THE UNIVERSITY OF NOTTINGHAM

Monitoring Population Changes in the Rhizosphere of a Range of Hydroponic Tomato Crops, Featuring Various Water Treatment Methods Across the 2014/2015 growing season.

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Abstract

A member of the solanaceous family of plants, tomato is the eighth most economically important agricultural product, and the fourth most economically important crop worldwide with around 164 million tonnes produced yearly. Europe dominates the highest yielding production systems, with the UK being the third highest yielding producer worldwide. Much of the modern day British production uses soilless systems housed in glasshouses. Glasshouses allow for greater control over the environmental factors that affect production, from pests and diseases, to temperature fluctuation and water scarcity. Using a soilless system adds to the control the grower has over the crop production, being able to adjust water and nutrient supply as and when needed. However, due to environmental impacts and high costs, it is becoming common to use a recirculating water system, rather than systems where the water runs to waste. With this closed system approach, issues arise regarding the recirculation, and the potential for rapid dispersal of potentially pathogenic organisms amongst whole crops. This can have obviously devastating effects upon grower's production and therefore it is now common place for growers to incorporate a water treatment step to remove such organisms or at least reduce their abundance.

In this research, microbial populations were monitored at separate points throughout commercial tomato production systems, at quarterly time points throughout the season. The populations were assessed by means of a small scale microarray, developed to contain probes targeting organisms commonly associated with commercially grown tomato rhizospheres. Five commercial nurseries were monitored through the season, each featuring contrasting water treatment systems; pSSF (part slow sand filter), fSSF (full slow sand filter), heat treated, UV treated and a final nursery that was instead an open system using a RTW (run to waste) method. Nurseries each supplied five water sample sets from separate sampling points throughout the loops, as well as a set of root samples at each

quarterly sampling point. On top of this, five more nurseries supplied root samples at two time points.

The first aim of the research was to monitor the population changes as the water passes through each system loop, including a pre and post treatment sample. The second aim of the research was to monitor the microbial population changes associated with the roots of each system throughout the season and build a base of knowledge of the common trends and features of the population through the year and if the treatments affected this. The final research aim was to develop and validate a range of qPCR assays for four commonly detected pathogens; *Colletotrichum coccodes, Plectospharella cucumerina, Pythium aphanidermatum* and *Pythium myriotylum.*

Many differences were demonstrated in the water samples throughout the various systems. The RTW system exhibited the lowest species richness of them all, whilst the fSSF exhibited the highest. In each system, peak species richness was usually found in either the slab solution samples or from the pre-treatment samples. From the species diversity scores, all treatments seemed to result in a reduction in diversity following treatment, and upon further inspection both pSSF and fSSF systems exhibited a significant difference. Throughout the season, two bacterial species *Nitrospira* and *Pseudomonas* were commonly detected as well as a range of *Pythia* species.

From the root observations, again the *Pythia* were very common, as was *Nitrospira, Pseudomonas, Xanthomonas* and *Erwinia*. These were common across the five main nurseries as well as the additional nurseries. Most systems throughout the season showed a general decline in abundance of species towards the end. Again, the fSSF system scored the highest species richness, whilst the heat treated nursery featured the lowest species richness. In the RTW system, two crops were grown, a crop on non-grafted roots, and another on a grafted rootstock. It was found that over the season the grafted roots, despite starting with higher species

richness, had a reduced organism number on their roots, and also in the water.

Finally, for the q-PCR assays developed, both the *C. coccodes* and the *P. cucumerina* assays were successfully validated and tested and showed positive results, whilst the *P. aphanidermatum* and *P. myriotylum* assays also showed promising results in their preliminary tests. Unfortunately the latter two assays could not be tested further due to time constraints.

From the findings of this research it was realised that it is important to use the microarray in conjunction with other techniques, and that the microarray should not be used alone to infer causality. As well as this, organisms such as *Pythia* were found to be consistently prevalent, across systems, sample types and the season. Further research should be carried out to characterise their habits and develop methods to effectively reduce their prevalence throughout the season. On top of this, samples from the system utilising the fSSF water treatment system generally yielded the highest species richness over the season whilst the pSSF seemed more effective at reducing this number. Little is known about the way in which SSF systems work, in terms of if they are a physical treatment or biological, and these results emphasise their complex attributes. Further research is needed into the organisms that may inhabit the filters, and whether potentially pathogenic species will remain dormant in them between seasons.

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Chapter 1

1.1: Tomatoes from a Historical Perspective: Classification and Domestication.

Tomatoes are one of more than 3000 solanaceous plant species, a family of plants that includes various other important species such as potato, pepper and tobacco (Wu & Tanksley, 2010). This dicotyledonous family, also known as the nightshade family, is extremely diverse, including annual and perennial herbs, vines, shrubs epiphytes and trees, amongst others. As well as a wide range of types of plants, the diversity of the family is reflected in the number of uses they have, from staple food crops, to medicinal and ornamental plants. Solanaceous plants have a worldwide distribution, particularly in temperate or tropical regions, whilst the greatest biodiversity can be found in the western hemisphere (Olmstead *et al.*, 2008).

Tomato originates from the Andean regions of South America, now made up of Chile, Peru, Ecuador, Bolivia and Columbia and it is suspected that the site of original domestication was in Mexico, although this is not known for certain (Bauchet & Causse, 2012). Subsequent to this initial domestication, it was then continued in North America. Following on from the earliest domestication, tomato was then taken to Europe in the 15th century where, despite being considered toxic like other members of the night shade family at first, further intense domestication took place in the 18th to 19th century (Sims, 1980). The intense domestication of tomatoes, distinct from divergent natural selection due to using an extremely narrow range of traits to select from such as fruit shape and size (Rodríguez *et al.*, 2011), has been commonplace in the past and has therefore resulted in a genetic bottleneck, due to a very narrow base of genetic variation. Domestication syndrome, as it is known, is commonplace in plants that are regularly selected for a collection of traits that are suited to humans rather than the natural environment and eventually results in deceleration

in crop improvement (Aflitos *et al.*, 2014) and therefore a decrease in genetic diversity.

1.2 The Modern Day Tomato.

At the beginning of the 20th century, domesticated tomatoes still maintained a reasonable degree of variation, particularly between families or communities where it was common for them to cultivate their own landrace varieties. With the onset of commercial production of tomatoes however, specific hybridisation of varieties rose to the fore in tomato breeding, replacing the randomness of open-pollination commonplace within the landrace varieties. The first commercially produced variety was 'Single-Cross' released in 1946 (Dorst, 1946). Since this point, the majority of breeding has been between hybrid varieties, worsening the effect of the domestication syndrome. Whilst it has been recognised that wild relatives now do hold a source of variation that is much needed to broaden the tomato genetic basis, and particular beneficial genes for introgression, producers have been reluctant to make use of them. This is due to the potential detrimental genes that may also be introduced, due to linkage drag, and the resultant time that would be required to remove them through conventional breeding (Bai & Lindhout, 2007). More recently, in a move away from the traditional traits that breeders aimed for, such as fruit colour and shape, the focus has been towards traits related to higher productivity and tailoring to particular cultivation methods. Examples of these traits include improved self-pruning, height and earliness of fruiting (Rodríguez *et al.*, 2011; Bauchet & Causse, 2012).

In the present day, solanaceous plants have become the third most economically important plant family overall, and the most important in terms of vegetable crop plants (Wu & Tanksley, 2010). In terms of tomato production, approximately 164 million tonnes are produced worldwide on over 4,800,000ha of land (FAOSTAT, 2012). Due to the tomatoes fairly simple genome, easy cultivability and close relation to other important

vegetable crops, it has been used extensively for research purposes as the model fruit bearing plant (Kimura & Sinha, 2008). In 2012, the complete genome of the Heinz 1706 cultivar was first published in Nature (The Tomato Genome Consortium, 2012) revealing the genome to be approximately 900 megabases (Mb) in size across 2n=24 chromosomes.

1.3 Tomato Production.

As mentioned previously (section 1.2), over 168 million tonnes of tomatoes are produced worldwide. The majority of this production total is gained from Asia (Fig. 1a.), whilst Africa, Americas and Europe produce similar totals of 11.4%, 15% and 12.8% respectively. The dominance of Asia's tomato industry is clearly reflected in the top producers worldwide, with China producing over 29 million tonnes whilst India produces just over 9 million tonnes (Fig. 1b.) (FAOSTAT, 2012). However, if world tomato producing figures are looked at in terms of yield rather than by total produced, European production dominates the top producers (Fig. 1c). This trend has been associated with European nation's preference towards using glasshouse production systems.

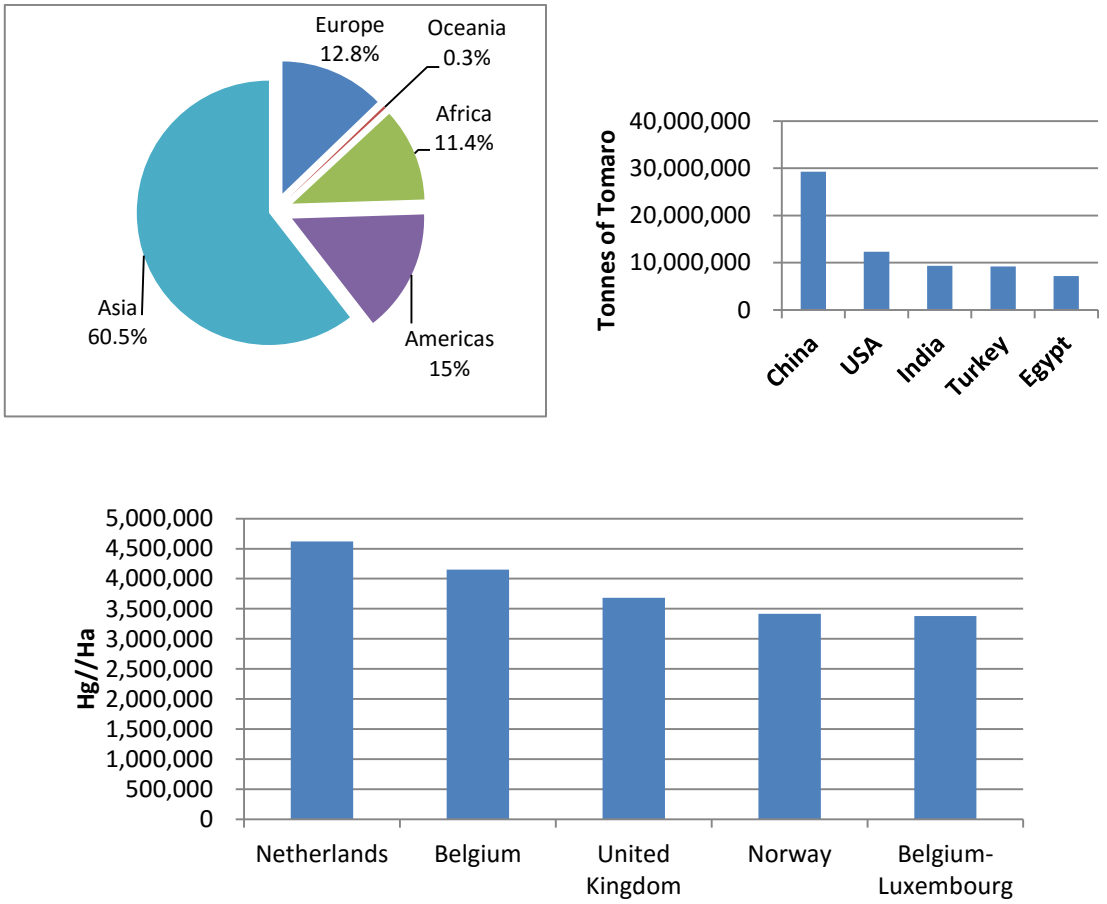


Figure 1.1: (From top left to right) a) Proportion of tomato production worldwide by region. b) Top 5 producing nations. c) Top 5 yielding nations. Adapted from (FAOSTAT, 2012)

1.4 British Tomato Production

1.4.a Inside the Glasshouses

Within the UK, the majority of commercial production of tomatoes uses heated glasshouses. These glasshouses are a way to circumvent the sub-optimal environmental conditions within the UK for tomato production. As a result, one of the key benefits provided is the extension of the natural growing season in the UK. Without the heated glasshouses the natural growing season within the UK would be from February to July/October, providing good conditions in the summer. However, with the use of heated glasshouses, the season can be extended further to November thus improving ripeness and other aspects of growth regardless of the external conditions. A step further from extending the growing season, heated

glasshouses may even offer the opportunity to eliminate seasonal growing and allow for year round production, with the addition of lighting systems. As well as facilitating beneficial modification to natural growing seasons, glasshouses are also able to protect the plant from various other biotic and abiotic stresses, allowing for the plant growth to be fully controlled.

This use of glasshouses in the UK has seen yields start to far outperform those of other nations that adopt alternative growth procedures. For example, in Spain it is common to grow tomatoes under polythene; with this method it would take five times the area of that of UK glasshouses to produce the same quantity of tomatoes.

This control over the external conditions, provided by the glasshouse, makes production very manageable and repeatable allowing consistent yields and fruit quality (Brooking, 1976) within the UK.

1.4.b Soilless Production

With the majority of the external abiotic and biotic stresses, above ground, being addressed by the glasshouse, soilless culturing aims to address some of the below ground related issues. It is believed that soilless production truly dates back as far as 4000 years to the ancient Egyptians, but in terms of commercial plant production it only really began gathering support in the 1970's. Until this point it was still believed that, although it worked, production in soil was far superior (Raviv & Lieth, 2008).

However, once the nutrient solution that the plants were cultured in was specifically formulated to the individual plants growth and a method to maintain this solution was proposed where pathogens were removed, soilless culturing increased in popularity (Raviv & Lieth, 2008). Hydroponics then offered a production system that offered improved control over the nutrient inputs but also importantly circumvented some of the issues soil-based production faced with persistent soilborne pathogens which was primarily the reason for the development of soilless culturing.

By definition, hydroponics initially used to only encompass culturing in water without any substrate medium. It has since come to encompass all methods of culturing in water including those using a substrate to stabilise and act as an anchor for the plant's roots. Now the original difference is defined as liquid hydroponics which has no substrate and aggregate hydroponics which uses inert substrates to support the roots (Stanghellini & Rasmussen, 1994). A key difference in hydroponic systems now is based on the use of the water following circulation. In the crudest form of hydroponic culturing, 'open systems' the water (also known as either fertigation solution [FS] or nutrient solution [NS]) will merely be drained away following one circulation, hence the name, 'run-to-waste' (RTW). However in a 'closed' hydroponic system the NS is re-circulated around the system. This recycling therefore requires the NS to be topped up and also sometimes undergo treatment to prevent build-up of potentially pathogenic bacteria or fungi (Stanghellini & Rasmussen, 1994). In terms of cost and benefits there are many associated with having either open or closed systems. Although in the open system you clearly reduce any chance of re-circulation of any pathogens, it also directly wastes a lot of fertilisers and nutrients, as well as water. This was clearly demonstrated when Grasselly et al. (2004) showed that the volume of NS added to the hydroponics water is usually 20-30% higher than that of which the plant can even take up, and therefore going to waste in a RTW system. However this cannot be solved by lowering the input of NS to the system as this margin is required to accommodate for the machinery, as well as variable crop uptake. As well as the economic downsides to this 20-30% of all NS added being lost, it also has significant environmental effects as well. The NS run-off would maintain high concentrations of nutrients such as Phosphorus (P), Magnesium (Mg) and Nitrogen (N), to name a few. In terms of Nitrogen, the concentration of the run-off may be in the range of 105-500 mg/L^{-1}. These sorts of concentrations of nitrates can often lead to increased nitrate leaching from the soils and then go on to act as a potential pollutant in the associated soils and groundwater if it is regularly

discharged (Grasselly *et al.*, 2004). Despite some of its benefits, these are some of the reasons why open systems are generally less popular now.

Closed systems are generally now regarded as the more economical and efficient systems to choose and are generally preferred. It's predicted (van Os, 1999), that these closed systems can save up-to 30% of the water used and 40% of nutrients and fertilisers. In spite of the efficiency benefits provided by a closed system over an open system, the recirculation of NS can lead to different threats. Within the commercial glasshouses the temperature is kept warm and often the plants are all genetically very similar. This means that, should an infectious pathogen be introduced to the water, it will quickly be dispersed amongst all the plants, whilst being able to proliferate quickly as a result of favourable conditions. This may then result in very high losses (Stanghellini & Rasmussen, 1994). As a result of this, growers will generally have some form of water treatment facility integrated into the circulation.

1.5 Recirculated Water Treatments

As previously mentioned (1.4.b), closed hydroponic systems have the ability to act as a vehicle for rapid dispersal of rhizosphere-borne pathogens, which can result in major crop losses if not dealt with. With the banning in recent times of traditional pesticides, much work has been dedicated towards developing new disease management systems. Much of this work has been directed to integrated pest management systems (IPS) serving the above ground portion of the plant, with advances in the use of biological control agents (BCA's) such as *Cycloneda sanguinea* which predate on common disease vectors such as aphids being met with relative success (Picanço *et al.*, 2007). However, not as much work has been dedicated to the protection of the below ground portion of the plant. The best mode of action at present is a stringent protocol for observation and removal of any harmful organisms.

However, it is still important to mention that, despite the potential for recirculating systems to allow the dispersal of potentially pathogenic organisms, organisms that are potentially beneficial in supressing the effects of plant pathogens may also be able to build up. For example *Pythium oligandrum* biocontrol properties within the rhizosphere including mycoparasitism, antibiosis or competition for nutrients and space with the pathogens (Le Floch *et al.*, 2005).

In closed system hydroponics, certain water treatment techniques have been adapted and added in to the systems so as to avoid recirculating any potentially harmful organisms.

1.5.a Heat Treatment

Heat treatment, or pasteurisation of water, is already a very common water treatment technique, and is the most commonly used method for water recirculation in the Netherlands (van Os, 2000). In this treatment the recirculated water is collected in a large pre-treatment tank, which is then fed through pipes to one of two heat exchangers. This first heat exchanger makes use of the residual heat from the post-treatment water passing out of the second heat exchanger and heats the nutrient solution to around 80°C. Following this initial pre-heating, the water is then fed onto the second heat exchanger where it is then heated up to 95°C for 30 s. This treatment has shown success rates of up to 100% effectivity (van Os, 2000; Stewart-Wade, 2011). To avoid high costs of heating large volumes of water to 95°C, other studies have looked at whether it was possible to achieve similar results from lower temperatures. It's since been found that heating water to 60°C for 3 min can be nearly as effective against fungi, bacteria and nematodes whilst using 68% of the energy (Runia & Amsing, 2001).

1.5.b UV Treatment

UV treatment, although less popular than heat treatment of recycled water, has been shown to have similar levels of effectiveness (Stewart-Wade, 2011). For this treatment, the recirculating water fills a reactor cylinder with a lamp at the centre. When filled, the lamp emits UV-C at a wavelength of 254 nm. This is absorbed by the microorganisms, causing mutation and damage to the DNA, killing the exposed organisms. The dosage can also be altered depending on the suspected pathogens in the solution; 100 mj/cm^2 is usually suitable for most microorganisms, however to eliminate viral pathogens 250 mj/cm^2 is more effective (Runia, 1993a,b).

1.5.c Slow Sand Filter

Slow sand filters (SSF) are a relatively newer form of water disinfection treatment; however popularity is rising rapidly. Using a SSF for treating recirculated water was first developed in 1993 (Wohanka, 1993, 1995) and was an alternative to other more expensive treatments such as the physical treatments; heat and UV treating and the chemical; chlorination an bromination, which are also losing popularity. As a disinfection method, SSF's are difficult to categorise, as in certain aspects they cover both physical and biological filtration (Wohanka & Brand, 2000; Stewart-Wade, 2011). The SSF itself acts as a physical sieve of the solution being passed through; however, it also is a habitat to certain microbial populations which are then able to interact with any microorganisms in the solution being passed through (Wohanka & Brand, 2000).

1.6 Grafting and Rootstocks:

The grafting of fruit and vegetable crops has been common throughout Europe and Asia over the last century, first developed commercially in Asia in the late 1920's to grow watermelon from a gourd root stock (Ashita, 1927). The initial use of this grafting was to attempt to overcome the yield losses in watermelon incurred by Fusarium wilt. However, since then, grafting has been adopted for many more benefits, such as disease

resistance (Grimault & Prior, 1994; Rivard *et al.*, 2010; Buller *et al.*, 2013), temperature tolerance (Venema *et al.*, 2008), salt tolerance (Estañ *et al.*, 2005) as well as for general growth promotion and improved yields (Yasinok *et al.*, 2009).

Grafting of plants allows scions (shoots/buds) with certain characteristics to be combined with a rootstock that often has contrasting characteristics to that of the scions natural root. This means that the grower is then able to 'tailor' the plants rootstock to one that suits their conditions best in terms of; growing season, cultivation method, soil quality and environment as well as by the type of crop and cultivar (Lee, 1994).

There is a wide range of grafting methodologies at the disposal of the modern grower, often now being selected based upon past success and personal preference. The two key types of grafting are those that are manually done and those that are mechanically. One basic but popular grafting technique is the 'tongue approach grafting'. In this method two plants are needed in close proximity; the scion plant and the rootstock. Angled incisions are made in both that are opposing and complimentary. These angled incisions thus form 'notches' that are able to fit together between the two plants and form a grafting union. This union is then supported by taping together to allow it to heal. Once healed the parts of the opposing plants that are no longer needed are removed; any part of the rootstock above the graft union is removed, and any part of the scion below the union (Lee *et al.*, 2010b). A more recent manual method of grafting that has proved successful is the use of tube grafting. Tube grafting involves the cutting of the scion and rootstock at an angle. Once both cut they are placed in either end of a small silicone tube or clip, till they meet in the middle. This method is very simple and low cost whilst having a high success rate; the only real disadvantage is that the acclimation conditions following must be very strictly regulated (Oda, 1995). More recently, in countries where grafting is extremely popular, such as Korea and Japan, the use of grafting robots is becoming more popular. These robots are capable of grafting plants at a rate far superior

to that of normal manual methods. Manually, it could be expected for a person to graft approximately 1000 plants/day, where as a simple machine with two operators can produce 600 grafts an hour, and a complex robot alone can graft 600-1200 plants/hour (Lee *et al.*, 2010a)

1.7 Roots and the Rhizosphere.

1.7.a Introduction to the Rhizosphere

The rhizosphere is formed gradually, alongside roots as they develop. As they are growing they secrete a variety of compounds into the media helping to form the rhizosphere, including ions, free oxygen and water, mucilage and compounds that act as attractants and repellents to certain soil borne microorganisms (Estabrook & Yoder, 1998; Bais *et al.*, 2001). These secretions are generally referred to as root exudates. The volume of these exudates that the plant releases should not be underestimated with approximately 5%-21% of all of the plant fixed carbon being secreted into the rhizosphere (Marschner, 1995). Following the initial growth of the roots, and thus secretion of root exudates, there is a sudden rapid rise in the growth of indigenous soil borne microbes in the rhizosphere, as a result of the large abundance in substrates and space for colonization. However, after this initial colonization, there is a drop in growth within the rhizosphere as it begins to reach its microbial maximal capacity (Bennett & Lynch, 1981; Kent & Triplett, 2002). The rhizosphere is made up of several components (Fig. 1.2), with two key regions making up the full rhizosphere; the ecto and endorhizosphere. The endorhizosphere, consists of the root cells from the cortex to the endodermis, and the ectorhizosphere spans from the root surface (rhizoplane) as far as >20mm into the media (McNear Jr., 2013). It is important to not overlook, however, that the size and shape of the rhizosphere, the ectorhizosphere in particular, is not able to truly be defined. The margin of the rhizosphere is better described as a chemical, biological and physical gradient. As well as the indefinable nature of its margin, the rhizosphere is also sensitive to biological, chemical and physical changes, exemplified by

a typical rhizosphere being able to range from <1-2mm to >20mm (Gregory, 2008).

The variable nature of the rhizosphere, depending upon the interactions between plants and microbes often lead individuals to overlook the growing media, which forms an integral part of an interacting trinity of the three constituents making up the rhizosphere area (Lynch, 1990; Deery, 2012). The media can have a strong effect upon the rhizosphere by affecting both physical properties as well as structural. The media is essential to temperature, PH as well as water and O_2 availability. In terms of structural effect, if the media is compact it can often affect the root growth, slowing it or dictating the way it grows, as well as impacting the physical properties. For example, for a media such as soil, that is highly compacted, water infiltration is poor resulting in little water being accessible to microbes or the root, penetration resistance will restrict root development and oxygen diffusion will be poor (Arshad & Coen, 2009). These media conditions have a clear detrimental effect upon the other constituents of the trinity, showing just how important a role the media plays on the rhizosphere.

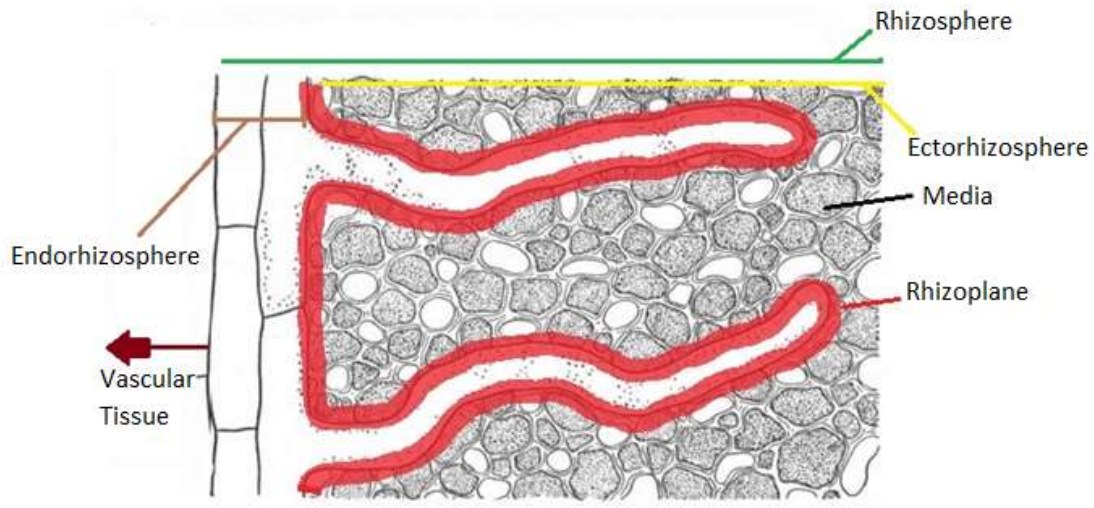


Figure 1.2: Schematic diagram of the root rhizosphere structure, including a root hair within its surrounding media.

1.7.b Inhabitants of the Rhizosphere.

Due to the nature of the rhizosphere, it is inhabited by a wide range of organisms that include bacteria, fungi, nematodes, protozoa, algae and micro-arthropods (Lynch, 1990). Some organisms can be of benefit to the plant, forming a mutualistic relationship. These include N-fixing bacteria, endo or exomycorrhizal fungi, and growth promoting rhizobacteria and fungi. There are also detrimental organisms, such as pathogenic fungi, oomycetes, bacteria and nematodes (Raaijmakers *et al.*, 2009). Despite the root exudates containing repellents, some pathogenic organisms are still able to overcome this.

Of the four groups of plant pathogens previously mentioned, fungi/oomycetes (often considered together due to significant similarities) and nematodes are considered to be the major soil borne threats. Bacteria are often overlooked as plant pathogens as a result of only a few species being considered truly soil borne, as they often require special conditions to survive and infect in the soil (Raaijmakers *et al.*, 2009). Fungi and oomycetes are however, when facing adverse soil conditions, able to survive through the formation of resting propagules such as chlamydospores, conidia and oospores which can withstand a variety of adverse abiotic conditions such as temperature and water stress in the absence of plant material and thus a rhizosphere (Bruehl, 1987). Therefore these groups are considered to be the key soil borne plant pathogens (see table 1). Within the soil these pathogens are almost exclusively necrotrophic, in contrast to many above-ground pathogens which are biotrophic, and often have a wide host range meaning they are not restricted to particular plant species.

Table 1.1: Common fungal/oomycete soil borne tomato pathogens, adapted from (Deery, 2012) **and** (Blancard, 2012)**. Orange= Fungal Blue= Oomycete.**

Soil borne fungi/ oomycete pathogens	Disease/ symptoms
Colletotrichum coccodes	Root rot/ Black dot
Fusarium lycopersici* f. sp. *lycopersici	Fusarium wilt
Fusarium lycopersici* f. sp. *radicis-lycopersici	FORL (Fusarium crown and root rot)
***Phytophthora* spp. (18 species)**	Similar to *Pythium*
Pyrenochaeta lycopersici	Corky root
***Pythium* spp. (19 species)**	Damping off, foot rot, root rot, Buckeye rot
Rhizoctonia solani	Damping off, fruit rot
Sclerotinia minor	Sclerotinia drop, watery soft rot
Sclerotinia sclerotiorum	Sclerotinia drop, watery soft rot
Sclerotium(Athelia) rolfsii	Sclerotium stem rot, Southern wilt
Thielaviopsis basicola	Black root rot
***Verticillium* (5 species)**	Verticillium wilt

Identification and diagnosis of different pathogens eliciting disease symptoms can be particularly difficult. One reason for this is as a direct result of the inaccessibility of the roots to visual examination. Within soilless growing systems this difficulty is partially eliminated, however, problems still remain in that many of the symptoms exhibited are difficult to distinguish between different pathogens and abiotic stressors, and great care is needed to be taken to do so accurately (Blancard, 2012).

For beneficial microorganisms within the rhizosphere, there are a variety of ways in which they may act. There are two distinct groups of beneficial microbial inhabitants: Microorganisms that have a direct positive effect upon the plant, and those that indirectly benefit the plant by inhibiting the effects exerted by pathogens (Raaijmakers *et al.*, 2009). Directly

beneficial microorganisms either work by inducing a systemic response (Pieterse *et al.*, 2003); this initiates the immune response partially protecting the root from later infection. They may also work by enabling the plant to either improve its nutrient uptake or improve the availability of nutrients to the plant (Richardson *et al.*, 2009). Beneficial microorganisms that act indirectly, aid the plants by inhibiting the negative effects of detrimental microorganisms. These are commonly now referred to as biocontrol agents (BCA's), and are being intentionally applied to the rhizospheres of plants as an alternative to pesticides and fungicides.

1.8 Tomato Pathogens of Note

1.8.a Colletotrichum coccodes

Colletotrichum coccodes is a fungal ascomycete that is pathogenic towards tomato and potato. Worldwide *C. coccodes* is a common pathogen reported in many countries within every continent in all forms of vegetable propagation, whether it be soil grown in the field or protected in a soilless production system within a glasshouse (Blancard, 2012). Pathogenic isolates taken from tomato and potato are morphologically indistinguishable and furthermore it's been found that those isolates taken from potato are also able to elicit infection on the tomato fruit (Illman *et al.*, 1959). In tomato *C. coccodes* is able to readily infect all parts of the tomato plant, resulting in anthracnose on the fruit, as well as causing black dot and brown rot on the roots. The damage inflicted upon the fruit is regarded as the key concern raised by *C. coccodes*, and in processed tomato production, if not controlled, anthracnose can lead to losses of approximately 70% of the yield (Blancard, 2012). Anthracnose starts initially as brown flecks on the surface of the fruit that will later progress to become large circular concave spots with brown centres where the flesh becomes discoloured and grainy (Dillard, 1989). As well as infection of the fruit, recently increasing attention is being directed towards root infection. *C. coccodes* was once just regarded as a secondary pathogen, associated with *Pyrenochaeta lycopersici*, however it has gathered more interest due

to the potential damage it may cause, especially in intensive soil grown systems.

C. coccodes can readily survive in the soil on plant debris, or in the form of sclerotia, which survive in the soil for up to 8 years (Dillard & Cobb, 1998), but predominantly it acts as a rhizosphere inhabiting organism. *C. coccodes* infects by the sclerotia forming either mycelium, or acervuli when the conidia contacts the surface of the fruit. These then adhere to the surface of the plant by means of an appressoria and can penetrate directly through the cuticle or through openings such as wounds (Blancard, 2012).

Root rot, as a result of *C. coccodes,* was a lesser known disease of tomato; however, since the reduction in use and replacement of many fungicides and pesticides, such as methyl bromide in 2005, there has been a rise in traditionally less common diseases such as *C. coccodes* (Garibaldi & Gullino, 2010). Due to *C. coccodes* being regarded as little threat prior to the ban of traditional fungicides, most tomato rootstocks commonly used commercially had no resistance to it. Following the widespread bans of traditional pesticides sudden over reliance on rootstocks for certain crops, such as tomato, for more common diseases aided *C. coccode's* increase in prevalence. Now *C. coccodes* needs to be taken into account when selecting rootstocks to use to prevent further losses as a result of root infection (Gilardi *et al.*, 2014).

In terms of control and prevention of *C. coccodes*, it must be very carefully observed throughout the season and any rise in prevalence within the soil must be managed immediately. Once the symptoms are observed on plant roots it is too late to eliminate it but plants with visible symptoms should be removed as soon as possible to prevent further spread. If this occurs in recycling systems such as hydroponic systems with no water treatment, the system should also be thoroughly cleaned and disinfected to prevent the reoccurrence of any residual *Colletotrichum*

following the removal of infected material. The current irreversible nature of infection is the reason for the importance that is placed upon the monitoring of levels of *C. coccodes*, until a durable resistance is available or an effective management method is developed.

1.8.b Fusarium oxysporum:

The *Fusarium* genus is made up of saprophytic and pathogenic ascomycetes with an extremely wide host range spanning across commercially produced plants from cereals to fruit. *Fusarium* is extremely common worldwide, so much so that it has a consistent presence within fungal communities in soils and is regarded as a normal constituent in a plants rhizosphere (Burgess, 1981; Gordon & Martyn, 1997). Fusarium can survive in soils as deep as 80cm for up to 10 years, through; maintaining mycelia on plant debris, micro and macro conidia and strong walled chlamydospores (Garrett, 1970; Blancard, 2012). In terms of *Fusaria* associated with tomato rhizospheres, there are several species described; *Fusarium oxysporum* and *Fusarium solani* are two that are generally considered the key ones.

Upon tomato's exposure to pathogenic strains of *F. oxysporum* it can result in tracheomycosis (vascular wilt) as well as root and crown rot (Fravel *et al.*, 2003) depending upon the strain of *Fusarium* infecting. Fusarium pathogens are highly specific in the host range they can infect, dependent upon the host species and cultivar. This high level of host specificity thus forms the basis of a further classification within *Fusarium oxysporum* separating into *formae speciales* (ff. spp.) and races. Of *Fusarium oxysporum* strains that are pathogenic, there are approximately 120 *formae speciales* (Armstrong & Armstrong, 1981). There are two *formae speciales* that are pathogenic towards tomato; f.sp*. lycopersici* and f.sp*. radices-lycopersici*.

1.9 Pathogen Detection Methods

1.9.a 'Pre-Molecular' Technologies

Unlike in the identification of human pathogens, detection methods within the field of plant pathology have been relatively slower to develop. Prior to the development of molecular techniques, detection and identification of plant pathogens was predominantly carried out by means of morphological assessment using a variety of specific culturing media and observations of visual symptoms. This, as an identification method, is not wholly suitable due to several factors such as; the analysis is a time consuming, labour intensive process and requires an individual with extensive taxonomical knowledge to carry out an accurate identification (Lievens & Thomma, 2005). As well as this, many plant pathogenic microbes are still very difficult to culture and often the species that are culturable have non-pathogenic and pathogenic strains which are difficult to distinguish between using morphological assessment and varying culture media alone such as with *F. oxysporum* and the various similar *formae speciales*. To distinguish between these organisms differences are looked for in symptomology, but this is made extremely difficult by the overlapping nature of many plant pathogen's symptoms as well as some of the symptoms also being common to those caused by many abiotic stresses. It should also be remembered that if symptoms have begun to be expressed in plants, although it may aid identification of the pathogen, it is then usually too late to treat it effectively.

1.9.b Molecular Based Detection

More recently, with the development of polymerase chain reaction (PCR), traditional methods of pathogen detection and identification have become less frequently used, whilst molecular methods have risen to the fore. Molecular methods of plant pathogen identification offer solutions to many of the lacking features of traditional methods such as; rapidity, specificity and accuracy (Schaad & Frederick, 2002). This also means that it is no longer necessary to wait for plants to demonstrate disease symptoms

before it's possible to identify the pathogen. Instead, molecular methods allow us to identify pathogens quickly, even on an asymptomatic plant, and to thus act accordingly preventing the disease progressing further or even at all.

Early molecular detection methods were generally based upon dot blotting techniques. These were extremely specific, making use of specific DNA hybridization probes. Despite the high specificity, this method initially struggled with low sensitivity. It was also often time consuming, and would also require expert knowledge to carry out (Schaad & Frederick, 2002).

However, during the 1980's, any issues regarding sensitivity in these techniques were largely eradicated due to the development of PCR. PCR allows for even extremely low concentrations of genetic material to be amplified to a relatively high and usable concentration. This advance unlocked new opportunities in molecular biology utilising specific primers that could specify between samples down to a strain level. This ability to produce ample genetic material has also led to the onset of developing more technologies related around the same principles as PCR.

Q-PCR's development is based on the same essential principles as normal PCR, however it takes it a step forward by quantifying the amount of target genetic material in the original sample. It achieves this by utilising PCR's cyclical denaturing, re-annealing and extension of DNA using fluorescent probes and dyes to calculate the rate of amplification. Due to the theoretical principle that amplification should double target strand number each cycle, the cycle number when a certain level of fluorescence is first emitted should be proportional to the logarithm of the original quantity within the sample.

1.9.c Microarray Analysis of Samples

The basis of microarray analysis in pathogen detection is very similar to that of Southern blotting but on a larger scale, testing for a potentially limitless amount of pathogens at once. Microarrays are more commonly

used for gene expression analysis (Schena *et al.*, 1995), however their uses have been expanded and adapted to be used phylogenetically. DNA microarrays make use of short, specific hybridization probes to identify within a sample if any complimentary target sequences are present. These are in the form of probes ≤25 bp long with sequences heterologous to that of the target sequences being looked for, bound to a specific membrane, such as a glass slide. To use the microarray, the specific genetic target sequence within a sample must be amplified, whilst incorporating uracil nucleotides. Once the sample is amplified it is hybridised with the microarray probes, along with the addition of uracil-specific-enzyme-linked antibodies which will bind to the previously incorporated uracil. To visualize successful hybridization to the probes, a substrate is added to the array which is cleaved by the uracil-specific-enzyme-linked antibody causing localised colour change. This colour change is then recorded and the degree to which the colour changes can be used in a semi-quantitative measure in some cases.

One of the key benefits microarrays have to offer as a diagnostic tool is to identify a large number of organisms to a very high level of specificity, all at once. This specificity can be to the level of individual base pairs, which in closely related microbial species is important as the difference can be as small as a single nucleotide polymorphism (SNP)(Lievens & Thomma, 2005). This slight difference between related microbial populations means that the region of DNA that is selected as a probe is very important. As well as having a high degree of specificity, microarrays also have much improved sensitivity when compared to some Southern blot techniques. Previously when samples were tested using Southern blot, there would be false negative results due to low sensitivity. As these microarrays involve DNA amplification, they can detect pathogens even if they were only in scarce quantities in the original sample.

1.9.d DNA Barcodes

DNA barcodes are sequences of DNA that can be used to differentiate between different species and strains. These DNA barcodes ideally should

contain high levels of interspecific variation whilst maintaining a low level of intraspecific variation to be reliable and specific in sample identification. The mitochondrial *cytochrome C oxidase subunit 1* (*CO1*) was initially the *de facto* barcode selection across all eukaryotes (Hebert *et al.*, 2003). However, despite the *CO1* region still being used reliably in species identification in a variety of animals it has now all but been discarded as a DNA barcode in fungi due to unreliability and variations within fungal species. It has now been replaced by nuclear rDNA (Schoch *et al.*, 2012). The nuclear rDNA cistron is made up of three 3 key sections; the 18S subunit (small subunit), the 5.8S subunit, and the 26/28S subunit (large subunit) (Fig. 1.3). Following transcription of the whole unit by RNA polymerase, the RNA undergoes post-transcriptional splicing, resulting in the removal of the entirety of the middle section. This includes the highly conserved 5.8S subunit as well as two highly variable internally transcribed regions; ITS1 and ITS2 (Schoch *et al.*, 2012). It is within these ITS regions that sufficient interspecific variation has been found that makes them a promising target for DNA barcoding of particular pathogens. The reasons for such variation within the ITS genes is due to them being non-coding sequences, which are quickly spliced out following transcription. Sometimes, the ITS regions alone are not sufficient enough to offer a full confirmation of the species and it is therefore useful to use other housekeeping genes as confirmation. Genes which have attracted interest in this field as discriminatory sequences thus far include; beta-tubulin genes (Fraaije *et al.*, 1999), actin (Weiland & Sundsbak, 2007) and elongation factor-1-alpha(O'Donnell *et al.*, 1998).

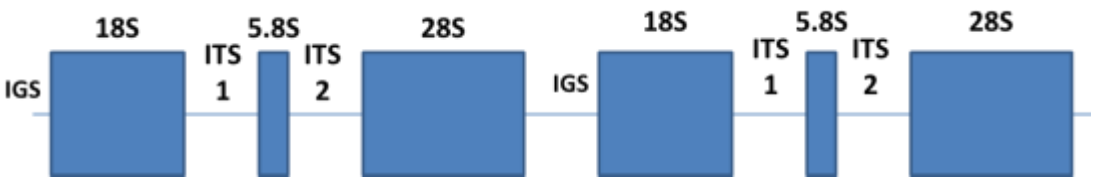


Figure 1.3: Graphic representation of the rDNA region, featuring the 18S, 5.8S and 28S exons (blue), and the IGS, ITS1 and ITS2 exons

1.10 Aims of the Study

Prior to this project, Deery (2012) conducted research into monitoring the microbial populations associated with the rhizosphere of commercially grown tomatoes. This was initially conducted utilising terminal restriction fragment length polymorphisms (T-RFLP's). This research was later followed up and complimented by Devine (2013) who developed a microarray featuring probes identifying a variety of microorganisms commonly associated with tomato production, pathogenic and beneficial. This microarray was employed by Scott (2013) to expand on the rhizosphere population studies of Deery (2012), since the improved technology not only increased sensitivity of the tests, but also the frequency of sampling. This study focusses on three separate rock wool tomato crops, using three contrasting water treatment systems.

The aim of this research was to build up more information on microbe population variation throughout the tomato growing season. This was facilitated by five parts to the research: (1) Monitoring five nurseries featuring different water treatment systems including heat, UV pSSF (part-slow-sand-filter), SSF, and RTW (Run-to-waste). Each of these nurseries was sampled at four separate time points throughout the year, and each nursery also had water sampled from five pre-designated points in the hydroponic system, so that as well as the general population being monitored, general variation throughout the system could also be observed. (2) Similar to in Scott's (2012) research, root samples were collected from each nursery and a further four different nurseries. This was to observe general population changes as well as the effect of certain microbes in the water on the populations within the roots. (3) A greenhouse trial was conducted to test the effects of two common tomato pathogens; *Fusarium oxysporum* and *Colletotrichum coccodes*, at a range of spore concentrations, as well as simultaneously comparing the effect these pathogens had on plants grown on their own roots to those cultured on traditionally more hardy rootstocks. (4) The final part of the project

was to develop and validate q-PCR primers for four common tomato pathogens: *Colletotrichum coccodes, Plectospharella cucumerina, Pythium aphanidermatum* and *Pythium myriotylum.* Once these had been validated they could then be employed to quantify the amount of specific pathogens within water and root samples, rather than relying on the microarrays semi-quantitative aspect.

Chapter 2

2.1 General Methods

2.1.1 DNA Extraction of Plated Samples

Fungal pathogens were maintained on a mixture of slope tubes and plates. *Fusarium oxysporum* was cultured on potato dextrose agar medium, whilst *Colletotrichum coccodes* was cultured on oatmeal agar medium. All the plates were stored at 25ºC for sporulation. The extraction of DNA from the plated samples provided from ADAS first required the loading of screw-cap Eppendorf tubes with 8-12 2 mm glass beads that had been autoclaved prior to use. The sample collected was 120 mg of scrapings of the fungi from the top of the agar plate using a sterilised spatula, trying to avoid the collection of excess agar with it. This was conducted under a class II biological safety cabinet; the 120 mg ±5 mg of sample was weighed out inside the sterile screw-cap Eppendorf tubes. Once 120mg were collected the samples were then homogenized; the samples were placed in liquid nitrogen for 2 min, after which they were then placed into the Fastprep FP120 (Thermo-Savant) at 6.5 ms^{-1} for 45 s. The samples were then rested before repeating the previous step. This sequence was repeated 3 times for each sample. Once the samples were homogenized they were then extracted using the 'DNeasy plant mini kit' (QIAGEN) following the standardised protocol provided. Upon extraction the DNA samples were then stored at -20ºC.

2.1.2 Polymerase Chain Reaction of Samples ITS Regions:

DNA samples for either genetic sequencing or for microarray analysis were amplified using PCR. Two sets of universal primers were used to amplify the eukaryotic ITS region, and the prokaryotic IGS sequence, (Table 2.1). These primers were produced by Sigma-Aldrich®, and were diluted to a stock solution of 100 µM and then when used diluted further to a working concentration of 10 µM, using sterile distilled water (SDW) and stored at -20ºC.

Table 2.1: Eukaryotic and prokaryotic ITS primers used, developed in (Sequerra *et al.*, 1997)**,** (Ranjard *et al.*, 2001) **respectively.**

Primers	**Sequence** (5'-3')	**Primer target**
Eukaryotic		
- **2234C**$^{\text{Forward}}$	GTTTCCGTAGGTGAACCTGC	3' of 18S subunit
- **3126T**$^{\text{Reverse}}$	ATATGCTTAAGTTCAGCGGGT	5' of 26/28S subunit
Prokaryotic		
- **S-D-Bact-1522-b-S-20**$^{\text{Forward}}$	TGCGGCTGGATCCCCTCCTT	3' of 16S subunit
- **L-D-Bact-132-1-A-18**$^{\text{Reverse}}$	CCGGGTTTCCCCATTCGG	5' of 23S subunit

For the PCR a master mix solution was produced of the reagents to a volume of n+1. This solution was composed of 1X 0.5 µl of both forward and reverse primers, 12.5 µl of 2X MangoMix (Bioline), and 10.5 µl of SDW. The PCR products that were to be analysed by the microarray had the further addition of 0.5 µl of 2x Biotin 16-dUTP (Roche) per reaction into the master-mix. The master-mix was then divided into 24 µl aliquots and distributed to autoclaved 50 µl single reaction PCR tubes. Then to each of these individual aliquots, 1 µl of sample DNA was added. The samples would then be placed in the BioRad® S1000TM thermal cycler, and set to the following programmes: Eukaryotic; 94°C for 2 min followed by 35 cycles of; 94°C for 30 s, 53 °C for 1 min and 72°C for 1 min; with a final extension of 72°C for 5 min. The prokaryotic amplification programme was: 94ºC for 3 min followed by 35 cycles of; 94ºC for 1 min, 55ºC for 30 s and 72ºC for a min; following cycling there was a final extension phase at 72ºC for 5 min.

Following amplification of the samples, products were separated on 1% agarose gels and stained using ethidium bromide. Once the products were separated on a gel it was then visualised using the UV camera InGenius3 on software platform GeneSys, both manufactured by Syngene, UK.

2.1.3 PCR Product Purification

Following the amplification of extracted DNA the PCR product would then require purification. This was done through the use of the QIAGEN "QIAquick PCR Purification Kit" and followed the manufacturer's protocol. The DNA samples to be micro-arrayed did not require purification.

2.2 Observation of Fertigation Solutions and Root Samples using Microarrays:

Throughout the 2014/15 growing season four nurseries, using five separate fertigation solution treatments, each had both water and root samples taken at four separate time points: January, April, July and October. On top of this a further five nurseries were sampled only for roots at two separate time points: July and October.

2.2.1 Sampling

All of the sampling from each of the nurseries was carried out by Sarah Mayne, of ADAS, under the instruction of Tim O'Neill.

2.2.1.a Root Sampling

For the sampling of roots, a scalpel and pair of scissors were sterilised prior to sampling. Approximately 2g of roots were collected for each sample. Throughout the season as the roots grew they were collected from different points. First they were collected from the bottom of the rock wool cube (pre slab contact). Following the roots reaching the rock wool slab they were collected from the slab for up to 4 weeks, and then finally for the rest of the season the root samples were collected from the corners of the slabs. Following the sampling of the roots the slab would then be re-sealed with tape. At each sampling point a new slab would be sampled from

For the first five main nurseries, three replicate root samples were collected at each of the four time points. Each nursery had a separate water treatment method, these were: pSSF, fSSF, heat, UV and RTW. An

additional five nurseries were sampled at two time points late in the season. These nurseries had one of two treatments; RTW (x2) or heat treatment (x3).

2.2.1.b Fertigation Solution Sampling

For each sampling section, a suitable point to take the sample was first located, with the assistance from a member of the nursery. There were generally three places to take samples; taps, tanks and slabs. For the sampling from taps, the water would be run for approximately ten seconds to clear the standing water, and then 250 ml of sample was run into a bottle. For sampling involving collection from a tank the sample bottle was dipped into the water until full. Finally, for the slab sampling, the plastic cover was slightly torn to allow access to the slab and then a sterile syringe would be used to take 250 ml. This 250 ml produced two replicates from each position. Each nursery has 5 pre-designated sampling sections, for the four systems using water treatment these were generally similar, whereas for the run-to-waste nursery (RTW) it was slightly different. For the RTW nursery, instead of pre and post treatment sampling, there were two different slabs to be sampled from. One slab accommodated tomatoes grown on their own roots, whilst the other slab contained plants that were instead grown on a hardier rootstock. For each of the nurseries, the sampling points used are listed in table 2.2.

Table 2.2: Water sampling points for each of the 5 nurseries. FSSF- full slow sand filter. pSSF- part slow sand filter. RTW- Run to waste. WS- Water source. BH- Bore hole. CT- Collection tank. M- Mains water. S- Slab. PRT- Pre-treatment. GS- Grafted root slab. POT- Post treatment. US- Un-grafted slab.

Sampling Point	FSSF	pSSF	UV	Heat	RTW
1	WS	WS	WS	WS	BH
2	CT	CT	CT	CT	M
3	S	S	S	S	CT
4	PRT	PRT	PRT	PRT	GS
5	POT	POT	POT	POT	US

2.2.2 DNA Extraction of Root Samples

For the root samples provided by the nurseries, extraction of DNA followed much the same protocol as the DNA extraction from plated samples. Root samples were first examined on sterile filter paper, removing much of the excess water, where the younger looking roots were selected. Once 120 mg ±5 mg was selected they were placed inside an autoclaved screw-cap Eppendorf tube containing 8-12 2 mm glass beads. This, as before, was then homogenised in a sequential process of being placed in liquid nitrogen and a Fastprep machine as outlined in 2.1.1). Following homogenizing, the protocol followed that of the 'DNeasy plant mini kit' (QIAGEN).

2.2.3 DNA Extraction of Fertigation Samples

Upon receiving the samples, the DNA was extracted as soon as possible. The sample would be separated into two replicates for each sampling location of 100 ml and were passed through a Nalgene™ reusable 250 ml vacuum filter unit that held a sterile 47 mm, 0.2 µm Supor®-200 PES Membrane Disc Filter (Pall product). Once 100 ml of fertigation solution was passed through, the filter was washed using 100 ml of sterile distilled water (SDW) to remove any excess salts that remained on the filter. Once the water had been passed through, the filter was then removed by cutting into thin strips using a sterile scalpel and placed in a micro Bio-Spin® chromatography column. Once samples were placed in this column, 400 µl of lysis buffer (AP1 from the DNeasy plant mini kit) and 4 µl of RNase (also from DNeasy mini kit) were added to the columns before incubation at 65ºC for 10 min within a water bath with 2-3 inversions. Once the incubation was complete the columns stopper at the bottom was removed and then placed in a receiver tube where it was then centrifuged for 7 min at 6000 rpm. Following this, the column was then discarded and the DNA extracted using the 'DNeasy plant mini kit' protocol.

2.2.4: Microarray Analysis of Samples

Once the sample DNA was amplified, it was then possible to analyse it using the small scale microarray. The microarray itself was manufactured by Alere™, Germany following the design developed by Devine (2013), with the potential to detect a wide range of organisms (listed in appendix 1). Throughout the protocol, a Thermomixer Comfort ® (Eppendorf) dry heat block was used. In the first stage of the protocol the ArrayTube itself first needs conditioning. This first means that 500 µl of SDW must be added to the tube, and is kept in the thermomixer agitating at 500 rpm for 5 min at 50ºC. Once the 5 min was finished the contents were discarded and replaced by 500 µl of Nexterion® Hyb which is again agitated at 500 rpm for 5 min at 50ºC. Once finished it was too, discarded. Once the microarray had been prepared a new 80 µl of Nexterion® Hyb was added to the array and kept at 55ºC whilst the Purified PCR products were heated to 95ºC for 5 min in the BioRad® s1000™ thermal cycler. This heating of the DNA allows the DNA strands to dissociate from each other. Twenty µl of both the prokaryotic and eukaryotic biotin incorporated samples were then added to each microarray and allowed to hybridise with the arrays probes whilst kept at 55ºC and agitated at 500 rpm for 1 hour and 15 min.

Once the hybridisation steps were finished the micro array underwent 3 stages of cleaning; first 500 µl of wash buffer 1, WB1 (2X SSC [saline sodium-citrate], 0.01 % Triton X100) was added and then agitated at 500 rpm for 5 min at 20ºC. Following this, the WB1 was discarded and replaced by 500 µl of WB2 (2X SSC) and kept in the same conditions as WB1 for 5 min. This was then repeated one final time following the discarding and replacement of WB2 with 500 µl of WB3 (0.2X SSC).

Once the cleaning was finished the next step of the preparing the microarray for analysis was to block the spots on the microarray where there is no DNA-probe binding. This was done with the addition of a freshly produced blocking solution (n+2) made up of 200 µl PBS

(phosphate-buffered saline), 4 mg of skimmed milk powder, and 1 µl of 1% Triton X100. Once vortexed 100 µl of this solution was added to each microarray and kept at 20ºC for 15 min before being discarded. The next step; conjugation, also required a freshly prepared solution. The solution was made at a quantity of n+2 with 100 µl of blocking solution and 1 µl of HRP (horse radish peroxidase) linked anti-uracil antibodies (Cell Signalling Technology®). This solution was then gently mixed and 100 µl added to each of the microarrays. This was again incubated at 20ºC for 15 min. The microarray then underwent another washing stage where 500 µl aliquots of PBST (1X PBS, 0.2% Tween 20) were added to each microarray, agitated at 500 rpm at 20ºC for 5 min and discarded. This was repeated three times. For the final step the array was stained with a 100 µl aliquot of SeramunGrün® chip (Seramun®). This is an o-Dianisidine substrate and is then cleaved by the horse radish peroxidase enzyme linked to the antibodies. This then results in a localised colour change that can then be recorded by the array-reader. The substrate was left to incubate in the ArrayTube for precisely 16 min at room temperature before being placed in the ArrayTube transmission reader-03 (Alere™) whilst utilising the software programme Iconoclust v4.1 (Alere™) to record the images of the array. These images were then subsequently analysed using a bespoke experimental template (10429_AT_myco_2_1.0, Alere™) which aligns probes with their respective organisms. The intensities of the probes were then normalised and corrected in terms of the local background intensity, using the equation:

$$i = \frac{Average\ spot\ intensity}{Local\ background\ intensity}$$

2.2.5 Microarray Data Analysis

From the results gathered through the use of Iconoclust V4.1, the data was first filtered by removing any ambiguous results or results due to background noise by using intensity cut off value of 0.1. This value was selected as it is double that of any recorded results recorded from a blank array. For all of the organisms detected on the array, there were two probes. This is to prevent lack of detection as a result of anomalous results or by damage to one probe. These two probes would then be averaged.

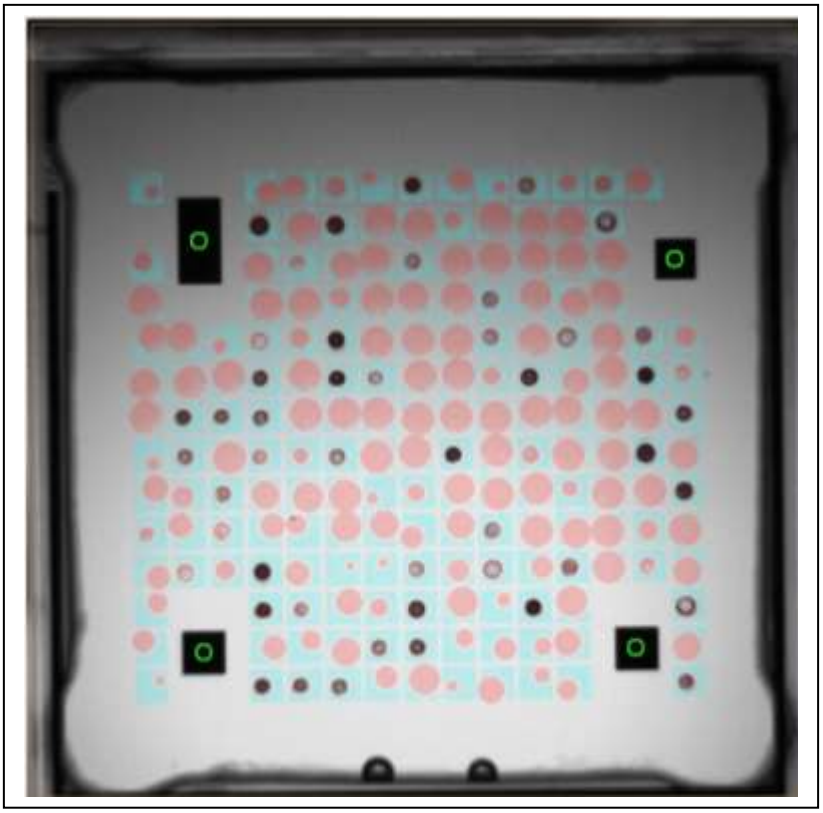


Figure 2.1: Microarray image, with template overlay.

2.2.6 Species Diversity

To maintain continuity with previous projects (Scott, 2013), the same method of calculating species diversity was maintained, using an adapted form of the Shannon-Wiener index (H'). All the taxa on the microarray were divided into four categories for this analysis: total array, fungi and

oomycetes, saprophytic fungi, and bacterial. Universal probes, such as the fungal or bacterial probe were regarded as independent species and allocated to their associated groupings. For each category the species index was calculated and recorded following the adapted formula:

$$H' = -\sum \left[\left(\frac{a}{b}\right) ln \left(\frac{a}{b}\right) \right]$$

Where a= average intensity of a single species on the array, b= sum of all average intensities on the array.

2.3 Validation of q-PCR Primers

2.3.1 Development of Primer Sequences

An initial literature search was conducted for any primers that already exist for the target organisms. This yielded primers for *Colletotrichum coccodes, Plectosphaerella cucumerina, Pythium aphanidermatum* and *Pythium myriotylum* (Table 2.3).

2.3.1 TaqMan multiplex assay

For *C. coccodes, P. cucumerina, P. aphanidermatum* and *P. myriotylum* the aim was to develop a q-PCR assay that could be multiplexed. This meant that each probe required a fluorophore of a different fluorescence wavelength range.

Table 2.3: List of TaqMan primers: [1](Cullen *et al.*, 2002)**, [2]**(Atkins *et al.*, 2003)**, [3]**(Li *et al.*, 2014)**.**

Primer	Sequence (5'-3')	Target DNA	Product size (bp)
***C. coccodes*[1]**			
- **CcTqF1**	TCTATAACCCTTTGTGAACATACCT AACTG	ITS 1	145
- **CcTqR1**	CACTCAGAAGAAACGTCGTTAAAAT AGAG		
- **Taqman probe**	CGCAGGCGGCACCCCCT		
***P. cucumerina*[2]**			
- **PcRtF1**	GTGCCCGCCGGTCTC	ITS 1	72
- **PcRtR1**	GACAGTTCGCTAAGAACACTCAGAA GT		
- **Taqman probe**	TCAGAATCTCTGTTTTCGAACCCGA CGA		
***P. myriotylum*[3]**			
- **PyuniTqF1**	CTGTTCTTTCCTTGAGGTG	ITS 2	149
- **PmTqR1**	GGAGCCGAAACTCTCACAAGAC		
- **Taqman probe**	TCCCAAATTGGTGTTGCCTTCTTTA CCC		
***P. aphanidermatum*[3]**			
- **PyuniTqF1**	CTGTTCTTTCCTTGAGGTG	ITS 2	163
- **PaTqR1**	GCGCGTTGTTCACAATAAATTGC		

Chapter 3 Observation of microbial population changes within fertigation solutions undergoing different fertigation treatments throughout the 2014/2015 growing season.

3.1 Introduction

As mentioned previously (Section 1.4), much of the European production of tomato is now conducted using hydroponic systems within glasshouses. Due to the outlined risks with disease within hydroponic systems (See section 1.4.b), and the cost of having an open system, a water treatment step in recirculating hydroponics is vital. Commercially, three types of water treatments are commonly used; pasteurisation, UV treatment and slow sand filters (SSF), all outlined previously (See section 1.5).

Whilst each of these has its benefits, each comes with its own separate disadvantages often being the factor that determines the user's choice. For example, despite pasteurisations having a potential effectivity of 100%, it is an extremely high cost treatment to set up and use. To heat 10,000 L of fertigation solution (FS) to 95° C, it needs approximately one gigajoule of gas energy (Rolfe *et al.*, 2000; Yiasoumi, 2005). This is clearly a high cost undertaking, and in a world where there are varying local gas prices, for many regions this will be unfeasible.

In terms of UV treatment a key issue effecting efficacy of the treatment is the clarity of the water undergoing treatment. If there is anything in the solution; whether it is plant debris, suspended or dissolved solids or even planting media, these will either absorb or reflect the UV radiation being emitted. For the treatment to really be effective the water needs clarity of approximately 60% transmissibility (Mebalds *et al.*, 1996). All things present in the solution have the potential to reduce the propagules exposure to the UV emission, even if it is just intermittently, which then affects the dosage administered to the solution as: Dosage= UV radiation x Time exposed (Downey *et al.*, 1998). This means for UV treatment to be effective, it requires the solution to first undergo flocculation and filtration prior to the treatment (Runia, 1993a).

A major issue encountered with both UV and heat treatment is how both exert their effect indiscriminately. In terms of microorganisms, there are many that are beneficial as well as pathogens (Stewart-Wade, 2011), but these treatments kill both types. As well as indiscriminately effecting microorganisms, heat and UV treatment have also been found to have undesirable affects upon certain chemicals and substances. In a system recycling its fertigation solution in an attempt to reduce losses of nutritional inputs this is counterintuitive. Not only does it break down some of these substances, it has also been found that UV treatment has been linked to the downstream reduction in plant growth as a result of UV radiation generating ozone and free radicals (Schwartzkopf *et al.*, 1987).

SSFs are still relatively new in terms of water treatment technology. As mentioned in section 1.5.c, much is still unknown about their efficacy or even their mode of action, as to whether they act as a physical treatment or if the disinfection is a result of a biological reaction between the microbial populations within the filter. It is proposed that the filter in physical terms acts more as just a crude filter removing larger organisms and debris, whilst the biological influence is responsible for the disinfection. This is supported by reports that the effectivity of the filter is reduced as a result of factors such as oxygen deprivation, low temperatures (Ellis, 1985; Vankuik, 1994; Brand & Alsanius, 2004), and if the filter has been sterilised and not allowed to ripen (Mine *et al.*, 2003; Furtner *et al.*, 2007). However, one reason that it is believed to outperform other physical treatments is that it is more discriminant and may also aid beneficial organisms to proliferate (Cafa, 2012).

The aim of this piece of research was to monitor the microbial populations within fertigation solutions from five separate commercial nurseries. Each of the nurseries utilised contrasting water treatment systems: Slow sand filters (SSF), part slow sand filters, UV treatment, pasteurisation and one that was a run-to-waste that has two different rootstocks. These nurseries were monitored at four separate time points throughout the 2014/2015 growing season. On top of monitoring over different time points, each

nursery was sampled from five different points in the system to monitor the fluctuation in microbial populations. The observations were facilitated by a small scale microarray developed by Devine (2013), that built upon the work of Scott (2013) and Deery (2012).

3.2 Methods

Over the 2014/2015 growing season four time points for sampling were spread between January and October. Sarah Mayne (ADAS) carried out sampling after first identifying points that could be resampled from throughout the season (Section 2.2.1)

Once samples had been collected, they needed to be filtered, collecting the microorganisms in the sample before the DNA was extracted as outlined in section 2.2.3. The DNA samples were then amplified using both prokaryotic and eukaryotic primers (Section 2.1.2) before undergoing microarray analysis as outlined in section 2.2.4 and 2.2.5. The data gathered was then used to work out the species diversity (Section 2.2.6).

3.3 Results

Table 3.1 shows a brief overview of the overall species richness observed over the growing season. This includes results from all fertigation samples from all sampling points in each system. Species richness gives a count of different individual species recorded at any point.

Table 3.1: Total species richness over the growing season in each of the different hydroponic systems using different water treatment methods.

	pSSF	fSSF	UV	Heat	RTW
Total Array	25	34	28	26	22
Pathogenic Fungi and Oomycetes	13	13	10	9	11
Saprophytic Fungi and Oomycetes	6	12	11	12	6
Bacterial Organisms	6	9	7	5	5

Over the whole season there were 15 species that were detected in every system in at least one time point, these are listed in table 3.2. As well as this there were also certain species that were detected uniquely to one system. There were 15 of these species unique to individual systems and these are listed with their associated system in table 3.3.

Table 3.2: List of organisms that appeared in all fertigation systems in at least one time point.

Pathogenic fungi/oomycetes	**Saprophytic fungi/ oomycetes**	**Bacteria**
P. irregulare	*A. flavus*	*Erwinia* spp.
P. myriotylum	*Cladophora* spp.	*Nitrospira* spp.
P. paroecandrum	*E. pisciphila*	*Pseudomonas universal*
F. oxysporum	*Penicillium* spp.	*Xanthomonas* spp.
S. subterranea		*Yersinia* spp.
V. nigrescens		

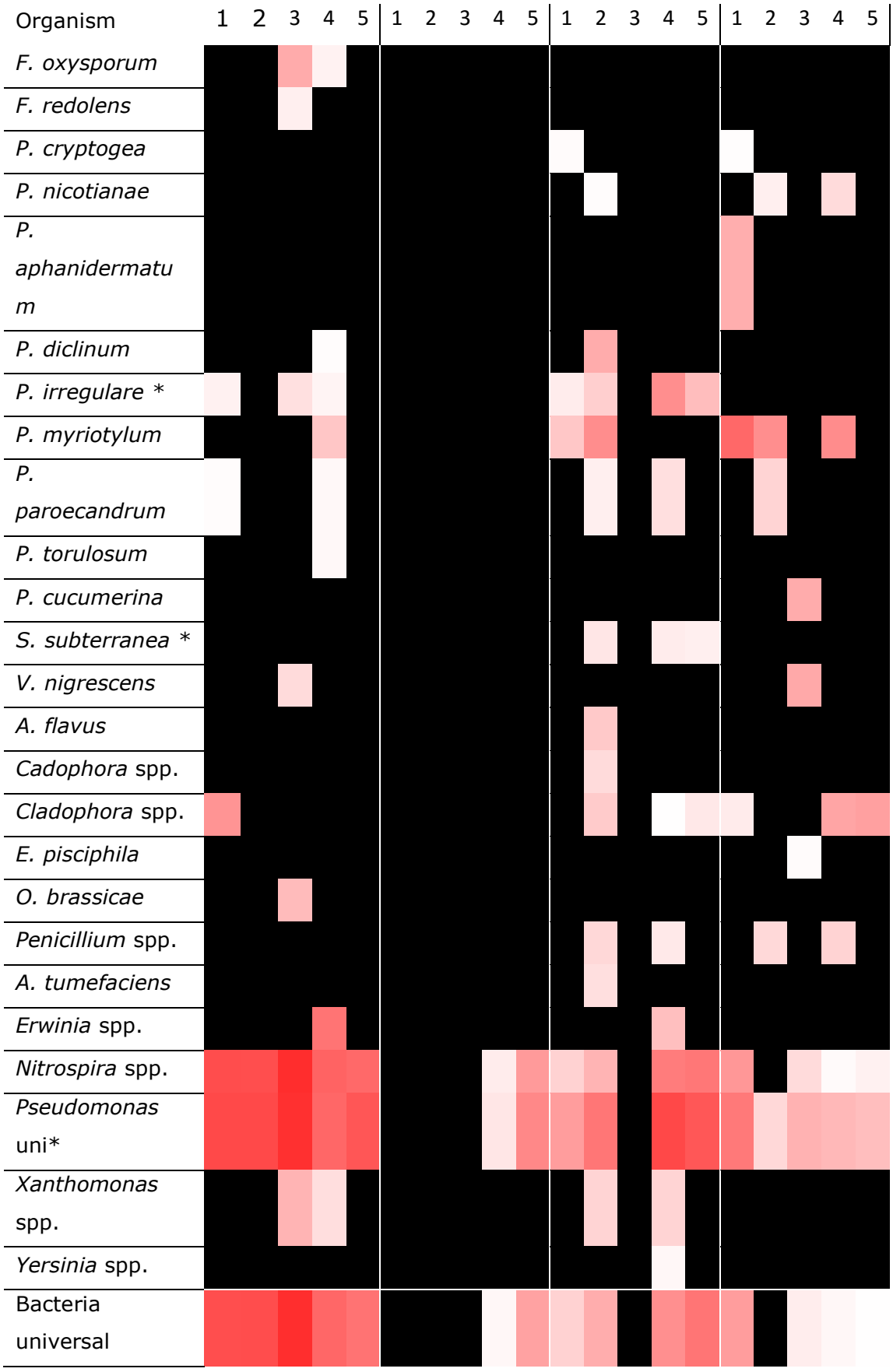
Table 3.3: Species that were detected uniquely to an individual system.

pSSF	fSSF	UV	Heat
P. torulosum	*P. echinulatum*	*A. solani*	*D. microsporus*
	P. megalacanthum	*P. debaryanum*	*P. brevicompactum*
	T. basicola	*R. solanacearum*	
	A. rhizogenes	*Alternaria* spp.	
	E. coli		
	Cephalosporium spp.		
	Chaetomium spp.		
	P. griseofulvum		

3.3.a Part-Slow sand filter

The part SSF filtered approximately 10 % of the FS being recirculated. In Figure 3.1 all results from each of the five sampling points, at each of the four time points, are presented in a heat map.

Overall the only organisms present at all four time points were *Nitrospira* spp. and *Pseudomonas* species. The most commonly detected fungal or oomycete species detected over the separate time points were the *Pythium* species *P. myriotylum* and *P. paroecandrum,* which were both detected in three of the four time points. It is worth noting that in both circumstances, neither was detected in their relevant post-treatment samples. It is also worth noting that for the two bacterial species the quantities detected seemed to increase post treatment.


Organism
1 2 3 4 5 1 2 3 4 5 1 2 3 4 5 1 2 3 4 5
F. oxysporum
F. redolens
P. cryptogea
P. nicotianae
P. aphanidermatum
P. diclinum
P. irregulare *
P. myriotylum
P. paroecandrum
P. torulosum
P. cucumerina
S. subterranea *
V. nigrescens
A. flavus
Cadophora spp.
Cladophora spp.
E. pisciphila
O. brassicae
Penicillium spp.
A. tumefaciens
Erwinia spp.
Nitrospira spp.
Pseudomonas uni*
Xanthomonas spp.
Yersinia spp.
Bacteria universal

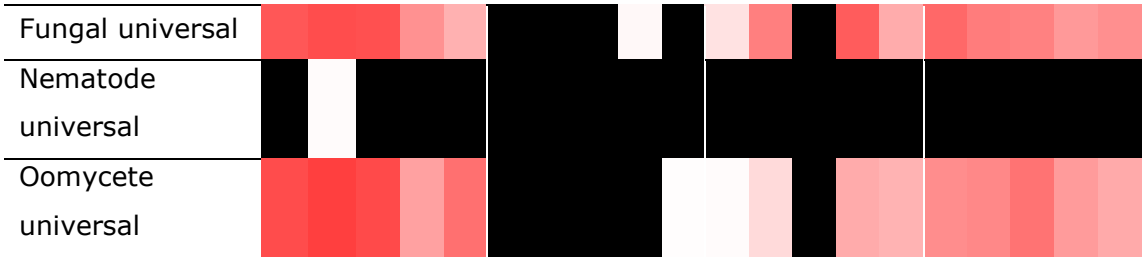


Figure 3.1: Heat map of all detected species throughout the part SSF system (1-5), at each time point (each block of 1-5 represents a time point), and their approximate abundance detected is denoted by colour intensity from white= low to red= high (black represents no detection). This was a system utilising a p-SSF as its water treatment technique. (*= Erroneous results possible from these probes)

Figure 3.2 represents the species richness detected at each FS sampling point in the system at the four separate time points. Three of the four results show a clear reduction in species number recorded in the post-treatment samples (5) when compared to their pre-treatment equivalents (4).

Figure 3.3 however, represents the average species diversity recorded at each sampling point in the system, for each class of organism on the array, including the universal probes. From this figure, it is clear that most classes, except for the universal probes, show a reduction in diversity following treatment. In terms of reduction in biodiversity, the reduction in pathogenic fungi and oomycetes seems to be significantly more than that of the bacteria.

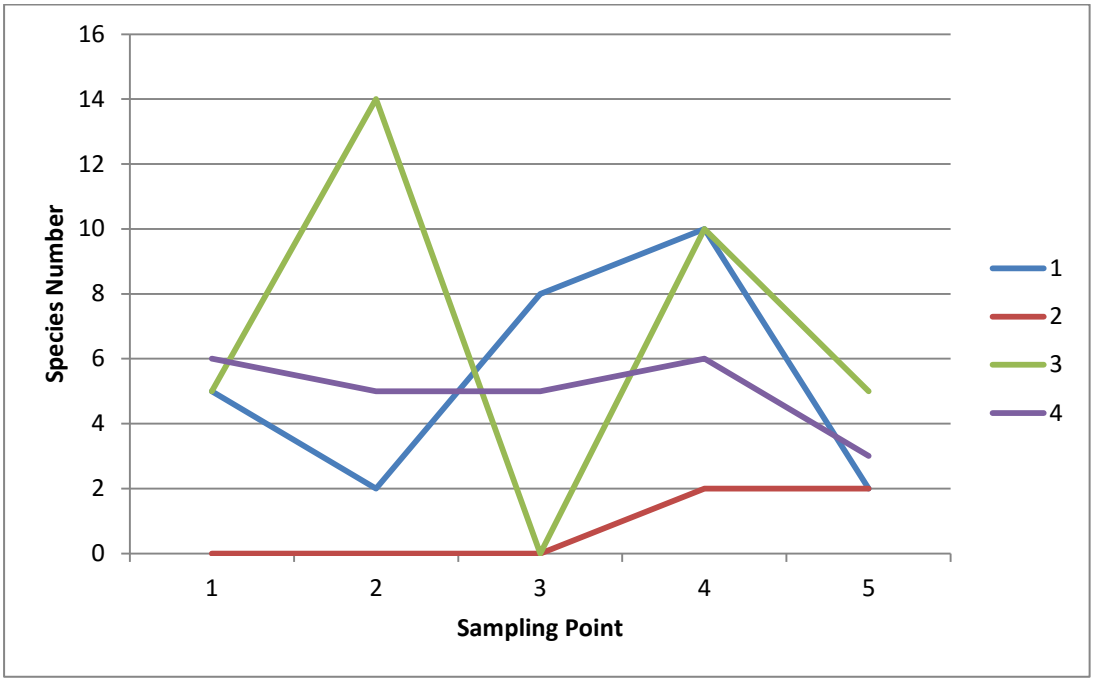


Figure 3.2: Species richness throughout the system using a p-SSF water treatment method, at all four time points (1-4 in key).

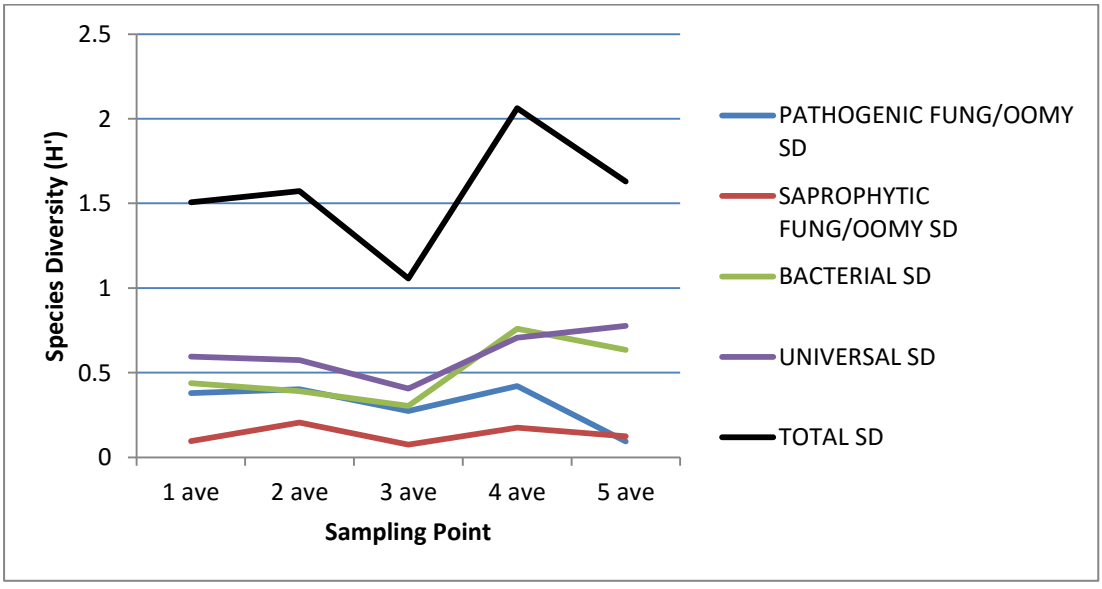


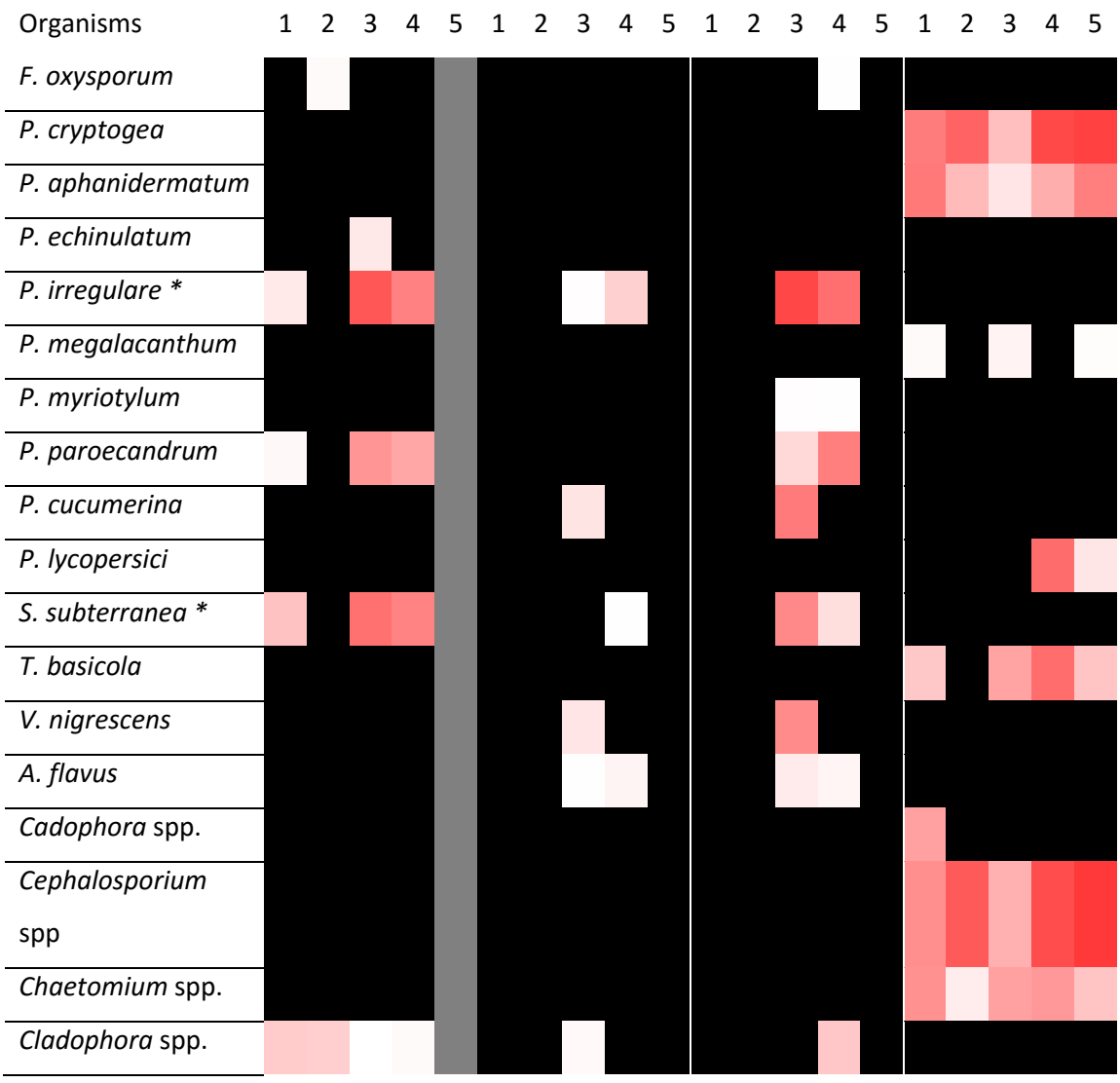
Figure 3.3: Average (ave) species diversity (H') throughout the system using a p-SSF water treatment method.

3.3.b Full-Slow Sand Filter

The fSSF, utilised the same basic system as the pSSF except, as the name suggests, on a much larger scale, filtering all recirculated water.

Overall, this system recorded the highest total species richness detected out of the five hydroponic systems observed (Table. 3.1), detecting 34 different individual species.

However when looking at figure 3.4, despite the higher species richness, no species was recorded at all four time points except for *Yersinia* spp., which was detected in each at fairly low quantities. Again, *Nitrospira* and *Pseudomonas* were both relatively common and recorded in high quantities till the final sampling point, where none were recorded. In terms of fungal or oomycete organisms a range of *Pythium* species were again fairly common throughout the season.



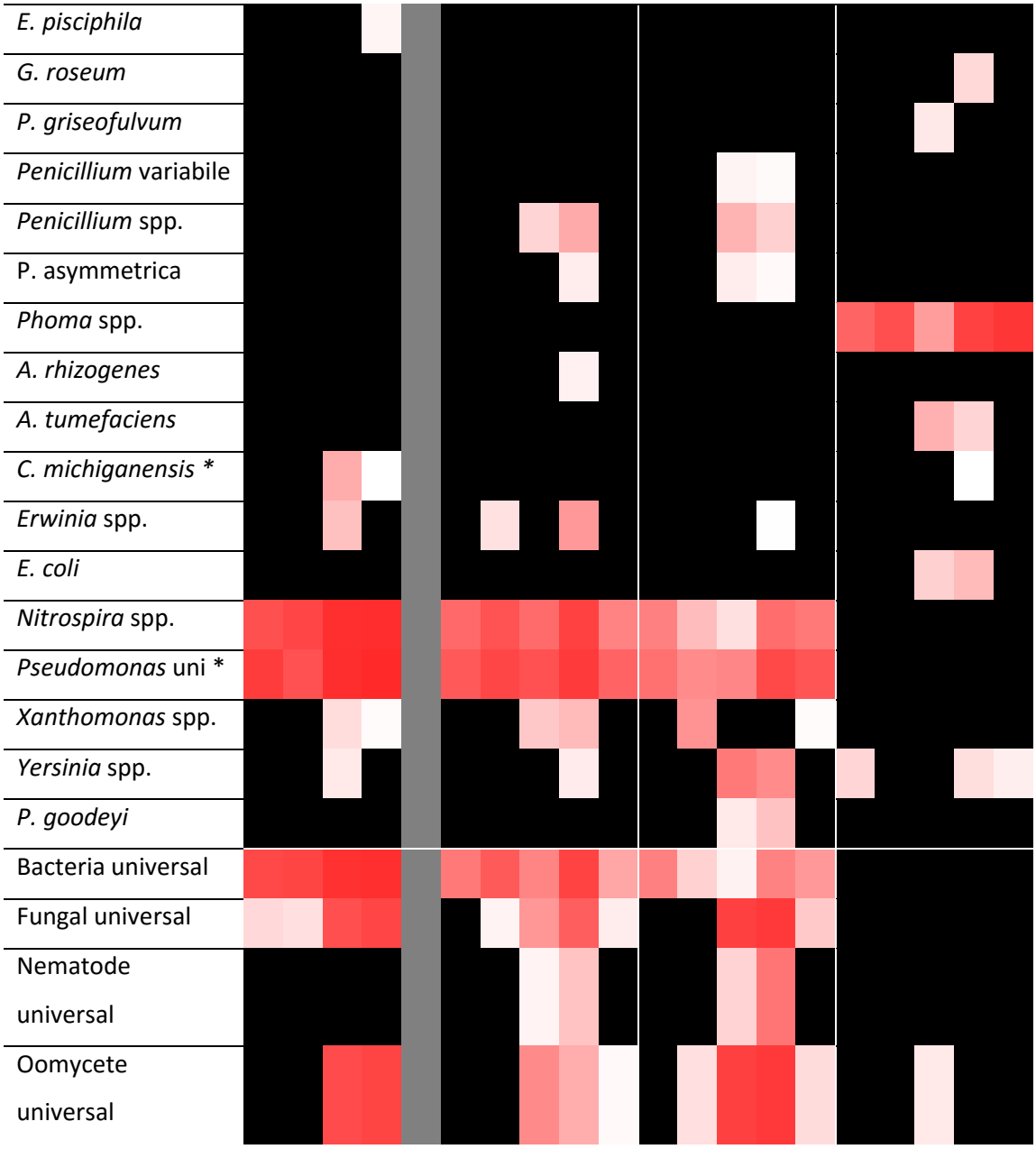


Figure 3.4: Heat map of all detected species, and their approximate abundance detected is denoted by colour intensity from white= low to red= high (black represents no detection; grey represents no sample set received). This was a system utilising a f-SSF as its water treatment technique. (*= Erroneous results possible from these probes).

Figure 3.5 shows the varying species richness throughout each system at each time point. In all sample sets where a post-treatment sample set was received (this excludes sample set 1) there was a clear reduction in the number of species present in the post-treatment sample when compared to that of the pre-treatment samples.

From figure 3.6 it seems to imply that, unlike the pSSF, the fSSF was equally as effective in reduction of bacterial organisms as it was in reducing the fungal or oomycete organisms from the solution.

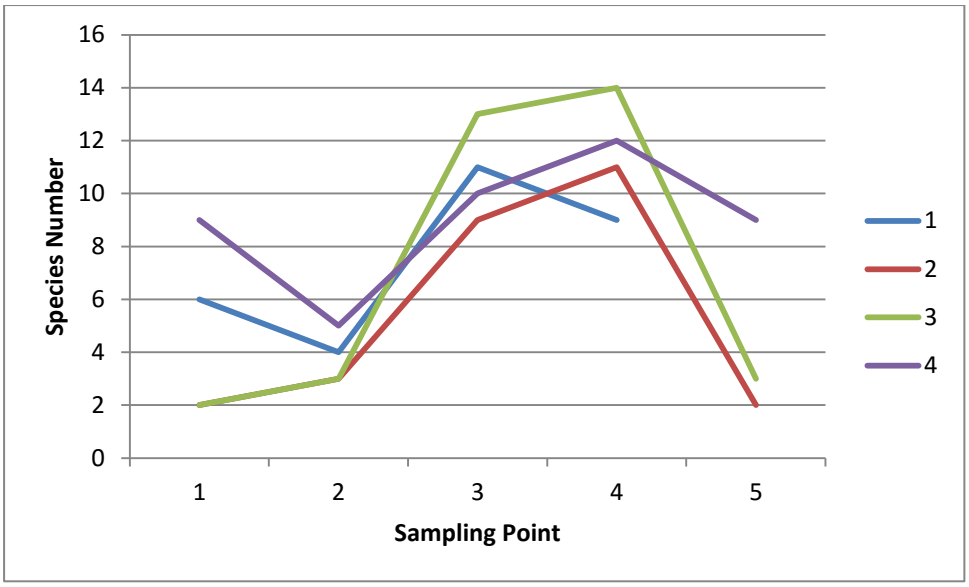


Figure 3.5: Species richness throughout the system using a f-SSF water treatment method at four time points.

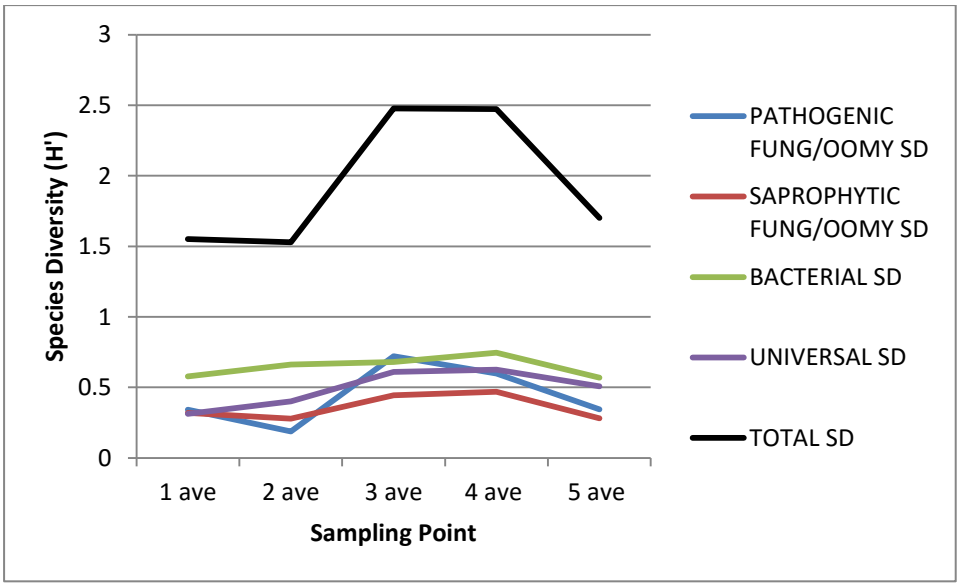
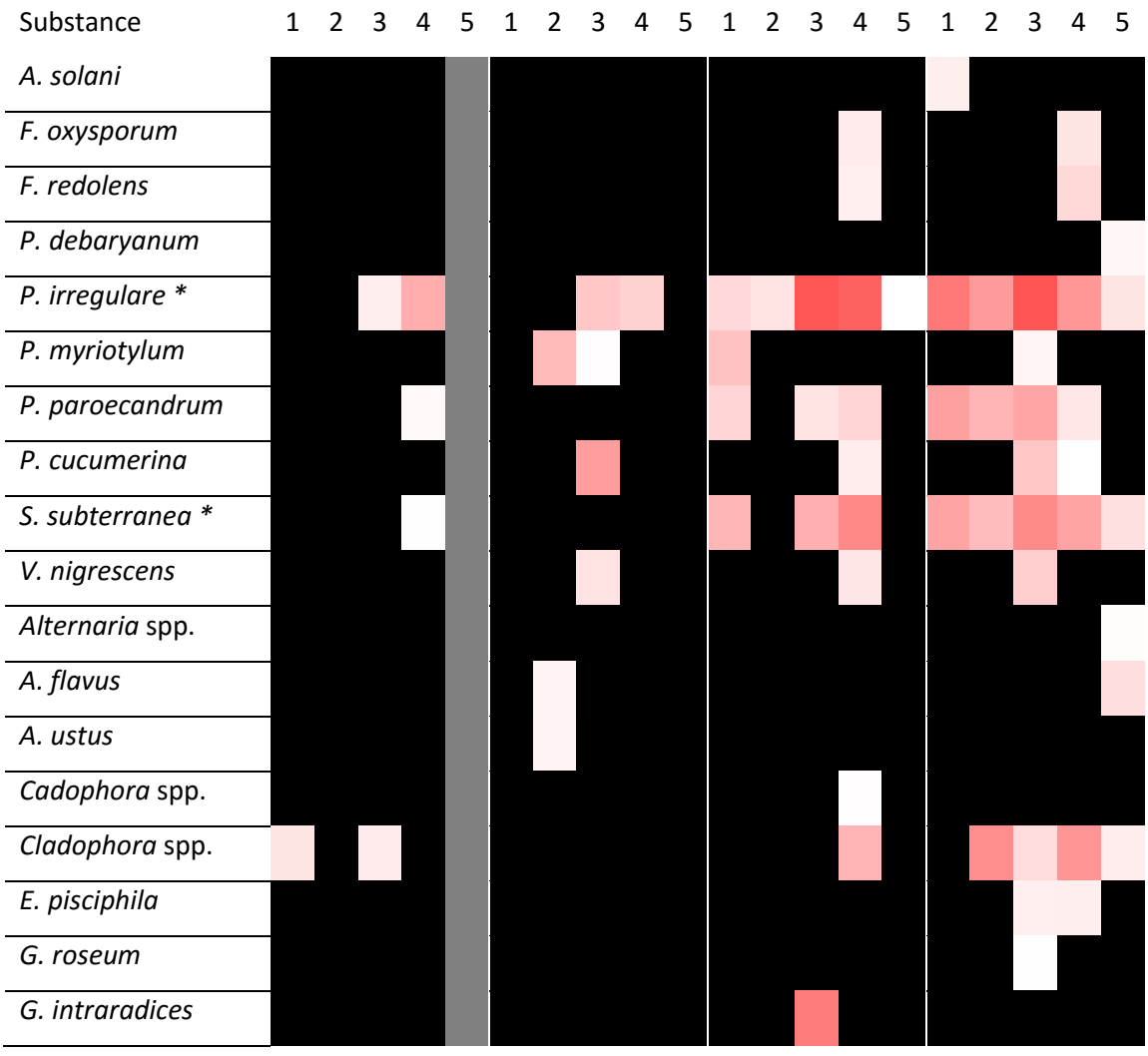


Figure 3.6: Species diversity (H') averages throughout the system using a f-SSF water treatment method.

3.3.c UV Water Treatment

The nursery using UV water treatment also had high species richness with a wide variety of organisms present. *Pythium irregulare* was the only fungal or oomycete organism to appear at all four time points over the season. Two more *Pythia*; *P. myriotylum* and *P. paroecandrum* were both relatively common being detected at three of the four time points along with *Plectosphaerella cucumerina* and *Spongosphora subterranea* also being detected at three points (see fig. 3.7).

In terms of bacterial organisms; *Erwinia* spp., *Nitrospira* spp. and *Pseudomonas* universal were all detected at each time point. However neither *Nitrospira* spp. nor *Pseudomonas* universal seemed to show reduction in detection despite treatment of the FS.



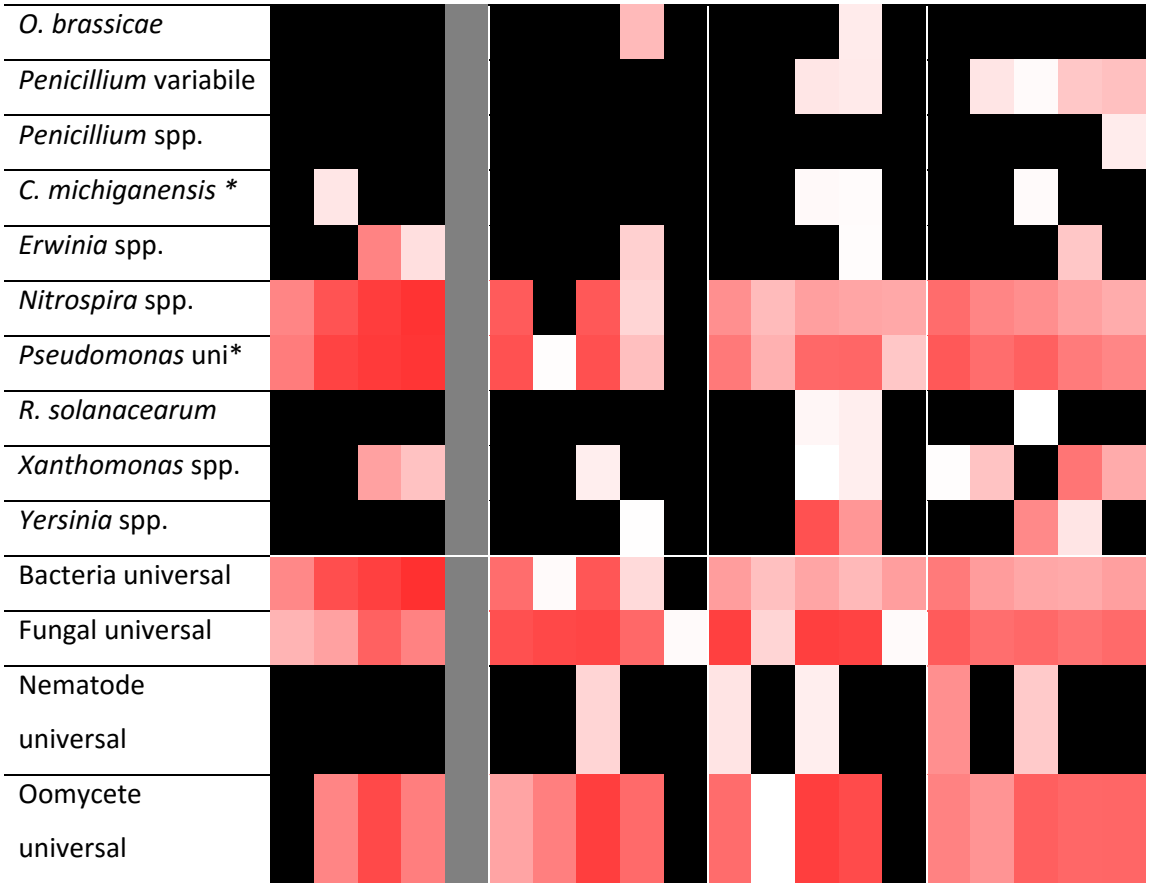


Figure 3.7: Heat map of all detected species, and their approximate abundance detected is denoted by colour intensity from white= low to red= high (black represents no detection; grey represents no sample set received). This was a system utilising UV as its water treatment technique. (*= Erroneous results possible from these probes).

Despite the aforementioned bacterial organisms seeming to show no effect as a result of the treatment, the trend was not similar with the total species richness. In figure 3.8 the resultant species richness reduction following the UV treatment seemed relatively successful. In figure 3.9 this effectiveness of treatment seems to be reflected by an average reduction of all classes of organisms following treatment, including bacterial organisms.

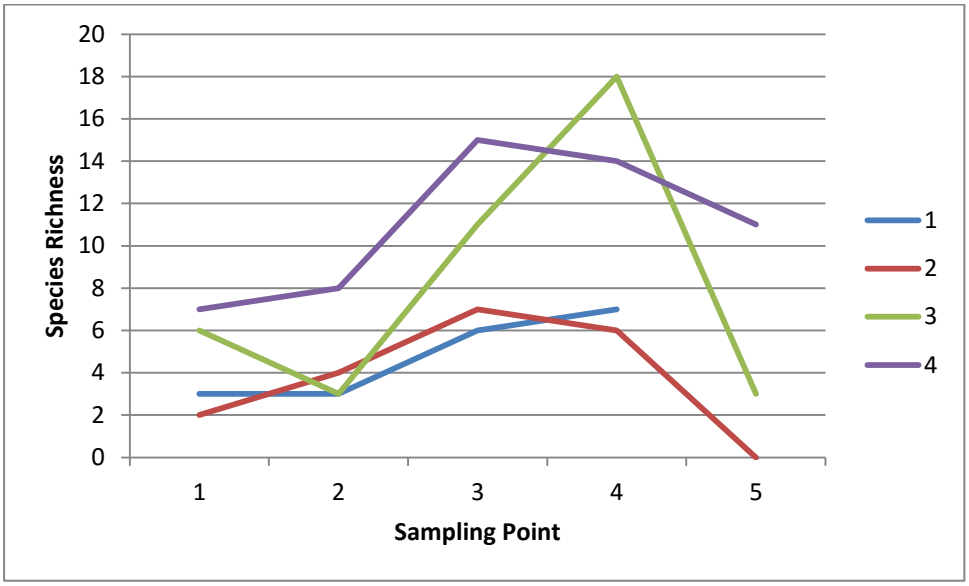


Figure 3.8: Species richness throughout the system using a UV water treatment method, at all four time points.

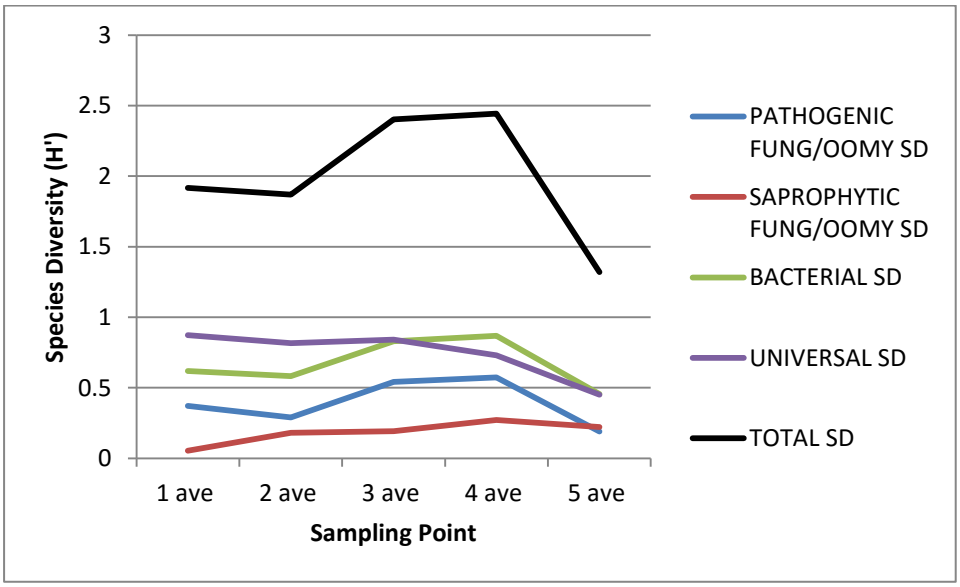


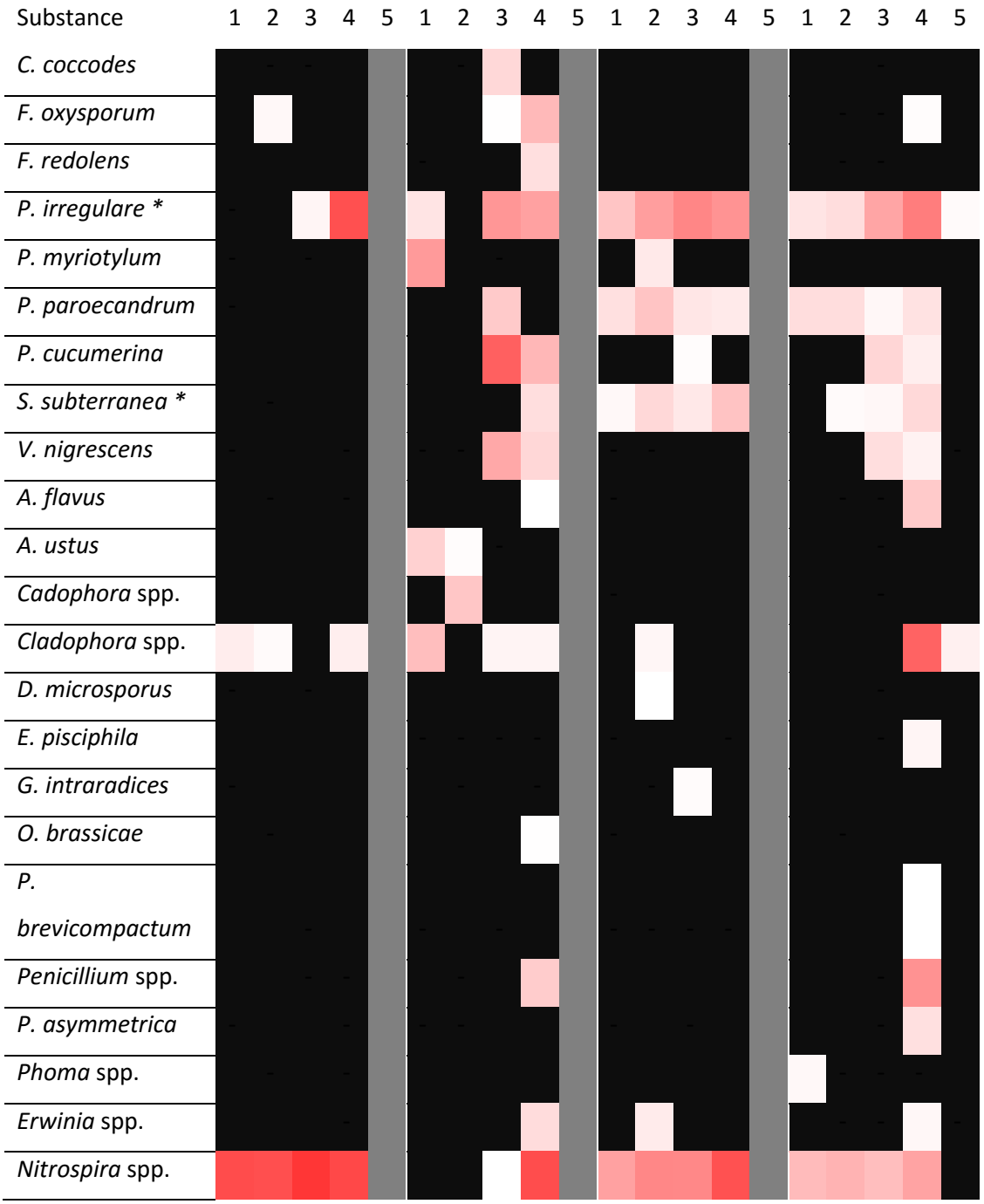
Figure 3.9: Species diversity (H') averages throughout the system using a UV water treatment method.

3.3.d Heat water treatment

Unfortunately for the majority of the season the nursery's pasteuriser was not functioning, which is why only the final sampling set has the post treatment sample results. Again, of all the fungal and oomycete organisms, only *Pythium irregulare* was detected at each time point.

However, other fungi and oomycetes were also common, with *P. paroecandrum* occurring at three time points, as well as *Plectosphaerella cucumerina and Spongospora subterranean.*

Of the bacterial organisms, *Nitrospira* spp., *Pseudomonas* universal and *Xanthomonas* spp. were also all detected at each time point (see fig. 3.10).



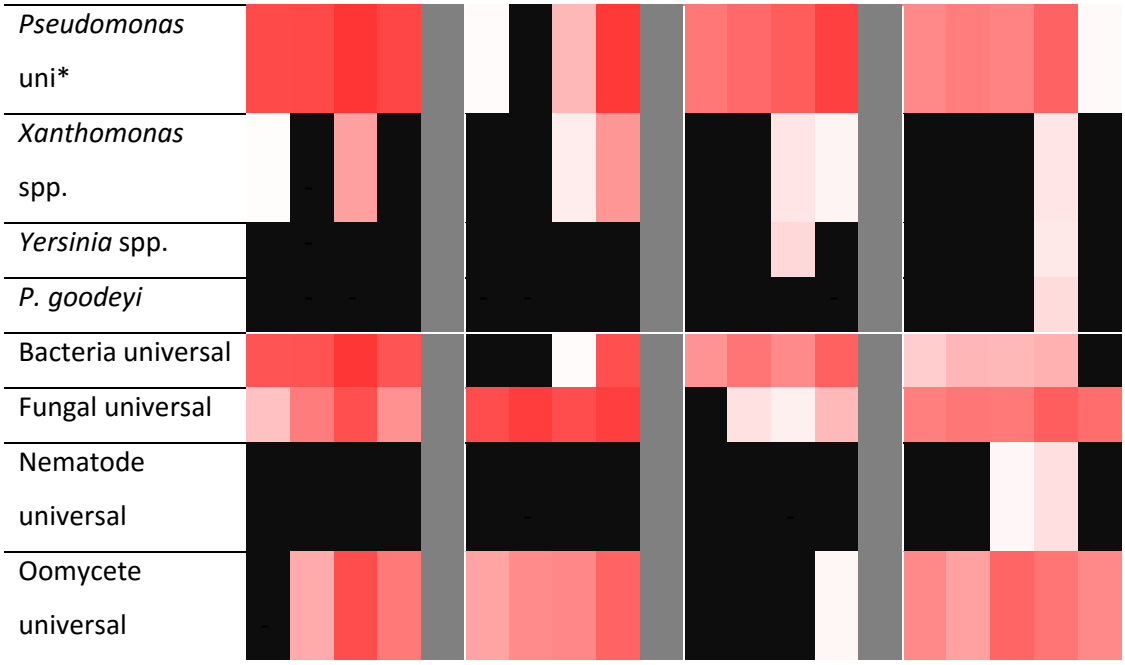


Figure 3.104: Heat map of all detected species, and their approximate abundance detected is denoted by colour intensity from white= low to red= high (black represents no detection; grey represents no sample set received). This was a system utilising heat as its water treatment technique. (*= Erroneous results possible)

From the only set of results featuring the pre and post-treatment samples, the result of the treatment seems successful in terms of reduction of diversity and richness (Figure 3.11 and 3.12 respectively).

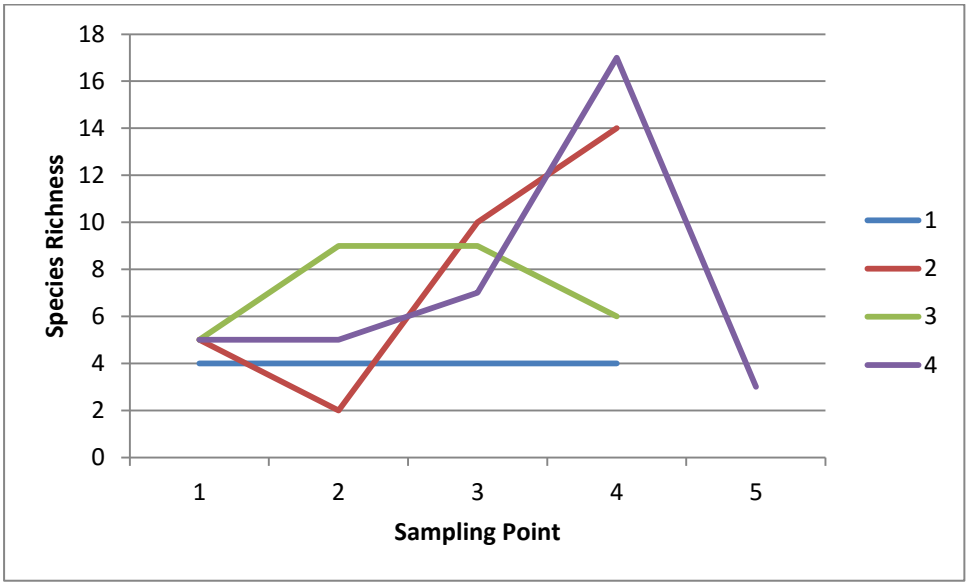


Figure 3.11: Species richness throughout the system using a heat water treatment method, at all four time points.

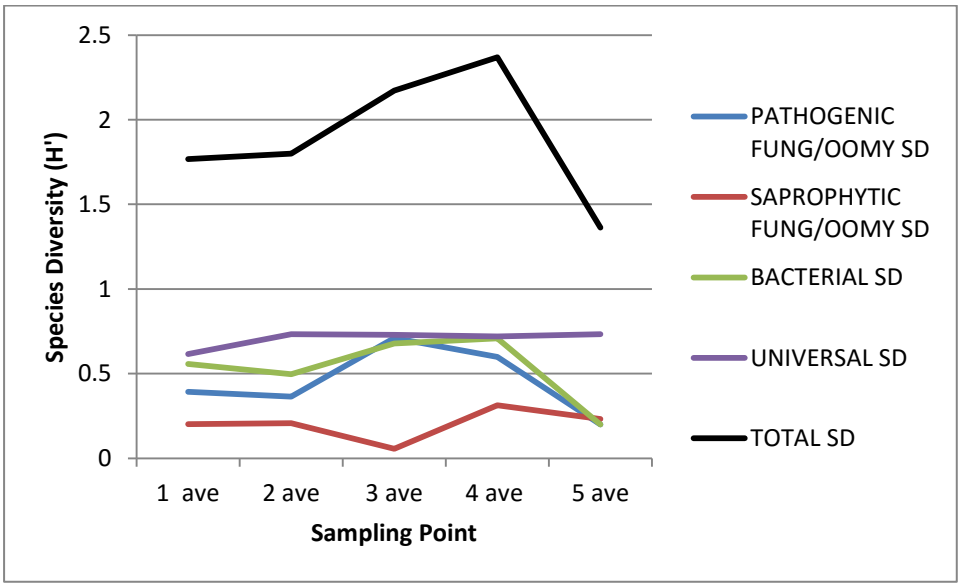


Figure 3.12: Species diversity (H') averages throughout the system using a heat water treatment method.

3.3.e Run to waste

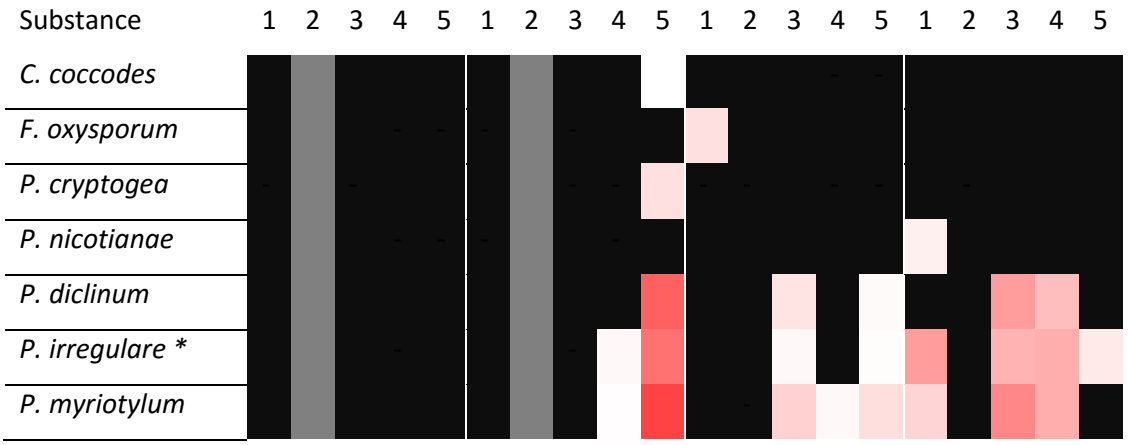
In the RTW system, the sampling points were organised differently to the 4 preceding nurseries. The five sampling points were: 1) the water source; at the first two time points this was mains water while the third and fourth sample points were rain collection, 2) collection tank of water

supply (when the water from rain collection was utilised, this is where the mains and rain water merged), 3) the mixing tank (where the mains and rain water would be mixed and nutrient solutions added), 4) slab solution from grafted root stock plants, 5) slab solution from the un-grafted plants.

From figure 3.13 it can be seen that there was a clear build-up of microbial populations in the system; from the early sample featuring a very low level of organisms, even in the slab solutions, to a comparatively diverse population throughout the system. It is also clear when rain water began being supplied to the systems. At time points 1 and 2 it was supplied from mains only, featuring few microbial populations in the sample points preceding the slab solutions. From the third time point, once the rain water collection tank began providing water (Fig. 3.13), it seems it allowed the introduction of more microorganisms to the early sampling points, some of which had not appeared before.

No fungal or oomycete organisms were present at all four sampling points over the season. Again, however, the *Pythia* were relatively common, with; *P. diclinum, P. irregulare* and *P. myriotylum* all occurring at three of the four timepoints, along with *Plectosphaerella cucumerina* and *Verticillium nigrescens* also present at the same three time points.

Of the bacterial microorganisms, again *Pseudomonas* and *Nitrospira* were the most common detectable at every sampling point, including the first, where they were the only organisms detected.



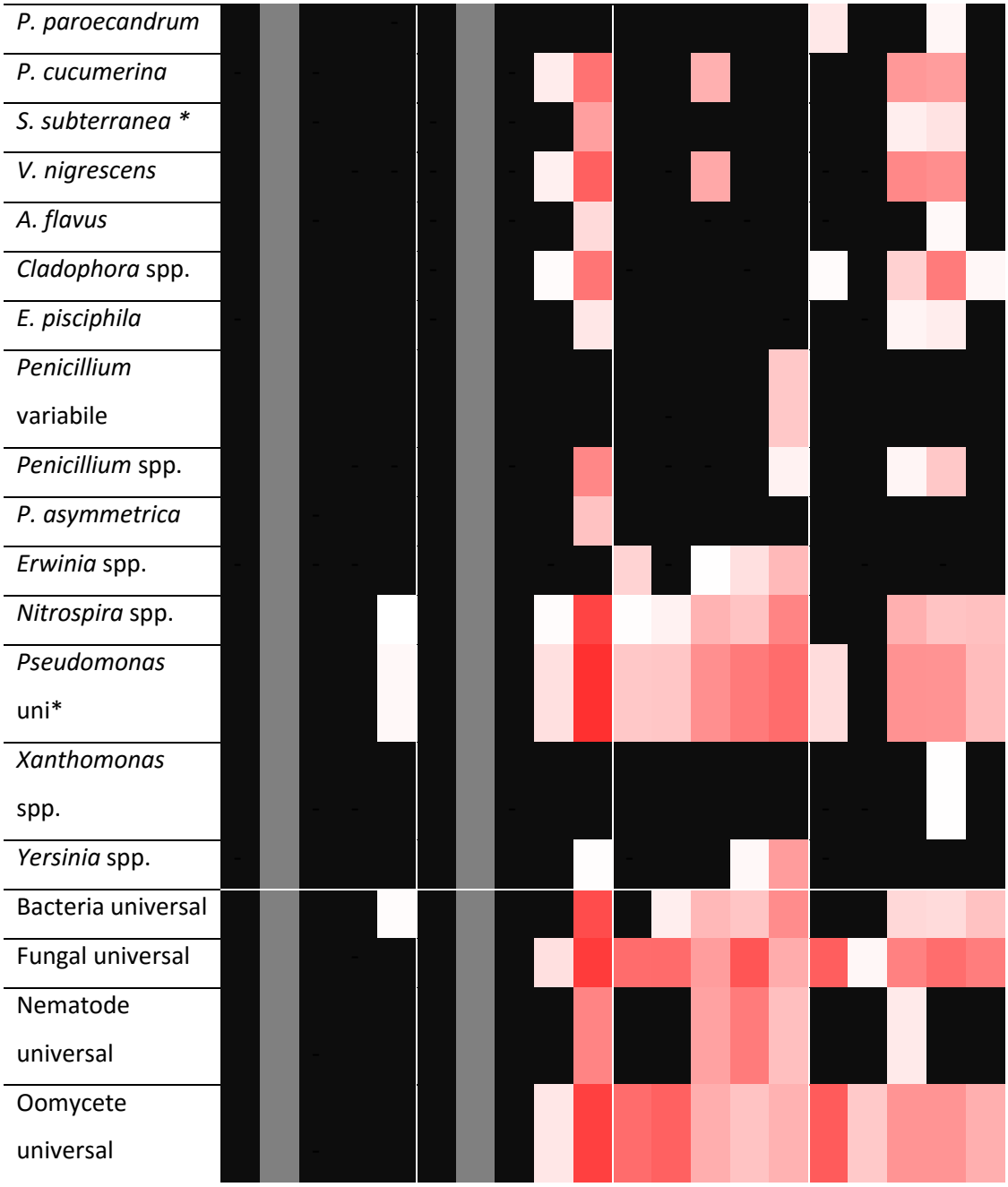


Figure 3.13: Heat map of all detected species, and their approximate abundance detected is denoted by colour intensity from white= low to red= high (black represents no detection; grey represents no sample set received). This was a system utilising no water treatment technique and running to waste. (*= Erroneous results possible from these probes).

When looking at the species richness total of the season (Table 3.1) and the individual totals throughout the system (Table 3.14), the RTW seemed no more prone to microorganisms than the other systems.

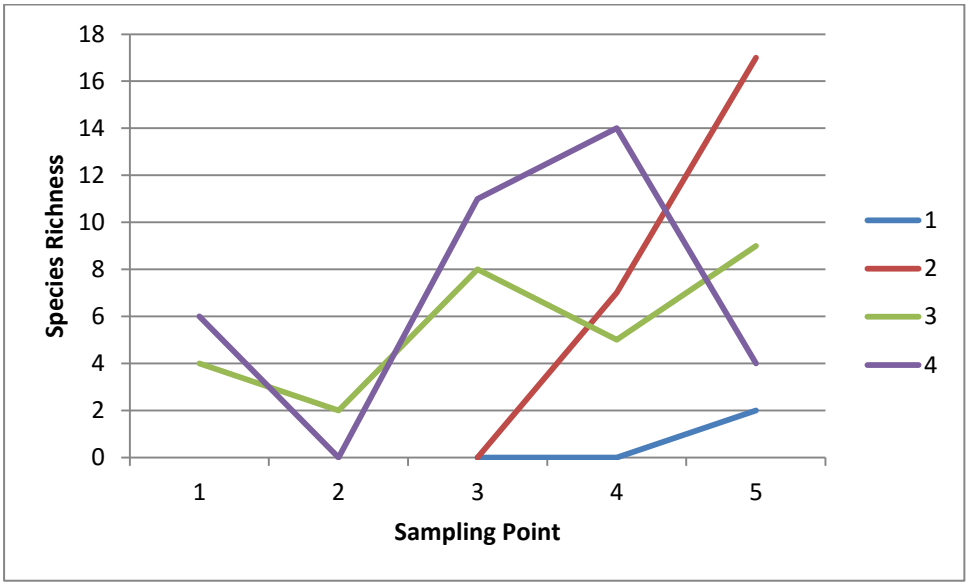


Figure 3.14: Species richness throughout the system using a no water treatment method, at all four time points. Point 4 was a slab solution from a grafted root stock, and 5 were from the un-grafted root stock.

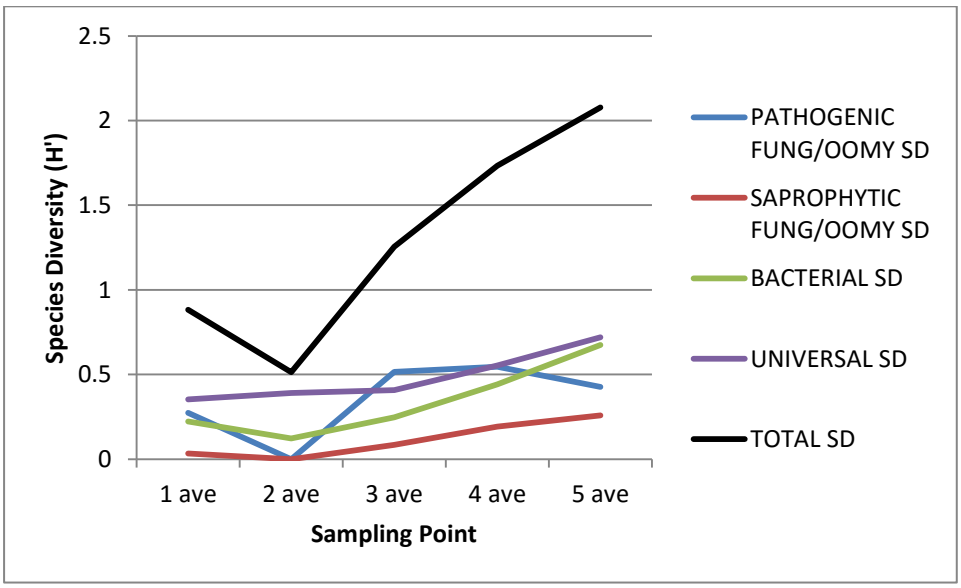


Figure 3.15: Species diversity (H') averages throughout the system using a no water treatment method. Point 4 was a slab solution from a grafted root stock, and 5 were from the un-grafted root stock.

3.3.f *t*-test of water treatment effectivity

For assessment of the efficacy of the water treatment systems, only the fSSF, pSSF and UV could effectively be tested. This was because for the heat treated samples, only one sample set was complete with pre and post treated solutions. Two of the three treatments produced significant results (Table 3.4). The effectiveness was tested by comparing the total species diversity of the pre-treatment solutions to the total species diversity of the post-treatment solutions.

The reduction in terms of pathogenic fungi and oomycetes, saprophytic fungi and oomycetes, and bacterial organisms could not be tested separately without a larger number of samples.

Table 3.4: Summary of the resultant *t*-test P-values, analysing the difference between the species diversity exhibited within the pre-treatment solution samples to that of the post-treatment solution samples of three commonly used water treatment techniques. A significance level of P<0.05 was used, if P>0.05 the null hypothesis was accepted.

Water Treatment	P-Value
pSSF	0.0271
fSSF	0.0372
UV	0.0753

Both the pSSF and fSSF produced significant results. However the total array species diversity produced positive results in terms of both these SSF methods. UV produced a value close to a significant p-value; however, the null hypothesis is accepted that it had no significant effect.

3.4 Discussion

From the cumulative species richness data, spanning across the growing season (Table 3.1), the nursery with the lowest total species richness observed was the one utilising a RTW system. This result is consistent with the past research conducted by Scott (2013). The nursery that

recorded the highest species richness was the one using a fSSF treatment system. This suggests that there may be an element of reintroduction of organisms that remain dormant, from the previous season, within the SSF back into the fertigation system at the start of the season as well as inefficient removal.

As well as just the shear species richness, table 3.3 lists the organisms whose detection was unique to only one nursery. Again, fSSF featured the most species unique to itself, with eight; whilst the RTW system featured no unique species. Of the eight species unique to the fSSF system, three were pathogenic fungi or oomycetes, four bacterial species and two were saprophytic fungi or oomycetes. For the rest of the nurseries; the pSSF, UV treatment and heat treatment nurseries detected one, four and three unique species respectively. These unique species may also support the idea that some of these species may have already inhabited the system prior to any treatment, and merely recycling the water may allow them to proliferate further.

Besides from the species that were unique to individual systems, there were certain species that over the course of the whole growing season were common to all nurseries and detected in each at least once (listed in table 3.2). Of these species; six were pathogenic fungi or oomycetes, four were saprophytic fungi or oomycetes and five were bacterial. Of the six pathogenic fungi or oomycetes, three were species of *Pythia*; *P. irregulare, P. myriotylum* and *P. paroecandrum.* These three were particularly prevalent throughout the season, in each of the systems, where in all but two cases each was present in at least two time points. However, despite the relative prevalence of the *Pythia* species over the season, the heat maps (Fig. 3.1, 3.4, 3.7, 3.10, 3.13) which show the relative abundances of each species, seem to suggest each of the water treatment techniques being used were relatively successful at reducing species abundance from pre-treatment sample to post-treatment. This suggests that the *Pythia* may be hosted on the slab or roots as, looking at the abundances from the heat maps; they are at their peaks in the slab

and pre-treatment solutions. Regardless, the inability of the treatment systems to remove *Pythia* may potentially put the crops at risk of associated diseases, including damping-off, and certain root rots.

It is important to note that the information on abundance gathered from the microarray is semi-quantitative, and results are only relative on an individual basis. The binding of organisms to different probes may be at differing rates or efficiency so should not be used relative to other species.

Aside from the *Pythia,* the other pathogenic fungi or oomycetes that were common across all systems were *F. oxysporum, S. subterranea* and *V. nigrescens* (now known as *Gibellulopsis nigrescens*). These are each common pathogens of tomato, but in comparison to the *Pythia* observed, were not quite so prevalent over the season. They also seemed to show similarities in peak abundances, at the slab point, and showing reduction, if not disappearance following treatments. Overall, the pathogenic fungi and oomycetes, as well as the bacterial organisms, generally tended to be more persistent across the season than the saprophytic fungi and oomycetes, which seemed to make more sporadic appearances at single time points rather than across several consecutive time points. The most persistent of all organisms, however, was *Pseudomonas* spp. and *Nitrospira* spp., which were present together in all but one time point for one system over the whole season. These also, commonly, demonstrated little effect from water treatments and at times their abundance looked independent of treatment sometimes increasing post treatment. Overall however, using Figures 3.1,3.4,3.7,3.10 and 3.13, it is clear that the net abundance of them both decreases as the season progresses. These are commonly expected to be in high abundance in the hydroponic rhizosphere (Hawkes *et al.*, 2007; Norström *et al.*, 2008). It has even been noted in past research (Cafa, 2012) that SSF's can act as a source of organisms such as *Nitrospira* and *Pseudomonas*, allowing them both to proliferate.

A key issue with the microarray in terms of use like this, is that the probes are often designed to recognise barcoding regions such as the ITS

regions, which are adept for detection purposes; however, they are often unable to identify the difference between pathogenic and non-pathogenic isolates within a species. So in the case of *Pythia* being prevalent in each system throughout the season, it may not be the case that the plants are at risk from associated disease if the oomycete lacks the appropriate pathogenicity genes. As well as this, often when these plants do exhibit symptoms of disease from organisms such as *Fusaria,* it is often difficult to differentiate between that and other pathogens as the symptoms are often indistinguishable.

In terms of efficacy of the water treatment systems, general remarks can be assumed from the abundance displayed in figures 3.1, 3.4, 3.7, 3.10 and in the data showing the average biodiversity for each sampling point (Figs. 3.3, 3.6, 3.9 and 3.12). All the evidence suggests a general reduction in the number and abundance of organisms detected in post-treatment samples than those in the pre-treatment samples. To further support this, the total-array species diversities for pre-treatment and post treatment samples were compared using the *t*-test. This showed that for both the pSSF and the fSSF the reduction in levels of species diversity, as a result of treatment, produced a significant P value. For UV water treatment the result was close, however not significant, but with further research and a greater sample set, this may be able to produce a significant result. Unfortunately, due to the lack of post-treatment data, the nursery using heat treatment could not be tested.

There are varying modes of introduction for new organisms to each system, such as any coming in with the plants roots when the initial rock wool cube is added to the slab, as well as from varying water sources. In the RTW subset of samples, the early season set of samples, 1 and 2, seemed to detect relatively low levels of microorganisms particularly in the early sampling points in the circuit. As a RTW system, if any microorganisms start being detected in the sampling points prior to the slab, it may be sensible to assume these were brought in with the water. Following the system beginning to utilise rain water as a source from the

third time point on, rather than mains water, microorganisms began being detected in the early sampling points of the circuit, and then subsequent sampling points throughout the system. This suggests that the introduction of these pathogens may be due to the new water source and that in future the secondary water sources should be more thoroughly monitored, as they may be the reason for some of the variations in diversity that were recorded.

Chapter 4 Observations of microbial population changes within root samples over the 2014/2015 growing season.

4.1 Introduction

With the current rise in popularity of hydroponic growth systems, the monitoring of organisms associated with the rhizosphere of the plants is of vital importance to truly characterise the inhabitants of the root rhizosphere correctly. Although the hydroponic system has the ability to act as a medium for the transport of microorganisms across whole crops, it does not guarantee that the microorganisms will be able to inhabit, or infect, the actual root systems. To truly be able to identify the microorganisms which will pose realistic threats in the hydroponic setting, the microorganisms that are able to inhabit this environment once introduced, need to be described. To identify these species, first a consistent general overview of species that are common to the systems must be built up, along with common seasonal trends in changes. Then information gathered based on points of introduction, the efficacy of removal procedures and the comparisons of the microbial populations of FS can be compared to that of the microbial populations of roots.

For this work, each of the five nurseries (See chapter 3) also had root samples taken at each time point. In addition, a further four nurseries were sampled at two time points. The purpose of these samples was to build up a general overview of microorganisms that were common over the growing season, as well as demonstrating if the water treatment system in the first five nurseries had any noticeable effects. This also allowed a comparison of the different microbial populations between plants grown on two separate root stocks, in the samples from the RTW system. Following this observation, there was then the opportunity to compare the slab solution results gathered in the previous chapter (Chapter 3) to the associated root sample results. This shows which microbial species in the immediate vicinity of the roots are then able to inhabit the roots.

4.2 Methods

Samples were again taken from the first five nurseries at four separate time points. Additionally to these five nurseries, five more sample sets were gathered from four additional nurseries, at two time points in the season. The samples were in the form of three replicate root samples from each nursery according to section 2.2.1. Once the roots had been collected DNA was extracted (section 2.2.2), and amplified using eukaryotic and prokaryotic primers as in section 2.1.2. Once amplified the DNA then underwent microarray analysis described in 2.2.4 and 2.2.5. These pieces of data were then used to determine species diversity as outlined in section 2.2.6.

As well as the roots sampled from the five main nurseries, at four time points, a further five were sampled at two time points towards the end of the season. These were sampled from nurseries using two water treatment systems; heat (x3), and RTW (x2). These additional nurseries are labelled as; AD1 heat, AD2 heat, AD3 heat, AD4 RTW and AD5 RTW. Another important thing to note is that, of the main nurseries, the RTW system yielded two sample sets, non-grafted plants and grafted plants.

4.3 Results

4.3.1 Overview of the season's microbial populations within roots from commercial nurseries.

From observation of the total species richness results in Table 4.1, it can be seen that the fSSF system was the system that resulted in the highest number of detected species. However, despite this, the number of pathogenic fungi or oomycetes was relatively similar to the other systems. Consistent with the results of the fertigation solution sampling (Table 3.1), the fSSF systems species richness is bolstered by a relatively high bacterial count compared to the rest of the systems.

The UV system, however, had a species richness score that was similar to that of the fSSF, but with a larger proportion of that being potentially

damaging pathogenic fungi or oomycete species. As a general trend there is much in common with the species richness scores detected in the fertigation solutions and the roots themselves being relatively similar in regard to the differing systems.

Table 4.1: List of species richness totals detected over all four time points in the root samples from the five main nurseries. *: Un-grafted, **: grafted

	pSSF	fSSF	RTW Un*	RTW G**	Heat	UV
Total	23	28	17	23	21	27
Pathogenic Fungi and Oomycetes	10	8	7	8	8	10
Saprophytic Fungi and Oomycetes	3	5	2	3	4	7
Bacteria	5	8	4	7	4	4

The results from the additional nurseries are much more sporadic and variable, probably as a result of only having two sampling time points. Again the system that scored the lowest species richness was one utilising a RTW method of water treatment. However, in contrast to the rest of the results gathered so far, the other RTW system also scored the highest level of species richness, although this may just be due to insufficient samples gathered to put together a really representative measure.

Table 4.2: List of species richness totals detected over both time points in the root samples from the five additional nurseries.

	AD1 Heat	AD2 Heat	AD3 Heat	AD4 RTW	AD4 RTW
Total	10	10	15	8	15
Pathogenic Fungi and Oomycetes	5	5	7	4	7
Saprophytic Fungi and Oomycetes	0	1	4	2	4
Bacteria	5	4	4	2	4

Looking at the heat maps of the main nurseries, it should be noted there seems to be a reduction in abundances of most species towards the final time point.

In Figure 4.1, which shows the results from both the pSSF and the fSSF system, the fSSF seems to have more difficulty in overall removal of organisms, with the pSSF having removed all but a few bacterial species by the third time point, with nothing detected in the final sample. In contrast, several pathogenic fungi or oomycete species are still detected in the fSSF, although at lower abundances in the third time point and one species is still being detected at the final time point.

This heat map gives a clear indication of general reduction in abundances over the season with seemingly high abundance at the start of the season to low, or absence in the final sample set.

Nitrospira, Pseudomonas and *Xanthomonas* spp. all seem generally common throughout, particularly in the fSSF system. As well as these, in the fSSF *Verticillium nigrescens* is very common, being detected in samples at all time points despite showing relative reduction. *Plectosphaerella cucumerina* is also relatively common, being detected in

the first three time points. A point to note is that, in the fSSF, both *V. nigrescens* and *P. cucumerina* seem to peak at the second time point.

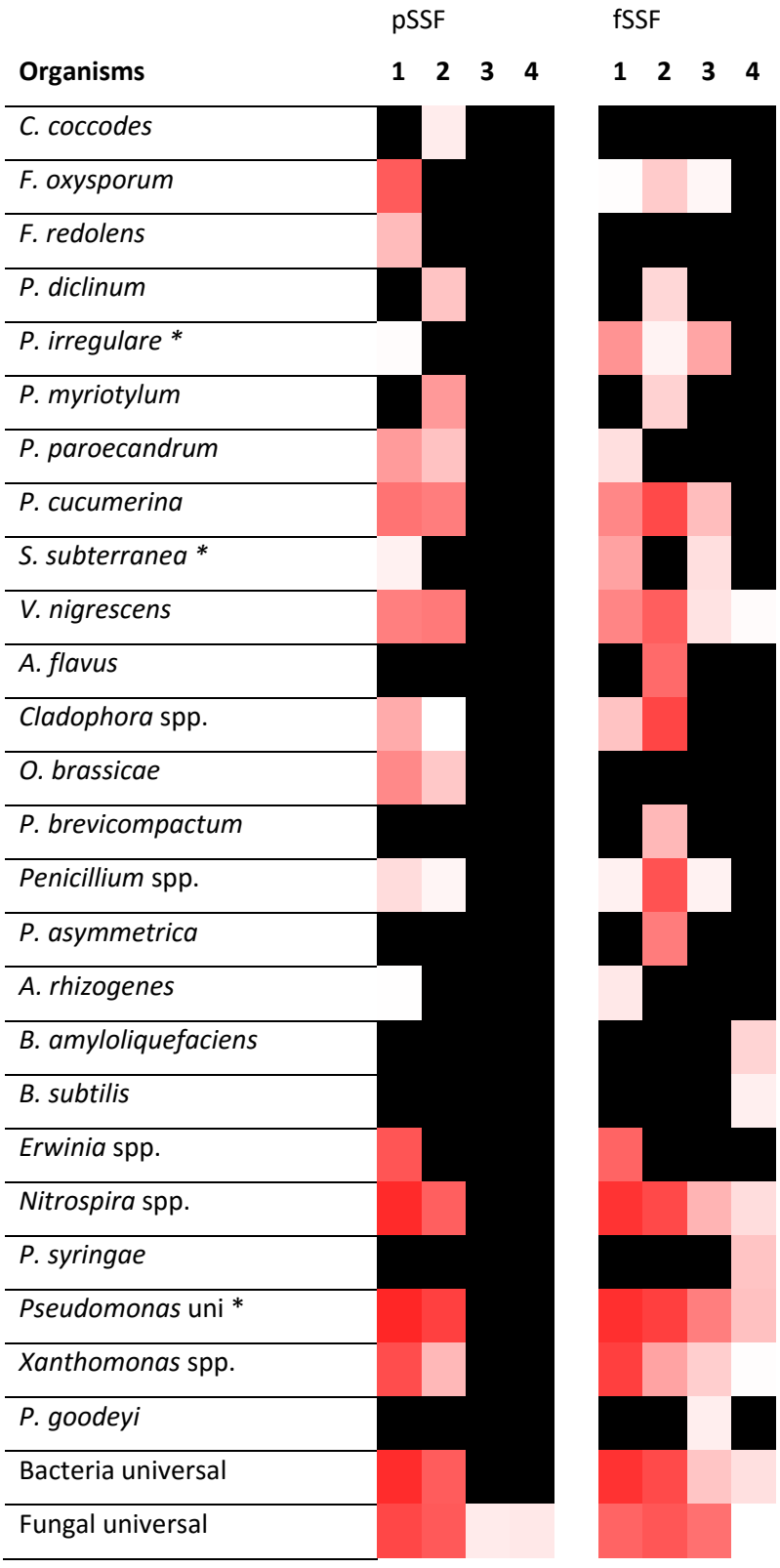




Figure 4.1: Heat map of the organisms detected in the roots from the pSSF and fSSF systems, at each time point (1-4). Their approximate abundance detected is denoted by colour intensity from white= low to red= high (black represents no detection). These systems each utilised one of two SSF water treatment systems; one that filtered all its water (fSSF) and one that just filtered 10%. (*= Erroneous results possible from these probes)

In the two root samples from the RTW system (Fig. 4.2) it is clear that the samples have a much lower level of organisms detected than in the previous samples , and this is reflected in the species richness (Table 4.1). However, where the differing species richness scores for the grafted and non-grafted roots is misleading, is that despite the grafted root samples having a higher species richness, the majority of these occur in the first week and are mostly gone for the rest of the season. However, in the non-grafted samples, many of the organisms continue into the second sample set.

Again *Nitrospira, Pseudomonas* and *Xanthomonas* were relatively common throughout the season, but in terms of fungi or oomycetes, none were really persistent throughout the season. However, *V. nigrescens, Cladophora* and *Penicillium* all make a late appearance, being detected in the final sample set, after not being detected since the first time point in the grafted roots.

In terms of the levels of organisms present on the grafted roots, when compared to those on non-grafted roots, it implies that the organisms may be brought in with the roots, as they are quickly lost following propagation in the RTW system.

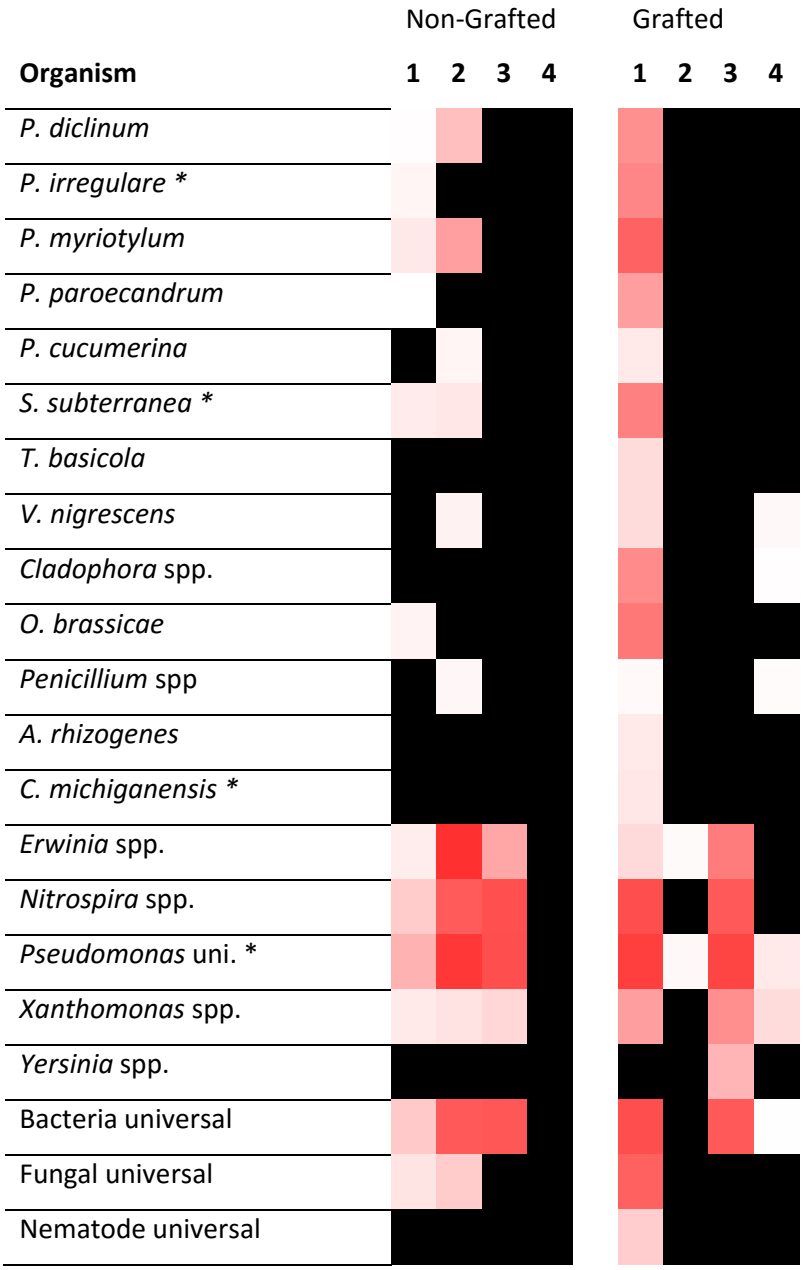


Figure 4.2: Heat map of the organisms detected in the two root samples sets yielded in the RTW system, Grafted rootstocks and those on their own roots, at each time point. Their approximate abundance detected is denoted by colour intensity from white= low to red= high (black represents no detection). This was a system with no water treatment technique that instead RTW. (*= Erroneous results possible from these probes)

In the two systems utilising physical water treatment methods, heat and UV, the distribution of organisms was more spread out over the growing season, particularly in the UV water treatment system. Introduction of organisms seem sporadic throughout with new organisms being

introduced at each time point. Heat treatment seemed more effective, with considerably reduced species richness by the final sampling point; however, the UV system seems to have relatively equal species richness throughout in terms of abundance.

Other than *Pseudomonas* and *Nitrospira* spp.*,* in the heat treatment system, no other organisms were present throughout all sampling points. However, in the UV treatment system, besides from the aforementioned organisms, there was also *P. irregulare, P. cucumberina* and X*anthomonas* at all sampling points*.*

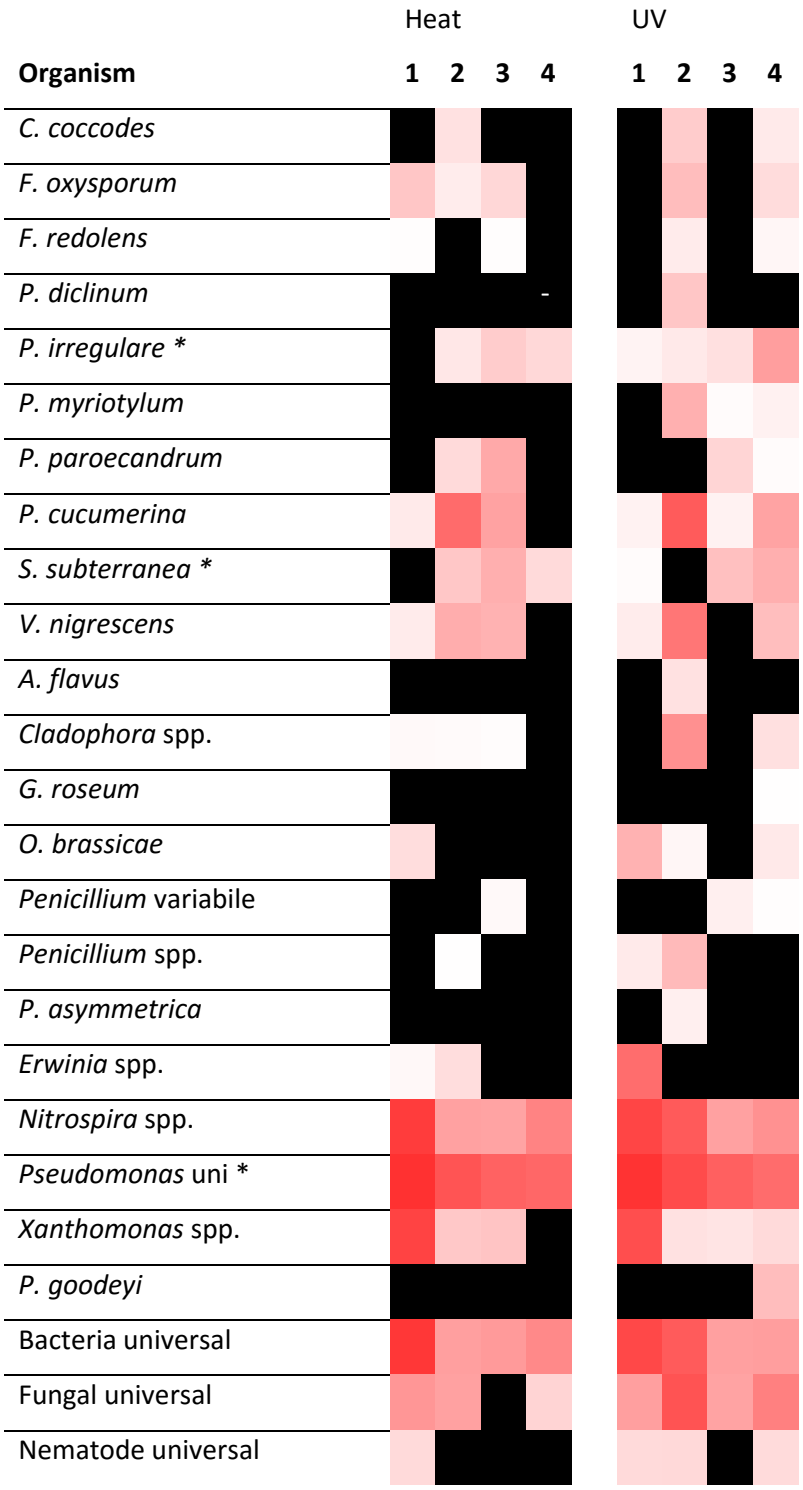


Figure 4.3: Heat map of the organisms detected in two nurseries, at every time point, utilising two different physical water treatments; Heat and UV. Their approximate abundance detected is denoted by colour intensity from white= low to red= high (black represents no detection). These two sets of samples are received from two systems utilising different physical water treatment systems; heat treatment and UV water treatment. (*= Erroneous results possible from these probes)

Table 4.3 lists the organisms that were present for the entirety of the season in certain systems. These organisms are similar to those that were also persistent in the water sampling (Fig. 3.1, 3.4, 3.7, 3.10, 3.13). It is of note that the non-grafted plants grown in the same RTW system as the grafted plants featured no persistent organisms at all. There were also no persistent organisms detected in the pSSF system.

Table 4.3: Organisms persistent at each time point throughout the season.

fSSF	**RTW (Grafted root)**	**UV**	**Heat**
V. nigrescens	*Pseudomonas* uni.	*P. cucumerina*	*Nitrospira* spp.
Nitrospira spp.		*P. irregulare*	*Pseudomonas* spp.
Pseudomonas uni.		*Nitrospira* spp.	
Xanthomonas spp.		*Pseudomonas* uni.	
		Xanthomonas spp.	

Table 4.4 lists all the organisms that were seemingly unique to only one system. Both the pathogens detected in the RTW system were again, detected at the first sample time point. These were then not detected again, possibly supporting the idea that they may have been brought in to the system with the roots, as the treatment was clearly effective in removing it.

Table 4.42: Organisms unique to individual systems.

fSSF	RTW (Grafted root)	UV
P. brevicompactum	*T. basicola*	*G. roseum*
B. amyloliquefaciens	*C. michiaganensis*	
B. subtilis		
P. syringae		

Table 4.5 shows all the organisms that were common to all systems, appearing at least one time point in each different system. These, again, are very similar to those gathered from the fertigation solution tests (Table 3.2) except for the detection of *Plectosphaerella cucumerina.*

Table 4.5: Organisms that were detected in all systems in at least one time point sample.

Pathogenic fungi or oomycetes	Saprophytic fungi or oomycetes	Bacteria
P. irregulare	*Penicillium* spp.	*Erwinia* spp.
P. paroecandrum		*Nitrospira* spp.
P. cucumerina		*Pseudomonas* uni.
S. subterranean		*Xanthomonas* spp.
V. nigrescens		

In Figure 4.4, the results from the additional five nurseries are shown. Of all the organisms' only one unique organism, *P. cryptogea,* was detected, that was not detected on any of the other root samples. This list helps to confirm which of the organisms were common over the season. In terms of the pathogenic fungi it was predominantly made up of various *Pythia* species as well as *P. cucumerina* and *V. nigrescens*.

Again the species such as *Nitrospira, Pseudomonas* and *Erwinia* were very common throughout.

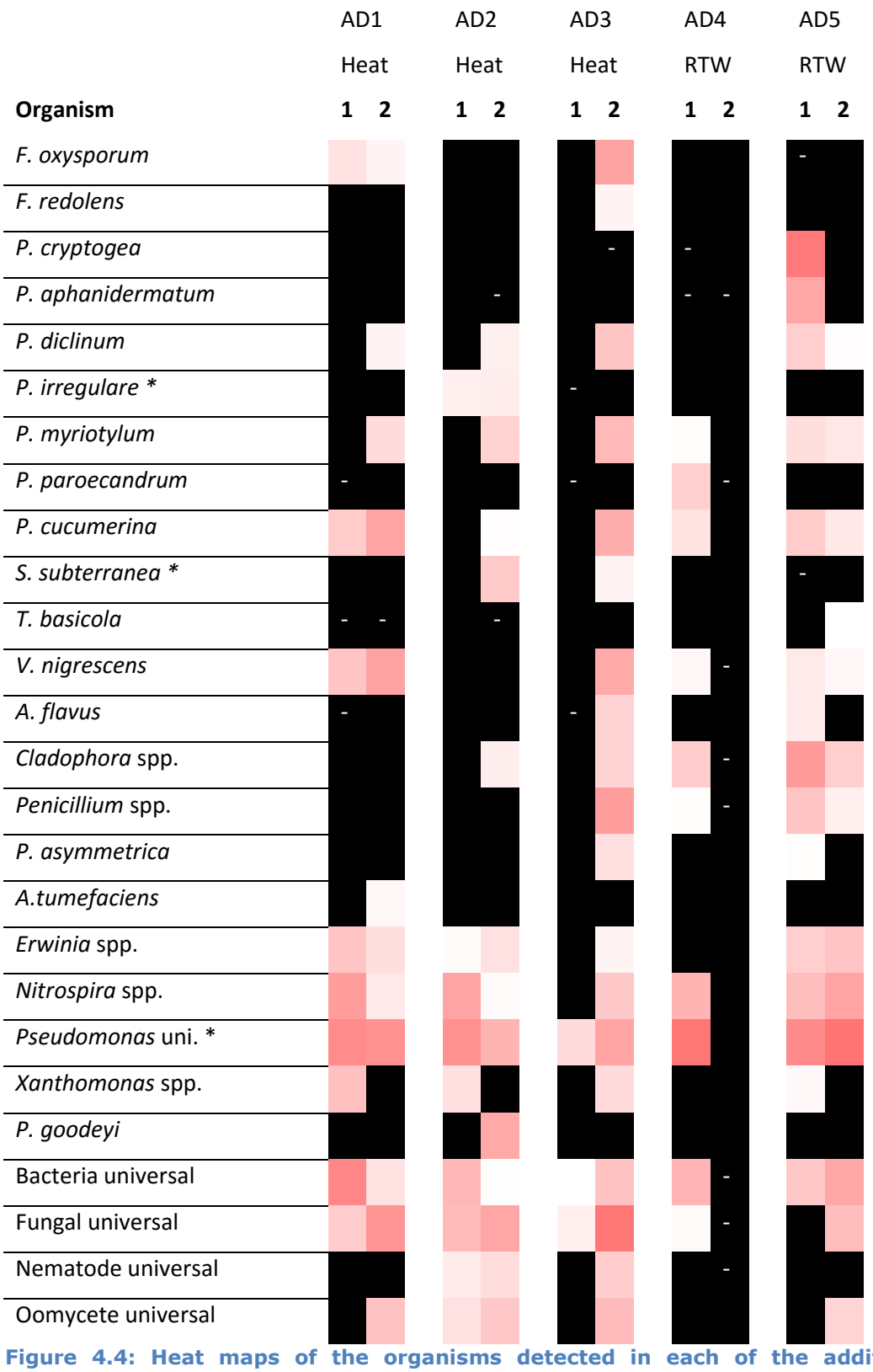


Figure 4.4: Heat maps of the organisms detected in each of the additional nurseries at their two time points(1-2).

4.3.2 Comparison of root microbial populations to that of the associated slab fertigation solution.

4.3.2.a pSSF

Figure 4.5 directly compares the root sample detection results to that of the FS taken from the slab where the roots were grown. From the first two sampling time points in the season, it seems that many more microorganisms are being detected in the root samples, as reflected in Figure 4.6, which graphically represents the species richness of the two samples. However, the water species richness is also relatively high for that point in the season when compared to other systems previously mentioned. However the species richness then drops, before rising back up again by the end of the season. This also includes new organisms not previously detected on the root such as *Exophiala pisciphila* which was newly detected in the week four water sample.

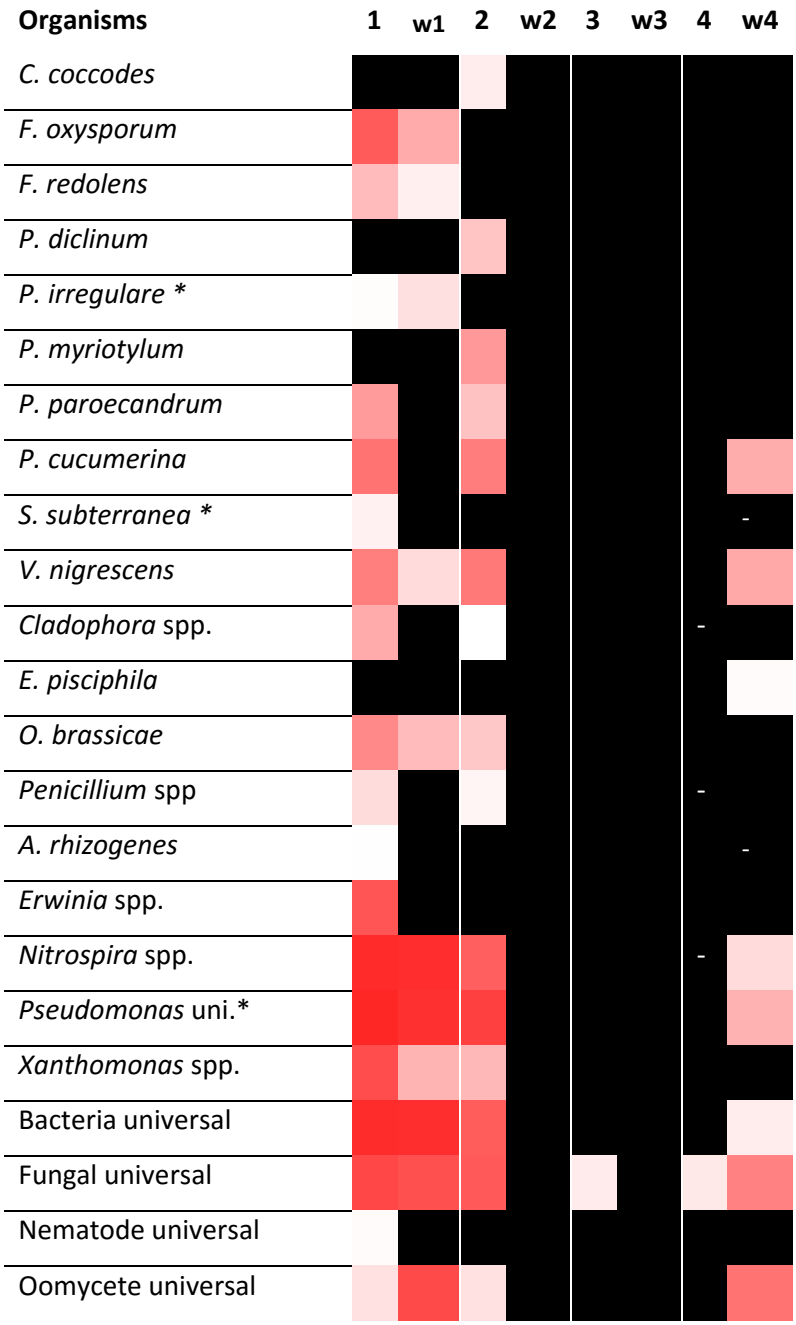


Figure 4.5: Comparison of the slab solutions (w1-w4) to their associated root samples (1-4) from the same time point. These are the samples gathered from the fertigation system utilising a pSSF as the water treatment.

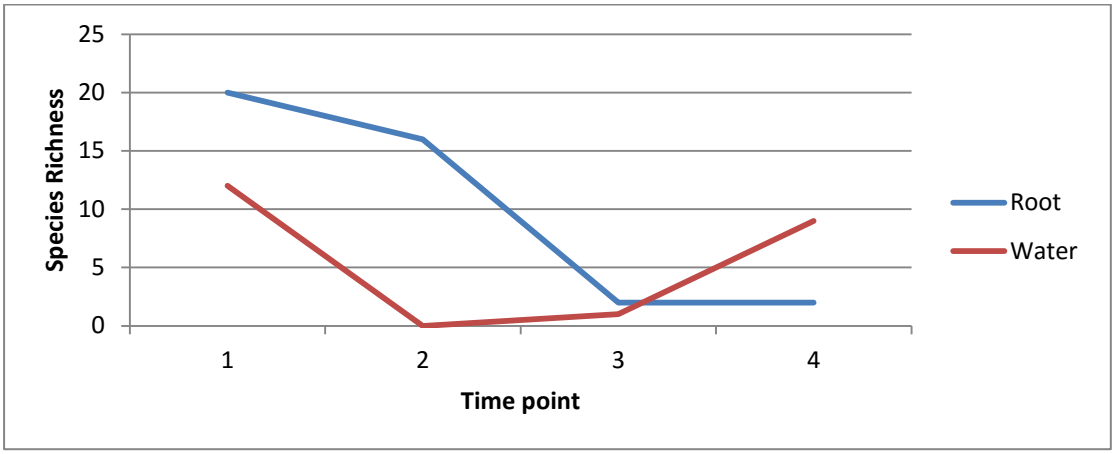
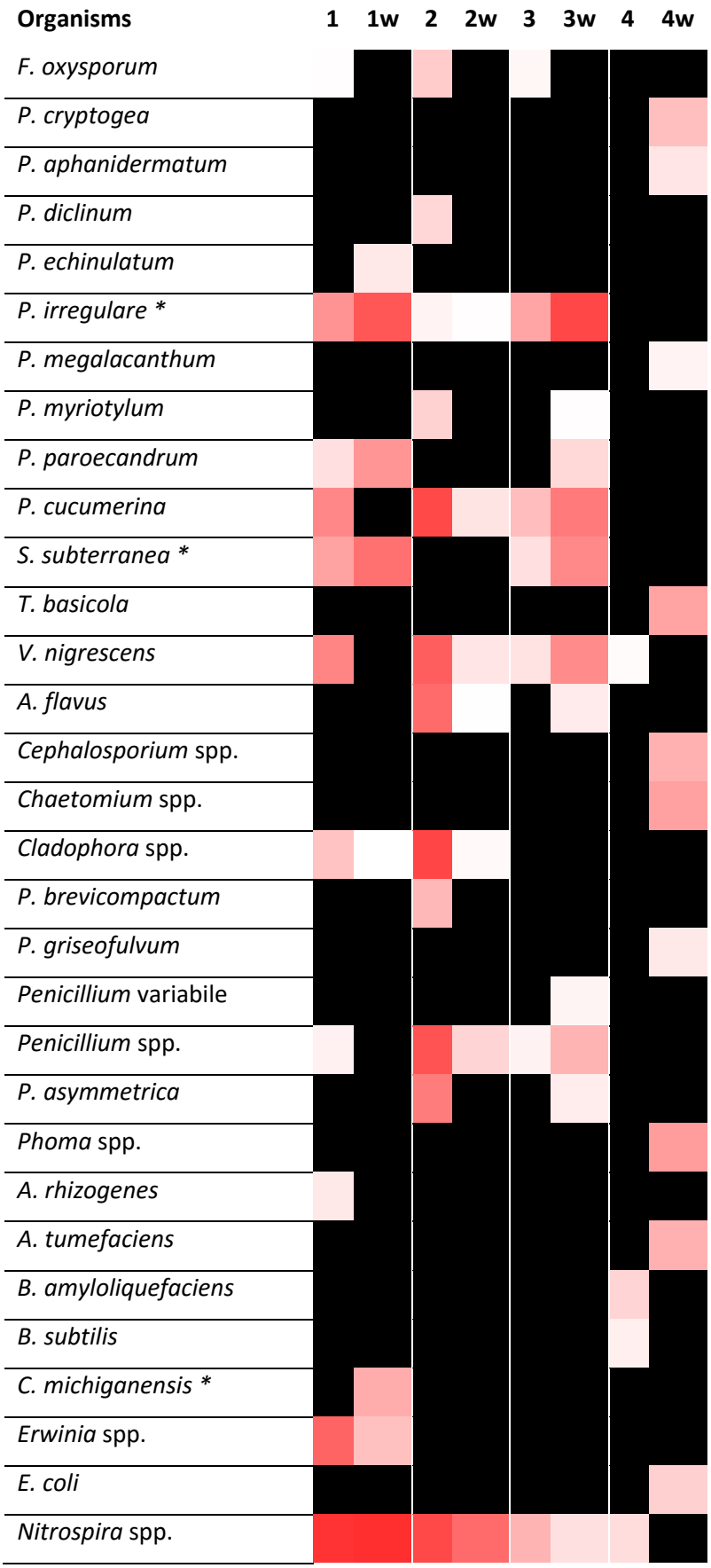


Figure 4.6: Comparison of species richness detected in slab solution compared to that of the associated root sample.

4.3.2.b fSSF

The fSSF showed a relatively consistent species richness throughout the season, eventually resulting in equal species richness at the final sampling point (Fig. 4.8). However, it is worth noting that at the start of the season the detection levels of fungi and oomycetes, along with bacterial organisms, were fairly similar but later in the season many more fungal species seemed to be detected in the water, whereas the roots featured a mix of organisms (Fig. 4.7). It should also be noted this has one of the highest species richness within the water at the first sampling point; this then doesn't reduce through the season.


Organisms
1
1w
2
2w
3
3w
4
4w
F. oxysporum
P. cryptogea
P. aphanidermatum
P. diclinum
P. echinulatum
P. irregulare *
P. megalacanthum
P. myriotylum
P. paroecandrum
P. cucumerina
S. subterranea *
T. basicola
V. nigrescens
A. flavus
Cephalosporium spp.
Chaetomium spp.
Cladophora spp.
P. brevicompactum
P. griseofulvum
Penicillium variabile
Penicillium spp.
P. asymmetrica
Phoma spp.
A. rhizogenes
A. tumefaciens
B. amyloliquefaciens
B. subtilis
C. michiganensis *
Erwinia spp.
E. coli
Nitrospira spp.

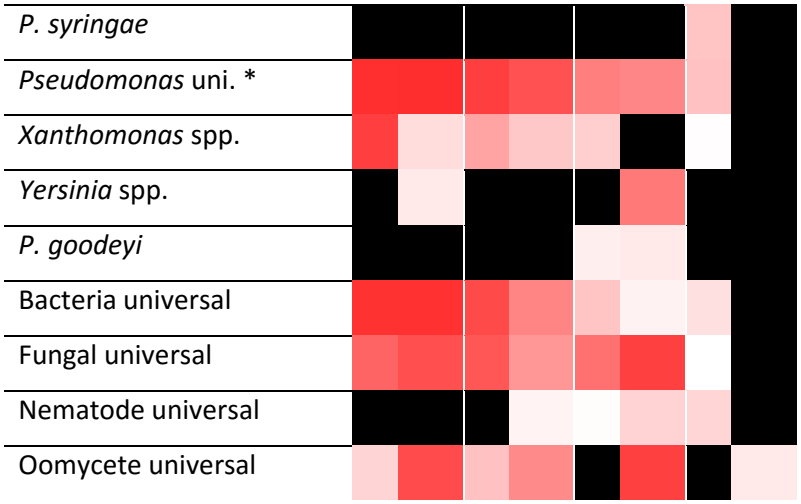


Figure 4.5: Comparison of the slab solutions (w1-w4) to their associated root samples (1-4) from the same time point. These are the samples gathered from the fertigation system utilising a full SSF water treatment.

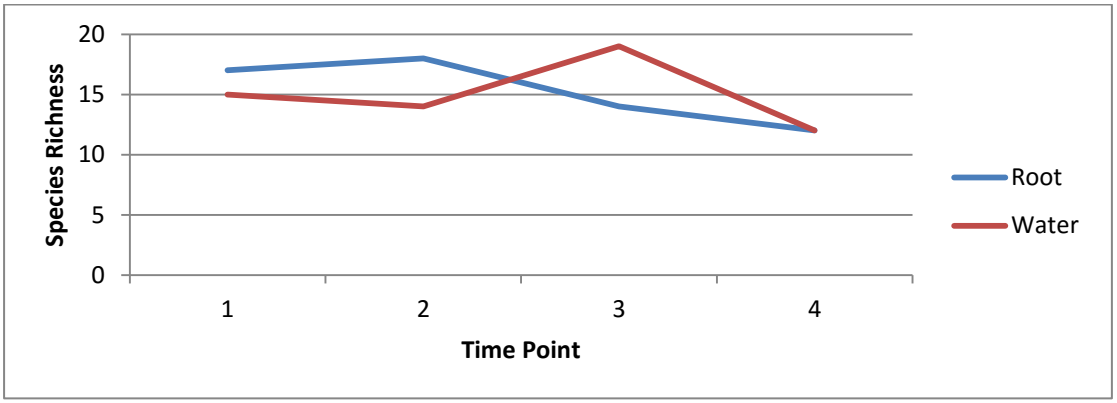


Figure 4.8: Comparison of the species richness in root samples and slab solution samples, in a system using a fSSF water treatment, over the growing season.

4.3.2.c RTW Non-grafted

Comparison between the FS samples and non-grafted root samples from a RTW system (Fig. 4.9) further emphasises the change throughout the season of where detection occurs. High levels of microorganisms were again detected on the roots in the early stages of the season, before the change to a high level of microorganisms being detected in water samples

when compared to the roots later in the season (Fig. 4.9). This is very clearly reflected in the graph of the species richness (Fig. 4.10).

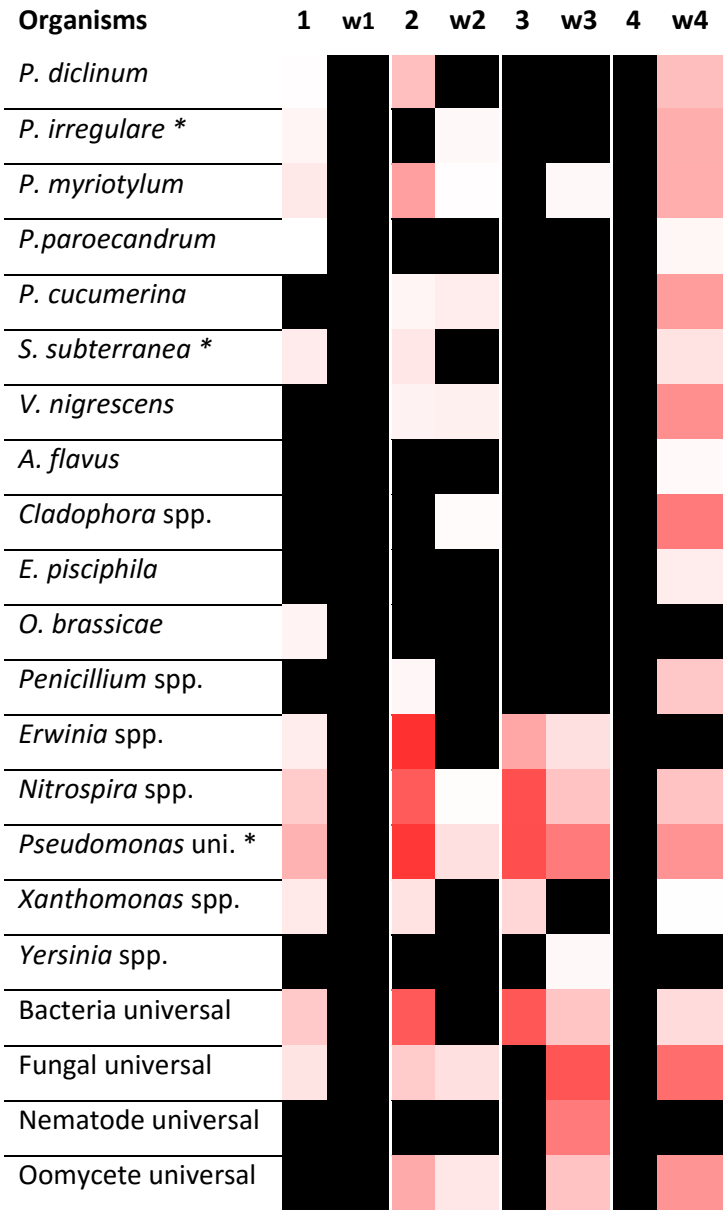


Figure 4.9: Comparison of the slab solutions (w1-w4) to their associated root samples (1-4) from the same time point. These are the samples gathered from the fertigation system utilising no water treatment, but instead RTW whilst propagated plants grown on their own root.

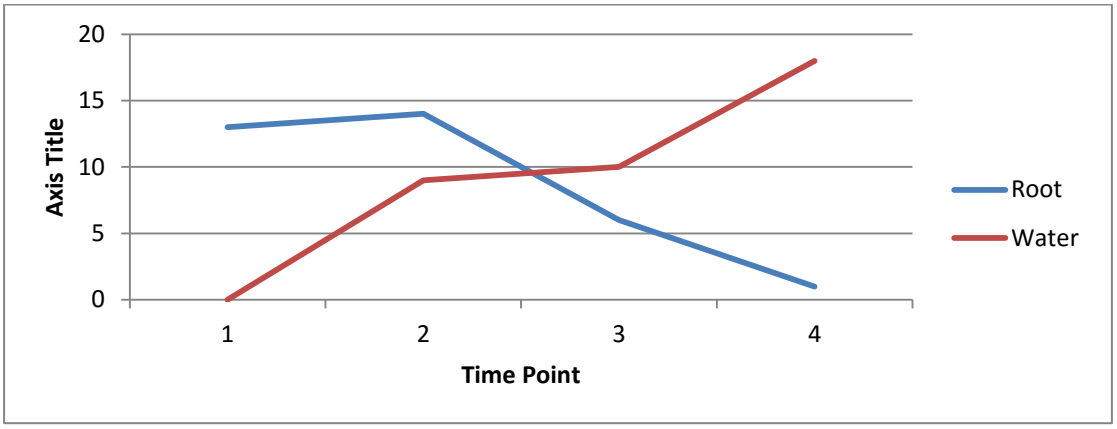


Figure 4.10: Comparison of the slab solution species richness compared to that of the root sample.

4.3.2.d RTW Grafted

In the samples where grafted roots were grown in a RTW system the results were similar to those of the non-grafted plants; however, the reduction in detection of root microorganisms was far quicker. Following the first sampling time point the microorganisms were almost completely eradicated from future root samples, much quicker than from the non-grafted root stocks. Then towards the end of the season the species richness of the water also declined to a similar level to that of the root samples (Fig. 4.12). The first root sample of the grafted plants featured the highest species richness for a single sample, out of all the roots. This then rapidly reduced, in contrast to the water samples, which then by the third sampling point began to equal out.

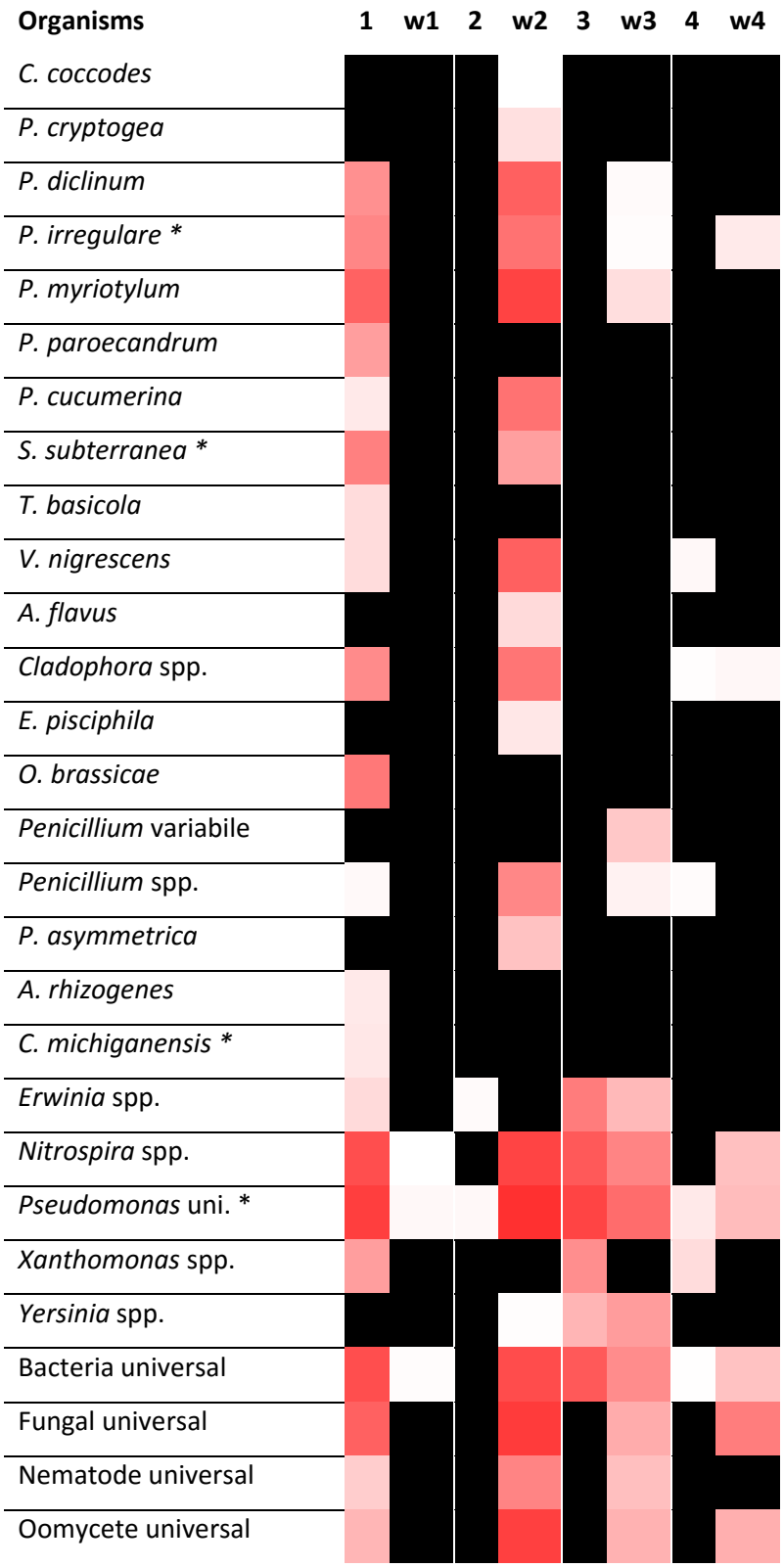


Figure 4.116: Comparison of the slab solutions (w1-w4) to their associated root samples (1-4) from the same time point. These are the samples gathered from the fertigation system utilising no water treatment, but instead RTW, whilst propagated plants are grown on different rootstocks.

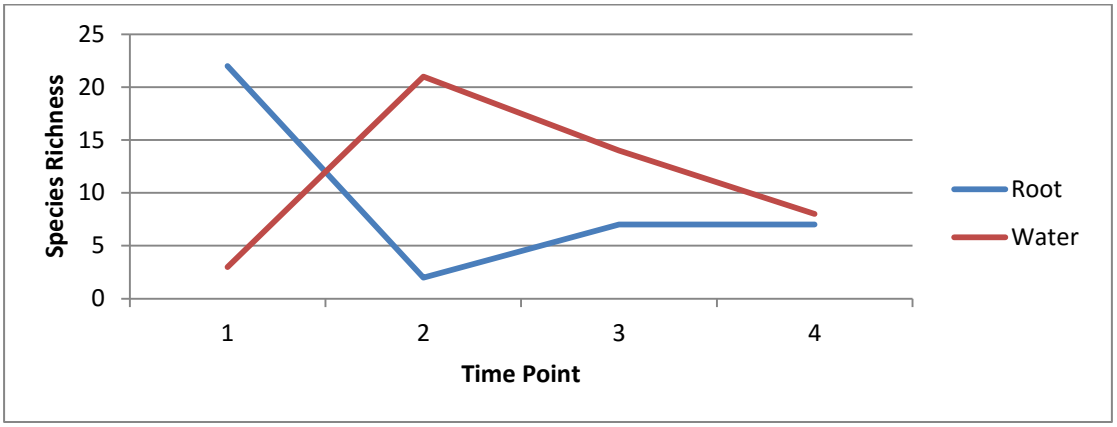


Figure 4.12: Comparison of the slab solutions species richness to that of the root samples in a system using a RTW water treatment method and grafted plants.

4.3.2.e Heat treatment

Again with the heat treated system, the roots start the season with a high level of organisms whilst the water samples stay relatively low. However, over the course of the season, the detection of microorganisms decreases, but relatively slowly. By the end of the season the detection rate of organisms in the water samples rises above that of the roots but they are both still relatively similar (Fig. 4.13).

In this system, the *Pythia* species seem to be relatively prevalent throughout the season, in both water samples and roots (Fig. 4.14). Again *Nitrospira, Pseudomonas* and *Xanthomonas* spp. were common throughout the season in relatively high abundances, although abundance in both water and roots seemed to decrease overall throughout the season.

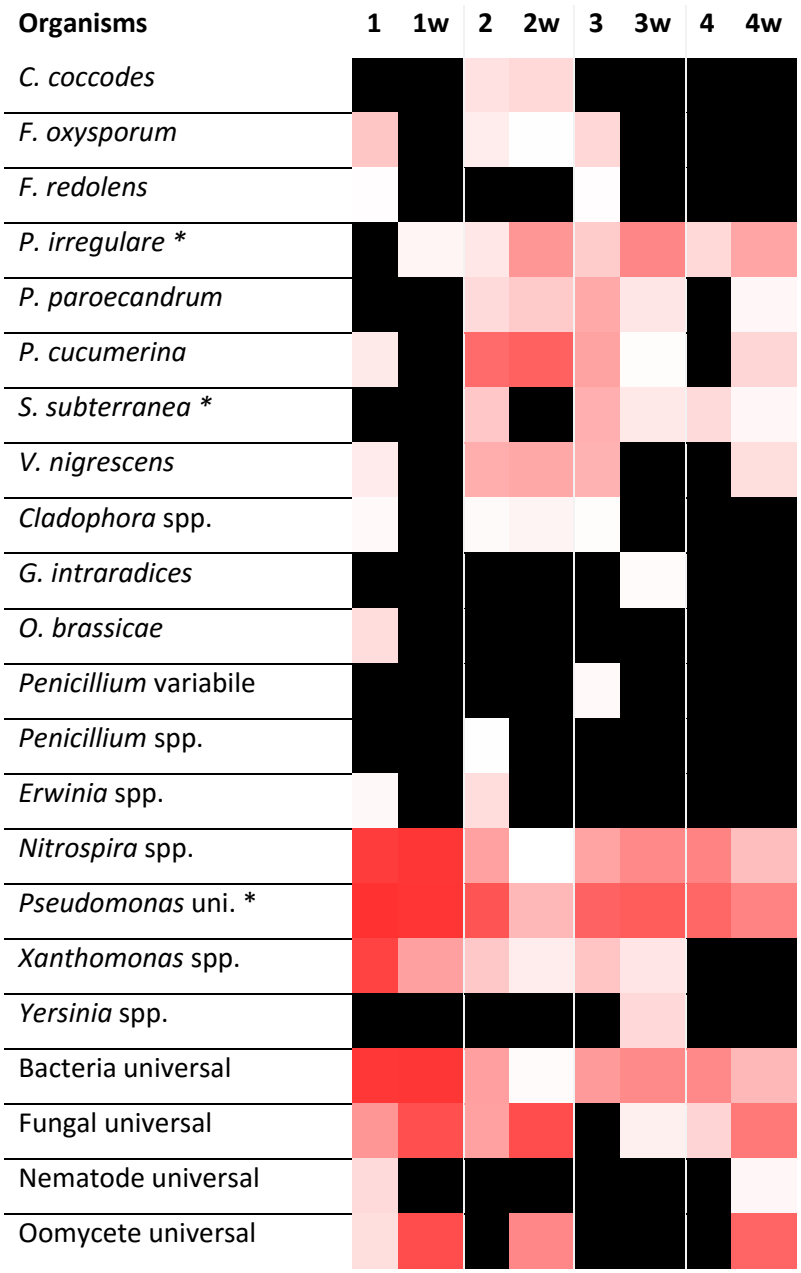


Figure 4.13: Comparison of the slab solution (1w-4w) to its associated root sample at the same time points (1-4). These samples were gathered from a system using heat to treat the water.

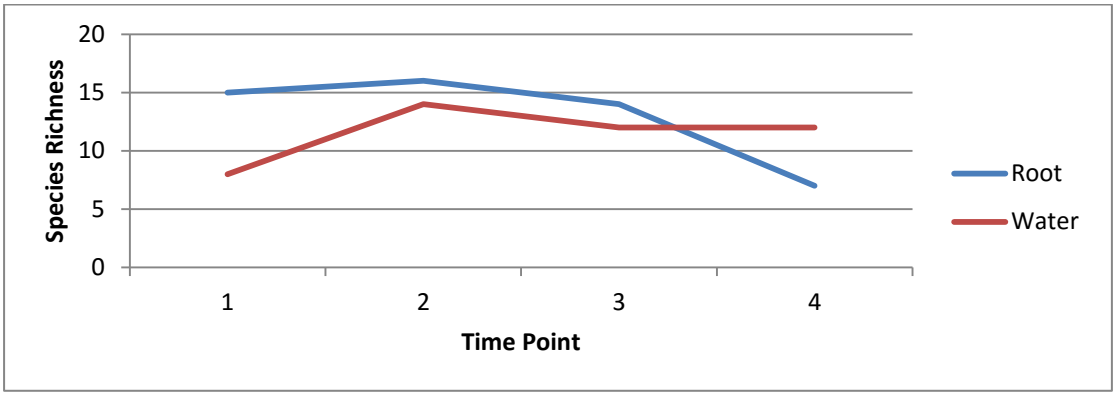
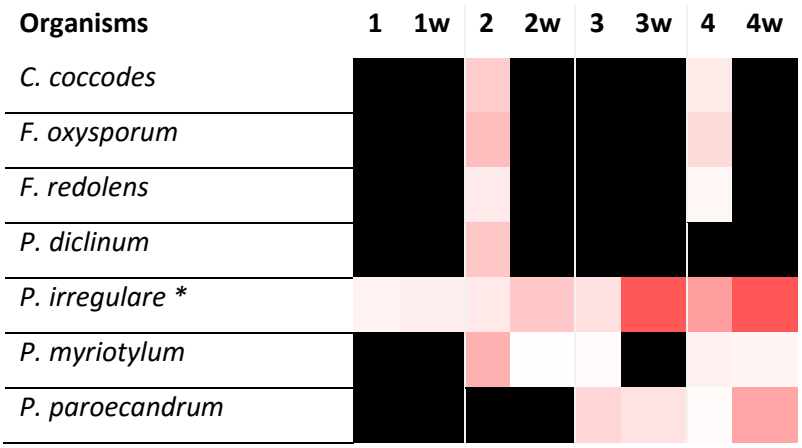


Figure 4.14: Comparison of the slab solution samples to the root samples at each time point, in a system using a heat water treatment.

4.3.2.f UV treatment

Of all the nurseries, the one which applied a UV water treatment to treat the FS featured one of the highest levels of species richness consistently throughout the season. Many of the organisms consistently detected were detected both in water and root samples. Often the occurrence of detected organisms seemed sporadic, with no clear patterns, whilst species such as *P. cucumerina, P. irregulare, P myriotylum, S. subterranean and V. nigrescens* were fairly consistently present and abundances of each were also fairly consistent.

Overall from observing Figure 4.16 there does not seem to be an obvious pattern or difference in species richness detected within roots compared to water samples.



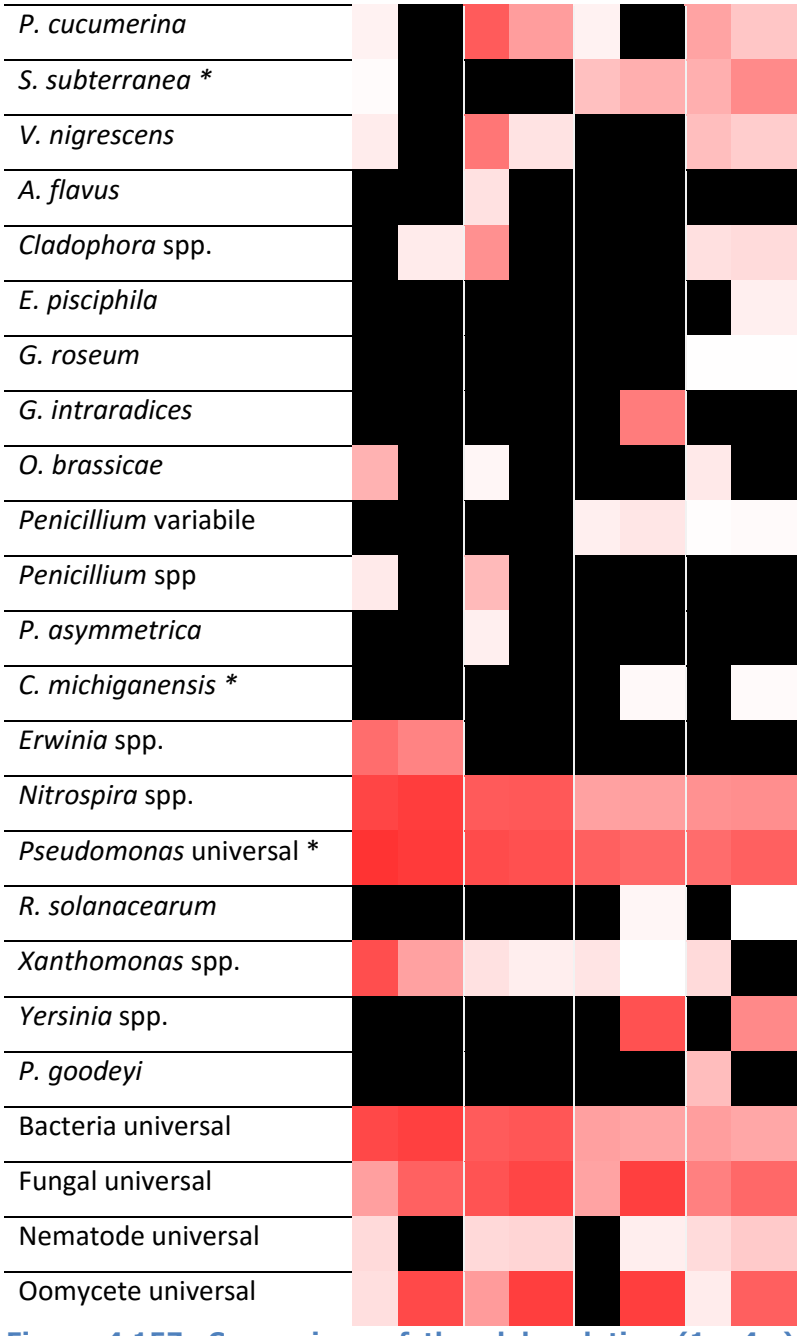


Figure 4.157: Comparison of the slab solution (1w-4w) to its associated root sample at the same time points (1-4). These samples were gathered from a system using UV to treat the water.

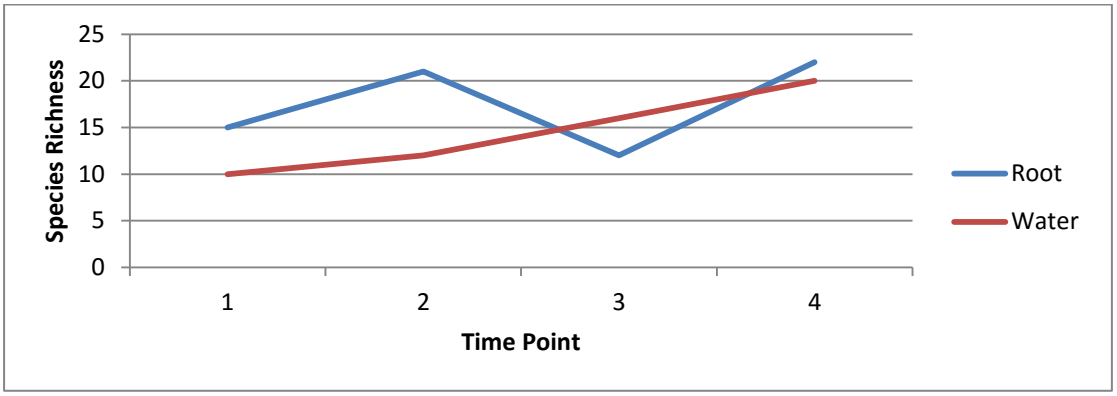


Figure 4.16: Comparison of the slab solution samples to the root samples at each time point, in a system using a heat water treatment.

4.4 Discussion

From the total species richness data gathered over the season (Table 4.1) it was clear to see the nurseries utilising the heat water treatments or the RTW systems showed good efficacy in terms of much reduced cumulative species richness on the roots, when compared to nurseries using treatments such as UV and fSSF.

The apparent inefficiency in the SSF's reduction of species richness may be a result of the SSF not only acting as a mode for reintroduction of organisms from previous seasons, but also as a medium for the further proliferation of organisms that may be introduced as suggested by Cafa (2012). In terms of comparing the season's results of the pSSF and fSSF (Fig. 4.1), despite more pathogenic fungi or oomycetes being detected in the pSSF, the fSSF system seemed to have more organisms that were persistent throughout the season. The pSSF nursery had no specific organisms that were present for more than two time points, whereas the nursery using the fSSF featured eight separate organisms that were persistent for three or more time points, of which four were potentially pathogenic fungi or oomycete species. This persistence of detection in the fSSF system may be important in the pathogens potential for eliciting

disease, whereas the pSSF seemed, over the course of the season, was perhaps more effective in removing organisms from the roots.

The fSSF nursery also featured the most species that were unique to only that system (Table 4.4) suggesting that the system is not only ineffective at removal of organisms but also may provide conditions favourable to the widest range of organisms. The only other nurseries to feature organisms unique to certain systems were the grafted roots from the RTW nursery and the nursery using UV water treatment, which featured two and one unique species respectively. Regarding the unique organisms that were detected on the grafted roots in the RTW system, they were both only detected at the first sampling time point (Fig. 4.2). This suggests they may have been brought in to the nursery with the plants and, since they were then no longer detected following the first time point, that the RTW system was adequate in their removal. However, the nursery using a UV water treatment system had its unique species introduced to the nursery on the final sampling time point which suggests there was some means of introduction of new organisms late in the season. Also, due to the late introduction, it was not possible to judge the UV system's effectiveness of removal.

The nurseries that utilised a heat water treatment system and a RTW system, in terms of cumulative species richness over the season, showed the lowest number of detected species. Of the two RTW systems, the non-grafted plants showed no species unique to the samples over the season, as did the samples from the heat treating nursery (Table 4.4). In terms of the RTW system, this may show that if organisms are introduced to the roots they are unable to stay for any period of time and proliferate as they are very quickly removed. This may be further supported by the fact that no fungal or oomycete organisms were able to persist for more than two time points (Fig. 4.2) in either RTW sample set.

To draw comparisons between the grafted and non-grafted sample sets that were propagated in the RTW system, it seems that despite the higher cumulative species richness of the grafted roots (Table 4.1), the grafted

roots seemed more effective in preventing persistence of organisms on the roots. From Figure 4.2, it is clear to see that the grafted roots higher cumulative richness is a result of early introduction of organisms at the first time point but, unlike the non-grafted roots, these were mostly eradicated for the rest of the season. This early introduction of organisms on grafted roots may be a result of the nature of grafted plants involving more external interference in early stage of growth and therefore more opportunity for introducing organisms. It is important to remember that the grafted and non-grafted plants were grown on the same water supply, and therefore at the early time point's detection of organisms is a result of introduction with the roots. This efficacy of the grafted roots is to be expected and a good demonstration of the benefits offered from a hardier and vigorous rootstock providing improved resistance to rhizosphere organisms (King *et al.*, 2008).

The detection of organisms in both the heat treatment and the UV treatment nurseries were both very sporadic over the course of the season (Fig. 4.3). The UV nursery featured the second highest number of species detected in the roots over the season, recording twenty-seven, whilst the heat treatment nursery recorded one of the lowest number of species at only twenty-one (Table 4.1). However, both were sporadic in the time points where organisms were detected, with no real clear time point where the majority of organisms occurred. The organisms also seemed to appear and then reappear at non-consecutive time points in several cases. This sporadic occurrence, with no clear point of introduction, possibly suggests that the heat treatment at the nursery is effective in terms of limiting the number of species to just twenty-one throughout the season.

There were several species of organisms detected that were common across the main nurseries (Table 4.5). *Pythia* such as *P. irregulare, P. paroecandrum, P. diclinum* and *P. myriotylum* were common across almost all nurseries at at least one sampling point. As well as these, other pathogenic fungi or oomycetes such as *P. cucumerina, S. subterranean*

and *V. nigrescens* were also very common across the season. In terms of bacterial species, *Erwinia, Nitrospira, Pseudomonas* and *Xanthomonas* were all detected on at least one of the root samples for the five main nurseries, often being persistent. These commonly occurring organisms are similar to those found by Scott (2013).

The five additional nurseries that were observed at two time points were an opportunity to gather further data of commonly detected organisms from samples elsewhere in the UK to further support the five main nurseries. From the collected data of detected organisms (Fig. 4.4) it is clear that the previous data collected seems to be consistent within the additional nurseries. *Pythia* are again common across all the nurseries, with the bacterial organisms *Nitrospira, Xanthomonas, Pseudomonas* and *Erwinia* being detected in almost all of the additional nurseries together, with some being persistent at both sampling time points.

When looking at the comparisons between the root samples to that of the associated water samples from the same time point, from chapter 3, there seems to be some exchanges between which sample acts as a source of the organisms. Figure 4.5 compares the pSSF results of individual species between the root and slab solution samples, whilst Figure 4.6 compares the total species richness between the root and water samples at each time point. From Figure 4.5 it may be hypothesised that the species that appear in the water samples may be residual from those that appeared in the roots from the first sample point. Both samples have low levels of organisms in the middle of the season, which may show that the pSSF system was successful in its complete control of organisms present in the system, and the rise in the final sampling point is just a result of random introduction.

Figures 4.7 and 4.8 seem to show no real exchange in organisms detected in the fSSF system roots and water samples. Both have relatively similar species richness number in Figure 4.8 throughout the season with no real drop in water or bacterial samples. This may imply that in terms of removal of organisms from both roots and water this system is poor.

Both of the SSF systems have a high species richness total in the first water sampling point, when compared to the other systems. This suggests that it is the filters reintroducing organisms from the previous season that have been allowed to proliferate and then lay dormant within the filter. The fSSF system has a higher species richness, which is to be expected in regards to the fSSF being significantly bigger than that of the pSSF which filters just 10% that of the fSSF.

From the results from the RTW system that utilised non-grafted roots (Figs 4.9 and 4.10) there appears to be a clear change midway through the season where the roots start as the clear host of the majority of organisms, yet by the end of the season the water samples seem to be the host to the majority of species. In a RTW system, as the water is not being recycled there should be minimal species in the water, so it may be possible to assume that by the end of the season the system is starting to get dirty, and able to host organisms. This may be a more reasonable explanation to assume than that the organisms from the early roots just transferred to the water, as there are many differing species from the early root samples to the later water samples. These differing species of organisms are more likely to have been introduced by random introduction throughout the season and then been able to inhabit the system. The roots may develop reduced species richness as any species exposed to the roots are quickly washed away with the system, therefore not allowing any organisms to inhabit the root system.

In Figure 4.11 and 4.12 it can be seen that the species richness within the grafted root sample is the highest recorded of any of the samples tested. This may suggest that the plant when arriving from the propagators is already carrying a substantial number of species in the roots. Figure 4.12 then seems to suggest an exchange of organisms from the roots to the water. However, it is then followed by an overall reduction in species richness in both sample sets. This may be a demonstration of the efficacy of both the RTW system and of grafted rootstocks.

As previously mentioned, in both the heat and UV results the organisms detected seem to be random and sporadic. This is further reflected in both sets combined water and roots results (Figs 4.13 and 4.15). Both systems seem to have organisms that inhabit the water and roots at points that are more persistent than those in the previously mentioned nurseries. Figures 4.14 and 4.16 suggest there is no real reduction throughout the season in the species richness of both root samples and water samples, whilst from the relative abundances in Figures 4.13 and 4.15 the levels of the organisms do not seem to be reduced either.

Chapter 5: Development and validation of qPCR primers for four common tomato root pathogens

5.1 Introduction

As mentioned in chapter 1 (Section 1.9.b) the development of PCR-derived technologies such as qPCR are becoming much more common place and are popular in detection of microbial populations due to not only specific detection, but also the ability to quantify the amount present. This, in terms of pathogen detection, is a very important aspect as inoculum level often needs to be closely monitored in relation to disease prevention. There are two popular methods used for qPCR, both commonly used for separate reasons; SYBR green fluorescence or 5' nuclease assays also known as TaqMan. Both of these have a similar basic principle in that they rely on the formation and denaturing of ds-DNA. During each cycle the number of amplified products should theoretically double, providing the primers are 100% efficient, and therefore the number of double strands will increase exponentially at each annealing and extension stage causing the fluorescence to increase proportionally. As this fluorescence increases it will eventually reach a threshold at where it is able to be recorded; this is known as the 'cycle threshold' (Ct), which is given on the readout in terms of cycle number. This Ct score is proportional to the logarithm of the initial amount in the original sample (Ponchel *et al.*, 2003). However for the quantification to be sufficiently accurate and specific it requires the assay to be optimal which can depend on the method used.

SYBR green is a commonly used method; however, it compromises sensitivity for price when compared to TaqMan probe assays, which are a much more expensive option. SYBR green works as a reporter dye by purely binding to any ds-DNA. This leads to loss in sensitivity as it will bind indiscriminately to any ds-DNA including non-specific PCR products such as primer dimers (Ponchel *et al.*, 2003). Despite being less sensitive than the TaqMan systems, SYBR green protocols are often sufficiently

sensitive for approximate quantification, whilst also being comparatively simple and inexpensive (Ponchel *et al.*, 2003).

TaqMan assays however, make use of a dual-labelled fluorogenic probe instead of an unspecific florescent reporter dye. The probe has a complimentary sequence to a region within the target template DNA, with a reporter fluorophore attached to the 5' end, and a quencher attached to the 3' end. The probe does not fluoresce whilst complete due to the presence of the quencher. However, following the denaturing of the DNA strands, the probe will attach to the target region of the template DNA. When *Taq* polymerase encounters the probe, during the extension phase of the reaction, its nuclease activity hydrolyses the non-extendable probe releasing the 5' fluorophore allowing fluorescent emission (Heid *et al.*, 1996; Huang & Li, 2009). This method is much more reliable and although primer dimers can still form and mildly affect the amplification efficiency, it is not really an issue until towards the end of amplification when primer concentration becomes a limiting factor. These primer dimers do not affect sensitivity like in SYBR green methods however, as they do not cause fluorescence. Another major benefit presented as a result of using TaqMan is the potential to produce small scale multiplex assays. This can be achieved by using a variety of different reporter fluorophores with separate signal wavelengths; however there are only a limited number of reporter wavelengths that can be used without overlapping and interfering with each other so multiplexing is still relatively on a small scale.

In this study the aim was to develop and validate qPCR assays for four fungal and oomycete organisms identified as either common inhabitants of fertigation solutions or common threats to tomato crops in previous work (Devine, 2013; Scott, 2013). Once the primers had been developed, they were validated and tested using DNA from cultures of each organism, where identity had been confirmed by ITS sequencing. The end objective was to then be able to use the assays in conjunction with microarrays to test fertigation solutions more sensitively and to verify the semi-quantitative nature of the array results.

5.2 Methods

Four microorganisms were identified for assay development, based on their perceived importance and prevelance as root-infecting pathogens: *Colletotrichum coccodes, Plectospharella cucumerina, Pythium aphanidermatum* and *Pythium myriotylum.* Potential primers were then identified through a search of the literature some further modification of the primers. Common barcoding genes were appropriate to use, targeting either the ITS 1 or ITS 2 region (Table 5.1). These primers were then validated using cultures from a variety of sources including ADAS's culture collection and the CBS culture collection. DNA was extracted from these cultures, the rDNA amplified using universal primers, and the resultant DNA sequenced as stated in 2.1.1, to confirm their identity. For each q-PCR assay reaction mixture, Promega GoTaq® Probe qPCR systems ready-to-use 2x master mix was used and each well volume was made up to 20 µl. The primer and probe working concentrations are listed in Table 5.2. For each assay 2 µl of DNA sample was added. Assays were carried out on Roche LightCycler® 480 white 384 well plates, in a Roche LightCycler® 480 II.

Assays were then validated using as many corresponding pure culture DNA samples as possible for each assay. These pure culture DNA samples were in a range of concentrations: one fold, tenfold and hundredfold. The serial dilution of the samples enables the assay efficiency to be tested when using the standardised amplification programme (Table 5.3). An assay with 100% efficiency of amplification should have a Ct difference of 3.32 per 10 fold dilution. The efficiency of the test assay is worked out by taking the actual Ct difference score per 10 fold dilution and working it out as a percentage of 3.32, where efficiencies of 90-110% are generally accepted as usable. Specificity for just the corresponding organisms was then tested by the use of negative control samples, containing wide ranges of non-target organisms to confirm it only amplified the specific target organisms DNA. In this testing, it was important to test closely related organisms to ensure specificity of primers, for example with the many closely related *Pythia* species it is important to find primers that do

not amplify similar species. For each sample used, there were three replicates and an average calculated.

Following initial validation of the primers they were then used on the same fertigation solution samples and root samples from the microarray analysis. This was to test the sensitivity of the microarray to varying levels of target DNA in samples, from low to high concentrations of target DNA.

The final stage was to test the quantification abilities of both the microarray and qPCR assays in tandem. This involved the ranking of microarray scores low, medium and high. This was done by ranking microarray scores from 0.1-0.3 as low concentration, 0.3-0.7 medium and 0.7-1.0 as high. A sample set including all three levels was tested using the qPCR assay to assess the reliability of the semi-quantitative rankings.

Table 5.1: List of TaqMan primers: [1](Cullen *et al.*, 2002)**, [2]**(Atkins *et al.*, 2003)**, [3]**(Li *et al.*, 2014)**.**

Primer	Sequence (5'-3')	Target DNA	Size of product (bp)	Fluorophore
***C. coccodes*[1]**				
CcTqF1	TCTATAACCCTTTGT GAACATACCTAACTG	ITS 1	145	
CcTqR1	CACTCAGAAGAAACG TCGTTAAAATAGAG			
Taqman probe	CGCAGGCGGCACCC CCT			Cy5
***P. cucumerina*[2]**				
PcRtF1	GTGCCCGCCGGTCTC	ITS 1	72	
PcRtR1	GACAGTTCGCTAAGA ACACTCAGAAGT			
Taqman probe	TCAGAATCTCTGTTT TCGAACCCGACGA			Fam1
***P. myriotylum*[3]**				
PyuniTqF1	CTGTTCTTTCCTTGA GGTG	ITS 2	149	
PmTqR1	GGAGCCGAAACTCTC ACAAGAC			Tex Red
Taqman probe	TCCCAAATTGGTGTT GCCTTCTTTACCC			
***P.aphanidermatum* [3]**		ITS 2	163	
PyuniTqF1	CTGTTCTTTCCTTGA GGTG			Hex
PaTqR1	GCGCGTTGTTCACAA TAAATTGC			
TaqMan probe	CATTTGCCCAGACCA TTGCCTC			

Table 5.2: qPCR Primers and probes working concentrations.

Assay		Working concentration
C. coccodes	Primer	0.3 µM
	Probe	0.1 µM
P. cucumerina	Primer	0.3 µM
	Probe	0.23 µM
P. aphanidermatum* and *myriotylum	Primer	0.5 µM
	Probe	0.25 µM

Table 5.3: Standardised PCR protocol

Stage	Temperature (ºC)	Time (s)	Cycles
Pre-incubation	50º	120	
	90º	120	1
Amplification	95º	15	
	60º	60	45
Cooling	40º	30	1

5.3 Results

5.3.a Validation of primers, probes and amplification protocol.

For the organisms, *Colletotrichum coccodes; Plectospharella cucumerina; Pythium aphanidermatum* and *Pythium myriotylum* a TaqMan assay was developed, where all four probes had different fluorophores so it was possible to measure them on the same plate under different wavelengths. However, some filter had slight overlaps in recording that may lead to difficulty in using them in the same well.

All assays successfully amplified the pure culture DNA samples for their target organism. The numbers of pure cultures used to test each assay set are listed in Table 5.4.

Also from Table 5.4, the overall efficiency of the programme was sufficient to be used. The efficiency of *C. coccodes* on its own falls just outside the sufficient efficiency range of 90-110%, so needs further refining; the rest fell within the sufficient range. Efficiencies are not certain values and are more estimates as a result of them being derived from serial dilutions which contain uncertainty. This is the reason for theoretically impossible efficiencies being derived >100% so a relatively wide range of 10% either side of 100% is permitted. All confirmed pure cultures were successfully detected and amplified.

Table 5.4: Amplification efficiency of assay protocol. Efficiencies based on the presumption that 90%-110% is acceptable and 100% is a Ct difference of 3.32 for a tenfold dilution.

Primer set	Number of Pure Cultures Used	Average Ct Difference	Amplification Efficiency
C. coccodes	3	3.76	113.6%
P. cucumerina	3	3.12	94.0%
P. aphanidermatum	2	3.18	102.1%
P. myriotylum	1	3.39	95.78%
Overall average		**3.36**	101.8%

5.3.b Microarray validation in conjunction with q-PCR primers: *Colletotrichum coccodes*

Table 5.5 compares microarray results to the same samples being analysed using the *C. coccodes* qPCR assay. The ranging concentrations derived from the microarrays seem to be well supported in this case by the qPCR amplification with clear definition between each of the samples.

There is also evidence for insufficient sensitivity in the microarray, as one sample that tested negative by the microarray, was amplified using qPCR. This amplification was relatively late in terms of qPCR amplification with a Ct of 33.21; however, this still suggests it was present at a low

concentration. Relative to the microarray that recorded low concentrations of *C. coccodes,* the false negative concentration is approximately 100 fold lower.

Table 5.5: Result of the Comparison of microarray results for detection *C. coccodes* to that of the qPCR results.

Test Samples	Concentration	Amplification expected/actual	Average Ct score	Average Ct difference
Negative Microarray result	n/a	-/+	33.21	
	n/a	-/-		
Low to high microarray score	Low	+/+	27.05	
	Medium	+/+	24.94	2.11
	High	+/+	22.80	2.14
No template control	n/a	-/-		

Plectosphaerella cucumerina

Again, in the *P. cucumerina* assay, there is a suggestion of lack of microaarray sensitivity with both supposed negative samples amplifying (Table 5.6). The result of the validation of the semi-quantitative side of the array gave inconclusive results with no real clear defined order.

Table 5.6: Results of the Comparison of microarray results for detection of *P. cucumerina* to that of the qPCR results.

Test Samples	Concentration	Amplification expected/actual	Average Ct score	Average Ct difference
Negative Microarray result	n/a	-/+	28.37	
	n/a	-/+	32.28	
Low to high microarray score	Low	+/+	22.45	
	Medium	+/+	25.16	-2.71
	High	+/+	24.23	0.93
No template control	n/a	-/-		

Pythium aphanidermatum

P. aphanidermatum tests were limited by a lack of positive microarray samples to retest; however, the investigation of negative microarray samples suggests there was wasn't any of this pathogen at even low concentrations throughout the season (Table 5.7).

Table 5.7: Result of the Comparison of microarray results for detection of *P. aphanidermatum* to that of the qPCR results.

Test Samples	Concentration	Amplification expected/actual	Average Ct score	Average Ct difference
Negative Microarray result	n/a	-/-		
	n/a	-/-		
No template control	n/a	-/-		

Pythium myriotylum

P. myriotylum showed inconclusive results. Unfortunately over the course of the season there were no pure cultures available. Despite this the *P. myriotylum* detected in root and fertigation solution samples using the microarray, the qPCR assay was unable to amplify any target sequence. This implies that either the qPCR assay was ineffective, or that there may have been fault in the microarray probe for *P. myriotylum* (Table 5.8).

Table 5.8: Result of the Comparison of microarray results for detection of *P. myriotylum* to that of the qPCR results.

Test Samples	Concentration	Amplification expected/actual	Average Ct score	Average Ct difference
Negative Microarray result	n/a n/a	-/- -/-		
Low to high microarray score	Low Medium High	+/- +/- +/-	n/a n/a n/a	
No template control	n/a	-/-		

5.4 Discussion

From the preliminary validation results, the primer assays seem to have good specificity. Both *C. coccodes* and *P. cucumerina* could be validated using three separate strains of pure culture to ensure the specificity of the primers were sufficient to identify separate isolates. However neither of the *P. aphanidermatum* or *P. myriotylum* assays were tested with as many samples, so, despite initial positive results, more tests may be needed to confirm reliability . *P. myriotylum* in particular did not seem to amplify

anything in samples that had been positively detected using the microarray, so needs further assessment using more pure culture strains to identify the reason for this result whether it is due to experimental error, ineffective primers or whether the microarrays probe was specific enough and not detecting other *Pythia* species. But it seems the use of the ITS regions for each of the primers was sufficient for specific and accurate identification. On the whole, from the serial dilutions the assays seemed to have sufficient efficiency falling within the guideline range, with the exception of *C. coccodes*. Just futher refining of the protocol and mastermixes is probably required for *C. coccodes.*

Despite the use of separate fluorophores to multiplex the assay, all but Fam 1, were affected by overlapping wavelengths. Therefore if the primer and probe sets were used collectively the overlapping wavelengths may result in false positives. For the pupose of this test where separate wells were used for each assay this is fine; however, if a multiplex assay was to be developed with them together in a well different fluorophores would be necessary for it to be effective with no background disturbance from overlapping signals.

One of the key findings in this research was the apparent lack of sensitivity of the microarray. This was clear in the results presented from the many negative samples tested by the array, showing late amplification with the qPCR assay, suggesting low amounts of target being present. This is a major flaw in solely using the microarray as a detection system for potentially pathogenic organisms. If the array was to be used further as a detection system it is advised it be used in conjunction with a more sensitive method such as qPCR to double check negative array results more stringently. The use of the two in combination, should circumvent both techniques shortfallings with qPCR being sensitive but difficult to multipex on a scale anywhere near similar to that of the microarray. Therefore the microarray could be utilised as a crude test for a general overview of the sample population, and qPCR developed to test for a more select group of organisms with greater sensitivity

Despite limited sensitivity of the microarray, demonstrated in the *C. coccode* assay, qPCR showed that the array has the capacity to be reasonably accurate as a semi-quantitative approach for monitoring pathogen populations. For the arrays semiquantitative side to be used more accurately more research is needed to define its capabilities, as shown by the *P. cucumerina* result. However if shown to be accurate and repeatable, it could be possible to develop approximate quantifications for each defined group; low medium and high.

Chapter 6 Summary, Final Conclusions and Future Research

Chapters three and four involved the utilisation of a small scale microarray to observe microbial populations associated with the FS and roots of tomato crops grown in soilless systems. Chapter three was directed towards monitoring the FS and focussed on the population changes throughout the different sampling points, through the system, and the effect of five separate methods of water treatment. These treatment strategies included pSSF, fSSF, heat treatment and UV treatment. The final water treatment strategy was a RTW system that included two separate crops; one with non-grafted plants and the other featuring plants grafted on to hardy, resistant rootstocks.

Overall in terms of cumulative species richness, it was found that over the season the RTW system detected the lowest species richness within its FS samples. The system that featured the highest number of detected species was the fSSF. In terms of the efficacy of the water treatments, using average species diversities, the scores tended to show a reduction in species diversity following treatment. Upon deeper investigation, both pSSF and fSSF were able to show significant reductions. The UV treatment was not able to attain the correct degree of significance and unfortunately the heat treatment results appeared to decrease diversity, but not enough post treatment samples were received to test its significance.

Over the season a total of 15 separate species were detected in each nursery FS, at least once. Of these, six were potentially pathogenic fungi or oomycetes, four were saprophytic fungi or oomycetes and five were bacterial species. Three of the commonly appearing potential pathogenic fungi or oomycetes were *Pythia* species; *P. irregulare, P. myriotylum* and *P. paroecandrum.* These three species were particularly prevalent throughout the season, in each of the systems, where in all but two cases each was present in at least two time points. However, despite the relative prevalence of *Pythia* over the season, it was found that all the water treatments seemed to show relative success in reducing the abundance or eliminating these organisms from pre to post-treatment

samples. This implies that despite the treatments being effective, the *Pythia,* along with other species, are able to be hosted, or even proliferate elsewhere in the system such as on the slab or in the plant roots throughout the season. As well as these fungal species, bacterial species that were common to each nursery included *Erwinia, Nitrospira, Pseudomonas, Xanthomonas and Yersinia.*

Chapter four also used the small scale microarray to monitor microbial populations on the roots of samples from the five main nurseries as well as a further five nurseries, across the season. Again, the RTW system root samples featured the lowest species richness, whilst the highest species richness was from the fSSF system roots.

The sampling of both the grafted and non-grafted roots from the RTW nursery also allowed comparisons to be made between the changes in microbial populations in each sample. In both RTW crops the microbial population was reduced quickly; however, despite the grafted rootstock starting with higher total species richness, it was able to reduce this much quicker than that of the non-grafted roots, clearly demonstrating the efficacy of the resistant rootstocks.

In terms of the other nurseries, the pSSF system had the second lowest species richness, whilst the fSSF system had the highest. The pSSF system also had no organisms that were persistent for more than two time points, whereas the fSSF system had eight that were persistent for three or more, four of these species being potentially pathogenic fungi or oomycetes. This suggests that the pSSF which filters just 10% of the water has a greater efficacy than the fSSF which filter 100%.

The seasons results of the roots grown in the heat and UV systems were relatively similar, both had relatively sporadic results in terms of appearance of organisms.

The second part of chapter four involved the comparisons of the root samples to their associated slab FS sample. From these observations it was, in some cases, possible to make assumptions of how some

organisms were introduced. Both systems using SSF had higher species richness in the water sample from the first time point, with the fSSF having more than the pSSF. This suggests that the SSFs may act as a source for organisms that may remain dormant within the filter from the previous season. With the grafted roots in the RTW system, it was instead the roots which held the highest species richness from the first sample point. This suggests that upon arrival to the nursery from the propagator the plants may already have a high number of organisms already on the roots which are then introduced to the water in the system. Both the heat treated and UV treated nursery had sporadic detection of organisms throughout the season, with roots and water samples having similar levels of species richness detected.

In both chapter three and four, the research was enabled by the use of the microarray. One of the key benefits put forward for the use of the array is its ability to easily multiplex detection of a wide range of rhizosphere related species on one array with a relatively simple protocol. The microarray development was a step forward from the older T-RFLP methods of characterising the microbial populations (Deery, 2012) and has been met with relative success allowing for more specific identification.

However, in terms of interpretation of the results it is important that this is done with caution, and regarded more as crude results. The microarray itself, along with interlinking methods, has a lot of external influence that may result in experimental bias meaning the results generated cannot be used to directly infer causality, but instead should be used to provide focus for further research.

One of the first points where bias may be introduced to the methodology is in the DNA extraction, which is commonly associated with bias especially when utilised in population analysis (Miller *et al.*, 1999; Martin-Laurent *et al.*, 2001). The efficiency of extraction between and even within samples may be very variable due to a variety of reasons including kit type, sample type or even population constituents. In this study, there

are three key classes of organisms being observed; fungal, oomycete and bacterial. These each differ in various aspects including cellular and cell wall structure. It would therefore be extremely unlikely that these would all share equal extraction efficiencies. Therefore the extraction method will be favourable to certain organisms over others. Once the DNA is extracted it must undergo amplification which provides more sources of bias (Anderson *et al.*, 2003; Anderson & Cairney, 2004). When utilising universal primers and protocols, it does not take into account the individual species optimal amplification requirements and sequence requirements. The amplification of organism sequences is therefore based on a compromised protocol and primer sequence which will, again, result in unequal rates of amplification. One of the final sources of bias found in the microarrays protocols is due to the sample DNA's binding efficiency to the correct probes. This again can be affected by sequence and conditions and insufficient specificity can result in cross-hybridisation (probes where cross hybridisation may occur are marked in lists with "*") as experienced in the research of Devine (2013) and Scott (2013).

Many of the aforementioned issues contribute to the microarray's difficulty in quantifying results, and are why if they are to be used in a semi-quantitative manner the probes should only be used relative to themselves and not compared to other probes, as the bias should be fairly consistent on an individual basis. However, as a new technology, the manufacturers are developing various ways of resolving some of these issues to improve data reliability. Each organism has two probes, which can then be averaged, reducing the effects of cross hybridisation and improving reliability. As well as this the manufacturers are improving the quality of probe printing improving the reproducibility of the experiments. In addition to manufacturer improvements, there are experimental improvements that can be made to also aid the reliability of the microarray. In respect to extraction and amplification it is advisable to use the the same extraction kits, primers, protocols and if possible when carrying out amplification to use the same machine. This should therefore improve consistency of results.

Besides issues associated with the microarray, there are some experimental issues that need to be considered in the future. One of the biggest problems with use of the current microarray is the lack of ability to identify if an isolate is pathogenic or not. For example, in terms of *Fusarium oxysporum* there are one-hundred and twenty pathogenic *formae speciales*, and of these only two are pathogenic to tomatoes (Armstrong & Armstrong, 1981). Often the difference between pathogenic isolates and non-pathogenic is small and would only be possible to identify by targeting pathogenicity factors. However, this is difficult as often this is not a singular target and would require separate assays to identify the presence of pathogenicity factors. On top of pathogenicity of organisms, another issue with the microarray is in regards to the microarrays inability to discriminate between viable and nonviable organisms. In this, mRNA may provide a clearer idea to viability due to RNA having a much shorter half-life to DNA and therefore will be more likely to detect viable organisms (Josephson *et al.*, 1993; Sheridan *et al.*, 1998). However, this also adds a complex step, involving reverse transcriptase, to a previously simple protocol.

On top of all these factors, the environment can still play the biggest role with external conditions able to affect the system in a beneficial or antagonistic manner. As well as affecting the system, the environment can also affect the rhizosphere population separately; this is a dynamic population on its own, with the ability to self-regulate itself.

Chapter five looked at the development of four qPCR assays for *C. coccodes, P. cucumerina, P. aphanidermatum* and *P. myriotylum*. The development and validation of the q-PCR assays was so that these may be used in conjunction with techniques such as the microarray. This should then circumvent some of the issues surrounding the quantification of the results.

For each target species, a TaqMan assay was developed with a separate fluorophore probe, so that the assay could potentially be used as a multiplex assay detecting different organisms at once. Unfortunately, the

fluorescence wavelengths overlapped too much, which if used together would lead to false positives.

During validation, each assay was tested using a combination of both pure cultures as well as positive and negative samples from nurseries. One of the key findings of the results was the apparent lack of sensitivity associated with the array. Whilst testing with the qPCR assays, many of the supposedly negative nursery samples exhibited late amplification, implying the presence of target sequences in very low quantities. However, despite the sensitivity issue, when the semi quantitative aspect of the array was tested using nursery samples of varying abundances it was found that the results from the array were relatively positive. Therefore, the array is clearly a useful tool for population observations, but the qPCR work further clarified its limitations in sensitivity and its need to be used in conjunction with other techniques.

From all the findings of chapter three, four and five many areas are highlighted for future research. One of the key issues is related to the use of the array and methods that can improve the characterisation of microbial populations. The qPCR work suggests that a more accurate and sensitive tool for quantification of organisms would be needed to truly observe the effects of various water treatments and test for total removal. It would also be beneficial to be able to truly quantify organisms and be able to compare organisms, rather than using the arrays semi-quantitative results, where the probes semi-quantitative nature is only relative on an individual species basis. As well as development of more assays for quantifying purposes, it would also be beneficial to develop a system to test a population's pathogenicity. In the research by Scott (2013), Loop mediated isothermal amplification (LAMP) assays were developed for some selected rhizosphere associated pathogens, one of which was *Fusarium oxysporum.* For this assay the primers were designed to be race specific to identify isolates pathogenic to tomato by targeting the pathogenicity genes. Unfortunately this assay was not fully tested and validated for use; however, methods like this should be considered for the

identification of pathogenic isolates in samples. LAMP is a single tube technique for DNA amplification which, unlike PCR, is carried out at a constant temperature throughout of 60-65 °C. The technique is advantageous as a screening method as it is relatively inexpensive, rugged, and a much faster and simpler method of molecular identification than regular PCR, which in conjunction with the microarray may be useful for identifying several key pathogens.

In terms of the population observations over the course of the season there were several areas raised that may require further research. From both the research on roots and water samples, it seemed that the pSSF outperformed the fSSF on both occasions. With the pSSF being relatively effective, whilst the fSSF was the least effective, the mode of action that causes the pSSF's efficacy would be important to understand. As well as this, the fSSF hosted the largest proportion of unique organisms throughout. It is important to know whether there is an aspect of the fSSF that encourages the proliferation of a wide range of organisms or if it was just a result of the fSSF being exposed to more organisms by chance.

For the heat treating site, it was not possible for the majority of the season to characterise the efficacy of the treatment, due to it not working. From the one post treatment sample received, it seems very effective and so it would be interesting to observe a full season to monitor its effect throughout. As well as this, the results from both the heat and UV nursery were puzzling, with sporadic introduction of new organisms through the season. In the RTW system the majority of organism introductions were within the first quarter of the season. It is important for both the RTW and the physical treatments to be able to characterise the mode of introduction of organisms, whether the RTW systems results were related to the roots from the propagators, or if the introductions in the heat and UV nurseries were caused by organisms in the water supply. Once the mode of introduction is known it will be much easier to develop a preventative protocol to minimise pathogen introduction than to have to use a treatment to eradicate the organisms.

Preventative measures are important in the context of the modern nursery; with the current reduction in treatment methods such as herbicides and pesticides and the high cost of crop losses, prevention is key. Installing water treatment facilities is effective, but at present is still outperformed by open hydroponic systems as demonstrated in the RTW systems tested in this research. However, for many an open system is not an affordable option, as well as coming with wider issues such as environmental factors. Introduction of organisms to a closed system is an unavoidable inevitability; however, if more importance was placed upon the prevention of as many organisms as possible being introduced, the existing treatment systems may be sufficient enough as they are.

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Appendices

Appendix 1: List of organisms detectable by the microarray developed by Devine, 2012.

Pathogenic Fungi/ Oomycetes
Alternaria solani
Armillaria mellea
Colletotrichum acutatum
Colletotrichum coccodes
Didymella lycopersici
Fusarium oxysporum
Fusarium redolens
Fusarium solani
Phytophthora arecae
Phytophthora cinnamomi
Phytophthora cryptogea
Phytophthora nicotianae
Pythium aphanidermatum
Pythium arrhenomanes
Pythium debaryanum
Pythium diclinum
Pythium echinulatum
Pythium irregular *
Pythium megalacanthum
Pythium myriotylum
Pythium oligandrum
Pythium paroecandrum
Pythium torulosum
Plectosphaerella cucumerina
Pyrenochaeta lycopersici
Rhizoctonia solani
Spongospora subterranea *
Thielaviopsis basicola
Verticillium albo-atrum
Verticillium dahliae
Verticillium nigrescens
Saprophytic Fungi/ Oomycetes
Alternaria spp.
Aspergillus flavus
Aspergillus sydowii
Aspergillus terreus
Aspergillus ustus
Cadophora spp.
Cephalosporium spp.
Chaetomium cochliodes
Chaetomium spp.

Cladophora spp.
Doratomyces microsporus
Exophiala pisciphila
Exophiala xenobiotica
Gigaspora rosea
Gliocladium roseum
Glomus intraradices
Myrothecium roridum
Olpidium brassicae
Paecilomyces lilacinus
Penicillium brevicompactum
Penicillium chrysogenum
Penicillium griseofulvum
Penicillium variabile
Penicillium spp.
Petriella asymmetrica
Phoma spp.
Rhizopus oryzae
Trichoderma viride
Trichoderma harzianum
Trichoderma spp.
Bacteria
Agrobacterium rhizogenes
Agrobacterium tumefaciens
Bacillus amyloliquefaciens
Bacillus subtilis
Clavibacter michiganensis *
Erwinia spp.
Escherichia coli
Nitrospira spp.
Pseudomonas syringae
Pseudomonas universal *
Ralstonia solanacearum
Rhizobium spp
Xanthomonas spp.
Yersinia spp.
Nematodes
Meloidogyne incognita
Pratylenchus goodeyi
Pratylenchus spp.
Universal Probes
Bacteria universal
Fungal universal
Nematode universal
Oomycete universal

*= Probe may present potential false positives as a result of known cross hybridisation.