enzymes discussed later in the chapter. In appendix A Table 9.2 shows the temperature and pH profiles generated for the inherent enzymes from the literature. These profiles indicate the behaviour of the inherent enzymes in the operational window of a WWTP. The Michaelis-Menten model is the most widely adopted approach when studying enzyme kinetics, as activity can be observed as a function of substrate concentration and the saturation point for the active site can also be determined. Other observations include finding the maximal velocity of a given substrate and comparison between enzyme affinity for different substrates. Figure 2.6 shows a typical Michaelis-Menten graph.

$$v1 = \frac{Vmax\,[\mathrm{S}]}{Km + [\mathrm{S}]}$$

Equation 1 Michaelis-Menten Equation

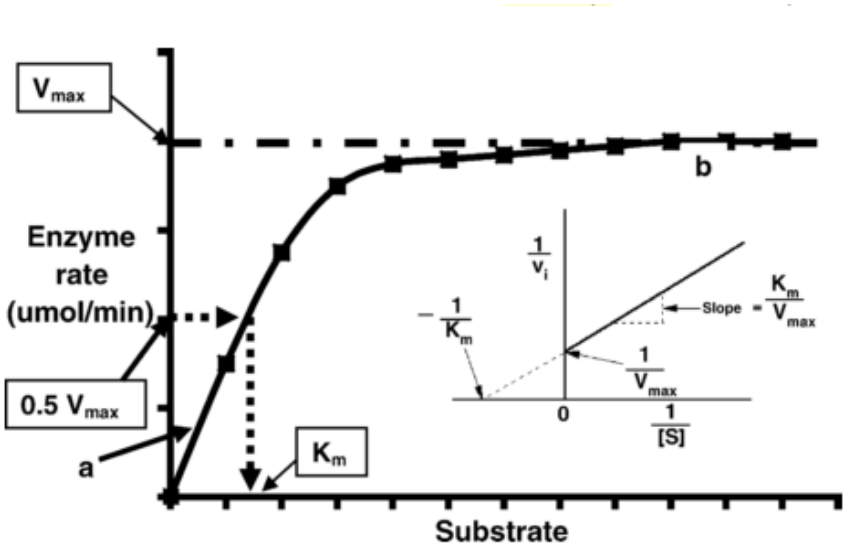


Figure 2.6 Michaelis-Menten graph showing the change in enzyme velocity against substrate concentration (Whiteley and Lee, 2006).

From the Michaelis-Menten equation V_1 is equal to the rate of the enzyme catalysed reaction and [S] is substrate concentration. For the other kinetic parameters K_m is the Michaelis-Menten constant and V_{max} signifies the maximum reaction rate. From Figure 2.6 it can also be seen that K_m is equal to the substrate concentration at 50% V_{max}. At low substrate concentrations the rate of reaction is first order as it is proportional to substrate concentration. At higher substrate concentrations, rate of reaction is zero order as almost all the enzymes become bound with substrate and so is concentration independent. The Lineweaver and Burk plot also presented in Figure 2.6 avoids having to determine the kinetic parameters from a curvilinear graph when 1/v is plotted against 1/[S] (Whiteley and Lee, 2006). The kinetic term k_{cat} details the number of catalytic cycles an enzyme can turnover substrate in a unit of time. Equation 2 shows how kinetic parameters determined by the Michaelis-Menten model can inform on the required contact time for an enzyme to remove a substrate of interest to a set concentration.

$$t = \frac{Km}{Vmax} \times \ln \frac{1}{1 - X} + \frac{X\,[S]_0}{Vmax}$$

t = batch reaction time
$X = ([S]_0 - [S])/[S]$; target removal efficiency for the substrate
$[S]_0$ and [S] = initial and final substrate concentration

Equation 2 Calculation for enzymatic residence time.

Temperature influences can be modelled by the Arrhenius equation that is shown within ppendix (page 228). As shown in Figure 2.7 the increase in temperature results in a greater fraction of molecules that are able to overcome the activation energy to give an enzymatic reaction. Temperature and enzyme activity do not increase indefinitely as enzymes are proteins, so become denatured at high temperatures typically above 50 ^{o}C. As already mentioned, the average temperature range for a WWTP in the UK will not lead to enzyme denaturation. A general rule associated with the Arrhenius equation is that a temperature increase of 10 ^{o}C will result in the rate of reaction to increase by a factor of between 2.2 and 2.5 termed Q10.

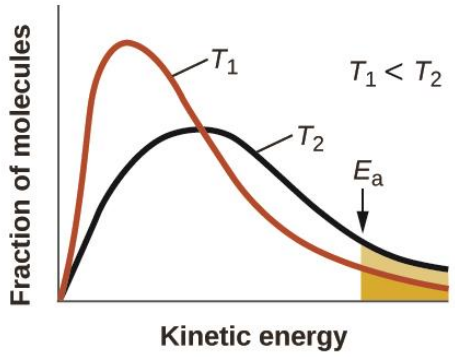


Figure 2.7 Distribution of molecular speed

Enzymes have an optimal pH as the ionisation state of the acidic and basic groups of the amino acids surrounding the active site influence the values of K_m and k_{cat}. Optimal pH is between p*K*a values of two or more catalytic amino acids (Buchholz and Kasche, 2006). First principles are commonly applied to enzymes that are in a clean water matrix whereas wastewater exhibits other enzyme influences apart from temperature and pH. Wastewater composition can impact activity shown by the inhibitory influence of metal ions and salinity. In conclusion first principles may inform on enzyme activity but the inherent complexity of wastewater may not result in the predicted effect to occur.

2.6 Variability Arising from the Wastewater Environment

Wastewater is highly variable mainly because there is less control than other biological processes. There is a range of input factors that contribute to the variability of wastewater. For example the complexity of wastewater is further increased by people using and abusing pharmaceutical drugs (Collado et al., 2013). Drugs that have expired or are no longer needed are commonly thrown away in the bin. The waste from bins

when discarded at a landfill site can leach and subsequently run off into the sewage system. People also throw away drugs by disposing them down the sink or toilet resulting in there being another entry point for pharmaceuticals into wastewater (Jelic et al., 2011). Wastewater variability is linked to a number of socioeconomic factors not usually replicated in other chemical processes (Castiglioni et al., 2014). If the degree of variability can be understood in a highly complex matrix like wastewater then the same approach could be successfully applied to other biotechnological processes.

To understand how a complex process environment impacts on enzyme activity the enzyme response to different environmental and process variables needs to be measured as described in Section 2.1 "Enzymes in Industrial Biotechnological Processes". The enzymes in wastewater are responsible for the biological degradation of organic pollutants including bioactive chemicals. The catalytic potential of enzymes can be enhanced or hindered by a wide range of different factors. Additionally an environment that has a complex mixture of pollutants can have a varying effect on enzyme activity (Rao et al., 2010). Seasonal temperature changes have a significant impact on wastewater variability. WWTPs are less effective at removing pollutants when the temperature is reduced. As the temperature decreases so does the metabolic rate of microorganisms. Enzymes also become far less efficient at breaking down pollutants. Measuring enzyme activity provides critical information on how the biological system is responding to an individual variable or a combination of variables (Koh et al., 2008) .

A recent paper by Balestri et al. (2013) aimed to develop a new quality index for wastewater by analysing enzyme activity. Ten enzymes that included tyrosinase formed the multi-enzymatic sensor that informed on how the taken wastewater samples influenced activity. Samples from different locations could be identified by their unique enzymatic quality index. Furthermore the authors recommended the enzymatic quality index as a parameter to observe quality improvements. Organic compounds and ionic species were also investigated to understand their impact on enzyme activity. The enzyme activities for all the enzymes were determined from analytical substrates and not EDC degradation applicable to tyrosinase. There is additionally no mention of any measured standard water parameters such as temperature, COD or TSS that could help to inform on the cause for any observed

improvement in water quality. It is also difficult to say if temporal variability was analysed.

The amount of rainfall also has an impact on wastewater variability as pollutants are concentrated to a greater extend in a time of a drought. Kirk et al. (2002) reported estrogen removal to be greater when there had been a substantial rainfall compared to their having been no rain at all. Another variable that impacts on enzyme activity is pH. The pH of a typical WWTP ranges between 6.5 and 8.5 (Tchobanoglous et al., 2003). All enzymes and microorganisms have an optimal pH value. However, it is impossible for a WWTP to operate at pH that will be optimal for them all, therefore pH is a compromise that at particular times will either impede or enhance micropollutant removal (Burgess and Pletschke, 2008).

Wastewater is highly variable because of its complex matrix of different components. Wastewater treatment is also extremely variable with many factors reported to influence removal efficiency. Metal ions are also known to have a significant impact on biological systems (Fischer and Majewsky, 2014). Variability is undesirable for any process due to the complications in achieving a consistent output. The processability of wastewater tends to fluctuate daily as numerous factors can have a positive or negative effect on the removal efficiency of pollutants. The contaminants within wastewater also change on a daily basis leading to different enzyme activity fingerprints (Fischer et al., 2013). Contaminants influence enzyme efficiency as the interactions between the two could enhance or inhibit enzyme activity. A change in pollutant concentration could also result in more competition for binding to enzyme active sites. Table 2.8 shows the variability in constituent concentrations for E1 and DCF between WWTPs in the same country and a comparison between different countries.

Table 2.8 Occurrence and concentration of estrone (E1) and diclofenac (DCF) in different water matrices across Europe.

Bioactive Chemical	Matrix	Country	Concentration	Reference
Estrone (E1)	Wastewater Treatment Plant	England	50 ng/L	(Rodgers-Gray et al., 2000)
		Germany	Influent: 188 ng/L Effluent: 12.6 ng/L	(Zuehlke et al., 2005)
		Italy	Influent: 15-60 ng/L Effluent 5-30 ng/L	(Yang et al., 2006)
		Luxembourg	Influent: 0.3-9 ng/L Effluent: 0. 3-14 ng/L	(Pailler et al., 2009)
		Portugal	Influent: 2484 ± 15 ng/L Effluent: 25 ± 2 ng/L	(Salgado et al., 2010)
		Spain	100–1,500 ng/L	(Pedrouzo et al., 2010)
Diclofenac (DCF)	Ground Water	Germany	590 ng/L	(Cleuvers et al., 2004)
	Hospital Effluent	Spain	60-1900 ng/L	(Ferrari et al., 2003)
	Surface Water	England	195 - <8 ng/L	(Thomas and Hilton., 2004)
		Germany	26-72 ng/L	(Ferrari et al., 2003)
			42-67 ng/L	(Ferrari et al., 2003)
	Wastewater Treatment Plant	England	380-1700 ng/L	(Hilton and Thomas., 2003)
			Upstream: <20 ng/L Effluent: 599 ng/L	(Ashton et al., 2004)
		Greece	Influent: 12-560 ng/L Effluent: 10-365 ng/L	(Schwaiger et al., 2004)
			Influent: 1.6-75 µg/L Effluent: 0.41-0.8 µg/L	(Samaras et al., 2013)
		Luxembourg	Influent: 2-43 ng/L Effluent: 0.3-78 ng/L	(Triebskorn et al., 2007)
		Spain	Influent: 3.02 µg/L Effluent: 2.51 µg/L	(Heberer., 2002)
			Influent: 200-3600 ng/L Effluent: 140-2200 ng/L	(Cleuvers., 2004)
			Influent: 160 ng/L Effluent: 40 ng/L	(Jelena et al., 2007)
			5–2,000ng/L	(Pedrouzo et al., 2010)
			Influent: 232 ng/L Effluent: 220 ng/L	(Rosal et al., 2010)
			Influent: 0.72 µg/L Effluent: 0.09-0.74 µg/L	(Martin et al., 2012)
		Switzerland	Influent: 1300-2900 ng/L Effluent: 1300-2400 ng/L	(Cleuvers., 2004)
			1900 ng/L	(Tauxe-Wuersch et al., 2005)
		United Kingdom	Influent: 901-1036 ng/L Effluent: 261-598 ng/L	(Ferrari et al., 2003)

2.7 Inherent Enzymes in the Wastewater System

The literature on inherent wastewater enzymes has mainly centred on lab scale treatment designs. Experimental treatment plants have been set up and the inherent enzyme activity assayed to inform on biological efficiency for degrading organic pollutants. The largest constituents of wastewater are organic pollutants such as proteins, carbohydrates and lipids. To mineralise these pollutants the microorganisms in the biological treatment stage utilise hydrolytic enzymes to break them down (Cortés-Lorenzo et al., 2012). Hydrolases are hugely critical in the treatment of wastewater and as a result are the class of enzymes selected to feedback on the biological efficiency of activated sludge. Activated sludge is the concentration of bacterial flocs that degrade wastewater pollutants in the biological treatment stage.

Hydrolases are not just specific to traditional organic pollutant removal as a recent paper by Krah et al. (2016) investigated the degradation of 20 micropollutants in activated sludge samples. The micropollutants were all pharmaceuticals including some examples of their more dominant excreted metabolites. Removal was observed to varying degrees for all 20 micropollutants spiked into whole activated sludge samples. When the activated sludge samples were lysed to produce cell-free enzyme extracts high activities of the following inherent enzymes; acid phosphatase, β-galactosidase, and β-glucuronidase; were all recorded. Only six of the 20 micropollutants spiked into the cell-free lysate were degraded to the same rate as observed in the whole activated sludge samples.

Nevertheless, the cell-free lysate showed the potential of inherent hydrolases to additionally breakdown bioactive chemicals. Some of the removal was presumed to occur by an oxidation reaction therefore the enzymes responsible for this are oxidoreductases (Krah et al., 2016). The hydrolytic enzyme sulfatase commonly present in the wastewater matrix (Fischer et al., 2013) (Kreutz et al., 2016) cleaves EDC metabolites with a sulfate conjugate resulting in the parent compound entering the environment (Khanal et al., 2006). Linking this to a tertiary treatment step with laccase or tyrosinase would then negate this route of environmental entry by deconjugated EDC compounds.

2.7.1 Hydrolytic Activity in Activated Sludge

Four prominent hydrolases were chosen and studied under the effect of different electron acceptor conditions by Goel et al. (1998). Activities of the enzymes acid phosphatase, alkaline phosphatase, protease, α-glucosidase and dehydrogenase were all analysed from a lab scale anaerobic-aerobic activated sludge sequencing batch reactor (SBR). The enzyme assay methodology developed within this paper has been widely adopted by the literature ever since. Enzyme activities were analysed during the anaerobic and aerobic phases of the SBR to investigate potential variations when under different electron acceptor conditions. They concluded that there was no significant difference between the activities during the two SBR phases. Localising enzyme activity in the taken activated sludge samples was also performed and subsequently shown to be primarily associated with the cell floc. Three activated sludge fractions were analysed i.e. (1) enzymes associated with the cells, (2) enzymes in the bulk solution and (3) enzymes entrapped in the flocs or loosely associated with the cells (Goel et al., 1998). More publications have since studied activity localisation in greater detail and investigated numerous cell lysis methods.

Esterase is another important hydrolase linked to organic pollutant degradation and was first studied in detail by Boczar et al. (2001). Similar to Goel et al. (1998) the distribution of esterase in the activated sludge was also investigated. Activated sludge from three separate municipal wastewater treatment plants (WWTPs) was sampled and characterised by their esterase activity. The assay method used by Boczar et al. (2001) for determining esterase activity has been adapted by a number of later papers as well. The wastewater feed into the sampled WWTPs comprised of different domestic and industrial wastewater mix ratios. The first WWTP was a small residential facility with the influent 100% domestic, the second WWTP was a medium sized residential facility that received >95% domestic waste and the final WWTP was a large municipal facility with 50% of its incoming flow originating from heavy industrial sources. Characterisation of the three WWTPs by esterase activity was the outline for future studies that investigated and compared larger sets of enzyme activities, typically as enzymatic fingerprints.

A range of different model substrates were utilised by Boczar et al. (2001) to assess the substrate that would generate the greatest response: C_2-C_{18} monoesters of *p*-nitrophenol, and C_2-C_8 diesters of fluorescein. The highest esterase activities were

recorded for the short alkyl chains (C_4) and activity was significantly reduced for chain lengths above this. The model substrates were also used to analyse esterase specificity in the particulate and freeze-thaw fractions from the activated sludges of each WWTP. The particulate fraction referred to centrifugation of the cell flocs and re-suspended in buffer whereas the freeze-thaw fraction only contained periplasmic space esterases. Extracellular esterase activity was additionally assayed for in the filtrate of filtered activated sludge samples but there was no measurable activity. Esterase activity was solely associated with either the cell floc or lysed cellular material. The WWTP with an influent flow consisting of 50% industrial waste showed exceedingly higher particulate activities compared to the other WWTPs for all the *p*-nitrophenol substrates. Higher protein concentrations were highlighted by the authors for the approximate 3 fold activity increase.

2.7.2 Cell Lysis of Activated Sludge Samples

Cadoret et al. (2002) also performed a series of experiments that focused on a range of substrates to determine enzyme activity. Unlike Boczar et al. (2001) the research aim was to investigate the extent of diffusion for high molecular weight compounds through the extracellular polymeric substance (EPS) layer. EPSs are secreted by bacteria and they are critical in the formation of activated sludge cell flocs to enable successful wastewater treatment. Reduced enzyme activities would be exhibited for substrates with diffusion limitations as less substrate would be available for the enzymes contained in the activated sludge flocs. The enzymes studied were Leu-aminopeptidase, α-glucosidase, α-amylase and protease. Ultrasound and cation ion exchange were both used separately and combined to disperse the activated sludge cell flocs. The cation ion exchange removed the bivalent cation Ca^{2+} resulting in the EPSs bridging to be disrupted causing the cell flocs to break apart.

Sludge floc dispersion had varying impacts on the enzymes. Combination of ultrasound and a cation exchange resin had no effect on protease activity compared to the whole sludge sample. However, the combination of the two dispersion techniques had a five-fold increase on α-amylase activity when compared against whole sludge samples. The activity of α-glucosidase appeared to be noticeably reduced by the two dispersion methods. All enzymes were assayed from activated sludge samples collected on three different days. There is not enough data to conclude any temporal

patterns as the focus was on floc dispersion and the enzymes were assayed on varying days within a set period. High molecular weight compounds do not have reduced availability to the tested enzymes and are not impeded by the EPS layer. Dispersion greatly improved α-amylase activity.

Publications by Yu et al. (2007) and Krah et al. (2016) have both expanded on enzyme activity from lysed activated sludge flocs. Yu et al. (2007) quantified enzyme activity in EPSs and cell floc pellets fractions. Both reflected the work by Cadoret et al. (2002) in performing several different extraction methods. The EPS extracted from the cell flocs were sub-divided into two categories either loosely bound (LB) and tightly bound (TB) fractions. As mentioned previously by other papers extracellular activity is negligible within the bulk solution and almost all enzyme activity is observed in the sludge cell flocs. Detailing the distribution of enzyme activity furthers the understanding on the probable contact between enzyme and substrate helping to inform on treatment efficiency (Molina-Muñoz et al., 2007).

Protease, α-amylase and α-glucosidase were the enzymes investigated by Yu et al. (2007) through different extraction processes. Along with the control there were seven different extraction processes tested. The extraction processes were the following; ultrasonication, cation exchange resin (CER), ethylenediaminetetraacetic acid (EDTA), formaldehyde, formaldehyde + ultrasonication, formaldehyde + NaOH. Ultrasonication resulted in the highest enzymes activities to be exhibited for all the enzymes tested. Additionally there is a minimal risk of intercellular material being released as shown in their study that examined DNA content compared to a control. This contradicts the results presented by Cadoret et al. (2002) that showed α-glucosidase to be negatively influenced by ultrasonication. Distribution of enzyme activity was not consistent for the enzymes. Both α-amylase and α-glucosidase were concentrated in the LB-EPS region with minimal amounts in the TB-EPS and pellet fractions. Protease activity was revealed to be completely associated with the pellet region. As noted by Yu et al. (2007) the activity locations indicate that protease is an ectoenzyme (bound to the cell surface) while α-amylase and α-glucosidase are exo-enzymes (immobilised in the EPS matrix in the free form). Each enzyme was differently affected by the extraction processes with formaldehyde commonly inhibiting activity with activities far lower than the control.

A recent paper by Krah et al. (2016) additionally examined the impact various lysate methods had on enzyme activity for acid phosphatase, β-glucosidase and β-glucuronidase. Furthermore, twenty different micropollutants were spiked into cell-free lysates to better understand the degradation of target compounds. Colorimetric substrates are almost always used to assay inherent wastewater enzyme activity to capture biological treatment efficiency. However, micropollutants are the pollutants of true concern and colorimetric substrates do not always reflect the capability of inherent enzymes to degrade these compounds. All twenty micropollutants were spiked into cell-free lysates and intact whole activated sludge samples to compare removal.

From the presented data, eighteen out of the twenty micropollutants were biodegraded in the intact sludge samples. Only six micropollutants were able to be degraded at rates similar to those found in cell-free lysates. These were acetaminophen, N-acetylsulfamethoxazole, atenolol, bezafibrate, erythromycin and 10,11-dihydrogen-10-hydroxycarbamazepine. Explanation for the difference in results was the impact of the additional steps undertaken to produce the cell-free lysate. Sludge pre-treatment, peptidase inhibitors, ammonium sulfate addition all affected the tested hydrolases differently. Pre-treatment of sludge through combinations of EDTA addition, ultrasonication and pressure filtration improved acetyl-SMX removal in the lysate whereas atenolol and bezafibrate degradation was inhibited. Erythromycin degradation was highlighted as lysates that had been filtered or unfiltered showed opposing removal rates. No significant erythromycin removal was recorded for the filtered lysate but the unfiltered showed >96% degradation. From this finding the authors commented that the glass microfiber prefilters within the used syringe filters were strongly retaining the enzymes responsible for erythromycin biotransformation (Krah et al., 2016).

2.7.3 Oxidoreductase Analysis

Also touched upon by Krah et al. (2016) is that oxidoreductases as well as hydrolases are responsible for micropollutant degradation. They mention the biotransformation of 10,11-dihydro-10-hydroxycarbamazepie to 9-carboxylic acid acridine (9-CA-ADIN) was the result of biologically-mediated oxidation. (Grekova-Vasileva and Topalova, 2009) are one of the few papers that investigated inherent oxidoreductases in a wastewater treatment environment. The WWTP of focus treated textile industry waste

discharges and as a consequence the incoming feed constituted of highly complex organic pollutants and xenobiotics resistant to biotransformation. Along with studying the enzymes catechol 1,2-dioxygenase (C12DO), catechol 2,3-dioxygenase (C23DO), protocatechate 3,4-dioxygenase (P34DO), succinate dehydrogenase (SDH) and unspecific azoreductase (AzoR) microbial identification was also performed. The response of the oxidoreductases and microbial evolution to a series of WWTP alterations were studied. Behaviour of the oxidoreductases was studied after the WWTP had been shock-loaded with azo compounds, recovery after the xenobiotic shock, incorporation of an additional aerobic reactor, and breakdown of facility exploitation after five months. Oxidoreductases compared to hydrolases do not inform so greatly on overall WWTP efficiency and therefore not selected to be analysed in Chapter 6.

2.7.4 Inherent Hydrolytic Enzyme Profiles

As mentioned in the introduction, the majority of the papers published on inherent wastewater enzymes have been focused on experimental treatment designs, that is, lab scale processes that have been assessed for their pollutant removal. Enzyme activity has been used an indicator to inform on activated sludge efficiency in removing the pollutants already in the matrix or added into the influent. Molina-Muñoz et al. (2007) investigated the enzyme response to volatile suspended solid (VSS) concentration in a submerged membrane bioreactor (SMB). The utilisation of membrane bioreactors mitigates the need for a secondary clarifier. This type of treatment process is typically employed in areas where space is limited. However, challenges, such as membrane fouling, and scalability to treat large wastewater population equivalents still need to be overcome. High VSS concentrations can be achieved in SMBs irrespective of the organic load and were therefore studied by Molina-Muñoz et al. (2007) to understand the impact on microbial composition and enzymatic activity. Operating the system with increased VSS concentrations encourages slow-growing bacteria to manifest usually characterised as ammonia and nitrate oxidisers important in toxic nitrogen removal.

The activated sludge microbial population between a SMB and conventional plant share similarities but the environmental pressures exert different biological adaptions. SMB recycles the biomass and as result bacteria are restricted by a substrate-limited

environment slowing growth rates. As a consequence, enzyme activity and species composition will vary. The pilot scale SMB set up by Molina-Muñoz et al. (2007) in treating real domestic wastewater was run in two different seasons of the year. The influence of VSS concentration was the central focus of the study. The first experiment ran between spring and summer with the second experiment going from autumn to winter. Weekly activated sludge samples were taken and the following enzymes analysed; acid/ alkaline phosphatase, glucosidase, protease, esterase and dehydrogenase. The maximum numbers of samples taken was 11 in the first experiment and 10 in the second experiment. For the second set of experiments, activity is not available for all enzymes when there was a significant increase in VSS concentration at the end of the sampling period.

The conclusions from the paper were that the performed experiments showed activity for all the enzymes though time in most cases to reduce when VSS concentration increased as shown in Figure 2.8. Reasoning for this was the accumulation of end products from the recycled biomass inhibited enzyme activity. For example, high levels of dissolved inorganic phosphate have been shown to inhibit phosphatase activity in the aquatic environment.

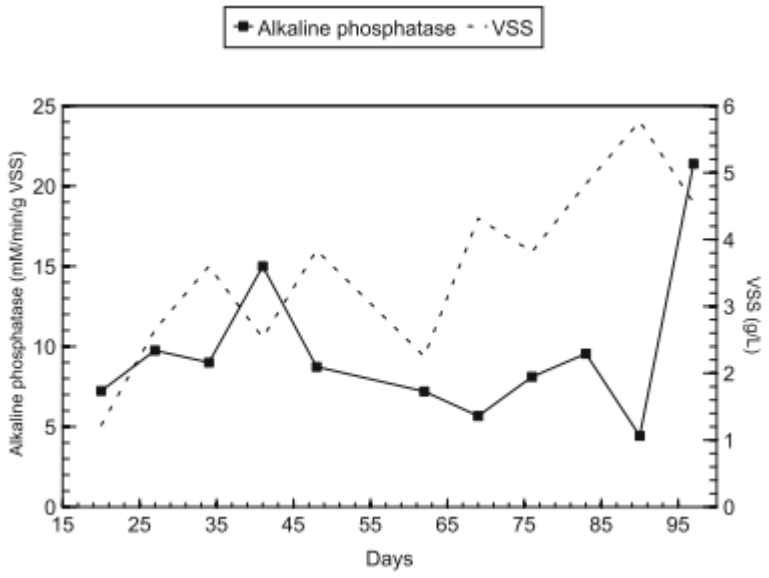


Figure 2.8 Enzyme activity for alkaline phosphatase analysed with VSS concentration (Molina-Muñoz et al., 2007)

The lower temperature throughout experiment 2 did not favour the nitrifying bacteria and is believed to result in the build-up of toxic nitrogen species, also negatively

influencing enzyme activity. Planned future work by the authors will explore this further and conclude the relationship between high VSS concentration and low temperatures on the nitrifying process. VSS concentrations over 8 g/L were concluded to decrease enzyme activity but the premature end to experiment 2 does not help to strengthen this conclusions.

Molina-Muñoz et al. (2010) expanded on their earlier work by investigating the effects of both increasing VSS and total suspended solids (TSS) on enzyme activity. Other process factors were also investigated including temperature and inflow rate. A pilot scale SMR with an operating volume of 225 L was yet again used for the experimental work, highlighting the limited work performed for full scale WWTP operations. The previous work had shown that enzyme activity decreased when VSS increased during long term operation potentially reducing the capability of the activated sludge to removal pollutants. The same six hydrolases were monitored and their activity compared against the earlier highlighted process and statically analysed by one-way or multifactor analysis of variance (ANOVA). The pilot-scale MBR was run in four stages with the TSS and VSS concentrations controlled to be at varying values. The experiments were conducted for a period of one year so the temperature fluctuated as well. Stage 3 was operated for the shortest period and as a result only four results are given for each of the monitored enzymes. For the other experimental stages activity was analysed up to seven times during the operational window.

When the enzyme activities were compared to the measured MBR conditions at the time of sampling, no significant correlations were shown between the two variables. VSS concentration informs on biomass concentration, as both are typically interlinked. An increase in biomass would be reflected by higher VSS readings. Therefore it would be expected for enzyme activities to increase with VSS concentration. Molina-Muñoz et al. (2010) yet again reported that increased VSS concentration resulted in the reduction of some enzyme activities. Furthermore VSS increases showed no relationship to biomass concentration as total heterotrophic platable bacteria (THPB) remained stable or decreased. The unique ability of a MBR system to recycle the biomass is responsible for this observation as the high retention time leads to competition for nutrients lowering growth rates. Increased VSS concentration was additionally explained to have a negative impact on oxygen transfer due to the sludge becoming more viscous and subsequent rheological changes. Dissolved oxygen is an

essential factor for hydrolysis and so limiting enzyme activity if not readily available. Composition and production rate of polysaccharides (EPS) from the activated sludge microorganisms is influenced by both VSS and dissolved oxygen concentration. Enzymes that are located within the EPS layers are adversely affected (Guellili et al., 2001).

The developmental treatment processes studied within the literature are rarely beyond the laboratory scale allowing for unrestricted alterations to be made. An example would be the publication by Calderón et al. (2012) that investigated the aeration of a MBR using pure oxygen. The advantages of utilising pure oxygen are clearly made and can be easily adapted to the lab scale MBR being used. However, the real life application of pure oxygen would require considerable justification as handling and storing pure oxygen is extremely hazardous and costs are substantial. Pure oxygen for aeration increases organic pollutant removal and also helps to reduce membrane fouling. The effect of aerating with pure oxygen on both microbial diversity and enzyme activity was studied in two experimental stages. First stage was between 23rd March 2009 to 20th April 2009 and second stage was between 15th June 2009 and 11th July 2009. Acid/ alkaline phosphatase, glucosidase, protease and esterase were the inherent enzymes analysed with ten data points collected for the first experimental stage and seven for the second.

The hydrolases chosen by Calderón et al. (2012) are crucial in enabling microorganisms to mineralise organic pollutants within the wastewater matrix and therefore excellent indicators to assess the biological efficiency of the activated sludge. From the collected enzymatic data and measured operational parameters a Monte Carlo permutation test was performed to determine the factors responsible for the observed variations in enzyme activity. From the statistical analysis, VSS concentration and temperature were significant variables that influenced activity for all enzymes apart from esterase. VSS concentration showed a negative correlation with enzyme activity, and temperature had a positive correlation with enzyme activity, but no correlations were observed for esterase. Chemical oxygen demand (COD), biological oxygen demand (BOD_5), pH were the other variables tested but there was no correlation with any of the analysed enzymes.

As well as modifying experimental MBRs to change treatment conditions, the influent

feed has also been manipulated to examine changes in wastewater composition. Salinity can have a negative influence on hydrolytic activity as discussed by Cortés-Lorenzo et al. (2012). Four experiments were setup with the wastewater feed altered to be at different NaCl concentrations being 0, 3.7, 24.7 and 44.1 g/L. For urban wastewater the salt concentration was said to range between 0.4 and 1.5 g/L. This study was directed towards countries that produce saline wastewater through seawater toilet flushing and also industries that generate highly saline wastewater such as diary, fish processing and leather production. Spikes in salinity are detected in wastewater during winter when salt is used to prevent pavements and roads icing over. The lab scale bioreactor used to treat the saline wastewater was a submerged fixed bed bioreactor (SFBBR) where the bacteria grew on a supporting matrix. The following enzymes were assayed; acid/ alkaline phosphatase, glucosidase, protease, and esterase from the recovered biofilm growth.

The five hydrolases were greatly affected when the salinity levels were increased to 24.7 and 44.1 g/L. From the other wastewater parameters measured pH, TSS, sampling day COD, BOD_5 only salinity and sampling day had a significant effect on enzyme activity from the performed Monte Carlo permutation test. The conclusion of salinity negatively influencing enzyme activity conflicted with earlier publications that reported opposite findings. As Cortés-Lorenzo et al. (2012) highlighted it is important to look at the details of the methods before making any solid comparisons. For example Hamoda and Al-Attae (1995) presented data that showed high NaCl concentrations to not inhibit biological activity. However, the wastewater used by Hamoda and Al-Attae (1995) was synthetically produced and, as a result, both the composition and inherent properties were hugely different to real wastewater. Anupama et al. (2008) reported an increase in alkaline phosphatase activity during the treatment of urban wastewater through continuous and fed batch anaerobic bioreactors when salinity exceeded 15 g/L. Compared to Cortés-Lorenzo et al. (2012) the bioreactors used were unalike as well as the increased activity was observed under anoxic and food starved conditions. These comparisons between the papers shows the importance in obtaining results from real world wastewater.

Fischer et al. (2013) was the first paper to investigate inherent enzyme activity at multiple full scale WWTPs. The biological degradation of organic pollutants is entirely reliant on enzymatic processes and therefore critical to the performance of any WWTP.

Enzyme activity is an important parameter that is not often considered when investigating pollutant removal. Highlighted from the start of the paper is that enzymatic activity can vary widely and that this characteristic can be utilised to generate novel enzymatic fingerprints. Activated sludge samples from each WWTP were collected over a set time period and eight hydrolases were analysed; esterase, α-/β-glucosidase, alkaline phosphatase, phosphodieaterase, phosphotriesterase, sulfatase and L-alanine-aminopeptidase. These hydrolases were selected as to cover a broad range of reactions along with the potential degradation of certain micropollutants (Demarche et al., 2011). Activated sludge samples were taken from six WWTPs all varying in size, treatment processes and influent composition. From the activity data collected, each WWTP could be characterised by a unique enzymatic fingerprint. One WWTP could be recognised from its enzymatic fingerprint due to its elevated levels of β-glucosidase, the result of treating high volumes of diary waste.

One WWTP was chosen for the enzyme activities to be analysed throughout the whole treatment process including the influent and effluent. Wastewater samples were only collected over a four day period between 18th April 2011 and 21st April 2011 with two samples taken each day in the morning and afternoon. In total eight samples were assayed for the different enzyme activities. This work was the foundation for the experimental work performed in Chapter 6 for determining spatial and temporal enzyme variability. A later paper by Kreutz et al. (2016) followed on from the work presented in Fischer et al. (2013) and assayed the activity of the same inherent enzymes from the activated sludge of a single WWTP. Instead of only analysing enzyme activity, multiple water quality measurements were also taken and correlated against activity with Spearman's rho. Significant relationships between activity and the wastewater characteristics were highlighted.

Seasonal variation in the hydrolytic enzymes acid/ alkaline phosphatase, α-glucosidase and protease was investigated by Gómez-Silván et al. (2013) in a full scale membrane bioreactor fed with real wastewater. Activated sludge samples were taken from the two bioreactors operated under different conditions one anoxic and the other anaerobic. The experimental work was divided into three stages covering the following seasons spring (28th April 2009to 21st May 2009), summer (22nd June 2009 to 20th September 2009) and autumn (21st September 2009 to 22nd December 2009). Samples were collected twice a week during the different experimental stages. Measuring hydrolytic

activity, important in breaking down organic pollutants to facilitate microbial assimilation, has rarely been performed for a full scale MBR. The MBRs usually studied are lab scaled, run for extremely short periods of time, and are fed with synthetic wastewater.

The spring duration was only three weeks whereas the summer and autumn seasons spanned a period over thirteen weeks. Considerably more data was collected over the last two seasons and needs to be taken into account when comparing the various data sets. The collected enzyme activity data was extremely dynamic with each enzyme showing complex and ranging patterns of variation. Greatest variation between the seasons was observed for α-glucosidase. When the activities were compared between the aerated and anoxic bioreactors there were no significant differences. The correlation of temperature with enzyme activity was most prominent in autumn. For summer the temperatures remained fairly consistent throughout the sampling period so any influence on enzyme activity was minor. Other variables such as TSS and VSS were shown to be positively correlated to acid phosphatase and α-glucosidase activity. The positive correlation reported for VSS is in contrast to earlier discussed results (Molina-Muñoz et al., 2007) (Molina-Muñoz et al., 2010).

2.8 Summary of the Literature Review

The discussed literature review has highlighted issues and gaps in knowledge associated with the planned experimental work that are linked with the aims and objectives in Section 1.3. These are summarised in the text below:

- Only a few recent studies within the literature have investigated laccase and tyrosinase behaviour in real world wastewater samples. Limited information is available on EDC removal by tyrosinase.
- The impact of temporal wastewater variability on laccase and tyrosinase performance has not been studied. Experimental conditions fail to reflect the wastewater environment, such as aerating the treatment processes with pure oxygen.
- Influence of inhibitors, at relevant wastewater concentrations, on laccase and tyrosinase performance through EDC removal has not been performed in the literature.

- Published inherent enzyme activities have mainly come from activated sludge samples with only one paper determining activity throughout an operational WWTP.
- There is a gap in knowledge on the wastewater factors responsible for variable inherent enzyme activity across a WWTP.

CHAPTER 3

Materials and Methods

This chapter describes all the utilised chemicals and reagents during this work and the details of the methodologies.

3.1 Materials

The EDC substrates estrone (E1) (>99%, CAS 53-16-7) and diclofenac (DCF) (>98%, CAS 15307-79-6) were acquired from Sigma-Aldrich (Poole, UK). Laccase from *Trametes versicolor* (≥ 10Units/mg) (CAS 80498-15-3) and tyrosinase from mushroom (≥ 1000Units/mg) (CAS 9002-10-2) were obtained from Sigma-Aldrich. UK). The analytical substrates, 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) (CAS 30931-67-0) and L-tyrosine (CAS 60-18-4), used for determining laccase and tyrosinase activity were additionally purchased from Sigma-Aldrich (Poole, UK).

The buffer reagents ammonium acetate (CAS 631-61-8), glacial acetic acid (CAS 64-19-17), potassium phosphate monobasic (KH_2PO_4) (CAS 7778-77-0) and potassium phosphate dibasic (K_2HPO_4) (7758-11-14) were purchased from Sigma-Aldrich (Poole, UK). Phosphoric acid (CAS 7664-38-2) and boric acid (CAS 10043-35-3) were purchased from Sigma-Aldrich (Poole, UK) to prepare the Britton-Robinson buffer with acetic acid.

The investigated inhibitors for laccase and tyrosinase activity were copper sulfate ($CuSO_4 \cdot 5H_2O$) (CAS 7758-99-8) and sodium chloride (NaCl) (CAS 7647-14-5) obtained from Fisher Scientific (Loughborough, UK). Hydrochloric acid (HCl) (CAS 7647-01-0) and sodium hydroxide (NaOH) both used to inactivate the exogenous enzymes were also purchased from Fisher Scientific. HPLC grade water (H_2O), methanol (MeOH) (CAS 67-56-1) and acetonitrile (ACN) (CAS 75-05-8) used for HPLC analysis and EDC stock preparation were obtained from Fisher Scientific (Loughborough, UK).

Chemical oxygen demand (COD) cuvette test tubes for characterising wastewater samples were acquired from Hach Lange (Manchester, UK) at two concentration ranges; LCI500 (0 to 150 mg/L O_2) and LCI400 (0 to 1000 mg/L O_2). The higher O_2

concentration range was used for influent samples. Calibration solutions for pH 4, 7 and 10 were purchased from Fischer Scientific (Loughborough, UK) and used to calibrate the pH meter. Collected wastewater samples were initially passed through 1.2 µm glass microfiber filters to determine total suspended solids (TSS). Further filtration of wastewater samples was required before HPLC analysis and passed through a 0.2 µm regenerated cellulose membrane filter. Both filters were obtained from Fisher Scientific (Loughborough, UK).

The substrates used for analysing inherent enzyme activity were all obtained from Sigma-Aldrich (Poole, UK). The purchased substrates were 4-nitrophenyl-α-D-glucopyranoside (CAS 3767-28-0), 4-nitrophenyl-β-D-glucopyranoside (CAS 2492-87-7), 4-nitropheyl-phosphate (CAS 68189-42-4), 4-nitrphenyl-sulfate (CAS 6217-68-1) and fluorescein diacetate (CAS 596-09-08). Other reagents used for the assays include acetone (CAS 67-64-1) and sodium carbonate (Na_2CO_3) (CAS 467-19-8) purchased from Sigma-Aldrich (Poole, UK).

Commercial versions of the inherent enzymes were also purchased from Sigma-Aldrich (Poole, UK). The source of the commercial enzymes if possible was matched to the wastewater environment as closely as possible. Inherent enzymes purchased included α-glucosidase from *Bacillus stearothermophilus* (*B. stearothermophilus)* (CAS 9001-42-7), β-glucosidase from *Almond* (CAS 9001-22-3), alkaline phosphatase from *Escherichia coli* (CAS 9001-78-9), esterase from *B. stearothermophilus* (CAS 9016-18-6) and sulfatase from *Helix pomatia* (CAS 9016-17-5). Milli-Q ultrapure water (Milli-Q, 18.2 MΩ-cm resistivity, Millipore, Bedford, MA, USA) supplied in the laboratory was used to reconstitute powdered enzymes and for preparing buffers.

3.2 Sampling of Wastewater

At the Old Ford WWTP wastewater samples for the influent, screened sewage, mixed liquor, returned activated sludge (RAS) and MBR output were collected in 500 ml plastic bottles as shown in Figure 3.1. Temperature, dissolved oxygen, pH and electrical conductivity were measured by a multi-meter probe (Hach HQ30D, (Manchester, UK).



Figure 3.1 Wastewater samples collected from the Old Ford WWTP. (1) Raw Sewage (2) Screened Sewage (3) Mixed Liquor (4) Returned Activated Sludge (RAS) (5) Post Membrane Bioreactor (MBR).

Effluent from the Stoke Bardolph WWTP was collected from the sample point in Figure 3.2 by using a stainless-steel container. Grab samples of the effluent were collected in 2.5 L clean amber glass Winchesters and transported to the University's laboratory. The temperature of the final effluent, its pH and dissolved oxygen (DO) were measured on site using a DO meter (Jenway 970, Staffordshire, UK) and a thermocouple thermometer (Digi-Sense, Cole-Parmer Instrument Ltd., UK).

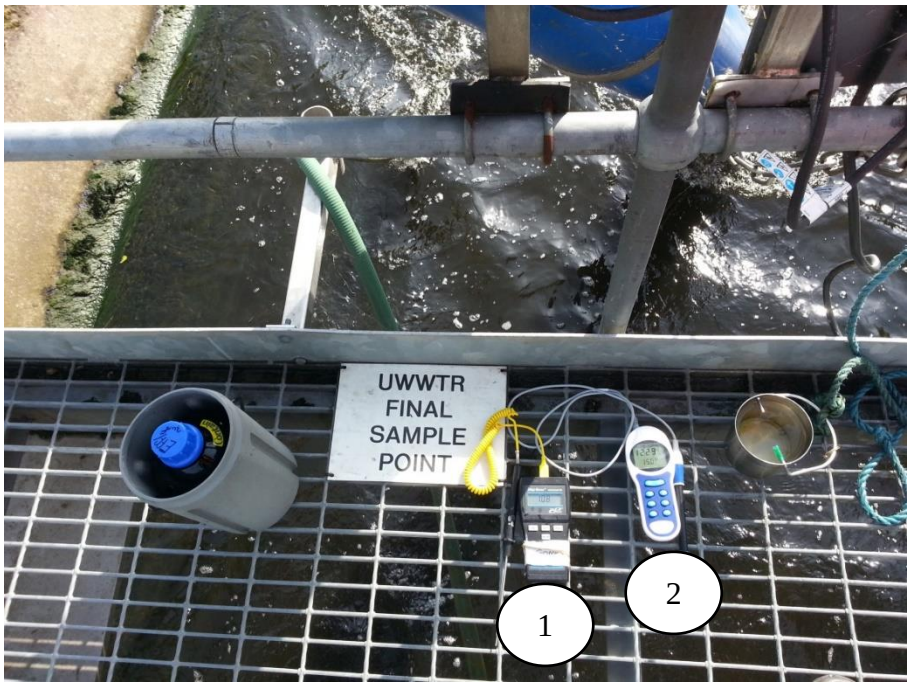


Figure 3.2 Final wastewater effluent sampling point and the used equipment to collect and characterise the sample on site e.g. (1) Thermometer and (2) Dissolved Oxygen Meter.

In the laboratory, part of the sample was used for total suspended solids (TSS) analysis, the remaining part was filtered through 1.2 µm glass microfiber membrane (GMF) (Fisherbrand) and passed for further analysis: chloride content and chemical oxygen demand (COD) analysis.

3.2.1 Total Suspended Solids (TSS)

For total suspended solids (TSS) analysis, half a litre (0.5 L) of well-mixed final effluent sample was measured using a clean volumetric flask. The content of the flask was filtered through 1.2 µm GMF filter paper using a vacuum filter kit. The filter paper retained on its surface all the suspended solids larger than 1.2 µm within the sample. The filter paper was then placed into a preheated oven at 105 ˚C till complete dryness (3 hrs). The dried filter paper was weighed out, giving a value of W_T. The weight of the filter paper before the filtration (W_0) was recorded at the start of the experiment. The concentration of the TSS within the sample was calculated using the following equation:

$$TSS_{(mg/l)} = ((W_T - W_0)/V_{sample})$$

TSS: Total Suspended Solids (mg/l) in the final wastewater effluent
W_0 : the weight of the filter paper before filtration (mg)
W_T: the weight of the filter paper after filtration and oven drying at 105 ˚C (mg)
V_{sample}: The volume of the filtered sample (L)

3.2.2 Chloride Concentration

The concentration of chloride ions in wastewater effluent was determined by titrating it against a standard silver nitrate ($AgNO_3$) solution. A standard silver nitrate solution of 0.0141 M was prepared by dissolving 1.1976 g of $AgNO_3$ in 500 ml of deionised water. A burette (25 ml) was filled with $AgNO_3$ and a conical flask with 50 ml of filtered effluent and 10 drops of potassium chromate indicator was placed under the burette. The flask was placed on a magnetic stirring plate to ensure the homogeneity of the solution during the titration process. The titration process was started by adding $AgNO_3$ solution into the conical flask and the end point of the titration was reached when the mixture changed its colour from yellow into orange. The experiment was performed in triplicate and the used volume of $AgNO_3$ was recorded after each titration.

3.2.3 Chemical Oxygen Demand (COD)

Chemical oxygen demand (COD) is the water quality parameter used to identify the capacity for oxidation of organic pollutants in wastewater. It represents a parameter that is frequently mentioned in various wastewater legislations and acts as a design basis and performance indicator in many WWTPs around the world. Utilising pre-dosed COD test tubes makes this test simple, accurate and environmentally friendly. The COD test was performed by adding 2 ml of the tested matrix e.g. filtered wastewater effluent into the purchased COD test tube (0 -150 mg/l) which was then placed inside a LT200 thermostat (Hach Lange, UK) for 2 hours at 148 ˚C. By the end of the contact time, the sample was allowed to cool down to room temperature. The COD of the cool sample was measured using DR 2800 spectrophotometer (Hach Lange, UK). A control sample with deionised water was performed simultaneously with the wastewater sample and used to represent COD = 0 mg/l and calibrate the used spectrophotometer.

3.3 Enzyme Preparation

Laccase was added to the enzymatic degradation studies in its powder form whereas tyrosinase was added as a solution. Therefore stock solutions of tyrosinase were prepared from the purchased solid powder to a concentration of 1 mg/ml. Laccase and tyrosinase were both stored in a -20 ^{o}C freezer. Activity was regularly checked to monitor any degradation.

3.3.1 Determination of Laccase Activity

The activity of laccase from *Trametes Versicolor* was measured by the oxidation of ABTS. The assay reaction mixture consisted of 5.0 mM ABTS in 0.1 M ammonium acetate buffer, with a suitable amount of laccase solution to give a total volume of 1.0 ml. One unit of enzyme activity (U) was defined as the amount of laccase that catalyses the oxidation of 1 µmol of ABTS per minute at 37 ^{o}C. Oxidation of ABTS was analysed by the increase in absorbance at 420 nm, Shimadzu UV-2600 UV/Vis spectrophotometer, with an extinction coefficient (e) of 3.6 x10^4 M/cm. The specific activity of laccase, the number of laccase activity units per gram, was calculated using Beer Lambert Law.

$$\text{ABTS concentration (M/min)} = \frac{\text{A/min}}{\varepsilon} = \frac{S \times 60}{36000}$$

$$\text{Specific activity of laccase (U/mg)} = \frac{ABTS_{Oxi}}{E}$$

Molar absorptivity (Ɛ) of ABTS is 36000 L/(M/cm)
S is the slope of the line in the "Absorbance vs Time" graph
$ABTS_{Oxi}$ is the amount of ABTS oxidised in the cuvette per minute (µmol/ min)
E is the amount of laccase in the cuvette (mg).

3.3.2 Determination of Tyrosinase Activity

The method used to determine tyrosinase activity was adopted from Sigma-Aldrich and Brar et al (2009). A reaction mixture was prepared that consisted of deionized water, 50 mM potassium phosphate buffer (pH 6.5) and 1 mM L-tyrosine. Added to this reaction mixture was an appropriate amount of tyrosinase solution. The final volume of the reaction mixture was 1 ml in the cuvette. One unit of tyrosinase was defined as the increase in A_{280nm} of 0.001 per minute at pH 6.5/ 25 ^{o}C. The linear part of the reaction was used to determine the absorbance at the rate of 280 nm/min.

3.4 Buffer Preparation

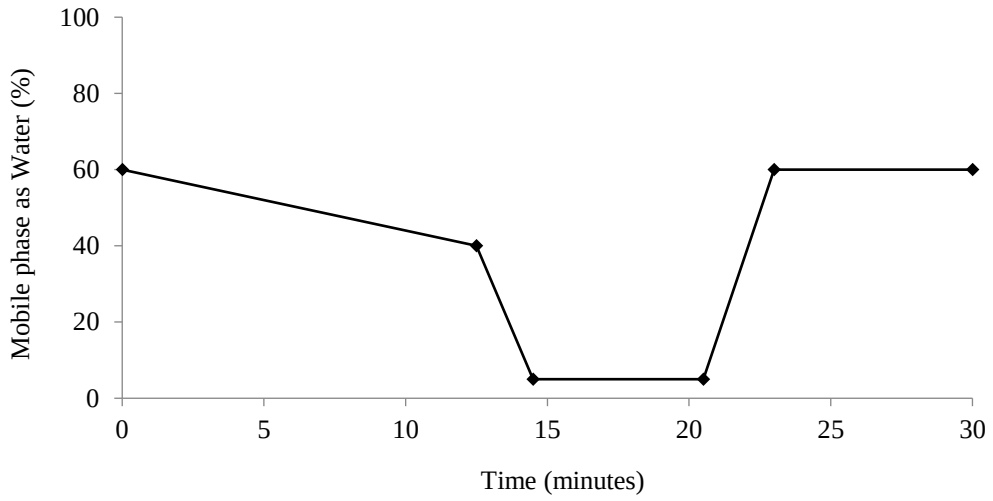
Phosphate buffers were prepared by mixing two salt solutions together; potassium phosphate monobasic (KH_2PO_4) and potassium phosphate dibasic (K_2HPO_4) at the same molar concentration. During the mixing, pH was continuously measured and adjusted by altering the quantities of salt solutions.

Ammonium acetate (AC) buffer pH 4.5 (0.1 M) was prepared using two salt solutions: 0.1 M of acetic acid solution and 0.1 M of AC solution. After preparing both solutions, a beaker with AC solution was placed onto a magnetic stirring plate and the acetic acid solution was used to adjust its pH into pH 4.5. The change in the pH was continuously monitored using a calibrated pH meter. The prepared AC buffer was stored in the fridge at 4 ˚C ready for use.

3.5 High Performance Liquid Chromatography with UV Detection

E1 and DCF concentrations before and after enzymatic treatment were analysed by high performance liquid chromatography (HPLC) (Agilent 1260 HPLC system, Berkshire, UK) coupled to an Agilent multiple wavelength detector (MWD). UV absorbance of E1 and DCF was measured at 200 nm. Detection of E1 in the

degradation studies where E1 was the only substrate was performed using a SunFire C18 column (250 mm x 4.6 mm x 5 µm) (Water, Hertfordshire, UK). Mobile phase flow rate was 1 ml/min and column temperature was 27.5 ºC. A binary mobile phase of water and ACN with a gradient elution was applied. The gradient elution was as follows; 60% water decreased to 40% over 12.5 minutes, then to 5% over 2 minutes, remained constant for 6 minutes, then increased to 60% again over 2.5 minutes and then stayed constant for the next 7 minutes. The total run time was 30 minutes as exhibited in Figure 1. Limit of quantification (LOQ) for E1 on this analytical equipment was 0.01 mg/L.



Combined analysis of E1 and DCF in mixed substrate degradation studies was performed using a SunFire C18 (150 mm x 4.6 mm x 5 µm). Mobile phase flow and column temperature were kept as before. The mobile phase was a binary mixture of water with 0.1% acetic acid v/v and ACN. Table 3.1 details the applied gradient elution.

Table 3.1 *Gradient HPLC elution for E1 and DCF detection.*

Time (minutes)	Water with 0.1% acetic acid v/v (%)	Acetonitrile (%)
0	85	15
4	85	15
5	55	45
9	55	45
10	45	55
18	45	55
21	5	95
27	5	95
28	85	15
35	85	15

3.6 Filter Adsorption of the EDC Substrates

E1 and DCF solutions prepared in deionised water to a concentration of 1 mg/L were passed through a 0.2 µm regenerated cellulose membrane filter. Substrates from their stock solutions (0.5 g/L dissolved in ACN or MeOH) were diluted to the correct concentration using deionised water. Samples for HPLC-UV analysis were taken before and after filtration to calculate the adsorption percentage (%).

$$\text{Adsorption (\%)} = (C_0\text{-}C_F/\ C_0)*100$$

Adsorption (%) is the abiotic removal percentage for the substrate
C_0 is substrate concentration before filtration (mg/l)
C_F is substrate concentration after filtration (mg/l)

Performed in triplicate, the E1 and DCF solutions at 1 mg/L were first drawn up a syringe to 10 ml and then passed through the 0.2 µm regenerated cellulose syringe filters to investigate adsorption. Finally a 1 ml aliquot was taken from the substrate solutions before and after filtration and placed into 2 ml glass HPLC vials ready for HPLC-UV analysis.

3.7 Laccase and Tyrosinase Inactivation

To analyse the performance of HCl and NaOH to inactivate laccase from *Trametes versicolor* and tyrosinase from mushroom the ability to oxidise the analytical substrates ABTS and L-tyrosine was determined under the following conditions: laccase concentration = 5 U/ml, tyrosinase concentration = 100 U/ml, contact time = 15 minutes, temperature = 20 ± 1 ˚C, matrix = 0.1 M phosphate buffer at pH 7. A trial and error approach was utilised to determine the suitable amount of HCl and NaOH to achieve complete inactivation both enzymes.

3.7.1 Stability of Substrates after Enzyme Inactivation

The impact of the enzyme inactivation methods on substrate stability was evaluated by comparing substrate degradation between 1 ml solutions treated with either 10 µl HCl (37%) or 15 µl 5 M NaOH and solutions having had no inactivation treatment. Solutions of E1 and DCF were prepared in deionised water to a concentration of 2.5 mg/L and 1 ml aliquoted into a glass HPLC vial ready for HCl or NaOH addition. Duplicates of substrate solutions, with or without HCl or NaOH, were analysed by HPLC-UV to observe any difference in substrate concentration.

3.8 Exogenous Enzymatic Parameters

The exogenous enzymatic parameters for laccase and tyrosinase were performed in filtered effluent that had been passed through a 1.2 µm glass microfiber filter first. Experimental conditions were as follows; 5 U/ml laccase concentration, 40 U/ml tyrosinase concentration, temperature = 20 ^{o}C, contact time = 1 hour and E1 concentration = 0.5 mg/L. The filtered effluent samples were spiked with an E1 standard solution (prepared in ACN) to give a final E1 concentration of 0.5 mg/L. From an E1 stock solution at 1 mg/ml prepared in ACN 250 µl of this solution was added to 249.75 ml filtered effluent to obtain the final E1 concentration of 0.5 mg/L.

A 10 ml sample was taken from the (E1+filtered effluent) solution, filtered through a 0.2 µm regenerate cellulose membrane filter, and mixed with 25 µl of HCl (37%) per 1 ml sample and placed into a HPLC vial ready for analysis. The reaction was started by either the addition of laccase powder or tyrosinase solution into (E1+filtered wastewater) solution, then the flask with the reaction mixture was placed onto a magnetic stirring plate inside an incubator for 1 hour set to 20 ^{o}C. By the end of the contact time, a 10 ml sample was withdrawn, filtered through a 0.2 µm regenerated cellulose filter membrane and mixed with 25 µl of HCl (37%) per 1 ml sample ready for analysis. All exogenous enzymatic parameter experiments were performed in duplicate and the equation below shows how the E1 removal rate (%) was calculated.

$$R_{Exp}\% = \frac{C_0\text{-}Ct}{C_0} \text{x}100$$

R_{Exp}%: the experimental removal efficiency of E1 as a percentage, C_0 and Ct are E1 concentrations at time=0 and time=t, respectively.

3.8.1 Chloride and Copper Inhibitory Influence and Diclofenac Mixture Effect

Experimental conditions were the same as described for the exogenous enzymatic parameters in section 3.8. As well as testing in filtered effluent, the experiments were also repeated in a 0.1 M phosphate buffer at pH 7.0. Final reaction concentrations of chloride were 100, 200 and 500 mg/L and the tested concentrations of copper were 0.05, 0.1 and 10 mg/L. These experiments were all inactivated with 25 µl HCl (37%) per 1 ml of sample whereas the E1 and DCF experiments were inactivated with 15 µl 5 M NaOH per 1 ml sample. The concentration of E1 and DCF was quantified by HPLC-UV before laccase and tyrosinase addition and after 1 hour contact time. The

percentage of the caused inhibition by the anion or metal ion was determined by calculating the difference between E1 removal efficiency in the control experiment ($R\%_{control}$) (in the absence of the inhibitor) and E1 removal efficiency in the presence of certain inhibitor concentration ($R\%_{I}$). All the HPLC-UV experiments were performed in duplicate.

3.9 Inherent Hydrolytic Enzyme Analysis

Table 3.2 outlines the assay methodology for analysing the activity of the selected inherent hydrolases.

Table 3.2 Assay methodology for the inherent hydrolytic enzymes.

Enzyme	Analytical Substrate	Incubation	Wavelength (nm)
α-Glucosidase	4-Nitrophenyl-α-D-glucopyranoside	30 minutes at 30°C	400
β-Glucosidase	4-Nitrophenyl-α-D-glucopyranoside	30 minutes at 30°C	400
Alkaline phosphatase	4-Nitrophenyl-phosphate	15 minutes at 30°C	400
Sulfatase	4-Nitrophenyl-sulfate	15 minutes at 30°C	400
Esterase	Fluoresceine diacetate	1 hour at 20°C	490

Activated sludge samples were first subjected to 5 minutes of sonication at 35 kHz. Sonicated samples of mixed liquor and returned activated sludge (RAS) were diluted by a dilution factor of 1:5 for analysing α-glucosidase, β-glucosidase and sulfatase activity. A dilution factor of 1:10 was used for analysing alkaline phosphatase and esterase activity. Samples were diluted with 0.14 M NaCl. The influent, screened sewage and MBR output samples required no dilution. Then 1 ml of analytical substrate solution was added to 10 ml wastewater samples (40 µl for esterase). The substrate solutions were prepared by dissolving 10 mg of substrate in 10 ml 0.14 M NaCl for all enzymes apart from esterase where 20 mg of substrate was dissolved in 10 ml acetone. Samples were then incubated in a water bath for their required times and the reactions stopped by the addition of 1 ml Na_2CO_3 solution (esterase not inactivated). After inactivation, samples were centrifuged at 3000 g for 10 minutes, before measuring absorbance on a UV/Vis spectrophotometer.

3.9.1 Pearson's Correlation of Hydrolytic Activity

The inherent enzyme activities were correlated to each other and to the standard water quality measurements using the software programme Minitab. To confirm the data was normally distributed the Ryan-Joiner test was first used before performing Pearson's correlation.

3.9.2 Commercial Hydrolase Activities

Purchased commercial hydrolases were reconstituted with deionised water to a concentration of 1 mg/ml. Wastewater samples were first filtered before the commercial hydrolases were added to the matrix by a 1.2 µm glass microfiber filter. The methodology described in Section 9.3 for preparing substrate solutions was adopted for the commercial hydrolases. Activity was additionally measured in deionised water and 50 mM phosphate buffer at pH 7.0. Control assays of the wastewater samples were performed first to determine any inherent activity, whereby the substrate was added with no commercial hydrolase. Activity in the control was a benchmark to calculate the net activity of the commercial hydrolases. Different temperatures were tested, ranging from 17 to 30 ^{o}C, reflecting optimal and wastewater conditions.

CHAPTER 4: Results & Discussion

Scope, Criteria and Selection of Substrates, Enzymes and Methods

4.1 Introduction

This section provides the justification and criteria used selecting the bioactive chemicals, as well as enzymes to be investigated for their removal in a complex wastewater environment. The development of an analytical method for the quantification of bioactive chemical removal is discussed as well as early control experiments. Gaps in knowledge were identified from the literature so that the performed research improved the understanding for both exogenous and inherent enzymes.

4.2 Highlights

- Justification for the selection of EDC substrates, exogenous and inherent enzymes to investigate enzyme performance in a wastewater environment.
- Development of a methodology to assess the degradation of bioactive chemicals estrone and diclofenac using the exogenous enzymes laccase and tyrosinase.

4.3 Substrate Selection Criteria

The main focus of this work was on endocrine disrupting compounds (EDCs). EDC removal from the wastewater matrix is a high priority for the water industry and governing bodies. The presence of EDCs within the aquatic environment are an ecological risk as concentrations even as low as a few ng/L can interfere with the endocrine system, which has resulted in male fish feminisation (Ahmed et al., 2017). Consequently, the adverse impact of EDCs highlights the need to tackle their elimination from the environment. As discussed in the literature review, the main source of EDCs entering the environment is from WWTPs unable to remove these compounds. EDCs pass through WWTPs unaltered and enter the surrounding water bodies through the discharged effluent (de Mes et al., 2005). New technological adaptions to the treatment process need to be made, such as tertiary treatment with exogenous enzymes.

The criteria used for selecting the EDC substrates had to satisfy three points. Firstly, they had to be prevalent in the effluent released from WWTPs resulting in high environmental occurrence. Secondly the EDCs of choice also had to represent a potential legislative and/or regulatory issue for the water industry otherwise the research had no future application. Finally, the literature had to show examples of EDCs being degraded by enzymatic treatment. Estrone (E1) was the main EDC selected to investigate laccase and tyrosinase performance, with diclofenac (DCF) chosen later to study mixture effects.

E1 was selected as it is a strong EDC that exhibits high estrogenicity known to contribute to male fish feminisation. From a legislative viewpoint E1 and DCF are EDCs currently on the EU Watch List, therefore their occurrence and removal is of interest to various stakeholders. Other known EDC compounds on the Watch List include 17α-ethinylestradiol (EE2) and 17β-estradiol (E2). E1 was selected to represent this group of estrogens as the first breakdown product of E2 is E1. As a result, the concentration of E1 is higher within the wastewater system than E2 (Manickum and John, 2014).

EE2 is a synthetic estrogen used in birth control pills. Even though EE2 is far more potent than E1, the estrogenic contribution of EE2 in wastewater is limited. Figure 4.1 shows the contribution of different groups of the Dutch population to estrogen levels in wastewater. Only 1% originates from oral contraceptives (EE2) where the rest is naturally excreted estrogens (de Mes et al., 2005). DCF, being the other EDC on the EU Watch List, was additionally investigated for studying EDC mixture influences. Table 2.2 within the literature review highlighted the poor removal of DCF from conventional WWTPs and need for alternative treatment technologies (Deblonde et al., 2011).

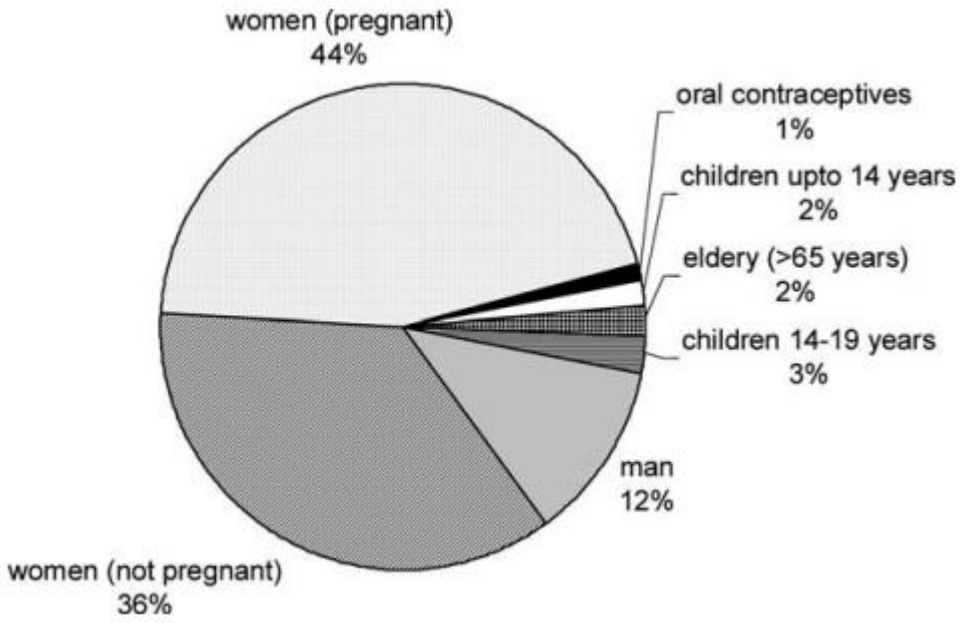
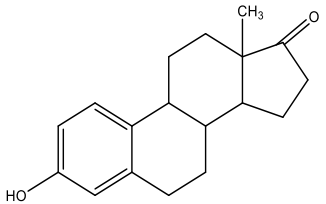
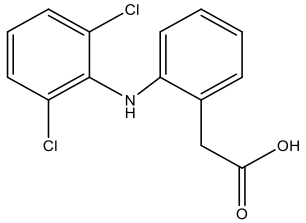


Figure 4.1 Contribution of different groups of the Dutch population to the amount of total excreted natural estrogens and 17α-ethinylestradiol; based of the total population figures of 2001 (de Mes et al., 2005).

4.4 Physicochemical Properties

Table 4.1 details the main physicochemical properties for the selected EDC substrates that inform on their fate in a WWTP (Das et al., 2017). Furthermore the described physicochemical properties aid with the development of the experimental work. The solubility of the substrates indicates the feasible concentration range that can be utilised. The bioactive chemicals need be added to clean water or wastewater matrices without undergoing precipitation. The hydrophobicity of a compound informs on its ability to be either biodegraded or adsorbed by organic solids, mainly bacterial flocs.

Table 4.10 Physicochemical properties of estrone (E1) and diclofenac (DCF).

Substrate	Structure	Acidic/ Basic	pK_a	Log K_{ow}[a]	Log K_{oc}[b]	Henry's Law Constant	Solubility
Estrone (E1) *Hormone*		Weakly Acidic	10.4	3.13	3.02	$3.80x10^{-10}$	Water: 1.3mg/L
Diclofenac (DCF) *Anti-inflammatory*		Weakly Acidic	4.0	4.51	2.61	$4.73x10^{-12}$	Water: 2.4mg/L

[a] Octanol-Water Partition Coefficient (K_{ow}) – relates to water solubility, soil adsorption coefficients and biococentration factors for aquatic life.

[b] Soil Adsorption Coefficient (K_{oc}) – indicates the chemical's ability to be adsorbed by the organic content of ground soil.

[c] Henry's Law Constant – is the measure of a chemical in air over its concentration in waster (informs on chemical volatility)

The low K_{ow} values for the EDC substrates show that biodegradation is their main removal pathway (Gardner et al., 2013). This explains the poor removal of the substrates by WWTPs as they are minimally adsorbed to organic solids and not biodegraded. Tertiary treatment by exogenous enzymes is critical to the future degradation of EDCs and preventing their occurrence in the environment. The wastewater matrix experiments with added E1 and DCF will still contain a minor fraction of organic solids after filtering. Once laccase and tyrosinase are added to the wastewater matrix the observed substrate removal (%) can be confirmed to be solely enzymatic as organic solid adsorption is minimal.

The soil adsorption coefficient (K_{oc}) based on K_{ow} and water solubility informs on the chemical's ability to be adsorbed by the organic content of ground soil and activated sludge. The two substrates, E1 and DCF, exhibit low K_{oc} coefficients indicating minimal sludge adsorption and high mobility through soil. DCF in landfill sites from unused medication being thrown away in bins is another source of environmental entry as its able to leach through the soil (Bound and Voulvoulis, 2005). The volatility of a chemical is characterised by the Henry's law constant and a high volatilization tendency is signified by a value between 10^{-2} and 10^{-3}. Volatilization for micropollutants is generally negligible and is the case for the substrates in Table 4.1 (Luo et al., 2014). Regarding the experimental work, any removal from the reactions with the substrates and exogenous enzymes can be assumed to not occur through volatilization.

4.5 Selected Exogenous Enzymes

The degradation of bioactive chemicals can occur either through hydrolysis or by oxidation (Krah et al., 2016). Table 2.1 in the literature review showed enzymes capable of degrading organic pollutants (Eibes et al., 2015). Oxidases and peroxidases showed the widest substrate ranges that include phenolic and aromatic compounds. Estrogens, strong EDCs, are phenolic compounds and so susceptible to degradation by these enzymes. The selection of exogenous enzymes was narrowed down to oxidases and peroxidases to investigate E1 and DCF removal, due to their potential EDC removal.

As shown in Table 2.1 peroxidases require hydrogen peroxide to function whereas oxidases only need oxygen. Hydrogen peroxide is not a common constituent of

wastewater, unlike oxygen, therefore oxidases are far more favourable in WWTP application, as hydrogen peroxide would have to be otherwise additionally purchased. Consequently, oxidases were the selected exogenous enzymes to explore EDC removal. The oxidases in Table 2.1 were laccase and tyrosinase. Tables 2.4 and 2.6 in the Chapter 2 summarised previous studies performed for these oxidases. Laccase was shown to be an already established treatment for removing numerous EDCs.

From Table 2.4 the literature had shown that E1 and DCF had both been able to be degraded by laccase, justifying its selection further. Mediators had also been used with laccase in some studies to increase the redox potential and in turn improve EDC removal. However, this is not feasible when scaled up and therefore not adopted when investigating E1 and DCF removal (Nguyen et al., 2014). Electron donating groups (EDGs) are vulnerable to oxidative attack whereas the electron withdraw groups (EWGs) are more resistant. The hydroxyl group in E1 that forms the phenolic compound is an EDG and so susceptible to enzymatic attack (Yang et al., 2013). For the DCF the molecular structure contains a carboxylic acid, which is a strong EWG, however the aromatic amine is classed as an EDG. The conflict between the electron groups reduces the redox potential of the compound, resulting in lower removal for DCF compared to E1 (Lloret et al., 2013).

Tyrosinase from the literature review had not been shown to degrade E1 but the removal of bisphenol A (BPA) had been reported by Xu and Yang (2013). BPA, similar to E1, is a phenolic compound therefore the hydroxyl group is most likely attacked by tyrosinase. Furthermore DCF had not been shown to be degraded by tyrosinase but the literature had highlighted the removal of anilines (Wada et al., 1995) (Muñoz-Muñoz et al., 2011). Aniline removal was improved by the presence of phenols in the tyrosinase reactions performed by Wada et al. (1995) leading to E1 and DCF mixtures being studied.

Additional reasons for the selection of the desired enzymes include their wide commercial availability. One of the most used phenol oxidases is laccase, which has been widely applied to the paper and pulp industry to degrade lignin (Demarche et al., 2012). The ability of laccase to degrade lignin led to laccase being developed as an alternative treatment method for removing EDCs. This was due to lignin and many of the EDCs sharing similarities in their molecular structure (Li et al., 2015). Tyrosinase

has been investigated in the promising use within the food, pharmaceutical and environmental industries (Zaidi et al., 2014). Laccase and tyrosinase were chosen as they are ideally suited to EDC removal and shown to be incorporated into industrial processes.

4.6 Inherent Enzymes

Enzymes belonging to the wastewater system as discussed in section 2.6 of the literature review have been previously analysed to feedback on overall treatment efficiency. Inherent enzymes were investigated alongside exogenous enzymes to provide a complete understanding on enzyme response to wastewater complexity. Laccase and tyrosinase were exogenous enzymes, therefore not present in the wastewater system but externally added to enhance EDC removal. Inherent activity has mostly been studied from activated sludge samples, where the activity of several inherent enzymes has been shown to vary between WWTPs and in the same WWTP over time (Fischer et al., 2013). Data for inherent activity throughout a large scale WWTP is limited.

From the literature review the two types of enzymes mostly studied for inherent activity have been hydrolases and oxidoreductases. The initial paper on hydrolytic activity was by Goel et al. (1998) and researchers have since adopted the methods presented within this publication. Oxidoreductases in the wastewater system have recently been highlighted by Krah et al. (2016) to degrade a mix of bioactive chemical metabolites. The paper by Grekova-Vasileva and Topalova (2009) is one of the few studies that has investigated inherent oxidoreductase activity. In total, five different oxidoreductases were assayed in the treatment of textile wastewater containing high amounts of azo compounds from the used dyes. Hydrolases, on the other hand, have been studied in far more detail but gaps in knowledge still exist, specifically identifying and determining enzyme variability throughout a large scale WWTP.

The hydrolases most commonly studied are excreted outside the cells requiring minimal sample preparation as the cells do not need to be lysed. The oxidoreductases analysed by Grekova-Vasileva and Topalova (2009) required 90% of the activated sludge cells to be first disrupted before analysing activity. Measuring hydrolases has shown to be reproducible and efficient. Furthermore hydrolases are involved in a wider range of degradation reactions as they are often the rate limiting step for the

removal of proteins, carbohydrates and lipids (Goel et al., 1998). Organic pollutants are first broken down by hydrolases to facilitate their microbial assimilation (Gómez-Silván et al., 2013). The already established methods for assaying hydrolytic activity and coverage of a broad range of enzymatic reactions resulted in hydrolases to be investigated instead of oxidoreductases. From the literature, common hydrolases were chosen to enable data comparison. Only Fischer et al. (2013) has shown temporal and spatial variability but the variation was not analysed by characterising the properties of the sampled wastewater.

The studied inherent hydrolases were α-glucosidase, β-glucosidase, alkaline phosphatase, esterase and sulfatase as they had shown to be highly prevalent in the wastewater environment. To add to the literature, all five hydrolases were analysed across a WWTP and correlations made with measured standard water quality parameters. This was the first example of such a study being performed that investigated activity across a WWTP with a membrane bioreactor (MBR) and standard water quality correlations. Kreutz et al. (2016) during this time performed a similar study but only focused on activated sludge samples that did not incorporate a MBR system.

4.7 Control Experiments

The literature showed different methods for inactivating laccase and tyrosinase at the end of a set contact time. For example, the addition of methanol to inactive laccase was carried out in high ratios to the sample (1:1 volume), resulting in the matrix being highly diluted. Heat denaturation performed by Cobas et al. (2015) took 20 minutes to complete, therefore extending the reaction period further. The rapid inactivation offered by HCl and the minimal sample dilution were advantageous over the other inactivation methods. Therefore, it was decided to test the inactivation of laccase and tyrosinase by using HCl.

The activities of laccase and tyrosinase were assessed in the presence and in the absence of the inactivation agent using the standard activity assays described in the materials and methods chapter. Laccase had previously shown to be inactivated by 25 µl of 37% HCl added to 1 ml volume of buffer solution containing the enzyme at a concentration of 5 U/ml (Youla J & Gomes L. R, submitted). Tyrosinase inactivation was unknown but, once evaluated, was shown to require a significantly lower molar

concentration of HCl. As exhibited in Figure 4.2, tyrosinase was inactivated by 10 µl of 1 M HCl added to 1 ml of buffer solution where the enzyme concentration was 100 U/ml. Laccase favours acidic conditions (optimum activity = 4.5), and can retain activity until pH 3 (Lloret et al., 2013), whereas tyrosinase has an optimum activity pH of 6.5 explaining the difference in HCl.

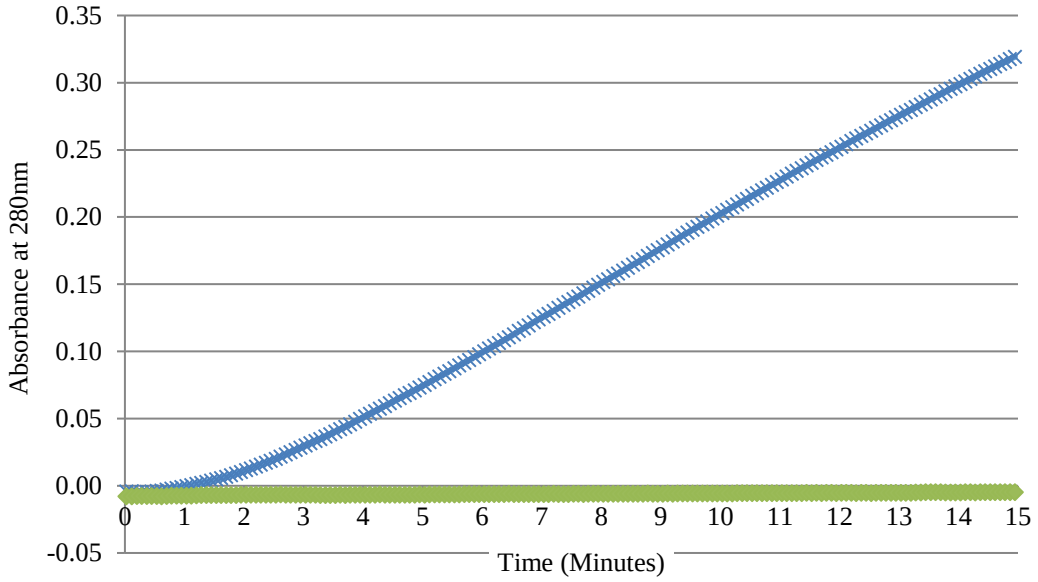


Figure 4.2 Enzymatic activity assay for tyrosinase (100 U/ml), before (×) and after (◆) inactivation with 10 µl of 1 M HCl to 1 ml of sample. Activity was assayed by the oxidation of L-tyrosine, as described in the methods.

The stabilities of E1 and DCF were examined after the addition of 10 µl of 37% HCl to a 1 ml buffer solution with the substrates prepared to a concentration of 2.5 mg/L. This work was performed to confirm that the HCl inactivation method did not also contribute to substrate degradation. DCF concentration was significantly reduced by almost 51% with the addition of 10 µl of 37% HCl compared to the control as shown in Table 4.2.

Table 4.2 Addition of 10 µl HCl (37%) to 1 ml samples of E1 and DCF each at a concentration of 2.5 mg/L. Stability of the substrates examined after undergoing laccase inactivation (n=2).

Substrate	Substrate Concentration (mg/L)		Difference (%)
	Control (No HCl Added)	+ 10 µl HCl (37%)	
E1	2.61	2.51	2.3
DCF	2.51	1.24	50.9

Therefore an alternative method was required for the degradation experiments investigating the mixed removal of E1 and DCF. From the literature review, other investigated inactivation methods included heat denaturation, addition of NaOH and glacial acetic acid. When these other inactivation methods were tested, heat denaturation was limited to five minutes so that the enzymatic reaction was not extended considerably past the set contact time. Laccase activity was still present for heat denaturation and also when 60 µl glacial acetic acid was added to a 1 ml volume of enzyme solution therefore neither were pursued any further. As shown in Figure 4.3, the addition of 15 µl 5 M NaOH to a 1 ml volume of buffer enzyme solution (5 U/ml) successfully inactivated laccase. The tested substrate stability in Table 4.3 showed NaOH to not impact on measured E1 and DCF concentration.

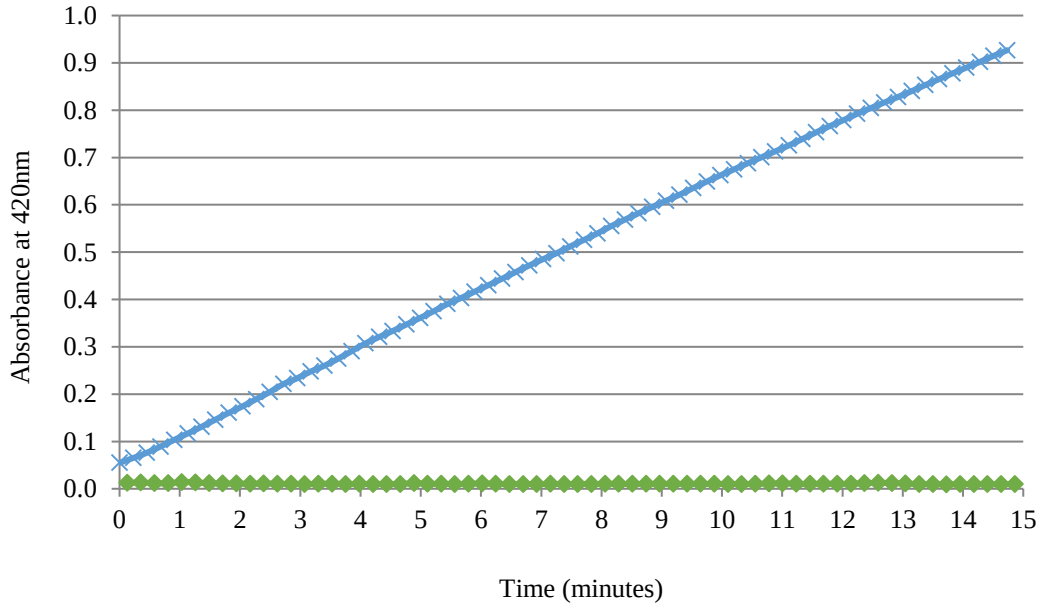


Figure 4.3 Enzymatic activity assay for laccase (5 U/ml), before (×) and after (◆) inactivation with 15 µl of 5 M NaOH to 1 ml of sample. Activity was assayed by the oxidation of ABTS, as described in the methods.

Table 4.3 Addition of 15 µl of 5 M NaOH to 1ml samples of E1 and DCF each at a concentration of 2.5 mg/L. Stability of the substrates examined after undergoing laccase inactivation (n=2).

Substrate	Substrate Concentration (mg/L)		Difference (%)
	Control (No NaOH Added)	+ 15 µl 5 M NaOH	
E1	2.59	2.59	0.3
DCF	2.52	2.50	0.9

Wastewater matrix samples need to be filtered prior HPLC-UV analysis to remove particulates that could block the column or internal tubing. The filters chosen were 0.2 μm regenerated cellulose membranes. They had demonstrated minimal E1 adsorption from previous work undertaken within the research group (Youla J & Gomes L. R, submitted) at 0.5 mg/L. The chosen substrate concentration was 1 mg/L for analysing E1 and DCF adsorption both prepared in deionised water. Table 4.4 displays the adsorption results obtained with adsorption not exceeding 5% for both substrates.

Table 4.4 Adsorption of the substrates E1 and DCF onto the filters used prior to HPLC analysis (0.2 μm regenerated cellulose membranes) at a concentration of 1 mg/L (n=3).

Substrate	Measured concentration of non-filtered sample (mg/L)	Measured concentration of filtered sample (mg/L)	Adsorption (%)
E1	1.13 ± 0.02	1.07 ± 0.01	5.0
DCF	1.39 ± 0.02	1.36 ± 0.04	2.2

4.7.1 HPLC Substrate Detection Method

Table 4.5 gives a summary on the various HPLC-UV methods used in the literature to separate and detect EDCs. All used reverse phase HPLC and the majority utilised a C18 column. Chapter 2 highlighted the basics of reverse phase HPLC. A HPLC method already adopted within the research group to analyse E1 was modified to detect DCF as well (Youla J & Gomes L. R, submitted). Lloret et al. (2010) ran two different methods when analysing a mixture of non-steroidal anti-inflammatory drugs and estrogens. Another bioactive chemical was analysed when developing the HPLC method for E1 and DCF. The inclusion of sulfamethoxazole (SMX) provided a HPLC method that could be run to detect a wide range of compounds from different EDC classes.

Table 4.5 HPLC-UV methods from the literature used to separate and detect EDC substrates.

Substrate/s	Column	Mobile Phase	Elution	Filtration	References
Acetaminophen, Caffeine, Carbamazepine, Ketoprofen, Naproxen, DCF, Ibuprofen	RP-C_{18} (125 x 4mm, 5µm)	Solvent A: MeOH Solvent B: ACN Solvent C: 50mM KH_2PO_4	Gradient Flow → 1ml/min	0.45µm glass fibre membrane (analysed wastewater matrix)	(Santos et al., 2005)
E1 E2 EE2	RP-C_{18} (250 x 4.6mm, 5µm)	15mM phosphate buffer: ACN (30:70%)	Isocratic Flow → 1ml/min	—	(Tanaka et al., 2009)
DCF	RP-C_8 (250 x 4.6mm, 5µm)	ACN/H_2O (50:50 v/v) with 0.1% TFA	Isocratic Flow → 1ml/min	0.45µm nylon filter (analysed wastewater matrix)	(Alahmad and Alawi, 2010)
E1, E2, EE2, DCF, NPX	RP-C_{18} (250 x 4.6mm, 5µm)	Estrogens ACN:H_2O (50:50 and 90:10) NSAIDs 50mM phosphate buffer: ACN (55:45%)	Estrogens Gradient NSAIDs Isocratic Flow → 0.8ml/min	—	(Lloret et al., 2010)
E1 E2	RP-C_{18} (150 x 4.6mm, 5µm)	50mM phosphate buffer: ACN (60:40%)	Isocratic Flow → 1ml/min	—	(Lloret et al., 2012)
E1	RP-C_{18} (150 x 4.6mm, 5µm)	ACN/H_2O (45:55 v/v)	Isocratic Flow → 0.8ml/min	0.22µm PES membrane (analysed wastewater matrix)	(Fonseca, 2013)

DCF NPX	RP-C_{18} (250 x 4mm, 5µm)	50mM phosphate buffer: ACN (55:45%)	Isocratic Flow → 0.8ml/min	—	(Lloret et al., 2013)
BPA, DCF, MFA, TCN	RP-C_{18} (300 x 3.9mm, 15-20µm)	Solvent A: H_2O with 0.1% acetic acid (pH 3.3) Solvent B: MeOH	Gradient MeOH increased from 40 to 65% (v/v) Flow → 1ml/min	—	(Margot et al., 2013)
BPA EE2	RP-C_{18} (150 x 4.6mm, 2.7µm)	Solvent A: H_2O Solvent B: MeOH/ACN (10/90 v/v)	Gradient Flow → 1.4ml/min	—	(Maryšková et al., 2016)
DCF, DHQ, β-NP, 2,4-DCP	RP-C_{18} (150 x 4.6mm, 5µm)	Solvent A: ACN Solvent B: 10mM phosphate buffer	Gradient Flow → 1ml/min	—	(Rodríguez-Delgado et al., 2016)

The gradient of elution was first altered to improve the separation between the three EDCs. Next the C18 column was changed to a shorter version to improve resolution. From the reviewing the literature is was noticed that a modifier such as acetic acid had been added to the HPLC solvents on some occasions (Margot et al., 2013). The addition of 0.1% acetic acid to the mobile phase reduces substrate dissociation. Additionally, papers that had analysed EDC concentration in a real wastewater matrix had filtered samples prior to HPLC injection. This removed any particulates large enough to create a blockage within the HPLC system. The control experiments on filter adsorption were important to carry out to show that EDC elimination in a wastewater matrix was by the result of the enzymes and not by abiotic removal.

Figure 4.4 details the result of the first HPLC method used and as can be seen from the chromatogram only E1 was well resolved. DCF and SMX appeared to co-elute and both had overlapping retention times. The standard concentration of 0.5 μg/ml utilised for the three substrates was too low for the initial HPLC developmental work. As result of this the concentration was increased to 100 μg/ml so that an easily visible response could be observed. To separate DCF and SMX from one another the starting ratio of water to ACN was lowered from 60:40 to 85:15. This resulted in visible substrate separation as shown in Figure 4.5. Nevertheless, SMX was still not being as sharply resolved as the other substrates. In method 3 Figure 4.6 a shorter column (250 mm to 150 mm) was used to improve the SMX resolution. A shorter column leads to the substrates being eluted at a faster rate because of the lower retention times. Even though the peak for SMX did begin to sharpen peak splitting still occurred as shown in Figure 4.6. The optimised method for detecting all three substrates included the addition of a modifier to the H_2O mobile phase. Adding 0.1% acetic acid directed SMX to its acidic form preventing substrate dissociation to occur as shown by the tighter SMX peak in Figure 4.7. The H_2O with 0.1% acetic acid / ACN gradient profile was also altered so that the peaks appeared on a flat section of the baseline and not on a slope which represents the substrate changing mobile phase conditions.

Time (minutes)	H_2O (%)	Acetonitrile (%)
0	60	40
12.5	40	60
22.5	5	95
28.5	5	95
31	60	40
38	60	40
38	60	40

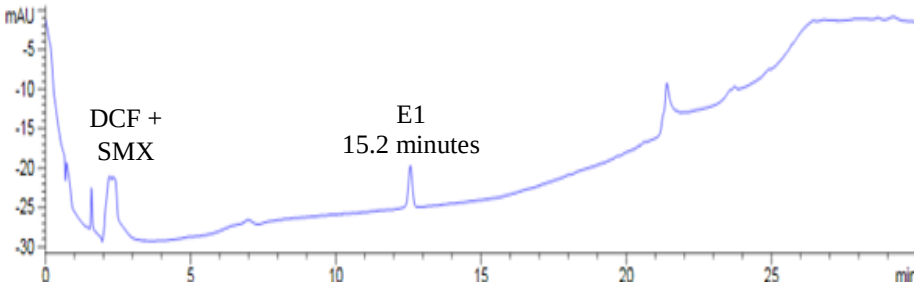


Figure 4.4 HPLC Method 1 - mixed substrate standard; estrone (E1), diclofenac (DCF) and sulfamethoxazole (SMX) at 0.5µg/ml; wavelength = 200nm.

Time (minutes)	H_2O (%)	Acetonitrile (%)
0	85	15
6	85	15
8	40	60
14	40	60
16	5	95
22	5	95
23	85	15
30	85	15

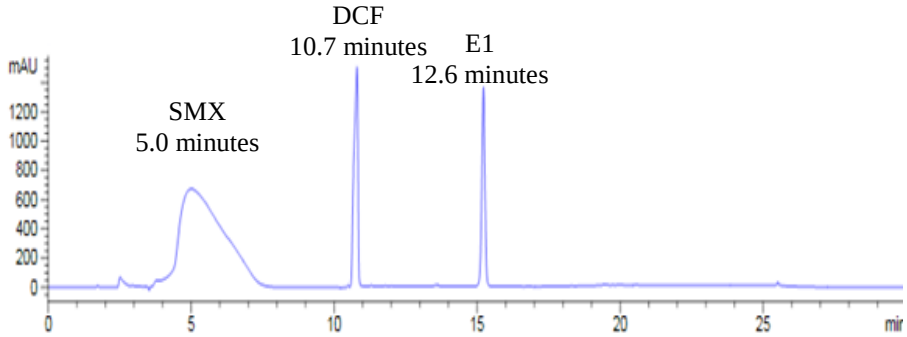


Figure 4.5 HPLC Method 2 - mixed substrate standard; estrone (E1), diclofenac (DCF) at 100µg/ml + sulfamethoxazole (SMX) at 500µg/ml, wavelength = 200nm.

Time (minutes)	H_2O (%)	Acetonitrile (%)
0	90	10
6	90	10
8	50	50
14	50	50
16	5	95
22	5	95
23	90	10
30	90	10

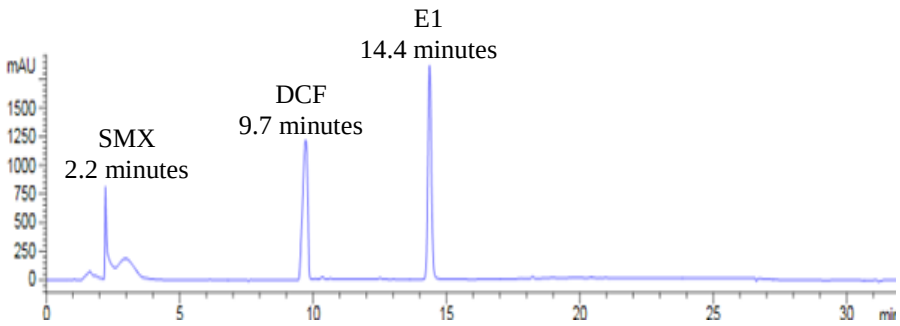


Figure 4.6 HPLC Method 3 - mixed substrate standard; estrone (E1), diclofenac (DCF) and sulfamethoxazole (SMX) all at 100µg/ml, wavelength = 200nm.

Time (minutes)	H_2O with 0.1% Acetic Acid v/v (%)	Acetonitrile (%)
0	85	15
4	85	15
5	55	45
9	55	45
10	45	55
18	45	55
21	5	95
27	5	95
28	85	15
35	85	15

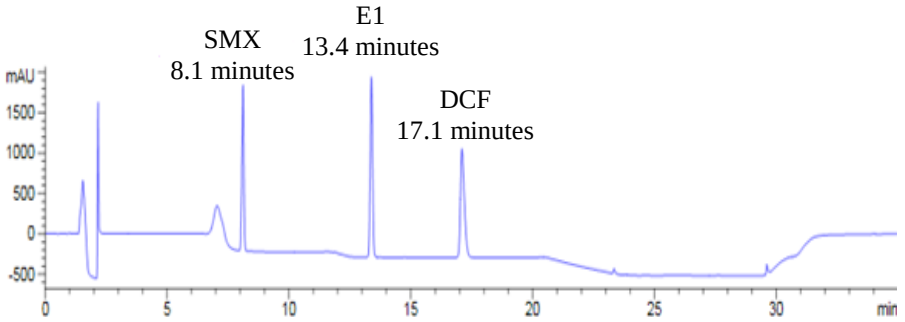


Figure 4.7 HPLC Method 4 - mixed substrate standard; estrone (E1), diclofenac (DCF) and sulfamethoxazole (SMX) all at 100µg/ml, wavelength = 200nm.

4.8 Conclusions

- Environmental regulations in particular the EU Watch List and the harmful nature of EDCs led to the selection of E1 and DCF as substrates. The prominence of E1 in the wastewater environment from human excretion and E2 breakdown resulted in its selection over the other natural and synthetic estrogens.
- Chosen exogenous enzymes were laccase and tyrosinase both shown to have promiscuous substrate specificity and so capable of removing a wide range of EDCs. Industrial application made the two enzymes readily available and applicable as an alternative treatment technologies.
- From the literature it was identified there was a gap in knowledge on the performance of laccase and tyrosinase in a wastewater matrix. Furthermore, the influence the matrix has on EDC removal at conditions reflective of a WWTP environment is not discussed in detail.
- The developed inactivation methods for laccase and tyrosinase were the addition of HCl or NaOH. Compared to the other techniques in the literature these methods inactivated the exogenous enzymes rapidly and did not greatly dilute the matrix distorting substrate concentration.

CHAPTER 5: Results & Discussion

Development and Application of Enzymatic Water Quality Parameters to Characterise Wastewater Variability Relating to Bioactive Chemical Removal

5.1 Introduction

From the literature review in Chapter 2, the complexity associated with wastewater was highlighted. Wastewater can be influenced by a wide range of factors that leads to matrix variability. A range of different standard parameters can be used to characterise wastewater and demonstrate this complexity. From Chapter 4, two exogenous enzymes and endocrine disrupting compounds (EDCs) were selected to investigate the influence the varying wastewater matrix has on enzyme behaviour. Development of this exogenous enzymatic parameter was novel and advanced the understanding for incorporating this technology into a WWTP. The exogenous enzymatic parameters analysed estrone (E1) removal from effluent samples taken from the Stoke Bardolph WWTP. Though standard water quality indicators such as chemical oxygen (COD) exist, there are none that relate the biological state to EDC removal. Directly measuring E1 removal through an enzymatic parameter tracks the influence of treatment conditions and matrix composition on the performance of the enzyme to remove EDCs. Such a water quality enzymatic parameter is therefore highly fundamental for 1) understanding the variability of the system 2) relating to how a current or developing process responds and removes the pollutant to that window process variability.

From the literature review in Chapter 2, it was highlighted that similarly classified enzymes may respond independently of each other, for example, in the case of hydrolases originating from wastewater (Gómez-Silván et al., 2013). As a result, in Chapter 4 the studied oxidoreductases, laccase and tyrosinase, were analysed to see if their responses were independent of each other. The enzymatic parameters were additionally adapted to compare E1 removal in a clean buffer matrix, and sampled effluent, with the common inhibitors Cl^- and Cu^{2+}. Mixture effect was additionally investigated by adding diclofenac (DCF) as an additive. The majority of studies investigating inhibitors for laccase and tyrosinase activity utilise the analytical

substrates rather than EDC substrates. Furthermore, experimental conditions and concentrations rarely reflect the wastewater environment. These various issues were addressed in this research to provide a detailed investigation on wastewater inhibitors and the environmental impact on exogenous enzyme performance. The applicability of using a clean buffer matrix model to predict enzyme performance in wastewater was also discussed.

5.2 Highlights

- Exogenous enzymatic parameters established to dynamically analyse estrone (E1) removal from wastewater to understand the impact wastewater complexity has on exogenous enzyme performance.
- For the first time the removal of E1 by the exogenous enzyme tyrosinase is reported in both a clean buffer matrix and sampled effluent from an operational WWTP.
- The exogenous enzymatic parameters informed on the biological state of the sampled wastewater to remove an EDC specifically E1 over time to demonstrate temporal variability.
- The wastewater characteristics, measured by the standard water quality parameters, were correlated to the exogenous enzymatic parameters to identify significant environmental and process factor relationships.
- The impact of known inhibitors Cl^- and Cu^{2+} on laccase and tyrosinase activity were analysed through the enzymatic parameters as well investigating EDC substrate mixtures. Previous knowledge had focused on analytical substrates not relevant to EDC removal.

5.3 Exogenous Enzyme Performance in Wastewater

In the literature there are conflicting reports on the impact wastewater has on exogenous enzyme performance but the majority do agree that performance is inhibited when compared to a clean water model. An early paper on this area of research by Auriol et al. (2007b) evaluated the ability of the exogenous enzyme horseradish peroxidase (HRP) to remove E1, E2, E3 and EE2 in synthetic and real wastewater matrices. The synthetic wastewater used was a 0.1 M sodium phosphate buffer and the wastewater was effluent collected from a local WWTP. It was noted

that, for removing the same concentration of estrogens in both of the matrices, a far greater amount of HRP was required for the wastewater matrix. In the synthetic wastewater 0.032 U/ml of HRP removed all four estrogens whereas 8 to 10 U/ml of HRP was required for the wastewater matrix. The need to use H_2O_2 with HRP increases costs and has the potential to inactivate the enzyme (Aktas et al., 2001) (Auriol et al., 2007b).

Auriol et al. (2007a) performed a similar investigation but the exogenous enzyme used was laccase instead of HRP. The removal of E1, E2, E3 and EE2 by laccase in synthetic and real wastewater matrices was evaluated in a bioreactor maintained at 25 ^{o}C. Unlike HRP, the effluent wastewater matrix did not strongly inhibit laccase performance as estrogenic removal was similar between the two matrices showing varied responses.

Following on from these two papers Auriol et al. (2008) then compared the performance of laccase and HRP to remove the four estrogens in the same study. Tested matrices were again synthetic water and wastewater. The same result was obtained for HRP as the activity was increased from 0.032 U/ml to 10 U/ml in wastewater to achieve estrogenic activity removal exceeding 80%. Only a slight difference between estrogen activity removal percentages for laccase in the two matrices was reported. At the same experimental conditions and laccase activity of 20 U/ml estrogenic activity removal was 100% in the synthetic water and 97% in effluent wastewater. It was concluded that HRP was far more influenced by the wastewater constituents than laccase (Auriol et al., 2008). Important to note that temporal variability was not considered in these papers and the literature has since shown wastewater to negatively impact on laccase.

For example Garcia et al. (2011) investigated the oxidation of oxybenzone in municipal wastewater (primary effluent) by laccase. Different mediators such as ABTS and ACE were added to improve removal. The wastewater matrix, products formed from the oxidation of the mediator by laccase and pH were the three main factors identified to impact on enzyme activity. The pH of a solution is known to influence enzyme activity as shown by the pH profiles in Figure 5.1.

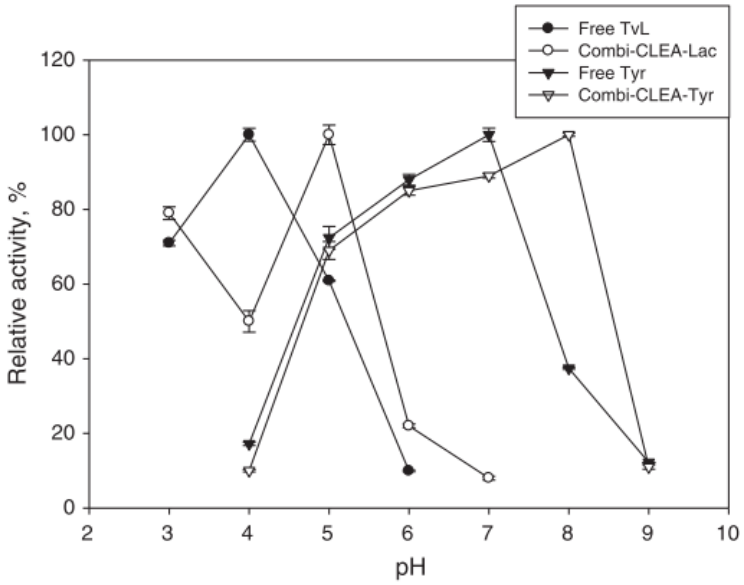


Figure 5.1 Activity of free and insoluble laccase (TvL) as well as free and insoluble tyrosinase (Tyr) at different pH values. Temperature of the assays kept constant at 20 ^{o}C (Ba et al., 2014).

Greater enzyme activity was also recorded in ultrapure water compared to the wastewater matrix (Garcia et al., 2011). Lloret et al. (2013) evaluated the performance of an enzymatic membrane reactor (EMR) to continuously remove E1, E2 and EE2. The EMR at a laccase concentration of 100 U/ml was initially fed with a phosphate buffer solution before investigating the impact of real wastewater (filtered secondary effluent). Laccase removal efficiency was reduced in the wastewater matrix compared to the buffer solution. The reason given for this observed loss was the presence of other compounds in wastewater that are more easily oxidised and slight biocatalyst inactivation. This work was also cited by Taboada-Puig et al. (2015) that showed versatile peroxidase (VP) to be negatively affected by real wastewater. The degradation rates for E1, E2, EE2, BPA and triclosan (TCS) were all reduced in wastewater compared to distilled water.

The removal of BPA by a laccase EMR was also carried out by Arca-Ramos et al. (2015). Similar to Lloret et al. (2013) synthetic water and real wastewater (filtered secondary effluent) were fed into a ceramic EMR. For the two matrices both the residual BPA and laccase activity (%) were compared over an operational time of 50 hours. At 10 hours BPA removal was 99.5% in the phosphate buffer (pH 6) and in wastewater (pH 7.2) removal was 94.6%. Laccase performance was lower in the wastewater matrix but the difference in laccase activity was far more pronounced

between the two matrices. When the buffer experiment was stopped at 50 hours the activity had remained above 850 U/L from the starting concentration of 1000 U/L. Additional laccase had to be injected into the wastewater experiment at 32 hours to maintain activity at 1000 U/L as the initial activity had decreased by almost 50% detailed in Figure 5.2.

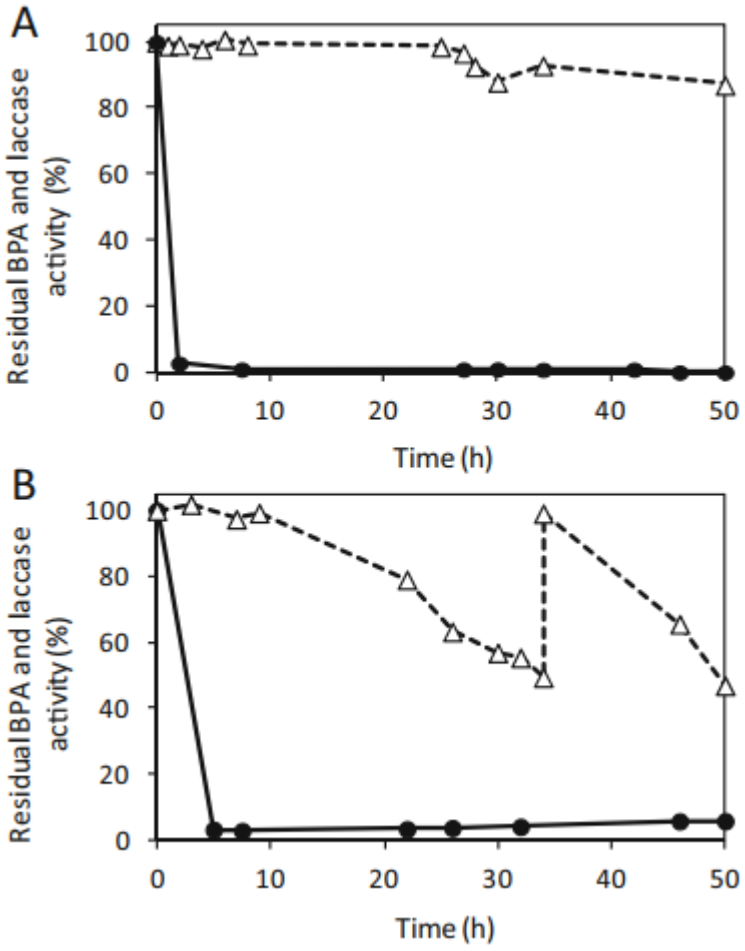


Figure 5.2 Removal of bisphenol A (BPA) (●) and loss of residual laccase activity (▵) in a continuously operated enzyme membrane reactor (EMR) with a hydraulic retention time of 10 hours (Arca-Ramos et al., 2015). (A) 0.1 M phosphate buffer at pH 6 and (B) real treated wastewater.

The literature shows that laccase is inhibited by a wastewater matrix either through its performance in degrading EDC substrates or activity. In addition to this the inherent wastewater conditions and composition never remain constant leading to temporal variability. Minimal work has been carried out in the literature investigating laccase performance and less so for tyrosinase in wastewater over an extended time period spanning days and months. Furthermore, understanding the cause of this variability

through characterising the wastewater by its standard water quality parameters will highlight the responsible factors.

5.4 Standard Water Quality Parameters

To demonstrate the inherent complexity of wastewater a range of parameters were used to characterise the effluent taken from the Stoke Bardolph WWTP. Common parameters for characterising wastewater include analysing the concentration for biological oxygen demand (BOD), chemical oxygen demand (COD), dissolved oxygen (DO), total suspended solids (TSS), ammonia, temperature, metals and pH (APHA, 2012). A selection of these parameters also form part of the discharge consents required to be met by WWTPs for example BOD, TSS and ammonia (COM, 2016). The released effluent has to meet the requirements of local, national and European legislations specifically the Wastewater Treatment Directive (UWWTD). The quality of the effluent is therefore already monitored to alert for discharge consent violations. Water quality parameters can also be utilised to indicate the temporal variability of wastewater and the impact this has on both laccase and tyrosinase EDC based treatments.

The effluent collected from the Stoke Bardolph WWTP between June 2016 and November 2016 was characterised by several different parameters to understand wastewater variability and the influence on enzyme behaviour. The following water quality parameters were measured for each effluent sample taken; temperature, DO, pH, COD, TSS, total dissolved solids (TDS), electrical conductivity (EC) and chloride (Cl). Later on within this chapter the water quality parameters are correlated to E1 removal (%) to establish the impact on laccase and tyrosinase performance through EDC removal. Table 5.1 shows the recorded values for the measured standard water quality parameters.

Table 5.1 Measured standard water quality parameters for effluent samples taken from the Stoke Bardolph WWTP between June 2016 and November 2016.

Sample Period	Temp (ºC)	DO (mg/L)	pH	COD (mg/L)	TSS (mg/L)	TDS (g/L)	EC (mS/cm)	Cl (mg/L)
June - July 2016	16.6 (±1.2)	4.2 (±1.0)	7.2 (±0.1)	17.2 (±5.1)	6.9 (±2.5)	0.4 (±0.1)	0.9 (±0.1)	104.1 (±18.5)
August - September 2016	16.7 (±1.6)	3.4 (±0.4)	7.1 (±0.2)	17.6 (±3.9)	5.8 (±1.0)	0.5 (±0.1)	0.9 (±0.1)	122.8 (±21.9)
October - November 2016	14.6 (±1.3)	2.5 (±1.4)	6.9 (±0.1)	20.9 (±4.3)	7.0 (±2.9)	0.5 (±0.0)	1.0 (±0.1)	135.6 (±16.9)

The water quality parameters shown in Table 5.1 are not necessarily interlinked with each other as daily variability and constituents within the wastewater may result in different relationships forming. As a result, one parameter cannot be used to predict the value of another parameter. However, increases in TDS concentrations are typically reflected by increased EC values, as inorganic salts measured by TDS release ions that increase electrical conductivity of wastewater. From Table 5.1 the highest concentrations of TDS and EC are both observed between October and November 2016. Furthermore, Cl concentration was the highest during the same sampling period. The presence of additional Cl^- ions will contribute to EC from dissolved salts like sodium chloride. Between these parameters there is a potential relationship.

Table 5.1 additionally highlights the temporal variability of the parameters and the deviations observed during the sampling periods. Temperature remained consistent between June and September 2016 before falling by almost 2 ºC in the months transitioning from summer to autumn. There is a seasonal change in temperature for the autumn sampling, which is expected due to the colder temperatures experienced. The decrease in temperature coincided with a decrease in DO concentration as well between October 2016 and November 2016. Other factors apart from temperature are known to influence DO concentration such as salinity, organic matter presence and the turbulence of the sampled matrix (Eddy, 2003) (Manahan, 2011). As a result, it is difficult to determine if the temperature decrease is significant to DO concentration. Variability in DO also arises from oxygen consumption by the biodegradation of carbonaceous materials and microorganism respiration (Eddy, 2003).

COD and TSS concentration increased slightly as the temperature dropped and this might have resulted from lower microbial activity in the activated sludge, and so less efficient in degrading the organic contaminants. TDS, EC and pH exhibited little

deviation between the different sampling periods and were all relatively consistent. The pH of the sampled effluent from the Stoke Bardolph WWTP was stable and remained close to neutral. Incoming wastewater into Stoke Bardolph is mainly municipal therefore pH extremities observed with industrial wastewater are not typically observed. Wastewater that is either highly acidic or basic is often treated beforehand to neutralise the pH as the microorganisms within the activated sludge function best between 6 and 9 (Eddy, 2003). Consequently, pH is a tightly controlled factor for process optimisation and minimising any negative impacts on the environment. Permitted pH range for effluent to be released into surrounding water bodies is between pH 6.5 and 8.5 (Eddy, 2003), demonstrated by the pH values recorded from the Stoke Bardolph WWTP.

The discussed water qualities, detailing the physicochemical properties of the sampled wastewater and later correlation to E1 removal, can identify the interactions that influence enzyme performance, However, Table 5.1 first highlights the complexity of wastewater and associated temporal variability. The introduction of laccase and tyrosinase as EDC treatment technologies needs to consider this variability as it will impact on enzymatic EDC removal. Constituents such as Cl^- ions in the wastewater matrix can also inhibit enzymatic activity shown within the literature for laccase. Two mechanisms are believed to be responsible for laccase inhibition by Cl^- and other ions including hydroxide ions (OH^-) and halide anions (F^- and Br^-). Binding of these ions to one of the internal laccase coppers can prevent electron transfer. The binding near another copper results in substrates being unable to enter the active site (Margot et al., 2015). Consequently, increased Cl concentrations would be expected to reduce laccase activity and in turn reduce E1 removal.

5.5 Enzymatic Parameter and Wastewater Variability

The previous section highlighted the complexity of wastewater and the temporal variability for the matrix by the measured standard water quality parameters. Water quality parameters alone do not inform on the biological state of the matrix and effectiveness to remove EDCs. The development of exogenous enzyme parameters informed on the capability of the sampled wastewater to remove EDCs and also identified factors that influence enzymatic EDC removal. The exogenous enzymes laccase and tyrosinase, not originating from the wastewater system, were chosen to

investigate EDC removal, specifically E1. Tables 2.4 and 2.6 in Chapter 2 detail their literature scope on EDC removal and application within wastewater. A few papers have touched upon the impact wastewater has on laccase behaviour (Auriol et al., 2007a) (Auriol et al., 2008) (Garcia et al., 2011) (Lloret et al., 2013) (Arca-Ramos et al., 2016) (Becker et al., 2017) with considerably less available for tyrosinase (Pigatto et al., 2013) (Martínková and Chmátal, 2016). The inherent complexity of wastewater from both its variable composition and environmental influences impacts on how enzymes perform regarding pollutant degradation. This requires evaluation in wastewater and with consideration that wastewater changes temporally and spatially.

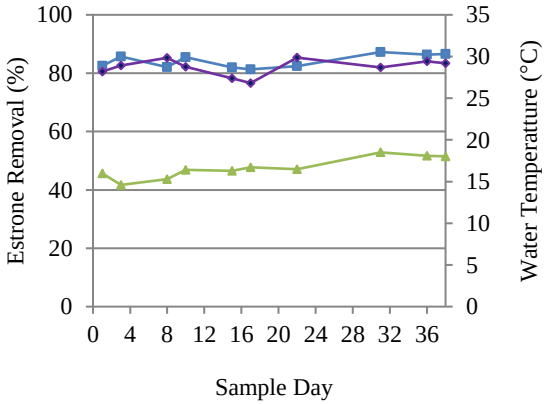
The development of the exogenous enzyme parameters fulfilled this missing evaluation on temporal variability. Wastewater can alter depending on rainfall, seasonal temperature changes and influx of industrial waste to provide examples. Exogenous enzymes investigated were laccase and tyrosinase to study the temporal removal of E1. Laccase, an already established EDC treatment option was compared against tyrosinase another promising oxidoreductase for EDC removal but less well studied. The selected EDC substrate was E1 as it contributes heavily to overall estrogen levels in wastewater and is harmful to aquatic wildlife. The standard water quality parameters for each wastewater sample collected from the Stoke Bardolph WWTP were measured. Correlation of the standard water quality parameters with E1 removal by the exogenous enzymes identified factors responsible for variable enzymatic performance.

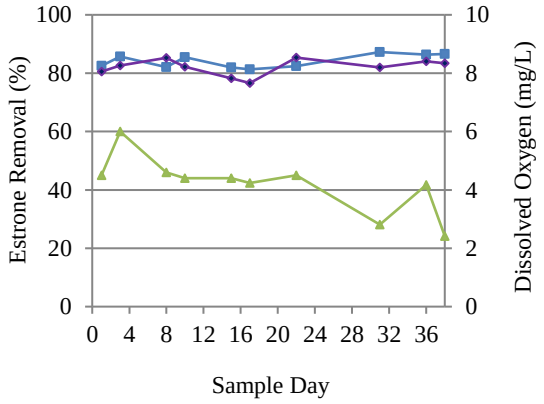
In addition to studying wastewater influences on enzymatic behaviour constituents within the wastewater matrix can also impact on enzyme activity. Tables 2.4 and 2.6 in Chapter 2 highlighted inhibitors that reduce enzyme activity. A selection of these inhibitors can be found in the wastewater environment such as common salts and heavy metals. Their impact on laccase and tyrosinase activity in removing an EDC substrate from real sampled wastewater at relevant wastewater concentrations and conditions is unknown. Therefore this was another area for the exogenous enzymatic parameters to fulfil by studying the influence of two common wastewater inhibitor pollutants copper (Cu^{2+}) and Cl^- ions. The exogenous enzymatic parameters were all carried out with sampled effluent from the Stoke Bardolph WWTP, because the application of laccase and tyrosinase has mostly been propositioned as a tertiary treatment step.

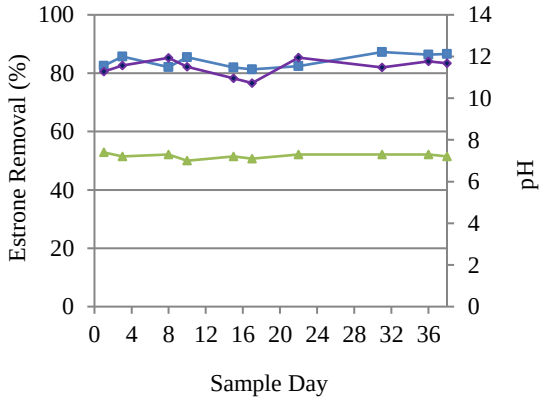
The laccase parameter was the first exogenous enzymatic parameter developed to investigate E1 removal in effluent samples from the Stoke Bardolph WWTP. Table 2.7 in Chapter 2 highlights two papers where laccase and tyrosinase were combined to remove chemical pollutants including acetaminophen an EDC (Krastanov, 2000) (Ba et al., 2014). The combination of tyrosinase with laccase contributed to the inclusion of tyrosinase as an additional enzymatic parameter. The literature before this had also shown tyrosinase to be a promising option for EDC removal and optimal conditions reflected a WWTP (Ba et al., 2014). The exogenous enzymatic parameter was performed separately for laccase and tyrosinase to determine any differences in response to the wastewater matrix. A gap in knowledge was prevalent for understanding exogenous enzyme response to wastewater conditions and the wastewater matrix through EDC removal.

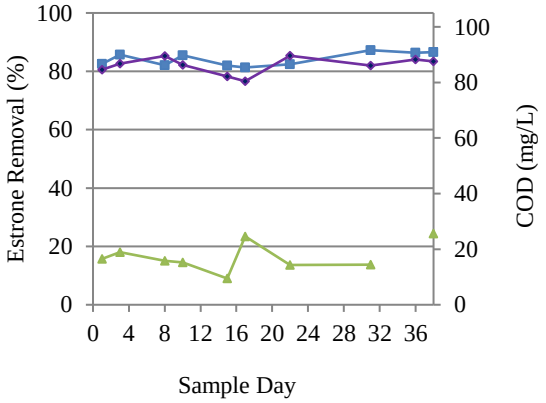
5.6 Initial Evaluation of the Laccase Enzymatic Parameter

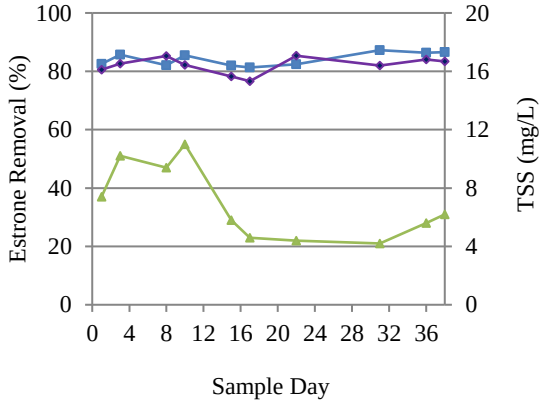
As mentioned previously, the laccase enzymatic parameter was developed before tyrosinase. An initial laccase enzymatic parameter was performed for the sampled effluent from the Stoke Bardolph WWTP between June and July 2016. The experimental conditions for the laccase enzymatic parameter described within the materials and methods were kept constant, but the effluent matrix varied for each sampling day. Figure 5.3 shows the results of this early work with E1 removal (%) compared against the measured water quality standards over this sampling period of two months.

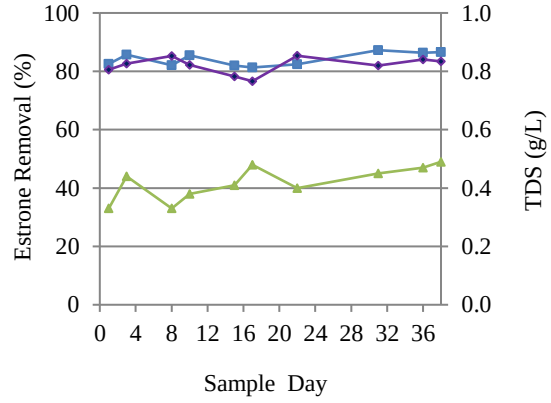

Estrone Removal (%)
Water Temperatture (°C)
Sample Day


Estrone Removal (%)
Dissolved Oxygen (mg/L)
Sample Day


Estrone Removal (%)
pH
Sample Day


Estrone Removal (%)
COD (mg/L)
Sample Day


Estrone Removal (%)
TSS (mg/L)
Sample Day


Estrone Removal (%)
TDS (g/L)
Sample Day

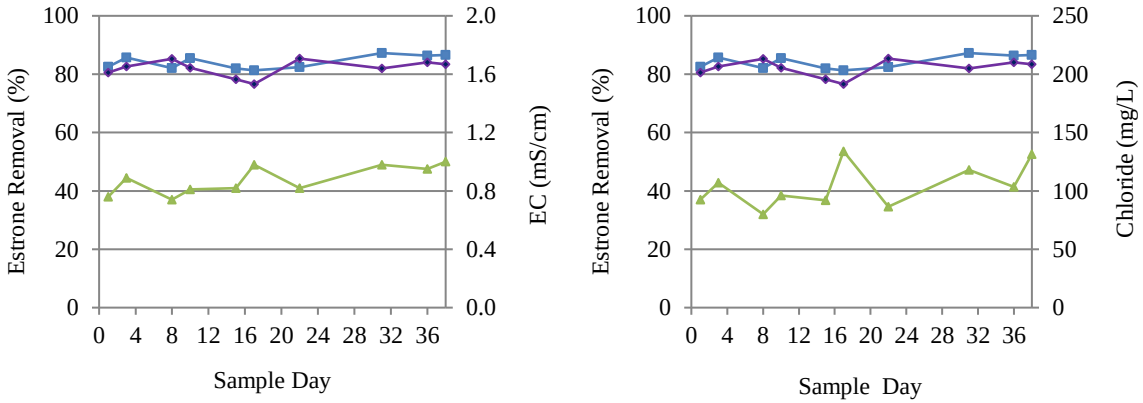


Figure 5.3 Laccase (5 U/ml) benchmark results for the removal of added estrone (E1 at 0.5 mg/L) into effluent samples collected from the Stoke Bardolph WWTP between June and July 2016. E1 removal (%) plotted with individual measured standard water quality parameters. Benchmarks performed in duplicates (n=2). Laccase benchmark R1 (■); laccase benchmark R2 (◆); and wastewater quality parameter (▲).

The results in Figure 5.3 show that variation in the wastewater characteristics directly impact on the performance of laccase to remove E1. Instead of only investigating a single sample snapshot common within the literature temporal variability was considered for the first time in analysing EDC removal over time. E1 removal (%) ranged between 79.0% and 85.2%, presented in Table 9.3 of appendix B, over this sampling period demonstrating variability. Experimental conditions of the enzymatic parameter were selected to achieve appropriate removal of E1 that ranged between 70% and 90%. The average E1 removal (%) was 83.1% (± 2.1%). From Figure 5.3 no clear correlations can be made between variability in E1 removal and the measured water quality parameters. A longer sampling period is required to understand any seasonal changes on the water quality parameters and, in turn, enzymatic removal of E1.

The application of laccase needs to be investigated in real environmental samples rather than clean water samples. Already from Figure 5.3 variability in wastewater to affect enzyme performance has been observed but not previously acknowledged before. Industrial application would require high E1 removal rates, therefore the chosen experimental conditions reflected this requirement as previously detailed in the paragraph before. Section 5.2 within this chapter discussed papers in the literature that investigated laccase performance in wastewater but with temporal variability not factored in. For example Lloret et al. (2013) mentioned they sampled effluent from a WWTP but are ambiguous to how many samples were taken and any subsequent impact on laccase performance. The correlation between E1 removal (%) and the measured standard water qualities in Figure 5.3 cannot be reliably determined visually from the graphs. Instead, a statistical approach is needed. A statistical approach utilising Pearson's correlation was later applied to accurately identify any significant relationships.

Pearson's correlation is a common approach utilised to evaluate liner correlation between two variables (Sun et al., 2014). The two variables in this work are E1 removal (%) and the standard water quality parameters. Pearson's correlation was utilised to calculate the strength of correlation between these two variables. The distribution of the data informs on which correlation method should be used. If the data is not normally distributed the statistic correlations that can be used are either Spearman's *rho* or Kendal's tau-b. However, normally distributed data satisfies the

application of the Pearson's correlation. The generated Pearson coefficients range between -1 and +1. A coefficient value of +1 indicates perfect positive correlation whereas -1 represents perfect negative correlation. For a positive correlation both variables increase or decrease together whereas a negative correlation indicates opposing trends.

5.7 Laccase and Tyrosinase Enzymatic Parameters

A tyrosinase enzymatic parameter was later adopted to also inform on E1 removal in wastewater. As well as laccase, the enzyme tyrosinase has also begun to be proposed as a remediation technology and its performance in wastewater relating to EDC removal is unknown. Comparing against laccase provided a benchmark for EDC removal. As both laccase and tyrosinase are copper containing oxidoreductases it was hypothesised that the enzymes would respond similarly to temporal changes in the wastewater matrix. During the development of the tyrosinase enzymatic parameter described in the materials and methods section, tyrosinase behaviour was shown to be variable between the tested matrices. This is reported in Figure 5.4.

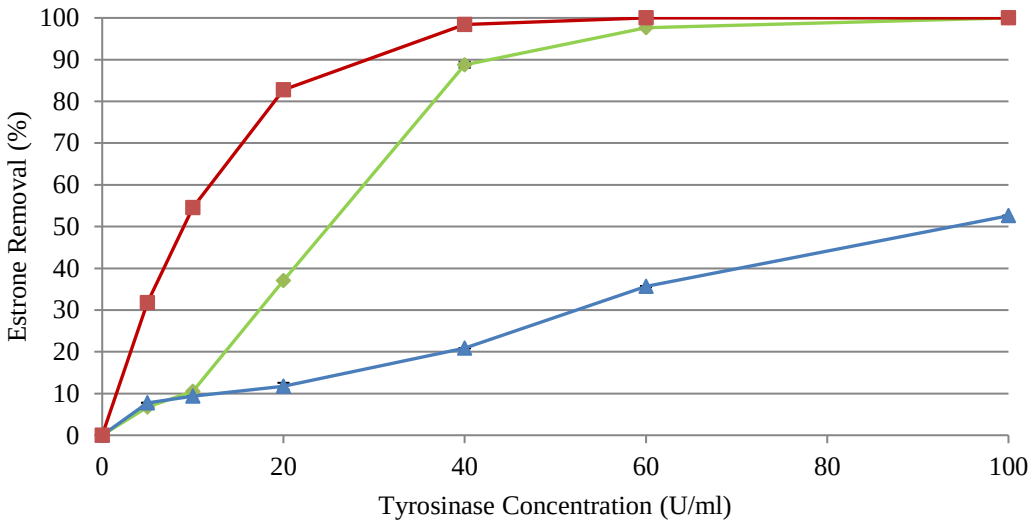


Figure 5.4 Tyrosinase (5 to 100 U/ml) benchmark scoping experiments for the removal of added estrone (E1 at 0.5 mg/L) in clean and wastewater matrices. Influent and effluent samples taken from the Stoke Bardolph WWTP. Experiments at 40 U/ml performed in triplicate for the influent and effluent. Triplicates performed for the buffer at 20 U/ml. All other experiments n=1. Influent (■); effluent (◆); and Britton-Robinson buffer at (pH 7.0) (▲).

Influent, effluent and a clean buffer matrix were selected in the scoping study to compare the impact matrix composition had on tyrosinase behaviour. From Figure 5.4

the chosen tyrosinase concentration for applying to the enzymatic parameter in effluent was 40 U/ml as this produced E1 removal (%) between 70% and 90%. Sampled effluent is the main focus as noted before as this is where the enzymatic treatment technologies will be applied to remove EDCs. At a 40 U/ml tyrosinase concentration there is a noticeable difference in E1 removal between the influent, effluent and clean buffer matrices. The buffer matrix represents a minimal composition containing no present inhibitors or mediators. Effluent compared to the influent has been treated and as result contains a lower concentration of organic contaminants that may inhibit or enhance tyrosinase activity. E1 removal in the influent was close to 100% at 40 U/ml whereas for the effluent it was shown to be near 90%. The buffer matrix showed considerably less removal as E1 removal was only 20% indicating reduced performance in a minimal matrix composition. Tyrosinase activity for the first time was shown to be enhanced in wastewater potentially the result of present mediators.

A previous paper by Ba et al. (2014) had indicated that the removal of the EDC acetaminophen by the combined treatment of laccase and tyrosinase was greater in ultrapure water compared to municipal wastewater. Furthermore, the enzymes had been crosslinked together. The transformation rate of acetaminophen in ultrapure water was 99% and 80% in sampled municipal wastewater. However, the starting acetaminophen concentration in the municipal wastewater was double of that in the ultrapure water. The authors additionally highlighted similar transformation of acetaminophen by free laccase in hospital wastewater measured to be at pH 7.7. Tyrosinase activity is favoured more by neutral pH levels whereas acidic pH levels favour laccase activity. The acetaminophen transformation rate of 98.4% was not expected for laccase due to the neutral pH. A possible explanation for this enhanced laccase response in wastewater was the presence of natural mediators in the wastewater matrix such as colloids, natural organic matter and humic substances promoting laccase activity (Cañas et al., 2007) (Feng et al., 2013). This could additionally help in understanding the observed increase in tyrosinase activity in wastewater for Figure 5.4.

5.7.1 Temporal Behaviour of the Exogenous Enzymatic Parameters

The behaviour of laccase and tyrosinase in wastewater cannot be concluded from a single wastewater sample as the matrix is never constant as shown by the temporal variability of the standard water quality qualities within Figure 5.3. This resulted in variable enzyme performance and in turn variable E1 removal. The laccase and tyrosinase enzymatic parameters were developed and performed to highlight this variability as well as determine the possible factors responsible for this. With both enzymes being copper containing oxidoreductases it was believed their responses would be similar. Even though E1 removal cannot be directly compared between the two enzymes the temporal removal trends can be correlated to examine if increases and decreases mirror each other. Figure 5.5 exhibits the results obtained for the performed laccase and tyrosinase enzymatic parameters during the sampling period between August 2016 and December 2016.

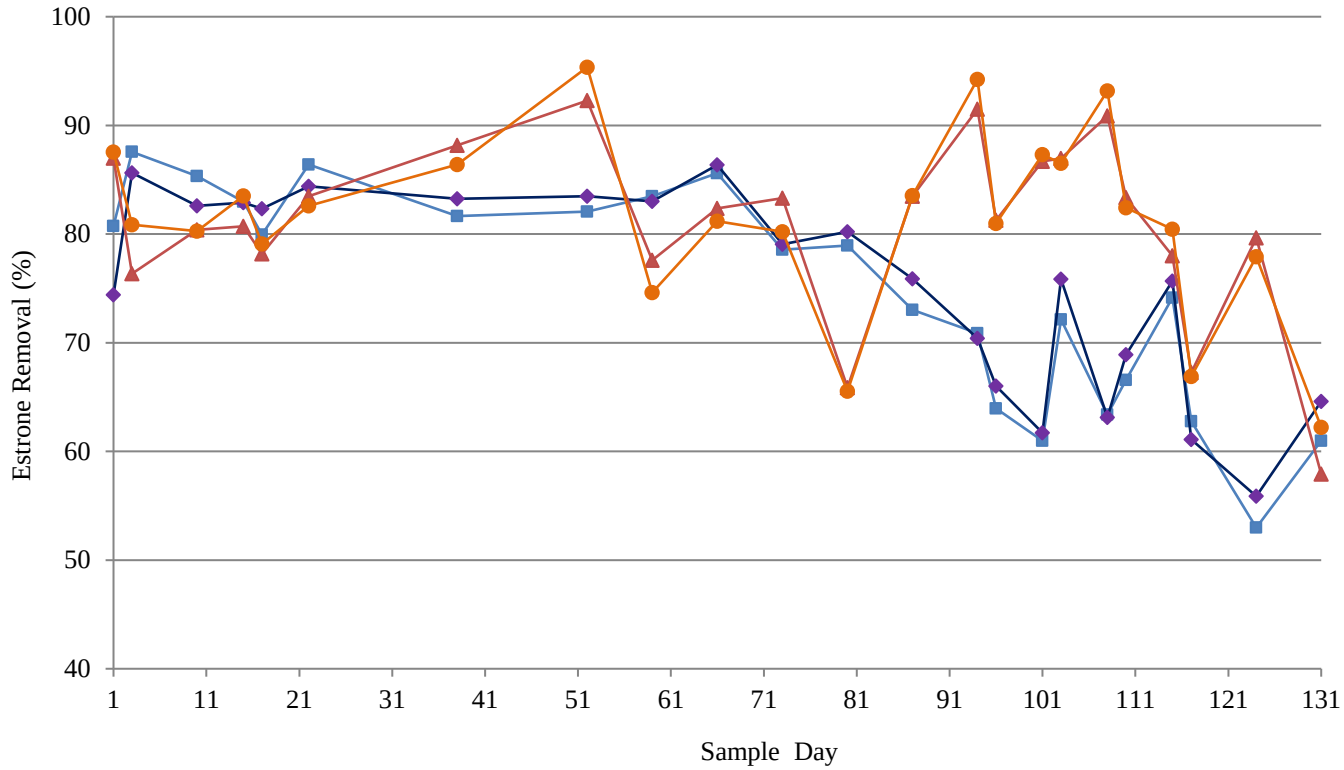


Figure 5.5 Laccase (5 U/ml) and tyrosinase (40 U/ml) benchmark results for the removal (%) of added estrone (E1 at 0.5 mg/L) in effluent samples taken from the Stoke Bardolph WWTP between August and December 2016. Laccase and tyrosinase benchmarks performed in duplicates (n=2). Laccase benchmark R1 (■); laccase benchmark R2 (◆); tyrosinase benchmark R1 (▲); and tyrosinase benchmark R2 (●).

Figure 5.5 is the first example of laccase and tyrosinase enzymatic parameters being used to investigate temporal variability. The initial results of the two enzymatic parameters exhibit high E1 removal (%) as removal is consistently greater than 75% before sample day 52. The months that correspond to the first 52 sample days are August and September whereas the remaining sampling days represent the months between October and December. This point of difference between these months is roughly the transition from summer to autumn where temperatures begin to reduce. The average E1 removal for laccase was 82.9% (± 2.6%) between August and September and for tyrosinase average E1 removal was 83.0% (± 5.6%) in the same sampling period. From these average E1 removal (%) values laccase and tyrosinase appear to behave similarly with tyrosinase showing the largest deviation and so the greater variability between results.

For the second half of the sampling period that was between October and December the average E1 removal for laccase was 69.6% (± 8.7%) and for tyrosinase E1 removal was 80.0% (± 9.6%). From Figure 5.5 it can be clearly seen that there was increased variability for E1 removal during this period. However, the average E1 removal for tyrosinase was close to the reported value in the first half of the sampling period. Important to note that the deviation increased from ± 5.6% to ± 9.6% once again detailing increased variability. Laccase performance was greatly reduced in the sampling period between October and December as shown by the close to 13% reduction in E1 removal. From October onwards there is little correlation between E1 removal for laccase and tyrosinase.

The disparity between E1 removal especially the difference in trend for October to December (sample days 59 to 131) for laccase and tyrosinase, would suggest that wastewater influenced the enzymes differently. As a result this not in agreement with an earlier hypothesis that said the two exogenous enzymes would be expected to behave similarly as both are copper containing oxidoreductases. The difference in enzymatic mechanism for the two enzymes could be a factor in their contrasting behaviour as laccase generates radicals and tyrosinase produces *o*-quinones. To identify the potentially contrasting relationships with the sampled effluent several standard water quality parameters were additionally measured and their influence on E1 removal discussed.

5.7.2 Standard Water Qualities Correlation to Exogenous Enzyme Parameters

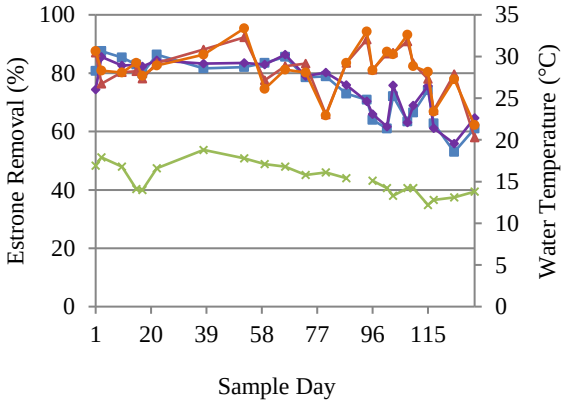
Previous papers in the literature have identified factors that influence and tyrosinase activity mostly being the first principles pH and temperature (Lloret et al., 2010) (Palma et al., 2013) (Ba et al., 2014) (Nguyen et al., 2014). Process and environmental factors concerning the WWTP are often overlooked such as the standard water quality parameters. Dissolved oxygen concentration and supply has been a factor investigated by select papers in the literature. However, aerating with pure oxygen as performed in some of these papers is unfeasible when scaled up, because of the expense involved and the health and safety surrounding the storage and transport of the flammable gas. Increasing the oxygen supply in a membrane reactor improved the degradation of E1 and E2 by laccase (Lloret et al., 2012).

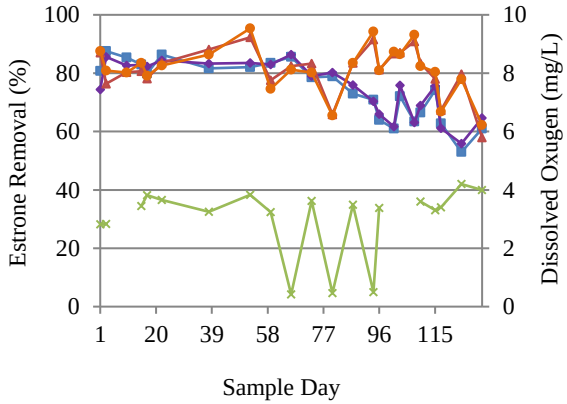
The same authors noted that an earlier paper by Dasgupta et al. (2007) showed no increased removal of phenolic compounds by laccase. A later paper by Lloret et al. (2013) showed the removal of E1, E2 and EE2 to improve by injecting oxygen into the treatment system. The injection of pure oxygen resulted in the maintained saturation level of 8 mg/L to temporarily increase to 35-38 mg/L. For the Stoke Bardolph WWTP the average DO value over the sampling period between August 2016 and December 2016 was 3.4 mg/L (± 1.2 mg/L). The mixture of air into the wastewater system impacts on DO concentration for the effluent from the Stoke Bardolph WWTP. There is little information available for similar studies performed with tyrosinase and the influence on EDC removal. As both enzymes are classed as oxidoreductases the availability of oxygen can be a rate limiting step therefore increased oxygen availability can improve enzymatic activity and in turn enhance EDC removal.

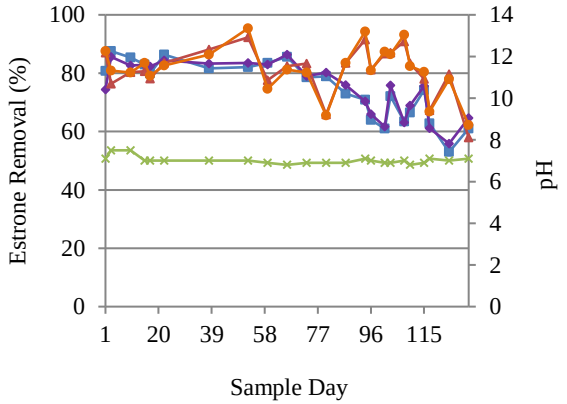
Other wastewater factors that have been considered to influence enzyme behaviour include natural organic matter (NOM) (Xia et al., 2014). The removal of E2 by laccase was investigated at different NOM concentrations. From these results increased NOM concentrations were shown to have an adverse impact on laccase as E2 removal was reduced. A paper by Lu and Huang (2009) had previously studied the influence varying concentrations of NOM had on the laccase catalysed removal of acetaminophen where an enhanced effect was instead shown. The reason for the improved removal was said to be the NOM compounds containing phenolic moieties and their transformation by laccase increasing the number of radicals enhancing

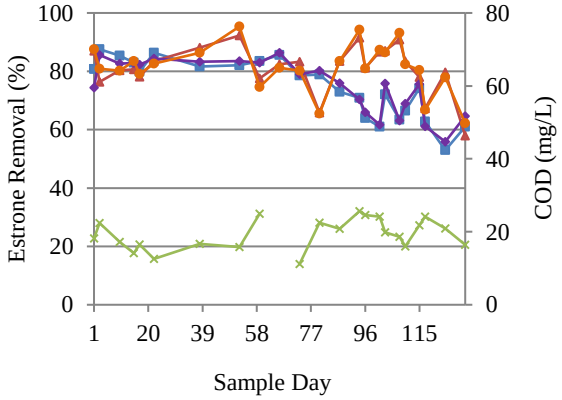
acetaminophen removal. DO and NOM have shown to be important factors that can potentially influence laccase and tyrosinase performance but require further understanding at concentrations relevant to a WWTP.

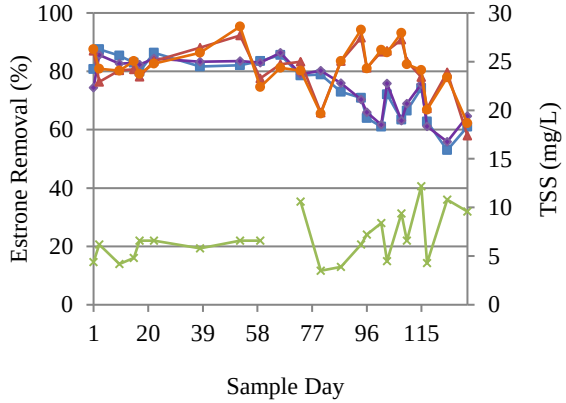
The concentration of NOM within the effluent sampled from the Stoke Bardolph WWTP was indirectly analysed through the chemical oxygen demand (COD) tests. Total chemical amount within the sampled wastewater is measured by COD that informs on the oxygen required to oxidise all organic matter. Temperature, DO, pH, and COD in Tables 2.4 and 2.6 in Chapter 2 were all shown to influence enzymatic performance. Figure 5.6 displays the recorded standard water qualities against the obtained E1 removal percentages.

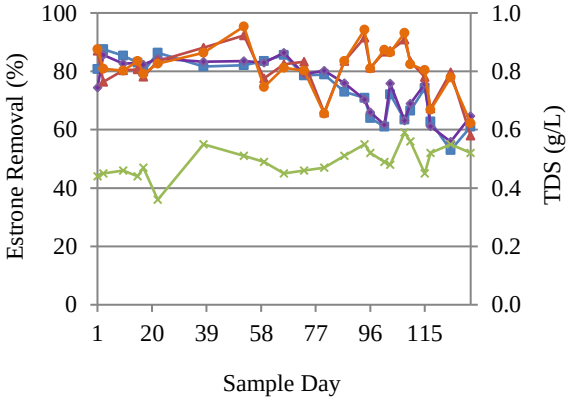

Estrone Removal (%)
Water Temperature (°C)
Sample Day


Estrone Removal (%)
Dissolved Oxugen (mg/L)
Sample Day


Estrone Removal (%)
pH
Sample Day


Estrone Removal (%)
COD (mg/L)
Sample Day


Estrone Removal (%)
TSS (mg/L)
Sample Day


Estrone Removal (%)
TDS (g/L)
Sample Day

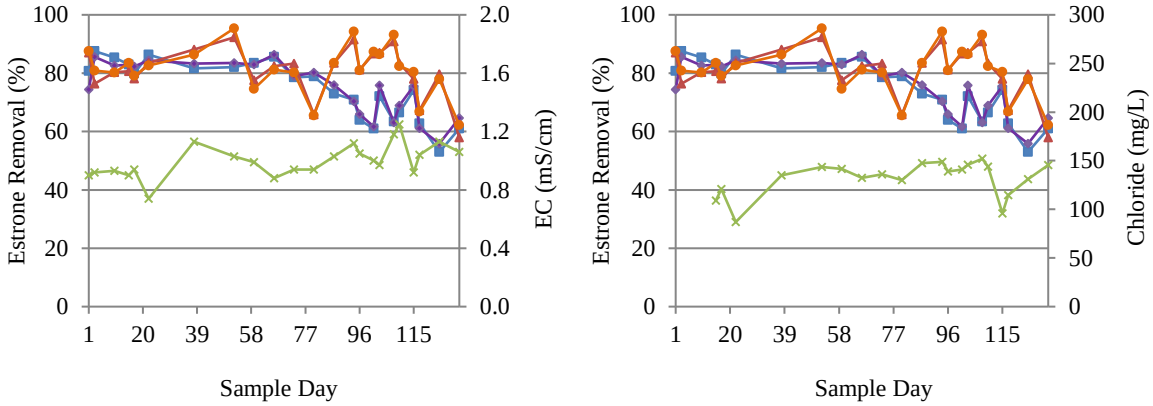


Figure 5.6 Laccase (5 U/ml) and tyrosinase (40 U/ml) benchmarks performed in effluent samples taken from the Stoke Bardolph WWTP between August and December 2016 plotted with the measured standard water quality parameters. Laccase and tyrosinase benchmarks performed in duplicates (n=2). Laccase benchmark R1 (■); laccase benchmark R2 (◆); tyrosinase benchmark R1 (▲); tyrosinase benchmark R2 (●); and wastewater quality parameters (×).

Figure 5.6 once again highlights the dynamic nature of wastewater and temporal variability. The pH value remained close to neutral during the sampling period as represented by the average pH value of 7.1 (± 0.2). The other standard water quality parameters showed greater variation. As shown in Table 5.1 temperature decreased between the summer and autumn months. The average temperature from August to September 2016 was 16.8 ^{o}C (± 1.2 ^{o}C) and for October to December 2016 the temperature was 14.3 ^{o}C (± 1.4 ^{o}C). This trend in temperature was expected as the temperature is lower in the months closer to winter and potentially correlates to the reduction in E1 removal described earlier for the second half of the sampling period between October and December 2016.

COD and DO averages between August and December 2016 were 19.33 mg/L (± 4.2 mg/L) and 3.0 mg/L (± 1.2 mg/L) respectively. Both of these factors in the literature had shown to influence enzymatic performance and the reported deviations exceeded 20% of the average value. This variability most likely contributed to the variable performance of the two enzymes. The only standard water quality parameters where there appeared to be a similarity in temporal trend was for EC, Cl and TDS concentrations up until the first sample day in October 2016 (sample day 108). All three parameters can be interlinked as discussed earlier in the chapter therefore it is not surprising to observe a shared trend. Nevertheless, there are no solid conclusions that can be extracted from Figure 5.6 to explain the difference in E1 removal (%) for laccase and tyrosinase. As a result, a statistical approach specifically Pearson's correlation was adopted to determine positive and negative correlations between E1 removal and standard water quality parameters.

5.7.3 Pearson's Correlation of the Exogenous Enzymatic Parameters

Examples of Pearson's correlation used within the literature include the paper by Molina-Muñoz et al. (2010) that sought to define the relationship between volatile suspended solids (VSS) concentration and the activities of six different inherent enzymes. Analysing E1 removal is an indirect assay for determining laccase and tyrosinase activity. The correlation between exogenous enzyme activity through E1 removal and a standard water quality reflects the study by Molina-Muñoz et al. (2010). A similar investigation by Kreutz et al. (2016) also analysed the impact of wastewater parameters to understand the influence on enzymatic activity. As the produced data

was non-parametric the authors had to instead apply Spearman's *rho* rather than Pearson's correlation. More complex statistical methods exist such as principal component analysis (PCA) (Avella et al., 2011). However, from the papers investigating similar enzymatic relationships, Pearson's correlation has shown to be adequate in determining significant relationships. Larger data sets are typically analysed by PCA as shown by Avella et al. (2011) where up to 16 different factors were simultaneously analysed. Table 5.2 details the generated Pearson's correlation coefficients from the data presented in Figures 5.3 and 5.6.

Table 5.2 Pearson's coefficients for the correlation of estrone (E1) removal for the laccase and tyrosinase benchmarks with the measured wastewater quality parameters. Wastewater Temperature (Temp); Dissolved Oxygen (DO); Chemical Oxygen Demand (COD); Total Suspended Solids (TSS); Total Dissolved Solids (TDS); Electrical Conductivity (EC); and Chloride (Cl). **p* value < 0.05.

Coefficient Range	-1.000 -0.800	-0.799 -0.600	-0.599 -0.400	-0.399 -0.200	-0.199 0.199	0.200 0.399	0.400 0.599	0.600 0.799	0.800 1.000
Correlation Strength	(-) Very Strong	(-) Strong	(-) Moderate	(-) Weak	No Correlation	(+) Weak	(+) Moderate	(+) Strong	(+) Very Strong

	Temp (ºC)	DO (mg/L)	pH*	COD (mg/L)	TSS (mg/L)	TDS (g/L)	EC (mS/cm)	Cl (mg/L)
Laccase (Jun-Jul 2016)	0.296	-0.227	0.200	0.028	0.199	0.122	0.170	-0.076
Laccase (Jun-Dec 2016)	0.637*	-0.073	0.346	-0.307	-0.252	-0.536*	-0.557*	-0.467
Laccase (Aug-Dec 2016)	0.593*	-0.374	-0.024	-0.419	-0.401	-0.583*	-0.581*	-0.334
Tyrosinase (Aug-Dec 2016)	0.103	-0.111	-0.176	-0.279	-0.002	0.195	0.195	0.368

The coefficients are classified by strength using the scaling system described by Evans, (1996). From the Pearson's correlation coefficients and subsequent heat map produced in Table 5.2 it is far easier to identify any significant relationships between E1 removal (%) and the measured standard water quality parameters. The initial laccase enzymatic parameters carried out between June and July 2016 showed that there were weak positive correlations for E1 removal and pH and for wastewater temperature. As pH and temperature increased so did E1 removal however, the pH range for this sampling period was narrow as the average pH value was 7.2 (± 0.1) Temperature on the other had exhibited greater variation as the average temperature was 17.2 ºC (± 5.1 ºC). When analysing the sampling period between June and December 2016 wastewater temperature was significantly positively correlated with

E1 removal. This is reflected in Figure 5.6 as E1 removal does show to decrease with the lower recorded temperatures representing the seasonal change from summer to autumn.

Interestingly the enzymatic parameters were always performed at 20 ^{o}C with all the samples brought to the correct temperature before adding the enzymes to negate any temperature variations. However, the temperature recorded on site at the Stoke Bardolph WWTP had an impact on E1 removal (%) by laccase. The temperatures recorded between August and September 2016 were the highest as the average was 16.8 ^{o}C (± 1.2 ^{o}C). This coincided with the highest E1 removal (%) to be reported for laccase as the average E1 removal was 82.9% (± 2.6%) during this sampling period. Higher temperatures lead to greater metabolic activity of the activated sludge microorganisms increasing inherent enzymatic activity resulting in a cleaner matrix (Gómez-Silván et al., 2013). Laccase activity is shown to be enhanced in a matrix containing less organic contaminants. This can additionally be observed in Figure 5.6 where the lower recorded values for COD and TSS were also observed during the sampling period between August and September 2016.

From Table 5.2 there is indication of a negative correlation with E1 removal (%) and COD and TSS concentrations for laccase. As the concentrations for these standard water quality parameters decrease E1 removal by laccase instead increases. Once again confirming the enhancement of laccase activity in a cleaner matrix containing less organic pollutants. This is a similar finding to Xia et al. (2014) where increased NOM concentrations reduced E2 removal by laccase. On the other had tyrosinase exhibited no correlation with wastewater temperature but did show a weak negative correlation with COD. Figure 5.4 had previously shown tyrosinase activity to be enhanced in matrices less treated.

Contrasting relationships were additionally highlighted for chloride concentration where tyrosinase showed a positive correlation and laccase a negative correlation. From Tables 2.4 and 2.6 in Chapter 2 chloride has been shown to be an inhibitor for both enzymes. Laccase for the sampling period between August and December 2016 was inhibited by increasing chloride concentrations unlike tyrosinase where E1 removal was slightly enhanced. This highlights the difference in response between the

two enzymes and difficulties in predicting behaviour from only one enzyme and then applying that to similarly classified enzymes.

Laccase displayed a greater number of correlations with the recorded standard water qualities than tyrosinase and most were moderately negative. Significant negative correlations were observed for TDS and EC between August and December 2016. Chloride had already shown to inhibit laccase activity and EC is a measure of salts with many dissociating to form Cl^-. Higher salt concentrations are also reflected by increased total dissolved solids therefore these three parameters share some direct association. Therefore as chloride concentration showed a negative correlation it would be expected for EC and TDS to also show a similar trend. The laccase enzymatic parameters recorded between June and July 2016 only showed weak correlations for temperature, DO and pH. Extension of the sampling period to December 2016 included a change in seasons resulting in more correlations to be observed. Temporal variability in E1 removal by laccase and tyrosinase become further evident after sample day 59, representing the transition to autumn. Consequently, temporal variability and the correlations with the measured standard water quality parameters become more prominent as the sampling period was extended beyond a couple of months.

Table 5.3 used Pearson's correlation to show the correlation between E1 removal for laccase and tyrosinase. It had been earlier hypothesised that the behaviour of the enzymes would be similar in wastewater. Removal is unlikely to be identical due to the difference in enzyme and chosen activities but the removal trends can be compared.

Table 5.3 Pearson's coefficients for the correlation of the laccase benchmarks with the tyrosinase benchmarks in the enzymatic removal of added estrone (E1) between August and December 2016. August-September (Aug-Sep) 2016 (n= 9); October-November (Oct-Nov) 2016 (n=12); and August-December (Aug-Dec) 2016 (n=24). *p value < 0.05.

Coefficient Range	-1.000 -0.800	-0.799 -0.600	-0.599 -0.400	-0.399 -0.200	-0.199 0.199	0.200 0.399	0.400 0.599	0.600 0.799	0.800 1.000
Correlation Strength	(-) Very Strong	(-) Strong	(-) Moderate	(-) Weak	No Correlation	(+) Weak	(+) Moderate	(+) Strong	(+) Very Strong

	Tyrosinase (Aug-Sep 2016)	Tyrosinase (Oct-Nov 2016)	Tyrosinase (Jul-Dec 2016)
Laccase (Aug-Sep 2016)	0.692		
Laccase (Oct-Nov 2016)		-0.177	
Laccase (Jul-Dec 2016)			0.233

From Table 5.2 it was already shown that laccase and tyrosinase behave differently regarding their response to variable chloride concentration. For the sampling period between August and September 2016 the correlation between the enzymes was strongly positive, therefore as E1 removal increased for one enzyme so did removal for the other enzyme. The standard water quality parameters recorded during this sampling period (59 sample days) varied less compared to the later sampling days resulting in more stable E1 removal.

After sample day 59 the start of October 2016 both E1 removal and the measured standard water quality parameters became far more variable as shown in Figure 5.6. Furthermore, there is no longer a positive correlation between the enzymes in the second part of the sampling period that ranged from October to November 2016. The correlation as a result over the complete sampling period of July to December 2016 only exhibited a weak positive correlation. These results highlight the importance in measuring temporal variability over an extended time duration that spans a few months rather than a few weeks to understand the true removal pattern. This was only performed at a single WWTP but does show the complex variability of an external enzyme applied as a tertiary treatment step over a sustained treatment period.

Furthermore, the results show that the earlier hypothesis was false as laccase and tyrosinase behave differently from one another in the wastewater environment. Tyrosinase appeared to be less susceptible to wastewater influences compared to laccase as the average E1 removal for tyrosinase between August and December 2016 was 83.0% (±. 8.3%) whereas the average was 74.8% (± 9.6%) for laccase. As well as both enzymes being classified as copper containing oxidoreductases they also share similar substrates highlighting further similarities that do not lead to shared enzyme behaviour.

5.8 Matrix Influence on Exogenous Enzyme Performance

The previous work analysed a range of different environmental and process WWTP factors and the influence they had on laccase and tyrosinase performance through E1 removal. Composition of the wastewater is another variable that can impact on enzymatic activity. This was highlighted in Section 5.3 where reduced laccase performance was observed in wastewater samples compared to a prepared buffer solution in the paper by Lloret et al. (2013). Reasons for this reduction in laccase activity included (1) presence of other compounds in the wastewater that are more easily oxidised (2) inhibitors present within the wastewater that prevent substrates binding to the enzyme (3) mediators present within the wastewater that enhance activity. Tables 2.4 and 2.6 in Chapter 2 of the literature review detail known inhibitors for laccase and tyrosinase. The majority of these inhibitors and mediators have also been identified in the wastewater matrix such as NaCl and Cu^{2+}, a common salt and metal, respectively. The NaCl salt dissociates in water to form the anion Cl^- that is known to inhibit laccase activity (Margot et al., 2015). The standard water quality parameters do not directly inform on these influences however, measured TDS, EC and Cl^- will inform on Cl^- concentrations.

For many of the papers that have studied the inhibitors and mediators for laccase and tyrosinase activity a clean buffer solution has been used. The influence in a real wastewater sample has not been previously explored. Consequently, it is unknown how well a clean buffer matrix models inhibitory behaviour of both laccase and tyrosinase in wastewater. Figures 5.3 and 5.6 have already highlighted the temporal variability associated with wastewater not shown before by clean buffer matrices. Figure 5.7 details the difference in E1 removal for laccase and tyrosinase between a clean buffer solution and sampled effluent. The results of the control experiments performed in the inhibitory study were compared.

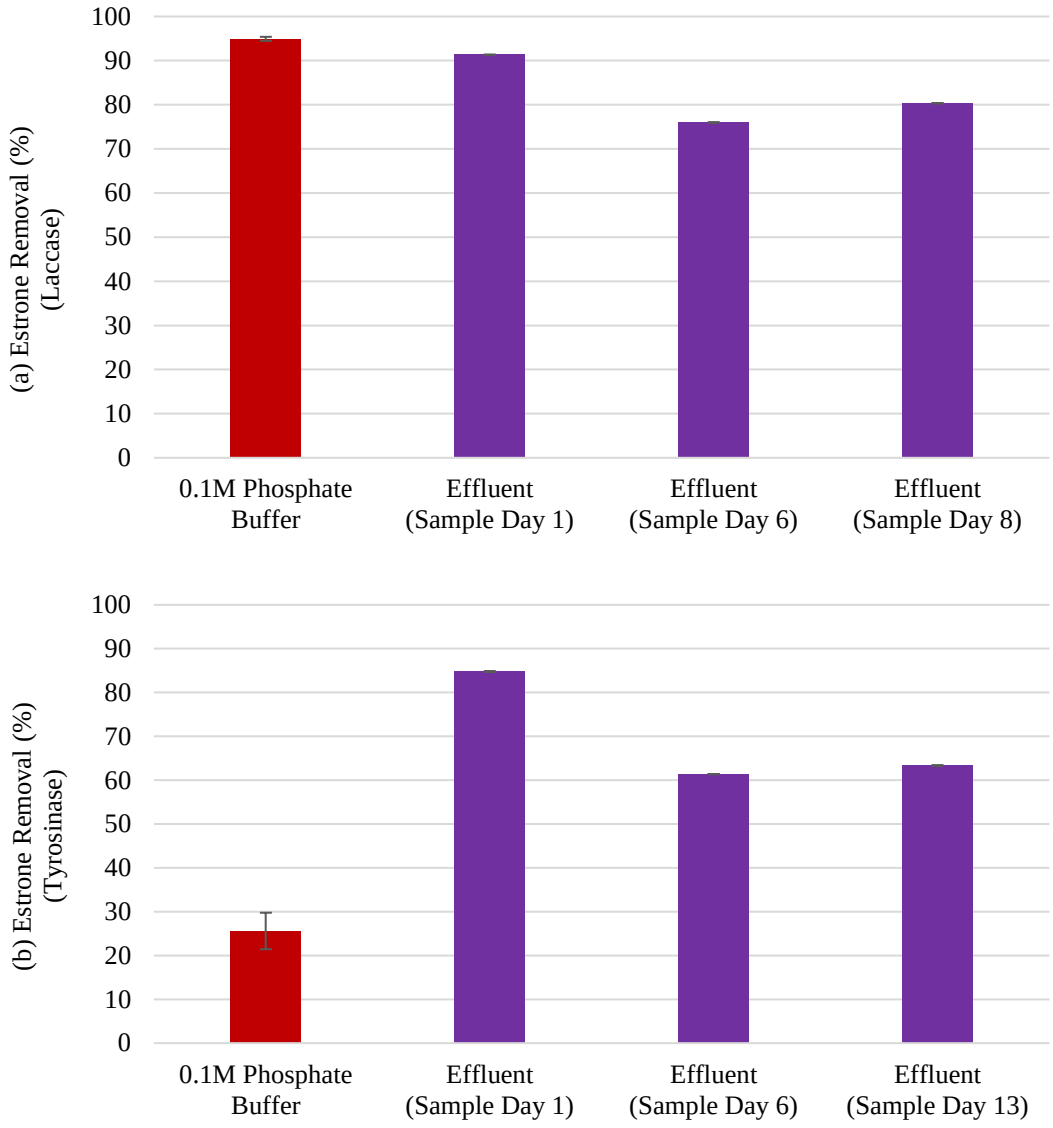


Figure 5.7 The (a) laccase and (b) tyrosinase benchmark controls performed alongside the additive experiments (Cl^-, Cu^{2+} and diclofenac), without these compounds added, for the sampled effluent compared against the same data for the phosphate buffer matrix. Estrone (E1) removal (%) for the phosphate buffer matrix averaged for the repeated experiments performed with the error shown (n=3). Effluent experiments were carried out in duplicates with less than 4% difference between samples.

The performance of laccase was reduced in the effluent compared to the buffer solution with the greatest reduction in E1 removal being 20% on sample day 6 compared to the control. This is similar to the previous findings provided by Lloret et al. (2013). As previously discussed additional inhibitors within wastewater such as Cl^- ions may restrict laccase activity. Tyrosinase on the other hand showed a contrasting result. The wastewater matrix as shown before in Figure 5.4 enhanced tyrosinase performance. Mediators within the effluent most likely enhance the oxidation of E1

not present in the clean buffer solution. On sample day 1 the effluent for the laccase and tyrosinase enzymatic parameters gave the highest E1 removal percentages. It should be noted that there had been heavy rainfall the night before resulting in the overspill channel to be operational that is located near where the effluent is sampled from. Consequently, the overspill dilutes the effluent matrix, enhancing enzymatic performance.

From Figure 5.7 the average difference in E1 removal (%) for tyrosinase between the buffer solution and the lowest recorded E1 removal for the effluent was 40% on sample day 6. This is a noticeable difference not influenced by pH as the recorded values for the effluent were close to the prepared buffer. The average pH for the effluent samples was 7.2 (± 0.2) and the phosphate buffer was prepared to pH 7.0. Additionally, the used phosphate buffer has not been reported in the literature to be detrimental to tyrosinase activity and is a widely utilised buffer as well. A potential cause for enhanced tyrosinase activity in wastewater could be the organic matter containing chemicals with phenolic moieties, and so behaving as mediators for improved E1 removal in wastewater. An early paper by Wada et al. (1995) showed the recalcitrant removal of aniline compounds by tyrosinase improved with added phenol.

Only a few papers have investigated the difference in enzyme response between a clean water matrix, typically a buffer solution, and sampled wastewater. All of these studies have focused on laccase activity with no similar information currently available for tyrosinase. Auriol et al. (2007b) and Auriol et al. (2008) showed no significant difference between laccase activity in buffer matrices and collected wastewater samples for the removal of four estrogens. The study by Garcia et al. (2011) commented that oxybenzone removal was reduced in the wastewater sample compared to the ultrapure water matrix a similar pattern observed in Figure 5.7. However, it is hard to make any direct comparisons as laccase concentration is different as well as experimental conditions. It cannot be concluded that the removal of EDC substrates by laccase or tyrosinase in a clean water matrix such as a buffer would be replicated in the wastewater matrix as highlighted by the differences in Figures 5.4 and 5.7.

5.8.1 Impact of Chloride and Copper Ions on Exogenous Enzyme Performance

The anion Cl^- and the metal Cu^{2+} ion concentrations were selected to investigate their influence on laccase and tyrosinase performance. These inhibitors were chosen from Tables 2.4 and 2.6 in Chapter 2 with criteria that they had to be known pollutants in the wastewater system as well. Tables 9.3 and 9.4 within appendix B show the typical chloride concentrations measured from the Stoke Bardolph WWTP effluent. The chloride concentrations selected to study were 100, 200 and 500 mg/L to reflect municipal wastewater. The average chloride concentration in Table 9.4 in appendix B was 122.7 mg/L (± 22.3 mg/L). The extremity would be 500 mg/L but wastewater released from the textile industry has been reported to exhibit concentrated levels of NaCl resulting in increased Cl^- concentrations as the salt dissociates (Enaud et al., 2011). Salt spread onto highways can also lead to similar increased Cl^- concentrations as the salt dissociates.

The concentration of copper was not determined for any of the collected effluent samples, unlike chloride concentration. However, the literature such as the paper by Gardner et al. (2012) has previously investigated common pollutant concentration ranges. The authors as part of the chemical investigation programme analysed an extensive range of pollutants from effluent samples taken from over 162 WWTPs. From this study total copper concentration was shown to be between 3 µg/L (5 percentile) and 28 µg/L (95 percentile). The concentration for Cl^- was reported to be between 64 mg/L (5 percentile) and 272 mg/L (95 percentile) reflecting the chosen concentrations in this work. For analysing the influence of Cu^{2+} on laccase and tyrosinase performance the selected Cu^{2+} concentrations were 0.05, 1 and 10 mg/L. Copper concentration between 0.05 mg/L and 1 mg/L represented the wastewater environment with the higher concentration of 10 mg/L testing any further industrial increases.

The salts used to analyse the impact of the two ions Cl^- and Cu^{2+} were NaCl and $CuSO_4$. An earlier paper by Kim and Nicell (2006) had concluded that these secondary ions do not additionally impact on laccase activity. Figure 5.8 shows the results obtained from the addition of Cl^- into the laccase and tyrosinase enzymatic parameters. Difference in E1 removal (%) was compared against a control with no chloride added. The sampled effluent for this work investigating Cl^- inhibition was taken in the

morning where there had been heavy rainfall the night before. When the chloride concentration was measured for the sampled effluent it was unusually low at 57.5 mg/L indicating dilution of the effluent matrix. The buffer control chloride concentration was 37.5 mg/L.

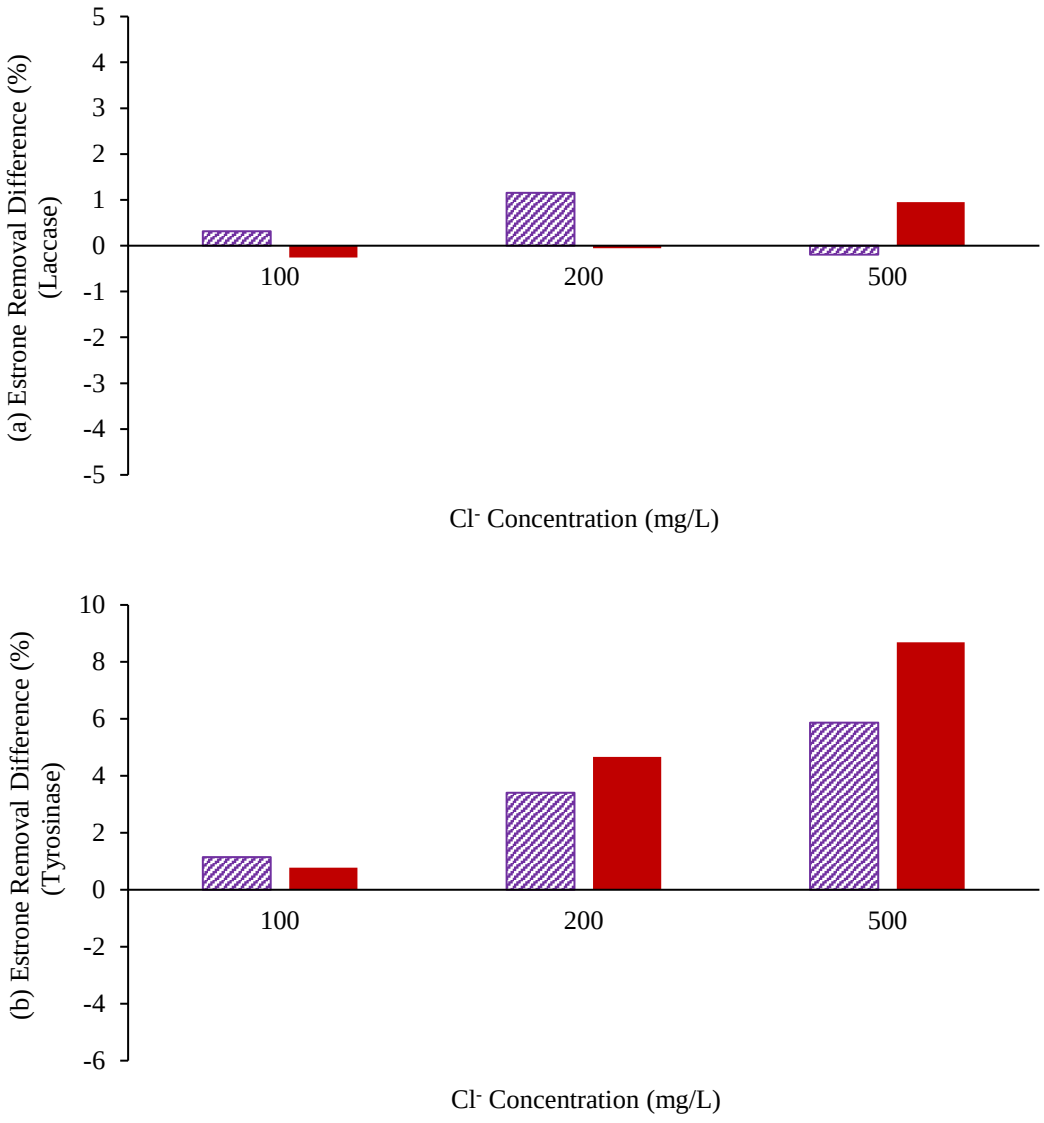


Figure 5.8 The difference in estrone (E1) removal by the (a) laccase and (b) tyrosinase benchmarks at increasing concentrations of added Cl^- compared to a control with no Cl^- added. Positive values indicate an increase in E1 removal. The influence of Cl^- compared between (▨) effluent (pH 7.0) sampled from the Stoke Bardolph WWTP (■), and 0.1 M phosphate buffer (pH 7.0). All experiments performed in duplicates (n=2) with the difference between samples less than 3%.

The laccase enzymatic parameters showed little difference in E1 removal with the addition of 100, 200 and 500 mg/L Cl^-. Both matrices showed no stark contrasts. Tyrosinase on the other hand showed chloride concentration to have a larger impact

on E1 removal. A similar removal trend in E1 removal was observed for the two matrices as E1 removal increased with Cl^- concentration with the effect more pronounced in the buffer solution. Overall E1 removal appeared to increase with chloride concentration as at 500 mg/L E1 removal was 6% and 8% higher than the control for the effluent and buffer matrices respectively.

Table 5.2 had highlighted a similar pattern as the Pearson's correlations showed E1 removal for laccase to be negatively correlated and for tyrosinase it was positively correlated. Therefore increases in chloride concentration for the sampled effluent tended to result in greater E1 removal for tyrosinase. The literature indicated that higher chloride concentrations for both laccase and tyrosinase would inhibit their activities. However, from the results presented in Figure 5.8 the same cannot be conclusively said as laccase showed no negative effects with increasing Cl^- concentration, and tyrosinase exhibited greater E1 removal. From the literature Margot et al. (2013) showed a 20% reduction in laccase activity from *T. versicolor* when NaCl concentration was 5 mM, 175 mg/L Cl^-, in an acetate buffer at pH 4.5, compared to a control. The oxidation of ABTS was investigated whereas other papers for example Kim and Nicell, (2006) have studied the impact of Cl^- concentration on BPA removal.

The paper by Kim and Nicell (2006) analysed the conversion of BPA at varying concentrations of NaCl (0 to 200 mM) by laccase from *T.versicolor*. At a NaCl concentration of 25 mM that is equal to 876 mg/L Cl^- BPA conversion was reduced from 59.5% in the control to 45.6%. This was a difference of 13.6% lower than the inhibition reported by Margot et al. (2013) at a far higher Cl^- concentration. Secondly, the BPA conversion experiments were all similarly performed in acetate buffer but at pH 5. Laccase concentration used by Kim and Nicell (2006) was over 10 times higher than that used for Margot et al. (2013) where concentrations were 0.15 U/ml and 0.01 U/ml respectively. From these observations and the results in Figure 5.8 it would appear that the influence of chloride is more pronounced at pH levels that are more acidic for laccase. The experiments performed in Figure 5.8 were all at neutral or close to neutral pH. ABTS oxidation was also shown to be more responsive to Cl^- concentration with 175 mg/L Cl^- greatly lowering laccase activity by 20% (Kim and Nicell, 2006). In Figure 5.8 for laccase at 200 mg/L Cl^- the removal of E1 showed a ± 1% difference to the controls with no inhibition occurring. This highlights that

inhibition results obtained from ABTS oxidation do not accurately reflect EDC removal.

The use of ABTS as a substrate provides immediate indication for determining inhibitors and mediators but does not give the full story for EDC removal in a wastewater matrix. Laccase catalysed elimination of an anthraquinonic acid dye (ABu62) was tested at different concentrations of NaCl by Enaud et al. (2011). Concentration ranged from 0.5 to 100mM NaCl equating to Cl^- concentrations between 0.02 and 3.5 g/L. The inhibition of laccase from *T.versicolor* by NaCl was investigated through the oxidation of ABTS with and without ABu62. The ABTS oxidation rates typically increased when ABu62 was present meaning chloride inhibition was reduced. Yet again showing the activity of laccase measured by EDC removal cannot be inferred from ABTS assays. Additionally, there has been little experimental work performed comparing such inhibition between clean water and wastewater matrices.

Fewer inhibition studies have been carried out for tyrosinase compared to laccase for chemicals relating to the wastewater environment. There have been no publications that have investigated the inhibition of tyrosinase and the effect on EDC removal relating to the wastewater matrix. Two tyrosinases from different bacterial sources specifically actinomycetes were investigated by Le Roes-Hill et al. (2015) for the effect of different inhibitors and metals on their activity measured by L-DOPA oxidation. The NaCl concentrations tested were 25 and 50 mM equating to 0.9 and 1.8 g/L Cl^- far higher than that tested in Figure 5.8. At 0.9 mg/L Cl^- the difference in residual activity for one of the tyrosinase showed no change to the control and the other tyrosinase showed a reduction of 15% in activity. Tyrosinase activity was not enhanced by the addition of Cl^- ions but the tested concentrations were significantly above 500 mg/L and do not reflect the wastewater matrix. It is difficult to directly compare with laccase but the literature did report higher inhibition for laccase at far lower Cl^- concentrations. At the Cl^- concentrations between 100 and 500 mg/L a different relationship may exist for tyrosinase as the literature reports no strong inhibition to occur.

Similar to the chloride inhibition study Figure 5.9 details the results with added Cu^{2+} ions instead. Compared to the chloride results, strong inhibition was observed for both laccase and tyrosinase especially at 10 mg/L Cu^{2+}.

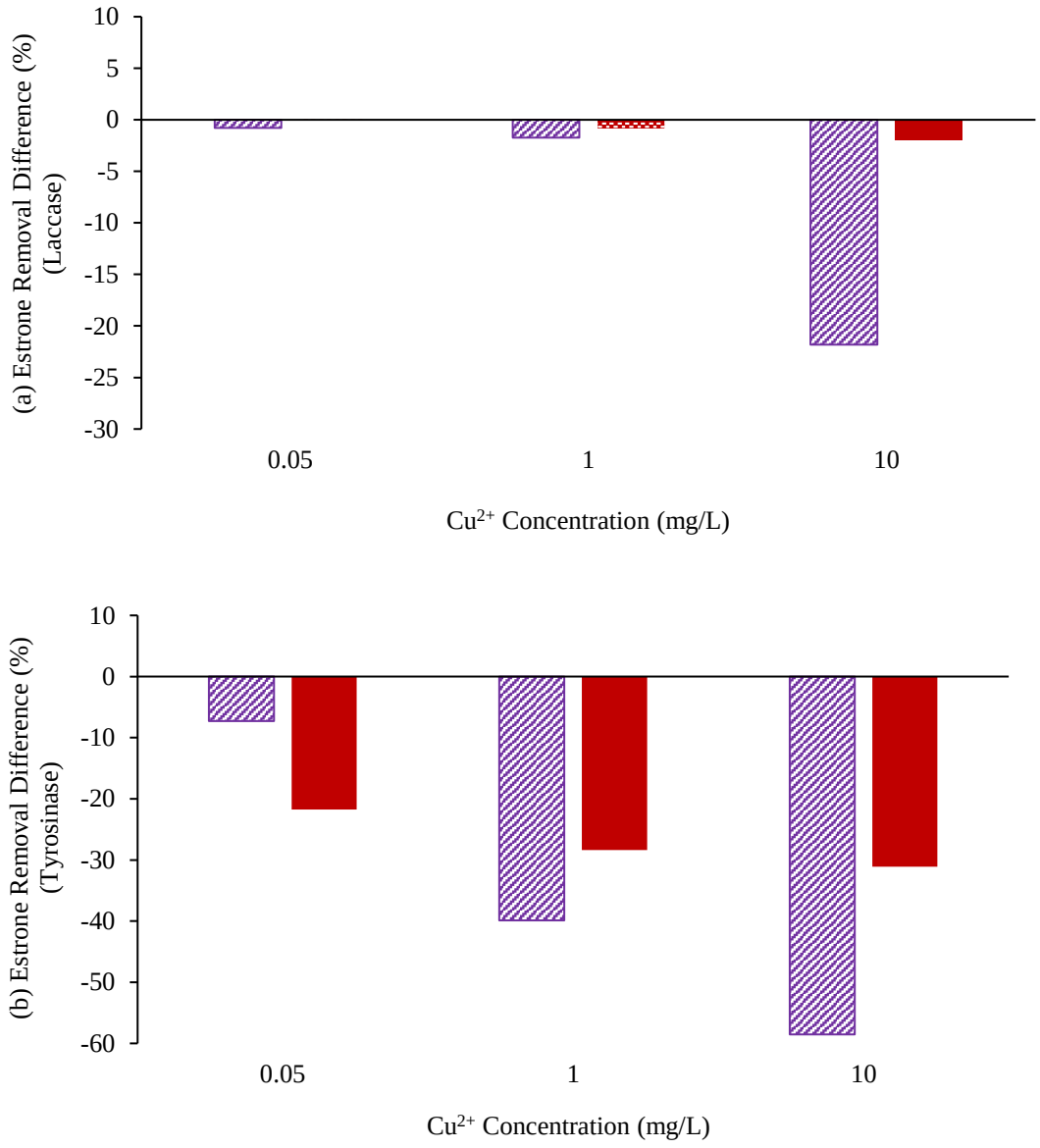


Figure 5.9 The difference in estrone (E1) removal by the (a) laccase and (b) tyrosinase benchmarks at increasing concentrations of added Cu^{2+} compared to a control with no Cu^{2+} added. Negative values indicate inhibited E1 removal. The influence of Cu^{2+} compared between (▨) effluent (pH 7.2) sampled from the Stoke Bardolph WWTP, and (■) 0.1 M phosphate buffer (pH7). All experiments performed in duplicates (n=2) with the difference between samples less than 3%.

Firstly laccase showed less than -3% E1 removal difference at 0.05 and 1 mg/L Cu^{2+} across the buffer and effluent matrices. At Cu^{2+} concentrations that reflect the wastewater environment inhibition was shown to be minimal not exceeding a 5% difference from the control. However, at 10 mg/L Cu^{2+} the inhibition effect was pronounced in particular for the effluent matrix where the difference in E1 removal was close to -25%. For the phosphate matrix at the same Cu^{2+} concentration, the E1 removal difference was shown to be below -5%. Laccase in the sampled effluent was significantly inhibited by 10 mg/L Cu^{2+}. The application of laccase as a tertiary treatment step would not be heavily influenced by the averaged Cu^{2+} concentrations presented by Gardner et al. (2012) however any large spikes in Cu^{2+} could result in inhibited EDC removal. Industrial wastewater typically contains higher levels of heavy metals. For periods where Cu^{2+} concentration exceeds 1 mg/L, the subsequent reduction in EDC removal could be combated by increasing laccase concentration or removing the copper before laccase contact.

Tyrosinase showed greater inhibition to Cu^{2+} than laccase. Adaptions to a WWTP, similar to that discussed for laccase, would need to be made for the use of tyrosinase at Cu^{2+} concentrations equalling or higher than 1mg/L. As Cu^{2+} concentration increased so did the level of inhibition with the difference in E1 removal decreasing from -7% to -59% for the effluent compared to the control. The differences observed for the buffer solution to the control were less prominent as removal ranged between -20% and -30%. Tyrosinase performance in the phosphate buffer was always lower than that reported for the sampled effluent as previously discussed. This once again shows the importance of analysing the effects of laccase and tyrosinase in wastewater as a clean buffer matrix commonly used in the literature will inaccurately report enzyme behaviour.

Examples from the literature all show copper to be an inhibitor for both laccase and tyrosinase in keeping with the results presented in Figure 5.9 (Kim and Nicell, 2006) (Le Roes-Hill et al., 2015). The impact of heavy metals on laccase activity from *T.versicolor* was analysed by Lorenzo et al. (2006). The inhibition of laccase activity was assayed through ABTS oxidation in a sodium acetate buffer at pH 4.5. At the highest tested Cu^{2+} concentration of 80 mM (5 g/L) inhibition was reported to be 80%. In the phosphate buffer at pH 7 the influence of copper even at twice the concentration of Lorenzo et al. (2006) still did not show similar levels of inhibition with the E1

removal difference less than -5%. Acidic pH levels increase the inhibition observed for laccase as first discussed for chloride (Margot et al., 2013). The previously highlighted study by Kim and Nicell, (2006) additionally analysed the effect Cu^{2+} ions had on BPA removal laccase. Added concentrations of Cu^{2+} ranged between 0.1 and 1 mM equal to 6.4 mg/L and 64 mg/L. The reduction in BPA was minimal with 64 mg/L Cu^{2+} only leading to a decrease in BPA removal by 5%. Therefore the effect of Cu^{2+} on E1 removal similar to BPA is less adverse then when compared to ABTS oxidation.

For the first time the increased inhibition of laccase performance by almost 20% was reported in the sampled effluent compared to the buffer matrix at 10 mg/L Cu^{2+}. This is explained by copper and iron ions that disrupt the electron transport system of laccase leading to reduced rates of substrate conversion (Torres et al., 2003). The inhibition mechanism for tyrosinase has not been explored but Cu^{2+} ions may additionally impact the electron transport system as both enzymes are copper containing oxidoreductases. As well as studying the impact of chloride on tyrosine activity, (Le Roes-Hill et al., 2015) also analysed the effect of Cu^{2+} ions. Concentrations tested were 2 and 5 mM, equating to 127 and 318 mg/L, and similar to Figure 5.9 inhibition occurred. At the lowest Cu^{2+} concentration residual tyrosinase activity was reduced to 71% and 53% for the two bacterial enzyme sources. Even at the lower concentrations of Cu^{2+} in Figure 5.9 there is still a difference in E1 removal showing inhibition. Testing the inhibitory effects in wastewater still remains a novel approach for both laccase and tyrosinase. Copper from the discussed results would be of a higher concern than Cl^- when incorporating external enzymes into a WWTP because the environmental concentrations have shown to inhibit tyrosinase activity, by 20% at the lowest concentration.

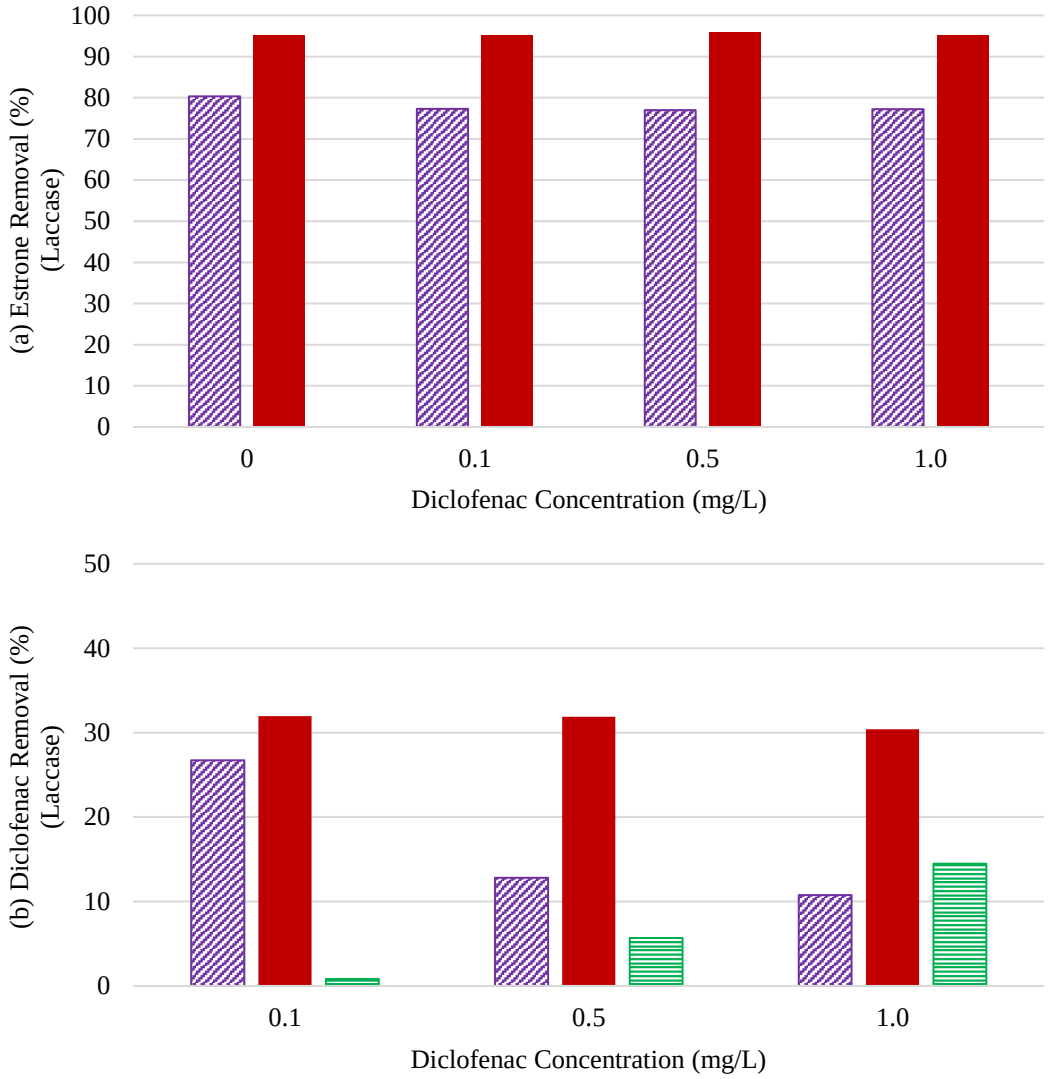
5.8.2 Mixed Estrone and Diclofenac Substrate Exogenous Enzyme Influence

Environmental concentrations of E1 and DCF are given in Table 2.8 within Chapter 2 of the literature review. From this table E1 and DCF concentrations can be seen to vary between WWTPS in different countries. Effluent concentrations are of most interest as this is where enzymatic remediation technologies will be applied in EDC removal. E1 and DCF are on the European Watch List therefore they are EDCs of concern to numerous stakeholders from water companies to environmental agencies. For many of the papers summarised in Tables 2.4 and 2.6 in Chapter 2 the removal of

EDC substrates by laccase and tyrosinase has been investigated as single substrates. Few papers have investigated the influence mixed EDC substrates have on both laccase and tyrosinase behaviour. The recent paper by Margot et al. (2013) showed that salts and metals are not the only constituents that can influence enzyme activity. The wastewater matrix does not solely constitute of a single EDC but a mixture of pharmaceuticals, hormones and personal care products. As a result there can also be an unknown additive effect.

The experimental work carried out by Margot et al. (2013) focused on this additive effect. The removal of the following EDC susbtrates DCF, BPA and mefenamic acid (MFA) by laccase was investigated in single and combined EDC substrate solutions. Sodium phosphate prepared to a pH of 7.2 was the selected reaction matrix and each pollutant was added to a final concentration of 20 mg/L. Removal was analysed over a 10 hour period with laccase at a concentration of 730 U/L. DCF as a single susbtrate showed only 25% removal at the end of this reaction period. The mixed substrate solution of MFA, BPA and DCF resulted in greater DCF removal. MFA and BPA are known to be readily oxidised by laccase leading to the formation of unstable radicals that then can interact with DCF. Two means of reaction occur either the radical cross-links with DCF creating a larger moelcule that is susceptible to precipation, or the radicals act as mediators. It was noted by the authors that precipitates were clearly visible at the end of the reaction period. The laccase and tyrosinase enzymatic parameters with E1 were altered to additionally test the additive effect of DCF reported for the first time in sampled wastewater.

The concentrations of DCF investigated were 0.1, 0.5 and 1 mg/L that represented a concentration range that was higher and lower than E1. The E1 and DCF concentrations used in the enzymatic parameters are not truly reflective of the wastewater environment as shown in Table 2.8 within Chapter 2. As substrate concentration is analysed by HPLC-UV there is a limit of detection that prevents environmental concentrations to be utilised. As well as analysing the additive effect of DCF on E1 removal the influence of E1 on DCF removal was also studied. Experiments only containing DCF were performed to compare against the substrate mixture of E1 and DCF. The additive effect on removal for E1 and DCF is first presented for laccase in Figure 5.10.



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Figure 5.10 Laccase benchmarks with estrone (E1) concentration set at 0.5 mg/L carried out with increasing concentrations of diclofenac (DCF) in (▨) effluent (pH 7.3) sampled from the Stoke Bardolph WWTP, and (■) 0.1 M phosphate buffer (pH7). Additive removal effect investigated for both (a) E1 and (b) DCF. The removal of DCF without E1 added was analysed in both the (▤) buffer and effluent matrices. DCF removal was not observed without E1 in the effluent so not reported. All experiments performed in duplicates (n=2) with the difference between samples less than 4%.

Firstly the removal of E1 by laccase was not influenced by the addition of DCF for both the buffer and effluent matrix. E1 removal observed in the control was the same for the reactions with DCF. Investigating the relationship E1 has on DCF removal provided greater findings. There was no DCF removal by laccase in the sampled effluent matrix. However, the addition of E1 led to DCF removal occurring in the

wastewater matrix. Furthermore, the DCF removal was not consistent between the studied concentrations as removal decreased as DCF concentration increased. For example at 0.1 mg/L DCF removal was 27% and at 1.0 mg/L removal was 11%. The buffer additionally showed greater DCF removal when E1 was present. For all three DCF concentrations in the buffer matrix, removal was close to 30% in a mix with E1. Without E1 the highest DCF removal was 15% at 1.0 mg/L whereas at 0.5 mg/L removal was considerably lower at 6%. The removal relationship with DCF concentration contrasted between the two matrices.

The presence of E1 in the effluent matrix resulted in greater DCF removal to occur. E1 is more readily oxidised by laccase as shown in Figure 5.10 by E1 removal being consistently above 70%. The production of free radicals from E1 oxidation led to greater DCF removal either from radical cross-linking resulting in the formation of large molecules that precipitated out of solution, or the radical acting as a mediator (Margot et al., 2013). The effluent matrix had a negative impact on DCF removal compared to the buffer, which has been discussed before in Section 5.8 for E1 removal. Tyrosinase, unlike laccase, exhibited no DCF removal in either the wastewater or buffer matrices with or without E1. As a result Figure 5.11 only shows the influence DCF had on E1 removal.

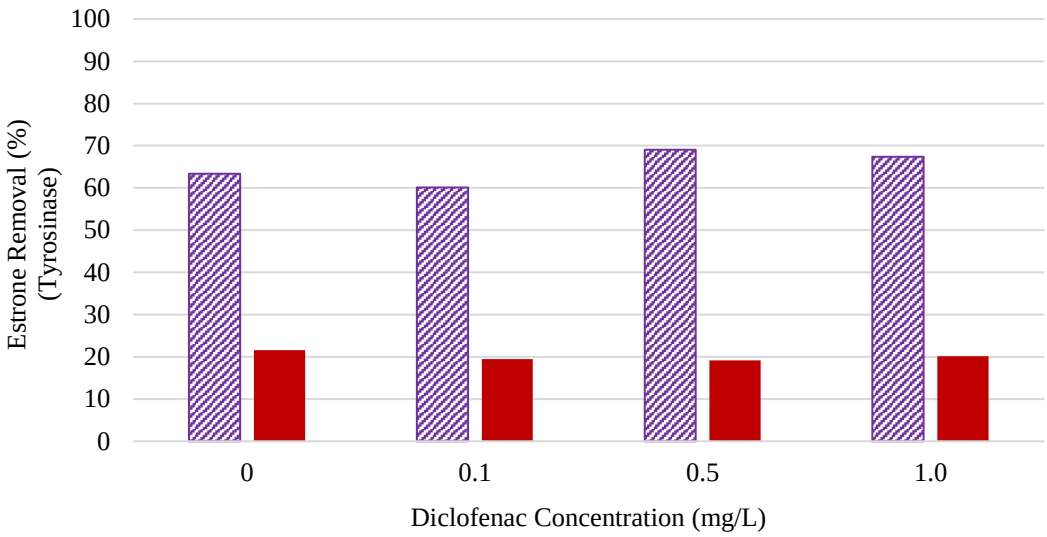


Figure 5.11 Tyrosinase benchmarks with estrone (E1) concentration set at 0.5 mg/L carried out with increasing concentrations of diclofenac (DCF) in (▨) effluent (pH 7.2) sampled from the Stoke Bardolph WWTP, and (■) 0.1 M phosphate buffer (pH7). Additive removal effect investigated for E1 and DCF. Removal of DCF without E1 was also analysed but no DCF degradation was seen in either the clean water or effluent matrix so not presented. All experiments performed in duplicates (n=2) with the difference between samples less than 3%.

There was no observed enhancement in E1 removal in the presence of DCF. Tyrosinase had shown to be unable to degrade DCF when studying DCF removal with E1. Consequently, DCF is not transformed into a free radical that can enhance E1 removal. The literature is limited on EDCs that are known to be degraded by tyrosinase with this work reporting E1 removal by tyrosinase for the first time. Furthermore, E1 removal by tyrosinase was studied in sampled effluent from an operational WWTP. Phenol was investigated as an additive for the tyrosinase catalysed removal of various compounds by Wada et al. (1995). The aniline compounds were of most interest as they did not have a hydroxyl group similar to DCF.

Removal was poor for all these compounds especially 3,4-dichloroaniline where 3% removal was observed. The presence of phenol increased this to 100% removal showing that the addition of a compound with a phenolic moiety can significantly enhance tyrosinase activity. E1 and DCF did not replicate such a result in removal. Oxidization of phenol results in the formation of quinones that easily react with nitrogen in an amino group. The compounds then undergo cross linking to form dimers, trimers and tetramers that still remain water soluble. Therefore a coagulant

needs to be added to precipitate these cross linked pollutants and then remove by filtering (Wada et al., 1995). The molecular structure of DCF has no amino group or free nitrogen, therefore explaining the absence of DCF removal even in combination with E1.

5.9 Conclusions

- Wastewater is highly dynamic and inherently complex leading to variable laccase and tyrosinase performance in E1 removal. High temporal variation was observed over the sampling period stretching from August 2016 to December 2016.
- Standard water quality parameters highlighted the temporal variability of wastewater. Pearson's correlation showed laccase to be significantly influenced by wastewater temperature, electrical conductivity and total dissolved solids. Laccase and tyrosinase showed no correlation with each other in removal over the sampling period of four months.
- Influence of two known wastewater inhibitors on laccase and tyrosinase activity was investigated. The selected inhibitors were chloride (Cl^-) and copper (Cu^{2+}). E1 removal was reduced with added Cu^{2+} for laccase and tyrosinase showing that the treatment process would benefit from removing copper first.
- Tyrosinase for the first time was shown to remove E1 in a buffer and wastewater matrix. Laccase was able to remove DCF, unlike tyrosinase. Removal of DCF improved with the addition of E1 for laccase, showing an enhanced mixture effect. E1 removal by tyrosinase was far greater in the effluent than the buffer matrix.

CHAPTER 6: Results & Discussion

Characterisation and Profiling of Inherent Hydrolytic Activity at Different Wastewater Treatment Stages

6.1. Introduction

The following work focuses on enzymes that are inherently present in a wastewater treatment process, either in the input stream, or coming from microorganisms during the biological treatment stage. In particular, hydrolases are critical in breaking down the organic pollutants in wastewater (Nybroe et al., 1992). Wastewater is an extremely complex matrix that continually evolves in composition over time and varies through a treatment process. On one hand, enzymes that are present in wastewater can be influenced by a wide range of different factors such as temperature and pH, resulting in a variable output in enzyme activity (Whiteley and Lee, 2006). On the other hand, microorganisms that comprise the biological process e.g. activated sludge itself might react differently to external stimuli, excreting different levels of hydrolytic enzymes.

Therefore, analysing inherent enzyme activity coupled to parameters can provide information about the biological efficiency for a given wastewater treatment system. The treated effluent released from a WWTP is required to meet regulations and legislation that meet, or exceed certain specification to prevent the receiving aquatic environment and flora/fauna within from being negatively impacted. Previous studies have assessed the performance of different wastewater treatments by determining the activity of various inherent enzymes. However, the variability of enzyme activity over time and significance of generated enzyme profiles are rarely expanded upon (Fischer et al., 2013). This chapter builds on the work presented by Fischer et al. (2013) to address this missing knowledge. For the first time, spatial and temporal inherent activity is analysed and correlated to several measured wastewater characteristics.

Two WWTPs, one at Old Ford and the other at Stoke Bardolph, were sampled to investigate spatial and temporal variability. Figures 6.1 and 6.2 show the contrasting process configurations. The Old Ford WWTP, located next to the Olympic Park Site in London was established as part of the Olympic Games 2012 sustainable water strategy to reduced demand for potable water by 40% (Knight et al 2012) and incorporates a membrane bioreactor (MBR) within its treatment process. Wastewater

treated at Old Ford directly enters a non-potable water system on the Olympic site, purposed for toilet flushing and irrigation. The MBR setup allows for highly effective wastewater treatment, as the solids retention time (SRT) is separated from the hydraulic retention time (HRT), allowing for high sludge ages. In a conventional process, as detailed in Figure 6.2, separation between the treated sewage and sludge occurs in the secondary settling tank driven by gravity. Filtration by the MBR is a far more efficient process leading to greater sludge retention (Kaya et al., 2013). The MBR can also be installed on sites where space is limited and further expansions cannot be made. The Stoke Bardolph WWTP, situated just on the outskirts of Nottingham, has a conventional activated sludge process and the effluent is discharged into the River Trent. Recent upgrades to the WWTP have improved both phosphate and ammonia removal as phosphate is now recovered as a fertiliser.

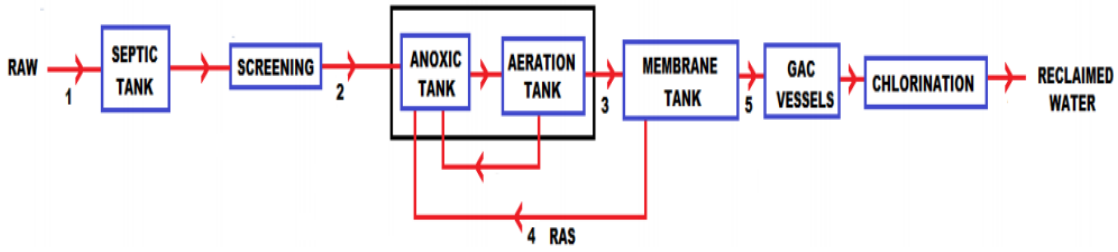


Figure 6.1 Old Ford WWTP process diagram (Purnell et al., 2016). Sample locations were (1) influent (raw sewage) (2) screened sewage (3) mixed liquor (4) returned activated sludge (RAS) (5) membrane bioreactor (MBR) output.

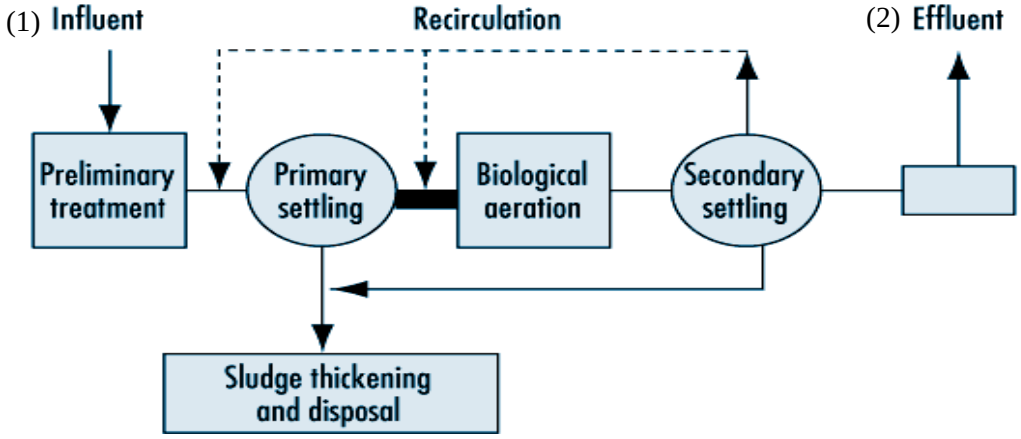


Figure 6.2 Process diagram for a conventional WWTP similar to Stoke Bardolph. Sample locations were (1) influent (after preliminary treatment) (2) effluent (before entering the River Trent).

From the literature, five hydrolases were chosen to investigate inherent enzyme activity. The justification for the selection of these five enzymes are given in Chapter

4 and relate to their high prevalence in wastewater and importance in breaking down the majority of organic pollutants (Burgess et al., 2008) (Fischer et al., 2013). Instead of only sampling the activated sludge this research explored the entire treatment process from influent to released effluent. As previously mentioned the majority of studies exploring inherent enzyme activity have far the most part solely focused on activated sludge. Little is known about inherent activity throughout a WWTP apart from the initial work carried out by Fischer et al. (2013). Greater information is needed on spatial and temporal enzyme variability to better understand overall treatment performance.

Pearson's correlation was used to identify any significant relationships between enzyme activity and measured wastewater characteristics taken for each sample. The measured wastewater characteristics analysed the impact of both process and environmental influences on enzyme activity. For example total dissolved solids (TSS), chemical oxygen demand (COD), temperature and pH are factors governed by environmental inputs and conditions. Temperature and pH can also be considered to be process factors. Acids and bases are in some cases added to wastewater to neutralise strong pH levels and the treatment process may regulate temperature. Chloride is another environmental influence that enters the wastewater system from used cleaning products and water softeners. Salt used in the winter to prevent roads from freezing over is another variable source of chloride pollution. Mixed liquor suspended solids (MLSS) and dissolved oxygen (DO) are process factors that can be operationally altered. By measuring a range of different influences, a greater understanding on enzyme behaviour can be achieved.

The aim of this chapter was to highlight the spatial and temporal variability of inherent enzyme activity at an operational WWTP. Alongside this environmental and process related wastewater characteristics were measured and correlated with enzyme activity. In addition to showing spatial and temporal enzyme activity the measured wastewater characteristics allowed for the reasons of this occurrence to be determined. Previous literature often fails to comment on the significance between enzyme activity and wastewater characteristics. The work in this chapter analysed inherent enzyme activity at five different process locations at the Old Ford WWTP as shown in Figure 6.1. These sampling locations were chosen as they represented the main stages of the treatment process. Samples from each process location were collected over a time

period spanning almost four weeks. Inherent activity was analysed to identify spatial and temporal variability and for the first time inherent activity was reported for returned activated sludge (RAS) samples. Wastewater characteristics also referred to as standard water qualities were measured as well. This experimental work was later repeated at the sampled Stoke Bardolph WWTP but only influent and effluent samples were collected as seen in Figure 6.2. Wastewater samples from the influent and effluent streams were taken over an extended time of almost four months. Access at the Stoke Bardolph WWTP limited the number of sampling locations.

6.2 Highlights

- Inherent enzyme activity analysed through an operational WWTP showed temporal and spatial variability.
- Activity for five inherent hydrolytic enzymes shown to vary temporally and spatially both within and between two WWTPs.
- Returned activated sludge samples were for the first time analysed for inherent activity not previously discussed in the literature.
- Wastewater samples characterised by standard water quality parameters and correlated to inherent activity. Determined the influence of varying process and environmental factors on enzyme behaviour that varied between the different sampled wastewater matrices.
- Activity of commercial hydrolases added to different matrices buffer, deionised water, influent and effluent showed variable responses between clean water and wastewater matrices.

6.3 Analysis of Hydrolytic Activity in Filtered Wastewater: Stoke Bardolph

In the first set of experiments the activities of α-glucosidase, β-glucosidase and esterase were tested in wastewater from Stoke Bardolph WWTP. To keep this early scoping work feasible and confirm variability in activity from collected influent and effluent samples only three hydrolases were tested. Furthermore, the applicability of the methodology adopted from the published study by Fischer et al. (2013) was also analysed. This methodology was utilised as the authors had reported variation in activity for a range of different inherent enzymes between multiple WWTPs, and within the same the WWTP at various sampling points over a one week period. Therefore, the activities of three hydrolases in influent and effluent samples were

assayed in a similar way. Activities were evaluated spectrophotometrically, by monitoring the release of the coloured product (p-nitrophenolate for the two glucosidases and fluorescein for esterase), over a period of time (Section 3.9 in materials and methods).

Influent and effluent samples taken from the Stoke Bardolph WWTP were passed through a 1.2 µM glass microfilter before assaying for activity. This reduced the turbidity of the samples allowing absorbance of the formed coloured products to be accurately measured over time. On the other hand Fischer et al. (2013) measured absorbance at the end of reaction period and separated the solids from the assayed liquid phase through centrifugation. Consequently, the recorded inherent enzyme activities by Fischer et al. (2013) are for unfiltered wastewater samples. The early scoping work for the influent and effluent samples taken from the Stoke Bardolph WWTP was performed over a period of 17 days. The sampling trips to the Stoke Bardolph WWTP were organised between Severn Trent and the University of Nottingham, and occurred on days 1, 3, 8, 15 and 17 as shown in Figure 6.3. Not all three hydrolases were analysed on the same sample day as the focus was also on refining the experimental procedure. For all six sample days, α-glucosidase activity was measured whereas β-glucosidase activity was analysed on days 8, 15 and 17. Esterase activity was measured on all the sample days apart from the last so daily comparisons cannot always be made.

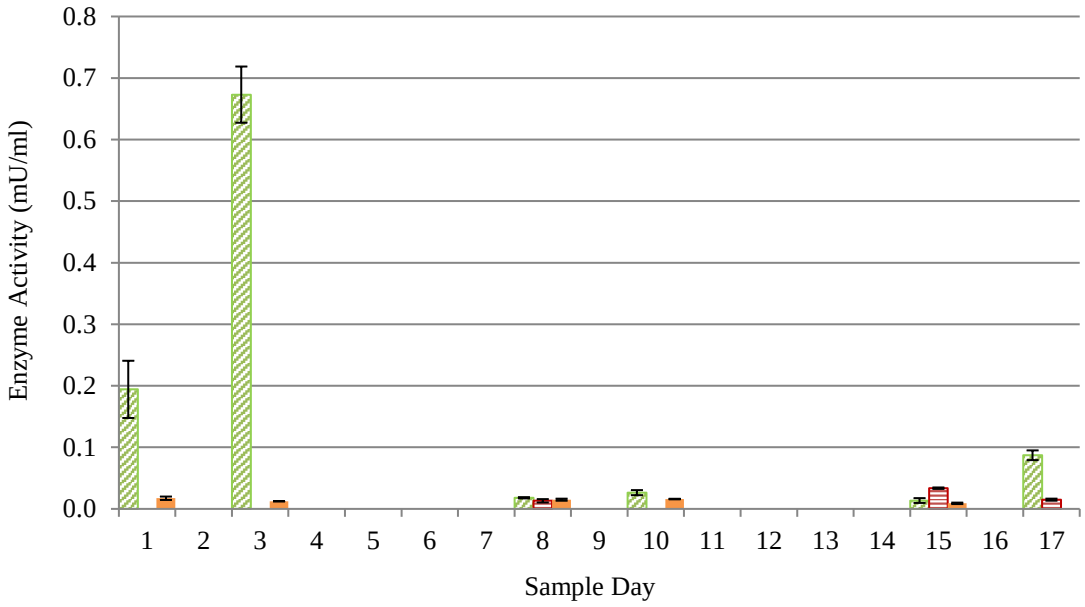


Figure 6.3 Inherent activities of the following hydrolases α-glucosidase (▨), β-glucosidase (▤) and esterase (■) for the influent samples collected from the Stoke Bardolph WWTP between January and February 2016 (n=3).

The results presented in Figure 6.3 show that the activities of the three enzymes varied between the multiple sampling days. The highest enzyme activity values were associated with α-glucosidase followed by β-glucosidase and then esterase. The activity values obtained were significantly lower than those presented by Fischer et al. (2013). Figure 6.3 only gives the inherent activities for the influent samples collected from the Stoke Bardolph WWTP. There was no activity observed for any of the hydrolases in the effluent samples taken. Compared to Fischer et al. (2013) the same three hydrolases had been detected to varying degrees in the effluent of a sampled WWTP with a population equivalent of (PE) of 100,000. On some occasions no activity had been reported for α-glucosidase and β-glucosidase but activities as high as 2.26 and 0.87 mU/ml had also been recorded for the two respective enzymes. For esterase the highest obtained activity was 0.87 mU/ml. This was a similar pattern to the influent results with α-glucosidase yet again exhibiting the highest activities for Fischer et al. (2013). The minimum activity recorded for the three hydrolases was consistently above 0.50 mU/ml concerning the influent samples. Even though the results within Figure 6.3 do not compare with Fischer et al. (2013) there is a similarity with α-glucosidase having the greatest activity.

One explanation for the activities being so low could be that the wastewater samples were first passed through a 1.2 µM glass microfiber filter paper before any analysis was carried out. Compared to the literature, most studies do not filter the wastewater before inherent enzyme analysis (Goel et al., 1998) (Molina-Muñoz et al., 2010) (Fischer et al., 2013). The influent and effluent samples were filtered to allow absorbance of the formed coloured products to be measured over time. Measurement of absorbance would be impacted by the presence of the solids so they had to first be removed. The removed colloidal and suspended solids included bacterial flocs, which also exhibit enzymatic activity (Yu et al., 2008). Bacterial flocs are held together by a matrix of extracellular polymeric substances (EPS) that contain excreted enzymes. Hydrolases are commonly observed in this binding matrix as they are essential in breaking down the organic pollutants so that they can enter the bacterial cell and be mineralised (Burgess and Pletschke, 2008). The work following on from this scoping study moved onto analysing unfiltered wastewater instead to account for total enzyme activity.

To test the hypothesis that wastewater filtration reduced enzyme activity, a comparison was made between filtered and unfiltered wastewater samples. This investigation was carried out for influent and effluent samples with the addition of commercial α-glucosidase to a final concentration of $5x10^{-4}$ mg/ml. Wastewater samples were filtered through a 1.2 µM glass microfiber filter and α-glucosidase activity was measured. Unfiltered wastewater was also analysed for added α-glucosidase activity. Figure 6.4 shows a clear difference in activity between the unfiltered and filtered samples, suggesting that the wastewater particulates filtered off prior to the experimental work may have an enhancing influence on enzyme activity. There was a significant difference between activity for unfiltered and filtered as shown by the T-test p value (<0.05). Therefore the following work on inherent hydrolytic activity from wastewater centred on unfiltered wastewater samples in order to obtain a true understanding of enzyme activity with the WWTP.

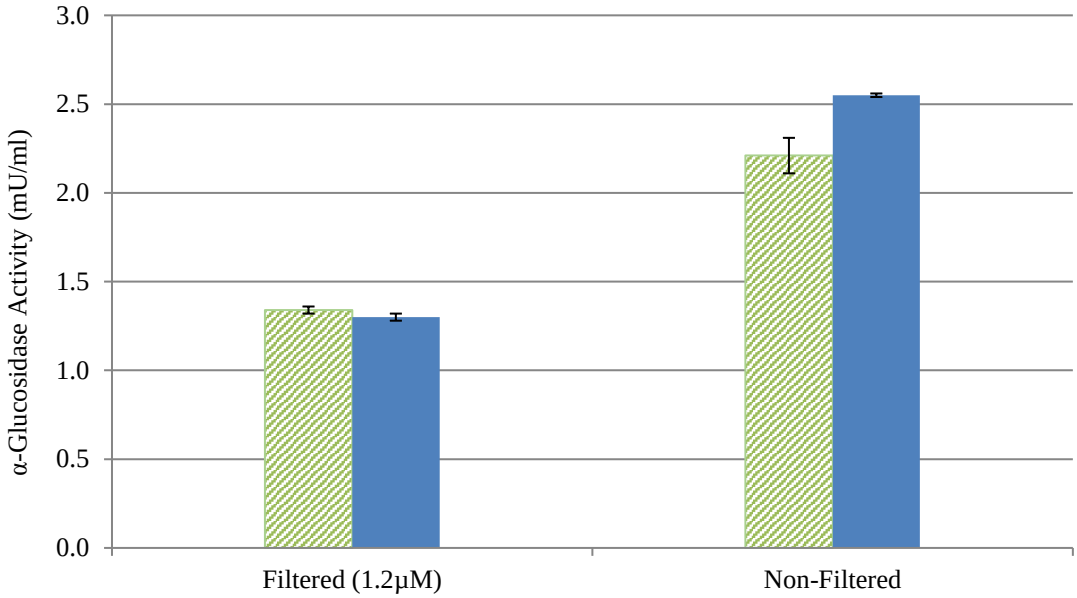


Figure 6.4 Activity of commercial α-glucosidase added to (▨) influent and (■) effluent samples taken from the Stoke Bardolph WWTP. Final enzyme concentration was $5x10^{-4}$ mg/ml in the wastewater samples. Influence of wastewater particulates on enzyme activity (mU/ml) was investigated (n=3).

Another potential cause for the low activities observed in the Stoke Bardolph influent and effluent was the use of 0.14 M NaCl to prepare the stock substrate solutions as per Fischer et al. (2013) and Kreutz et al. (2016), resulting in a concentration of 12.7 mM (0.74 g/L) NaCl in the final assay cuvette. From Table 6.1 it can be seen that the NaCl prepared substrate solutions had no effect on enzyme activity compared to the deionised waster dissolved substrates. The calculated *p* values only showed a significant difference in results for alkaline phosphatase as the activity was slightly higher for NaCl.

Table 6.1 Stoke Bardolph influent sampled on 17/02/17. The influence of adding 1 ml substrate solution, prepared in either deionised (DIW) or 0.14 M NaCl, to a 10 ml influent sample was studied (n=3).

Enzyme	Enzyme Activity (mU/ml)		T-test (*p value*)
	Substrate in 0.14 M NaCl	Substrate in DIW	
α-Glucosidase	0.05 (±0.00)	0.05 (±0.00)	0.772
β-Glucosidase	0.12 (±0.01)	0.12 (±0.01)	0.630
Alkaline Phosphatase	1.83 (±0.04)	1.71 (±0.01)	0.030
Sulfatase	0.02 (±0.01)	0.02 (±0.01)	0.935

6.3.1 Influence of Sonication on Enzyme Activity in Mixed Liquor and Returned Activated Sludge Samples

Before the inherent activity of the activated sludge samples could be analysed they first needed to be sonicated. This procedure was performed by Fischer et al. (2013) and Kreutz et al. (2016). The reason for sonicating the activated sludge samples is unclear in both of these papers. Other papers have not sonicated when analysing inherent activity for activated sludge samples (Li and Chróst, 2006). Activated sludge samples from conventional WWTP tend to be sonicated. Though the literature does not state why sonication was performed, it is hypothesised that sonication of the activated sludge samples most likely helped to break down these bacteria flocs, so that the enzymes contained in the extracellular polymeric substance matrix are more readily exposed. Table 6.2 shows for the four inherent enzymes their activity after sonication, compared to the sample not being sonicated.

Table 6.2 Comparison between sonication and no sonication on inherent enzyme activity (mU/ml) for mixed liquor and returned activated sludge samples taken from the Old Ford WWTP (n=3).

Mixed Liquor (Old Ford WWTP)			
Inherent Enzyme	Activity After Sonication (mU/ml)	Activity After No Sonication (mU/ml)	T-test (*p value*)
α-Glucosidase	2.99 (±0.10)	3.06 (±0.10)	0.455
β-Glucosidase	1.85 (±0.06)	1.80 (±0.03)	0.302
Alkaline Phosphatase	14.33 (±0.16)	13.37 (±0.28)	0.012
Sulfatase	4.48 (±0.17)	4.42 (±0.17)	0.706
Returned Activated Sludge (Old Ford WWTP)			
Inherent Enzyme	Activity After Sonication (mU/ml)	Activity After No Sonication (mU/ml)	T-test (*p value*)
α-Glucosidase	3.89 (±0.13)	4.02 (±0.05)	0.204
β-Glucosidase	2.47 (±0.01)	2.43 (±0.06)	0.397
Alkaline Phosphatase	20.91 (±0.23)	18.64 (±0.31)	0.001
Sulfatase	5.84 (±0.18)	6.02 (±0.14)	0.258

From Table 6.2 the difference in activity between mixed liquor and RAS samples is reported for the first time. Activity was constantly determined to be higher in the RAS than mixed liquor. Previous work had overlooked inherent activity derived from RAS so the effect of sonication was unknown. Comparing enzyme activity between sonication and no sonication also confirmed the increase in activity observed for the

RAS was due to the matrix composition and not sonication. All the enzymes apart from alkaline phosphatase showed no significant difference between sonication and no sonication as the T-test *p* values were higher than 0.05. Therefore sonication had no enhanced effect on inherent activity for the two glucosidases and sulfatase in the mixed liquor or RAS.

Alkaline phosphatase on the other hand did show a significant difference between sonication and no sonication as the *p* values were 0.012 and 0.001 for mixed liquor and RAS respectively. The difference between activities were 0.96 mU/ml for the mixed liquor and 2.27 mU/ml for the RAS. Calculated as percentage increases the differences equate to 7.2% and 12.1%. This shows that sonication leads to a similar increase in activity for the mixed liquor and RAS. The increase in RAS activity compared to the mixed liquor is due to matrix characteristics rather than the result of sonication. The activated sludge properties from a conventional WWTP are different to a MBR treatment process.

Bacterial floc sizes vary greatly between the two processes as the MBR system typically exhibits flocs sizes that range between 10 and 100 µm. A conventional WWTP system produces bacterial flocs that range in size between 100 and 500 µm (Cirja et al., 2008). The results given in Table 6.2 are from the Old Ford WWTP that incorporates a MBR system so the bacterial flocs are smaller and the enzymes more readily exposed. This could explain the little impact sonication had on the various samples apart from alkaline phosphatase. A greater difference would most likely by observed for wastewater samples taken from a conventional WWTP such as Stoke Bardolph where the bacterial flocs are larger and need to be disrupted to expose the enzymes. However, the hydrolytic activities from the Stoke Bardolph WWTP were only analysed from the sampled influent and effluent where sonication was not required.

6.3.2 Linearity of the Inherent Enzymatic Assays

The method for analysing inherent enzyme activity was performed by taking an assay sample at the end of the incubation period, which varied between 15, 30 and 60 minutes. It was unknown whether the absorbance was constantly linear during this period. This was important to consider, as the calculation for converting the end absorbance into enzyme activity presumes linear correlation. To check linearity,

kinetic analysis of the inherent enzymes was performed in the following samples influent, mixed liquor and RAS from the Old Ford WWTP. Multiple assays for the same enzyme were incubated at varying lengths of time with absorbance then measured, as shown in Figure 6.5 for α-glucosidase. This was also repeated for β-glucosidase, alkaline phosphatase and sulfatase. The results in Figure 6.5 were similar to that for all the other enzymes tested. No plateau in absorbance was seen with absorbance close to linear for all wastewater matrices confirming the reliable application of the activity calculations.

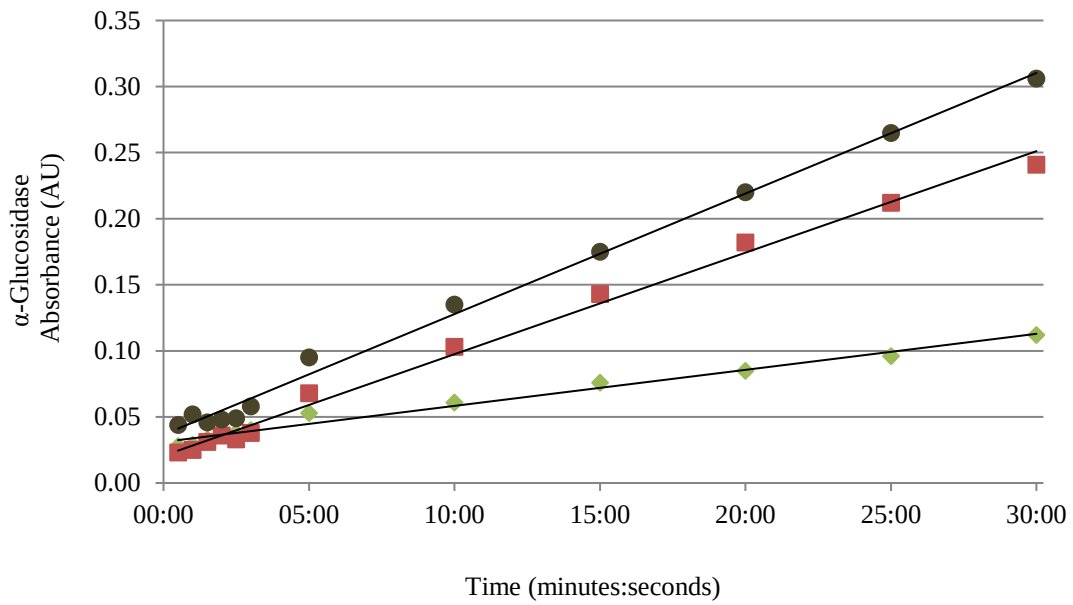


Figure 6.5 Inherent kinetic analysis of α-glucosidase in the (◆) influent, (■) mixed liquor and (●) returned activated sludge samples taken from the Old Ford WWTP on 06/05/2016 (n=1).

6.4 Influence of Wastewater and Clean Water Matrices on Commercial Inherent Enzymes

For each of the enzymes tested previously, the hydrolase activity detected in wastewater samples depends on the amount of enzyme present in the sample, and on the fraction of the enzyme that is active under the assay conditions. It is difficult to discriminate between these two parameters. Both the amount of enzyme present in the medium, and the enzyme activity have a separate dependence on wastewater conditions, which are complex and variable. To decouple activity from amount of enzyme, it is useful to determine the impact of wastewater on the activity of a known

amount of enzyme, externally added to the assay. Therefore, this section of work investigates the variable influence of wastewater on commercial hydrolases.

In wastewater, the source of hydrolases is mixed and depends on the microbial community in the process, as well as on the influent (Burgess and Pletschke, 2008). Throughout this section, when assessing the influence of wastewater on commercial hydrolases, the assumption is that hydrolases from the different sources will have similar response to changes in the environment. Wherever possible, the commercial hydrolases selected here are from microbial origin, to have the highest similarity to the one predominant in wastewater.

Activities of commercial hydrolases were determined in buffer, in deionised water and in wastewater samples from Stoke Bardolph and Old Ford WWTPs. The samples were taken on different days and at different points in the process, to assess the variability of the wastewater impact on enzyme behaviour. A control assay was performed to determine the background activity in the wastewater sample, and this subtracted from the sample activity in the results reported here. The same concentration for each enzyme was added to each tested activity assay. Phosphate buffer used was at pH 7.2, the average pH measured between the influent and effluent samples taken from the Stoke Bardolph WWTP.

6.4.1 Stoke Bardolph Added Inherent Enzyme Experimental Work

The results obtained with α-glucosidase are presented in Figure 6.6 and Table 6.3. For deionised water, specific activity could not be calculated as absorbance plateaued immediately resulting in no satisfactory linear absorbance. This shows that enzyme performance was poor in an unbuffered system. Similar activities were observed in influent and effluent samples. The activity in the phosphate buffer at pH 7.2 was slightly lower than the wastewater samples, for which pH was similar. From the literature there are no papers that suggest phosphate inhibits α-glucosidase activity. The procedure by Sigma-Aldrich to determine α-glucosidase activity is performed at a lower pH of 6.8 with a phosphate buffer.

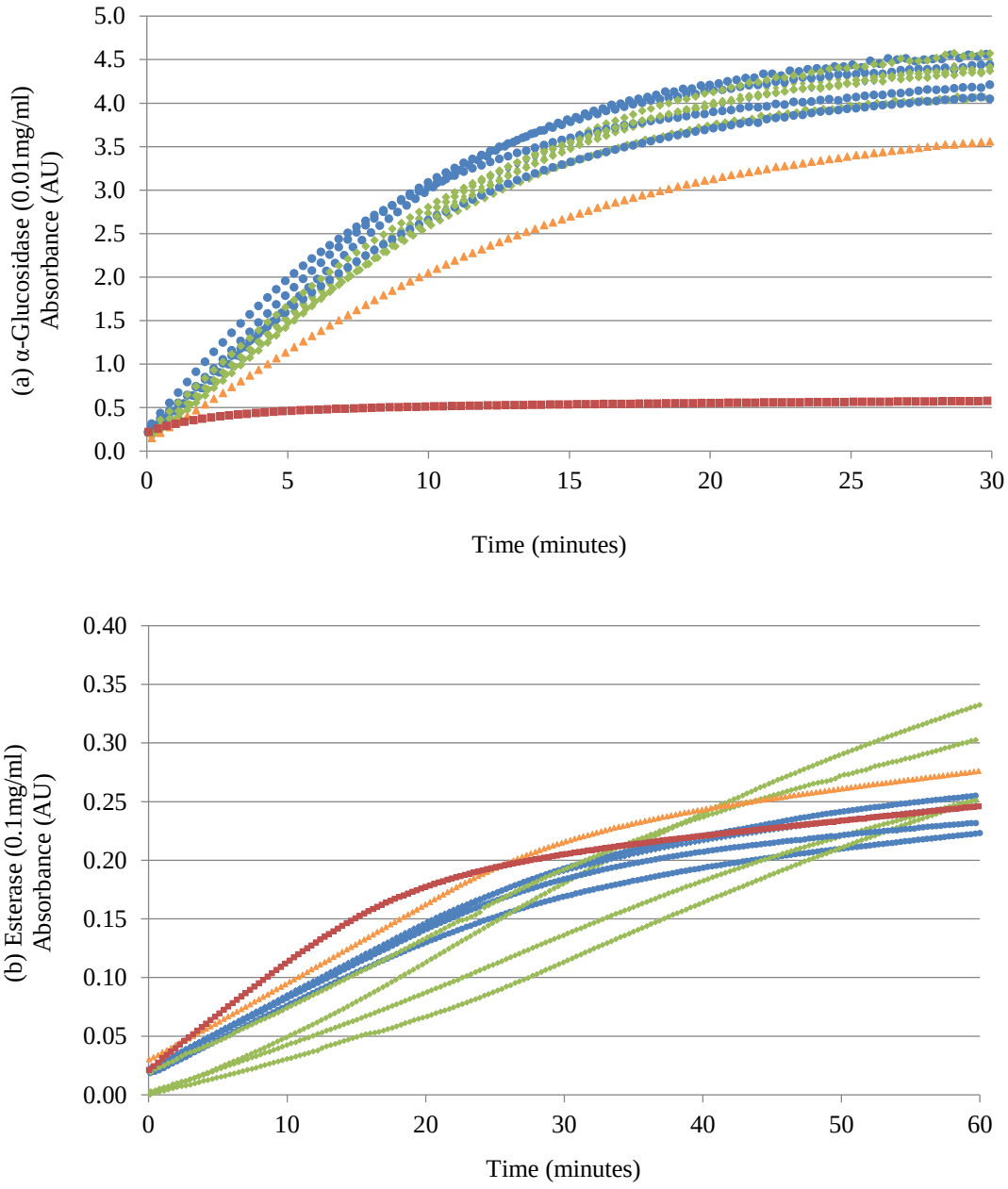


Figure 6.6 Abosrbance curves for commerical (a) α-glucosidase and (b) esterase added into wastewater samples collected from the Stoke Bardolph WWTP between January and Feburary 2016. Final enzyme concentration was 0.01 mg/ml and 0.1mg/ml respectively in all (◆) influent, (●) effluent, (▲) buffer and (■) deionised water samples. The influent and effluent absorbance curves are the average of triplicate assays carried out for each sampling day. Deionised water and buffer absorbance curves are the average for all assays performed in triplcate throughout the sampling period.

Table 6.3 Specific activity (mU/mg) for commercial α-glucosidase added into wastewater samples, deionised water and buffer solution at a final concentration of 0.1mg/ml (n=3). The effluent control showed no α-glucosidase activity without an external enzyme source.

Sample Date	α-Glucosidase (0.01 mg/ml) Specific Activity (mU/mg)			
	Influent	Effluent	Deionised Water	Buffer
25/01/2016	1.62 (±0.03)	1.59 (±0.02)	0.00 (±0.00)	1.15 (±0.11)
27/01/2016	1.51 (±0.03)	1.56 (±0.05)		
01/02/2016	1.55 (±0.01)	1.79 (±0.17)		
08/02/2016	1.46 (±0.05)	1.56 (±0.10)		
10/02/2016	1.68 (±0.04)	2.09 (±0.08)		

The acquired results for α-glucosidase indicate that both the influent and effluent samples exhibit a buffering potential. Furthermore, the buffering salts in the wastewater samples enhance α-glucosidase activity. Other mediators in the sampled wastewater could also be responsible for the observed increase in activity compared to the clean phosphate buffer matrix. From the five wastewater samples taken in Table 6.3 there is little difference in activity for the added commercial α-glucosidase between sampling locations and sampling days. Therefore inherent activity of this enzymes is more likely to vary because of the amount present in the medium rather than its intrinsic activity to wastewater conditions.

Figure 6.6 and Table 6.4 detail the results obtained with added commercial esterase. Unlike α-glucosidase, the specific activity for esterase was higher in deionised water than the buffer. This shows that esterase is less dependent on a buffering solution to achieve high activity. Additionally the effluent and buffer activity values were very similar. The influent for all sampling days had lower activity than the clean water matrices indicating an inhibitory action occurring. Table 9.1 in appendix A highlights potential inhibitors for esterase that will exhibit greater concentrations in the influent than the effluent. However, from Figure 6.6 it can be seen that the influent velocities remained almost linear during the 60 minutes time measurement. On the other hand the velocities decreased over time for both the effluent and clean water samples and they also began to plateau. It is difficult to establish a hypothesis on why the esterase added to the influent is not similarly inhibited considering the lower recorded activities.

Table 6.4 Specific activity (mU/mg) for commercial esterase added into wastewater samples, deionised water and buffer solution at a final concentration of 0.1mg/ml (n=3). The effluent control showed no esterase activity without an external enzyme source.

Sample Date	Esterase (0.1 mg/ml) Specific Activity (mU/mg)			
	Influent	Effluent	Deionised Water	Buffer
25/01/2016	0.66 (±0.05)	0.76 (±0.12)	1.27 (±0.14)	0.83 (±0.04)
27/01/2016	0.63 (±0.02)	0.85 (±0.02)		
01/02/2016	0.49 (±0.03)	0.84 (±0.01)		
08/02/2016	0.73 (±0.05)	0.84 (±0.03)		

Between the wastewater samples, the effluent activities varied far less than that exhibited for the influent. Whereas the wastewater matrix appeared to enhance α-glucosidase activity, the opposite occurred for esterase especially within the influent. When investigating inherent enzymes in the wastewater system Kreutz et al (2016) remarked on the significant difference in esterase behaviour to the other analysed hydrolases such as the two glucosidases and sulfatase. This trend was once again repeated with the enzyme being added to wastewater samples instead. Alkaline phosphatase was the next enzyme analysed and similar to α-glucosidase, showed greater activities in the wastewater samples than deionised water. To conclude, the wastewater environment would seem to have a non-negligible influence on esterase activity due to the variability observed between the different sampling days and locations.

Table 6.5 shows the results obtained for commercial alkaline phosphatase. The enzyme was only added to influent and effluent samples collected on a single sample day. Therefore no significant conclusions can be made. Nevertheless, specific activity in the buffer solution was considerably higher than the wastewater and deionised water samples. Similar to α-glucosidase, the activity of alkaline phosphatase improves in a buffered environment. The buffering potential for the influent and effluent samples was less optimal, compared to the phosphate buffer solution. Additional pollutants present in the wastewater samples may have had an inhibiting effect on activity. Yet again, specific activity in deionised water was significantly reduced. The influent and effluent activities did not differ by more than 10% between the two recorded values. Esterase showed the greatest disparity between the influent and effluent samples. From one sample day variability cannot be dismissed or confirmed. More wastewater

samples are required to determine the influence, if any, the wastewater environment has on alkaline phosphatase activity.

Table 6.5 Specific activity (mU/mg) for commercial alkaline phosphatase added into wastewater samples, deionised water and buffer solution at a final concentration of 0.005mg/ml (n=3). The effluent control showed no alkaline phosphatase activity without an external enzyme source.

Sample Date	Alkaline Phosphatase (0.005 mg/ml) Specific Activity (mU/mg)			
	Influent	Effluent	Deionised Water	Buffer
17/02/2016	0.75 (±0.00)	0.68 (±0.01)	0.16 (±0.02)	2.04 (±0.01)

The previous work discussing added commercial inherent enzymes was conducted at optimal assay conditions. In particular the temperature for the assays exceeded the typical temperature range associated with a WWTP in the UK. For example the esterase assay was performed at 20 ^{o}C whereas the assays for α-glucosidase and alkaline phosphatase were both carried out at 30 ^{o}C. The average temperature for sampled effluent from the Stoke Bardolph WWTP over a year period was 17 ^{o}C. Consequently, the used optimal assay temperatures do not accurately reflect the wastewater environment. Therefore the enzymatic assays were modified so that the added commercial inherent enzymes were analysed at conditions replicating an operational WWTP for both clean water and wastewater samples. The specific activities presented in Table 6.6 were derived from enzymatic assays all performed at 17 ^{o}C. In addition to this, the phosphate buffer solution represented by the "Wastewater Replicate" in Table 6.6 was lowered to a pH of 7.0. This change reflected the average pH for the sampled effluent at the Stoke Bardolph WWTP.

Table 6.6 Specific activities (mU/mg) for the commercial glucosidases, alkaline phosphatase, esterase and sulfatase added into wastewater samples taken from the Stoke Bardolph WWTP August 2016. "Wastewater Replicate" assays performed in 50mM phosphate buffer at pH 7 and at a temperature of 17 ^{o}C to replicate Stoke Bardolph wastewater conditions. All assays were carried out in triplicates (n=3). Effluent controls showed no activity for any of the five tested enzymes without an external enzyme source.

Sample Date	Enzyme	Concentration (mg/ml)	Specific Activity (mU/mg)		
			Influent	Effluent	"Wastewater Replicate"
01/08/16	α-Glucosidase	$0.5x10^{-3}$		7.41 (±0.03)	10.67 (±0.45)
	β-Glucosidase	0.02		–	–
	Alkaline Phosphatase	0.01		0.44 (±0.00)	0.13 (±0.00)
	Esterase	$0.5x10^{-3}$		3.33 (±0.64)	5.12 (±0.06)
	Sulfatase	0.1		0.11 (±0.00)	0.02 (±0.00)
03/08/16	α-Glucosidase	$0.5x10^{-3}$	8.77 (±0.02)		11.78 (±0.02)
	β-Glucosidase	0.02	–		–
	Alkaline Phosphatase	0.01	1.13 (±0.00)		0.16 (±0.00)
	Esterase	$0.5x10^{-3}$	4.88 (±0.06)		3.75 (±0.01)
	Sulfatase	0.1	0.18 (±0.01)		0.03 (±0.00)
08/08/16	α-Glucosidase	$0.5x10^{-3}$		–	–
	β-Glucosidase	0.02		1.39 (±0.01)	1.59 (±0.02)
	Alkaline Phosphatase	0.01		0.55 (±0.00)	0.11 (±0.00)
	Esterase	$0.5x10^{-3}$		–	–
	Sulfatase	0.1		0.03 (±0.00)	0.03 (±0.00)
10/08/16	α-Glucosidase	$0.5x10^{-3}$	–		–
	β-Glucosidase	0.02	1.15 (±0.01)		1.20 (±0.01)
	Alkaline Phosphatase	0.01	0.98 (±0.01)		0.17 (±0.00)
	Esterase	$0.5x10^{-3}$	–		–
	Sulfatase	0.1	0.07 (±0.00)		0.02 (±0.00)

The results presented for α-glucosidase in Table 6.6 conflict with the earlier data given in Table 6.3. Enzyme concentration was reduced in Table 6.6 from 0.01 to $0.5x10^{-3}$ mg/ml. Activity for α-glucosidase was higher in the "Wastewater Replicate" than the wastewater samples. Furthermore, there was a greater difference in activity between influent and effluent samples. A higher activity for α-glucosidase was recorded in the influent compared to the effluent. This result showed that when analysing at corrected conditions the wastewater environment did have a variable influence on enzymatic activity for the different sampling locations. From Table 9.1 in appendix A, magnesium and manganese have both shown to enhance α-glucosidase. The higher presence of such mediators in the influent could be responsible for the observed activity increase. The next enzyme investigated was β-glucosidase and from Table 9.1 in appendix A activity was shown also to be enhanced by magnesium and manganese.

However, the activities reported in Table 6.6 show little variation between the matrices with the influent in fact exhibiting the lowest activity.

Alkaline phosphatase showed far less activity in the "Wastewater Replicate" buffer solution compared to the higher temperature used within Table 6.5. There was however, a greater distinction in specific activity between the influent and effluent samples as activity was almost double in the influent. The higher pH associated with the influent is closer to the optimal assay condition for alkaline phosphatase. From Table 9.1 in appendix A optimal pH for alkaline phosphatase was determined to be at pH 9.5 by Janeway et al. (1993). Similar to α-glucosidase higher concentrations of mediators in the influent may also enhance activity. Unlike the previous results in Table 6.4, commercial esterase showed greater activity within the influent than the effluent. Only two sample days are available for added esterase so no repeating trend can be demonstrated. Interestingly, significant sulfatase activity was only observed in the wastewater samples. Higher activity was recorded in the influent but the effluent samples also showed greater buffering potential than the "Wastewater Replicate". To summarise, analysing at conditions reflective of a WWTP led to greater variability being observed.

For pure enzymes, the impact of the temperature on the activity can be estimated from first principles, using Arrhenius Law (see Equation 1 in appendix C). Temperature has a significant influence on enzymes as increasing the temperature up to a certain point before denaturation also increases activity. The Arrhenius equation detailed in appendix C can be used to predict the effect of a change of temperature on the rate constant and therefore activity. The theoretically calculated rate constant ratios between the different temperatures tested were compared to the experimentally observed rate constant ratios. For example, α-glucosidase, by applying a known activation energy of 38.3 kJ/mol determined using starch as a substrate in a sodium citrate buffer at pH 5 (Jadhav and Singhal, 2014), the change in activity can be estimated from 30 ^{o}C (optimal temperature) to 17 ^{o}C (WWTP environment) using the values presented in Table 6.7. The two temperatures were first converted to kelvin and then placed into Equation 2 (see appendix C), along with the activation energy to first calculate the rate constant ratio shown below.

$$\frac{k2}{k1} = e^{\frac{38300J/mol}{8.314J/mol}\left(\frac{1}{303K} - \frac{1}{290K}\right)}$$

$$\frac{k2}{k1} = e^{(-0.68)} \qquad \frac{k2}{k1} = 0.51$$

Figure 6.7 Calculation for the rate constant ratio for α-glucosidase from a starting temperature of 303K (30 ^{o}C) to 290K (17 ^{o}C).

From Figure 6.7 the theoretical rate constant ratio between k2 at 290K (17 ^{o}C) and k1 at 303k (30 ^{o}C) was calculated to be 0.51. The temperature difference is just over 10 ^{o}C and from the theoretical calculated rate constant ratio the reaction rate at 17 ^{o}C would be expected to be half that observed at 30 ^{o}C. A generalised rule associated with the Arrhenius equation is that for a temperature increase of 10 ^{o}C the rate of reaction doubles. This is reflected in the calculated rate constant of 0.51 as the decrease in temperature by slightly more than 10 ^{o}C indicated that the reaction rate would halve instead. The activity of α-glucosidase can be predicted at 17 ^{o}C from the activity obtained at 30 ^{o}C.

Table 6.7 Arrhenius equation used to calculate the theoretical rate constant ratio between the optimal assay temperature at 30 ^{o}C and averaged wastewater temperature at 17 ^{o}C for the commercial inherent enzymes. The experimentally observed rate constant ratio between the two temperatures was also calculated for comparison.

Enzyme	Concentration (mg/ml)	Matrix	pH	Temperature (^{o}C/ Kelvin)	Activity (mU/mg)	Activation Energy (kJ/mol)	Reaction Rate (k) (µM/s)	k2 (290K) / k1 (303K) (Theoretical)	k2 (290K) / k1 (303K) (Observed)
α-Glucosidase	$0.5x10^{-3}$	WW Replicate*	7.0	30^{o}C / 303K	17.72	38.3	0.15	0.51	
		WW Replicate*	7.0	17^{o}C / 290K	10.67		$87.6x10^{-3}$		0.58
		Influent	7.5	17^{o}C / 290K	8.77		$72.8x10^{-3}$		0.47
		Effluent	7.1	17^{o}C / 290K	7.41		$61.6x10^{-3}$		0.41
β-Glucosidase	0.02	WW Replicate*	7.0	30^{o}C / 303K	2.17	32.2	$71.6 x10^{-3}$	0.56	
		WW Replicate*	7.0	17^{o}C / 290K	1.29		$40.9x10^{-3}$		0.57
		Influent	7.4	17^{o}C / 290K	1.15		$38.6x10^{-3}$		0.54
		Effluent	7.4	17^{o}C / 290K	1.39		$46.3x10^{-3}$		0.65
Alkaline Phosphatase	0.01	WW Replicate*	7.0	30^{o}C / 303K	0.20	41.4	$33.7x10^{-3}$	0.47	
		WW Replicate*	7.0	17^{o}C / 290K	0.13		$22.2x10^{-3}$		0.65
		Influent	7.5	17^{o}C / 290K	1.13		0.19		5.64
		Effluent	7.1	17^{o}C / 290K	0.44		$72.7x10^{-3}$		2.15
Esterase	$0.5x10^{-3}$	WW Replicate*	7.0	30^{o}C / 303K	7.50	23.3	0.12	0.66	
		WW Replicate*	7.0	17^{o}C / 290K	3.33		$27.8x10^{-3}$		0.23
		Influent	7.5	17^{o}C / 290K	4.88		$79.3x10^{-3}$		0.66
		Effluent	7.1	17^{o}C / 290K	5.12		$85.6x10^{-3}$		0.71

*Wastewater Replicate (WW Replicate) was a 50mM phosphate buffer at pH 7.0

In Table 6.7 the activity of α-glucosidase in the "Wastewater Replicate" at 30 ºC was calculated to be 17.7 mU/mg. Therefore the activity of α-glucosidase can be estimated to be 9.0 mU/mg if added into the same phosphate buffer matrix but at a lower temperature of 17 ºC that reflects a WWTP environment. The experimentally determined activity for α-glucosidase at the same conditions was 10.7 mU/mg. The activation energy used in Figure 6.7 was derived from starch as the used substrate whereas the assays in Table 6.7 used 4-nitrophenyl-α-D-glucopyransoide as the substrate. This difference will cause a slight error in the calculations as the activation energies are unlikely to be identical.

As previously discussed the wastewater matrix is highly complex leading to variability. The Arrhenius Law can reliably model the response of enzymes to temperature changes in clean water matrices. However, the complex composition associated with the wastewater matrix and variation in pH interferes with the temperature kinetics. As well as calculating the theoretical rate constant ratios the experimentally observed rate constant ratios were additionally calculated from the reaction rates given in Table 6.7. The values were compared to see how similar they were. Differences in theoretical and experimentally observed rate constant ratios would indicate other wastewater influences impacting on activity. The greatest similarity was seen for esterase in Table 6.7 between theoretical and observed rate constant ratios. There was a significantly larger ratio difference for the change in temperature for the buffer samples than wastewater.

Greater variability was exhibited for the other enzymes especially between the influent and effluent for the experimentally observed rate constant ratios. This would suggest activity to be influenced by other factors such as pH variations, evolution of the wastewater through the treatment process and presence of mediators and inhibitors in the matrix. Alkaline phosphatase activity was shown to be considerably higher in the wastewater samples than the phosphate buffer matrix in Table 6.7 even when compared to activity analysed at 30 ºC. This is reflected by the experimentally observed rate constant ratios that were 5.64 and 2.15 for influent and effluent respectively. The Arrhenius Law was not followed as activity increased in the wastewater samples as temperature decreased compared to the "Wastewater Replicate". Behaviour of alkaline phosphatase cannot be predicted by the Arrhenius equation due to the significant impact of the wastewater matrix. Consideration has to

be taken into account that the Arrhenius parameters selected from the literature have used different buffers, substrates and enzyme sources that differ from the ones used in this study. Nevertheless, the Arrhenius equation can be used to indicate the expected impact of temperature on enzyme activity and identify where there is a contribution from other wastewater factors.

6.4.2 Commercial Hydrolases Added to Old Ford Wastewater

To assess whether the results obtained from the sampled Stoke Bardolph wastewater were representative of other WWTPs, the activity of commercial hydrolases was additionally analysed in wastewater samples taken from the Old Ford WWTP, which has a MBR system. The incubation temperatures were 20 ^{o}C for esterase and 30 ^{o}C for the other inherent hydrolases. The average absorbance curves were similar to those shown in Figure 6.6 and so the specific activities given in Table 6.8 are calculated from the initial linear part of the reaction. In addition to the influent and effluent samples, the screened sewage matrix was also investigated. The activity of β-glucosidase was not determined in these experiments, with the focus on a single glucosidase.

Similar to the results obtained from Stoke Bardolph in Table 6.6, there was a clear difference between the influent (raw sewage) and effluent (MBR output) in enzyme activity for α-glucosidase. However, Table 6.6 showed α-glucosidase activity to be higher in the influent at 8.77 mU/ml compared to 7.41 mU/ml in the effluent. A contrasting result is given in Table 6.8 as the influent (raw sewage) is instead the inhibiting matrix. Enzyme activity in the effluent for α-glucosidase was over double that recorded for the influent at the Old Ford WWTP. The other three commercial hydrolases also showed activity to be higher in the effluent than the influent and screened sewage. The differences between activities for alkaline phosphatase and esterase were less pronounced than for both α-glucosidase and sulfatase. This is highlighted in the percentage differences between the influent and effluent samples that are 3% and 20% for alkaline phosphatase and esterase respectively. The other hydrolyses α-glucosidase and sulfatase exhibit percentage differences that exceed 50%.

The results presented in Table 6.8 show that activity of the commercial hydrolases varied between the different sampled wastewater matrices and the two WWTPs. Results were obtained from a single sampling day so any continuing activity trends cannot be concluded. Sulfatase activity was never detected when analysed in the deionised water or buffer samples. Alkaline phosphatase also showed a significant reduction in activity by almost a factor of 10 when added to deionised water. From Table 6.6, a similar result was reported for sulfatase with greater activity seen in the wastewater samples from the Stoke Bardolph WWTP especially in the influent. Sulfatase activity in Table 6.8 increased as the wastewater matrix became cleaner progressing from the influent to screened sewage and then finally the MBR output. This is hard to explain as Table 6.6 showed an opposite result but the effluent (MBR output) from the Old Ford WWTP is treated to a high water quality reflected by the incredibly low COD and TSS readings. Therefore the MBR output would be similar to deionised water due to the minimal compositions. However, greater activity is shown in the MBR output. A buffering potential provided by the MBR output most likely increases enzyme activity, unlike with deionised water. Inhibitors within the influent may reduce the expected activity here. Compared to the Stoke Bardolph WWTP results in Table 6.6 the influent here would suggest that mediators are instead present and enhanced activity.

Table 6.8 Specific activities (mU/mg) for commercial α-glucosidase, alkaline phosphatase, esterase and sulfatase added into various wastewater samples collected from the Old Ford WWTP in May 2016. Only the influent showed activity in the control assays without an external enzyme source. All the sulfatase assays were performed in triplicates including alkaline phosphatase within the influent sample (n=3). The remaining enzymatic assays were all carried out in duplicates (n=2).

Enzyme	Specific Activity (mU/mg)				
	Influent (Raw Sewage)	Screened Sewage	Effluent (MBR Output)	Deionised Water	Buffer
α-Glucosidase (0.001 mg/ml)	1.46	–	3.05	2.00	4.00
Alkaline Phosphatase (0.005 mg/ml)	1.65 (±0.03)	–	1.70	0.18	–
Esterase (0.1 mg/ml)	0.53	–	0.66	–	–
Sulfatase (0.1 mg/ml)	0.014 (±0.000)	0.030 (±0.001)	0.039 (±0.002)	0.004 (±0.002)	0.000 (±0.000)

6.5 Inherent Enzyme Profile of the Old Ford WWTP

The results obtained previously with commercially added enzymes showed that the reaction environment, in this case the difference between the sampled influent and effluent matrices, can influence the activity of enzymes externally added into the system. This influence depends on the type of wastewater used (between WWTPs, or between different sampling points in the same WWTP), as well as on the time of sampling. In addition to the enzyme activity, the amount of enzyme present in the wastewater matrix is also influenced by these spatial and temporal factors. Therefore, in order to build a picture of how enzyme activity varies in a WWTP, an extensive investigation of hydrolases activity profile was performed at Old Ford. Additionally, enzymatic activities were correlated with wastewater parameters.

6.5.1 Enzyme Activity Distribution in the Activated Sludge Samples Taken Before and After the MBR System

The literature contains numerous studies investigating the distribution of inherent enzyme activity in activated sludge samples. Activated sludge samples are typically fractionated into several different parts to localise the major source of inherent hydrolytic activity. The different fractions include the supernatant, slime, loosely-bound EPS (LB-EPS), tightly-bound EPS (TB-EPS) and pellet (Yu et al., 2008). The majority of this work has focused on conventional WWTPs with far less on MBR systems. Furthermore, most of the published results have only investigated mixed liquor and not RAS. As a result the distribution of activity in the solid and liquid fractions of the RAS was analysed for alkaline phosphatase, sulfatase and the two glucosidases. Results were compared to the literature to see if the distribution of activity reflected mixed liquor. The RAS is concentrated mixed liquor with a higher solids concentration as shown in appendix D, Tables 9.8 to 9.9, therefore more activity would be expected to be associated with the solid fraction.

Figure 6.8 details the results of the study into the distribution of activity between the solid and liquid phases for sampled RAS at the Old Ford WWTP. The activity of the two phases was compared to the activity measured for the whole samples taken. Additionally, the difference in activity was compared between sonicated and non-sonicated samples. The reason for performing this comparison was to see if sample sonication broke down the bacterial flocs including the EPS matrix, releasing more

enzymes into the liquid phase. Figure 6.8 shows that there was no significant increase in activity for the liquid phase after sonication for any of the tested hydrolases. Furthermore, the majority of the activity was associated with the solid phase that represents the bacterial flocs and EPS matrix. It can be concluded that activity almost completely derives from the solid fraction therefore as the solid concentration increases so should enzyme activity. This observation compared well to the literature that also stated most extracellular enzymes are bound with cell flocs or organic matter in the sludge flocs (Li and Chróst, 2006).

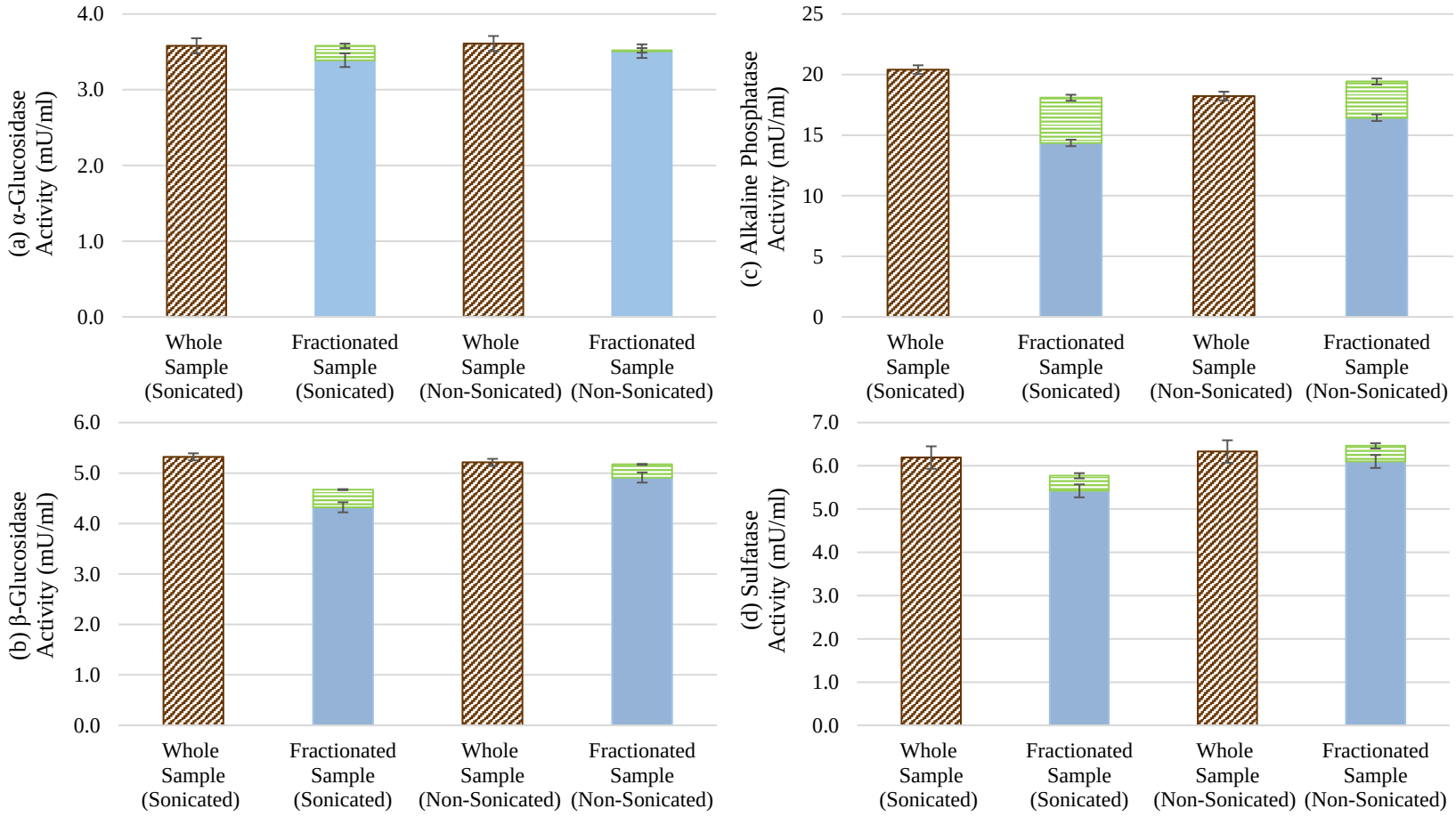
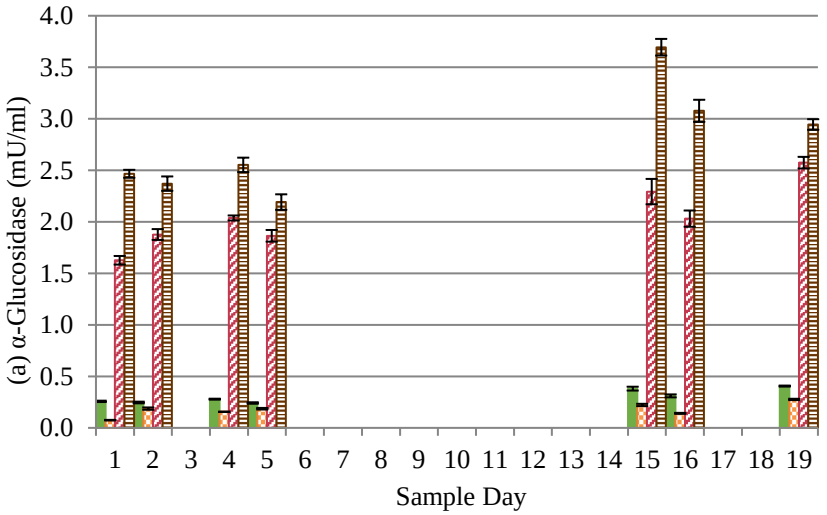


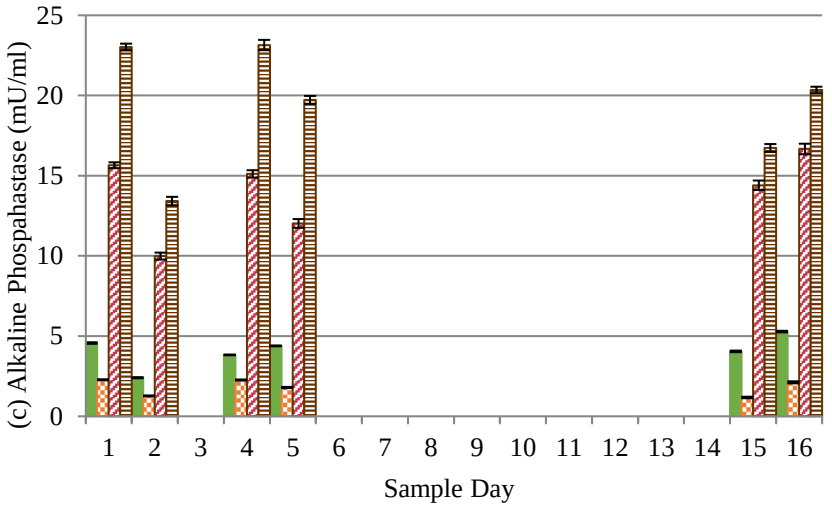
Figure 6.8 Inherent enzyme activity (mU/ml) distribution in the (■) solid and (▤) liquid fractions from a (▨) whole sample of returned activated sludge taken from the Old Ford WWTP in May 2016. The enzymes analysed were (a) α-glucosidase, (b) β-glucosidase, (c) alkaline phosphatase and (d) sulfatase (n=3).

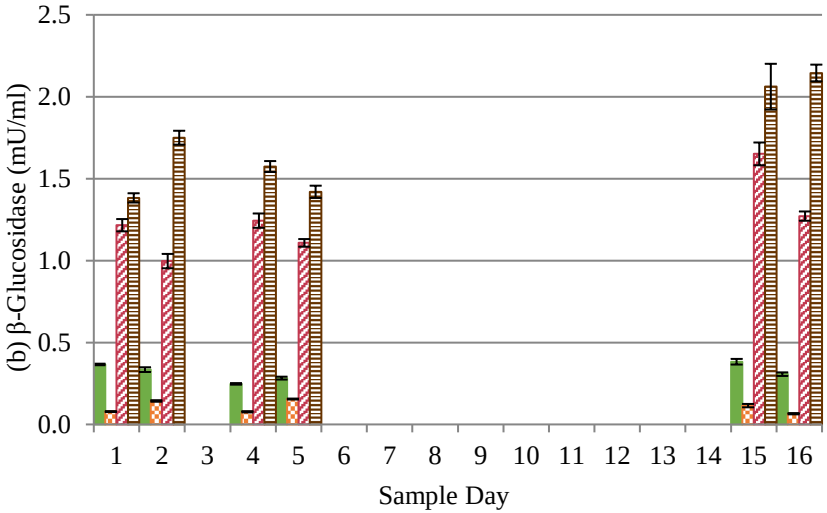
Alkaline phosphatase in Figure 6.8 showed the highest percentage of activity in the liquid phase. However, the contribution to overall enzyme activity was minimal with the other enzymes showing the liquid phase to contribute less than 5% to overall enzyme activity. A previous paper by Yu et al. (2008) investigated the distribution of extracellular enzyme activity for sludge flocs collected from 14 different WWTPs. One of those WWTPs had a MBR system and the enzymes analysed were protease, acid and alkaline phosphatase, α-amylase and α-glucosidase. Two of those enzymes were analysed in Figure 6.8. Both α-glucosidase and alkaline phosphatase activities originated mainly from the TB-EPS fraction agreeing with the results in Figure 6.8. Minimal amount of activity was seen in the supernatant or liquid phase. Acid phosphatase interestingly showed high presence in the supernatant accounting for almost 20% of overall enzyme activity.

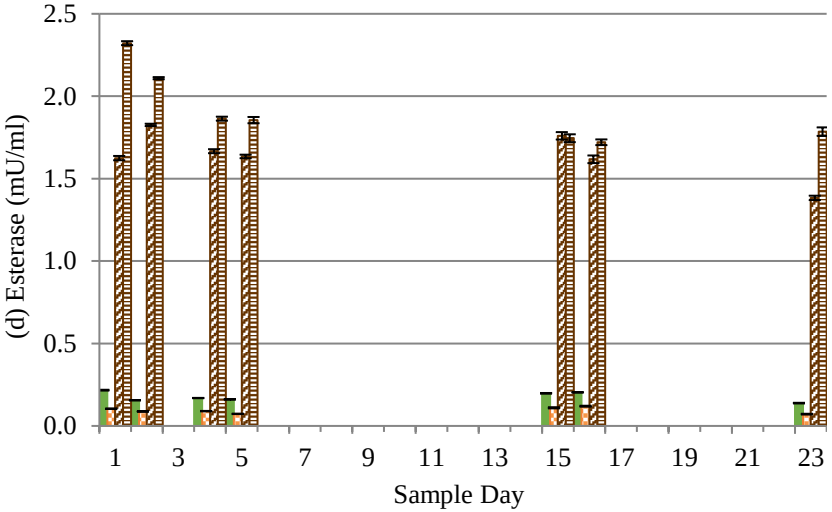
6.5.2 Spatial and Temporal Variability

This section compiles the previous experimental work in the chapter to enable the analysis of spatial and temporal variability in inherent enzyme activity. Five hydrolases; α-glucosidase, β-glucosidase, alkaline phosphatase, esterase and sulfatase, were all selected to investigate inherent enzyme activity. The activities of these hydrolases were for the first time analysed throughout a WWTP that had a MBR system being Old Ford. The five sampling locations were the influent, screened sewage, mixed liquor, RAS and MBR output. Activity profiles for each inherent enzyme were generated for the different sampling days and locations. Figure 6.9 shows that alkaline phosphatase consistently gave the highest enzyme activity readings also reported by Fischer et al. (2013) and Kreutz et al. (2016). The trend in activity for the sampling locations followed as RAS > mixed liquor > influent > screened sewage > MBR output. Sulfatase activity was never observed in the influent or screened sewage, but the activities recorded in mixed liquor and RAS were usually greater than α-glucosidase and β-glucosidase. Unlike sulfatase, the other inherent enzymes all exhibited activity that stemmed from the sewage, either from a bacterial source or human discharge, as activity was regularly detected in the influent. Sulfatase activity only originated from the activated sludge. For all enzymes spatial and temporal variability was observed, highlighting the complex nature of wastewater.


(a) α-Glucosidase (mU/ml)
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19
Sample Day


(c) Alkaline Phospahastase (mU/ml)
25
20
15
10
5
0
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
Sample Day


(b) β-Glucosidase (mU/ml)
2.5
2.0
1.5
1.0
0.5
0.0
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
Sample Day


(d) Esterase (mU/ml)
2.5
2.0
1.5
1.0
0.5
0.0
1 3 5 7 9 11 13 15 17 19 21 23
Sample Day

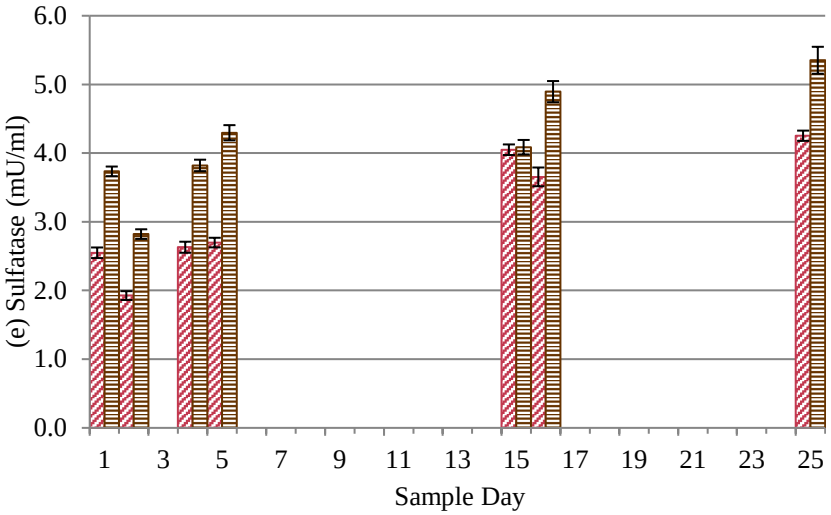


Figure 6.9 Inherent enzyme activity (mU/ml) profiles for the sampled wastewater (■) influent (▩) screened sewage (▨) mixed liquor (▤) returned activated sludge at the Old Ford WWTP between May 2016 and June 2016 (n=3). Sulfatase activity was not detected in any of the influent or screened sewage samples. The inherent enzymes analysed were (a) α-glucosidase, (b) β-glucosidase, (c) alkaline phosphatase, (d) esterase and (e) sulfatase.

The next series of tables compare the activities for each inherent enzyme across the sampling period and treatment process. All samples were taken between May 2016 and June 2016 over a 3.5 week period. In total there are 6 sample days when the inherent enzymes were all assayed on the same day between days 1 and 16. For α-glucosidase, esterase and sulfatase additional activity measurements were taken. Sampling days were arranged when the WWTP was fully operational and planned around other experiments. As well as showing enzyme activity as the suspended volume assayed (mU/ml), the activity was also corrected against solid concentration for mixed liquor and RAS samples (mU/mg TSS). The previous section had shown that the solids in both of these matrices contributed significantly to enzyme activity. Correcting for the solid concentration normalised the activity values, as RAS not previously investigated would be expected to show higher activity due to a greater solids concentration. If the corrected activities for the mixed liquor and RAS are not the same then other factors are influencing enzyme activity and not the solids concentration.

Figure 6.9 shows the generated enzymatic profile for α-glucosidase over the different sampling days. It can be seen that RAS activity is far higher than the mixed liquor activity especially on days 15 and 16. This can in part be explained by a higher solid concentration as detailed within appendix D. By correcting the activity for solid concentration in Table 6.9 the impact of this factor is negated. Is the ratio between solid concentration for mixed liquor and RAS reflected in the recorded enzyme activities? Table 6.9 shows that enzyme activity corrected against solid concentration narrows the difference between the two matrices. Performed T-tests showed that there was no significant difference between the matrices on day 2, 4 and 5. Therefore, difference in activity in Figure 6.9 for the matrices on those days was mainly due to an increased solids concentration. Difference in activity for the other sample days was due to other influences. Measuring the standard water qualities in addition to analysing inherent activity allows correlations to be made against environmental and process influences. These correlations are discussed later in the chapter but for this section the trends in spatial and temporal variability are first highlighted.

Table 6.9 The activities for α-glucosidase corrected against the measured solid concentrations (mU/mg TSS) for both mixed liquor and returned activated sludge (RAS) samples collected from the Old Ford WWTP between May and June 2016 (n=3).

Inherent Enzyme		Sample Day (Old Ford WWTP)						
		1	2	4	5	15	16	19
Mixed Liquor α-Glucosidase	Solids Concentration (mg/L)	6020	5916	6186	6360	7150	7442	7474
	Activity (mU/mg TSS)	1.35 ±0.03	1.59 ±0.04	1.65 ±0.02	1.47 ±0.04	1.60 ±0.09	1.36 ±0.05	1.72 ±0.04
RAS α-Glucosidase	Solids Concentration (mg/L)	7344	7592	8080	7574	9104	9900	9486
	Activity (mU/mg TSS)	1.68 ±0.03	1.56 ±0.05	1.58 ±0.04	1.45 ±0.05	2.03 ±0.04	1.55 0.05	1.55 ±0.03
RAS Activity Increase (%)		24.3	-1.6	-4.1	-1.3	26.5	13.9	-9.9
T- Test (*p* value)		0.00	0.54	0.10	0.65	0.00	0.01	0.00

Figure 6.9 shows the results obtained for β-glucosidase over the conducted sampling period. Similar to α-glucosidase the highest RAS activities were observed towards the end of the sampling period on days 15 and 16. This higher activities coincide with increasing solid concentration throughout the sampling period, as detailed within appendix D. The TSS concentrations are also lower in the screened sewage, compared to the influent, reflecting this trend in activity. The removal of TSS also removes any bacterial flocs that contribute to activity. Table 6.10 shows the activities corrected against solid concentration for the mixed liquor and RAS. The T-tests showed that there was no significant difference for sample days 4 and 15. For α-glucosidase there was also no significant difference reported for sample day 4, confirming that solid concentration was a significant contributor to the increase in activity between the mixed liquor and RAS on that day. Other influences were impacting on enzyme activity for the other days. The correlations discussed later on identify any shared enzyme behaviour.

Table 6.10 The activities for β-glucosidase corrected against the measured solid concentrations (mU/mg TSS) for both mixed liquor and returned activated sludge (RAS) samples collected from the Old Ford WWTP between May and June 2016 (n=3).

Inherent Enzyme		Sample Day (Old Ford WWTP)					
		1	2	4	5	15	16
Mixed Liquor β-Glucosidase	Solids Concentration (mg/L)	6020	5916	6186	6360	7150	7442
	Activity (mU/mg TSS)	1.01 ±0.03	0.84 ±0.04	1.01 ±0.04	0.87 ±0.02	1.15 ±0.05	0.85 ±0.02
RAS β-Glucosidase	Solids Concentration (mg/L)	7344	7592	8080	7574	9104	9900
	Activity (mU/mg TSS)	0.94 ±0.02	1.15 ±0.03	0.97 ±0.02	0.94 ±0.02	1.13 ±0.08	1.08 ±0.03
RAS Activity Increase (%)		-6.8	36.8	-3.1	7.6	-1.9	26.8
T- Test (*p* value)		0.04	0.00	0.27	0.02	0.69	0.00

Alkaline phosphate activity, as presented in Figure 6.9 showed that this enzyme gave the highest recorded activities. Compared to the other enzymes at the same sampling locations, the activity was almost tenfold higher. Unlike for α-glucosidase and β-glucosidase, the highest enzyme activities relating to the RAS matrix were observed nearer the start of the sampling period. Even when the results are corrected for solid concentration in Table 6.11 enzyme activity is still greater for the sampling days 1, 4 and 5. This is an interesting result as RAS inherent activities have not been previously published. Alkaline phosphatase activity was higher at the lower solid concentrations whereas the glucosidases showed greater activity at the higher solid concentrations. Furthermore, the two glucosidase showed less variation in mixed liquor and RAS activity. On day 2 in Table 6.11 there is a noticeable drop in enzyme activity compared to the surrounding days. No reasons can be given for this large decrease in activity until correlations are made with the measured standard water qualities later on in this chapter.

Table 6.11 The activities for alkaline phosphatase corrected against the measured solid concentrations (mU/mg TSS) for both mixed liquor and returned activated sludge (RAS) samples collected from the Old Ford WWTP between May and June 2016 (n=3).

Inherent Enzyme		Sample Day (Old Ford WWTP)					
		1	2	4	5	15	16
Mixed Liquor Alkaline Phosphatase	Solids Concentration (mg/L)	6020	5916	6186	6360	7150	7442
	Activity (mU/mg TSS)	26.00 ±0.30	16.88 ±0.37	24.43 ±0.37	18.91 ±0.43	20.15 ±0.41	22.40 ±0.44
RAS Alkaline Phosphatase	Solids Concentration (mg/L)	7344	7592	8080	7574	9104	9900
	Activity (mU/mg TSS)	31.35 ±0.29	17.68 ±0.35	28.65 ±0.40	26.05 ±0.33	18.37 ±0.27	20.55 0.21
RAS Activity Increase (%)		20.6	4.7	17.3	37.8	-8.8	-8.2
T- Test (*p* value)		0.00	0.05	0.00	0.00	0.01	0.01

Esterase activities from Figure 6.9 appear to be less variable compared to the other enzymes over the sampling period. The literature has commented before that esterase behaviour is different to the other commonly assayed inherent hydrolases such as alkaline phosphatase, glucosidases and sulfatase (Kreutz et al., 2016). This is further illustrated in Table 6.12 with the activities corrected for solid concentration. Unlike the other enzymes mixed liquor activity is consistently higher than RAS activity with the exceptions being on days 1 and 23. No significant difference was reported for day 23 between the two matrices. From these results alone it would it seem that esterase activity is enhanced in the mixed liquor matrix. This is surprising as the difference in matrix between mixed liquor and RAS should not be that contrasting. The Old Ford WWTP stages are tightly contained, therefore the sampling locations are close together and do not cover large distances. Consequently, there is not enough time for matrices to dynamically evolve in the distribution network, for example, conditions becoming anaerobic. Once again highlighting the importance in characterising wastewater to better understand the cause for such a difference to be seen.

Table 6.12 The activities for esterase corrected against the measured solid concentrations (mU/mg TSS) for both mixed liquor and returned activated sludge (RAS) samples collected from the Old Ford WWTP between May and June 2016 (n=3).

Inherent Enzyme		Sample Day (Old Ford WWTP)						
		1	2	4	5	15	16	23
Mixed Liquor Esterase	Solids Concentration (mg/L)	6020	5916	6186	6360	7150	7442	8250
	Activity (mU/mg TSS)	1.35 ±0.01	1.54 ±0.01	1.35 ±0.01	1.29 ±0.01	1.23 ±0.02	1.09 ±0.02	0.84 0.01
RAS Esterase	Solids Concentration (mg/L)	7344	7592	8080	7574	9104	9900	10786
	Activity (mU/mg TSS)	1.58 ±0.01	1.39 ±0.00	1.15 ±0.01	1.22 ±0.01	0.96 ±0.01	0.87 ±0.01	0.83 ±0.01
RAS Activity Increase (%)		17.1	-10.0	-14.4	-4.7	-22.1	-20.0	-1.3
T- Test (*p* value)		0.00	0.00	0.00	0.00	0.00	0.00	0.27

Sulfatase as shown by Figure 6.9 only showed activity in the mixed liquor and RAS samples. There was no activity observed in either the influent or screened sewage. Therefore it would imply that sulfatase originates from the wastewater biological treatment stage being the activated sludge. As a consequence, sulfate bioactive conjugates in the sewage network will remain whole until the compound comes into contact with the activated sludge, resulting in the parent compound to be released. In Table 6.13, for the corrected solid concentrations there is a significant increase in mixed liquor activity over RAS on sample day 15. The percentage increases on that sample day was -21%. Esterase was the only other hydrolase to show such high percentage decreases. Later correlations may explain the cause for this unusual percentage decrease compared to the two glucosidases and alkaline phosphatase. From the performed T-tests there was no significant difference between activities on sample day 23. The reported differences for the other sample days was due to additional influences apart from solids concentration.

Table 6.13 The activities for sulfatase corrected against the measured solid concentrations (mU/mg TSS) for both mixed liquor and returned activated sludge (RAS) samples collected from the Old Ford WWTP between May and June 2016 (n=3).

Inherent Enzyme		Sample Day (Old Ford WWTP)						
		1	2	4	5	15	16	25
Mixed Liquor Sulfatase	Solids Concentration (mg/L)	6020	5916	6186	6360	7150	7442	8236
	Activity (mU/mg TSS)	2.12 ±0.06	1.63 ±0.05	2.13 ±0.07	2.12 ±0.06	2.83 ±0.05	2.46 ±0.09	2.58 ±0.05
RAS Sulfatase	Solids Concentration (mg/L)	7344	7592	8080	7574	9104	9900	10346
	Activity (mU/mg TSS)	2.54 ±0.05	1.86 ±0.05	2.36 ±0.05	2.84 ±0.07	2.24 ±0.06	2.47 ±0.08	2.59 ±0.09
RAS Activity Increase (%)		20.1	14.1	11.2	33.7	-20.7	0.7	0.2
T- Test (*p* value)		0.00	0.01	0.01	0.00	0.00	0.82	0.95

The literature can be used to inform on factors that might be responsible for enzyme variability over time at the different process locations. Even though the majority of literature is focused on activated sludge (mixed liquor), findings may also reflect the results from Old Ford WWTP. The only paper to have analysed inherent activity throughout an WWTP before was (Fischer et al., 2013) but compared to Old Ford the WWTP did not have MBR system and RAS was not analysed. Measuring RAS inherent activity is a novel addition compared to other published studies and is important to consider especially for an MBR system. In a conventional treatment process and an MBR system the RAS is recycled back into the system. The higher efficiency of ultrafiltration offered by the MBR system prevents any of the sludge separating. Consequently, a greater amount of enzyme activity is retained, therefore it is important to understand its behaviour in the treatment process and later impact on removing organic pollutants.

The paper by Reboleiro-Rivas et al. (2013) investigated inherent activity for the treatment of real urban wastewater by a moving bed membrane bioreactor (MRMBR system). Hydrolases studied were acid phosphatase, alkaline phosphatase and α-glucosidase for both suspended and attached biomass. Multiple parameters were analysed alongside this work including COD, biological oxygen demand at 5 days (BOD_5), mixed liquor volatile suspended solid concentration (MLVSS), biofilm total solids (BTS), hydraulic retention time (HRT), pH, carrier filling ratios (CFR) and

temperature. From those listed factors CFR, MLVSS, BTS, COD, temperature, and HRT were all identified to have a significant relationship with enzyme activity. Relating to the next section wastewater parameters or standard water qualities were analysed at Old Ford to allow such an evaluation to be made. Figures 6.6 and Tables 6.9 to 6.13 only give a partial story to enzyme variability. The publication by Gómez-Silván et al. (2013) took a similar approach. Seasonal variation was observed for the hydrolytic activities of acid and alkaline phosphatase, α-glucosidase and protease sampled from aerated and anoxic phases of a full-scale MBR. Variation in inherent activity was highlighted but the following factors temperature, composition of wastewater, and biomass accumulation in bioreactors were said to be responsible for this variation occurring.

6.5.3 Old Ford Inherent Enzyme Activities Correlated with the Measured Operational Wastewater Parameters

For every sample collected at the Old Ford WWTP, a set of standard water quality parameters were also measured. Pearson's correlation was used to correlate the inherent hydrolytic activities to the corresponding standard water qualities. Temperature, dissolved oxygen (DO), chemical oxygen demand (COD), total suspended solids (TSS), non-temperature corrected electrical conductivity (EC) and pH were the measured standard water qualities. These various parameters in the literature have been noted as key process conditions that inform on the overall operational state of a WWTP. Furthermore the results from these standard water quality tests could be generated on the same day of sampling. Correlating inherent hydrolytic activity to the standard water quality parameters furthers the understanding on the biological performance of a WWTP to remove organic pollutants including bioactive chemicals (Dorival-García et al., 2014).

Pearson's correlation was used to correlate the two variables together as well as looking at any relationships between the enzymes. The literature informed on the statistical approach to be taken as similar papers had used Pearson's correlation to correlate similar variables. For example the paper by Molina-Muñoz et al. (2010) correlated volatile suspended solid (VSS) concentration with inherent activity by Pearson's correlation. As well as Pearson's correlation, Spearman's rank can also be utilised to perform a similar function. The recent paper by Kreutz et al. (2016) used

Spearman's rank to correlate inherent activity with several process factors. For choosing between the two approaches the data first needs to be tested to see if it is normally distributed. If the data is normally distributed than Pearson's correlation is selected over Spearman's rank as is the case for the Old Ford generated data. The calculated Pearson's correlation coefficients can also be classified to inform on the strength of correlation from 'weak' to 'very strong' first detailed by Evans (1996). Defining correlation strength on the scale proposed by Evans (1996) allows for a heat map to be produced as shown in Tables 6.14 to 6.16.

Table 6.14 Pearson's coefficients for the correlation of inherent enzyme activity with measured standard water quality parameters for the influent (RS), screened sewage (SS), mixed liquor (ML) and returned activated sludge (RAS) sampled at the Old Ford WWTP between May and June 2016. The measured standard water quality parameters were pH, temperature (Temp), dissolved oxygen (DO), chemical oxygen demand (COD), total suspended solids (TSS) and electrical conductivity (EC). *p value < 0.05

Coefficient Range	-1.000 -0.800	-0.799 -0.600	-0.599 -0.400	-0.399 -0.200	-0.199 0.199	0.200 0.399	0.400 0.599	0.600 0.799	0.800 1.000
Correlation Strength	(-) Very Strong	(-) Strong	(-) Moderate	(-) Weak	No Correlation	(+) Weak	(+) Moderate	(+) Strong	(+) Very Strong

Old Ford WWTP (α-Glucosidase)						
	pH	Temp (ºC)	DO (mg/L)	COD (mg/L)	TSS (mg/L)	EC (µs/cm)
RS	0.565	0.648	-0.614	0.369	0.770*	0.790*
SS	0.337	0.433	-0.156	0.693	-0.216	0.225
ML	0.802*	0.731	-0.555	–	0.779*	-0.274
RAS	0.408	0.818*	-0.679	–	0.773*	0.057

Old Ford WWTP (β-Glucosidase)						
	pH	Temp (ºC)	DO (mg/L)	COD (mg/L)	TSS (mg/L)	EC (µs/cm)
RS	0.734	0.382	-0.911*	-0.052	0.493	0.292
SS	-0.458	-0.471	-0.722	0.191	-0.303	-0.381
ML	0.352	0.811*	-0.539	–	0.662	0.120
RAS	0.712	0.736	-0.579	–	0.915*	0.196

Old Ford WWTP (Alkaline Phosphatase)						
	pH	Temp (ºC)	DO (mg/L)	COD (mg/L)	TSS (mg/L)	EC (µs/cm)
RS	0.342	0.700	-0.289	0.954*	0.815*	0.873*
SS	-0.100	-0.254	0.829*	-0.096	-0.114	0.184
ML	0.416	0.619	-0.492	–	0.539	-0.008
RAS	-0.099	-0.178	0.029	–	-0.048	-0.322

Old Ford WWTP (Esterase)						
	pH	Temp (ºC)	DO (mg/L)	COD (mg/L)	TSS (mg/L)	EC (µs/cm)
RS	0.796*	0.772*	-0.942*	0.589	0.092	0.841*
SS	0.241	0.856*	-0.198	0.139	0.073	0.779*
ML	-0.141	0.676	0.147	–	-0.690	0.154
RAS	-0.901*	0.099	0.140	–	-0.733	0.478

Old Ford WWTP (Sulfatase)						
	pH	Temp (ºC)	DO (mg/L)	COD (mg/L)	TSS (mg/L)	EC (µs/cm)
ML	0.593	-0.034	-0.557	–	0.935*	0.079
RAS	0.560	-0.375	-0.428	–	0.817*	-0.210

The Pearson's correlations coefficients in Table 6.14 were calculated by the statistical software package Minitab allowing relationships between the inherent activities and standard water quality parameters to be evaluated. From Table 6.14 it can be seen that most of the determined correlations are positive, indicating that enzyme activity increased as the parameter values increased. A negative correlation details an inverse relationship where activity increases as the parameter value decreases. To expand on the discussion given in section 7.5.2 esterase activity was noticed to increase when TSS concentration decreased for both the mixed liquor and RAS matrices. Table 6.14 confirms this trend as the four other hydrolyses show a positive correlation with TSS concentration. Esterase on the other hand exhibits a strong negative correlation with TSS concentration therefore demonstrating that esterase activity does not benefit from an increased bacterial count. Increased VSS concentration and its effect on inherent enzyme activity has been investigated by Molina-Muñoz et al. (2007) and Molina-Muñoz et al. (2010) in a pilot scale MBR system. VSS concentration is linked to biomass concentration and therefore enzyme activity was expected to increase with VSS.

The inherent enzymes analysed in both papers were acid and alkaline phosphatase, glucosidase, protease and esterase. However, for all these enzymes the associated activity tended to decrease as VSS concentration increased. Reasons given for this were enzymatic decay at high VSS concentrations and inhibition feedback from the increased products formed with a larger biomass. Esterase activity at the Old Ford WWTP may be influenced by similar mechanisms as described in these papers. The later paper by Molina-Muñoz et al. (2010) also analysed TSS concentration but no correlations were made with the recorded inherent activities. Another interesting conclusion from this paper was that the optimal temperature for the tested enzymes was between 17 ^{o}C and 23 ^{o}C. The only exception to this observation was for α-glucosidase that showed higher activity at lower temperatures ranging from 7 ^{o}C to 13 ^{o}C. All the work performed by Molina-Muñoz et al (2007 & 2010) analysed activated sludge samples.

From Table 6.14 temperature showed no negative correlation with α-glucosidase and in most other cases activity increased with temperature. The only occasions were negative temperature correlations were observed was for β-glucosidase and alkaline phosphatase in screened sewage and sulfatase in the RAS. Correlation strengths for

alkaline phosphatase and sulfatase were both weak whereas β-glucosidase was moderate. These correlation strengths indicate no significant influence. Temperature over the sampling period did not vary widely with the highest and lowest values not differing by more than 2 degrees. The influent did however exhibit a greater temperature variation with 16.4 ^{o}C being the lowest recorded temperature and 19.5 ^{o}C the highest. From these measurements temperature is more variable for the influent compared to the other matrices. Once the wastewater had entered the Old Ford treatment process the temperature became more consistent and regulated. Sampling was not carried the whole year through so the change in process temperatures between seasons was unknown.

Other published studies have analysed inherent activities throughout the different seasons to determine the associated variability. Acid and alkaline phosphatase, α-glucosidase and protease activities were analysed from activated sludge samples collected from a pilot scale MBR during spring, summer and autumn (Gómez-Silván et al., 2013). The temperature change was noticeable between seasons especially for summer and autumn where the average temperature difference was 10 ^{o}C. The temperature increase led to higher enzyme activities. To extrapolate these results to Old Ford and from the correlation coefficients within Table 6.14 activity is heavily influenced by temperature therefore lower activities would have been observed in winter for example. This effect on inherent activities may be more emphasised in the influent, as there is less process control for this matrix. A longer sampling period is required to accurately inform on seasonal variation.

Gómez-Silván et al. (2013) additionally showed that, from the enzymes analysed, the generated activity profiles were dynamic and independent of each other. Therefore analysing only one hydrolytic enzyme would give a poor overview on WWTP performance. Positive correlations were observed for α-glucosidase with both VSS and TSS as well as for acid phosphatase not studied at Old Ford. Table 6.14 shows α-glucosidase to be positively correlated to TSS concentration as well. The VSS correlation conflicts with the results presented within the earlier papers by Molina-Muñoz et al. (2007 & 2010). Similar wastewater parameters measured by Cortés-Lorenzo et al. (2012) were correlated to the hydrolytic activities of acid and alkaline phosphatase, glucosidase, protease and esterase in a submerged fixed bed bioreactor. From the factors measured salinity and sampling day were the only factors that

significantly contributed to the observed enzyme variability. Other parameters tested were pH, TSS, COD and BOD_5. It is difficult to make a comparison between these different papers as the process configurations and size are not alike. However, it does appear that the influence of the different measured wastewater parameters is not always shared between WWTPs.

The most intriguing result from Table 6.14 was that increased dissolved oxygen (DO) appeared to decrease enzyme activity. Especially prominent for β-glucosidase and esterase activity in the influent. Both glucosidases showed negative correlations for DO across the different matrices sampled. Alkaline phosphatase activity in screened sewage was the only case where a significant positive correlation with DO was observed. Sulfatase additionally exhibited negative correlation with DO for both mixed liquor and RAS. Two papers have been published that investigated the source of aeration on inherent activity. Dorival-García et al. (2014) studied inherent activity before and after an MBR had been injected with pure oxygen. The improvement in spiked BRA removal was also analysed. For the enzymes tested acid and alkaline phosphatase, α-glucosidase and esterase to a smaller extend positively responded to pure oxygen aeration. For acid and alkaline phosphatase and α-glucosidase activity was close to 100% higher in the pure oxygen aeration compared to air aeration. The use of pure oxygen was said to increase microbial activity.

The other paper by Calderón et al. (2013) additionally investigated the influence of inherent activities in a MBR supplied with either air or pure oxygen. The same hydrolases were assayed as in the paper by Dorival-García et al. (2014). Acid phosphatase was the only enzyme to show a significant increase in activity when aerated with pure oxygen over air. The other enzymes performed showed no significant differences either positive or negative. The authors also commented on an earlier paper by Goel et al. (1998) that analysed α-glucosidase and protease activities in the aerobic, anoxic and anaerobic phases of an activated sludge bench-scale system that also showed no significant differences. However, factors found to influence activity by Calderón et al. (2012) were as previously mentioned in other studies temperature and VSS. The literature does not explain or even give examples of increased oxygen levels decreasing hydrolytic activity. The negative correlation is repeated across the different matrices in Table 6.14 showing it is not specific to one sample location.

Other significant correlations that can be viewed from Table 6.14 include the negative correlation of pH with esterase activity in the RAS. Esterase activity and pH also showed a significant correlation in the influent but this was positive and not negative. The influent parameters within appendix D show that the pH was more alkaline compared to the other matrices as their values were closer to neutral pH. As a result esterase activity appears to favour alkaline conditions. The general pH trend for the inherent enzymes in Table 6.14 was that activity increased with pH. From appendix D, Tables 9.5 to 9.9 it can be seen that the pH ranges for the sampled matrices did not widely fluctuate. Over such a tight pH range enzyme activity is not going to change that dramatically. As well as the measuring the standard water quality parameters the composition of the wastewater can also result in enzyme variability. Certain humic acids and heavy metals are known to influence extracellular enzyme activity by inhibitory mechanisms (Gómez-Silván et al., 2013).

6.5.4 Correlation between the Inherent Enzymes at the Various Old Ford Sampling Locations

The papers by Fischer et al. (2013) and Kreutz et al. (2016) highlight the limited analysis performed in the literature on correlating activity between the different inherent hydrolases. No other published studies have assayed inherent hydrolytic activity for RAS samples. Therefore, the enzyme correlations presented in Table 6.15 are unique for RAS. In a study by Gómez-Silván et al. (2013) α-glucosidase activity was shown to be negatively correlated to protease activity. Protease activity was not analysed at the Old Ford WWTP but all the other enzymes can be correlated against each other. Table 6.15 shows that the correlations between the enzymes varied for each sampled matrix. For example the influent solely showed positive correlations with strong correlations exhibited for esterase between alkaline phosphatase and β-glucosidase. The screened sewage had a larger proportion of negative correlations represented by alkaline phosphatase showing strong negative correlations with both glucosidases. In addition to this there was also a strong negative correlation between esterase and β-glucosidase. This is in contrast to the strong positive correlation reported for the influent. Different influences within the two matrices impact on enzyme activity.

Table 6.15 Pearson's coefficients for the correlation of inherent enzymatic activity in the sampled influent, screened sewage, mixed liquor and returned activated sludge from the Old Ford WWTP between May and June 2016. The inherent enzymes analysed were α-glucosidase (α-GLU), β-glucosidase (β-GLU), alkaline phosphatase (ALP), esterase (EST) and sulfatase (SUL). *p value < 0.05

Coefficient Range	-1.000 -0.800	-0.799 -0.600	-0.599 -0.400	-0.399 -0.200	-0.199 0.199	0.200 0.399	0.400 0.599	0.600 0.799	0.800 1.000
Correlation Strength	(-) Very Strong	(-) Strong	(-) Moderate	(-) Weak	No Correlation	(+) Weak	(+) Moderate	(+) Strong	(+) Very Strong

Old Ford WWTP (Influent)					
	α-GLU	β-GLU	ALP	EST	SUL
α-GLU		0.456	0.243	0.459	–
β-GLU	0.456		-0.004	0.677	–
ALP	0.243	-0.004		0.600	–
EST	0.459	0.677	0.600		–
SUL	–	–	–	–	

Old Ford WWTP (Screened Sewage)					
	α-GLU	β-GLU	ALP	EST	SUL
α-GLU		0.632	-0.795	-0.210	–
β-GLU	0.632		-0.673	-0.707	–
ALP	-0.795	-0.673		0.026	–
EST	-0.210	-0.707	0.026		–
SUL	–	–	–	–	

Old Ford WWTP (Mixed Liquor)					
	α-GLU	β-GLU	ALP	EST	SUL
α-GLU		0.722	0.139	0.355	0.720
β-GLU	0.722		0.521	0.024	0.879*
ALP	0.139	0.521		-0.675	0.602
EST	0.355	0.024	-0.675		-0.192
SUL	0.720	0.879*	0.602	-0.192	

Old Ford WWTP (Returned Activated Sludge)					
	α-GLU	β-GLU	ALP	EST	SUL
α-GLU		0.817*	-0.178	-0.556	0.396
β-GLU	0.817*		-0.419	-0.653	0.366
ALP	-0.178	-0.419		0.075	0.470
EST	-0.556	-0.653	0.075		-0.658
SUL	0.396	0.366	0.470	-0.658	

Furthermore, the correlations between enzymes in the mixed liquor and RAS were additionally contrasting. A significant positive correlation was observed for β-glucosidase and sulfatase in the mixed liquor. This result was not reflected within the RAS matrix similar to the strong negative correlation seen for esterase and alkaline phosphatase activity. However, for both matrices a strong positive correlation was observed between the two glucosidases and was significant in the RAS. A similar strong correlation between the two glucosidases was reported in the study by Kreutz et al. (2016). This result was expected due to the high functional relationship of the enzymes. Alkaline phosphatase also exhibited strong correlation with the other enzymes tested by Kreutz et al. (2016) including α-glucosidase, β-glucosidase and phosphodiesterase. These discussed inherent enzyme activities were obtained from activated sludge samples. The same correlation for alkaline phosphatase cannot be seen in Table 6.15 as there is no enzyme that is strongly correlated with all enzymes apart from sulfatase in the mixed liquor. These results yet again show the dynamic nature of wastewater and how environmental and process influences can differ throughout a WWTP.

6.6 Stoke Bardolph Inherent Enzymatic Profile

Temporal and spatial inherent enzyme variability was also investigated at the Stoke Bardolph WWTP. Unlike at Old Ford only influent and effluent samples were collected from Stoke Bardolph. The sampling period was also considerably longer to account for greater changes in the measured standard water quality parameters. The sampling period as detailed in Figure 6.10 for the recorded alkaline phosphatase activities in the influent was over a four month period between February 2017 and June 2017. The sampling trips were once again organised between Severn Trent and the University of Nottingham depending on personnel availability. The extended sampling period allowed for any seasonal variation to be observed as the seasons transitioned from winter to spring. However, the change in temperature for influent was no more than 4^{o}C as the average temperature in February was 10.7 ^{o}C and 13.9 ^{o}C for May. This can explained by the samples always collected in the morning before 10 am representing the cooler part of the day. For all the hydrolases tested no activity was observed in the effluent similar to at Old Ford. Additionally no sulfatase activity was detected in any of the influent samples collected. Temporal variability demonstrated by alkaline phosphatase in Figure 6.10 was also observed for α-glucosidase, β-

glucosidase and esterase in the influent matrix. The activity for alkaline phosphatase was yet again the highest.

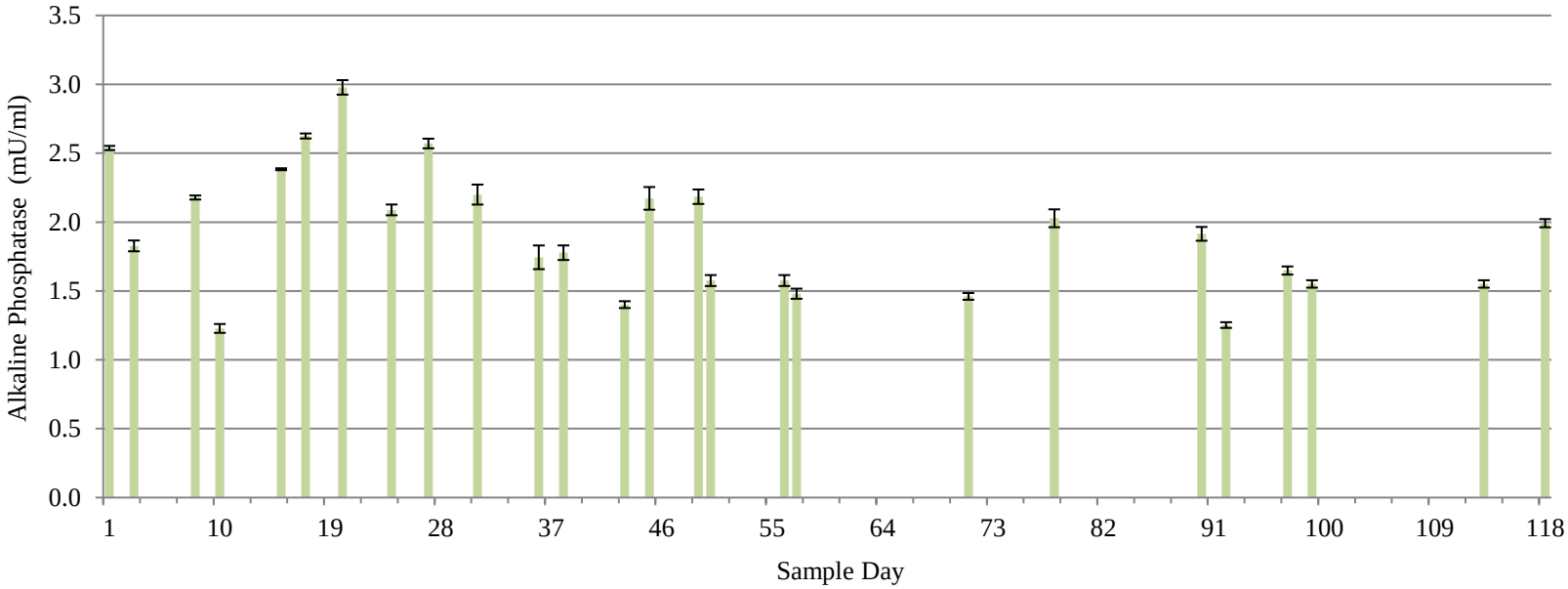


Figure 6.10 Alkaline phosphatase activity (mU/ml) profile for the influent sampled from the Stoke Bardolph WWTP between February and June 2017 (n=3).

Pearson's correlation was also performed between the inherent enzyme activities and measured standard water quality parameters as shown in Table 6.16. There were no significant relationships observed for the influent characteristics and inherent activities. Compared to the work conducted at the Old Ford WWTP additional parameters were measured at Stoke Bardolph and these were chloride concentration (Cl) and total dissolved solids (TDS). Temperature and pH showed positive correlations for all the enzymes apart from alkaline phosphatase. Neither correlation was classified as strong but there was an inverse relationship between these two variables. Molina-Muñoz et al. (2010) had shown α-glucosidase to produce higher activity readings at temperatures below 13 ^{o}C. Nothing similar has been reported for alkaline phosphatase in the literature as most studies have shown activity to increase with temperature. As previously mentioned the temperature range for the sampled influent did not vary widely therefore the effects are not going to be drastic. A moderate positive correlation was seen for α-glucosidase activity and DO unlike Old Ford where a strong negative correlation was shown. The strongest correlations were calculated for TSD and EC. The two glucosidases showed negative correlation whereas alkaline phosphatase and sulfatase exhibited positive correlations. This once again confirms the dynamic nature of wastewater and the different influences on enzyme behaviour.

Table 6.16 Pearson's coefficients for the correlation of inherent enzymatic activity in the sampled influent from the Stoke Bardolph WWTP and with the measured standard water quality parameters between February and June 2017. Inherent enzymes analysed were α-glucosidase (α-GLU), β-glucosidase (β-GLU), alkaline phosphatase (ALP) and esterase (EST). The measured standard water quality parameters were pH, temperature (Temp), chemical oxygen demand (COD), total suspended solids (TSS), total dissolved solids (TDS), electrical conductivity and chloride concentration (Cl). *p value < 0.05

Coefficient Range	-1.000 -0.800	-0.799 -0.600	-0.599 -0.400	-0.399 -0.200	-0.199 0.199	0.200 0.399	0.400 0.599	0.600 0.799	0.800 1.000
Correlation Strength	(-) Very Strong	(-) Strong	(-) Moderate	(-) Weak	No Correlation	(+) Weak	(+) Moderate	(+) Strong	(+) Very Strong

Stoke Bardolph WWTP (Influent)				
	α-GLU	β-GLU	ALP	EST
α-GLU		0.200	-0.177	0.192
β-GLU	0.200		-0.105	0.211
ALP	-0.177	-0.105		0.044
EST	0.192	0.211	0.044	

Stoke Bardolph WWTP (Influent)								
Enzyme	pH	Temp (°C)	DO (mg/L)	COD (mg/L)	TSS (mg/L)	TDS (g/L)	EC (mS/cm)	Cl (mg/L)
α-GLU	-0.085	0.501	0.492	-0.023	0.019	-0.581	-0.585	-0.284
β-GLU	0.131	0.311	0.148	0.094	0.427	-0.481	-0.453	0.181
ALP	-0.343	-0.406	-0.396	0.440	0.167	0.433	0.382	0.339
EST	0.442	0.416	0.130	-0.028	0.230	0.477	0.463	0.036

The activities for each enzyme were also correlated against one another as first presented in Table 6.16 for the Old Ford WWTP. A positive correlation was observed for the two glucosidases as previously described for Old Ford and by Kreutz et al. (2016). Esterase and β-glucosidase activity was additionally shown to be a positive correlation similar to the strong correlation reported for Old Ford. However, these correlations for Stoke Bardolph were both classified as weak so conclusions are not completely reliable. If this research was continued then activated sludge samples would also be taken. The authors from the paper by Kreutz et al. (2016) have studied temporal variability of inherent hydrolytic activity from activated sludge samples collected a period of one year. The WWTP sampled had a conventional treatment process. They used Spearman's rank to correlate the enzyme activities for esterase (EST), α-glucosidase (α-Glu), β-glucosidase (β-Glu), L-alaine-aminopeptidase (LAA), phosphatase (PME), phosphodiesterase (PDE) and sulfatase (SUL) with measured process parameters.

As well as correlating the activated sludge hydrolytic activities to the process parameters of the analysed matrix Kreutz et al. (2016) additionally made correlations to the influent and effluent process parameters. Table 6.16 shows that the influence of the different matrix parameters on similar inherent hydrolases are dynamic and also independent of each other. Process parameters cannot be accurately applied to other sampled matrices to determine enzyme behaviour. Significant correlations for the activated sludge process parameters were observed for the following activities PME, PDE, SUL, LAA, α-GLU and β-GLU with sludge volume. All enzymes showed significant correlation with sludge dry matter. Other positive correlations included SUL activity with sludge index. As highlighted previously esterase appears to be behave differently to the other common hydrolases. The only negative correlation reported was for esterase with sludge index. It would be interesting to see if similar findings would be observed at the Stoke Bardolph WWTP. Characterisation of the wastewater performed in this chapters allows environmental and process factors to be distinguished on their overall influence on enzyme behaviour.

6.7 Conclusions

- Activity of the analysed inherent enzymes α-glucosidase, β-glucosidase, alkaline phosphatase, esterase and sulfatase was shown to be highly variable across a WWTP and between sampling days. The activity exhibited by the returned activated sludge (RAS) was investigated for the known first time at an operational WWTP.
- Separation of the RAS sample into liquid and solid phases identified the majority source of the inherent enzymes. The activities for all four enzymes stemmed almost completely from the solid fraction of the RAS sample. Consequently, the higher solids concertation associated with the RAS was expected to lead to higher enzyme activities. This was not seen for esterase where activity was higher in the mixed liquor than the RAS matrix. Previous literature had commented on the unusual behaviour of esterase compared to other hydrolases.
- Correction of inherent hydrolytic activity with the measured solids concentration suggested that other environmental and process factors influenced activity. The addition of commercial inherent hydrolases to wastewater samples also showed that the various wastewater matrices had a variable influence on enzyme activity. Added commercial sulfatase activity was enhanced in the wastewater matrix with no activity recorded in either deionised water or phosphate buffer.
- Characterisation of the sampled wastewater by the different standard water quality parameters highlighted WWTP influences that contribute to variable inherent activity temporally and spatially. Pearson's correlation identified positive and negative relationships between the standard water quality parameters and activity. These correlations showed each inherent enzyme behaved independently of each other and also each wastewater matrix had different influences on activity. Consequently, the standard water quality parameters had varying relationships to enzyme activity and so the effects could not be applied holistically. However, increased DO concentrations did result in decreased activity.

CHAPTER 7

Conclusions and Recommendations for Future Work

7.1 Conclusions

The main aim of this work was to investigate the influence the complex nature of wastewater has on enzyme response and behaviour, reflecting other biotechnological processes where a biological component is responsible for converting raw feedstock into a final product. Biological performance, in this case measured by enzyme activity can be affected by numerous environmental and process factors leading to variable output. Understanding this variability associated with enzyme activity allows processes to adapt accordingly and minimise variable biological performance. Enzymes external to the wastewater system were studied in addition to enzymes already present in the system. Laccase and tyrosinase were the external enzymes selected as both are being explored as alternative treatment technologies to remove endocrine disrupting compounds (EDCs). Hydrolases were chosen to represent the inherent enzymes as they are critical in breaking down the majority of organic pollutants.

Chapter 4 justified the selection of enzymes and substrates to identify and determine variable enzyme activity in the wastewater environment. For the external enzymes their activity was indirectly assayed by the removal of E1 to relate to their proposed application within a wastewater treatment plant (WWTP). Control studies were performed to confirm that any reported EDC removal was the result of the enzymes, rather than degradation by the inactivation methods or sample filtration. Inherent enzymes were introduced and a gap in knowledge was shown to exist for understanding how activity transpires across an operational WWTP. Almost all the literature previously had focused on activated sludge samples taken from benchscale sludge processes.

The laccase and tyrosinase enzymatic parameters performed in Chapter 5 showed that EDC removal and so enzyme performance varied temporally for the different effluent samples taken. Previous papers had not considered this temporal wastewater effect before when investigating the tertiary application of laccase and tyrosinase. Pearson's correlation with the standard water quality parameters indicated that temperature, total

dissolved solids (TDS) and electrical conductivity (EC) significantly influenced laccase activity. The removal of estrone (E1) by tyrosinase was shown to be enhanced with increased chloride (Cl^-) concentrations. For the first time E1 was shown to be removed by tyrosinase in both a clean buffer matrix and sampled wastewater. Removal of E1 in the clean buffer matrix was significantly reduced compared to the sampled effluent for tyrosinase, showing unknown mediator processes. Inhibitor and additive effects on laccase and tyrosinase activity were also investigated to determine their impact at conditions relevant to the wastewater environment. Copper (Cu^{2+}), for example at a concentration that reflected wastewater, inhibited tyrosinase activity. Therefore the extraction of Cu^{2+} from the wastewater before tyrosinase treatment would greatly improve EDC removal.

Inherent enzyme activity was analysed in Chapter 6 with activity assayed throughout the Old Ford WWTP. The measured enzyme activity for the returned activated sludge (RAS) had not been previously discussed in the literature. For the five hydrolases analysed their activity was shown to be regularly independent of each other. The standard water quality parameters taken for each sampled wastewater matrix also had varying effects on activity for each inherent enzyme. Therefore a single hydrolase cannot be used to inform on the behaviour of similarly classed enzymes. The same conclusion was given for laccase and tyrosinase where the two enzymes also shared very few similarities even though both are copper containing oxidoreductases. The addition of the commercial hydrolases to sampled wastewater from the two WWTPs also showed the wastewater characteristics to have a noticeable impact on activity. No variability in activity for the commercial hydrolases would have suggested inherent activity to be more indicative of the bacterial population. Pearson's correlation once again highlighted the significant relationships between activity and the standard water quality parameters. Interestingly, there appeared to be a repeating trend for dissolved oxygen (DO) where increased DO concentrations resulted in lower enzyme activities.

- **The developed laccase and tyrosinase inactivation methods did not result in further degradation of the chosen EDCs. Furthermore, E1 and DCF were shown not to be adsorbed by the used filters prior HPLC injection.**

The experimental procedure for determining EDC removal by laccase and tyrosinase had to first be evaluated to assure any reported EDC removal was the

result of enzymatic degradation. The used inactivation methods, addition of hydrochloric acid (HCl) or sodium hydroxide (NaOH), provided an extremely rapid means to stop the enzymatic reactions. However, the addition of HCl led to significant DCF degradation that was almost 51%. The addition of NaOH was adopted instead to prevent this happening. Other inactivation methods in the literature such as adding methanol or heat denaturation were not adopted as the enzymatic reactions do not stop instantly and the sample dilution is too great. Reliable inactivation methods were developed for analysing both E1 and DCF removal. The abiotic removal of the substrates by the used 0.2 µm regenerated cellulose filters was additionally analysed and shown to be minimal.

- **High temporal variability reported for the removal of E1 by laccase and tyrosinase in effluent sampled from the Stoke Bardolph WWTP over a sampling period of four months. Standard water qualities characterised the sampled effluent to identify the process and environmental factors responsible for the observed variability.**

The removal of E1 by laccase in a clean water and wastewater matrix had been well documented before in the literature. Nevertheless, very few papers have considered the temporal change in wastewater and its subsequent impact on enzymatic EDC removal. A novel finding was E1 removal by tyrosinase not previously discussed before in the literature. The measured wastewater characteristics, the standard water quality parameters, informed on the temporal change in wastewater. The E1 removal (%) studied alongside these parameters showed the variable response of laccase and tyrosinase to these changes.

E1 removal over the four month sampling period was shown to vary for both laccase and tyrosinase. For example the highest reported E1 removal for laccase was 86.6% and the lowest was 54.4% detailing a difference of 32.2%. Tyrosinase also exhibited a large difference between the highest and lowest E1 removal, which was 33.7%. Considerable variability in performance occurred, therefore a result taken from one wastewater sample on one day does not represent the overall removal rate.

- **Correlation of E1 removal by laccase and tyrosinase with the measured standard water quality parameters identified the environmental and process factors that influence enzyme performance, and result in high variability.**

A range of environmental and process factors were measured to determine their influence on laccase and tyrosinase activity. Determining the factors that significantly influence laccase and tyrosinase performance can inform on enzyme activity in real time. For example the temperature of the sampled effluent from the Stoke Bardolph WWTP had a significant relationship with laccase performance in removing E1. Increased temperature resulted in greater E1 removal exhibiting a positive correlation. The other significant correlations for laccase were both negative for TDS and EC. Tyrosinase on the other hand only showed weak correlations and they were for chemical oxygen demand and chloride (Cl^-). Correlation between laccase and tyrosinase E1 removal was additionally weak during this four month sampling period indicating that both enzymes respond differently to the wastewater environment. Consequently, laccase cannot be used to inform on tyrosinase behaviour as well as the other way around if combined enzymatic treatments are applied, even though they are both copper containing oxidoreductases.

- **Determined the impact two persistent wastewater pollutants, Cu^{2+} and Cl^- ions, have on laccase and tyrosinase activity including EDC mixtures. Enzyme performance was reduced by the presence of Cu^{2+} and Cl^- at environmentally relevant concentrations.**

Two known pollutants within wastewater, Cu^{2+} and Cl^-, had been shown to be inhibitors for laccase and tyrosinase within the literature. The majority of the work that exhibited this adverse effect had utilised analytical substrates and experimental conditions not representative of the wastewater environment, for example, acidic pH values and temperatures beyond 20 ^oC. The impacts of the Cu^{2+} and Cl^- ions were instead examined in sampled wastewater at relevant wastewater conditions. Tyrosinase with added Cl^- showed a slight increase in E1 removal with Cl^- concentration as there was a 6% E1 removal increase at 500 mg/L compared to the control and a 9% increase for the buffer. For tyrosinase there was high

inhibition, almost 20% less E1 removal compared to the performed control, at the copper concentration of 0.05 mg/L. Laccase showed similar inhibition but only at the highest copper concentration of 10 mg/L. Therefore the tertiary application of tyrosinase would heavily benefit from the removal of copper first from the wastewater matrix.

The removal of EDCs by laccase and tyrosinase is commonly analysed as single compounds. The removal of EDC mixtures are rarely studied and the additive effects overlooked. For example the addition of DCF to the laccase and tyrosinase enzymatic parameters already containing E1 showed improved removal for DCF with E1. This result was only observed for laccase as tyrosinase exhibited no removal of DCF with or without E1. The wastewater matrix contains multiple EDC substrates therefore this mixture effect needs to be further investigated for both enzymes. Finally the difference in E1 removal between a clean buffer matrix and sampled effluent wastewater was pronounced for tyrosinase. The wastewater matrix on one sample day showed almost 60% higher E1 removal than the phosphate buffer solution. The literature has not shown tyrosinase activity to be enhanced in wastewater before with the mediator properties needing to be determined.

- **Inherent hydrolytic activity associated with the sampled returned activated sludge (RAS) was discussed for the first time.**

The literature investigating inherent enzyme activity has mostly focused on activated sludge. Activity relating to the RAS has not been explored and is especially important for the Old Ford WWTP that incorporates a MBR system, recycling almost all the mixed liquor unlike conventional WWTPs. From the selected hydrolases the higher solids concentration associated with the RAS resulted in higher enzyme activities. This applied to all the enzymes apart from esterase that is known to behave differently to the other hydrolases (Kreutz et al., 2016). From examining where enzyme activity derived from in the RAS almost all originated from the solid fraction, therefore, initially indicating increased solid concentrations would lead to higher enzyme activities. Enzyme activity corrected for the solids concentration showed that additional factors influenced inherent

enzyme activity, otherwise the activities would have been identical between the mixed liquor and RAS.

- **Enzyme activities of five hydrolases were analysed throughout a WWTP with an incorporated MBR system and activity was shown to vary both temporally and spatially.**

Only one paper had previously shown inherent activity throughout a WWTP but only over four days. Furthermore, standard water quality parameters were not measured to understand the potential factors that result in temporal and spatial variability. Inherent activity was analysed at the Old Ford WWTP and the Stoke Bardolph WWTP. At the Old Ford WWTP activity was analysed throughout the treatment process whereas at the Stoke Bardolph WWTP influent and effluent samples were only analysed. For the effluent at the two WWTPs there was no inherent activity detected. Additionally sulfatase activity was not observed in the influent but solely for the mixed liquor and RAS at Old Ford. This suggests sulfatase does not propagate from the sewage system but originates entirely from activated sludge. The wastewater samples were characterised by the measured standard water quality parameters and correlated with the recorded activity to highlight significant factor relationships.

From the calculated Pearson's correlation coefficients, the inherent enzymes analysed at the Old Ford WWTP showed varying responses to the wastewater characteristics. For the different standard water quality parameters there was typically a positive correlation with inherent activity. However, higher DO concentrations resulted in lower inherent activity, as demonstrated by the trend of negative correlations for each enzyme. Relationships between activities for the inherent enzymes also varied. For example, in the influent, β-glucosidase and esterase showed a strong positive correlation but in the screened sewage this correlation was instead strongly negative. The activities for the analysed inherent hydrolases were shown to vary temporally and spatially with each sampled wastewater matrix, leading to different enzymatic behaviours and independent relationships.

7.2 Recommendations for Future Work

To advance the application of enzymatic EDC treatment technologies, further work is required on their performance in wastewater environments. Greater understanding on inherent enzyme activity has been achieved and activity profiles can be compared between different WWTPs.

- **Determining the capability of inherent enzymes to degrade bioactive chemicals from the wastewater system.**

  Temporal variability in enzyme activity was studied for the inherent hydrolases but analytical substrates were used instead of any bioactive chemicals. The literature had shown that cell free enzymes extracted from the activated sludge were able to degrade specific bioactive chemicals (Krah et al., 2016). Esterase for example was said to be able to degrade erythromycin, a common antibiotic. Furthermore, deconjugation of sulfate conjugated EDCs has not been studied before by sulfatase in the wastewater environment. These additional investigations would enhance the understanding for tertiary enzymatic treatments to remove EDCs.

- **Greater understanding on enzyme inhibitors within the wastewater system and their influence on inherent or exogenous enzymatic performance.**

  From the literature review in Chapter 2 it was additionally shown that zinc inhibited tyrosinase activity (Han et al., 2007). Zinc was on the chemical investigation programme (CIP), therefore known to be present in the wastewater matrix and important to consider in any future work (Gardner et al., 2012). Regarding the inherent enzymes the activities of both α-glucosidase and β-glucosidase have been shown to be enhanced by the metals magnesium and manganese (Deng et al., 2014) (Reshmi and Sugunan, 2013). Magnesium hydroxide is often added to the wastewater matrix to neutralise acidic pH levels. Future work should also consider investigating this influence, as shown with laccase and tyrosinase the different constituents of wastewater can greatly impact on enzyme activity.

- **Utilising a combined laccase and tyrosinase treatment technology to remove EDCs from wastewater.**

The recent paper by Ba et al., (2014) studied the combined treatment of laccase and tyrosinase to degrade acetaminophen. From the exogenous enzymatic parameters recorded in this work, laccase and tyrosinase were shown to respond differently to the measured characteristics of the sampled wastewater. Therefore the combined treatment of laccase and tyrosinase may result in a more consistent EDC removal output and a greater substrate range. The advantages of a laccase and tyrosinase treatment combination needs further investigation.

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9.0 Appendices

9.1 Appendix A - Hydrolase Literature Table Summary

Table 9.1 First principles shown in the literature for the inherent enzymes.

Enzyme	Organism	Substrate	Temperature	pH	Chemical + Metal Effects (% → relative activity against a control)	Reference
α-Glucosidase	*Thermophilic Bacillus*	*p-nitrophenyl-α-D-glucopyranoside*	40 - 70ºC Optimum = 60ºC *(Thermophilic)*	pH 6.0 - 8.5 Optimum pH = 6.0		(Suzuki et al., 1976)
α-Glucosidase	*Lactobacillus Acidophilus*	*α-p-nitrophenyl-β-D-glucopyranoside*	20 - 45ºC Optimum = 35ºC	pH 5 - 8 Optimum pH = 5.0 (*Acidophilus*)	Inhibited Activity: Mercury Cl (2.8%), Zinc Cl (61%), Nickel Sulfate (69%), Copper Cl (73%), Cobalt Cl (83%), Calcium Cl (84%), Potassium Cl (94%), Lead Acetate (97%), Barium Cl (98%) Enhanced Activity: Manganese Cl (168%), Magnesium Cl (103%)	(Li K-Y., 1983)
α-Glucosidase	*Saccharomyces Cerevisiae*	*p-nitrophenyl-α-D-glucopyranoside*	20 - 60ºC Optimum = 46ºC	pH 2.6 - 8.0 Optimum pH = 7.0	Inhibited Activity: Tris (<5%), Iron (III) Cl (<10%), Glycine (95%), Urea (95%), Cobalt Cl (100%), No Activity Effect: EDTA, Calcium Cl, Lithium Cl, Ammonium Cl	(Deng et al., 2014)

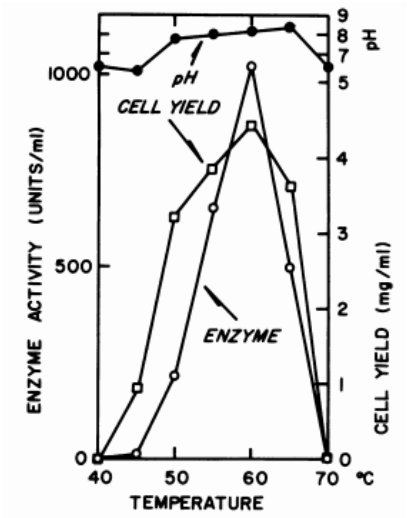
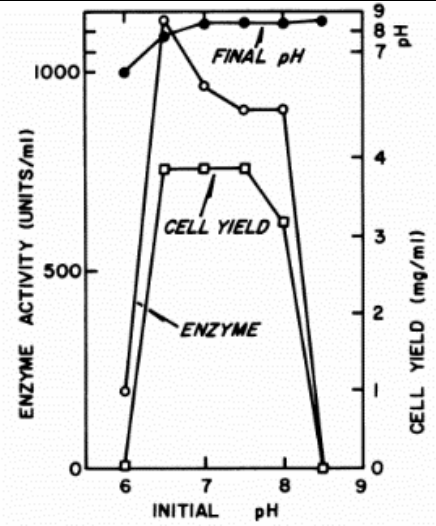
					Enhanced Activity: Magnesium Cl (115%), Manganese Cl (115%)	
α-Glucosidase	*Saccharomyces Cerevisiae*	*p-nitrophenyl-α-D-glucopyranoside*	25 - 55°C Optimum = 40°C (activity stable between 25-50°C)	pH 3 -9 Optimum pH = 6.0		(Dusan et al., 2014)
α-Glucosidase	*Aspergillus Niger*	*Starch*	30 - 80°C Optimum = 60°C	pH 3 - 10 Optimum pH = 5.0		(Jadhav and Singhal, 2014)
β-Glucosidase	*Aspergillus Niger*	*4-nitrophenyl-β-D-glucopyranoside*	37°C	pH 2 - 8 Optimum pH = 4.0		(Ortega et al., 1998)
β-Glucosidase	*Saccharomyces Cerevisiae*	*4-nitrophenyl-β-D-glucopyranoside*	20 - 60°C Optimum = 40°C (no significant difference between 40-50°C)	pH 3 - 7 Optimum pH = 4.0 (no significant difference between pH 4-5)		(Hernández et al., 2003)
β-Glucosidase	*Almond*	*4-nitrophenyl-β-D-glucopyranoside*	30 - 75°C Optimum = 50°C (large increase in activity from 30-50°C and then stabilises)	pH 3 - 8 Optimum pH = 6.0	Inhibited Activity: Nitrate (3%), Mercury Cl (4.8%), Copper Cl (48%), Silver Iron (II) Cl (72%), Zinc Cl (89%), Calcium Cl (91%), EDTA (98%), Cadmium Cl (98%), Enhanced Activity: Magnesium Cl (112%),	(Reshmi and Sugunan, 2013)

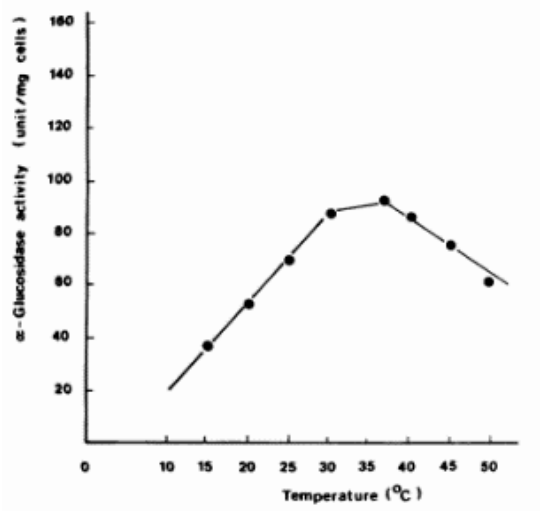
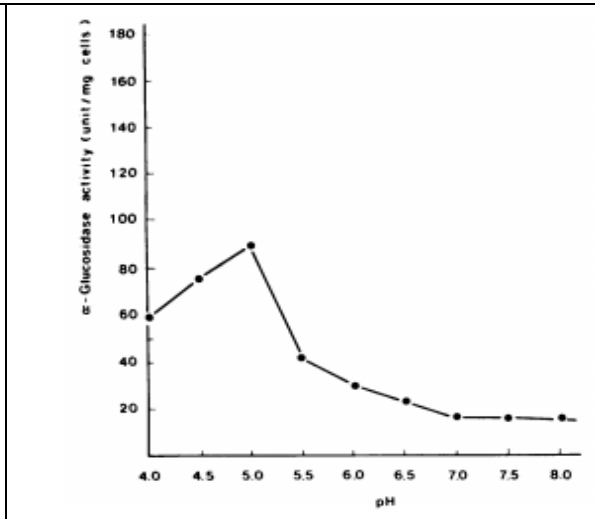
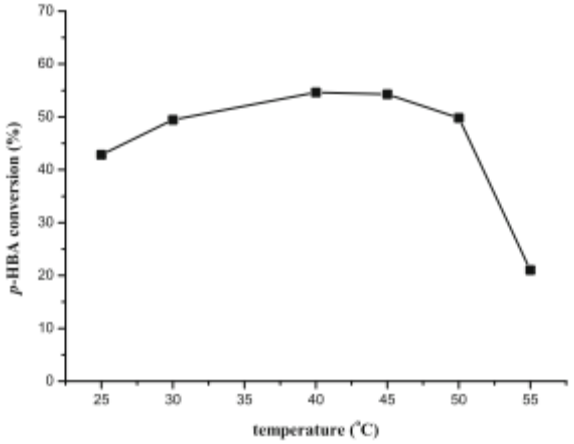
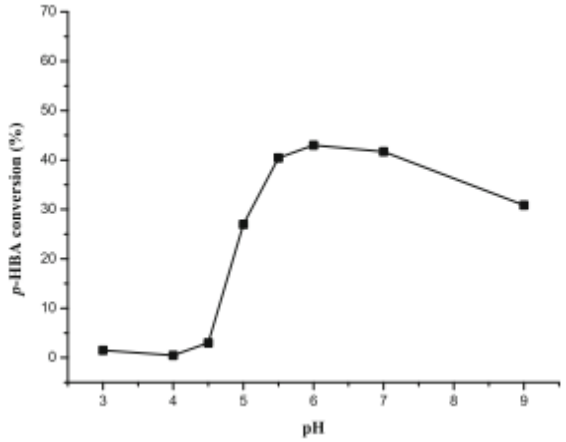
					Manganese Cl (110%), Cobalt Cl (110%)	
β-Glucosidase	*Muscat of Bornova Grape*	*4-nitrophenyl-β-D-glucopyranoside*	30 - 75ºC Optimum = 55ºC	pH 3 - 7 Optimum pH = 5.0		(Ümit Ünal et al., 2014)
β-Glucosidase	*Almond*	*Cellobiose*	15 - 70ºC Optimum = 50ºC (sharp drop in activity after 55ºC)	pH 3 - 7 Optimum pH = 5.0		(Vervoort et al., 2016)
β-Glucosidase	•*Almond* •*Streptomyces Griseus*	*4-nitrophenyl-β-D-glucopyranoside*	20 - 100ºC Optimum = 60ºC (*Almond*) 70ºC (*S.Griseus*)	pH 3 - 9 Optimum pH = 7.0 (from both organisms)	Inhibited Activity: Chloral Hydrate (10%), PMSF (10%), BME (10%), Cinnamic Acid (10%), Triton (60%), Guanidine HCl (80%), Quercetin (80%), Phenanthraquinone (95%), Deoxycholic Acid (95%), EDTA (90%), Enhanced Activity: SDS (105%)	(Kumar et al., 2017)
Esterase	*Bacillus Brevis*	*p-nitrophenyl butyrate*	40 - 70ºC Optimum = 60ºC	pH 7.0 - 8.5 Optimum pH = 7.5	Inhibited Activity: Phenylmethylsulfonyl fluoride (0%), Diisopropyl fluorophosphates (0%), Iodoacetamide (4%), p-Chloromercuribenzoic acid (8%), Copper Cl (13%), Zinc Sulfate (26%), Ethylenediaminetetraacetic acid (37%), Magnese Cl (56%), Sodium dodecyl sulfate (62%), Magnesium	(Amaki et al., 1992)

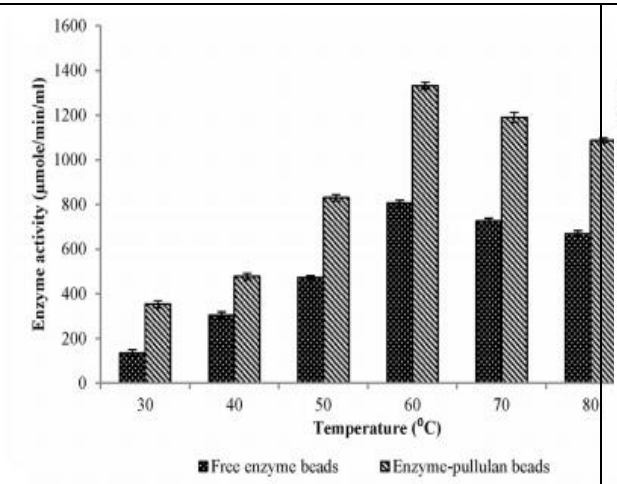
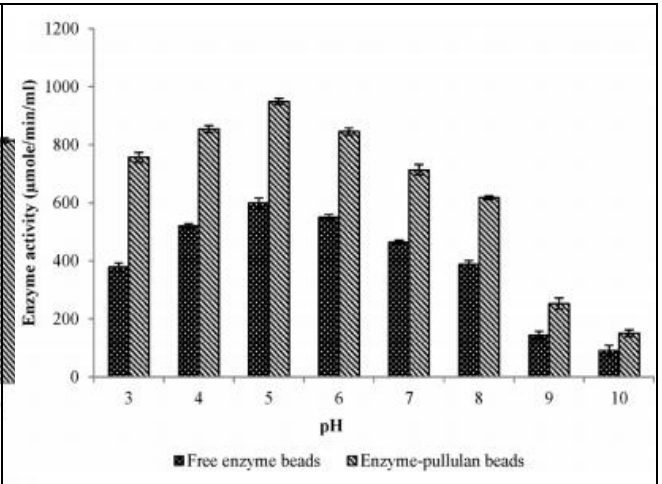
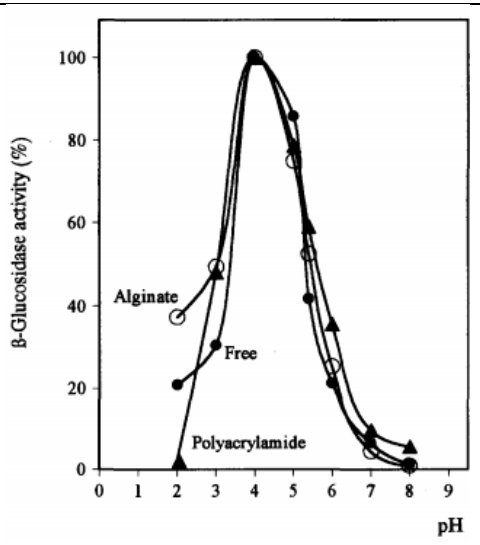
					Cl (73%), Calcium Cl (81%), <u>Enhanced Activity:</u> 2-Mercaptoethanol (114%), Dithiothreitol (101%)	
Esterase	*Bacillus Circulans (Thermophile)*	*p-nitrophenyl caprylate*	<u>20 - 80ºC</u> Optimum = 55ºC (*Thermophile*)	<u>pH 7 - 11</u> Optimum pH = 9.0	<u>Inhibited Activity:</u> Mercury Cl (0%), Phenylmethylsulfonyl fluoride (0%), p-Hydroxymercuribenzoix acid (0%), Esterine (0%) Ethylenediaminetetraacetic acid (84%), Diethylpyrocarbonate (93%) <u>14Enhanced Activity:</u> β-Mercaptoethanol (105%)	(Kademi et al., 2000)
Esterase	*Pseudomonas sp.*	*Erythromycin*	<u>20 - 80ºC</u> Optimum = 55ºC	<u>pH 5 - 9</u> Optimum pH = 7.0		(Kim et al., 2002)
Sulfatase	*Helix Pomatia*	*2-hydroxy-5-nitrophenyl sulfate*		<u>pH 5.5 - 8.0</u> Optimum pH = 6.6	<u>Inhibited Activity:</u> Sodium sulfite (5%), Sodium bisulfite (6%), Trisodium phosphate (24%), Hydroxylamine (34%), Sodium fluoride (47%), Phenylhydrazine (61%), Zinc Cl (77%), Maganese Cl (81%), Magnesium Cl (85%), Sodium sulfate (86%), Sodium citrate (91%), Semicarbazide (91%), Sodium cyanide (91%), Tetrasodium pyrophosphate (95%),	(Dodgson and Powell, 1959)

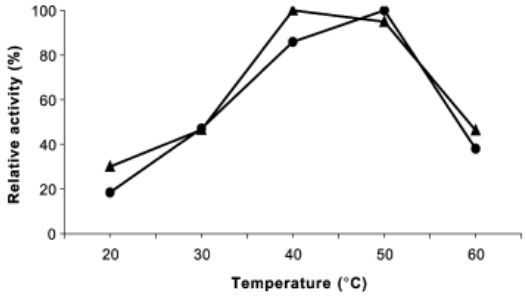
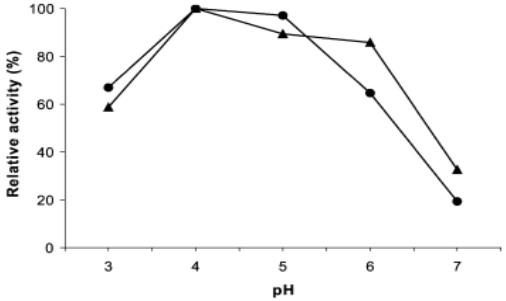
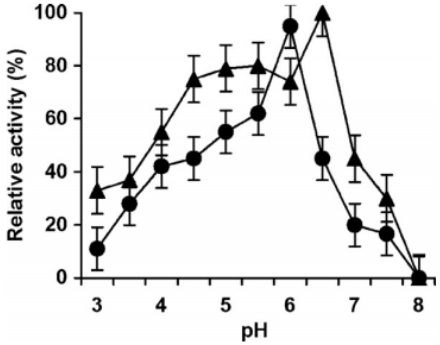
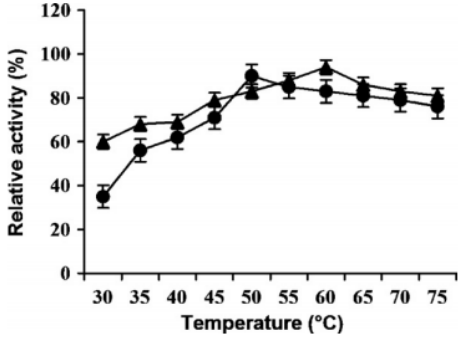
					Sodium benzene sulphonate (95%), Urea (100%), <u>Enhanced Activity:</u> Hydrazine (103%),	
Sulfatase	• *Helix Pomatia* • *Aerobacter Aerogens*	*p-nitrophenyl sulfate*	-	<u>pH 3 - 10</u> Optimum pH = 4-5 (*Helix Pomatia*) 7.0 (*A.Aerogens*)		(Gibby et al., 2004)
Sulfatase	*Pseudomonas sp.*	*SDS*	<u>10 - 100°C</u> Optimum = 70°C	<u>pH 4 - 11</u> Optimum pH = 8.0		(Li et al., 2016)
Alkaline Phosphatase	*Escherichia Coli*	*p-nitrophenyl phosphate*	-	<u>pH 7 -11</u> Optimum pH = 9.5		(Janeway et al., 1993)

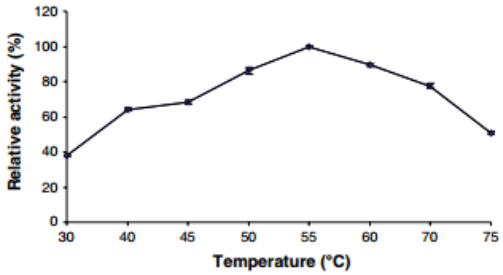
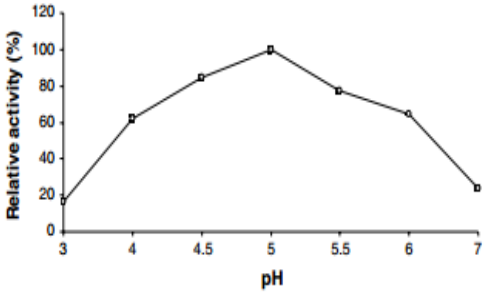
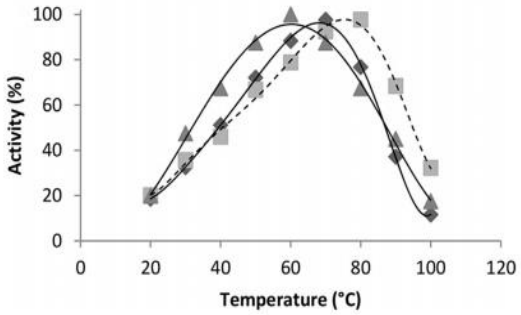
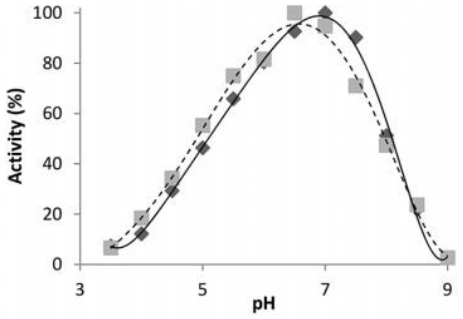
Table 9.2 Temperature and pH profiles of the inherent enzymes collected from the literature in regards to first principles.

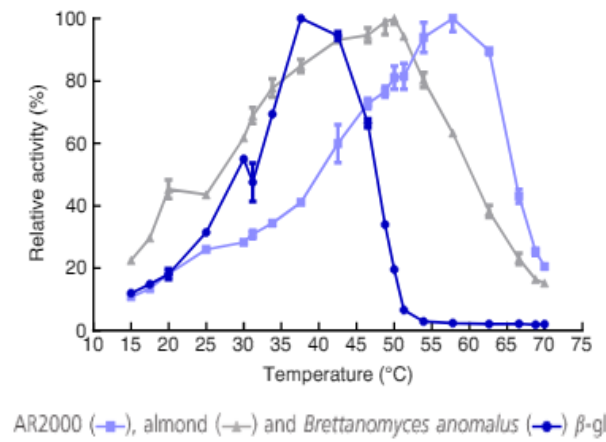
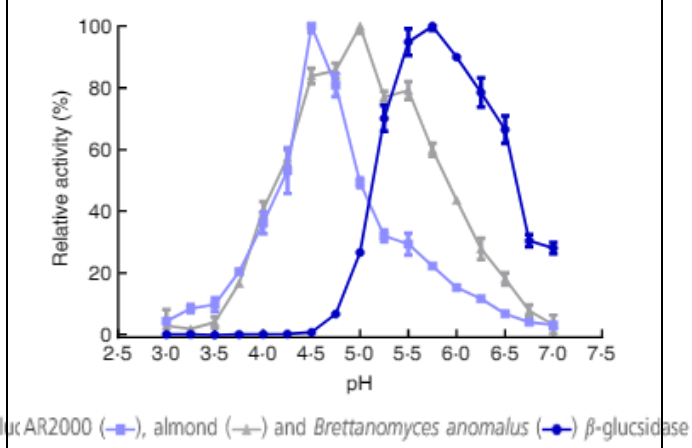
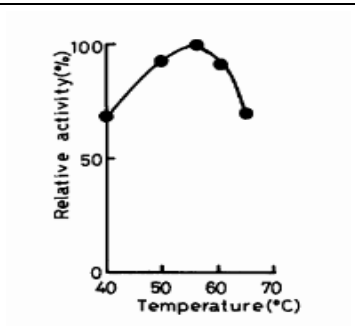
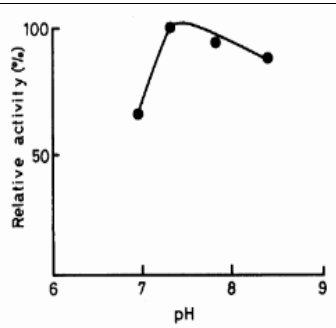
Enzyme	Reference	Temperature Profile	pH Profile
α-Glucosidase (*Thermophilic Bacillus*)	(Suzuki et al., 1976)		

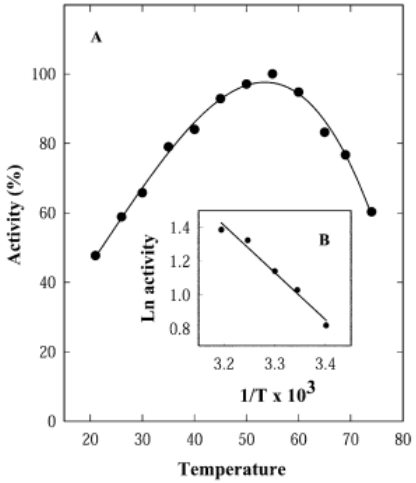
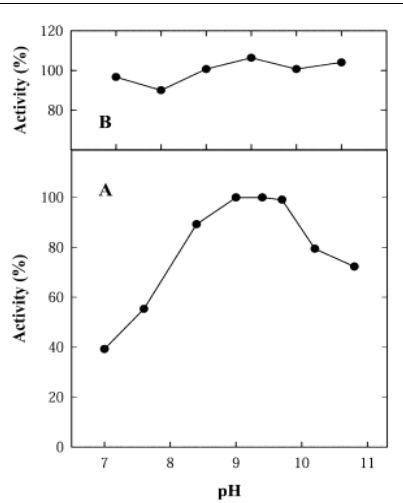
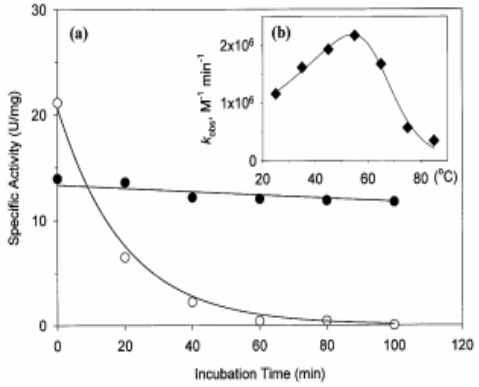
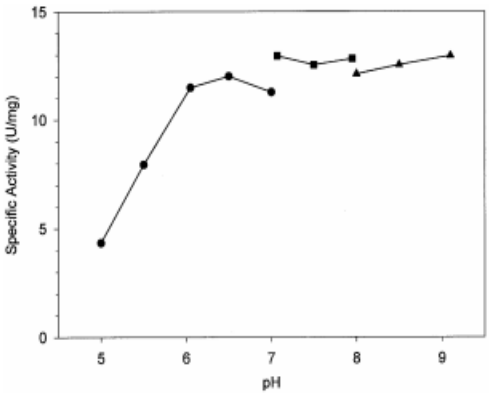
α-Glucosidase (*Lactobacillus acidophilus*)	(Li K-Y., 1983)		
α-Glucosidase (*Saccharomyces cerevisiae*)	(Dusan et al., 2014)		

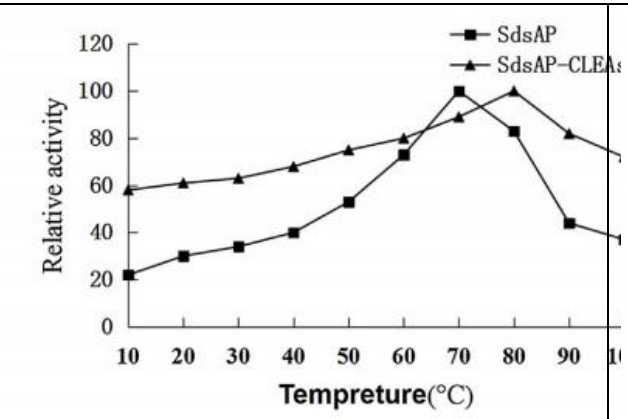
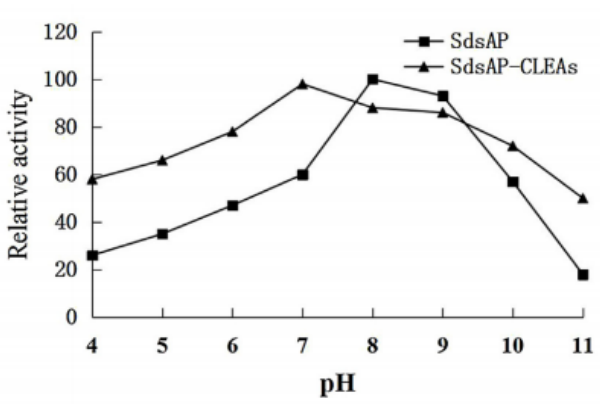
α-Glucosidase (*Aspergillus niger*)	(Jadhav and Singhal, 2014)		
β-Glucosidase (*Aspergillus niger*)	(Ortega et al., 1998)		

β-Glucosidase (*Saccharomyces cerevisiae*)	(Hernández et al., 2003)	 ● Supernatant from cell culture ▲Pellet from cell culture	 ● Supernatant from cell culture ▲Pellet from cell culture
β-Glucosidase (*Almond*)	(Reshmi and Sugunan, 2013)	 ● Free Enzyme ▲Immobilised Enzyme	 ● Free Enzyme ▲Immobilised Enzyme

β-Glucosidase (*Muscat of Bornova Grape*)	(Ümit Ünal et al., 2014)		
β-Glucosidase (*Strepomyces griseus*)	(Kumar et al., 2017)	 ◆ *Strepomyces griseus* ▲Commercial *Almond* ■ Immobilised *S.griseus*	 ◆ *Strepomyces griseus* ■ Commercial *Almond*

β-Glucosidase (*Brettanomyces anomalus*)	(Vervoort et al., 2016)		
Esterase (*Bacillus stearothermophilus*)	(Amaki et al., 1992)		

Esterase (*Bacillus circulans*)	(Kademi et al., 2000)	 A) Effect of pNPC8 activity B) Arrhenius plot	 A) Effect of pH on activity B) pH effect on stability (1hr treatment)
Esterase (*Pseudomonas sp. GD100*)	(Kim et al., 2002)	 (a) Thermal denaturation of esterase at (●) 35ºC and (○) 55ºC/ (b) effect of temperture on the observed enzymatic rate	

Sulfatase (*Pseudomonas sp. S9*)	(Li et al., 2016)	 ■ Free Enzyme ▲ Immobilised Enzyme	 ■ Free Enzyme ▲ Immobilised Enzyme

9.2 Appendix B - Stoke Bardolph WWTP Enzymatic Parameter Results

Table 9.3 Laccase benchmark results along with the recorded wastewater quality parameters for the sampling period between 20/06/16 and 27/07/16. Benchmarks performed in duplicate. Repeat 1 (R1) and repeat 2 (R2).

Stoke Bardolph WWTP														
Sample Day	Date	Day	Matrix	**On Site**		**University of Nottingham Laboratory**						**Laccase E1 Removal (%)**		
				WW Temp ˚C	DO mg/L	pH	COD mg/L	TSS mg/L	Cl mg/l	TDS g/L	EC mS/cm	R1	R2	**Avg.**
1	20/06/16	Monday	Effluent	16.0	4.5	7.4	16.5	7.4	92.5	0.33	0.76	82.5	80.6	**81.5**
3	22/06/16	Wednesday	Effluent	14.6	6.0	7.2	18.9	10.2	107.0	0.44	0.89	85.7	82.6	**84.1**
8	27/06/16	Monday	Effluent	15.3	4.6	7.3	15.8	9.4	80.0	0.33	0.74	82.1	85.2	**83.7**
10	29/06/16	Wednesday	Effluent	16.4	4.4	7.0	15.2	11.0	96.0	0.38	0.81	85.5	82.2	**83.8**
15	04/07/16	Monday	Effluent	16.3	4.4	7.2	9.5	5.8	92.0	0.41	0.82	82.0	78.3	**80.1**
17	06/07/16	Wednesday	Effluent	16.7	4.2	7.1	24.6	4.6	134.0	0.48	0.98	81.3	76.6	**79.0**
22	11/07/16	Monday	Effluent	16.5	4.5	7.3	14.3	4.4	86.5	0.40	0.82	82.4	85.3	**83.9**
31	20/07/16	Wednesday	Effluent	18.5	2.8	7.3	14.4	4.2	118.0	0.45	0.98	87.3	82.0	**84.6**
36	25/07/16	Monday	Effluent	18.1	4.2	7.3	-	5.6	103.6	0.47	0.95	86.3	84.1	**85.2**
38	27/07/16	Wednesday	Effluent	18.0	2.4	7.2	25.6	6.2	131.3	0.49	1.00	86.6	83.4	**85.0**

Table 9.4 Laccase and tyrosinase benchmark results along with the recorded wastewater quality parameters for the sampling period between 01/08/16 and 09/12/16. Benchmarks performed in duplicate. Repeat (R1) and repeat 2 (R2).

Stoke Bardolph WWTP																		
Sample Number	Sample Day	Date	Day	Matrix	On Site		University of Nottingham Laboratory						Laccase E1 Removal (%)			Tyrosinase E1 Removal (%)		
					WW Temp ˚C	DO mg/L	pH	COD mg/L	TSS mg/L	TDS g/L	EC mS/cm	Cl mg/L	R1	R2	**Avg.**	R1	R2	**Avg.**
1	1	01/08/16	Monday	Effluent	16.9	2.8	7.1	18.2	4.4	0.44	0.90	-	80.7	74.4	**77.6**	87.0	87.5	**87.3**
2	3	03/08/16	Wednesday	Effluent	17.9	2.8	7.5	22.4	6.2	0.45	0.92	-	87.6	85.6	**86.6**	76.4	80.9	**78.6**
3	10	10/08/16	Wednesday	Effluent	16.8	-	7.5	17.2	4.2	0.46	0.93	-	85.3	82.6	**84.0**	80.4	80.3	**80.3**
4	15	15/08/16	Monday	Effluent	14.1	3.5	7.0	14.1	4.8	0.44	0.90	109.0	83.0	82.9	**82.9**	80.7	83.5	**82.1**
5	17	17/08/16	Wednesday	Effluent	14.0	3.8	7.0	16.6	6.6	0.47	0.94	121.0	79.9	82.3	**81.1**	78.2	79.1	**78.6**
6	22	22/08/16	Monday	Effluent	16.6	3.7	7.0	12.5	6.6	0.36	0.74	87.0	86.4	84.4	**85.4**	83.5	82.6	**83.0**
7	38	07/09/16	Wednesday	Effluent	18.8	3.3	7.0	16.7	5.8	0.55	1.13	135.0	81.7	83.2	**82.5**	88.2	86.4	**87.3**
8	52	21/09/16	Wednesday	Effluent	17.8	3.8	7.0	15.8	6.6	0.51	1.03	143.5	82.1	83.5	**82.8**	92.3	95.4	**93.8**
9	59	28/09/16	Wednesday	Effluent	17.1	3.2	6.9	24.9	6.6	0.49	0.99	141.5	83.5	83.0	**83.2**	77.6	74.6	**76.1**
10	66	05/10/16	Wednesday	Effluent	16.8	0.4	6.8	-	-	0.45	0.88	132.5	85.6	86.4	**86.0**	82.4	81.2	**81.8**
11	73	12/10/16	Wednesday	Effluent	15.8	3.6	6.9	11.2	10.6	0.46	0.94	136.0	78.6	79.1	**78.8**	83.3	80.2	**81.8**
12	80	19/10/16	Wednesday	Effluent	16.1	0.5	6.9	22.5	3.5	0.47	0.94	130.0	78.9	80.2	**79.6**	65.9	65.6	**65.7**

13	87	26/10/16	Wednesday	Effluent	15.4	3.5	6.9	20.8	3.9	0.51	1.03	147.5	73.0	75.9	**74.5**	83.5	83.5	**83.5**
14	94	02/11/16	Wednesday	Effluent	-	0.5	7.1	25.7	6.2	0.55	1.12	149.0	70.9	70.4	**70.6**	91.5	94.2	**92.9**
15	96	04/11/16	Friday	Effluent	15.1	3.4	7.0	24.6	7.2	0.52	1.05	139.0	64.0	66.0	**65.0**	81.3	81.0	**81.1**
16	101	09/11/16	Wednesday	Effluent	14.2	-	6.9	24.2	8.4	0.49	1.00	141.0	61.0	61.7	**61.4**	86.7	87.3	**87.0**
17	103	11/11/16	Friday	Effluent	13.3	-	6.9	19.9	4.5	0.48	0.97	146.0	72.2	75.9	**74.0**	87.0	86.5	**86.7**
18	108	16/11/16	Wednesday	Effluent	14.2	-	7.0	18.6	9.4	0.59	1.18	152.0	63.4	63.1	**63.3**	90.9	93.2	**92.0**
19	110	18/11/16	Friday	Effluent	14.2	3.6	6.8	16.0	6.6	0.56	1.25	144.0	66.6	68.9	**67.7**	83.4	82.4	**82.9**
20	115	23/11/16	Wednesday	Effluent	12.2	3.3	6.9	21.8	12.2	0.45	0.92	96.0	74.1	75.7	**74.9**	78.0	80.5	**79.2**
21	117	25/11/16	Friday	Effluent	12.8	3.4	7.1	24.2	4.3	0.52	1.04	114.5	62.8	61.1	**61.9**	67.3	66.9	**67.1**
22	124	02/12/16	Friday	Effluent	13.1	4.2	7.0	20.9	10.8	0.55	1.13	131.0	53.0	55.9	**54.4**	79.7	77.9	**78.8**
23	131	09/12/16	Friday	Effluent	13.8	4.0	7.1	16.4	9.6	0.52	1.06	145.5	61.0	64.6	**62.8**	57.9	62.2	**60.1**

9.3 Appendix C - Enzyme Kinetic Formulas

$$k = A\ e^{-\frac{Ea}{RT}}$$

Equation 3 Arrhenius Equation where k = rate constant, A = pre exponential factor, Ea = activation energy, R = universal gas constant and T = temperature (kelvin).

$$\ln k = \ln A - \frac{Ea}{RT}$$

$$\ln\left(\frac{k2}{k1}\right) = \frac{Ea}{R}\left(\frac{1}{T1} - \frac{1}{T2}\right)$$

$$\frac{k2}{k1} = e^{\frac{Ea}{R}\left(\frac{1}{T1} - \frac{1}{T2}\right)}$$

Equation 4 Rate constant ratio calculation from the Arrhenius equation between two temperatures. T_1 = reference temperature; T_2 = investigated temperature; K_1 = rate constant for T_1; and K_2 = rate constant for T_2.

Table 9.5 Activation energies (Ea) in the literature for the inherent enzymes detailing both the source and substrate used.

Enzyme	Organism	Substrate	Activation Energy	Reference
α-Glucosidase	*Aspergillus niger*	Starch	38.3kJ/mol	(Jadhav and Singhal, 2014)
β-Glucosidase	*Aspergillus niger*	pNPG* Cellobiose	52.5kJ/mol 46.0kJ/mol	(Chirico and Brown, 1987)
β-Glucosidase	*Almond*	pNPG*	32.2kJ/mol	(Yan et al., 2010)
β-Glucosidase	*White rot fungi*	pNPG*	1.5kJ/mol – 33.6kJ/mol	(Mfombep et al., 2013)
Esterase	*Bacillus circulans* (Thermophile)	*p*-nitrophenyl caprylate (pNPC8)	23.3kJ/mol	(Kademi et al., 2000)
Sulfatase	Purified from Human Urine	4-nitrocatechol sulfate 4-methylumbelliferyl Cerebroside sulfate	51.9kJ/mol 36.0kJ/mol 90.0kJ/mol	(Stevens et al., 1975)
Sulfatase	Soil	*p*-nitrophenol sulfate	42..2kJ/mol	(Elsgaard and Vinther, 2004)
Alkaline Phosphatase	*Cenococcum graniforme*	*p*-nitrophenyl-phosphate	41.4kJ/mol	(Bae and Barton, 1989)

*pNPG = *p*-nitrophenyl-β-D-glucopyranoside

9.4 Appendix D - Old Ford WWTP Standard Water Qualities

Table 9.6 Measured standard water quality parameters for the influent sampled between May and June 2016 at the Old Ford WWTP.

Old Ford WWTP (Influent)							
Sample Day	Day	WW Temp ^{o}C	DO mg/L	pH	COD mg/L	TSS mg/L	EC µS/cm
1	Monday	19.1	0.47	8.55	572	404	1053
2	Tuesday	18.8	0.84	8.46	402	312	780
4	Thursday	19.0	1.42	8.09	540	311	1023
5	Friday	19.1	1.04	8.26	555	353	1014
15	Monday	19.5	0.56	8.56	-	397	1057
16	Tuesday	19.4	0.64	8.71	724	446	1071
19	Friday	19.2	0.24	8.69	579	565	1123
23	Tuesday	16.4	3.78	7.97	-	486	381
25	Thursday	18.5	0.62	8.20	-	312	988

Table 9.7 Measured standard water quality parameters for the screened sewage sampled between May and June 2016 at the Old Ford WWTP.

Old Ford WWTP (Screened Sewage)							
Sample Day	Day	WW Temp ^{o}C	DO mg/L	pH	COD mg/L	TSS mg/L	EC µS/cm
1	Monday	18.2	5.01	7.43	249	75	968
2	Tuesday	18.2	4.79	7.42	249	98	903
4	Thursday	18.3	5.10	7.35	227	85	941
5	Friday	18.1	4.67	7.35	291	68	971
15	Monday	18.6	4.45	7.59	-	81	992
16	Tuesday	18.5	5.22	7.74	296	102	993
19	Friday	18.5	5.22	7.69	357	72	1000
23	Tuesday	17.7	5.56	7.79	-	109	785
25	Thursday	17.6	4.77	7.93	-	103	1025

Table 9.8 Measured standard water quality parameters for the mixed liquor sampled between May and June 2016 at the Old Ford WWTP.

Old Ford WWTP (Mixed Liquor)							
Sample Day	Day	WW Temp ^{o}C	DO mg/L	pH	COD mg/L	Solids mg/L	NTC EC µS/cm
1	Monday	19.3	0.50	6.98	-	6020	871
2	Tuesday	19.2	2.06	7.05	-	5916	847
4	Thursday	19.3	3.47	7.12	-	6186	741
5	Friday	19.0	3.40	7.09	-	6360	803
15	Monday	19.6	0.24	7.14	-	7150	847
16	Tuesday	19.5	0.15	7.26	-	7442	843
19	Friday	19.6	0.14	7.27	-	7474	843
23	Tuesday	19.1	1.28	7.27	-	8250	838
25	Thursday	18.7	0.46	7.12	-	8236	816

Table 9.9 Measured standard water quality parameters for the returned activated sludge sampled between May and June 2016 at the Old Ford WWTP.

Old Ford WWTP (Returned Activated Sludge)							
Sample Day	Day	WW Temp ^{o}C	DO mg/L	pH	COD mg/L	Solids mg/L	NTC EC µS/cm
1	Monday	19.4	0.83	7.05	-	7344	954
2	Tuesday	19.3	3.00	7.20	-	7592	928
4	Thursday	19.2	4.26	7.27	-	8080	803
5	Friday	19.0	4.06	7.24	-	7574	891
15	Monday	19.6	0.67	7.25	-	9104	915
16	Tuesday	19.5	0.39	7.40	-	9900	913
19	Friday	19.6	0.17	7.42	-	9486	902
23	Tuesday	19.2	1.25	7.37	-	10786	933
25	Thursday	18.8	0.93	7.28	-	10346	876

Table 9.10 Measured standard water quality parameters for the influent sampled between February and June 2017 at the Stoke Bardolph WWTP.

Stoke Bardolph WWTP (Influent)							
Sample Day	Day	WW Temp ^{o}C	DO mg/L	pH	COD mg/L	TSS mg/L	Cl mg/L
1	Wednesday	10.5	1.7	7.4	222	115.8	-
3	Friday	10.4	1.9	7.0	90	60.6	101.5
8	Wednesday	11.9	0.1	6.8	196	63.8	104.5
10	Friday	10.3	0.1	7.1	194	52.0	104.0
15	Wednesday	10.5	2.0	7.0	253	153.2	144.0
17	Friday	10.3	2.0	7.1	365	164.0	181.0
20	Monday	11.0	-	6.6	222	77.6	117.0
24	Friday	10.9	-	7.0	240	97.0	112.0
27	Monday	11.0	-	7.2	192	134.2	98.5
31	Friday	11.3	-	7.0	208	137.0	101.5
36	Wednesday	11.1	3.0	7.0	163	67.0	117.0
38	Friday	11.2	3.2	6.9	152	84.8	94.0
43	Wednesday	11.7	3.0	-	136	136.2	96.0
45	Friday	11.6	2.5	7.2	179	123.4	90.0
49	Tuesday	12.3	2.5	7.7	276	62.2	154.0
50	Wednesday	11.8	2.5	7.3	132	48.8	84.5
56	Tuesday	12.4	2.4	7.3	250	108.8	103.0
57	Wednesday	12.3	2.6	7.2	170	136.4	93.5
71	Wednesday	12.3	2.7	7.4	280	110.4	103.0
78	Wednesday	13.2	2.8	7.3	298	303.0	145.5
90	Monday	13.9	2.6	7.3	168	134.4	117.5
92	Wednesday	14.2	2.9	7.2	175	154.0	111.5
97	Monday	13.8	2.4	7.6	191	115.4	202.0
99	Wednesday	14.3	2.7	7.3	170	127.4	119.5
113	Wednesday	14.1	3.9	7.3	155	37.6	72.5
118	Monday	14.7	2.7	7.2	252	117.4	111.5