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Linking Microbiology and Performance of Slow Sand Filters for Wastewater Treatment

A thesis submitted in partial fulfilment of the requirements for the degree of Doctor of Philosophy

by

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2018

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Firstly I would like to thank my supervisor Dr. Gavin Collins for offering me the opportunity and for providing me with his guidance, insight and patience throughout my PhD. I would also like to thank my co-supervisor Dr. Eoghan Clifford for his assistance and expertise in facilitating my work in his laboratory.

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Abstract

Slow Sand Filtration (SSF) is biologically mediated method of water treatment dependent upon the natural development of a complex microbial community within the filter-bed. Due to increasing water scarcity worldwide, the need for wastewater re-use is growing and the suitability of this environmentally friendly, adaptable, cheap and easy method for wastewater treatment needs to finally be determined. The aim of this thesis was to take advantage of the rapid progress of molecular microbial ecology techniques which now allow the characterisation such a complex community and to distinguish the microbes responsible for the biological contaminant removal. This knowledge would provide the platform to improve this centuries-old method of water treatment to meet the needs of the modern world. Through a highly replicated laboratory-scale study, the treatment performance of two SSF configurations, the Traditional SSF and the Manz SSF, were examined to determine the capacity for the secondary treatment of municipal wastewater. The microbial ecology of the filter-bed was determined through direct microscopy, Q-PCR, T-RFLP and MiSeq amplicon sequencing. Finally, sand from the surface layer, or schmutzdecke, was used to prepare microcosms for a targeted DNA-SIP analysis of the mechanisms of $^{13}$C-labelled *E. coli* removal.

Both configurations proved excellent at bacterial removal (>98%) as well as achieving varying levels of nutrient removal, all of which occurred predominantly within the sand surface, or schmutzdecke. Crucially, backwashing of the Manz SSF proved more effective at maintaining flow and produced a more biologically active schmutzdecke. The development of an indigenous microbial community within the filter-bed was associated with the increase in treatment performance. Backwashing of the Manz SSF was shown to prevent the excessive accumulation of larger metazoans such as nematodes and annelids in the schmutzdecke. DNA-SIP analysis demonstrated that the suppression of the dominant annelid *Aeolosoma hemprichi* population in the backwashed Manz SSF allowed other predatory eukaryotes to achieve significant $^{13}$C incorporation. Categorization of the microbial community based upon their association with the influent showed the potential to distinguish the indigenous microbes that were beneficial for overall SSF performance whilst the more direct, targeted approach demonstrated the effect that configuration had on the microbes involved in specific contaminant removal.
Scientific Communications

Oral Presentations

   “Slow sand filters in the 21st century”.
2. International Society of Protistology and European Congress of Protistology:  
   ISOP-ECOP Joint Conference. Seville, Spain. 2015.  
   “Understanding the Eukaryotic Microbial Community of Slow Sand Filters  
   and the Implications for Bacterial Pathogen Removal from Wastewater”.

Poster Presentations

   “A fresh look at Slow Sand Filters: Understanding and exploiting biofilm  
   mediated water treatment mechanisms”
   “Understanding and exploiting the microbial ecology of Slow Sand Filters”
   “Understanding the protozoan community of Slow Sand Filters and the  
   implications for bacterial pathogen removal from wastewater”
   “Understanding the protozoan community of Slow Sand Filters and the  
   implications for bacterial pathogen removal from wastewater”
5. FEMS, Valencia, Spain. 2017  
   “Re-engineering Slow Sand Filtration affects the total eukaryotic community,  
   but a keystone functional food web is responsible for pathogen removal across  
   alternative configurations”.
## List of Abbreviations

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<tbody>
<tr>
<td>ANOSIM</td>
<td>Analysis of Similarities</td>
</tr>
<tr>
<td>BOD</td>
<td>Biological oxygen demand</td>
</tr>
<tr>
<td>cDNA</td>
<td>complementary-Deoxyribonucleic Acid</td>
</tr>
<tr>
<td>CFU</td>
<td>Colony Forming Units</td>
</tr>
<tr>
<td>COD</td>
<td>Chemical Oxygen Demand</td>
</tr>
<tr>
<td>CO₂</td>
<td>Carbon Dioxide</td>
</tr>
<tr>
<td>DGGE</td>
<td>Denaturing Gradient Gel Electrophoresis</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic Acid</td>
</tr>
<tr>
<td>DNA-SIP</td>
<td>Deoxyribonucleic Acid - Stable Isotope Probing</td>
</tr>
<tr>
<td><em>E. coli</em></td>
<td><em>Escherichia coli</em></td>
</tr>
<tr>
<td>EPA.</td>
<td>Environmental Protection Agency</td>
</tr>
<tr>
<td>EU</td>
<td>European Union</td>
</tr>
<tr>
<td>FISH</td>
<td>Fluorescent <em>In Situ</em> Hybridisation</td>
</tr>
<tr>
<td>HRT</td>
<td>Hydraulic Retention Time</td>
</tr>
<tr>
<td>IndVal</td>
<td>Indicator Value</td>
</tr>
<tr>
<td>MAR-FISH</td>
<td>MicroAutoRadiography with Fluorescent in-situ Hybridization</td>
</tr>
<tr>
<td>MPN</td>
<td>Most Probable Number</td>
</tr>
<tr>
<td>mRNA</td>
<td>mitochondrial Ribonucleic Acid</td>
</tr>
<tr>
<td>NMDS</td>
<td>Non-Metric Multi-Dimensional Scaling</td>
</tr>
<tr>
<td>OTU</td>
<td>Operational Taxonomic Units</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase Chain Reaction</td>
</tr>
<tr>
<td>PERMANOVA</td>
<td>Permutational Multivariate Analysis of Variance</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>Q-PCR</td>
<td>Quantitative Polymerase Chain Reaction</td>
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<tr>
<td>RFLP</td>
<td>Restriction Fragment Length Polymorphism</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic Acid</td>
</tr>
<tr>
<td>rRNA</td>
<td>ribosomal Ribonucleic Acid</td>
</tr>
<tr>
<td>RT-QPCR</td>
<td>Reverse Transcriptase Quantitative Polymerase Chain Reaction</td>
</tr>
<tr>
<td>SEM</td>
<td>Scanning Electron Microscopy</td>
</tr>
<tr>
<td>SIP</td>
<td>Stable Isotope Probing</td>
</tr>
<tr>
<td>TRFLP</td>
<td>Terminal Restriction Fragment Length Polymorphism</td>
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<tr>
<td>TRFs</td>
<td>Terminal Restriction Fragments</td>
</tr>
<tr>
<td>TN</td>
<td>Total Nitrogen</td>
</tr>
<tr>
<td>NH$_4$-N</td>
<td>Ammonium Nitrogen</td>
</tr>
<tr>
<td>TON</td>
<td>Total Oxidised Nitrogen</td>
</tr>
<tr>
<td>TP</td>
<td>Total Phosphorus</td>
</tr>
<tr>
<td>PO$_4^{3-}$-P</td>
<td>Orthophosphate-Phosphorus</td>
</tr>
<tr>
<td>TOC</td>
<td>Total Organic Carbon</td>
</tr>
<tr>
<td>TIC</td>
<td>Total Inorganic Carbon</td>
</tr>
<tr>
<td>SO$_4$</td>
<td>Sulphate</td>
</tr>
<tr>
<td>SS</td>
<td>Suspended Solids</td>
</tr>
<tr>
<td>UV</td>
<td>Ultraviolet</td>
</tr>
<tr>
<td>WWTP</td>
<td>Waste-Water Treatment Plant</td>
</tr>
<tr>
<td>$^{13}$C</td>
<td>Carbon-13</td>
</tr>
<tr>
<td>$^{12}$C</td>
<td>Carbon-12</td>
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Chapter 1
1. General Introduction

Over four billion people experience temporary, but severe, water scarcity at some point each year (Mekonnen and Hoekstra, 2016). The reclamation of wastewater for irrigation, industry and even for the replenishment of drinking water reservoirs has increased rapidly in recent years and is predicted continue (W.H.O., 2006). Methods or reducing the quantity of potentially pathogenic bacteria of faecal origin from municipal wastewater destined for reuse is therefore of more relevant than ever (Sanders et al., 2013). The removal of excessive nitrogen, phosphorous, sulphur and other organic nutrients from wastewater is also a major issue, particularly in Ireland. The majority of Irish watersheds that have been designated as being of special concern (Water Framework Directive, 2000) have been so due to eutrophication, which is Ireland’s most serious environmental pollution problem in surface waters (Mockler et al., 2016). Tertiary technologies have progressed rapidly in recent years with the development of methods such as membrane filtration, ozone and U.V. irradiation which, though effective, are very expensive to operate and maintain. The choice of tertiary treatment is an important one and would depend on the specific contaminant removal required, safety requirements in the receiving water body, the environmental impact as well as socio-economic factors (Plakas et al., 2016).

One such choice for tertiary treatment is Slow Sand Filtration (SSF). Biological filters have been used for wastewater treatment throughout the world for hundreds of years (Haig et al., 2011). A greater awareness of the environmental impact of wastewater treatment combined with ever increasing efforts to reduce the amount of microorganisms and disinfection by-products has led to a renaissance in biological filtration over the last 20 years (Graham and Collins, 2014). SSF and other biological filters have demonstrated the capacity to remove organic and inorganic compounds, particulates and microorganisms from wastewater (Bomo et al., 2004b; Healy et al., 2010; Hijnen et al., 2007; Rodgers et al., 2005) despite an insufficient understanding of the biologically mediated methods employed.

To successfully optimize and adapt these bio-filtration technologies for modern use requires an understanding of exactly how the organisms in a community interact to produce this observed biological contaminant removal. Engineering advances can only proceed so far with a “Black Box” approach to the microbial ecology.
The tertiary treatment of a fluctuating, real-world municipal secondary treated effluent provides a considerable challenge. The filter biofilm community is subjected to a continuous yet equally inconsistent microbial community contained within the wastewater. The first step in understanding the ecology of such a complex system is to identify the stable core microbes that colonize and succeed despite such extreme exposure to outside competition.

In addition to an over-arching, understanding of the ecological factors affecting the colonization and succession of an indigenous SSF community, the desire to optimize this biotechnology towards a specific contaminant requires a more targeted functional approach. Modern techniques in functional microbial ecology such as Stable Isotope Probing (Radajewski et al., 2000) provide culture-independent means of identifying the keystone population within a complex microbial ecosystem that are actively involved the metabolisis of a specific substrate.

Discovering both the indigenous and the keystone microbes underpinning SSF wastewater treatment would hopefully provide the foundation for the improvement of this simple, cheap adaptable and environmentally friendly method of wastewater reclamation, just as the need for such technologies becomes increasingly relevant.

The thesis was organized into the following chapters:

**CHAPTER 2** reviewed the relevant literature regarding the relevant aspects of Slow Sand Filtration, the microbial ecology of biological wastewater treatment and the molecular microbial techniques available to study them.

**CHAPTER 3** presents the design, construction and operation of laboratory-scale SSF replicates from two separate configurations: The Traditional Slow Sand Filter and the Manz Slow Sand Filter. The detailed analysis of a suite of typical water quality parameters revealed the capacity of each SSF configuration for the removal of contaminants and thus the treatment of municipal wastewater. A comparison of the contaminant removal of each the SSF configurations allowed the direct comparison of the efficiency of these alternative configurations. The importance of filter depth was examined through wastewater analysis from a series of sampling ports over a gradient of depths to determine the optimal height for future designs.
CHAPTER 4 examined the effect of filter configuration and depth on the microbial community ecology of the filter-bed. The limited previous research into the microbial community of wastewater SSFs and in particular of the eukaryotic portion of these communities presented a substantial knowledge gap for such a commonly used wastewater treatment method. It also described the role of the influent community upon the development of an indigenous filter-bed community. The indigenous microbial community was shown to correlate strongly with treatment performance. This chapter also demonstrated the effect of back-washing in the Manz SSFs on the microbial community structure of the schmutzdecke.

CHAPTER 5 determined the microbes responsible for pathogenic *E. coli* removal in the schmutzdecke through Stable Isotope Probing and high-throughput amplicon-sequencing. Identification of the specific microbes responsible for the removal of individual contaminants is an essential step in the further development of this biotechnology. The role of back-washing in shaping community structure was again demonstrated, specifically the production of a more diverse community of active predators of bacteria.

CHAPTER 6 summarizes the conclusions from this thesis and gives recommendations for areas of future research.
Chapter 2
2. Reviewing our Understanding of the Microbial Foundations Underpinning Slow Sand Filtration

2.1. Introduction
A rapidly growing world population, facing a future of increasing global water scarcity, is placing a greater emphasis on wastewater treatment (Mekonnen and Hoekstra, 2016). Wastewater treatment protects the environment, human health and increases the reservoir of available freshwater.

Slow Sand Filtration is an energy-efficient, chemical-free, easy-to-use method capable of producing high quality wastewater treatment (Huisman and Wood, 1974). As a biologically mediated treatment technology, SSF relies upon the development of an indigenous microbial community upon sand of the filter-bed. Although widely acknowledged to responsible for the treatment capabilities of SSF, this indigenous microbial community has proven to be too dynamic and complex to be fully harnessed as a microbial biotechnology. The swift pace of development of molecular microbial ecology provides the means to finally characterise and explain such complex communities (Rittmann et al., 2006).

The aim of this chapter was to review the scientific knowledge of Slow Sand Filter technology, to define the current understanding of the role of microbes in biological filtration and to assess the methods of analyzing the microbial community.

2.2. Municipal Wastewater Treatment
In wastewater treatment plants (WWTP) municipal wastewater is collected in the sewer systems and supplied to the WWTPs for treatment prior to release into the environment. The raw wastewater undergoes primary, secondary and occasionally tertiary treatment. Primary treatment consists of separating out the least soluble constituents through a series of screening, settling and skimming steps. This primary treated effluent is then ready for further secondary treatment which consists of high rate biological processes designed to remove the suspended and dissolved organic matter. Secondary treatment usually consists of aerobic treatment methods such as activated sludge, biological trickling filters and rotating biological contactors. Such treatments are designed to harness the ability of microbial communities to metabolise the organic matter in the wastewater under aerobic conditions.
Tertiary treatment is not always necessary, but when applied is capable of targeting specific contaminants that could be harmful to the receiving waters. Tertiary treatments, often called “polishing” treatments, include: Nutrient removal - such as Nitrification/Denitrification and Phosphorous removal; Disinfection - such as U.V., Ozone and Chlorination; and Submerged Filters –consisting of a variety of media, flow directions and velocities, of which Slow Sand Filtration is one.

**2.3. Slow Sand Filtration**

Slow Sand Filtration is a method of water treatment that consists of a tank containing a bed of filter medium, a layer of gravel for support and an under-drain system (Fig. 2.1). Raw or pre-treated water is introduced from the top. A constant head of water, called the supernatant, is created above the filter bed. A system of control valves is used to control the rate of flow through the filter unit and maintain the required depth of supernatant, usually 1 to 1.5 meters. The pressure created by this head of water forces the water down through the filter medium. The filter medium is usually fine sand (0.2-2mm), and typically 1 to 2 meters deep. The supporting gravel and the under-drainage system allow the treated effluent water to be easily drained out the bottom.

![Figure 2.1: Slow sand filter design (Huisman and Wood, 1974)](image)

The flow rate through the system is typically slow, 0.1 – 1 m/h, in order to allow contaminant removal to occur through both mechanical (e.g. absorption, diffusion,
<table>
<thead>
<tr>
<th>Influent type</th>
<th>Flow Rate (m$^3$/m$^2$/h)</th>
<th>Supernatant height</th>
<th>Filter Medium Type</th>
<th>Filter Medium diameter (mm)</th>
<th>Filter Size</th>
<th>Reference</th>
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<tr>
<td>Pre-filtered lake water</td>
<td>0.1 m h$^{-1}$</td>
<td>n/a</td>
<td>Glass beads</td>
<td>0.17 mm</td>
<td>Lab scale</td>
<td>Weber-Shirk and Dick (1999)</td>
</tr>
<tr>
<td>Pre-treated river water</td>
<td>0.15</td>
<td>0.0 m</td>
<td>Sand</td>
<td>0.3 mm</td>
<td>Full scale</td>
<td>Campos et al.</td>
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<tr>
<td>Untreated reservoir water</td>
<td>0.15</td>
<td>0.0 m</td>
<td>Plasterer’s sand 0.3mm</td>
<td>0.3 mm</td>
<td>Lab scale</td>
<td>Calvo-Bado et al. (2003)</td>
</tr>
<tr>
<td>Domestic wastewater treatment 2ndary effluent</td>
<td>0.15, 0.2, 0.3 and 0.4 m/hr</td>
<td>n/a</td>
<td>Sand</td>
<td>0.3 mm and 0.5 mm</td>
<td>Pilot Scale</td>
<td>Sadiq et al. (2003)</td>
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<tr>
<td>Pre-treated lakewater</td>
<td>0.5 - 1.2 m/hr</td>
<td>n/a</td>
<td>Sand</td>
<td>0.2 - 8 mm;</td>
<td>Full scale</td>
<td>Mauclaire et al. (2006)</td>
</tr>
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<td>Synthetic effluent (acetic acid, NO3 &amp; trace elements)</td>
<td>0.015 - 0.06 m/hr 0.0m headspace</td>
<td>5 cm</td>
<td>Sand</td>
<td>0.5 mm</td>
<td>Lab scale</td>
<td>Aslan and Cakici (2007)</td>
</tr>
<tr>
<td>Raw river water</td>
<td>0.2 - 0.6 m/hr</td>
<td>3 – 5 cm</td>
<td>Sand</td>
<td>0.39 mm</td>
<td>Lab scale</td>
<td>Unger &amp; Collins (2008)</td>
</tr>
<tr>
<td>Pretreated &amp; sterilized. No chemical disinfectant.</td>
<td>0.3 m$^3$/hr$^{-1}$</td>
<td>n/a</td>
<td>Sand</td>
<td>0.6 mm</td>
<td>Lab scale</td>
<td>McDowall et al. (2009)</td>
</tr>
<tr>
<td>Domestic wastewater treatment 2ndary effluent</td>
<td>0.1 - 0.5 m/hr 0.6 m</td>
<td>Silica sand</td>
<td>1-2 mm</td>
<td>Pilot scale</td>
<td>0.9 m</td>
<td>Zheng et al. (2010)</td>
</tr>
<tr>
<td>Roughing filtered wetland water</td>
<td>Not given</td>
<td>0.9 m</td>
<td>River sand</td>
<td>0.2 mm</td>
<td>Lab scale</td>
<td>Wakelin et al. (2011)</td>
</tr>
<tr>
<td>Secondary treated municipal wastewater</td>
<td>0.05 m / hr</td>
<td>0.3 m</td>
<td>Building sand</td>
<td>0.25 mm , 0.4 mm and 0.63 mm</td>
<td>Lab scale</td>
<td>Pfannes et al. (2015)</td>
</tr>
</tbody>
</table>
screening and sedimentation) and biological (e.g. predation, natural death and metabolic breakdown) activity (Weber-Shirk and Dick, 1997). The biological mechanisms result from actions of microbes, initially derived from the raw water, that colonize the sand particles and that create the “schmutzdecke”. The term schmutzdecke is commonly used to describe the layer of mainly organic material that builds up on the upper surface of the sand which creates another, vital, filter medium through which the water must pass. The schmutzdecke is both home to and made up of microorganisms that shall be described in detail in a later section.

SSFs were amongst the earliest engineered water treatment (Campos et al., 2006b) and are still being used throughout the world today. Large-scale operation was first developed by John Gibb in 1800’s and first incorporated for municipal use in London in 1827 by James Simpson (Baker, 1948). Alterations to the design and operation to optimize flow rates, capacity issues, control of influent and ease of operation have been minimal and the quality of the biological purification remains unchanged (Huisman and Wood, 1974).

SSF was the first method of treatment used for municipal potable water production and this remains the most common use today (Ellis and Wood, 1985; Gadgil, 1998). SSF technology is also used for treatment of municipal (Ellis, 1987; Langenbach et al., 2009; Pfannes et al., 2015; Sadiq et al., 2003), industrial (Baker, 1948; Sánchez-Martín et al., 2010; Zahirn and Hilal, 2013), agricultural, horticulture (Calvo-Bado et al., 2003; Wohanka, 1993) and aquaculture effluent prior to recharge (Sommer, 1988).

The use of SSFs was reduced through competition from rapid sand filters and other high rate filtration techniques due to their lower productivity, susceptibility to turbidity, large capital costs due to high land requirement and a lack of quick and easy method of sludge removal (Ellis and Wood, 1985). Technological advances also lead to a wide range of modern, high-tech methods of Tertiary treatment methods such as membrane filtration, U.V and ozonation.

A resurgence in SSF as a viable treatment option occurred due to increasing energy costs, increased awareness of environmental issues and in particular the effectiveness of SSF against protozoan cysts (DeLoyde et al., 2006). The low-cost, low-energy, chemical free nature also proved desirable in developing countries as the
need for safe and reliable water treatment increased worldwide (Hunter et al., 2009).

The treatment of municipal secondary clarifier effluent by slow sand filtration has shown to provide reliable removal of pathogenic bacteria prior to water re-use (Langenbach et al., 2009; Pfannes et al., 2015; Sadiq et al., 2003; Seeger et al., 2016). However, the ability to maintain hydraulic conductivity during treatment is difficult due to high turbidity. A solution to this problem is the use of the Manz Slow Sand Filter (Manz Engineering Limited- Biosand Filter ©) which combines the biological mechanisms of a traditional flow sand filter design with the lower sand-bed and backwashing capabilities of a Rapid Sand Filter (Manz et al., 2010).

Figure 2.2: Graphical illustration of the Manz SSF with a schematic description of the backwashing process from the Oasis filter International brochure (www.manzwaterinfo.ca).
The Manz SSF reports to provide all of the advantages while eliminating many of the disadvantages of SSF as a method of wastewater treatment through a fluidized bed backwashing mechanism for sludge removal.

2.4. Microbial Ecology

Microbial ecology involves determining which species are present, what kind of metabolic reactions are occurring a microbial community and how those species interact with each other and their environment (Rittmann et al., 2006). This environment, or ecosystem, consists of the microbial community and its interacting biotic factors - macro-organisms such as plants and animals; and abiotic factors - environmental factors such as pH, temperature, inorganic and organic nutrients (Xu, 2006b). The microbial community can range from low complexity, like acid mine drainage biofilms (Tyson et al., 2004), to incredibly high complexity such as marine (Venter et al., 2004) and soil (Torsvik et al., 1990). The complexity of the interactions depends on species numbers and the population structure, variation in nutrient supply and the geography of the habitat (Raes and Bork, 2008).

Microbiology has played a role in monitoring SSFs performance since 1885 when water supplies in London were first examined for the presence of Bacteria (Huisman and Wood, 1974). The idea that drinking water was responsible for the spread of disease was first suggested by John Snow in his essay On the Mode of Communication of Cholera in 1849 and the observation that SSF treatment prevented the spread of cholera in 1892 was a prominent early demonstration (Huisman and Wood, 1974). Since these early discoveries, there has been constant work to measure the pathogens that remain in SSF treated water. However, due to a large amount of different potential pathogens, a system of indicator organisms were generally used to measure pathogens in treated water (Harwood et al., 2005). This relied on the assumption that reduction of the indicator organism, typically coliform Bacteria, to an acceptable level corresponded to a similar reduction in all other pathogens. Harwood et al. (2005) found that a combination of multiple indicator organisms could replicate actual pathogen reduction through a treatment process.

The microbiology of the wastewater is also considered when examining the biological oxygen demand (BOD) which is a measure of the oxygen required for microorganisms to respire and grow upon the organic compounds contained in the
It is accepted that SSFs remove pollutants from the raw water by a mixture of physicochemical and biological actions (Weber-Shirk and Dick, 1997). Whilst civil engineers have been studying and improving the design and capabilities empirically from a physicochemical standpoint from the beginning, only recently have microbial ecologists had the means find out the very basic question of community composition (Tiedje et al., 1999). As SSFs were already performing their function of providing potable water before the existence of microbes was even discovered, the prevailing paradigm of the biological process of SSFs has been to treat it as a “BLACK BOX”, where all the elements entering and exiting the system are investigated in order to calculate its capacities without determining precisely how it was happening. As a result of this paradigm both the development and further understanding of SSFs have been restricted.

Identifying the specific microorganisms involved in contaminant removal is an extremely difficult prospect within a complex community such as a SSF due to the variety of species that may be involved and the variable environmental conditions in which they operate. Also, the nucleic acid based techniques required to identify the whole community were not available until very recently. Most of what is known about microbial diversity and microbial activity in natural microbial communities such as this was due to culture-based experiments performed in the laboratory. While such studies are still essential, recent developments have shown that much of what had been previously discovered about environmental microbiology was actually very biased (Amann et al., 1998; Amann et al., 1995; Tiedje et al., 1999; Torsvik et al., 1990). Calculating the bacterial concentrations, for example, rested on the most probable number (MPN) theory, where total bacteria concentrations were derived from the number of cultivable bacteria from serial dilutions. Given that up to 99.5% of the microorganisms present are uncultivable (Rappe and Giovannoni, 2003) such calculations could not accurately describe the natural population.

To optimize the biological filtration activity of SSFs or to tailor SSFs towards specific applications, a deeper understanding of the specific microorganisms involved in filter performance and the interactions between the members of the microbial community
is required. If this can be achieved it may be possible to design filter configuration with a desired species in mind or bio-augment existing filters with chosen communities. To achieve these aims the microbial ecology of wastewater SSFs must be unravelling to determine the main colonizers of the SSFs that form the backbone of a dynamic community and separate the desirable microbes from those that impede performance.

2.5. Microbial Ecology in Wastewater Treatment

Wastewater treatment is both the most common and the highest volume type of microbial biotechnology in the world today and yet it is the least understood ecologically (Wheatley, 1984). Despite being commonplace throughout the developed world, along with substantial costs and strict regulations, municipal waste-water treatment plants (WWTPs) still rely on the development of naturally forming microbial communities (Curtis and Sloan, 2006). WWTPs harness the capacity of these microbial communities to metabolise pollutants just as they would in natural ecosystems but they are engineered to achieve higher microbial density and activity whilst operating under severe conditions.

Microbially mediated secondary and tertiary treatment processes in municipal WWTPs include activated sludge, aerobic and anaerobic granular digestion, rotating biological contacters, trickling filters, constructed wetlands and Slow Sand Filters. These processes developed long before the advent of modern molecular techniques that now allow the characterization of the microbial communities. Whilst WWTPs have been effectively monitored and managed through trial and error and more recently using classical techniques such as microscopy and culturing, there are obvious limitations to these approaches. Microscopy is time consuming and unsuitable for distinguishing bacteria, whilst culturing is only relevant for the small proportion of microbes that are cultivable.

2.6. Indigenous Microbial Ecology of Biological Filtration

In the last 20 years, microbial ecology has benefited from the huge developments in genome-based methods of analysing microbial communities and the ecology of the wastewater SSFs has just begun to be unravelled with ever-increasing accuracy, through PCR (Calvo-Bado et al., 2003), Fatty Acid Chromatography Analysis (Mauclaire et al., 2006), High-Density Micro Array (Wakelin et al., 2011) TRFLP and
cloning (Pfannes et al., 2015). The species generally accepted to be involved in the biological purification include algae, protozoa, bacteria, archaea, viruses, diatoms, rotifers and plankton (Huisman and Wood, 1974). To determine which microorganisms are actively involved in biological purification, those microbes that are able to colonize and succeed, develop into an indigenous population, distinct from the continual competition from the influent wastewater microbial community, must first be identified. The ability to identify and track genomes capable of contaminant removal would allow us to delve deeper into the functional aspects of the biological filtration mechanisms than ever before.

2.6.1. Algae

Algal growth occurs in both the supernatant water and on the schmutzdecke (Brook, 1954; Campos et al., 2006a). Algae are typically autotrophic and thus require sunlight for photosynthesis. The filtration ability of the schmutzdecke is aided by the formation of a sludge-mat, containing algae and bacteria, on the surface of the sand water interface (Campos et al., 2006a). Algae use organic matter from the untreated water for growth within the SSFs (Huisman and Wood, 1974). At night algae remove oxygen from the schmutzdecke through anaerobic metabolism which may create conditions suitable for anaerobic decomposition. This can cause an unpleasant effect on drinking water. Coccoid, single cell, benthic-algae are often found within the filter bed due to their small size and propensity for life in sub-surface sediments (Brook, 1954; El-Taweel and Ali, 2000; Wakelin et al., 2011) where they are a potential food source for protozoa and metazoa and have been found in the guts of insects and worms (Brook, 1954).

2.6.2. Protozoa

Single-cell eukaryotes, commonly known as protozoa, are a major cause of bacterial mortality in many environments (Matz and Jürgens, 2005; Matz and Kjelleberg, 2005b; Pernthaler, 2005; Sherr and Sherr, 1994) and have been demonstrated to be involved in eliminating bacterial pathogens in drinking water SSF (Haig et al., 2015b; Weber-Shirk and Dick, 1997; Weber-Shirk and Dick, 1999). Weber-Shirk and Dick (1999) demonstrated that augmentation of a SSF with an unspecified chrysophyte cultured from lake water caused an increase in bacterial removal. Haig et al. (2015b)
added $^{13}$C-labelled *E. coli* to a SSF to determine that the choanoflagellate *Monosiga brevicollis* and ciliate *Tetrahymena* spp. were directly involved in the majority of bacterial grazing. Ciliates and flagellated phagotrophic cercozoan species have been identified in clone libraries created from waste-water and storm-water SSF communities and have been suggested as potential protozoan grazers of bacteria (Pfannes et al., 2015; Wakelin et al., 2011). Protozoa are not just bacterial grazers however; they are also algivores, parasites of metazoa, predators of smaller protozoa, symbionts, autotrophs as well as mixotrophic combinations of each of these possibilities.

**2.6.3. Metazoa**

Macro-invertebrates such as annelid and nematode worms, as well as insect larvae and micro-crustaceans are commonly found in the rich environment of the SSF schmutzdecke (Hurley and Wotton, 2006; Jellison et al., 2000; Mauclaire et al., 2006). They are thought to be generally confined to the upper 20cm and assist in maintaining hydraulic conductivity by their movement through the schmutzdecke (Castaldelli et al., 2005; Mauclaire et al., 2006). Wastewater SSF should produce a higher nutrient environment with greater clogging which should provide a more suitable environment for metazoans than drinking water SSFs.

**2.6.4. Bacteria**

The bacterial community of SSFs for wastewater or re-use has been examined by a number of researchers using different approaches. In a study of a SSF used for horticultural irrigation water (Calvo-Bado et al., 2003) the bacterial community was analysed by DGGE and dominant bands at different depths were sequenced and identified, namely *Bacillus infernus*, *Desulfovibrio*, *Porphyrobacter tepidarius*, *Blastomonas natatoria*, and *Lutibacterium anuloederans*.

A study by Mauclaire et al. (2006) into the effects of clogging on the microbial community in SSFs, through the Fatty Acids analysis and Scanning Electron Microscopy (SEM) showed gram-negative bacteria dominated the SSF community. Filter clogging led to significant changes in prokaryotic abundance and diversity,
specifically an increase in the detection of fatty acid biomarkers for anaerobic bacteria suggesting development of anaerobic niches.

Petry-Hansen et al. (2006) undertook a study involving PCR, Cloning and sequencing, FISH and RFLP to characterize the SSF microbial ecology. This study identified *Proteobacteria* as the dominant bacterial phyla, along with the *Cytophaga-Flavobacterium-Bacteriodes* group, *Nitrosospira*, *Planctomycetes*, Bacteria Division TM6 and *Chloroflexus*.

Renault et al. (2012) used clone libraries to identify the bacterial community in slow filters for recirculation of nutrient-rich, hydroponic plant feed. They compared the natural community that developed to the community from filters that were amended with particular strains. In the naturally developed community the best-represented phyla were *Proteobacteria* (40% of sequences) and *Firmicutes* (21%), while other significant phyla were *Planctomycetes* (10%), *Acidobacteria* (7%), and *Chloroflexi* (7%).

Wakelin et al. (2011) used a Phylochip-Microarray when examining the bacterial composition of the Schmutzdecke of a SSF treating stormwater run-off and identified 21 bacterial Phyla, 43 classes, 85 orders and 148 families. The dominant phyla were identified as *Proteobacteria* (43%), *Firmicutes* (24%), and *Acinobacteria* (16%). Pfannes et al. (2015) found that in SSF for tertiary treatment of municipal waste, that the *Bacteriodetes* were confined to the schmutzdecke whilst members of the *Alphaproteobacteria*, *Betaproteobacteria* and *Planctomycetes* were abundant in the schmutzdecke and the sand depths below. Despite all these studies, there has been no conclusive link between bacterial species and contaminant removal in wastewater SSFs.

2.6.5. Archaea

Whilst most cultivated Archaea are extremophiles such as methanogens, ammonia-oxidizers, halophiles and sulphur-metabolizers, an abundance of uncultivated Archaea have been found in marine, freshwater, soils, sediments and symbionts living within metazoans (DeLong, 1998). Bates et al. (2011) found that Archaea made up an average of 2% of total 16S rRNA gene sequences over a large range of different soil types.
Wakelin et al. (2011) found, through cloning and sequencing of 31 archaeal 16S rRNA gene clones, that the archaeal community of a SSF schmutzdecke was dominated by *Euryarchaeota*, and *Halobacteriales* in particular as well as sequences associated with *Methanomicrobiales*, *Methanosaeta* and *Crenarchaeota*. They subsequently hypothesized that Archaea were active in removal of dissolved organic C from the influent water.

Archaeal abundance has been found to correlate with C:N ratios in soil, with the proportion of Archaea to Bacteria decreasing with increased levels of nitrogen (Bates et al., 2011). This is thought to be due to Bacteria outcompeting Archaea due to their faster growth rate. With regards to SSF performance this would suggest Archaea would only be relevant ammonia oxidizers in low nitrogen conditions.

### 2.6.6. Viruses

The viral composition of the SSF microflora has not as of yet been thoroughly studied. Haig et al. (2015b) determined the role of the viral community in *E. coli* removal in drinking water SSFs. They found that viral lysis accounted for 0.14% of the total *E. coli* removal during the experiment and identified enterobacteria phages as playing an important role.

### 2.7. Techniques in Microbial Ecology

Microbial Ecology is a rapidly progressing field with continuous advancements in molecular techniques which allow culture-independent analysis of microbial communities. This section of the review will first deal with some basic techniques that have been used previously to study SSFs, and then move on to introduce some more recent techniques that have a potential to provide new insights.

#### 2.7.1. DNA extraction

Microbial Ecology has, in the last 25 years, become increasingly reliant upon environmental DNA extraction to illuminate the darkness surrounding the uncultivable majority of microorganisms. Extraction of DNA allows the investigator a much deeper insight into the community structure of a given sample than culture dependant methods (Torsvik et al., 1990).
Direct cell-lysis has become the main method for extracting the DNA from environmental samples. It involves splitting the cells whilst still attached to the sand/soil/sediment, either physically or chemically, then extracting the DNA using phenol or chloroform (Robe et al., 2003). Mechanical bead-beating has been shown to recover more diversity than chemical treatments (Niemi et al., 2001), though it causes higher fragmentation of the DNA. Recently, commercial kits have been developed that optimize yield and reproducibility, with the added benefits of speed and simplicity (Whitehouse and Hottel, 2007).

There are many imperfections regarding DNA extraction. Underestimation can occur due to inefficient lysis, whilst overestimation of viable cells can occur due to the persistence of DNA in the environment after cell death (Nocker et al., 2006).

2.7.2. PCR

The Polymerase Chain Reaction (PCR), which involves amplification of specified DNA sequences, has become an essential part of microbiology. PCR has been crucial in the understanding of microbial diversity through the ability to amplify the 16s ribosomal-RNA gene from environmental samples. Pace et al. (1985) introduced this cultivation-independent approach based on the extraction, amplification, cloning, and characterization of ribosomal-RNA genes directly from natural environments. The very first application of PCR in phylogenetic analysis of mixed microbial communities in ocean waters led to the discovery of ubiquitous and abundant groups of new micro-organisms (Giovannoni et al., 1990).

PCR allows for detection of target sequences and thus gives definitive answers regarding the presence or absence of the sequence of interest in the sample, but that is all it can definitively conclude as the proportions of amplified product do not necessarily reflect the proportions of genes in environmental DNA samples (Suzuki and Giovannoni, 1996).

The 16S ribosomal-RNA gene is highly conserved and present in all prokaryotes (Woese et al., 1990) and its use has facilitated the discovery of 13,000 new prokaryotes. Phylogenetic techniques are used to characterize the 16S ribosomal-RNA gene profile of a community based on databases of previously characterized organisms. Many techniques, including DGGE, RFLP, and T-RFLP, which shall be
expanded upon later, which require initial PCR amplification of a certain DNA sequence are hampered by their reliance on this uncertainty surrounding this representivity. An alternative target for PCR amplification include functional genes for cellular processes of interest, but smaller databases with fewer representative sequences available for such genes create even greater uncertainty.

Another drawback of PCR based approaches is the dependence on primers designed from already known species, producing an inherent bias against the discovery of novel organisms. Although random shotgun sequencing techniques now provide a viable alternative, PCR based approaches still have an important role to play in specifically targeting taxonomic or functional markers.

2.7.3. Quantitative-PCR

Real time or Quantitative-PCR (Q-PCR) is a more powerful iteration of the PCR technique. It not only amplifies the targeted DNA sequence, but also allows the calculation of the starting quantity of that sequence in the original sample through the measurement of fluorescence in the reaction after each cycle by a real time PCR machine capable of detecting the fluorescence. This fluorescence is created by either a double-stranded-DNA binding dye such as the SYBRgreen assay (Wittwer et al., 1997), or a fluorescent probe such as the Taqman probe system (Gelfand et al., 1996). Quantification is achieved by measuring the increase in fluorescence caused by amplification of double-stranded-DNA or by when taq polymerase cleaves the fluorescent label from the probe releasing it from the quencher.

The additional specificity created by the probe ensures nonspecific products do not affect the quantification as can happen with the double-stranded-DNA binding dye approach. Also multiple probes with different fluorescent signals can be used in a single multiplex reaction (Baldwin et al., 2003). The probe based approach is more expensive and requires an extra conserved site that may not always be present within the short target sequences required for efficient Q-PCR. Particularly if targeting divergent gene sequences. Whichever approach is chosen, Q-PCR offers a high degree of sensitivity with detection of as low as 2 copies of a gene from a sample (Fey et al., 2004).
Renault et al. (2012) has used Q-PCR to measure overall bacterial abundance at different filter depths in SSFs. This gives accurate quantification of total bacterial population, but the real power of this technique lies in the ability to quantify different subsets of the total population, either through targeting specific variations of the 16S rRNA gene or targeting specific functional genes. In order to understand how microbial communities function the diversity of species and genes in environmental samples and also the abundance and distribution of these genes in varying environmental conditions needs to be determined.

Q-PCR is ideally suited for experiments that investigate the effect that environmental conditions have on microbial populations and subsequently on the biologically driven environmental processes that these microbes are involved in. Biogeochemical cycling and bioremediation are examples of areas in which quantification of key microbial genes in situ is of major importance.

2.7.4. RT-QPCR

Reverse transcriptase creates DNA from a RNA template and the combination of this of Q-PCR allows quantification of mRNA and thus active gene expression of the community. RNA is more difficult to work with then DNA as it is a labile molecule with a potentially short half life (Grunberg-Manago, 1999) making mRNA extraction from environmental samples difficult. Also, sample dilution to prevent inhibition has been known to adversely affect reverse transcriptase (Chandler et al., 1998) making accurate quantification more difficult. Despite these hurdles RT-QPCR is preferable to Q-PCR as it can provide measurement of actual gene expression, through the mRNA transcripts of functional genes, not just potential activity as measured by functional gene Q-PCR.

2.7.5. T-RFLP

Terminal restriction fragment length polymorphism is a commonly-used fingerprinting technique in microbial ecology. As the name suggests, it allows the automated and accurate measurement of the size-polymorphisms of fragments of PCR product that have been created by restriction enzyme digestion. These fragment size polymorphisms, allow the creation of a community profile that can be compared against other profiles or used to provide estimates for diversity (Marsh, 1999).
The microbial community of sand filters have been frequently analysed with T-RFLP (Calvo-Bado et al., 2003; Pfannes et al., 2015; Ramond et al., 2013; Scholz et al., 2001) to reveal changes in diversity and dominance. However composition of the microbial community can not be identified with certainty, but can be estimated through further cloning or bioinformatics.

2.7.6. Cloning

Cloning is a common method of identifying bacteria and archaea from environmental samples, cloning involves amplification of fragments of a certain gene by PCR. These amplified fragments are then ligated onto plasmids and inserted into *E. coli*. When the *E. coli* are grown on agar plates each colony contains *E. coli* with replicates of an individual gene fragment from the environmental sample. This subset of individual gene fragments isolated with each *E. coli* colony can be used as a representation of the total population. Sequencing the isolated gene fragments can then allows us to identify the proportions of different microbes within this subset, which in turn is being used as an estimate of the total community.

A large variety of cloning systems are available to accommodate different types and sizes of DNA fragments. Vectors with large insert capacities are ideal for studying genome organizations of uncultivable microorganisms in the environment and metagenomics has benefited hugely from such cloning systems (Xu, 2006a). Wakelin et al. (2011) used cloning and sequencing to analyse 96 eukaryotic clones and 48 archaeal clones from a SSF treating storm-water.

2.7.7. Micro-arrays

Micro-arrays are a powerful, high throughput technology that allows the concurrent analysis of thousands of different genes. First used for whole genome gene expressions, they have recently been adapted for microbial community analysis (Zhou, 2003). Micro-arrays have demonstrated the ability characterize microbial communities in natural habitats (Wu et al., 2004). Wakelin et al. (2011) used PhyloChip, a high-density microarray to successfully characterize the bacterial community structure of a SSF at the phylum level. Recently, however, researchers have moved away from micro-arrays due to competition from next generation sequencing platforms.
2.7.8. MAR-FISH

MicroAutoRadiography combined with Fluorescent in-situ Hybridization (MAR-FISH) is a culture independent method of microbial identification with simultaneous monitoring of their activity and substrate uptake ability (Lee et al., 1999; Nielsen et al., 1999). It involves microscopic visualization of the uptake of radio-labelled substrates by communities of microbes that have been targeted in situ by fluorescent oligonucleotide probes. This ability to definitively prove if a specific group of microorganisms consume a certain substrate within their ecological niches has become an indispensable tool in microbial ecology. Nielsen and Nielsen (2002) identified functional groups of bacteria involved in activated sludge treatment of wastewater. However MAR-FISH is not possible in all environmental conditions, is limited by the number of fluorescent probes that can be used and is unable to quantify substrate uptake. MAR-FISH still has an important role to play in biofilm analysis when used in combination with metagenomic data analysis or SIP experiments for the testing or checking of theories and results.

2.7.9. Stable Isotope Probing

Stable Isotope Probing (SIP) is a popular method of identifying the active, functioning microorganisms in environmental samples. The addition of stable isotope labelled substrates allows us distinguish the microorganisms that metabolize and incorporate this substrate into their DNA from the rest of the community, providing a direct link between identity and function. The stable isotope labelled DNA is then separated from the rest of the total population DNA by ultracentrifugation and individuals from this sub-population can then be identified by various means FISH, Microarray, Cloning, qPCR or metagenomics (Neufeld et al., 2007a; Neufeld et al., 2007b; Whiteley et al., 2006; Whiteley et al., 2007). The separation of this sub-population makes it easier to identify less abundant species, which can be a problem with metagenomics. DNA-SIP allows the use of conditions similar to those experienced environmentally, preventing a reduction in diversity caused by artificially selecting for cultivatable microbes.

Substrate concentrations and timescales must be carefully calculated in order create an effective experiment using DNA-SIP. Relevant organisms could be inhibited by high substrate concentrations or inactivated if it is too low. Ideally substrate levels
must be kept as close as possible to in situ levels but this leads to low levels of the labelled substrate incorporation into DNA (Neufeld et al., 2007a).

2.7.10. Raman

Raman Microscopy is a vibrational, spectroscopic method that uses a laser-illumination to shift the energy of photons. This shift in energy gives information about the molecular composition of the single bacterial cell. In microbial ecology this technique is used to examine the molecular composition of in situ microbial communities (Wagner, 2009). By detecting differences in the spectral profile Raman allows the distinction between bacterial taxa, between species, between individuals of the same species, and between growth phases. It is of particular relevance in combination with stable isotope probing or FISH to measure the activity and uptake of substrates by microbial cells in complex microbial communities.

2.7.11. Genomics and ‘omics

Genomics was originally used to describe mapping, sequencing and analyzing the total genome of an organism. The term became more popular when a new journal called “Genomics” began in 1986. Since then the suffix –omics has caught on in the life sciences, used to describe large scale, high throughput, holistic, data driven, integrated system approach to a study. In Microbial Ecology this refers to the study of the genomes of all species or organisms in a sample. There are four fields of particular relevance: Metagenomics; Metatranscriptomics; Metaproteomics; and Metabolomics. The prefix Meta- in microbial ecology refers to the combined data produced by a mixed community.

2.7.11.1. Metagenomics

First coined by Handelsman et al. (1998) the term “Metagenomics” now describes the functional and sequence-based analysis of the total microbial genome contained in an environmental sample. It typically involves the use of high throughput genomic techniques for the study of communities of microbes directly in their natural environments, without isolation and laboratory cultivation. It refers to the idea of treating the study of the genomes of a whole community as if you were studying a single genome, a holistic approach that sees the total microbial community as one functioning ecosystem.
The rapid progress in automated sequencing combined with an increasing market has resulted in a massive decrease in the cost of sequencing. Due to revolutionary improvements in high-throughput DNA sequencing technologies, close to 30,000 microbial genomes from 50 phyla have been fully sequenced, and many more are nearing completion (Land et al., 2015).

Metagenomics is such a vast and rapidly growing field that all approaches cannot be fully reviewed in this thesis. In general there are two overarching approaches in modern microbial ecology: environmental single gene approach; and random shotgun approach. The single gene approach or amplicon sequencing does not technically constitute metagenomics. It is again reliant upon PCR amplification and the associated, inherent biases (Sanschagrin and Yergeau, 2014). The low cost and less extensive data analysis in comparison to shotgun sequencing has meant the vast majority of microbiome studies has adopted this approach (Ranjan et al., 2016). 16S rRNA gene amplicon sequencing has been used to characterize the bacterial community previously in drinking water SSFs (Haig et al., 2015a; Nitzsche et al., 2015; Pinto et al., 2012; White et al., 2012).

Metagenomics, on the other hand, is based on the shot-gun approach whereby random fragments of genome are sequenced without the use of primers. The many advantages of this approach include the lack of PCR bias, the fact that other genes can be predicted and monitored, increased detection of diversity, and more accurately identification at the species level (Ranjan et al., 2016). Haig et al. (2015b) demonstrated the potential of shotgun metagenomics within slow sand filters. The shotgun approach was used in combination with DNA-SIP to identify the complex multi kingdom foodweb responsible for the fate of *E. coli* biomass within slow sand filters.

In addition the field has branched out to create the fields of transcriptomics, proteomics and metabolomics.

### 2.7.11.2. Transcriptomics

Transcriptomics, or Meta-transcriptomics in microbial ecology, involves the sequencing of total mRNA expressed by a target community. Like shotgun metagenomics it avoids the constraints of PCR. While metagenomics provides a detailed list of the members of the community, meta-transcriptomics provides details
25
of the actual gene expression taking place in situ (van Elsas and Boersma, 2011). In particular comparative metatranscriptomics has huge potential for the understanding of microbial response to environmental changes, which microbes respond and which genes they use to make that response (Moran et al., 2013).

2.7.11.3. Metaproteomics
Metaproteomics is the study of all the proteins collectively expressed by an ecosystem at a certain moment. It is a potentially more insightful tool for describing the activity of microbial communities then metatranscriptomics, in that mRNA expression does not always lead to protein expression (Gygi et al., 1999) whilst each protein represents the end product of gene expression.

Metaproteomics involves 7 steps namely sample collection, recovery of the targeted fraction, protein extraction, separation, mass Spec. analysis, database search, and data analysis, and there are inherent difficulties in every one of these steps (Siggins et al., 2012). In particular the development of cheaper automated methods as seen in metagenomics as well as larger databases are required. These bottlenecks are preventing proteomics from taking its place at the centre of the rapidly moving field of microbial ecology. Still, metapromteomics has already been used to identify new functional proteins and keystone species for wastewater treatment (Narayanasamy et al., 2015; Püttker et al., 2015; Salerno et al., 2016; Wilmes and Bond, 2006).

2.7.11.4. Metabolomics
As mentioned, the gene, transcript and protein profile can estimate the potential metabolic activity within a microbial community but still lacks definitive data on the effect of this potential upon the actual rates at which the metabolic activities are occurring. Metabolomics is the measurement of multiple naturally-occurring, low molecular weight metabolites that result from cellular processes, through mass spectrometry or NMR spectroscopy (Bundy et al., 2008). Metabolic analysis has many advantages because metabolites are the functional entities used and produced by microbes and thus variations of their concentrations show the functionality of the microbes.

Metabolomics is the final step required for the integrated “omics” approach. The advances being made within the other “omics” fields are rapid and increase the need for metabolomic confirmation. The combined data from all these fields can provide a
complete picture of the metabolic pathways within a microbiome, such as nutrient and energy cycling. This approach is referred to as “Systems Microbiology”. The aim of which is to allow predictive modelling of microbial ecosystems.

Advances in robotics are paving the way for high-throughput metabolomics to facilitate this systems approach, but this process is still at the beginning. The vast range of possible metabolites makes it almost impossible to identify them and better databases and computational solutions are needed for the interpretation and integration of large scale meta-metabolomic data sets.

2.8. Scope of Thesis

To date slow sand filtration is one of the few microbial biotechnologies remaining in which the organisms responsible are still poorly understood and have yet to be exploited. For SSF to be a competitive method of wastewater treatment in the future this age-old biotechnology needs to be advanced and to do this it must first be better understood

This thesis aimed to address the following research questions:

- What is the treatment capacity of Traditional and Manz SSF configurations for tertiary treatment of municipal wastewater?
- What is the effect of configuration, design and engineering on treatment performance?
- Is the schmutzdecke as important in wastewater treatment as it is drinking water treatment or does the eutrophic wastewater environment lead to a stratified, active microbial community within the filter-bed below?
- What is the pattern of eukaryotic colonization - particularly the protozoa - of the filter bed?
- Does the influent-wastewater microbial community determine the development of the filter-bed community, and does an indigenous microbial community develop within the SSF?
- What is the effect of configuration, design and engineering on the microbial community structure and function?
- Does the microbial community affect treatment performance?
Can the microbes responsible for the removal of particular contaminants be identified? Does configuration affect these functional, contaminant removing microbes?

To achieve these aims a highly replicated laboratory scale trial was chosen to study the capacity of both configurations to provide tertiary treatment for real-world municipal wastewater. A wide range of typical wastewater parameters were analysed from a number of depths within the filter bed to determine exactly what contaminants SSFs were capable of treating and the exact location at which it occurred. The microbial community was then characterized over four different phases of filter development with equal attention given to the both the bacterial and eukaryotic communities. Finally, to focus on the mechanisms of removal of a single contaminant within the fluctuating, eutrophic environment, a targeted and controlled experiment was designed to understand the effect of the filter-bed microbial community on the removal of a contaminant that is relevant for public health or environmental protection.
2.9. References


3. Comparison of Slow Sand Filter Configurations  
   Demonstrates how Backwashing Reduces Clogging and  
   Increases the Performance of the Schmutzdecke.

Abstract

The performance of two laboratory-scale Slow Sand Filter (SSF) configurations, the  
Traditional SSF and the Manz SSF, was studied over 188 days to determine the  
factors affecting the tertiary treatment of secondary treated municipal waste.  
Configuration did not show a significant effect on over-all removal rates. 99.8%  
removal of coliform bacteria and E. coli, 95% removal of Suspended Solids and  
Turbidity was demonstrated in filters with 90% of total removal occurring in the  
surface layer of sand. Treatment of the typical chemical properties of the waste-water  
was less effective with removal of 75% COD; 30% organic carbon; 80% ammonium;  
and 10% oxidized nitrogen. Removal of all contaminants was higher in the  
schmutzdecke than throughout the rest of the filter combined. Manz SSF was less  
efficient in the beginning of the trial but achieved parity in the final stages. Depth  
resolved contaminant concentrations showed that the schmutzdecke of the Manz SSF  
also underperformed during the juvenile and ripening phases of filter development  
but eventually outperformed that of the Traditional SSF. Clogging of the filters  
increased over time, the mature Traditional SSF required daily unclogging and the  
Manz SSF required unclogging every 2 days. This increased frequency of draining  
and de-clogging in the Traditional SSF correlated with lower performance of the  
schmutzdecke relative to that of the Manz SSF. However the greater depth of the  
Traditional SSF meant that overall treatment efficiency was not lost. The decreased  
clogging frequency, the simplicity of de-clogging by backwashing, combined with no  
loss of treatment efficiency makes the Manz Slow Sand Filter was more suitable for  
tertiary wastewater treatment.
3.1. Introduction

Tertiary wastewater treatment has gained relevance worldwide due to a greater demand for freshwater sources (Mekonnen and Hoekstra, 2016; W.H.O., 2006). Typically implemented to remove microbial pathogens (Henze, 2008), tertiary wastewater treatment is also needed for the removal of chemicals from wastewater as treatment plant operators seek to meet ever stricter regulations. Eutrophication of many aquatic environments due to excessive nitrogen, phosphorous, sulphur and other organic nutrients has lead to the designation of watersheds of special concern (Water Framework Directive, 2000) with severe licensing criteria for wastewater release.

Tertiary wastewater treatment technologies have progressed rapidly in recent years with the development of methods such as membrane filtration, ozone and U.V. irradiation, which even though they have proven to be highly effective, are very expensive to operate and maintain (Sonune and Ghate, 2004) and the choice of tertiary treatment method would depend on many factors such as time, money, wastewater volume, discharge limits and wastewater strength.

Biological filters have been used for water treatment throughout the world for hundreds of years (Haig et al., 2011), and a greater awareness of the environmental impact of wastewater treatment combined with ever increasing efforts to reduce the amount of microorganisms and disinfection by-products has led to their renaissance in the last 20 years (Graham and Collins, 2014). SSFs and other biological filters have demonstrated the capacity to remove organic and inorganic compounds, particulates and microorganisms from waste-water (Bomo et al., 2004b; Healy et al., 2010; Hijnen et al., 2007; Rodgers et al., 2005) and pre-existing infrastructure is often already in place. Continuing to use, re-commissioning or re-engineering these pre-existing SSF facilities would be an extremely cost effective method of maintaining pace with the increasingly strict contaminant discharge limits.

The vast majority of studies on SSF have focused on its use as a method of drinking water production (Aslan and Cakici, 2007; Elliott et al., 2008; Haig et al., 2014, 2015a; Hijnen et al., 2004; Hijnen et al., 2007; Jellison et al., 2000; Mauclaire et al., 2006; Weber-Shirk and Dick, 1997) and the removal of microbial pathogens, such as *E. coli*, coliforms, *Aeromonas spp*, bacteriophage, poliovirus, gardia and
cryptosporidium oocysts, was identified as its main strength as a method of water treatment. Pathogen removal mechanisms were shown to be reliant upon biological activity as opposed to simply physical straining (Weber-Shirk and Dick, 1997). The schmutzdecke, or sand-water interface was determined to be the location of significant removal and Haig et al. (2015b) determined that protozoan grazing was responsible for over 99.5% of the observed removal a specific bacterial pathogen.

There have also been studies of SSF demonstrating similarly high rates of pathogen removal from waste-water (Pfannes et al., 2015; Sadiq et al., 2003; Seeger et al., 2016) as well from horticultural, aquacultural and agricultural effluent waters destined for re-use (Bomo et al., 2004a; Pfannes et al., 2015; Stevik et al., 2004). Whilst protozoan role in pathogen removal from SSF for production of potable water it is unlikely that the same organisms are responsible for pathogen removal in wastewater treatment environment due to the protozoan composition of water and wastewater being so highly dependent on the concentrations of organic compounds (Foissner, 1996).

Slow sand filtration is also capable of the treating important chemical waste-water parameters. Under various experimental conditions SSF of waste-water has demonstrated the reduction in the concentrations of such chemical parameters as: 85-100% removal of Suspended Solids; 25-35% removal of Organic Carbon; 50-70% removal of COD; Simultaneous Nitrification and denitrification (Aslan and Cakici, 2007; Bauer et al., 2011; Campos et al., 2006a; Ellis, 1987; Ellis and Aydin, 1995; Farooq et al., 1994; Grace et al., 2016; Langenbach et al., 2009; Nakhla and Farooq, 2003; Pfannes et al., 2015; Sadiq et al., 2003; Seeger et al., 2016; Timms et al., 1995; Tyagi et al., 2009a; Tyagi et al., 2009b; Wohanka, 1993; Zheng et al., 2010). However, if the removal of a certain chemical is required there is likely to be a specific designed system developed that would be more efficient than SSF.

As with many methods of tertiary wastewater treatment, SSFs are susceptible to clogging when treating turbid waters. Clogging reduces flow and eventually stops treatment altogether. In order for SSF to be used for tertiary wastewater treatment the wastewater need to be sufficiently pre-treated and the filter needs to be regularly de-clogged. In a Traditional SSF this de-clogging is performed by draining the supernatant and scraping or furrowing the schmutzdecke to remove excess sludge.
build-up. The Manz Slow Sand Filter (Manz, 2000) is a shallow SSF with specific grades of sand and a much smaller supernatant that allows de-clogging by means of backwashing. The biologically active schmutzdecke layer is maintained by eliminating the loss of schmutzdecke sand and biofilm to scraping or furrowing. The potential surface area for biofilm formation in this layer is increased by the use of a fine grade of sand (0.1 mm diameter) to increase biological activity. Backwashing of the Manz SSF provides an easy, potentially automated method of maintaining hydraulic conductivity which is ideally suited for tertiary waste-water treatment.

![Diagram of TSSF and MSSF with schmutzdecke layer highlighted](image)

**Figure 3.1:** Comparison of the heights of the Traditional SSF (TSSF) and the Manz SSF (MSSF). The schmutzdecke layer of the Manz SSF with fine sand (0.1 mm) and biologically active biofilm is highlighted in yellow.

The scope of this study was to compare the ability of two different SSF designs, the Manz SSF and the Traditional SSF, to provide efficient tertiary treatment of municipal wastewater.
The aims of this experiment were:

1. To compare the Traditional SSF configuration with the new Manz SSF configuration to find out exactly how design and engineering affects treatment.
2. To determine which aspects of configuration design had an impact on contaminant removal.
3. To test whether backwashing of SSFs was suitable for tertiary wastewater treatment.
4. To discover whether successive nutrient depletion would occur along a gradient of depth in the SSF beds and, if so, to understand how this affected contaminant removal.

The following hypotheses were tested:

- **Hypothesis 3.1**: The Manz SSF would outperform the Traditional SSF in terms of tertiary waste-water treatment efficiency.
- **Hypothesis 3.2**: The removal of bacteria would correspond to increased abundance of protozoa within the filter bed and in the effluent.
- **Hypothesis 3.3**: The eutrophic wastewater would lead to a stratified environment below the schmutzdecke with gradient of nutrient depletion.
- **Hypothesis 3.4**: The Manz SSF schmutzdecke would achieve higher performance than the traditional SSF schmutzdecke.
- **Hypothesis 3.5**: The deeper Traditional SSF-bed would support greater nutrient depletion below the schmutzdecke.

Based on these aims and hypotheses, the specific research questions for this experiment were:

- Which SSF configuration was the most suitable for tertiary treatment of waste-water?
  - Did schmutzdecke of the Manz SSF offer improved performance?
  - Did the greater depth of the Traditional SSF provide increased removal?
• Which contaminants were SSFs capable of treating from fluctuating real-world waste-water?
  o At which depths were each of these contaminants removed?
  o Did a gradient of nutrient depletion develop with depth in the filter bed?
• Did protozoa concentrations positively correlate with bacterial removal?
  o How did configuration affect protozoan concentrations?
• What factors of SSF design improved treatment performance?
3.2. **Materials and Methods**

3.2.1. Source of secondary treated wastewater

Raw water for treatment by the laboratory scale filters was collected weekly from Tuam Waste-water treatment facility, located 30 miles north of the National University of Ireland, Galway (Fig. 3.2).

![Figure 3.2](image.png)

**Figure 3.2:** The town of Tuam, Co. Galway, location of the wastewater treatment facility where the secondary treated wastewater was sourced.
The municipal wastewater treated at this facility includes household, industrial and storm-water drain effluent. The wastewater at this facility (Fig. 3.3) underwent primary settlement; followed by biological treatment by an activated sludge system, and finally a rotating trickling filter prior to discharge (a SSF was out of commission due to mechanical issues). Sterilized 25 litre plastic drums were filled with water from the final discharge point of the WWTP, covered from the light and brought to the university grounds within 1 hour of collection, where it was stored in a 200 litre container, covered from the light, continuously stirred at 200rpm and maintained at 11°C.

![Figure 3.3: Arial view of the Tuam municipal WWTP where the secondary treated wastewater used for the laboratory-scale SSF experiment was sourced.](image)

3.2.2. Design, construction and operation of laboratory-scale SSFs

Columns have been shown to produce realistic representations of full-scale slow sand filters (Haig et al., 2014). The experimental set-up comprised of 12 laboratory-scale SSF columns. Six Traditional SSF columns (labelled T1- T6) and six Manz SSF columns (labelled M1- M6) were set up in a temperature controlled room (11°C +/- 1°C). Both configurations were made from clear acrylic tubes of 1-cm-thickness and with a 4 cm internal diameter. The filter-bed area was covered from light but the supernatant water was uncovered.
The **Traditional SSFs** were two meters high and were designed for a 95 cm sand bed below an 80 cm head of supernatant water which was controlled by an outflow weir. Sampling ports, comprised of three-way Luer-lock stopcocks (EW-45502-80, Cole Palmer), were located 2.5 cm, 22.5 cm, 42.5 cm and 62.5 cm below the surface of the sand (Fig. 3.4). The sand bed contained 85 cm of sand (0.3 mm diameter) above a 10-cm-deep layer of gravel (0.5 - 2 cm diameter).

The **Manz SSFs** were one meter high and were designed for 58.5 cm of sand below a 10 cm head of supernatant water which was controlled by an outflow weir. Sampling ports comprised of three-way luer-lock stopcocks (EW-45502-80, Cole Palmer) were located 2.5 cm, 12.5 cm, 22.5 cm and 32.5 cm below the surface of the sand. The bottom of the sand bed contained a 10-cm-deep layer of gravel (0.5-2 cm diameter). Above this were a 25 cm layer of 0.3 mm diameter sand, then a 25 cm layer of 0.4 mm diameter sand, another 25 cm layer of 0.3 mm diameter sand and finally a 20 cm layer of 0.15 mm diameter sand.

**Figure 3.4:** Graphical representation of the experimental set-up of the laboratory-scale SSF trial.
All sand and gravel was acquired from Irwin’s quarry (Endenderry, Antrim, Northern Ireland, UK) and was sieved for confirmation of size, washed, sterilized by autoclave and dried. The SSF columns were filled with water prior to adding of sand to prevent air-pockets forming within the filter bed.

Peristaltic pumps were used to continuously feed all replicas of both filter configurations at the same hydraulic loading rate of 0.1 m³/m²/hr⁻¹. Hydraulic retention time was calculated by means of a Bromide tracer experiment. Sodium bromide was added to tap water and used as influent on the sterile sand Hydraulic retention time for the Traditional SSF was 9.4 hours and the Manz filter was 4.6 hours. Sludge removal in the Traditional SSF was achieved by draining the filter and scraping the sludge off the surface of the filter. Sludge removal in the Manz SSF was achieved by backwashing.

**Figure 3.5:** Images depicting **A)** the laboratory-scale SSF columns setting of the experiment complete with reservoir, peristaltic pump and reactor rig, and **B)** a close-up of the sampling ports on SSF columns during construction, in various stages of completion.
3.2.3. Sampling procedure

Three filters from each configuration were designated for sampling and the remaining three were designated as non-sampled in order to determine the effect of sampling on the filters. Liquid was collected from influent and effluent weekly (500 ml) from all twelve filters and twice monthly from the intermediate sampling ports (2 ml) of the six filters designated for sampling.

Short circuiting of the flow through the sampling ports occurred for one week at the end of the trial in order to achieve enough water to test SS, Dissolved Oxygen, pH, TOC, T.N. and T.P. This period of short circuiting was also used as an opportunity to confirm validity of low sample volumes for determining microbial concentrations.

3.2.4. Water quality analyses

3.2.4.1. Chemical Oxygen Demand (COD)
COD was measured using the Low range HACH TNT test vials (Item no: TNT821). Sample (2 mls) were added to the tubes and heated to 100°C for 2 hours in the presence of potassium dichromate causing oxidizable organic compounds to reduce dichromate ion to a green chromic ion. The amount of yellow Cr$^{6+}$ ions remaining was measured with the HACH DR/2000 spectrophotometer (Hach Company, Loveland, Colorado) at 420 nm. Results were expressed in mg/l of COD.

3.2.4.2. Total Organic Carbon (TOC) and Total Inorganic Carbon (TIC)
TOC from samples was determined with BioTector Analytical Systems Limited B7000 On-Line Analyser. 40 ml glass vials containing the samples were placed in an auto-sampler from which they were then pumped into the analyser. The sample injection valve automatically selected the appropriate sample volume for the chosen measuring range. In order to generate a TOC measurement, acid was added to lower the pH so that inorganic carbon was sparged off as CO$_2$ and measured as Total Inorganic Carbon (TIC). Hydroxyl radicals, generated by ozone and sodium hydroxide, oxidize the sample. CO$_2$ was removed from the oxidized sample, by lowering the pH again. The CO$_2$ was sparged and measured by a non-dispersive infrared CO$_2$ analyser and the result was displayed as Total Organic Carbon (TOC).
3.2.4.3. Total Nitrogen (TN)

TN from samples was determined with BioTector Analytical Systems Limited B7000 On-Line Analyser. Glass vials (40 ml) containing the samples were placed in an autosampler from which they were then pumped into the analyser. The sample injection valve automatically selected the appropriate sample volume for the chosen measuring range. After TOC analysis, performed as described previously, the oxidized sample liquid was transferred to a measuring cell. Wavelengths analysis for nitrates was measured by a photometer and the result was displayed as Total Nitrogen (TN).

3.2.4.4. Ammonium Nitrogen (NH₄-N)

NH₄-N was measured using a Thermo Clinical Labsystems, Konelab 20 Nutrient Analyser (Fisher Scientific, Waltham, Massachusetts). Ammonia was determined by spectrophotometrically measuring at 660 nm the absorbance of a blue complex formed when a sample was reacted a reagent containing sulphanilamide and N-(1-naphthyl)-ethylenediamine dihydrochloride. Results were displayed as mg NH₄-N/l.

3.2.4.5. Total Oxidised Nitrogen (TON)

NO₃-N was measured using a Thermo Clinical Labsystems, Konelab 20 Nutrient Analyser (Fisher Scientific, Waltham, Massachusetts). Nitrate ions were determined by spectrophotometrically measuring at 540 nm the absorbance of a azo coloured dye formed after the reduction of Nitrate to Nitrate using hydrazine sulphate followed by diazoitasisation with sulphanilamide and coupling with N- (1-naphthyl)-ethylenediamine dihydrochloride. Results were displayed as mg TON/l.

3.2.4.6. Total Phosphorus (TP)

TP from samples was determined with BioTector Analytical Systems Limited B7000 On-Line Analyser. Glass vials (40 ml) containing the samples were placed in an autosampler from which they were then pumped into the analyser. The sample injection valve automatically selected the appropriate sample volume for the chosen measuring range. After TOC analysis as discussed previously, the oxidized sample liquid was transferred to a TP boiler for acid boiling at 100ºC to break down the polyphosphate bonds into orthophosphates. The sample was reacted with TP reagent and transferred into the measuring cell where wavelength analysis for phosphates
was measured by a photometer and the result was displayed as Total Phosphorus (TP).

3.2.4.7. Orthophosphate-phosphorus (PO$_4^{3-}$-P)
PO$_4^{3-}$ -P was measured using a Thermo Clinical Labsystems, Konelab 20 Nutrient Analyser (Fisher Scientific, Waltham, Massachusetts). Orthophosphate was determined by spectrophotometrically measuring at 880 nm the absorbance of the complex formed when a sample was reacted with molybdate and antimony potassium tartrate under acidic conditions. Results were displayed as mg PO$_4$-P/L.

3.2.4.8. Sulphate (SO$_4$)
SO$_4$ was measured using a Thermo Clinical Labsystems, Konelab 20 Nutrient Analyser (Fisher Scientific, Waltham, Massachusetts). Turbidity was determined by spectrophotometrically measuring at 405 nm the absorbance of the complex formed when a sample was reacted in a strongly acid medium with Barium Chloride. Results were displayed as mg SO$_4$/L.

3.2.4.9. Total Alkalinity
Total Alkalinity was measured using a Thermo Clinical Labsystems, Konelab 20 Nutrient Analyser (Fisher Scientific, Waltham, Massachusetts). Alkalinity was determined by spectrophotometrically measuring at 600nm the absorbance of the complex formed when a sample was reacted with bromophenol blue in a pH 3.5 phthalate buffer. Results were displayed as mg CO$_3$/L.

3.2.4.10. pH
pH was measured using a WTW SenTix 21 pH electrode connected to a WTW pH 320 meter. The probe was inserted into samples as soon as they were taken from the sheets. The probe was calibrated before each use.

3.2.4.11. Suspended Solids (SS)
The non-filterable residue of the sample was measured in accordance with the Standard Methods for the Examination of Water and Wastewater (APHA, 2005), method 2540-D.

3.2.4.12. Turbidity
Turbidity was measured with the Hach HACH DR/2000 spectrophotometer (Hach Company, Loveland, Colorado), measured at 420 nm.
3.2.4.13. *E. coli* and total coliform bacteria

Colilert trays were used to measure total coliform bacteria and *E. coli*. Colilert analysis uses Defined Substrate Technology in combination with QuantiTray/QuantiTray2000 (IDEXX Laboratories, USA) for a Most Probable Number (MPN) multi-well procedure. Sample volume was dependant on treatment stage and dilutions, if any, were performed in PBS buffer. Incubation was for 24 h at 35 ± 0.5°C. The presence of coliform bacteria was determined by the colour change from yellow to magenta. The presence of *E. coli* was determined by the colour change from yellow to magenta combined with fluorescence when exposed to a 6-watt, 365 nm, UV light within 5 inches of the sample, in a dark environment.

3.2.4.14. Protozoa

The protozoa were enumerated using light microscopy and a haemocytometer. Water samples were fixed with formalin solution, loaded onto the haemocytometer counting chamber and visualized with a light microscope at 100X magnification following the method of Dehority et al., (1984). Results were expressed in protozoa cells/100 ml⁻¹.

3.2.5. Statistical analysis

Experimental results were compared using paired t-tests to determine significant differences before and after treatment, between depths, between configurations and between sampled and un-sampled replicas. Data from replicated experiments were determined to be statistical replicates and pooled after comparison with analysis of variance. Confidence intervals of 95% and significance levels of 0.05 were used.

3.2.6. Performance metrics

Due to the quantity of wastewater parameters studied, the development of the filter treatment ability over time and the fluctuating nature of the real-world municipal wastewater being treated, two practical multi-parameter metrics were developed. These metrics combined the many results obtained from a range of parameters and provided an easily understood measurement of the effectiveness of the SSF treatment based upon national and EU regulatory standards.
3.2.6.1. Water Quality Failure Metric

To determine the practical effect of the tertiary treatment of wastewater by each of
the SSF configurations the aggregated “Water Quality Failure Metric” was developed.
This multi-parameter metric was based on either the most stringent discharge limits
for Irish municipal WWTPs or the most stringent Irish bathing water quality
waste-water parameter analysed in this experiment was assigned an individual cut-
off point based on these most stringent national licensing limits (Table 3.1).

Table 3.1: Maximum water quality values used to determine pass or fail result for each
parameter which combined to give the “Water Quality Failure Metric” score.

<table>
<thead>
<tr>
<th>Wastewater parameter</th>
<th>Emission limit value</th>
<th>Corresponding regulatory source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Suspended Solids</td>
<td>10 mg / l</td>
<td>E.P.A. discharge licence, Swords WWTP, Fingal county council, 2012</td>
</tr>
<tr>
<td>Turbidity</td>
<td>10 mg / l</td>
<td>E.P.A. discharge licence, Swords WWTP, Fingal county council, 2012</td>
</tr>
<tr>
<td>COD</td>
<td>80 mg / l</td>
<td>E.P.A. discharge licence, Swords WWTP, Fingal county council, 2012</td>
</tr>
<tr>
<td>TOC</td>
<td>7.5 mg / l</td>
<td>E.P.A. discharge licence, Swords WWTP, Fingal county council, 2012</td>
</tr>
<tr>
<td>NH4 – N</td>
<td>0.2 mg / l</td>
<td>E.P.A. discharge licence, Ballyjamesduff WWTP, Wexford county council, 2008</td>
</tr>
<tr>
<td>T.O.N</td>
<td>7.5 mg / l</td>
<td>E.P.A. discharge licence, Coill Dubh WWTP, Kildare county council, 2012</td>
</tr>
<tr>
<td>T.N.</td>
<td>10 mg / l</td>
<td>E.P.A. discharge licence, Swords WWTP, Fingal county council, 2012</td>
</tr>
<tr>
<td>T.P.</td>
<td>0.5 mg / l</td>
<td>E.P.A. discharge licence, Drumshambo WWTP, Leitrim county council, 2011</td>
</tr>
<tr>
<td>Phosphate</td>
<td>0.2 mg / l</td>
<td>E.P.A. discharge licence, Tuam WWTP, Galway county council, 2016</td>
</tr>
<tr>
<td>Total coliform bacteria</td>
<td>500 cfu / 100 ml</td>
<td>Irrigation water regulation, B.O.E., Spain, 2007</td>
</tr>
<tr>
<td>E. coli</td>
<td>100 cfu / 100 ml</td>
<td>Irrigation water regulation, B.O.E., Spain, 2007</td>
</tr>
</tbody>
</table>
The scoring was calculated as follows: If the concentration of a waste-water parameter failed to meet that assigned quality limit for the given parameter it received a score of one. If the concentration of a waste-water parameter passed the assigned quality limit for the given parameter it was given a score of zero. The score from each parameter was added together to give a total score for the influent, the Manz SSF effluent and the Traditional SSF effluent.

The “Water Quality Failure Metric” was an aggregate of the number of water quality failures from all of the tested parameters available on that sampling day. Unfortunately all parameters were not available on each sampling day so the Water Quality Failure Metric was only useful as a comparison with the influent as to how many water quality standards it meets.

### 3.2.6.2. Trial Performance Capacity Metric

Another new parameter, the “Trial Performance Capacity Metric”, was created with the performance of the filters specifically in mind rather than the more arbitrary water quality limits used for the “Water Quality Failure Metric”. Again, this consisted of the combination of multiple parameters into one easy-to-understand metric. This metric was used compare the performance of the SSF configurations over time.

**Table 3.2:** Values for greatest percentage removal achieved for each parameter during the whole trial, by either configuration.

<table>
<thead>
<tr>
<th>Wastewater parameter</th>
<th>Maximum removal (%) recorded during trial</th>
</tr>
</thead>
<tbody>
<tr>
<td>Suspended Solids</td>
<td>96.5%</td>
</tr>
<tr>
<td>Turbidity</td>
<td>95.1%</td>
</tr>
<tr>
<td>COD</td>
<td>89.4%</td>
</tr>
<tr>
<td>TOC</td>
<td>59.4%</td>
</tr>
<tr>
<td>NH4 - N</td>
<td>96.6%</td>
</tr>
<tr>
<td>T.O.N</td>
<td>28.5%</td>
</tr>
<tr>
<td>T.N.</td>
<td>43.5%</td>
</tr>
<tr>
<td>T.P.</td>
<td>79.4%</td>
</tr>
<tr>
<td>Phosphate</td>
<td>59.9%</td>
</tr>
<tr>
<td>Total coliform bacteria</td>
<td>99.9%</td>
</tr>
<tr>
<td><em>E. coli</em></td>
<td>99.97%</td>
</tr>
</tbody>
</table>
The Trial Performance Metric uses the highest removal (%) achieved during the trial by either filter configuration as the maximum demonstrated in-trial performance capacity. The percentage removal of each parameter was then measured against this maximum demonstrated in-trial performance capacity (Table 3.2) to yield a removal capacity percentage for each parameter. These removal capacity percentages were then averaged on each day, for each SSF configuration, to give an overall “Trial Performance Capacity Metric” value.

### 3.3. **Results**

#### 3.4.1. Treatment capacity of Manz and Traditional SSF configurations

The ability of the SSFs to provide tertiary municipal wastewater treatment was demonstrated by the decrease of the twelve measured contaminants after treatment by both SSF configuration types (Figs. 3.6 and 3.7). After an initial 40 day period, during which time the microbial colonization was expected to take place upon the sterile sand (Haig et al., 2014), a consistent decrease in contaminants in the treated SSF effluent was demonstrated as the filters became biologically active (Figs. 3.6 and 3.7).

Results from the whole trial were divided into four different phases: the juvenile phase; the ripening phase; the optimal phase; and the mature phase. These phases represented the four distinct phases of filter development which were based upon the filter clogging frequency (Table 3.3), the water quality failure metric (Fig. 3.8), and the trial performance capacity metric (Fig. 3.9). Each of the phases provided a sufficient subsample of results to allow statistical comparison between phases.

**Table 3.3:** Increase in the frequency of de-clogging required to maintain a constant flow rate of 0.1 m$^3$/m$^2$/hr$^{-1}$ during each of the four phases of filter development.

<table>
<thead>
<tr>
<th>De-clogging frequency (days)</th>
<th>Juvenile phase</th>
<th>Ripening phase</th>
<th>Optimal phase</th>
<th>Mature phase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Traditional SSF</td>
<td>7</td>
<td>4</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td>Manz SSF</td>
<td>7</td>
<td>5</td>
<td>4</td>
<td>2</td>
</tr>
</tbody>
</table>
The typical wastewater parameters tested at WWTPs were commonly divided into the physical waste-water properties, chemical waste-water properties and microbiological waste-water properties. The physical waste-water properties as measured through suspended solids and turbidity were reduced by an average of ~80% by the biologically active SSFs. Suspended solids were reduced equally between configurations but the Traditional SSF configuration achieved significantly greater turbidity reduction than the Manz SSF. Both SSF configurations achieved highest removal efficiency for the microbiological properties of the wastewater (Fig. 3.6: C and D). Total coliform bacteria and *E. coli* removal was over 99.5% during the optimal and mature phases with no significant difference (p<0.05) observed between configurations. The efficiency of SSF treatment for the chemical properties of the waste-water (Figs. 3.6 and 3.7) did not follow the same progression over time as the physical and microbiological properties. Some chemical properties were reduced consistently by SSF treatment: COD was reduced by ~70%, ammonium by ~50%, TOC by ~30%, TN by ~15% and TON by ~10%. Other chemical properties were not consistently removed with no statistically significant (p<0.05) reduction of sulphate, orthophosphate, total phosphorous, or alkalinity by SSF treatment.

**Table 3.4:** Removal efficiency (%) of the physical waste-water properties achieved by each filter configuration during each of the four phases

<table>
<thead>
<tr>
<th></th>
<th>Juvenile phase</th>
<th>Ripening phase</th>
<th>Optimal phase</th>
<th>Mature phase</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Days 1-40</td>
<td>Days 41-80</td>
<td>Days 81-120</td>
<td>Days 121-188</td>
</tr>
<tr>
<td><strong>(% removal)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Suspended Solids</td>
<td>Manz 50.8%</td>
<td>Manz 76.3%</td>
<td>Manz 75.9%</td>
<td>Manz 86.6%</td>
</tr>
<tr>
<td></td>
<td>Trad 60.0%</td>
<td>Trad 82.9%</td>
<td>Trad 81.2%</td>
<td>Trad 86.5%</td>
</tr>
<tr>
<td>Turbidity</td>
<td>Manz 33.3%</td>
<td>Manz 55.4%</td>
<td>Manz 75.0%</td>
<td>Manz 76.5%</td>
</tr>
<tr>
<td></td>
<td>Trad 37.8%</td>
<td>Trad 73.1%</td>
<td>Trad 78.1%</td>
<td>Trad 85.7%</td>
</tr>
</tbody>
</table>

The treatment of the physical properties of the wastewater by SSF as measured by the removal of solids and reduction in turbidity by the SSFs demonstrated an increase in performance over time (Table 3.4.). The sterilized sand of both SSF configurations was immediately capable of over 50% removal of suspended solids during the juvenile phase by physical straining and adhesion to sand particles without the development of a microbial community. In the early ripening period, as the necessity for de-clogging increased in frequency, removal increased significantly...
Figure 3.6: Influent, effluent and percentage removal of A) Suspended Solids, B) Turbidity, C) Coliform Bacteria, D) E. coli, E) Chemical Oxygen Demand and F) Total Organic Carbon of each SSF configuration over the 188 day trial period. (Influent n=3; Effluent n=6)
Figure 3.7: Average influent, effluent and percentage removal of A) Ammonium, B) Total Oxidized Nitrogen, C) Total Nitrogen, D) Sulphate, E) Total Phosphorous and F) Orthophosphate of each SSF configuration over the 188 day trial period. (Influent: n=2; Effluents: n=6)
within the traditional filter, achieving over 75% removal efficiency. Further, slight increases occurred in the ripe and mature periods. Filter configuration was shown to effect SS removal in the first three time periods but becomes irrelevant in the mature period. Removal of turbidity was consistently higher in the Traditional SSF, but not significantly. The most efficiency removal produced by both SSF configurations was of the microbiological properties of the waste-water. The sterile sand of the juvenile SSFs were capable of trapping suspended solids and as such flocs of bacteria formed during previous treatment stages would likely be strained without the development of any biological mechanisms. Microbial removal rates from these first 40 days were 34.5% and 31.2% removal of total coliform bacteria, 63.9% and 60.2% removal of \( E. \ coli \) and 48.9% and 58.6% removal of protozoa. The ripening phase brought a large increase in microorganism removal. The optimal phase produced highest protozoan removal but the total coliform bacteria and \( E. \ coli \) removal increased even higher in the mature phase with total coliform and \( E. \ coli \) removal above 99.5% while protozoan removal fell. The only significant difference in the microbial removal ability between configurations occurred during the ripening phase where the Traditional SSF produced significantly protozoan removal.

Table 3.5: Removal efficiency (%) of the microbiological waste-water properties by each filter configuration during each of the four phases

<table>
<thead>
<tr>
<th></th>
<th>Juvenile phase</th>
<th>Ripening phase</th>
<th>Optimal Phase</th>
<th>Mature phase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Days 1-40</td>
<td>Manz</td>
<td>Trad</td>
<td>Manz</td>
<td>Trad</td>
</tr>
<tr>
<td>Total coliforms</td>
<td>34.5%</td>
<td>31.2%</td>
<td>91.5%</td>
<td>94.0%</td>
</tr>
<tr>
<td>( E. \ coli )</td>
<td>63.9%</td>
<td>60.2%</td>
<td>89.0%</td>
<td>91.9%</td>
</tr>
<tr>
<td>Protozoa</td>
<td>48.9%</td>
<td>58.6%</td>
<td>62.4%*</td>
<td>78.5%*</td>
</tr>
</tbody>
</table>

* = significant difference between configurations

There was limited and extremely variable removal of the chemical properties of the waste-water produced by both SSF configurations (Fig. 3.6: E and F; and Fig. 3.7). The capacity of SSFs to remove ammonium and reduce COD effectively from low strength wastewater was demonstrated. There was insignificant removal of COD and TOC by either configuration during the juvenile phase. During the ripening phase,
while significant COD removal of over 40% took place in both configurations, only the Manz configuration produced significant TOC removal at just over 20%. The ripening phase was the only time that there was a significant difference between filter configurations with the Manz SSF community proving faster to develop TOC treatment capabilities.

The optimal phase produced the highest COD (>75%) and TOC (>33.3%) removal in both configurations during the period of highest influent concentrations. The mature phase, which was characterized by frequent clogging with resultant periods of reduced flow, resulted in the highest ammonium, TON and TN removal.

Table 3.6: Average removal efficiency (%) of the chemical waste-water properties by each filter configuration, plus influent and effluent pH and alkalinity during the four phases.

<table>
<thead>
<tr>
<th></th>
<th>Juvenile phase</th>
<th>Ripening phase</th>
<th>Optimal phase</th>
<th>Mature Phase</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Days 1-40</td>
<td>Days 41-80</td>
<td>Days 81-120</td>
<td>Days 121-188</td>
</tr>
<tr>
<td>% removal</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>COD</td>
<td>-10%</td>
<td>*40.4%</td>
<td>*77.1%</td>
<td>*67.8%</td>
</tr>
<tr>
<td>TOC</td>
<td>4.8%</td>
<td><em>21.0%</em></td>
<td>*34.5%</td>
<td>*31.4%</td>
</tr>
<tr>
<td>TON</td>
<td>-4%</td>
<td>-1.2%</td>
<td>8.2%</td>
<td>12.8%</td>
</tr>
<tr>
<td>Nh4</td>
<td>-7.2%</td>
<td>*14.3%</td>
<td>*46.4%</td>
<td>*83.2%</td>
</tr>
<tr>
<td>TN</td>
<td>4.1%</td>
<td>2.5%</td>
<td>*13.2%</td>
<td>*18.7%</td>
</tr>
<tr>
<td>S</td>
<td>2.6%</td>
<td>0.0%</td>
<td>0.4%</td>
<td>1.2%</td>
</tr>
<tr>
<td>TP</td>
<td>21.6%</td>
<td>9.9%</td>
<td>16.9%</td>
<td>13.6%</td>
</tr>
<tr>
<td>PO4 (-P)</td>
<td>28.7%</td>
<td>19.5%</td>
<td>22.5%</td>
<td>30.8%</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>Influent</th>
<th>Effluent</th>
</tr>
</thead>
<tbody>
<tr>
<td>ALK mg/l</td>
<td>91.1</td>
<td>140.4</td>
</tr>
<tr>
<td></td>
<td>171.5</td>
<td>150.2</td>
</tr>
<tr>
<td>pH</td>
<td>7.5</td>
<td>8.2</td>
</tr>
<tr>
<td></td>
<td>8.0</td>
<td>7.9</td>
</tr>
<tr>
<td></td>
<td>7.3</td>
<td>7.7</td>
</tr>
<tr>
<td></td>
<td>7.7</td>
<td>7.9</td>
</tr>
<tr>
<td></td>
<td>7.8</td>
<td>8.3</td>
</tr>
<tr>
<td></td>
<td>7.8</td>
<td>8.2</td>
</tr>
</tbody>
</table>

* = significant removal (p <0.5)
3.3.1. Examining the total treatment using multi-parameter metrics

The Water Quality Failure Metric illustrated the development time required for biological SSF filtration (Fig. 3.8), with 40 days passed before the SSFs developed the ability to provide treatment that would be valuable in a real-world WWTP. After that initial juvenile phase, SSF provided an increase in overall water quality of the wastewater as shown by a decrease in the number of water quality failures from an average of 5 failures per sampling time point to an average of 2 failures per sampling time point.

![Figure 3.8: Water Quality Failure Metric](image)

**Figure 3.8:** Water Quality Failure Metric: Measurement of the number of potential failures of water quality licence limits by the waste-water before and after SSF treatment over the course of the trial.

The Trial Performance Capacity Metric (Fig. 3.9) also illustrates the limited removal capacity of the SSFs during the first 40 days. This metric more clearly demonstrates the gradual improvement in overall SSF performance over time by both configurations. No significant difference (t-test; p<0.05) was found between configurations. The Traditional SSF has consistently higher removal capacity (%) than the Manz SSF, but the Manz SSF achieves parity from there on.
Figure 3.9: Trial Performance Capacity Metric: The average of the proportional removal of all parameters, each proportional removal was determined against the greatest removal rate achieved for that parameter during this trial.

3.4.2. Depth resolved performance

To gain a comprehensive understanding of the demonstrated waste-water treatment capacity, wastewater from different depths was examined to determine the location of greatest removal and the effect of filter bed depth on SSF treatment. Protozoa, ammonium and total oxidized nitrogen were monitored at each depth over all four phases; total coliform bacteria and *E. coli* were monitored at each depth over three phases; COD, TOC, TN, SS, pH and dissolved oxygen were monitored at each depth during the mature phase by recirculation of the flow through the sampling ports. The juvenile phase proved too unstable to determine differences in the removal rates of the total coliform and *E. coli* between depths but demonstrated significant (p<0.05) protozoa removal in the schmutzdecke (Fig. 3.10 A and E). Total coliform and *E. coli* were removed but the results were too inconsistent and turbulent to produce any significant differences between depths during this phase. As a result, sampling for total coliform and *E. coli* was temporarily paused during the next phase a precautionary measure to prevent any potential filter-bed disturbance due to sampling and to allow the filter community to develop naturally during the ripening phase. It was still possible to calculate protozoa concentrations in the interstitial water due to the low volume required. Results from the ripening phase show that protozoa concentrations decreased in the effluent despite an accumulation in the
Figure 3.10: Depth resolved quantification of *E. coli*, total coliforms and protozoa within Traditional SSF during A) Juvenile phase B) Ripening phase C) Optimal Phase and D) Mature phase; and within Manz SSF during E) Juvenile phase F) Ripening phase G) Optimal Phase and H) Mature phase.
interstitial water from the upper portion of the filter which was also of a higher concentration than the effluent water (Fig. 3.10: B and F).

Full depth resolved sampling of total coliform and *E. coli* was resumed in the optimal phase (Fig. 3.10 C and F) and demonstrated that over 90% removal of total coliform bacteria and *E. coli* occurred in the schmutzdecke (Table 3.5). Bacterial removal continued gradually down through the filter and produced 99% cumulative removal through the filter bed. Protozoa concentrations in the interstitial water within the schmutzdecke remained stable with continued higher concentrations than in the influent water. Protozoan concentrations within the lower depths of the sand-bed also increased (Fig. 3.10 C and F) despite protozoa concentrations in the effluent demonstrating the highest rates (97% Manz; 98% Traditional) during the trial.

**Table 3.7:** Cumulative removal (%) with depth from waste-water sampled through sampling ports of designated filters

<table>
<thead>
<tr>
<th>Depth (cm)</th>
<th>Juvenile Phase Days 1-41</th>
<th>Ripening Phase Days 41-80</th>
<th>Optimal Phase Days 81-120</th>
<th>Mature Phase Days 121-188</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><em>E. coli</em></td>
<td>Coliform</td>
<td>Protozoa</td>
<td><em>E. coli</em></td>
</tr>
<tr>
<td>Traditional SSF</td>
<td>n=33</td>
<td>n=33</td>
<td>n=9</td>
<td>n=33</td>
</tr>
<tr>
<td>2.5</td>
<td>39%</td>
<td>35%</td>
<td>64%</td>
<td>-</td>
</tr>
<tr>
<td>22.5</td>
<td>72%</td>
<td>62%</td>
<td>67%</td>
<td>-</td>
</tr>
<tr>
<td>42.5</td>
<td>63%</td>
<td>20%</td>
<td>67%</td>
<td>-</td>
</tr>
<tr>
<td>62.5</td>
<td>74%</td>
<td>50%</td>
<td>64%</td>
<td>-</td>
</tr>
<tr>
<td>100 (effluent)</td>
<td>61%</td>
<td>45%</td>
<td>64%</td>
<td>-</td>
</tr>
<tr>
<td>Manz SSF</td>
<td>n=33</td>
<td>n=33</td>
<td>n=9</td>
<td>n=33</td>
</tr>
<tr>
<td>2.5</td>
<td>39%</td>
<td>20%</td>
<td>66%</td>
<td>-</td>
</tr>
<tr>
<td>12.5</td>
<td>72%</td>
<td>45%</td>
<td>69%</td>
<td>-</td>
</tr>
<tr>
<td>22.5</td>
<td>74%</td>
<td>73%</td>
<td>72%</td>
<td>-</td>
</tr>
<tr>
<td>32.5</td>
<td>61%</td>
<td>56%</td>
<td>74%</td>
<td>-</td>
</tr>
<tr>
<td>58.5 (effluent)</td>
<td>59%</td>
<td>57%</td>
<td>66%</td>
<td>-</td>
</tr>
</tbody>
</table>
The schmutzdecke continued to be the dominant depth for bacterial removal during the mature phase (Fig. 3.10 D and G) although the cumulative removal efficiency of subsequent depths increased to their highest levels. Cumulative bacterial removal exceeded 99% after 22.5cm of the filter-bed in both configurations. This coincided with protozoa concentrations within the interstitial water of the upper 22.5cm zone of the filter bed that exceed the protozoa concentrations found in the influent.

Depth resolved analysis of the chemical properties of the waste-water was limited to ammonium and total oxidized nitrogen over whole trial. COD, TOC, TN, SS, pH and DO analysis was performed during the mature phase only.

In the juvenile period, removal of ammonium (17%) occurred in the schmutzdecke of both configurations and coincided with no significant decrease in the oxidized forms of nitrogen. Below the schmutzdecke there were no further significant changes in ammonia. There was no significant change demonstrated in oxidized nitrogen with depth.

As the filter community developed during the ripening phase the rate of ammonium removal increased and remained localized to the schmutzdecke. There was still no significant change demonstrated in oxidized nitrogen with depth.

During the optimal phase there was significant removal of ammonium and oxidized nitrogen in the schmutzdecke. The Manz SSF schmutzdecke achieved greater removal of ammonium but the greater depth of the Traditional SSFs resulted in greater overall removal of oxidized nitrogen.

The mature phase saw both filter configurations achieve their maximum rates of ammonium and oxidized nitrogen removal. Ammonia oxidation and denitrification continued to occur simultaneously in the schmutzdecke. Ammonium removal by the SSFs was greatest in the mature phase due to the improved removal that occurred in the depths below the schmutzdecke where total oxidized nitrogen also continued to be removed within the traditional SSF.

For three weeks during the mature phase the filters were short-circuited for 24 hours through each of the sampling ports starting with the lowest depth and working sequentially upwards. This allowed retrieval of a sufficient volume of wastewater from each depth to analyse the pH, D.O., COD, T.N. and TOC. Results of this
Figure 3.11: Depth resolved concentrations of Ammonium and Total Oxidized Nitrogen: within the Traditional SSF during A) Juvenile phase B) Ripening phase C) Optimal Phase and D) Mature phase; and within Manz SSF during E) Juvenile phase F) Ripening phase G) Optimal Phase and H) Mature phase.
Figure 3.12: Bar-charts showing depth-resolved water quality analysis. The results of Traditional SSF treatment of A) Total Organic Carbon, B) Total Nitrogen, C) COD, D) Dissolved Oxygen and E) pH; and of Manz SSF treatment of F) Total Organic Carbon, G) Total Nitrogen, H) COD, I) Dissolved Oxygen and J) pH were from the mature phase of filter development.
analysis (Fig. 3.12) demonstrate that significant (p<0.05) removal of TN, COD and TOC occurred only in the schmutzdecke. Suspended Solids were below the detection limit at all depths, suggesting total removal of SS in the schmutzdecke. The dissolved oxygen profiles demonstrated a significant difference between SSF configurations (p<0.05). The level of dissolved oxygen within the filter bed of the deeper Traditional SSF was much higher than within the shorter Manz SSF (Fig. 3.12: D and I).

3.4.3. Schmutzdecke performance capacity metric

The surface layer, or schmutzdecke, of the sand bed was the main location of contaminant removal (Figs. 3.10; 3.11; 3.12). Using criteria from the trial performance capacity metric (Fig. 3.8), the removal rates achieved at a depth of 2.5 cm were further examined to evaluate the performance of the schmutzdecke alone. Results from the use of this multi-parameter metric demonstrated that, in both the Manz and Traditional SSFs, the schmutzdecke alone achieved performance levels similar to that of the total filters. The metric also clearly demonstrated the effect of configuration upon the schmutzdecke. The schmutzdecke of the Traditional SSF out-performs the Manz SSF schmutzdecke for the first 120 days (juvenile, ripening and optimal phases). However, during the mature phase, the Manz SSF schmutzdecke achieved greater overall performance. This coincided with the increased requirement for de-clogging and the subsequent increased frequency of scraping and furrowing of the Traditional SSF schmutzdecke.

Figure 3.13: Measuring overall treatment efficiency of all contaminant achieved by the schmutzdecke alone using the “Trial Performance Metric” criteria
3.4. Discussion

3.4.4. Evaluation of SSF for tertiary treatment of municipal wastewater

SSF is known for the ability to remove of microbes during treatment for the production of potable water (Hijnen et al., 2004; Hijnen et al., 2007; Timms et al., 1995). Results from this trial confirmed SSF is also a very effective method for the removal pathogens from wastewater with both SSF configurations achieving over 98% removal of both total coliform bacteria and E. coli from the secondary treated municipal wastewater over a sustained period of time. These results were consistent with previous studies of pathogen removal by SSF (Bomo et al., 2004a; Bomo et al., 2004b; Pfannes et al., 2015; Seeger et al., 2016; Stevik et al., 2004). This capability is highly desirable when the receiving water bodies are a source for irrigation, a source for potable water or are being used for recreation. Total Coliform and E. coli are common indicator organisms for monitoring potentially pathogenic bacteria of faecal origin (Noble et al., 2003) and are used determine water safety by regulatory bodies worldwide (W.H.O., 2006). The ability of a treatment method to remove these particular organisms would determine its effectiveness, regardless of the ability to remove other species.

SSF also produced removal rates exceeding 80% for ammonium, 70% for COD and 85% for suspended solids and turbidity (Table 3.6). Removal of excess nutrients from wastewater is an important in fighting eutrophication, which is Ireland’s most serious environmental pollution problem in surface waters (Mockler et al., 2016). Half of all Irish surface waters are currently failing EU guidelines, and the continued improvement in nutrient reduction needs to be maintained in order to meet the Water Framework Directive (Water Framework Directive, 2000). As was demonstrated by the “Water Quality Failure Metric” the treatment of wastewater by SSF prior to discharge can reduce otherwise excessive contamination below stringent limits for areas failing to meet the Water Framework Directive or areas designated as sensitive by the EPA.

Optimal nutrient removal rates were achieved during the mature period where clogging frequency was highest- which caused the flow rate to fall and supernatant to increase. The ability of SSF technology to provide adequate tertiary treatment of municipal wastewater is determined by the ability to treat of turbid, high solids
wastewater while maintaining the flow. This cannot be achieved without regular unclogging, so the ease at which the filters can be cleaned and returned to operation is fundamental to the suitability of SSF for tertiary wastewater treatment.

3.4.5. Importance of the schmutzdecke

To fully understand the contaminant removal capability of SSFs for wastewater the depth at which removal occurs must be discovered. This study was the first to measure such a broad range of wastewater parameters from multiple depths within the filter bed. Results from this in-depth, highly replicated examination reject the hypothesis that a stratification of nutrient removal exists within the filters (Hypothesis 3.3). There was no evidence of successive depletion of nutrients. The schmutzdecke was by far the predominant location for contaminant removal, being responsible for 78.9% of the total removal in the Traditional SSF and 89.4% in the Manz SSF during the mature phase (Fig. 3.14), and removal rates of all nutrients decreased with depth.

**Figure 3.14**: Proportion of the total removal that was achieved by the schmutzdecke alone in each SSF configuration.
Log removal of the bacterial indicator organisms was found to occur in the schmutzdecke and no statistically significant removal was shown to occur below 22.5cm. The greatest removal of nutrients also occurred in the schmutzdecke, which was the only individual depth which showed statistically significant removal rates.

The filter bed can effectively be divided into two parts: 1) The schmutzdecke; and 2) the total remaining sub-surface, which was the rest of the filter combined. Removal by the total remaining sub-surface demonstrated significant removal (t-test; p<0.05) of COD, TON and Ammonium. Unlike SSF treatment for potable water production the lower depths do provide small but significant removal of contaminants but the schmutzdecke alone was more efficient than the remainder of the filter bed below.

The increase in dissolved oxygen during flow through the schmutzdecke was contrary to results of previous study (Pfannes et al., 2015). This may be due to the release of oxygen from photosynthesis occurring in the supernatant water.

3.4.6. Correlation of protozoa and bacterial removal

This was the first study to examine the development of the protozoan community over time in slow sand filters. Results confirmed that higher protozoan concentrations in the filters coincided with improved removal of total coliforms and E. coli (hypothesis 3.2), as was previously found (Haig et al., 2015b; Weber-Shirk and Dick, 1999), adding to the growing body of evidence for the role of protozoa in the biological mechanisms of bacterial pathogen removal in wastewater SSF (Bauer et al., 2011; Pfannes et al., 2015; Ravva et al., 2010; Seeger et al., 2016). Concentrations of protozoa within the filter bed were much greater than effluent and even influent numbers confirming a build up or colonization of the filter-bed.

Very little is known about the eukaryotic community of SSFs. These may be signs of a heterotrophic eukaryotic community that has colonized the filter which was capable of consuming not only bacteria, but also nutrients and detritus, in order to multiply and grow and cause their progeny to be found in the effluent. Eukaryotic microbes tend to have a minimum concentration of prey bacteria below which it is not energetically favourable to graze in order to maintain cell function and remain in an active state (Sherr and Sherr, 1994). Our results found that the indicator organisms rarely get completely removed which was in agreement with previous studies (Haig et al., 2014; Haig et al., 2015b; Pfannes et al., 2015; Seeger et al., 2016).
The ability to achieve the high removal rates demonstrated may be due to the constant replenishment of new prey or the ability of heterotrophic eukaryotes to survive on other nutrients and detritus (de Vargas et al., 2015; Pernthaler, 2005) from the wastewater.

Despite the highly efficient removal rates of pathogen indicator organisms, the increase in the protozoan concentrations in the effluent over time could be a potential health risk if the filters are harbouring and propagating pathogenic eukaryotes in the sand bed. Whilst protozoan removal was shown to decrease in the mature period, there was still a total removal rate of over 80% from the wastewater, so until the identity of the protozoa that were being discharged in the effluent were determined this would still indicate that SSF provides a reduced risk of protozoan pathogens in the receiving water body.

3.4.7. Effect of configuration on SSF performance

The Manz SSF differs from the Traditional SSF due to its shallower depth, multiple grades of sand (most notably a very fine surface layer), smaller supernatant head of water (Fig. 3.1), and sludge removal by means of backwashing. The results of this trial show the impact of these differences on contaminant removal. Despite the reduced depth the removal efficiency Manz SSF was only significant lower for TON and turbidity. As hypothesised (hypothesis 3.4) the Manz SSF schmutzdecke proved to be more biologically active, providing higher removal of total coliforms, *E. coli*, COD, TOC, and ammonium. Significantly higher dissolved oxygen levels measured in the schmutzdecke of the Traditional SSF suggests that the much larger supernatant header of water leads to higher rates of photosynthesis.

Crucially, backwashing of the Manz SSF proved more effective at maintaining flow rates by improving hydraulic conductivity (Table 3.3). The time between de-clogging procedures in the Manz SSF was twice as long as that of the Traditional SSF during the mature phase. During this period where the Traditional SSF required daily draining, furrowing and refilling the Manz SSF schmutzdecke begins to out-perform the Traditional SSF schmutzdecke for overall removal (Fig. 3.14). During the laboratory-scale trial at the mature phase of the filter development, treatment time lost due to backwashing was seven minutes per week for backwashing the Manz SSFs and 90 minutes per week for draining, furrowing and refilling for a Traditional SSF.
Whilst this obviously was not representative of full scale operation, it does highlight the suitability of backwashing as a potentially automated mechanism for quick and easy sludge removal with effective maintenance of hydraulic conductivity.

### 3.5. Conclusions

This trial showed that bacterial pathogen removal by SSF was excellent demonstrating the suitability of this method for wastewater reclamation. The secondary-treated municipal wastewater being filtered was already low in chemical contaminants and the nutrient removal, whilst statistically significant, was erratic and incomplete. While both configurations were capable of similar levels of contaminant removal from municipal wastewater, only the Manz SSF was able to effectively maintain the hydraulic conductivity necessary to meet the needs of a constant operational environment of a WWTP. The Schmutzdecke was responsible for the majority of effective contaminant removal whilst the lower sand depths were inefficient. The Manz Schmutzdecke takes longer to reach maturity but once matured, was more efficient than the Traditional SSF schmutzdecke. Overall SSF can be effectively used to treat wastewater if the need to remove pathogens is sufficient to vindicate the cost and effort that is required for daily sludge removal.
3.6. References


Chapter 4
4. The Development of an Indigenous Microbial Community within the Slow Sand Filter-Bed Correlates with Treatment Performance

Abstract

The ability to characterise the microbial community of slow sand filters has been limited by a focus on Bacteria. In this study both the bacterial and the eukaryotic community structures of two Slow Sand Filter (SSF) configurations (Manz-SSF and Traditional-SSF) and of the influent and effluent wastewaters, were characterised simultaneously using T-RFLP, Q-PCR and MiSeq amplicon sequencing. An indigenous microbial community developed over time in the SSFs. An indigenous colonizer was defined in this experiment as a microbe that is found in sand from within the filter-bed despite being absent or extremely rare in the influent water samples. The eukaryotic filter-bed community contained a greater proportion of such indigenous colonizers than the bacterial filter-bed community, which more closely resembled the influent community. Configuration and depth were both more deterministic of the eukaryotic community structure. Configuration had a particular impact on metazoans. The Manz SSF community contained a far lower relative abundance of nematodes and annelids in the schmutzdecke layer, likely due to removal by backwashing. Categorizing microbes based on their association with the influent, filter-bed and effluent helped to glean a potential insight into the relationship between community structure and wastewater treatment capacity. Performance was linked to a category termed “Indigenous Strict Colonizers” from both the bacterial and eukaryotic communities, suggesting the importance of the Verrucomicrobia and Acidobacteria bacterial phyla and the Cercozoa and Chlorophyta eukaryotic super-groups in tertiary wastewater treatment.
4.1. Introduction

Slow sand filtration (SSF) is microbially mediated (Huisman and Wood, 1974; Weber-Shirk and Dick, 1997) yet due to the complexity of the microbial community, the microbes responsible for almost all aspects of the filter function are very poorly understood. The ecological web of SSFs consists of interactions between metazoa, protists, algae, fungi, bacteria, archaea and viruses. Compounding this complexity is the effect of localized environmental factors such as influent type, loading rates (Mauclaire et al., 2006), exposure to light (Campos et al., 2006a; Haig et al., 2015b; Iwase et al., 2006) and contamination from local species upon the community structure.

Evaluation of SSF performance in Chapter 3 showed that laboratory-scale SSFs were capable of removing over 98% of faecal indicator bacteria from secondary-treated municipal wastewater, as well as achieving significant levels of nutrient reduction. The schmutzdecke was found to be the location of the majority of contaminant removal (>90%) and the highest concentration of protozoan colonization. Comparing the schmutzdecke of the two SSF configurations, the Manz SSF and the Traditional SSF, found that higher removal of COD, TOC, Ammonia and E. coli occurred in the Manz SSF, which used backwashing for sludge removal and consisted of finer grade of sand. However, due to its greater depth, the Traditional SSF achieved comparable removal of those contaminants by the effluent stage, as well as significantly higher removal of Total-Oxidized Nitrogen and Total-Nitrogen. The much larger supernatant of the Traditional SSF also supported higher rates of photosynthesis which increased dissolved-oxygen throughout filter bed below. The “Trial Performance Capacity Metric” indicated that whilst the Traditional SSF schmutzdecke achieved higher over-all contaminant removal than the Manz SSF schmutzdecke for the first three phases of trial, when maintaining flow rates became an important issue in the final, mature phase, that the Manz SSF schmutzdecke displayed greater over-all contaminant removal. It was concluded, in Section 3.5, that the Manz SSF was more suitable for tertiary wastewater treatment due to an easy mechanism for sludge removal which did not impair removal efficiency.

Characterising the microbial communities of these two SSF configurations is challenging. Fluctuations in the strength and the associated microbial community of the municipal wastewater would constantly alter the SSF community. Influent would
play a large role in both the original seeding of the community in the sterile sand and the continual development and alteration thereafter. Tracking this development over time would allow us to distinguish the key microbes that play a consistent role from the temporary, less resilient microbes. Colonization of the filter indicates an ability to survive and multiply within that environment and microbes capable doing this are going to impact the filter performance. This impact may be positive or negative but shall be important regardless.

The interface between the sand and water, the schmutzdecke, is the most biologically active layer in SSFs (Elliott et al., 2011; Hijnen et al., 2007; Pfannes et al., 2015) particularly for drinking water SSFs. Previous studies have all found removal to be predominantly associated with the schmutzdecke and microbial characterization of the filters is typically done from only the schmutzdecke. Results from Chapter 3 showed that contaminant removal from wastewater had continued below the surface, albeit at a much reduced rate, so it was decided to examine the microbial community of the schmutzdecke and to compare it with the community from a series of depths below the surface in both configurations.

Pinto et al. (2012) found that sand filters contained a stable bacterial community in an otherwise highly variable drinking water system, and this community was integral in shaping the drinking water throughout the remainder of the distribution system. This observation was achieved through the grouping of bacterial taxa based on their association with the treatment stages. Due to the numerous factors that influence microbial communities such an approach could conceivably be used to help determine whether the communities that develop within SSF for municipal wastewater treatment are as stable as those found in the drinking water system. Understanding the microbial seeding and colonization processes in SSF community could help us to determine the permanent changes to the microbial community structure that were a result of the engineered environmental differences.

Despite the limitations that still exist, molecular microbial ecology offers the scope to study the majority of species that compromise SSF community. Previous molecular microbial studies of the SSF community tended to focus on the bacterial or prokaryotic community through examination of the 16S rRNA profile (Calvo-Bado et
Despite studies demonstrating the important role of eukaryotes in bacterial removal in SSFs (Weber-Shirk and Dick, 1997; Weber-Shirk and Dick, 1999), inclusion of Eukaryotes in molecular microbial community analysis of SSFs has been limited in comparison to Bacteria. To date this has consisted of a lab-scale study that sequenced 70 different 18S rRNA gene clones (Wakelin et al., 2011), a study of fatty acid biomarkers which included protists, Actinomyces and fungi analysis (Mauclaire et al., 2006), a study that combined T-RFPL, cloning and Q-PCR of 18S rRNA genes (Pfannes et al., 2015) and a key study was from Haig et al. (2015b), who used DNA-SIP to track 13C-labelled E. coli removal. This key study used Illumina HiSeq technology to sequence total microbial community (Eukaryotic, Prokaryotic and Viral) and determined that protozoan grazing was responsible for the consumption of 98% of labelled E. coli biomass in SSF for potable water production.

As shown in Chapter 3, the consistent removal of over 98% of potentially pathogenic bacteria was the greatest strength of SSF for tertiary treatment of municipal wastewater. Direct light-microscopy showed that protozoan numbers increased in the filter bed as bacterial removal increased. The location of bacterial removal correlated with highest protozoan concentrations and effluent protozoan concentrations far exceeded influent protozoan concentrations. With the knowledge that pathogen removal was driven by grazing eukaryotic bacteriovores in drinking water SSFs (Haig et al., 2015b), the characterization of the eukaryotic community in wastewater SSFs was an essential first step towards understanding the ecology of pathogen removal.

Other critical impacts of eukaryotes in SSFs include: clogging of the filter beds by diatoms, algae and yeasts (Devadhanam Joubert and Pillay, 2008; Gorczyca and London, 2003; Konno, 1993); reduction of excess biomass through the grazing of phagotrophic eukaryotes on these eukaryotic herbivores and bacteria (Sherr and Sherr, 1994) as well as dead or un-viable cells (Low and Chase, 1999); and creation of pathways for water to flow through the schmutzdecke by nematodes and filamentous algae (Iwase et al., 2006; Nakamoto and Kato, 2006).

There is a dearth of knowledge about the role of eukaryotic microorganisms in many of the biological wastewater treatment processes (Amann et al., 1998). Used mainly
as bio-indicators of activated sludge process performance, little is known about their function. Microscopic examinations have revealed the dominance of testate amoeba and attached-ciliate species in rotating biofilm contacters (Martin-Cereceda et al., 2002), the presence of crawling and attached microeukaryotes in trickling filters (Madoni, 2011) and implicated various protozoan ciliates in bacterial removal by constructed wetlands (Decamp and Warren, 1998; Papadimitriou et al., 2010). The evidence of the role of eukaryotes in pathogen removal in drinking water SSFs makes the limited understanding of the eukaryotes in tertiary wastewater treatment a major obstacle towards improving the pathogen removal capabilities of these technologies.

This study of the SSF community structure was carried out to challenge the following hypotheses:

**Hypothesis 4.1:** The influent community would affect the structure of the SSF biofilm community and the SSF community would in turn significantly influence the structure of the effluent wastewater community.

**Hypothesis 4.2:** The SSF-bed community would develop over time to form a stable community independent of the wastewater community.

**Hypothesis 4.3:** SSF configuration would play a role in shaping the structure of the microbial community. Backwashing in the Manz SSF would reduce the abundance of larger eukaryotes and increase the abundance of attached, biofilm-associated microbes.

**Hypothesis 4.4:** Depth would have a strong impact on the microbial community, with the schmutzdecke producing a community that is distinct from all the other depths.

**Hypothesis 4.5:** Higher microbial diversity and evenness in the SSF would correlate with higher SSF performance.

**Hypothesis 4.6:** Eukaryotes would demonstrate an equal capability as Bacteria for the active colonization and specialization based on depth and filter configuration

**Hypothesis 4.7:** Microbes that were dominant in the filter bed and the effluent were actively colonizing the filter-bed and so were important involved in nutrient cycling and contaminant removal.
With these hypotheses in mind, and with the aid of the molecular microbial techniques of Q-PCR, T-RFLP and high-throughput sequencing for gene and transcript analysis, the aims of this chapter were:

1. To provide a highly resolve description of the bacterial and eukaryotic communities of wastewater SSFs.
2. To describe the development of the filter-bed microbial community over time, including the effect of the influent community on this development.
3. To understand the changes to the microbial community structure created by re-engineering the SSF configuration.
4. To determine the difference between the community of the schmutzdecke and the community below the surface.
5. To link performance with community structure.
6. To determine whether the filter-bed community affected the effluent community.
7. To compare and contrast the prokaryotic and eukaryotic community and to develop hypotheses relating to the ecological food-web of a SSF for tertiary wastewater treatment.
8. To develop a method of examining SSF (or other biological wastewater treatment methods) communities over time in wastewater treatment plants that would allow us to determine the significant communities.

The specific research questions of this chapter were:

- How does the microbial community colonize and develop on the sterile sand grains of the SSF-bed during wastewater treatment?
- What impact did the influent microbial community had on the filter-bed microbial community?
- How was each of the bacterial and eukaryotic communities affected by SSF configuration?
- Could the keystone microbes be identified within a highly complex and constantly fluctuating natural community?
- Could correlations be shown between contaminant removal and the microbial community?
- Could the improved performance of the Manz SSF schmutzdecke be linked to community structure?
- Could T-RFLP fingerprinting be used for monitoring SSFs and explaining performance?
4.2. **Materials and Methods**

4.2.1. Source of biomass

The experimental conditions of the laboratory-scale SSFs were as described previously in Section 3.3.2. Influent and effluent samples (100mls) were collected on eight days (Fig. 4.2) over the course of the trial and stored at -20°C for DNA extraction. Filter bed sand samples (0.5 g) were retrieved from the sampling ports from three depths within both Manz and Traditional SSF configurations (Fig. 4.1) on four sampling days (Fig. 4.2) and stored at -80°C for DNA and RNA co-extraction. Four sampling ports were present at each depth and a different one was used on each occasion in order to reduce the impact of sampling on the microbial community. As described in the Chapter 3, only the three designated filters of each configuration were sampled for the first three time points. Prior to decommissioning (day 188) all filters were subjected to sand sampling.

![Diagram](image)

**Figure 4.1:** Experimental set up of the laboratory-scale SSF columns consisting of six replicas of the Manz SSF configuration and six replicas of the Traditional SSF configuration.
4.2.2. Nucleic acid extraction and preparation

Influent samples (100 ml) were filtered onto 0.22 µm Millipore nitrocellulose filter paper (Fisher Scientific Ireland Ltd.). Effluent samples (100 ml) from all six replica filters from each configuration were pooled for each of the eight sampling days and these pooled effluent samples were filtered onto 0.22 µm Millipore nitrocellulose filter paper (Fisher Scientific Ireland Ltd.). All filter papers were then cut into 16 pieces with sterile scissors. DNA was extracted from the dissected filter papers using a Maxwell 16 Tissue DNA Purification Kit and a Maxwell 16 Research Instrument System (Promega).

Total genomic DNA and transcriptomic RNA were co-extracted from the filter-bed sand biofilm using the PowerMicrobiome RNA isolation Kit (#UC-26000-50, Cambio, UK). Sand (0.5 g) was added to 1.5 ml centrifuge tubes containing glass beads (0.1 mm diameter) and 100 µl of phenol:chloroform:isoamyl alcohol pH 8.0. A chemical lysis buffer (650 µl) and β-mercaptoethanol (6.5 µl) were added and the centrifuge tubes were vortexed using the Vortex-Genie (Mobio, Catalogue#13111-V) and the Vortex adaptor (Mobio, Catalogue#13000-V1-24) for 10 min to lyse the cells. Nucleic acids were captured on spin columns using centrifugation before being washed and re-eluted with RNase-free water (50 µl).

The final volume of extracted nucleic acids was 50 µl, of which 25 µl were designated for each of downstream DNA- and RNA-based analyses. DNA and RNA quality was determined by using a Nano-Drop spectrophotometer (ThermoScientific, UK) and concentrations were quantified using a Qubit fluorometer (Invitrogen, UK). DNA designated samples were stored at -20°C for downstream analysis.

RNA-designated samples were snap-frozen in liquid nitrogen and stored at -80°C before being used for the synthesis of cDNA. DNA was removed by the addition of 1 µl of TURBO DNase and 2 µl of TURBO DNase buffer (Ambion, UK) to 18 µl of the co-extracted DNA and RNA, followed by incubation at 37°C for 30 min. The DNase was deactivated by the addition of 4 µl of 25 mM of Ethylenediaminetetraacetic acid and incubation at 75°C for 10 min.

DNA removal was confirmed by visualization after electrophoresis of a 5 µl aliquot on a 1.5% agarose gel stained with SYBR safe combined with a negative PCR. The
Figure 4.2: Schematic of the time and location of sampling from each SSF configuration. The extent and types of analyses performed on each sample are represented by the colours detailed in the legend.
remaining pure RNA template (21 µl) was combined with 0.75 µl of 50 mM MgCl2+, 2.25 µl of 60 µM Random Primer Mix (New England Biolabs) and 1.5 µl of 10 mM dNTP followed by incubation at 75°C for 4 min. The mixture was then placed on ice and 1.5 µl of M-MuLV Reverse Transcriptase (New England Biolabs) and 3 µl of M-MuLV Reverse Transcriptase Buffer (500 mM Tris HCl; 750 mM KCl; 30 mM MgCl2+; 100 mM DTT) were added. Reverse transcription was performed in a thermocycler with incubation at 25°C for 10 min, 50°C for 30 min and 85°C for 5 min. The resulting cDNA samples were then snap-frozen in liquid nitrogen and stored at -80°C for downstream RNA-based analysis.

4.2.3. T-RFLP

Microbial community fingerprinting of the bacterial and eukaryotic community was carried out through terminal restriction fragment length polymorphisms (T-RFLP). Pools of fluorescently-labelled fragments were created through PCR amplification of sample DNA and cDNA. 16s and 18S rRNA genes were each targeted by PCR with a FAM-6-carboxyfluorescein labelled forward primer in combination with an unlabelled reverse primer (Table 4.1). Total community DNA from filter-bed sand from three separate sampling depths were analysed from 4 sampling days (Fig. 4.2). Total community RNA was analysed from the uppermost filter-bed sand layer (2.5 cm) only due to insufficient RNA concentrations for cDNA below this depth. Total community DNA from the influent water and the effluent water of each filter configuration was analysed from 8 different sampling days, twice as many sampling days as the filter-bed to overcome the lack of replication of the single influent supply.

All labelled PCR products were cleaned with the GeneElute PCR clean-up kit (#NA1020-1KT, Sigma-Aldrich Ireland Ltd.). The labelled and cleaned PCR products of the bacterial domain were digested with Alu I restriction enzyme (Fermantas, Fisher Scientific Ireland Ltd.) and those from the eukaryotic domain were digested following the protocol from Kim et al. (2012), with Hae III (NEB, Brennan and Company). The digested fragments were precipitated with 0.25 µl of glycogen (20 mg/ml) and 75 µl of MgSO4·7H2O (0.2 mM) solution in 70% Ethanol, vortexed for 2 sec, and incubated at room temperature for 30 min. Samples were centrifuged at top speed for 15 minutes before the supernatant was removed and the washing process was repeated. The pellet was then air dried in the dark followed by resuspension in
nuclease free water. Restriction digests were analysed by Source BioSciences (Riverstown Industrial Estate, Tramore, Co. Waterford) using an ABI 310 capillary sequencer with a LIZ500-labelled internal lane standard. Fragment sizes were determined with Peakscanner software (Fisher Scientific Ireland Ltd.). Duplicate profiles were aligned using the T-align programme (Smith et al., 2005).

Statistical analysis was performed using Primer-6 (Clarke and Warwick, 2005) and PERMANOVA (Anderson et al., 2005) software (PRIMER-E Ltd.). Fragment data were square root transformed to down-weight the influence of the most dominant fragments in each sample before Bray-Curtis similarities were calculated. Bray–Curtis dissimilarity matrices were generated to differentiate samples by community composition based on terminal restriction fragments (T-RFs) abundance data. (Where: 0.0 = complete dissimilarity between communities, and 1.0 = samples sharing exactly the same community composition)

4.2.4. High-throughput amplicon sequencing

Influent DNA (1), effluent DNA (2), filter-bed sand DNA from two configurations at two depths (12) and cDNA from two configurations at two depths (12), all from the final sampling point of day 188 were standardized to a concentration of 2 ng/µl. Prokaryotic and eukaryotic communities were both targeted in each of the samples (Fig. 4.2). The V4-V5 region of the prokaryotic 16S rRNA gene was amplified from DNA and cDNA using the primer pair of 515-f and 806- r and the V9 region of the eukaryotic 18S rRNA gene was amplified from DNA and cDNA using the primer pair of Euk1391-f and EukB-r (Table 4.1). PCR amplification was performed in triplicate, confirmed with visualisation by agarose gel electrophoresis and pooled into a single sample. The amplified pooled samples were purified with QIAquick96 PCR purification kit (Qiagen, UK), the concentration was determined by Nanodrop ND-1000 ((Thermo-Fisher Scientific, UK) and then normalized before sequencing on the Illumina MiSeq platform at RTLGenomics (Lubbock, TX, U.S.A.).

MOTHUR 1.36.1 software was used to select for high quality sequences for both the V4-V5 region of the Prokaryotic 16S rRNA gene and the V9 region of the Eukaryotic 18S rRNA gene in accordance with the published method (Schloss et al., 2011) and the online MiSeq standard protocol (http://www.mothur.org/wiki/MiSeq_SOP). Contigs were assembled from pair end reads and trimmed to remove primers.
Sequences with ambiguous base-pairs were removed. 16S sequences less than 75 and greater than 400 base-pairs were eliminated. 18S sequences less than 75 and greater than 310 base-pairs were eliminated. The remaining sequences were then aligned with the SILVA reference alignment version 123 (Quast et al., 2013). The remaining sequences were trimmed to produce common starting and ending alignment coordinates and sequences containing over eight homopolymers were removed. Chimeric sequences were removed using UCHIME software (Edgar et al., 2011).

A Bayesian classifier was used to classify the sequences at an 80% bootstrap value cut-off. 16S rRNA sequences were classified against the SILVA database version 123 (Quast et al., 2013) and 18S rRNA sequences were classified against PR2 database (Guillou et al., 2013) as downloaded on the 8 August, 2016. For 16S rRNA analysis, all eukaryotic, mitochondrial and chloroplast sequences were excluded. For 18S rRNA analysis all bacterial, archaeal mitochondrial and chloroplast sequences were excluded. To normalize samples a sub-group was created in which each of the samples contained the same number of sequences. This was carried out separately for DNA and cDNA. Sub-sampling resulted in each sample consisting of 19,279 sequences for 16S DNA, 11,811 sequences for 16S cDNA, 13,946 sequences for 18S DNA and 36,140 for 18S cDNA. Finally, a “.biome” file was created for each of the four groups and further analysis was carried out using R (R:Core-Team, 2015).

4.2.5. Quantitative-PCR

Quantitative-PCR (Q-PCR) with a Lightcycler 480 instrument (Roche, Switzerland) was used to quantify 18S rRNA genes and transcripts, and 16S rRNA genes and transcripts. Internal standard curves were used for each assay for accurate gene fragment quantification using triplicate 10-fold dilutions of EcoR1 plasmid DNA containing cloned inserts confirmed by sequencing. All samples were tested in duplicate. Each 20 µl reaction contained 10 µl of GreenMaster SYBR green Mastermix, 1 µl of each primer (25 µM) and 2 µl of an appropriate dilution of either plasmid DNA, sample DNA or sample cDNA. Cycling conditions and primers were as outlined (Table 4.1). Melt curve analysis and agarose gel electrophoresis was performed after each assay to confirm that fluorescence was due to specific PCR products. The standard curves generated were linear (r² > 0.925) with high efficiency (E= 3.2 – 3.5).
Table 4.1: Primers and conditions used for domain based analyses of the microbial communities from wastewater and SSFs.

<table>
<thead>
<tr>
<th>Target Domain</th>
<th>Primers</th>
<th>Sequence (5′ – 3′)</th>
<th>Amplicon Size (bp)</th>
<th>Annealing Temperature</th>
<th>PCR cycles</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Q-PCR</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>16S rRNA</td>
<td>3338 f</td>
<td>ACTCCTACGGGAGGCAGCAGG</td>
<td>~465</td>
<td>57°C</td>
<td>45 cycles</td>
<td>Yu et al. (2005)</td>
</tr>
<tr>
<td></td>
<td>805 r</td>
<td>GACTACCAGGGGTATCTAA</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>18S rRNA</td>
<td>EUK345f</td>
<td>AAGGAAGGCAGCAGGCGAGG</td>
<td>~150</td>
<td>60°C</td>
<td>45 cycles</td>
<td>Zhu et al. (2005)</td>
</tr>
<tr>
<td></td>
<td>EUK499r</td>
<td>CACCAGACTTGCCCTCYAAT</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>T-RFLP</strong></td>
<td></td>
<td></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>16S rRNA</td>
<td>27f-FAM*</td>
<td>FAM*-AGAGTTTGATCMTGCTCAGCGGAACATGTGCGGG</td>
<td>~1,365</td>
<td>58°C</td>
<td>35 cycles</td>
<td>Lane (1991)</td>
</tr>
<tr>
<td></td>
<td>1392r</td>
<td>CGGAACATGTGCGGGG</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>18S rRNA</td>
<td>Euk-1A-FAM*</td>
<td>FAM*-CTGGTTGATCTGCAAGGCTATTGGAGCTGGAATAC</td>
<td>~600</td>
<td>59.5°C</td>
<td>35 cycles</td>
<td>Medlin et al. (1988); Weekers et al. (1994)</td>
</tr>
<tr>
<td></td>
<td>570r</td>
<td>GCTATTGGAGCTGGAATTAC</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>MiSeq</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>16S rRNA</td>
<td>515f</td>
<td>GTGCCAGCMGCGCGGTAA</td>
<td>~390</td>
<td>50°C</td>
<td>28 cycles</td>
<td>Caporaso et al. (2011)</td>
</tr>
<tr>
<td></td>
<td>806r</td>
<td>GGACTACHVGGGTWTCTAAT</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>18S rRNA</td>
<td>Euk 1391 f</td>
<td>GTACACACGCCCCGTC</td>
<td>~260</td>
<td>57°C</td>
<td>28 cycles</td>
<td>Amaral-Zettler et al. (2009)</td>
</tr>
<tr>
<td></td>
<td>Euk B r</td>
<td>TGATCCTTCTGCAAGGTACCTAC</td>
<td>+/- 50</td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>
4.3. Results

4.3.1. SSF treatment increases eukaryotic diversity of the wastewater

Before examining the community structure of the filter-bed, the influent and effluent communities were first examined in isolation. SSF is a treatment method specifically used for the removal of microbes and so the effect on the wastewater microbial community is of practical importance. Chapter 3 demonstrated that the SSFs were capable of 65-85% protozoan removal and over 98% bacterial removal. Despite the effectiveness of the removal it was not complete and with huge volumes of treated wastewater being discharged from sand filters into natural watersheds the associated microbial community that remains would still have a significant impact.

**Figure 4.3:** Two-dimensional MDS plots demonstrating the similarity of the influent and effluent communities based on a Bray-Curtis similarity matrix of the relative abundance of (A) 16S rRNA profiles and (B) 18S rRNA profiles of ribotypes (TRFs) from influent and effluent water. Sampling days are indicated by the numbers on the plot.
The differing effect of treatment upon the bacterial and eukaryotic community structure of the wastewater was demonstrated with Bray-Curtis similarity analysis of the TRF profiles. The eukaryotic community from effluent water clustered separately from that of the influent water (Fig. 4.3 B) while the bacterial community structure showed no distinct clustering for samples before and after SSF treatment (Fig. 4.3 A).

Figure 4.4: Bar-chart showing the average diversity of TRF profile from A) 16S and B) 18S rRNA community finger-printing analysis from influent and effluent profile using Simpson’s Index.

Diversity analysis of the TRFs, as calculated by the Simpson’s diversity index showed a significance increase in the diversity of the eukaryotic community in the effluent whilst the bacterial community of the effluent showed no significant difference (Fig. 4.4).
To determine the association between the microbial communities of the influent and effluent water the ribotypes were binned into four categories, namely: Influent Derived; Manz SSF effluent associated; Traditional SSF effluent associated; Both SSF effluent associated. This was done to estimate the changes in microbial community membership before and after SSF treatment.

Table 4.2: Categorisation of individual TRFs into four defined ribotype categories based on presence or absence in influent or effluent wastewater samples across eight sampling time-points.

<table>
<thead>
<tr>
<th>Ribotype Category</th>
<th>Colour</th>
<th>Category definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Influent Derived</td>
<td>Orange</td>
<td>Ribotype was detected in the Influent</td>
</tr>
<tr>
<td>Manz SSF Effluent Associated</td>
<td>Blue</td>
<td>Ribotype was only detected in Manz SSF effluent</td>
</tr>
<tr>
<td>Traditional SSF Effluent Associated</td>
<td>Red</td>
<td>Ribotype was only detected in Traditional SSF effluent</td>
</tr>
<tr>
<td>Both SSF Effluent Associated</td>
<td>Green</td>
<td>Ribotype was only detected in the effluent and from both SSF configurations</td>
</tr>
</tbody>
</table>

A TRF was categorised as an “Influent Derived” ribotype if it was found in the influent water at any stage over the course of the eight sampling time-points. A TRF was categorised as a “Manz SSF Effluent Associated” ribotype if it was only found in the effluent of the Manz SSF, and never in either the influent or the effluent of the Traditional SSF. A TRF was categorised as a “Traditional SSF Effluent Associated” ribotype if it was only found in the effluent of the Traditional SSF, and never in either the influent or the effluent of the Manz SSF. A TRF was categorised as a “Both SSF Effluent Associated” ribotype if it was found in the effluent from both the Manz and the Traditional SSFs, but never in the influent.
Tracking the origin of the bacterial and eukaryotic ribotypes found in the effluent wastewater after treatment by each of the SSF configurations and over the course of the trial (Fig. 4.6) shows the eukaryotic community of the effluent wastewater was dominated by ribotypes that did not originate in the influent. The high relative abundance, typically greater than 80%, of Influent Derived ribotypes in the effluent bacterial community from both configurations demonstrates the similarity of the bacterial community before and after SSF treatment. In contrast, the relative abundance of Influent Derived ribotypes amongst the eukaryotic community of the effluent was never greater than 60% and was shown to decrease over time in both configurations to less than 40% in the final three sampling time-points. Instead the eukaryotic community of the effluent was composed mainly of Both SSF Effluent Associated ribotypes whose relative abundance increased over time, replacing the Influent Derived ribotypes as the dominant category.

**Figure 4.5:** Graphical illustration of the parameters used for binning different categories of ribotypes from the SSF treated effluent water.
Figure 4.6: Column-charts displaying the cumulative relative abundance of TRFs designated to each of the four ribotype categories from pooled effluent wastewater bacterial and eukaryotic communities across eight sampling time-points. The total number of ribotypes per sample ranged from 16-44 for 16S rRNA and from 23-60 for 18S rRNA
4.4.1. Development of the filter-bed community over time

To discover whether these observed effect of SSF treatment on the effluent eukaryotic community could be linked to the filter-bed community structure, T-RFLP analysis of 16S and 18S rRNA genes and Q-PCR analysis of 16S and 18S rRNA genes and transcripts was carried out at three separate depths within the filter bed; and from both Manz and Traditional SSF configurations.

Figure 4.7: Line-chart showing the increase in performance index score (%) and the corresponding decrease in the length of time that filters could provide treatment without being de-clogged. This data from Chapter 3 was used for derivation of the four phases of filter development during the trial: A) Juvenile phase B) Ripening phase C) Optimal phase D) Mature phase.

This filter-bed community structure was tracked through each of the four phases determined in Chapter 3: the formation of a “juvenile” community on sterile sand;
the “ripening” phase as biological activity began to affect contaminant removal; the
“optimal” phase as performance increased and hydraulic conductivity was easily
maintained; and then finally the “mature” phase as the filters became heavily clogged
and frequent de-clogging disrupted performance (Fig. 4.7).

T-RFLP analysis of 16S gene profiles (Fig. 4.8[A]) from each of the four phases
showed that the filter-bed bacterial community of the juvenile phase showed high
similarity with the wastewater community, while the following “ripening”, “optimal”
and “mature” phases were dissimilar from the wastewater community and clustered
separately in a distinct cluster. The 18S gene profiles (Fig. 4.8[B]) also showed that
the eukaryotic community of the juvenile phase also showed high similarity with the
wastewater community. Unlike the bacterial community profiles however, the three
later phases demonstrated enough dissimilarity to suggest significant structural
changes were occurring.

The diversity of the 16S rRNA community profiles increased gradually over time at
all depths but the diversity of the 18S rRNA community profiles was more
complicated and variable, and was linked with both depth and configuration (Fig.
4.10).

The abundance of both bacterial and eukaryotic gene and transcript copy numbers
per gram showed a gradual increase in the total and active microbial community over
time (Figs. 4.10 and 4.11). Proximity to the surface also led to an increase in total
bacterial and eukaryotic gene and transcript abundance. Gene abundance was
consistently higher than transcripts and the reduction in transcript copy numbers
with depth was proportionally greater than the reduction in gene copy numbers.

As with sampling time, depth did not produce a change in visible effect on the
similarity of the bacterial community (Fig. 4.7) in the NMDS plots, but the NMDS
plots of eukaryotic community (Fig. 4.8) visually demonstrate the effect of depth on
similarity at each sampling time. The schmutzdecke (2.5 cm) eukaryotic
communities in particular showed high structural similarity at each given sampling
point.
**Figure 4.8 (A):** Two-dimensional MDS plots demonstrating the similarity values from bacterial community profiles of samples taken from influent water, filter-bed sand and effluent water. The arrow indicates the progression of time and associated filter development.
Figure 4.8 (B): Two-dimensional MDS plots demonstrating the similarity of eukaryotic community profiles of samples taken from influent water, filter-bed sand and effluent water. The arrow inside the panel indicates the progression of time and associated filter development.
Figure 4.9: Bar-charts illustrating the variation in the diversity of A) 16S rRNA ribotypes and B) 18S rRNA from TRFLP analysis of Manz SSF (blue) and Traditional SSF (red) from various depths in each of the four phases of the trial.
Figure 4.10: Box-plots illustrating 16S rRNA gene and transcript abundance from three depths and over four time-periods in 
A) Traditional SSF (Dark red= genes; light red= transcripts); and B) Manz SSF (Dark Blue= genes; light blue= transcripts). Boxes represent the interquartile range. n=3.
Figure 4.11: Box-plots illustrating 18S rRNA gene and transcript abundance from four depths and over four time-periods in A) Traditional SSF (Dark red= genes; light red= transcripts); and B) Manz SSF (Dark Blue= DNA genes; light blue= transcripts). Boxes represent the interquartile range. n=3.
PERMANOVA and ANOSIM analysis of community structure variance based on the relative abundance profiles (table 4.3) showed that depth was in fact a significant factor for bacterial community structure, but only when comparing schmutzdecke to other depths. Depth was greater factor in eukaryotic community structure as it produced significant differences over a greater number of depths.

**Table 4.3:** Examination of the significance of depth and configuration as factors influencing bacterial and eukaryotic community structure.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Bacterial community</th>
<th>Eukaryotic community</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PERMANOVA</td>
<td>ANOSIM</td>
</tr>
<tr>
<td></td>
<td>t</td>
<td>p-value</td>
</tr>
<tr>
<td>Traditional vs. Manz</td>
<td>1.241</td>
<td>0.129</td>
</tr>
<tr>
<td>2.5cm vs. 12.5cm</td>
<td>1.637</td>
<td>0.012*</td>
</tr>
<tr>
<td>2.5cm vs. 22.5cm</td>
<td>1.901</td>
<td>0.002*</td>
</tr>
<tr>
<td>2.5cm vs. 42.5cm</td>
<td>1.652</td>
<td>0.009*</td>
</tr>
<tr>
<td>12.5cm vs. 22.5cm</td>
<td>1.007</td>
<td>0.367</td>
</tr>
<tr>
<td>12.5cm vs. 42.5cm</td>
<td>1.343</td>
<td>0.082</td>
</tr>
<tr>
<td>22.5cm vs. 42.5cm</td>
<td>0.850</td>
<td>0.637</td>
</tr>
</tbody>
</table>

* = groups determined significantly different by PERMANOVA (p <0.05)
** = groups determined significantly different by ANOSIM (significance level <0.5%)

Configuration was found to affect the rate of eukaryotic colonization of the schmutzdecke. Concentrations of 18S rRNA genes and transcripts in the Manz SSF schmutzdecke increased steadily at each time point, whilst in the schmutzdecke of the traditional SSF they were found to peak at day 66 and stabilised thereafter (Fig. 4.10). This difference between configurations was not seen in the 16S rRNA genes or transcripts (Fig. 4.11).
Statistical analysis of T-RF similarity, by PERMANOVA and ANOSIM (Table 4.2), determined that the eukaryotic community structure was significantly altered by configuration but bacterial community structure was not.

Diversity analysis of T-RFs showed a broadly similar pattern for 16S and 18S rRNA, where diversity was greatest in the schmutzdecke and decreased with depth. Over time the effect of depth became less significant as the community below the surface continued to increase whilst the schmutzdecke community stabilised very early, by sampling day 66. However, the exception to this pattern was the eukaryotic community of the Manz SSF. Eukaryotic diversity of the Manz SSF schmutzdecke was significantly lower than that of the Traditional SSF schmutzdecke, and increased with depth.

To further understand how influent shapes the filter community and how this filter community in turn shapes the effluent community the previous binning strategy was adapted. This new strategy consisted of binning TRFs into categories depending on the presence or absence from the influent, filter-bed and effluent wastewater communities over all four time points (Fig. 4.13). Depth was not used as a distinguishing factor for binning as colonizer ribotypes were typically found in all depths just to varying degrees of relative abundance.

Table 4.4: Categorisation of individual TRFs into five defined ribotype categories based on presence or absence in influent, filter-bed or effluent samples across four sampling time-points.

<table>
<thead>
<tr>
<th>Ribotype Category</th>
<th>Colour</th>
<th>Category definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pass-through</td>
<td>Yellow</td>
<td>Ribotype was detected in the influent and effluent but not in the filter-bed</td>
</tr>
<tr>
<td>Influent-derived Strict Colonizers</td>
<td>Dark orange</td>
<td>Ribotype was detected in the influent and the filter-bed but not in the effluent</td>
</tr>
<tr>
<td>Influent-derived Leaky Colonizers</td>
<td>Light orange</td>
<td>Ribotype was detected in the influent, the filter-bed and the effluent</td>
</tr>
<tr>
<td>Indigenous Strict Colonizers</td>
<td>Light green</td>
<td>Ribotype was detected in the filter-bed but not the influent or the effluent</td>
</tr>
<tr>
<td>Indigenous Leaky Colonizers</td>
<td>Dark green</td>
<td>Ribotype was detected in the filter-bed and the effluent but not the influent</td>
</tr>
</tbody>
</table>
The relative abundance of each of the designated groups of the bacterial ribotypes from each phase was used to illustrate the over-all pattern of bacterial colonization of the SSFs (Fig. 4.14). The Influent-derived Leaky Colonizers proved to be the dominant bacterial category in the filter-bed during each phase, at each depth and in both configurations. The relative abundance of these Influent-derived Leaky Colonizers was greatest (>80%) in the juvenile phase at all depths and in both configurations but decreased sharply to between 35-65% in the Early-ripening phase and remained relatively stable during the following Optimal and Mature phases, typically above 50%.

Within the filter-bed the next most common bacterial category was the Indigenous Strict Colonizers whose relative abundance was least in the juvenile phase (<5%), but increased over time to 20-60%. Influent-derived Strict Colonizers bacteria were only significant (>5%) in the juvenile phase. Indigenous Leaky Colonizers remained relatively stable (10-20%) over time, depth and configuration.
Figure 4.14: Stacked column-charts indicating the cumulative relative abundance of all bacterial TRFs designated into each of the ribotype categories from three depths of each SSF configuration over the four phases of filter development.
Figure 4.15: Stacked column-charts indicating the cumulative relative abundance of all eukaryotic TRFs designated into each of the ribotype categories from three depths of each SSF configuration over the four phases of filter development.
The relative abundance of each of the designated groups of the eukaryotic ribotypes from each phase was used to illustrate the over-all pattern of eukaryotic colonization of the SSFs (Fig. 4.15). Unlike the bacterial communities, the eukaryotic community of the filter bed was not dominated by influent-derived ribotypes. Instead the filter bed was dominated by an indigenous eukaryotic community.

The Indigenous Leaky Colonizers had the greatest overall relative abundance of eukaryotes within the filter-bed over the course of the trial. The relative abundance of eukaryotic Indigenous Leaky Colonizers increased significantly with depth and also increased slightly over time. The eukaryotic Indigenous Strict Colonizers produced the greatest increase in relative abundance over time, but decreased in relative abundance with depth.

There was an inverse relationship between relative abundance of the Indigenous Colonizers (Leaky and Strict) and the Influent-derived Colonizers (Leaky and Strict). The Indigenous Colonizers increased in relative abundance over time and this resulted in a decrease in the relative abundance of the Influent-derived Colonizers. This decrease occurred at all depths and both configurations. The Influent-derived Leaky Colonizers had a higher initial relative abundance in the juvenile phase which led to a more marked decrease over time.

4.4.2. High resolution snap-shot of the mature community

MiSeq amplicon sequencing was performed on samples from the final day of the mature phase with appropriate primer sets (Table 4.1) that targeted the prokaryotic and eukaryotic communities. Results from this sequencing were used for a higher resolution analysis of the mature SSF microbial community.

Phylum level analysis of 16S rRNA genes and transcripts (Fig. 4.16) revealed that Proteobacteria were the dominant bacterial phylum in the influent and effluent wastewater and in the schmutzdecke of both SSF configurations. The greatest relative abundance of transcripts below the schmutzdecke belonged to the Planctomycetes phylum, particularly at 22.5cm depth in the Traditional SSF.

Phylum level analysis of 18S rRNA genes and transcripts (Fig. 4.17) found that eukaryotic community composition differed between configurations much more so than that of bacterial community composition. The schmutzdecke of the
Figure 4.16: Stacked bar-chart of the distribution of relative abundance of bacterial phyla from total community 16S rRNA genes from influent and effluent wastewater, plus total community 16S rRNA genes and transcripts from two depths within the filter-bed.
Figure 4.17: Stacked bar-chart of the distribution of relative abundance of eukaryotic super-groups from total community 18S rRNA genes from influent and effluent wastewater, plus total community 18S rRNA genes and transcripts from two depths within the filter-bed.
Traditional SSF was dominated by Metazoans (30-60% of gene and 30-70% of transcript relative abundance) to a much greater degree than the Manz SSF (2-30% of gene and 10-40% of transcript relative abundance). The Manz SSF eukaryotic community in turn consisted of higher relative abundance of the Archaeplastida (green algae) and Stramenopiles (diatoms). Below the schmutzdecke Metazoa were less abundant with a corresponding increase in Archaeplastida.

OTU level diversity analysis of MiSeq results (Fig. 4.18) shows a slight fall in bacterial diversity with depth in both configurations. Eukaryotic diversity was much lower than bacterial diversity and increases with depth in the Manz SSF whilst it decreases greatly in the Traditional SSF.

**Figure 4.18:** Depth resolved diversity determined by OTU level Illumina MiSeq amplicon sequencing of A) 16S rRNA from Manz and Traditional SSFs; and B) 18S rRNA from Manz and Traditional SSFs
To further understand the factors shaping the development of an indigenous microbial community within the SSFs through the identification of microbes potentially responsible for populating each of the colonizer categories, OTUs from MiSeq analysis were binned into the same categories that were used for ribotypes from T-RFLP analysis. The categories from the T-RFLP analysis were created on the presumption that although these ribotypes were not discovered in the influent over the eight sampling days, they must have been present but not detected due to either low sampling frequency or low resolution analysis. Due to the higher resolution provided by high-throughput MiSeq amplicon sequencing compared to T-RFLP ribotype analysis, the presence/absence based binning was changed to a 1% cut-off. This new cut-off still distinguished between indigenous OTUs with the ability to multiply within the filter and the transient influent-derived OTUs.

Table 4.5: Explanation of the rules for binning individual OTUs into each of the five defined categories based on the relative abundance of that OTU exceeding the 1% maximum cut-off in influent, filter-bed or effluent samples across four sampling time-points.

<table>
<thead>
<tr>
<th>OTU Category</th>
<th>Colour</th>
<th>Category definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pass-through</td>
<td>Yellow</td>
<td>OTU relative abundance exceeded 1% in the influent and effluent but did not exceed 1% in the filter-bed</td>
</tr>
<tr>
<td>Influent-derived Strict Colonizers</td>
<td>Dark orange</td>
<td>OTU relative abundance exceeded 1% in the influent and the filter-bed but did not exceed 1% in the effluent</td>
</tr>
<tr>
<td>Influent-derived Leaky Colonizers</td>
<td>Light orange</td>
<td>OTU relative abundance exceeded 1% in the influent, the filter-bed and the effluent</td>
</tr>
<tr>
<td>Indigenous Strict Colonizers</td>
<td>Light green</td>
<td>OTU relative abundance exceeded 1% in the filter-bed but did not exceed 1% the influent or the effluent</td>
</tr>
<tr>
<td>Indigenous Leaky Colonizers</td>
<td>Dark green</td>
<td>OTU relative abundance exceeded 1% in the filter-bed and the effluent but did not exceed 1% the influent</td>
</tr>
</tbody>
</table>

The relative abundance of each of the bacterial colonizer categories produced by MiSeq analysis from the final day (Fig. 4.19) was very similar to those produced by T-RFLP analysis, particularly from the corresponding time point (Fig. 4.14). Again the Influent-derived Leaky Colonizer bacteria were found to have been the dominant group of both the total and active bacteria of the SSF biofilm (37-53%) as well as the dominant group in the influent (60%) and effluent (Traditional SSF effluent 72%, Manz SSF effluent 49%). The Indigenous Strict Colonizers were again found to have
Figure 4.19: Stacked column-charts indicating the cumulative relative abundance of bacterial OTUs designated into each of the colonizer categories from the final day of the experimental trial. Influent and effluent water samples consist of the DNA profile only. Both DNA and RNA profiles are shown from filter-bed samples from two depths within of each SSF configuration.
Figure 4.20: Stacked column-charts indicating the cumulative relative abundance of eukaryotic OTUs designated into each of the colonizer categories from the final day of the experimental trial. Influent and effluent water samples consist of the DNA profile only. Both DNA and RNA profiles are shown from filter-bed samples from two depths within of each SSF configuration.
been the second most abundant colonizers of the filter-bed with a relative abundance ranging from 13-27% in the total and active SSF biofilm community. There was a noticeably lower relative abundance of active Indigenous Strict Colonizers from transcript analysis at lower depths in both configurations compared to total gene analysis.

In both SSF configurations, the Indigenous Strict Colonizers were the most abundant eukaryotic group as determined by MiSeq amplicon sequencing of the mature SSF biofilm (Fig. 4.20). The heat-map of the top 50 most abundant eukaryotic families (Fig. 4.21) showed that the Indigenous Strict Colonizers category included 6 families from the metazoa super-group of eukaryotes, 7 families from the Cercozoa super-group and 7 families from the Chlorophyta (green algae) super-group, two families from the Oligohymenophorea super-group and one family from the Excavata Super-group. The categorization six families of the Cercozoa super-group as Indigenous Strict Colonizers grouping, and of four different families of the Cercomonadida class in particular, was very noticeable due to their limited occurrence other categories.

The highly active eukaryotic Influent-derived Strict Colonizers that dominated the schmutzdecke of the Traditional SSF (Fig. 4.20) have been identified here as the microscopic metazoan annelid worms of the Aeolosoma family. The high relative abundance of Indigenous Strict Colonizer transcripts from 22.5cm below the surface in the Traditional SSF was shown to be caused by the high activity of a number of families of the Chlorophya Super-group.

The relative abundance of each of the eukaryotic colonizer categories for this final day MiSeq analysis was quite different from those produced by the T-RFLP analysis. The Indigenous Strict Colonizers again demonstrated a high relative abundance. Relative abundance in the total and active eukaryotic community was between 50 – 54% in the upper SSF-bed depth from both filters and between 67-86% in the lower SSF-bed depths. The increased relative abundance of Indigenous Strict Colonizers in the MiSeq analysis came at the expense of the Indigenous Leaky Colonizers which had been shown to be more dominant in the T-RFLP analysis. Influent-derived Strict Colonizers category also produced a much greater relative abundance when determined through MiSeq analysis. MiSeq transcript analysis also indicated that this category was highly active in the schmutzdecke of the Traditional SSF.
**Figure 4.21**: Heatmap relative abundance of the top 50 families of Bacteria. Taxonomy of the families is detailed and they are grouped according to the previously described colonizer groupings. Heat represents the relative abundance of these OTUs as described in the legend.
Table 4.22: Heatmap of heat map relative abundance of the top 50 families of eukaryotes. The taxonomy of the families is detailed and they are grouped according to the previously described colonizer groupings. Heat represents the relative abundance of these OTUs as described in the legend.
Grouping of OTUs from MiSeq came with the obvious added advantage being able to identify the organisms within each of the groups. The top 50 most abundant bacterial families were grouped as described previously and presented in a heat-map (Fig. 4.21). The Influent-derived Leaky Colonizers that dominate the bacterial community consist of 11 families of Proteobacteria; a Planctomycetaceae family; and one family each from the Actinobacteria and Bacteriodetes phyla. Interestingly, the Indigenous Strict Colonizers contained families from the phyla Verrucomicrobia (5), Acidobacteria (5) Chlorofexi (1), Gemmatimonadetes (1) and Nitrospira (1). These phyla were exclusively found in the Indigenous Strict Colonizers category. The families Acidobacteria GP3 and GP6, Caldilineaceae, Verrucomicrobiaceae and Nitrospiraceae in particular displayed high activity in the schmutzdecke.

**4.4. Discussion**

The results outlined in section 4.4 add to our understanding of the ecology of SSF, and more specifically to our understanding of the ecology behind the biologically driven tertiary treatment of wastewater that SSF provides. Chapter 3 had already described the treatment capabilities of each SSF configuration and demonstrated that the shallower Manz SSF was capable of matching the removal capacity of a

<table>
<thead>
<tr>
<th>Hypotheses of this study</th>
<th>True?</th>
</tr>
</thead>
<tbody>
<tr>
<td>4.1 The influent community would affect the structure of the SSF biofilm community which would in turn affect the structure of the effluent wastewater community.</td>
<td>✔</td>
</tr>
<tr>
<td>4.2 The SSF-bed community would develop over time to form a stable community independent of the wastewater community.</td>
<td>✔</td>
</tr>
<tr>
<td>4.3 SSF configuration would play a role in shaping the structure of the microbial community.</td>
<td>✔</td>
</tr>
<tr>
<td>4.4 Depth would have a strong impact on the microbial community, with the schmutzdecke producing a community that is distinct from all the other depths.</td>
<td>✔</td>
</tr>
<tr>
<td>4.5 Higher microbial diversity and evenness in the SSF would correlate with higher SSF performance.</td>
<td>✗</td>
</tr>
<tr>
<td>4.6 Eukaryotes would demonstrate a capability, equal to that of Bacteria, for the active colonization and specialization based on depth and filter configuration</td>
<td>✔</td>
</tr>
<tr>
<td>4.7 The dominant types of bacteria and eukaryotes associated with the effluent wastewater community would be actively growing in the filter and so be important for SSF treatment performance.</td>
<td>✗</td>
</tr>
</tbody>
</table>

Table 4.6: The hypotheses proposed prior to this study, as previously described in section 4.2 of this chapter.
Traditional SSF. The Manz SSF also provides the added benefit of easy sludge removal which is a necessity for wastewater treatment. The schmutzdecke of the Manz SSF took longer to achieve optimal performance than the Traditional SSF schmutzdecke but eventually provided better multi-parameter treatment capacity in the mature phase (Fig. 3.12). This high performing schmutzdecke compensated for the reduced contact of the wastewater with the sand-biofilm due to the lower total depth of the Manz SSF.

4.5.1. Configuration and depth influence community structure

One of the main aims of this chapter was to test the hypothesis that SSF configuration would affect the microbial community structure (hypothesis 4.3) and this was proven to be the case in the schmutzdecke. The Manz SSF schmutzdecke microbial community structure differed from that Traditional SSF in the following ways:

- Slower accumulation of eukaryotes
- Less diverse eukaryotic community
- Lower proportion of metazoa

The rate of eukaryotic colonization of the sterile sand of the schmutzdecke of each SSF configuration, as determined by the abundance of 18S rRNA genes and transcripts, was faster in the Traditional SSF schmutzdecke. (Fig. 4.11). Kasuga et al. (2007) have previously shown that backwashing leads to a temporary removal of total microbial biomass and also alters the structure of the eukaryotic community through the reduction of larger metazoan eukaryotes. The higher abundance of 18S rRNA genes and transcripts in the Traditional SSF schmutzdecke during the first three phases may have been caused by less efficient sludge removal that allowed a build up of larger eukaryotes in the upper 2.5cm of the filter bed.

Configuration also had an impact on diversity (Hypothesis 4.4) and again, the effect was greatest in the eukaryotic community of the Manz SSF schmutzdecke (Fig. 4.10). Bacterial diversity was highest in the schmutzdecke and showed slight increase across all depths over time (Fig. 4.9). This was also the pattern of eukaryotic diversity in the Traditional SSF but in the schmutzdecke of the Manz SSF it was found that eukaryotic diversity was at its lowest in the mature schmutzdecke (Figs. 4.10 B; 4.18 B). Many factors have been shown to affect microbial diversity in
wastewater treatment, such as pH, temperature, location, rainfall (Pinto et al., 2012) as well as backwashing (Feng et al., 2017; Kasuga et al., 2007; Liao et al., 2015).

The highly-clogged Traditional SSF schmutzdecke created a more diverse microbial community whilst backwashing of the Manz SSF produced a less diverse, but possibly more specialized, microbial community. The selective pressure caused by frequent backwashing (every two days) may have removed more inefficient species leading to a more functional and flexible community of facultative microorganisms capable of multiple trophic strategies which can compensate for lower overall diversity (Briones and Raskin, 2003). Feng et al. (2013) found a similar pattern of increasing bacterial community diversity with depth in a pilot scale GAC –Sand dual media filter that was cleaned by backwashing. Bacterial diversity in the backwashed Manz SSF in this study did not increase with depth, as observed by Feng et al. (2013), but the decrease in bacterial diversity with depth was reduced as the biofilm developed until there was no longer any significant decrease in the mature phase (Figs. 4.8 A; 4.13 A).

The schmutzdecke of the Manz SSF contained proportionally fewer metazoan sequences than the schmutzdecke of the Traditional SSF in the mature 188 day old SSFs as determined by MiSeq analysis (Fig. 4.12). This result was in agreement with hypothesis 4.3, that back-washing would change the community structure due to the removal of larger organisms. The metazoan community of the schmutzdecke of both filters contained crustacean family Maximillapoda, the nematode family Chrioriorhabditis, the platyhelminth family Stenostonum and unclassified annelids and nematodes that were derived from the influent (Fig. 4.22). A major difference however was the high relative abundance of the annelida family Enchytraeus and nematode family Curvidtis in the schmutzdecke of the Traditional SSF, two families of metazoan invertebrates that were absent from the influent on that day and also from the schmutzdecke of the Manz SSF (Fig. 4.22). Mauclaire et al. (2006) found that nematodes were a sign of clogged filters with low hydraulic conductivity, while Castaldelli et al. (2005) found that nematodes built up in the surface of filters but were removed by efficient backwashing. The presence of metazoans in the Manz SSF can be explained by their presence in the influent and the higher relative abundance of each of these groups Traditional SSF suggests build-up between backwashing.
Again, all of this points to a community of larger influent derived metazoans that was allowed to accumulate in the schmutzdecke of the Traditional SSF but was prevented from reaching excessive levels in the Manz schmutzdecke due to the efficiency of the backwashing. It was interesting to note that high number of metazoans in the Traditional SSF schmutzdecke was associated with higher diversity. Predators such as annelids and nematodes have been shown to increase overall microbial diversity (Brown, 1995; Krumins et al., 2006; Saleem et al., 2012; Saleem et al., 2013; Stork and Eggleton, 1992). Fewer metazoan predators in the Manz SSF schmutzdecke produced a less diverse eukaryotic community. Predators have been shown to cause increases in otherwise rare and slow growing species through the disproportionate grazing on the most abundant fast growing species (Gotthard, 2000; Huston, 1979). This theory would imply that reduction in metazoan predators in the Manz SSF schmutzdecke prevented the fastest growing species of bacteria and microeukaryotes from greater predation. There was a large number of eukaryotic families whose relative abundance, from both gene and transcript analysis, produced a strong (<-0.5) negative correlation with both the *Enchytraeus* and *Curvidtis* families in particular a number of families of green algae, ciliates and phagotrophs.

4.5.2. Development of an indigenous microbial community

Initial examination of the similarity (Fig. 4.3) and diversity (Fig. 4.4) of influent and effluent profiles created by T-RFLP analysis through showed that only the eukaryotic community of the wastewater changed significantly due to the treatment by the SSFs. As a result of this finding a method of binning OTUs that had been previously used by Pinto et al. (2012) to examine the influence of Sand Filters on the post filtration communities in drinking water systems was adapted to try to understand the dynamics of the wastewater SSF community. The microbial communities of the SSF effluent water were categorized based on their presence or absence of ribotypes in the influent and effluent. The cumulative relative abundance of these Effluent Associated eukaryotic ribotypes gradually increased over time while Influent-derived ribotypes decreased (Fig. 4.6). This finding suggested that the filter-bed was likely to be the source of these new eukaryotic ribotypes. Pinto et al. (2012) similarly describes a group of “Leaky Colonizers” that derive from a stable reservoir of biomass within the sand filter-bed that gets sloughed off into the effluent. Biofilm in SSFs is continually lost through due to sheer stress, decay, starvation and detachment.
(Rittmann, 1990). Meanwhile the bacterial community of the effluent water remained predominantly composed of influent derived ribotypes (Fig. 4.6). Due to the use of sterilized sand when building the laboratory-scale SSFs, the low resolution provided by the T-RFLP fingerprinting technique and the low sampling frequency it could be safely assumed that the influent was the original source of all of the effluent associated ribotypes but that they were present in too low a quantity or too infrequently to be detected.

These findings were used to inform a new set of categories to examine the filter-bed community. As was suggested by the effluent analysis, the bacterial community of the SSFs was dominated by Influent-derived Leaky Colonizers while the eukaryotic community of the SSFs was dominated by a combination of Indigenous Leaky Colonizers and Indigenous Strict Colonizers not discovered in the influent wastewater. A study by Pfannes et al. (2015) also showed that the three most abundant eukaryotic ribotypes within SSFs treating secondary wastewater were not detected in the influent but displayed a high relative abundance within the filter-bed and in the effluent.

Tracking the colonization of the filter-bed over time through T-RFLP (Figs. 4.14 and 4.15) it was shown that the initial colonization by Influent derived colonizers [Orange shades] was followed by succession of the Indigenous colonizers [Green shades] in both the bacterial and eukaryotic communities. This succession of Indigenous colonizers over time was indicative of the gradual formation of a stable specialized community unchanged by the fluctuating influent community, similar to the development of the microbiota of the gastrointestinal tract (Berg, 1996; Savage, 1977). The fact that an Indigenous bacterial community did not develop to the same extent as the Indigenous eukaryotic community in the SSF demonstrates the ability of certain eukaryotes to multiply within this niche environment at a greater rate (Freter, 1992). This finding suggests that eukaryotes may have a greater of a role to play in ecosystem functioning.

These indigenous eukaryotic ribotypes were too low or too infrequent to be detected in the influent over 8 separate sampling days suggesting they were not productive in the preceding activated sludge and trickling filters treatment but were highly competitive in the SSFs. The majority of microbes suspended in the influent were
likely to be those multiplying within the activated sludge as this is a treatment method that exploits planktonic microbes. After the activated sludge process the wastewater passes through trickling filters, which were a fixed biofilm treatment method, where it was more likely that these indigenous ribotypes originate.

Binning of OTUs from the single-time-point MiSeq analysis provided a higher resolution snapshot of these eukaryotic colonizer categories (Fig. 4.20). Indigenous Strict Colonizer were still the most dominant category overall but Influent-derived Strict Colonizers were much more prominent, particularly in the RNA transcript profile of the schmutzdecke of the Traditional SSF. The Indigenous Leaky Colonizer were much less prominent. Another important difference between the results from TRFLP and MiSeq was that the effluent on this sampling day contained 50% influent derived families. These differences may to be due to the higher resolution afforded by the high throughput sequencing. The most likely explanation for this was that the T-RFLP categorization of was based on the presence of these ribotypes in the effluent or effluent at any point over the course of the whole trial. Some of the eukaryotic families grouped as Indigenous Strict Colonizers by the single day MiSeq analysis were likely to have been present in the effluent on previous time points. Despite these differences, with both methods of analysis, a clear trend immerged of the eukaryotic community being dominated by a combination of the indigenous colonizers (Leaky and Strict).

The eukaryotic families identified as Indigenous Strict Colonizers (Fig. 4.22) included many from the super-groups of Cercozoa, Metazoa and Chlorophyta. Cercozoa are flagellate or amoeboid single-celled protozoa known to be heterotrophic and consumers of bacteria and algae (Howe et al., 2011). Previous studies of the eukaryotic community of SSFs using clone libraries (Pfannes et al., 2015; Wakelin et al., 2011) have suggested a dominant role of cercozoan species. Whilst this did not match high-throughput sequencing analysis from this study, there was an increase in cercozoan 18S rRNA transcript abundance in the Manz schmutzdecke and a strong negative correlation with the activity of metazoa which could indicate a role in bacterial and algal grazing in the absence of larger eukaryotes.
Larger multi-cellular metazoans such as Maxillopoda, Enchytraeus, Stenostonum and Curviditis were identified as amongst the most abundant Indigenous Strict Colonizers in the schmutzdecke (Fig. 4.22) and the Aeolosoma family of annelids as the cause of the high relative abundance of the Influent-derived Strict Colonizers category determined by MiSeq analysis.

The high abundance of various Chlorophyta families in the Indigenous Strict Colonizer category also indicates an ability to grow and multiply within the SSF. Although the majority of the Chlorophyta are strict photo-autotrophs, some are mixo-trophic and can survive on dissolved organic carbon. Various species of Sphaeropleales (Burrell et al., 1984), Ankistrodesmus (Giovanardi et al., 2014), Chlorella (Cheirsilp and Torpee, 2012), Stichococcus (Figueroa-Martinez et al., 2015; Martinez et al., 1987) have demonstrated mixo-trophic growth in wastewater for biodiesel production using various carbon sources and in the absence of light.

In contrast to the eukaryotic colonizer profile, the bacterial colonizer profile of the filter-bed was dominated by the Influent-derived Leaky Colonizers category with a low relative abundance of the Indigenous Leaky Colonizers category (Figs. 4.14 and 4.19). The successful colonization of the filter-bed by the dominant bacteria of the influent water suggests that the bacterial community of the SSFs are performing similar roles to those they performed in the preceding stages of wastewater treatment. The important bacterial families of the dominant Influent-derived Leaky Colonizers category consisted of 11 families from the Proteobacteria phylum and one family from each the Planctomycetes, Actinobacteria and Bacteriodetes phyla (Fig. 4.21).

The Proteobacteria are typically the most abundant phyla in SSF communities and the most abundant proteobacterial families identified in these SSFs were all previously identified as amongst the most abundant taxa in other SSF bacterial community analysis (Feld et al., 2015; Lautenschlager et al., 2014; Liao et al., 2015; Pfannes et al., 2015; Wakelin et al., 2011). Of the families of Proteobacteria identified amongst the Influent-derived Leaky Colonizers: Comamonadaceae and Sphingomonadaceae have previously been proposed as potential herbicide degraders in horticultural SSFs (Feld et al., 2015) while Rhizobiales and
Rhodobacteraceae have been proposed as degraders of organic nitrogenous compounds in drinking water SSFs (Liao et al., 2015).

The Planctomycetes were the most abundant phyla within the filter bed (Fig. 4.16). This phylum was shown to have been mainly comprised of members of the Planctomycetaceae family (Figs. 4.21). The Planctomycetaceae family are typically aerobic, motile, flagellated, chemo-organotrophs (Ward, 2015) and they are likely to play a role in the aerobic decomposition of organic compounds from the wastewater for use as sources of carbon and nitrogen, as well as nitrate and ammonium (Hedlund, 2010; Ward, 2015).

The Influent-derived Strict Colonizers and the Indigenous Leaky Colonizers were also comprised of mainly Proteobacteria, Actinobacteria and Bacteriodetes highlighting the importance of these phyla in this environment. Wakelin et al. (2011) found that Proteobacteria (43%) Firmicutes (24%) and Actinobacteria (16%) were the dominant phyla in SSF treatment of stormwater runoff for re-use in Australia. However drinking water distribution systems offer a very different environment to that of municipal wastewater treatment systems. Pfannes et al. (2015) found that in wastewater SSFs that Alphaproteobacteria, Betaproteobacteria and Planctomycetes were more abundant within the filter than in the influent, suggesting their suitability for colonizing SSFs in more eutrophic environments.

The most interesting bacterial category was the Indigenous Strict Colonizers whose relative abundance would suggest they played an greater role in the filter-bed than in the preceding treatment stages. Over the four phases of filter development there was a gradual increase in the cumulative relative abundance of the bacterial Indigenous Strict Colonizers category, particularly in the schmutzcdecke of the Traditional SSF (Fig. 4.14). The composition of this category was also very different to the other categories, uniquely consisting of families from the phyla Verrucomicrobia, Acidobacteria, Chloroflexi, Nitrospira and Gemmatimonadales. These findings suggested that these families were actively colonizing growing and multiplying within the filter-bed.

Members of the Verrucomicrobiaceae family, the most abundant family of the Verrucomicrobia found in these filters, are typically slow growing, aerobic freshwater chemoheterotrophs that specialize in carbohydrate degradation and
prefer complex natural polysaccharides (Hedlund, 2010). Their slow growth rate corresponds to the late development of Indigenous Strict Colonizers determined in this experiment (Fig. 4.14). Hunter et al. (2013) noted that despite an absence of Verrucomicrobia from all early SSF communities and they were a significant presence in all mature SSF communities.

In accordance with their designation as strict colonizers due to their low relative abundance in the effluent Poitelon et al. (2010) also identified both Verrucomicrobia and Acidobacteria in GAC filters from different locations but not in the subsequent distribution systems of each. Acidobacteria are ubiquitous across a range of habitats and display high phylogenetic diversity but yet we still have limited understanding of their ecology and activity due to difficulties in culturing. Of those Acidobacteria cultivated, many are heterotrophic, carbohydrate degraders while genomic studies suggest many may be involved in decomposition and the utilization of natural polymers (Kielak et al., 2016). Lautenschlager et al. (2014) also identified populations of both Acidobacteria and Nitrospira in biologically active filters. The Nitrospiraceae family identified are well known nitrite oxidizers in wastewater treatment (Galilee et al., 2017; Wilkinson, 2012). The Caldelineaceae family from the Chloroflexi phylum was identified as a nitrifier and phosphate accumulator in wastewater treatment (Kindaichi et al., 2013) that may have been involved in the ammonium and phosphate removal demonstrated by the SSFs. The Hyphomicrobiaceae family of the Proteobacteria phyla that was also designated as an Indigenous Strict Colonizer is a known degrader of nitrogenous organic compounds in waters and has been linked with the removal of dissolved organic nitrogen in SSFs (Liao et al., 2015).

The strategy of designation of ribotypes, OTUs and families into categories confirmed many of the findings of Pinto et al. (2012) who similarly identified a “Strict Colonizers” category mainly composed of members of the Proteobacteria, Bacteroidetes and Actinobacteria phyla in sand filters for drinking water systems. The bacterial orders categorized as “Leaky Colonizers” in that study, Rhizobiales, Rhodobacterales, Sphingomonadales and Burkholderiales, were also determined amongst the main Leaky Colonizers (both Influent-derived and Indigenous) in our systems.
4.5.3. Linking community structure and SSF performance

The ability to understand the development of the microbial community in SSFs was not just an exercise in microbial ecological theory but a necessary tool for the practical improvement of wastewater treatment. To determine the effect of community structure upon the treatment capacity of the SSFs, the Performance Indices developed in Chapter 3 were used. The use of the Performance Indices provided a holistic view of filter performance that compares the removal of a wide range of contaminants against the highest removal rate achieved for each and combines them to give an overall percentage for removal capacity.

The variation in total eukaryotic 18S rRNA gene abundance between configurations over time correlates strongly (Fig. 4.23) with the changing performance achieved by the schmutzdecke of the Manz SSF (Pearson’s r correlation coefficient = 0.88) and Traditional SSF (Pearson’s r correlation coefficient = 0.92), as measured by the Trial Performance Index. This result was in agreement with previous findings that eukaryotes such as protozoa are responsible for grazing upon bacterial pathogens in SSF (Haig et al., 2015b; Weber-Shirk and Dick, 1999).

Figure 4.23: Line graph showing a comparison between the schmutzdecke Performance Index with the abundance of 18S rRNA gene copies per gram of sand in the schmutzdecke of each SSF configuration over time.
High ecological diversity was typically associated with a more stable community and has previously been linked with better wastewater treatment performance (Briones and Raskin, 2003; Haig et al., 2014, 2015a; Siripong and Rittmann, 2007). Bacterial diversity was highest in the schmutzdecke and showed slight increase across all depths over time. Both of which showed a weak positive correlation with increasing overall filter performance in agreement with hypothesis 4.5. This was also the pattern of eukaryotic diversity in the Traditional SSF but in the schmutzdecke of the Manz SSF it was found, contrary to hypothesis 4.5, that eukaryotic diversity was at its lowest in the area of greatest performance. This finding suggests that the Manz SSF schmutzdecke environment produced a greater selective pressure and allowed the development of a leaner, less-diverse community through the removal of large and planktonic organisms by way of backwashing.

To examine whether the ecological categories defined in our study could add to our understanding of filter performance Pearson’s correlation coefficient was used to test whether there was an association between the cumulative relative abundances of each ribotype category and the Performance Indices (table 4.7).

There were strong positive correlations with eukaryotic Indigenous Strict Colonizers (0.77 and 0.50) and weak positive correlations with eukaryotic Indigenous Leaky Colonizers (0.25 and 0.18). Conversely the Influent-derived Colonizers categories all produced a negative correlation suggesting that their presence was detrimental to SSF performance. The majority of the total contaminant removal occurred in the schmutzdecke and the correlation coefficient with eukaryotes and performance Index at this active zone was consistently stronger, either positively or negatively. These findings further the idea that the eukaryotic Indigenous Colonizers (Both Strict and Leaky) were stable, specialized community that were consuming substrates and multiplying within the SSFs.

Despite the much lower influence of the bacterial SSF biofilm community on the bacterial effluent community and the lower relative abundance of the designated Indigenous bacterial colonization within the SSF biofilm, the bacterial Indigenous Strict Colonizers also had a strong positive correlation coefficient with the Performance Index at both the schmutzdecke level and over-all.
Table 4.7: Pearson’s r correlation coefficient between the relative abundance of each group and the increasing removal capacity as measured by the performance indices over four separate time points. The “Schmutzdecke Performance Index” and the “Total Performance Index” was calculated from removal rates at 2.5 cm and at the effluent stage.

<table>
<thead>
<tr>
<th></th>
<th>Bacterial</th>
<th>Eukaryotic</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Schmutzdecke</td>
<td>Total</td>
</tr>
<tr>
<td></td>
<td>Performance</td>
<td>Performance</td>
</tr>
<tr>
<td></td>
<td>Index</td>
<td>Index</td>
</tr>
<tr>
<td>Influent-Derived</td>
<td>0.30</td>
<td>-0.64 **</td>
</tr>
<tr>
<td>Strict Colonizers</td>
<td>0.33</td>
<td>-0.42</td>
</tr>
<tr>
<td>Influent-Derived</td>
<td>-0.76 **</td>
<td>-0.68 **</td>
</tr>
<tr>
<td>Leaky Colonizers</td>
<td>-0.49</td>
<td>-0.56 **</td>
</tr>
<tr>
<td>Indigenous Strict</td>
<td>0.78*</td>
<td>0.77 *</td>
</tr>
<tr>
<td>Colonizers</td>
<td>0.64 *</td>
<td>0.50 *</td>
</tr>
<tr>
<td>Indigenous Leaky</td>
<td>0.27</td>
<td>0.18</td>
</tr>
<tr>
<td>Colonizers</td>
<td></td>
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</tbody>
</table>

* = strong positive correlation  
** = strong negative correlation

The bacterial Influent-derived Leaky colonizers also gave a similarly strong negative correlation as their eukaryotic counterparts.

The weak positive correlation coefficient between performance indices and bacterial Influent-derived Strict Colonizers would not seem to be practically relevant due to the extremely low relative abundance of this category within the SSF bacterial community.
4.5. Conclusions

Using more traditional methods of community structure analysis such as quantitative, diversity and phylogenetic data in combination with less conventional binning strategy the microbial community of the SSFs was subdivided to focus attention on the indigenous, keystone community. The development and maintenance of an indigenous community was crucial in the biological treatment of wastewater due to the constant flux of non-indigenous microbes contained within the wastewater as well as the potential for wash-out. The Indigenous Strict Colonizers from both bacterial and eukaryotic domains exhibit disproportionately large impact on filter performance relative to their abundance, the defining trait of a keystone community.

Backwashing was shown to be crucial for use with municipal wastewater through the maintenance of hydraulic conductivity plus the additional benefit of producing a schmutzdecke community capable of higher removal rates. The reduction of Nematodes and Annelids brought about by the backwashing of the Manz SSF schmutzdecke was identified as the greatest structural change to the microbial community. Whilst removal of these metazoans greatly impacted the whole eukaryotic community, the identification of Indigenous Strict Colonizers as the keystone community indicates the potential role of eukaryotes from the Chlorophyta and Cercozoa super-groups in this improved performance.

Determining the specific microbes responsible for individual contaminant removal requires targeted studies such as the DNA-SIP experiment performed by Haig et al. (2015b) Understanding the factors that influence the colonization and succession of the Indigenous SSF microbes could still be of benefit to the continued modernize and improve this age-old, environmentally friendly method wastewater treatment.
4.6. References


5. Slow Sand Filter Configuration Impacts the Eukaryotic Predators Grazing on Bacterial Pathogens.

Abstract

Pathogen removal from wastewater makes SSF a suitable treatment method for water re-use (Seeger et al., 2016) and this removal occurs predominantly in the schmutzdecke layer. Chapter 3 demonstrated the Manz SSF produced a more efficient schmutzdecke than the Traditional SSF configuration and Chapter 4 illustrated the development of distinct microbial community structures within the schmutzdecke of each configuration. Microcosms, containing fresh sand from the schmutzdecke of laboratory-scale SSF column, representing the Manz and the Traditional SSF configurations, along with sterile wastewater, were prepared. In a controlled and replicated experiment these microcosms were amended with a $^{13}$C-labelled, pathogenic strain of *E. coli* at a concentration typically encountered, and eliminated, by the laboratory-scale SSFs during a twelve hour period. The $^{13}$C-labelled *E. coli* were virtually eliminated within 4 hrs by microcosms from both configurations. Stable-isotope probing, followed by 16S and 18S rRNA amplicon sequencing, was used to track $^{13}$C incorporation by the eukaryotic community. IndVal analysis of 18S rRNA community structure identified the organisms involved in this incorporation. The relative abundance of these $^{13}$C-labelled eukaryotes was shown to increase over time. *Aeolosoma hemprichi*, a microscopic bacteriovorous annelid, was the main predator of *E. coli* in both configurations, particularly in the Traditional SSF microcosms where it accounted for over 85% of the isotopically-labelled eukaryotic community. In the Manz SSF microcosms, however, *Aeolosoma hemprichi* accounted for less than 35% of the isotopically-labelled eukaryotic community after 24 hours. This reduced dominance allowed other bacterial predators to compete, with known other bacterial predators *Choriorhabditis cristae*, *Oligohymenophoreae* and *Chrysophyceae* also demonstrating significant $^{13}$C incorporation. This finding provides direct evidence that the alternative engineering of the Manz SSF, which was shown to reduce metazoan community in the schmutzdecke through backwashing, leads to a more diverse community of eukaryotic predators involved in bacterial pathogen removal.
5.1. Introduction

Water is a vital but limited resource, becoming ever more relevant with a rising global population (W.H.O., 2006). Reclaiming wastewater for irrigation, industry and even for the replenishment of drinking water reservoirs is being suggested and considered in a growing number of countries, having already been implemented in countries such as Australia, Saudi Arabia, Israel and Denmark (Mekonnen and Hoekstra, 2016). Reducing the quantities of potentially pathogenic bacteria of faecal origin from the wastewater destined for water reuse or entering freshwater and ground water sources (Sanders et al., 2013) is therefore of utmost importance. SSF is increasingly being considered as a method of pathogen removal for wastewater reuse (Seeger et al., 2016).

In order to optimize bio-filtration technologies for more efficient pathogen removal, exactly how the organisms in a community interact to achieve the observed biological contaminant removal must be understood. Engineering advances can only proceed so far with a “Black Box” approach to applying microbial consortia (Tiedje et al., 1999). A targeted approach is required to determine the portion of the complex microbial ecosystem involved in a specific functional activity.

The measurement of contaminant removal during the 188-day laboratory-scale trial in Chapter 3 indicated that the elimination of bacteria, specifically faecal indicators, was identified as the most significant aspect of SSF as a tertiary treatment method and so the process that has the greatest impact on public health. Q-PCR analysis and direct microscopy confirmed that protists were colonizing the filters in large numbers in the exact areas where the removal of these faecal indicator organisms was occurring. Analysis of 18S rRNA gene profiles in Chapter 4 demonstrated that backwashing delayed the development of the eukaryotic schmutzdecke communities, that this development correlated strongly with performance and identified colonization patterns of potential bacteriovores. Despite such correlations between ecology and performance, was still necessary to identify the species from the filter bed community involved in pathogen elimination. This knowledge can inform both the efficient operation of existing SSFs and the design of innovative improvements, but first the key functional species underpinning this specific task must be identified.
Micro-eukaryotes (protozoa or protists) have long been known to be the major regulators of bacterial mortality in soil (Aceae and Alexander, 1988; Aceae et al., 1988), ocean (Pernthaler, 2005) and freshwater habitats (Bettarel et al., 2003; Fenchel, 1986), and, as a result, were long presumed to play some role in the removal of bacteria by SSF. Early work to provide empirical evidence for bacterial removal by protozoan grazing in SSFs began with demonstrating that elimination of E. coli in SSFs was prevented by the use of the biological inhibitor Sodium Azide (Weber-Shirk and Dick, 1997). These researchers then proceeded to isolate a protozoan strain from sand filter effluent and proved its ability to reduce E. coli numbers in batch culture experiments (Weber-Shirk and Dick, 1999). Furthering this theory, a protozoan-specific inhibitor, cycloheximide, was shown to reduce the removal of the pathogenic bacterium Aeromonas hydrophila from SSF columns (Bomo et al., 2004b). Addition of bacterial culture to the influent of SSF columns during these studies, and others (Wand et al., 2007), has demonstrated the ability of SSF to reduce the numbers of the bacteria but increase the number of protozoa in the effluent. Viruses may also have a role to play in bacterial mortality in SSF but it has been shown to be less significant than that of the protists (Haig et al., 2015b). In an applied ecology context, where the aim is towards improving pathogen removal, that the protists would be of much greater relevance.

Stable Isotope Probing (SIP) is a culture independent method of identifying the microorganisms involved in metabolizing a specific substrate in a near natural environment (Radajewski et al., 2000). The cultivation of fully 13C-labelled E. coli (Lueders et al., 2006) allows the use of E. coli as the specific substrate. This allows the selective labelling and isolation of the DNA of the species responsible for the initial mineralisation, degradation or metabolisation. Haig et al. (2015b) examined a potable water SSF community and demonstrated the viability of this method of tracking the fate of 13C-labelled bacterial biomass in the SSF environment. This experiment, in which I was a collaborator, determined that within 4 hours of spiking, 99.86% of E. coli removal was due to grazing by protists.

Eukaryotic predators can be extremely specific when feeding on bacteria. Previous studies have demonstrated the importance of both the species and the concentration of bacterial prey in order for predator growth (Bettarel et al., 2003; Epstein and
Shiaris, 1992; Inamori et al., 1990; Pernthaler, 2005; Sherr and Sherr, 1994). The concentration of labelled substrate is critically important for SIP-based experiments (Neufeld et al., 2007a), and so the concentration of labelled bacteria must be within the normal range experienced by the filter to identify the correct species.

The aims of this experimental chapter were

1. To expand the use the DNA-SIP method to a different environment
2. To discover the predators of an isotopically-labelled bacterial pathogen in the highly eutrophic environment of wastewater SSFs.
3. To determine the effect of SSF design upon the community responsible for pathogen removal.

The hypotheses to be tested were:

Hypothesis 5.1: Pathogen removal in SSF is due to active grazing and the labeled E. coli biomass would be consumed and incorporated by predators the schmutzdecke microbial community.

Hypothesis 5.2: Protozoa were the primary grazers of the bacterial biomass in SSFs when treating municipal wastewater.

Hypothesis 5.3: Configuration has demonstrated the ability to change the eukaryotic community of SSFs and so it would affect the composition of the predator community grazing upon bacterial pathogens.

The specific research questions being asked were:

- Is the schmutzdecke community of SSFs capable of consuming E. coli?
- How long would the E. coli survive within the schmutzdecke community?
- Will configuration affect the rate of this removal?
- Which species were responsible for this key pathogen removal function?
- Does this functional community change due to SSF configuration?
- What is the pathogen removal food-web in SSFs treating wastewater?
5.2. Materials and Methods

5.2.1. DNA-SIP Microcosms

5.2.1.1. Schmutzdecke preparation
Sand was taken from the decommissioned laboratory-scale SSF columns used for the trial. The sludge build-up on the surface was removed prior to collection using the typical method for each configuration. The uppermost layers of sand (3 cm) from five replicas were pooled together for each configuration. This produced a total of 106 ml of biologically-active wet sand from each configuration. To provide a biologically-inactive control, wet sand from the Manz (35 ml) and Traditional (35 ml) was mixed together, sterilized by autoclave at 121°C for 25 min and allowed to return to room temperature.

The wet sand (20 ml) of the appropriate type was added to sterile glass vials in triplicate for each of the following conditions (Fig. 5.1): Amended Manz SSF schmutzdecke; Un-amended Manz SSF schmutzdecke; Amended Traditional SSF schmutzdecke; Un-amended Traditional SSF schmutzdecke; Sterilized schmutzdecke; 20 ml of sterilized wastewater was added to each vial, sealed with a screw cap lid and then placed in the dark, at 12°C, on a rotating shaker at 10 rpm for 24 hrs to allow for adaption to the experimental conditions.

5.2.1.2. Substrate preparation
*E. coli* strain Fa21 was isotopically labelled using a published protocol (Marley, 2001). *E. coli* FA21 was grown to stationary phase at 37°C at 150 rpm in M9-minimal media with filter sterilized 13C6-labelled glucose (Sigma, UK) as the sole carbon source. All six of the glucose carbons were 13C-labelled. This culture was measured with a spectrophotometer to determine concentration, centrifuged at 2000g for 10min and washed twice in PBS. Washed cells were resuspended in sterile secondary treated wastewater at 15°C and at a concentration of 6.0 x 10³ per ml

5.2.1.3. Schmutzdecke microcosms
After the 24 hour adaptation period, 20 ml of the wastewater was removed from all vials and replaced with 20 ml of either the 13C-labelled *E. coli* amended wastewater or the un-amended wastewater.
Microcosms types comprised of: Manz SSF schmutzdecke amended with $^{13}$C labelled *E. coli*; un-amended Manz SSF schmutzdecke; Traditional SSF schmutzdecke amended with $^{13}$C labelled *E. coli*; un-amended Traditional SSF schmutzdecke; and sterilized schmutzdecke control with $^{13}$C labelled *E. coli* (Fig. 5.1). Microcosms were incubated horizontally, in the dark, at 12 - 15°C on a rotating shaker at 10 rpm.

5.2.1.4. Microcosm sampling and extraction

Samples (0.5 ml sand plus 0.5 ml wastewater) were taken immediately upon the addition of the $^{13}$C labelled *E. coli* to the incubation, and again at 4 hrs, 9 hrs and 24 hrs. Half (0.25 ml sand plus 0.25 ml wastewater) was immediately snap frozen in liquid nitrogen and stored at -80°C for later DNA extraction. The other half was serially diluted in PBS used for *E. coli* enumeration using Colisure Quanti-Trays system (TECHNOPATH Distribution Ltd., Ireland) as described previously in Section 3.3.6.1.. DNA was extracted from the microcosm samples (0.25 ml sand plus 0.25 ml wastewater) using the PowerMicrobiome Isolation Kit (#UC-26000-50, Cambio, UK) as described previously in Section 4.2.2..
5.2.2. Separation of isotopically labelled DNA

In order to separate the $^{13}$C labelled DNA from the total community $^{12}$C DNA the protocol of Neufeld et al. (2007a) was adapted for the equipment available. Separation of labelled and un-labelled DNA involved the formation of a density gradient in a CsCl solution through ultracentrifugation, dividing the solution into small volumes of different density through fractionation and finally recovering the DNA contained within each fraction through precipitation.

5.2.2.1. Ultracentrifugation

Ultracentrifugation conditions were tested using DNA extracted from pure cultures of $^{13}$C-labelled and unlabelled *E. coli* to determine the conditions required to achieve separation of isotopically labelled DNA from un-labelled DNA. The distinct profiles created by the $^{13}$C-labelled DNA and the unlabelled DNA along with the formation of two peaks formed by the mixture of labelled and un-labelled DNA (Fig. 5.2) confirmed the correct conditions.

![Figure 5.2](image)

**Figure 5.2:** Confirmation of the ultracentrifuge conditions required to achieve separation of isotopically-labelled DNA from unlabeled DNA through quantification of 16S rRNA genes in density gradient based fractions.

A fixed concentration of DNA (3000 ng) was added to a 100% solution of CsCl (1 g/ml; density = 1.4186 g/ml). A gradient buffer was used to ensure the final density of the mixture was ~ 1.65 g/ml. This solution was added to 3.5-ml Quick-Seal ultracentrifuge tube (Beckman Coulter, UK), until flush with the neck of the tube.
Tubes were weighed to ensure a maximum weight differential of 10 mg was achieved and were then heat sealed. Tubes were then arranged in order to achieve equal distribution of the weight across the TLN-100 rotor (Beckman-Coulter, UK). Spacer-plugs were inserted, rotor-lids were replaced and tightened to 110 bars of pressure before rotor was placed in the Optima MAX-XP Bench-top Ultracentrifuge (Beckman-Coulter, UK), and spun for 48 h at 69,000 rpm at 20°C with maximum acceleration and minimum deceleration.

5.2.2.2. Fractionation

After ultracentrifugation the samples were removed carefully with a forceps and stored in a still, dark rack prior to being placed in a fraction recovery system (Beckman-Coulter, UK). The top and bottom of the tube was pierced, mineral oil was pumped through at a constant flow with a peristaltic pump and the CsCl₂ based density gradient fractions of 100 μl each were collected in 1.5-ml tubes. The fraction recovery system was cleaned with 0.1 M NaOH followed by molecular grade absolute ethanol before the next sample was inserted. After all samples were converted into fractions in this manner, the density of each fraction was determined using an AR200 refractometer (Reichert).

5.2.2.3. DNA precipitation

DNA was precipitated from the CsCl₂ by the addition of 1 μl of glycogen (20 ng/μl) for every 150 μl of DNA followed by 2 volumes of PEG. The solution was mixed well by inversion, left at room temperature for two hours to precipitate DNA, centrifuged at 13000 g for 5 min and the supernatant was then discarded. The pellet was washed with 500 μl of 70% ethanol, centrifuged at 13000 g for 5 min, supernatant removed and allowed to air dry for 15 min. the pellet was resuspended in 30 μl of sterile water.

5.2.2.4. Q-PCR

Precipitated DNA (2 μl) of each fraction was used for 16S and 18S rRNA gene Q-PCR assays as described previously (section 4.2.5).
Figure 5.3: 16S and 18S rRNA gene abundance profiles formed by density gradients from total community DNA within TraditionalSSF schmutzdecke microcosms A) 0 hours, B) 4 hours, C) 9 hours and D) 24 hours after exposure to 13C labelled E. coli. n=2. Shaded areas represent fractions chosen for amplicon sequencing.
Figure 5.4: 16S and 18S rRNA gene abundance profiles formed by density gradients from total community DNA within Manz SSF schmutzdecke microcosms A) 0 hours, B) 4 hours, C) 9 hours and D) 24 hours after exposure to 13C labelled *E. coli*. Shaded areas represent fractions chosen and pooled for amplicon sequencing, n=2.
5.2.3. High-throughput amplicon sequencing

Based on the DNA quantification by QBit and domain specific gene quantification by Q-PCR analysis, selected fractions were pooled together (Fig. 5.5) one pool representing the unlabelled $^{12}$C DNA and another representing the labelled $^{13}$C.

All samples were standardized to a concentration of 2 ng/µl. Illumina MiSeq analysis was then used to examine both the prokaryotic and eukaryotic communities in each of the designated pooled samples. Preparation and data analysis for 16S and 18S rRNA community sequencing were carried out as described in Section 4.2.4.

5.2.4. Statistical analysis

Statistical analysis and graphics were performed with R (R:Core-Team, 2015), version 3.3.3. For each SSF configuration, the most characteristic organisms for both the isotopically labelled and the un-labelled communities were determined using the IndVal method (Dufrene and Legendre, 1997) with the labdsv package (Roberts, 2013). This was performed firstly at genus and OTU level. The OTUs and Genera whose disproportionate abundance in the isotopically labelled pool was deemed significant (indicator values $>$0.66, p-values $<$0.05) were determined to be isotopically labelled. The organisms characteristic of each of the labelled and unlabelled communities at the genera level were further analysed with constrained correspondence analysis (CCA) using the vegan package (Oksanen et al., 2007).
5.3. Results

5.3.1. Schmutzdecke microcosm dynamics

After 4 hrs the concentration of *E. coli* in the un-sterilized sand microcosms had decreased by more than half. The concentration of *E. coli* in the sterilized sand produced no significant decrease after 4 hrs, but gradually decreased by 38.5% after the 24 hour experimental period. The rapid decrease in concentrations of *E. coli* was confined to the initial 4 hr time period, at which point they were lower than the background levels recorded in the un-amended control. Background levels of *E. coli* present in the schmutzdecke were demonstrated in the un-amended schmutzdecke microcosms (Figs. 5.6 and 5.7), and this background population decreased by over 75% over 24 hours. The sterilized sand microcosms produced the smallest reduction in *E. coli* concentrations with just 8.4% reduction occurring within 4 hours and a total of 38.5% over 24 hours.

![Figure 5.6: Line-graph illustrating the survival of *E. coli* (cfu) and its effect on total 18S rRNA gene numbers in *E. coli*-amended (red), un-amended control (dashed-line) and sterilized control (black) Traditional SSF schmutzdecke microcosms. n=3](image-url)
Figure 5.7: Line-graph illustrating the survival of *E. coli* (cfu) and its effect on 18S rRNA gene numbers in *E. coli*-amended (blue), un-amended control (dashed-line) and sterilized control (black) Manz SSF schmutzdecke microcosms. *n=3*

Total 18S rRNA gene concentrations in the un-amended schmutzdecke microcosms decreased over time, in contrast to the amended microcosms the 18S rRNA gene copies showed a slight increase. This resulted in significantly higher 18S rRNA gene concentrations in the amended versus un-amended microcosms after 9 and 24 hours in both configuration types.

Figure 5.8: Scatter-plot describing potential Predator–Prey interactions in Manz (blue) and Traditional SSF (red) schmutzdecke microcosms.
Initial analysis of the microcosms did not allow comparison of *E. coli* elimination rates between configurations due to the majority of removal occurring within 4 hrs. The response rate of each configuration’s total eukaryotic community, through 18S rRNA gene analysis, did show a contrast. Total 18S rRNA genes concentrations of Manz SSF microcosms demonstrated a slight increase after 4 hrs and peaked after 9 hrs. The total 18S rRNA genes in Traditional SSF microcosms did not show a response until 9 hrs with a further increase after 24 hrs.

5.3.2. Tracking 13C-labelled bacteria

The bacterial community structure of the Manz and Traditional SSF schmutzdecke microcosms responded very similarly to the amended *E. coli*. Whilst the unlabelled bacterial community structure showed no significant change over the 24 h time period, there were dramatic changes to the composition of the isotopically-labelled community. A very small number of bacterial genera were determined to be indicators for isotopically-labelled community (Figs. 5.9 and 5.10), namely *E. coli*, *E. shigella*, *Fusibacter* and an unknown genus of Clostridiaceae which were determined as isotopically-labelled in both SSF configurations.

Analyses at the OTU level (Figs. 5.11 and 5.12) provided higher resolution and found the initial isotopically-labelled community was composed almost completely (>99.5%) of the amended *E. coli*. After 4 hrs the *E. coli* were no longer present and had been replaced by *Fusibacter* OTUs, which also dominated the isotopically-labelled community after 9 h.

As was also the case with genera level analysis, the majority of OTUs were determined as indicators of the unlabelled community by IndVal analysis. Importantly, after 24 hrs the isotopically-labelled and the unlabelled bacterial communities were no longer significantly different with the labelled community composed of the same OTUs as the unlabelled community.

Comparing the bacterial community structure at OTU and genus level the isotopically-labelled communities were very similar between configurations, with the initial community being composed almost completely (>99.5%) by the amended *E. coli* replaced by one dominated by *Fusibacter* in Manz and Traditional SSF microcosms.
Figure 5.9: Constrained correspondence analysis demonstrating the effect of time on the abundances of bacterial genera in the “Light” and “Heavy” DNA. The shaded region highlights the $^{13}$C-labelled bacterial genera from Traditional SSF microcosms. Time and Carbon-Isotope were used as the environmental variables. $^{13}$C-labelled bacterial genera were determined by IndVal. Arrows indicate the direction of time.
Figure 5.10: Constrained correspondence analysis demonstrating the effect of time on the abundances of bacterial genera in the “Light” and “Heavy” DNA. The shaded region highlights the $^{13}$C-labelled bacterial genera from Manz SSF microcosms. Time and Carbon-Isotope were used as the environmental variables. $^{13}$C-labelled bacterial genera were determined by IndVal. Arrows demonstrate the direction of time.
Figure 5.11: Column chart depicting the relative abundance of bacterial OTUs determined to be indicators of A) un-labelled and B) ¹³C-labelled population of Traditional SSF microcosm communities by IndVal analysis. Shaded columns highlight the OTUs present in the “Heavy” ¹³C-labelled DNA pools. Un-shaded columns represent the “Light” un-labelled DNA pool.
Figure 5.12: Column chart depicting the relative abundance of bacterial OTUs determined to be indicators of A) un-labelled and B) $^{13}$C-labelled population of Manz SSF microcosm communities by IndVal analysis. Shaded columns highlight the OTUs present in the “Heavy” $^{13}$C-labelled DNA pools. Un-shaded columns represent the “Light” un-labelled DNA pool.
5.3.3. Eukaryotic incorporation of \textsuperscript{13}C-labelled \textit{E. coli} biomass

Q-PCR analysis demonstrated an increase in the concentration of 18SrRNA genes over time in the isotopically-labelled pool (Fig. 5.5). To resolve which eukaryotes were responsible for the incorporation of the \textsuperscript{13}C-labelled \textit{E. coli} biomass, the 18S rRNA profiles of the isotopically-labelled and un-labelled communities were examined. IndVal was used to determine the characteristic organisms within each of the labelled and un-labelled communities as described in Section 5.3.4.

As with the un-labelled bacterial community, the un-labelled eukaryotic community structure showed no significant change over the 24 h time period. Changes to the \textsuperscript{13}C-labelled community structure were subtle, in contrast to the complete shifts in dominant organisms found in the isotopically-labelled bacterial community over time. The community structure of the “Heavy” DNA was shown decrease in similarity to the “Light” DNA progressively at each time point in both configurations (Figs. 5.13 and 5.14). \textit{Aeolosoma} was the only organism determined to be an indicator for the isotopically-labelled community in both SSF configurations at genus level.

OTU level analysis of each time point (Figs. 5.15 and 5.16) found that and that the relative abundance of isotopically-labelled OTUs was greatest after 24 hours. \textit{Aeolosoma hemprichi} had the highest relative abundance of the isotopically-labelled OTUs in both Manz and Traditional SSF microcosms.

The isotopically-labelled eukaryotic community showed greater differentiation between configurations than the bacterial community. \textit{Aeolosoma hymprichi} was the most abundant \textsuperscript{13}C-labelled genus and OTU in both configurations; it was much more dominant in the “Heavy” pool of the Traditional SSF microcosms with 20.1\% of total OTUs in the “Heavy” pool after 24 hours but only 7.1\% in the “Heavy” pool of the Manz SSF microcosms after 24 hours. As a result the Manz SSF microcosms produced greater diversity \textsuperscript{13}C-labelled eukaryotes with OTUs classified as \textit{Oligohymenophorea}, \textit{Chrysophytes}, \textit{Choriorhabditis cristata}, \textit{Auxenochlorella protothecoides} and unclassified \textit{Opisthokonta} all displayed a much higher relative abundance in the Manz \textsuperscript{13}C-labelled community.
Figure 5.13: Constrained correspondence analysis demonstrating the effect of time on the abundances of eukaryotic genera in the “Light” and “Heavy” DNA. The shaded region highlights the $^{13}$C-labelled eukaryotic genera from Traditional SSF microcosms. Time and Carbon-Isotope were used as the environmental variables. $^{13}$C-labelled eukaryotic genera were determined by IndVal. Arrows demonstrate the direction of time.
Figure 5.14: Constrained correspondence analysis demonstrating the effect of time on the abundances of eukaryotic genera in the “Light” and “Heavy” DNA. The shaded region highlights the $^{13}$C-labelled eukaryotic genera from Manz SSF microcosms. Time and Carbon-Isotope were used as the environmental variables. $^{13}$C-labelled eukaryotic genera were determined by IndVal. Arrows demonstrate the direction of time.
Figure 5.15: Column chart depicting the relative abundance of eukaryotic OTUs determined to be indicators of A) un-labelled and B) $^{13}$C-labelled population of Traditional SSF microcosm communities by IndVal analysis. Shaded columns highlight the OTUs present in the “Heavy” $^{13}$C-labelled DNA pools. Un-shaded columns represent the “Light” un-labelled DNA pool.
Figure 5.16: Column chart depicting the relative abundance of eukaryotic OTUs determined to be indicators of A) un-labelled and B) $^{13}$C-labelled population of Manz SSF microcosm communities by IndVal analysis. Shaded columns highlight the OTUs present in the “Heavy” $^{13}$C-labelled DNA pools. Un-shaded columns represent the “Light” un-labelled DNA pool.
5.4. Discussion

5.4.1. *E. coli* elimination in the schmutzdecke microcosms

A crucial factor in DNA-SIP analysis is the volume of isotopically-labelled substrate. Substrate volume must be sufficient to allow 50% incorporation of $^{13}$C-labelled carbon into progeny DNA (Haig et al., 2015b). However, isotope incorporation can be low when substrate concentrations are kept low to mimic environmental values (Dunford and Neufeld, 2010). A total of roughly 120,000 CFUs of $^{13}$C-labelled *E. coli* were added into the microcosm experiment immediately before the first sampling point. Based on the flow rates used, this was comparable to the volume of coliform bacteria that the schmutzdecke of the SSF columns would be exposed to over a typical 12 hours period during the laboratory scale experiment (Chapter 3, fig 3.5). Protozoan grazing, however, is highly specific to both prey species and prey concentration (Frias-Lopez et al., 2009; Pernthaler, 2005). Therefore these experimental conditions may, as a result, be artificially selective towards predators capable of grazing on high concentration of this specific strain of bacteria. Starting concentrations were roughly ten-fold higher than the average influent coliform concentrations found during the laboratory scale trial but accumulation of sludge at the surface during physical straining by the sand filter produces elevated concentrations of coliform bacteria in the schmutzdecke compared to the influent (Pfannes et al., 2015). Quantification of *E. coli* in the un-amended microcosms (Figs. 5.6 and 5.7) showed that roughly 1,000 CFU per ml were still present at the beginning of the experimental time period, having already survived the 24 hr adaption period.

The schmutzdecke layer proved to be a highly eutrophic and competitive environment and the concentration of amended *E. coli* in the microcosms was rapidly reduced. The schmutzdecke communities of both SSF configurations caused a reduction of over 95% of the viable *E. coli* within 4 hours (Figs. 5.6 and 5.7) to below the normal background levels found in the un-amended control microcosms. Haig et al. (2015b) found that majority of $^{13}$C-labelled *E. coli* was eliminated from a drinking water SSF within 3 hrs. Ideally more frequent sampling points within the first 4 hours would have allowed us to compare the elimination efficiency of the schmutzdecke communities of each SSF configuration.
Sequencing of 16S amplicons from the designated $^{13}$C-labelled pool from each time point confirmed a 99.9% reduction in $^{13}$C-labelled *E. coli* sequences after 4 hours (Fig. 5.11 and 5.12). The combination of these results demonstrates that SSFs were not only capable of pathogen removal from wastewater through the physical acts of straining and trapping the bacteria, but was also capable of the rapid and effective elimination of viable bacteria that were exposed to the schmutzdecke community.

5.4.2. Selection of $^{13}$C-labelled fractions

DNA-SIP essentially involves separation of “Heavy” $^{13}$C-labelled DNA from background $^{12}$C DNA. However the contamination of the “Heavy” $^{13}$C-labelled DNA with detectable levels of unlabelled $^{12}$C DNA occurs even in pure culture experiments (Neufeld et al., 2007a). In natural microbial communities the variation in incorporation of the labelled substrate leads to a gradient of isotopically labelled DNA. This is due to GC content, the use of other available substrates, interactions between different trophic levels, length of experiment and the number of cell divisions in the presence of the labelled substrate (Lueders et al., 2006; Lueders et al., 2003; Neufeld et al., 2007a; Pepe-Ranney et al., 2016).

Despite our best attempts to isolate the “Heavy” $^{13}$C-labelled DNA from the unlabelled DNA, results show that the unlabelled DNA was also present in the fractions designated as labelled. This was clearly evident from the highly similar community structure found when comparing labelled and unlabelled 16S DNA from the 24-hour time-point (Figs. 5.11 and 5.12) and comparing labelled and unlabelled 18S DNA from the 0-hour time-point (Figs. 5.15 and 5.16). As a result only those OTUs with a statistically significant increase in their relative abundance in the “Heavy” DNA (Indicator values >0.66; p-values<0.05) were designated as the isotopically-labelled community. Such a varying gradient of heavy $^{13}$C-labelled DNA in DNA-SIP experiments has been shown to be a normal occurrence in natural communities due to varying rates of incorporation and G/C content (Neufeld et al., 2007b; Pepe-Ranney et al., 2016).

5.4.3. Major role of *Aelosoma hemprichi* in predation of *E. coli*

The eukaryotic OTUs determined to be isotopically-labelled, in both the Manz SSF and the Traditional SSF microcosms, were dominated by those assigned to
Aeolosoma hemprichi. A microscopic, transparent worm, typically less than 1 mm in length and commonly found in activated sludge, *Aeolosoma hemprichi* is known to feed on bacteria, organic content of detritus, suspended solids and sludge-flocs (Falconi et al., 2007; Liang et al., 2006; Ratsak and Verkuijlen, 2006). Explosions in populations of *Aeolosoma hemprichi* are very common in activated sludge in early summer. As the laboratory-scale SSFs were treating wastewater downstream from activated sludge during the summer it was unsurprising that high concentrations were found in influent and schmutzdecke samples.

The eukaryotic community structure showed that the relative abundance of the *Aeolosoma hemprichi* OTUs within the $^{13}$C-labelled fraction increased slightly after 4 hours, further still after 9 hours but was by far the highest after 24 hours (Figs. 5.15 and 5.16). Taking into account the quantity of total 18S rRNA genes retrieved in each of the time points (Fig.5.6 and 5.7) it was concluded that the increase in the *Aeolosoma hemprichi* population due to grazing on the $^{13}$C-labelled *E. coli* was predominantly after 24 hours. *Aeolosoma hemprichi* reproduction is typically through asexual fission (budding) and the population doubling time in activated sludge has previously been determined by Falconi et al. (2007) to be one day. A growth rate of 0.25 day$^{-1}$ has been found in when feeding upon a pure culture of *E. coli* (Inamori et al., 1990). With the alternative food sources available in the microcosms along with evidence of a small increase in relative abundance in the unlabelled community it was reasonable to expect that progeny produced after 9 hours would not be the result of grazing exclusively on the $^{13}$C-labelled *E. coli*

As well as the need for earlier, more frequent sampling points to determine the exact rate of *E. coli* elimination, it would also have been beneficial to have extended sampling points to ensure the impact of the isotopically labelled *E. coli* on the metazoan community was fully understood. Tellingly, after 24 hours isotopically-labelled OTUs *Choriorhabditis cristata* were identified. These microscopic nematodes are also roughly 1 mm in length (Shaw et al., 2011) and are members of the *Rhabditis* genus, many of which are known bacterial predators (Anderson and Coleman, 1982). Larger metazoans would have a longer generation time rendering their progeny outside the range of this experiment, but as a consequence of this their larger size, such metazoans were less likely to be bacterial-feeders.
Figure 5.17: Column chart showing quantity of the 16S and 18S isotopically-labelled OTUs in A) Traditional SSF and B) Manz SSF microcosms extrapolated from the total concentration of 16S and 18S rRNA genes quantified by Q-PCR combined with their relative abundance as determined by amplicon sequencing.
Aeolosoma hemprichi have also been shown to feed selectively on particular bacterial species including *E. coli* (Inamori et al., 1990). So while this experiment demonstrated that *Aeolosoma hemprichi* play a role in *E. coli* removal, this does not necessarily mean that *Aeolosoma hemprichi* were capable of eliminating other bacterial pathogens. This inability to grow on certain bacterial species also suggests that some bacterial species are capable of surviving ingestion by this invertebrate. Bacteria are known symbionts, colonizers and pathogens of annelids, nematodes and protozoa (Akhurst, 1993; Matz and Kjelleberg, 2005a; Ruehland et al., 2008).

Bacterial OTUs classified as *Fusibacter* species showed high rates of $^{13}$C incorporation and correlated positively with *Aeolosoma hemprichi*. Such species may be capable of surviving ingestion and be capable of growth within the internal structures of this invertebrate forming part of their gut microbiome.

**5.4.4. SSF configuration shapes the community of bacterial-grazing eukaryotes responsible for pathogen removal**

Comparing the response of the communities from the Traditional and the Manz SSF schmutzdecke microcosms to amendment with *E. coli* FA21, configuration was determined to play a role in shaping the eukaryotic community responsible for the removal of this particular pathogen. Predator-prey interaction (Fig. 5.8) estimation from total 16S and 18S rRNA gene quantification gave an initial suggestion of differing responses by the eukaryotic communities of each configuration.

In Chapter 4 (Figs 4.17 and 4.22), the eukaryotic community of the Traditional SSF schmutzdecke was dominated by *Aeolosoma hemprichi* (16.8% ±13.4 18S rRNA genes; 47.8% ±15.4 rRNA transcripts) but was much lower in the Manz SSF (6.5% ±7.8 18S rRNA genes; 26.0% ±16.0 rRNA transcripts). Chapters 3 discussed the effectiveness of backwashing in the Manz SSF to easily remove sludge build-up and Chapters 4 hypothesized that backwashing was also effective at removing excessive build-up of metazoans such as *Aeolosoma hemprichi*.

The starting concentration of *Aeolosoma hemprichi* in the microcosms varied between configurations (Traditional 3.5%; Manz 1.4%) despite removal of as much of the surface sludge as was possible when harvesting the schmutzdecke sand. This difference in the initial eukaryotic community structure helps to explain the eventual
difference in the relative abundance of *Aeolosoma hemprichi* between configurations (Traditional 85.2%; Manz 30.3%) amongst the designated isotopically-labelled OTUs.

Using the total 16S and 18S rRNA gene concentrations from the quantification of each pooled sample (Fig. 5.5), the relative abundance of the designated isotopically-labelled OTUs at each time-point can be examined in context (Fig. 5.17). Whilst there was a substantial increase in the relative abundance of certain eukaryotes after 4 hours the low concentration of 18S copy numbers at this time-point renders this difference irrelevant. After 9 hours the first significant signs of incorporation began, with the Manz SSF microcosms producing greater quantity and greater variation in isotopically-labelled OTUs. After 24 hours the isotopically-labelled OTUs from Traditional SSF microcosms were dominated by *Aeolosoma hemprichi* but the Manz SSF microcosms produce a more diverse and even group of isotopically-labelled OTUs.

The lower relative abundance of *Aeolosoma hemprichi* in the Manz SSF microcosms allowed other species to successfully compete for the available *E. coli*. The other isotopically-labelled OTUs in the Manz SSF were classified as:

- Unknown species of *Oligohymenophorea*: A class of ciliates with many identified species known as bacterial grazers in SSF and activated sludge (Anderson et al., 2013; Haig et al., 2015b; Moreno et al., 2010a; Pfannes et al., 2015; Wakelin et al., 2011).
- *Auxenochlorella protothecoides*: a free living mixotrophic micro-algae used for biodiesel production from wastewater (Yan et al., 2015; Zhao et al., 2014).
- Unknown species of *Chrysophyceae*-Clade C: Golden algae, phagotrophic flagellates, known grazers of bacteria in freshwater, marine and wastewater environments (Bird and Kalff, 1987; Epstein and Shiaris, 1992; Jones, 2000; Lin and Ju, 2017; Moreno et al., 2010b) as well as in a SSF (Weber-Shirk and Dick, 1999).
- *Choriorhabditis cristata*: A microscopic nematode from a genus of known bacterial predators (Anderson and Coleman, 1982)
- Unclassified *Opisthokonta*
- Unclassified Eukaryotes

The build-up of *Aeolosoma hemprichi* demonstrated in the schmutzdecke of the Traditional SSF (Chapter 4) may restrict the development of other eukaryotic species. This could explain why the Manz SSF responds better to the regular de-
clogging during the mature phase of the trial (Chapter 3). The high abundance of *Aeolosoma hemprichi* in the Traditional SSF suggests the ability to dominate the available nutrients causing the populations of other eukaryotic species to be lowered to unproductive levels.

Results of T-RFLP and MiSeq 18S rRNA analysis in Chapter 4 found that the Manz SSF had lower eukaryotic diversity than the Traditional SSF despite the Traditional SSF community being dominated by *Aeolosoma hemprichi*. Such dominance by a single species should decrease the overall diversity, however log-transformation of the data was performed to offset the importance of dominant species such as these. Also the presence of predators has often been shown to increase the diversity through the control of fast growing primary producers. *Aeolosoma hemprichi* would be capable of consuming other small eukaryotes such as micro algae but would be unable to consume larger protozoa such as ciliates.

5.5. **Conclusions**

This DNA-SIP microcosm assay demonstrated that the microbial community of the schmutzdecke was capable of not just simply straining of *E. coli* and trapping them in the schmutzdecke, but also of killing and consuming them. Previous studies have shown the importance of bacterial grazing by protozoa in SSFs (Haig et al., 2015b; Weber-Shirk and Dick, 1999) but this study was the first to demonstrate the important role of microscopic metazoan worms in SSFs for the removal of bacterial pathogens from wastewater. Incorporation of $^{13}$C by micro-algae and fusibacter also demonstrated the availability of a small portion of this $^{13}$C-labelled carbon for heterotrophic, osmotrophic organisms. The $^{13}$C-labelled *E. coli* cells may have burst due to viral lysis, fungal action of mechanical action, but as previously demonstrated in drinking water SSFs (Haig et al., 2015b) the vast majority of the $^{13}$C-labelled *E. coli* biomass was incorporated by eukaryotic phagotrophs.

The backwashing-based design of the Manz SSF allows quick and easy sludge removal plus the removal of excessive populations of larger metazoans which were shown here to be major grazers of potential pathogens. The reduction of the highly abundant *Aeolosoma hemprichi* in the Manz SSF leads to the increased importance of other eukaryotes including the small bacteriovorous nematode *Choriorhabditis cristae* plus well known bacterial grazers from the *Oligohymenophorea* and
Chrysophycaeae classes. Ultimately, more research is needed to determine to whether a dominant population of *Aeolosoma hemprichi* is harmful or beneficial to the overall SSF performance. Previous studies have found *Aeolosoma hemprichi* can reduce sludge volume by up to 65% in the laboratory (Liang et al., 2006) and counteract bio-fouling in membrane bioreactors through the reduction of excess biomass (Klein et al., 2016). Falconi et al. (2007) however stated their unsuitability for use in WWTPs due to uncontrollable population explosions and die-offs.

Results from Chapter 3 demonstrate that despite the potential of metazoans to reduce excess sludge and biofilm growth and to increase SSF porosity due to tunnelling movement (Hurley and Wotton, 2006), that the removal of sludge by backwashing in the Manz SSF was more effective at maintaining hydraulic conductivity of during the mature phase trial. To engineer improvements for SSF efficiency for tertiary wastewater treatment the consequences of alternative designs upon *Aeolosoma hemprichi* populations need to be considered.
5.6. References


Chapter 6
6. General Discussion and Future Recommendations

6.1. Introduction
SSF has been well studied as a method of drinking water production but its use as a method of wastewater treatment still requires a more comprehensive understanding. The engineering advances that allow more efficient use of SSF technology for wastewater treatment still need to be understood at a biological level. Despite the fact that successful studies linking SSF function and ecology have all shown that eukaryotes (Haig et al., 2015b; Weber-Shirk and Dick, 1999) were responsible for bacterial pathogen removal, the majority of ecological studies of SSFs continue to focus on the bacterial community.

An inherent difficulty in studying the microbial community of real world SSF of wastewater is the exposure to an external, constantly fluctuating microbial community within the wastewater. This microbial load is derived from previous stages of wastewater treatment and is therefore capable of contaminant removal. However for of this treatment method there would be species that are more readily suited to the specific environment of the SSF. This leaves us with the problem of distinguishing the independently fluctuating external community of very competitive microorganisms that densely saturate the influent wastewater from the indigenous community that has proved capable of colonizing and competing within this niche environment.

This thesis sought to build upon the foundation of previous research into Slow Sand Filtration by determining the factors affecting both the performance and the microbial community that drives it. To establish these factors a focus was placed upon the development of indigenous microbial community, the impact of alternatively engineered environments and the identification of keystone microbes responsible for contaminant removal.

6.2. Indigenous Community Development
Using an unconventional binning strategy, adapted from Pinto et al. (2012), the microbial community of the SSFs was subdivided into categories based upon association with the filter-bed, the influent and the effluent wastewater to focus attention on the indigenous, keystone community. The development and
maintenance of an indigenous community was crucial in the biological treatment of wastewater due to the constant flux of non-indigenous microbes contained within the wastewater as well as the potential for wash-out. The correlation between these Indigenous Strict Colonizers from both bacterial and eukaryotic domains and the performance based indices derived from overall contaminant removal suggested they exhibit disproportionately large impact on filter performance relative to their abundance, the defining trait of a keystone community.

6.3. Impact of Engineering

While both SSF configurations were capable of contaminant removal from municipal wastewater, the backwashing of the Manz SSF allowed it to effectively maintain the hydraulic conductivity necessary to meet the needs of a constant operational environment of a WWTP. As a result of the backwashing, the Manz SSF schmutzdecke takes longer to reach peak performance but the mature Manz SSF schmutzdecke was more efficient than the mature Traditional SSF schmutzdecke.

Backwashing had been previously shown to lead to temporary loss of biomass and long-term reduction in the abundance of larger metazoa (Kasuga et al., 2007). In line with this finding, this research demonstrated that the Manz SSF schmutzdecke was slower to accumulate eukaryotes than the traditional SSF schmutzdecke, and a lower proportion of the mature Manz SSF schmutzdecke were metazoa.

The removal of these nematodes and annelids greatly impacted the eukaryotic community structure of the Manz SSF schmutzdecke, decreasing overall diversity but allowing other species to thrive such as protozoa, green algae and golden-brown algae. In the DNA-SIP microcosms, the lower proportion of *Aeolosoma hemprichi* in the Manz SSF schmutzdecke was shown to allow these eukaryotes to successfully compete for the labelled *E. coli*. These backwashing-adapted eukaryotes included protozoa previously identified as bacterial grazers in SSFs, the ciliate class *Oligohymenophorea* (Haig et al., 2015b) and flagellate class *Chrysophycaeae* (Weber-Shirk and Dick, 1999).

6.4. Identification of Keystone Microbes

Indigenous Strict Colonizers were identified as the keystone bacterial and eukaryotic community due to their correlation with increased treatment performance over time.
Backwashing resulted in a higher relative abundance of indigenous bacteria and eukaryotes in the schmutzdecke of the Manz SSF.

Eukaryotes from the Chlorophyta, Metazoa and Cercozoa super-groups and bacteria from Verrucomicrobia and Acidobacteria, Firmicutes, Chloroflexi, Nitrospira and Gemmatimonadales phyla were implicated as the Indigenous Strict Colonizers potentially associated with this improved performance.

Not all aspects of filter performance are of equal importance. Bacterial pathogen removal was demonstrated to be the greatest strength of SSF treatment of secondary wastewater. DNA-SIP microcosms identified a variety of eukaryotes, including microscopic worms (Aeolosoma hemprichi and Choriorhabditis cristata), ciliates (Oligohymenophorea Class), golden-brown algae flagellates (Chrysophyceae-Clade C), and the mixo-trophic green algae (Auxenochlorella protothecoides) as the keystone species for the removal of this specific contaminant type.

The predominant role of Aeolosoma hemprichi in the removal of bacterial pathogens was unexpected. Aeolosoma hemprichi was the species most negatively impacted by backwashing in the Manz SSF and was categorized as an Influent-derived Strict Colonizer a group which correlated negatively with performance index and as such was associated with lower overall treatment performance. However it was clearly responsible for the incorporation of the vast majority of E. coli biomass in the Traditional SSF while also playing a lesser but still significant role in the Manz SSF.

6.5. Future Research

6.5.1. Improving SSF design

The physical mechanisms of SSF are well understood. This study confirms previous findings that have identified the schmutzdecke as the location of the majority of contaminant removal and the effectiveness of backwashing for maintaining hydraulic conductivity. Future designs ideally should try to maximize wastewater contact with the schmutzdecke through a series of shallow filters with backwashing clearly a necessary and effective mechanism for maintaining sufficient flow for wastewater treatment. The biological mechanisms, however, are the key to further progress and these are just beginning to be determined.
6.5.2. Indigenous populations

Understanding the factors that influence the colonization and succession of the Indigenous SSF microbes could be of great benefit to the continued modernize and improve this age-old, environmentally friendly method wastewater treatment. The development of this sub-population within the filter-bed and the correlation with treatment performance suggests that further research into the factors that would assist the colonization and growth of these particular species could be of benefit. Augmentation of new or existing SSFs with a consortium of these indigenous species could further elucidate the true value of these microbes.

A similar strategy of identification and augmentation of indigenous colonizers could also be used for SSF of other water types with different priorities, such as industrial and hospital effluents, or for recirculation in horticulture and aquaculture.

6.5.3. Role of metazoa

More research is needed to determine to whether *Aeolosoma hemprichi* is harmful or beneficial to the overall SSF performance. The suitability for wastewater treatment is unclear due to extreme population explosions and die-offs (Falconi et al., 2007) despite demonstrating a capacity for sludge reduction (Liang et al., 2006) and reduction of excess biomass in the membrane biofilms (Klein et al., 2016). The consequences of alternative SSF designs upon *Aeolosoma hemprichi* populations need to be considered further.
References


