The effect of filter bed length on the microbial communities within GAC biofilters and treated effluent water

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Abstract

The supply of clean drinking water to areas of rural Scotland is currently a challenging operation, costly in both financial and environmental terms. This is due to the widespread dispersal of the population across large rural areas. The current model of water treatment relies on many small, chemically and energy intensive water treatment plants spread across rural Scotland. The operation of these plants places a financial burden on Scottish Water and in turn the consumer and contributes to greenhouse gas emissions. Thus, the current model is not sustainable. An alternative solution may be found in a point of entry system of water treatment, purifying drinking water at household or community level. A low cost, low energy technology which could be utilised for a point of entry system is slow sand filtration (SSF). However, a drawback of SSF is that the biological communities and mechanisms of treatment are currently poorly understood. As such SSF can be unreliable and prone to failure. If a greater understanding of the microbial communities of SSF was obtained however, it may be possible to optimise the biological mechanisms and contaminant removal, improving the reliability of SSF.

To develop a better understanding of the microbial communities of SSF a series of lab-scale biofilters (90cm length) were designed and operated over 23 weeks. Biofilters were sacrificially deconstructed to allow depth resolved access to the communities of the filter bed at 4 timepoints during the filter run. Thus, allowing changes in the microbial communities of the filter bed to be monitored through depth and over time. Optimisation of the biological mechanisms of SSF may lie in the engineered design of the biofilter. One design parameter which is currently under-investigated is the effect of filter bed length on the communities of the filter bed and effluent water. To investigate the effect of bed length, short (30cm) and medium (60cm) biofilter columns were run in parallel to the long filter (90cm) columns. These were deconstructed after 23 weeks to compare the effect of column length on the communities of the filter bed. The influent and effluent water was also sampled weekly to compare the effect of column size on biofilter performance against chemical and biological contaminants and the effect it may have on the biological composition of the effluent water.

The results from this study found a diverse filter bed community which was shown to increase in biomass and activity over time. Biomass and diversity were found to be highest in

the top section of the filter bed and decreased with depth. Between column sizes, the top section of the filter bed was found to be very reproducible, harbouring similar biological communities. More differences were observed in the bottom sections of the three column sizes, with specific taxa seeming to be selected in the bottom section of the long filter such as *Bradyrhizobium* and *Rhodoferax*. The effluent water was found to be most similar to the influent water and bottom section of the filter bed. Effluent water between column sizes was found to be largely similar, though with higher abundances of certain taxa found in the effluent of the long filter thought to originate from the bottom of the filter bed. In terms of performance the long filter demonstrated a higher removal capacity for DOC and iron than the medium and short filters. No significant difference was observed between column sizes for biological removal of coliforms, *Legionella pneumophila* or total/intact cells measured by flow cytometry. The abundance of small cell sized Patescibacteria was also investigated, uncovering a diverse phylogeny which appears to be enriched by the filtration process.

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Declaration of originality

I certify that the thesis presented here for examination for a PhD degree of the University of Glasgow is solely my own work other than where I have clearly indicated that it is the work of others (in which case the extent of any work carried out jointly by me and any other person is clearly identified in it) and that the thesis has not been edited by a third party beyond what is permitted by the University's PGR Code of Practice.

I declare that this thesis does not include work forming part of a these presented successfully for another degree.

I declare that this thesis has been produced in accordance with the University of Glasgow's Code of Good Practice in Research.

I acknowledge that if any issues are raised regarding good research practice based on review of this thesis, the examination may be postponed pending the outcome of any investigation of the issues.

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Abbreviations

SSF	Slow Sand Filter
GAC	Granular Activated Carbon
BAC	Biological Activated Carbon
GF	Glass Fiber filter
ST	Sterivex filter
ATP	Adenosine Triphosphate
TOC	Total Organic Carbon
DOC	Dissolved Organic Carbon
BDOC	Biodegradable Dissolved Organic Carbon
AOC	Assimilable Organic Carbon
EPS	Extracellular Polymeric Substances
HNA	High Nucleic Acid
LNA	Low Nucleic Acid
ASV	Amplicon Sequence Variant
EBCT	Empty Bed Contact Time
HLR	Hydraulic Loading Rate
DWTP	Drinking Water Treatment Plant
CPR	Candidate Phyla Radiation
DBP	Disinfection By Products
THM	Trihalomethane
NTU	Nephelometric Turbidity Units

Chapter 1

Introduction and Literature Review

1.1. Introduction

Access to safe drinking water is vital to human survival and an essential component of societal health. Yet as of 2017, 785 million people lack even a basic form of drinking water treatment, many entirely dependent on untreated surface water collected from lakes, rivers and ponds (WHO and UNICEF, 2019). Furthermore, it is estimated that 2 billion people globally are consuming water contaminated with faecal matter (WHO and UNICEF, 2019). Water borne diseases such as cholera, dysentery, and typhoid lead to an estimated 829,000 preventable deaths annually (WHO and UNICEF, 2019). While pathogenic organisms pose the greatest risk to human health, chemical contaminants such as arsenic or lead may also lead to ill health, usually through long term exposure (Amrose et al. 2020). Further exacerbating the situation is encroaching climate change. Higher temperatures and less predictable weather patterns are thought to lead to a reduced availability of drinking water, indeed by 2050 it is estimated that around half the global population will be living in water stressed areas (Unesco 2018). Other areas are expected to see an increased risk of flooding events, potentially contaminating or disrupting the current water treatment infrastructure (Boretti and Rosa 2019). Further challenges can be encountered in the supply of safe drinking water to remote or rural areas. Such areas may lack the infrastructure to support large treatment facilities or efficient distribution networks (VanDerslice 2011), and even when they are present, the operational costs to treat water for smaller communities is greater.

Fortunately, Scotland does not suffer from a lack of availability of clean potable water. However, there are challenges in supplying safe and clean water to rural areas that are becoming more apparent. Around 98% of the land mass of Scotland is described as rural, with 70% described as remote rural i.e. more than a 30-minute drive to the nearest settlement with a population of 10,000 or more ("Scottish Government Urban Rural Classification - 2016," 2016). As a result, one fifth of the population resides in widely dispersed small settlements, making water treatment and distribution economically and environmentally costly and inefficient. As of now, the solution has been a large number of small treatment plants dispersed across the rural landmass, relying on energy and chemical intensive means of disinfection or expensive, energy intensive, membrane filtration. As a result, 90% of Scottish Water assets serve 10% of the population. Aside from the financial burden this places on the company and indeed the consumer, such means of water treatment are far from carbon neutral, using up to 10X the energy costs of centralised systems. In 2019, drinking water treatment accounted for 23% of Scottish Waters' greenhouse gas emissions ("Scottish Water

Sustainability Report", 2019.). As Scottish Water has pledged to be carbon neutral by 2045, the current model is simply not sustainable financially or environmentally.

A proposed solution is the design of a point of entry method of water treatment which would be capable of producing potable water at a household or community level. This would allow for a scaling back of chemically and energy intensive drinking water treatment plants which currently cater to remote rural communities. However, for such a solution to be feasible, a technology must be utilised which is capable of producing high quality, safe drinking water while maintaining a low carbon footprint. Such a solution may potentially be found in slow sand filtration.

Slow sand filtration is a process of water treatment which involves the passage of untreated influent water through a bed of biologically active sand or other particle media (Verma et al. 2017). As the untreated water passes through the filter bed, a combination of physical and biological mechanisms remove particle matter and contaminants both chemical and biological in nature (Guchi 2015; Ellis 1987). Traditionally slow sand filters have been operated at scale and are characterised by slow filtration rates and long empty bed contact times. The simplicity of the slow sand filter design allows for the low energy production of treated water while remaining inexpensive to operate and maintain (Verma et al. 2017). These traits make slow sand filtration a promising technology for a point of entry method of water treatment. However, the technology is not without its drawbacks. Slow sand filtration has previously been referred to as a “black box” method of water treatment, meaning the biological mechanisms of removal are poorly understood (Haig et al. 2015). A substantial knowledge gap exists concerning the microbial communities responsible for contaminant removal by slow sand filtration. As a result, slow sand filters have often proven unreliable and prone to failure. As the safety of the treated water is paramount, any unreliability found in a point of entry system would be unacceptable. As such, slow sand filtration in its current form would not be a suitable technology to utilise. However, if a greater understanding of the microbial communities involved could be achieved, it may be possible to optimise and intensify the biological process of contaminant removal leading to reliably produced, safe potable water.

Advancements in the field of molecular biology, such as high throughput sequencing, have allowed microbial communities to be investigated in higher detail than ever before (Di Bella et al. 2013). Current literature suggests that the microbial communities of slow sand filtration and biofiltration in general are highly diverse and have been shown to differ in composition

through the depth of the filter bed and with the passage of time (Chen et al. 2021; Haig et al. 2015; Wakelin et al. 2011; Campos et al. 2002). However, many questions remain regarding the development of the microbial communities associated with biofiltration and the selective processes which drive community composition. Biofilter community composition has been shown to be driven by both stochastic and deterministic forces (Vignola et al. 2018). As such, a potential route to optimisation may lie in alterations to the biofilter design parameters. Changing design parameters may alter the deterministic forces within the filter environment and select for different and potentially more beneficial community compositions. Design parameters such as media choice and temperature have previously been shown to have an influence on the community composition within biofilters (Moll et al. 1999; Nemani et al. 2016; Vignola et al. 2018; Ma et al. 2020). However, a design parameter which remains under-researched is the effect of filter bed length on the community of the filter bed or quality of the effluent water.

For the development of a point of entry method of water treatment, a reduced filter bed length would likely be beneficial. A smaller bed size would occupy less space which would be advantageous for household installations. However, it is important that a reduced filter bed size is not detrimental to the quality of effluent water produced. Current literature suggests that bed length is of less importance when considering the removal of biological contaminants (Freitas et al. 2021; Verma et al. 2019). It has been suggested that the majority of biological removal occurs in the top section of the filter bed, due to the filter environment being hostile to most pathogens and the presence of predatory grazers (Chen et al. 2021; Matuzahroh et al. 2020; Hijnen et al. 2021; Bellamy et al. 1985). However, for the removal of chemical contaminants there is evidence to suggest that longer filter beds are more effective, particularly against recalcitrant compounds (Jun et al. 2002; Hoang et al. 2008; De Vera et al. 2019). It remains unclear what effect filter bed length may have on the biological community within the filter bed. Different filter bed lengths may facilitate different nutrient or oxygen gradients leading to varying selective pressures and thus community composition (Boon et al. 2011). Furthermore, it remains unclear what effect filter bed depth may have on the biological composition of the effluent water. The microbial community of the filter bed has been shown to have some influence on the biological composition of the effluent water (Lautenschlager et al. 2014; Vignola et al. 2018). Thus, changes in the filter bed community attributed to filter bed length may have implications for the community composition and biological stability of the effluent water. Previous studies have also indicated an increased

abundance of LNA, or nanobacteria present in the effluent water following biofiltration (Lautenschlager et al. 2014; Vital et al. 2012; Chan et al. 2018). Recent advancements in metagenomic and single cells sequencing techniques have greatly expanded the bacterial domain of the tree of life to include CPR bacteria under a single phyla known as Patescibacteria (Hug et al. 2016; Parks et al. 2018). Characterised by small cell and genome size, these bacteria have been largely overlooked in biofilter studies.

The work in this thesis aims to take the initial steps in closing the knowledge gap required to develop an optimised point of entry method of water treatment for remote rural areas. Specifically, to investigate changes in the microbial composition of biofilters both through depth and over time and to investigate the effect filter bed length has on the filter bed community, effluent water quality and biological composition of the effluent water. This was achieved through the design and operation of several lab scale biofilters over 23 weeks. To determine the effect of filter bed length, three different column sizes, short (30cm), medium (60cm) and long (90cm) were run in parallel. These biofilters were designed to allow frequent (every second week) sampling of the effluent water to monitor for key chemical and biological contaminants and samples for molecular analysis. Further sets of 90cm columns were also run in parallel and sacrificially deconstructed at weeks 5, 9, 12 and 23 to gain access to various depths of the filter bed allowing a profile of community changes to be developed over time and through the depth of the filter bed. At these deconstruction timepoints, the influent and effluent water was also filtered through a 0.1µm membrane filter, to characterise the Patescibacteria which may be present.

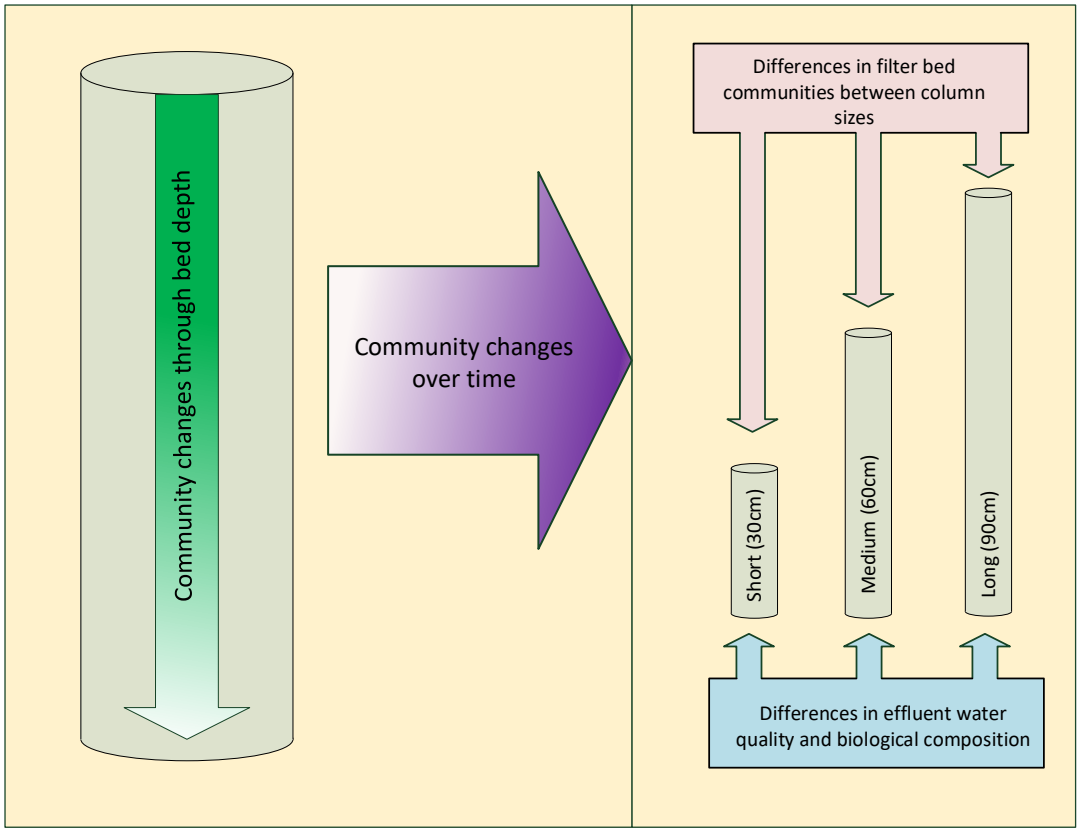


Figure 1.1. *Summary of the main areas of investigation provided through the running of lab scale GAC biofilters.*

While it would be possible to take media samples from full scale biofilters already in operation, the eventual endpoint of this research is seeking to introduce decentralised biofiltration units for rural areas, potentially to be set up in the homes of consumers. Thus, these communities may not be representative of those able to be cultivated in a reduced home filtration unit. This is due to various conditions which may influence community development, but which would not be replicated in a home unit. Conditions such as seasonal and weather changes, exposure to wildlife and extensive pre-treatment of influent water (Gerrity et al. 2018; Ma et al. 2020; Nájera et al. 2021). Lab scale filters have been shown to mimic the communities found in full scale slow sand filters and allow for much greater control of the experiment design (Haig et al. 2014). Aside from eliminating factors such as weather and seasonal changes, operating lab scale filters allows for wider scope in terms of sampling, media selection and influent water choice. Furthermore, initial community changes can be investigated in the early weeks of a filters lifespan which may not be possible when sampling from filters already in operation.

The biofilters operated in this study were constructed using PE80 and MDPE pipe fittings. These materials have been widely utilised in drinking water distribution systems and easy to obtain. The filters were operated at a flow rate of 1ml/min which is comparable to that of a full-scale slow sand filter (between 0.1 and 0.2m/h) and deemed manageable in terms of influent water requirements (Maurya et al. 2020). The media selected was granular activated carbon (GAC) which has been shown to harbour a greater density of biomass and diversity than sand and have been shown to outperform sand filters in terms of contaminant removal (Emelko et al. 2006; Reaume et al. 2015). This is likely due to the large surface area and porous structure of GAC allowing more space for bacterial attachment, and its high adsorption capacity. GAC has a high affinity for organic adsorption and is often used as a biologically independent form of water treatment (Jung et al. 2001). However, once the adsorption sites are saturated, the biofilm colonising the GAC becomes the main mechanism for water treatment, at this point it is known as biologically active carbon (BAC) (Lu et al. 2020). From a design perspective, the adsorption capacity of GAC may be advantageous to a home filtration unit as it would allow some form of water treatment to take place in the early days of a filter lifespan before the biofilm is fully developed. However, from an experimental perspective it may prove difficult to decouple adsorption from biological mechanisms of water treatment. Again, as the main overall objective is the design of a household point of use filtration system, the potential benefits of GAC were deemed more valuable than avoiding experimental complications.

1.1.1 Research Aims and Objectives

The aim of this research is to utilise a series of lab scale GAC biofilter columns to address several objectives set out below.

- To investigate the changes in the microbial community of the filter bed over time and spatially through the depth of the filter bed.
- To investigate the effect of column length on effluent water quality against key biological and chemical contaminants.
- To investigate the effect of column length on the biological composition of the effluent water and how it may change with time.
- To investigate and characterise the community of Patescibacteria found in the influent water, effluent water and filter bed.

1.1.2. Thesis Outline

Chapter 1: Introduction and Literature review – The remainder of this chapter contains a detailed literature review outlining the process of slow sand filtration and the biological communities involved in biofiltration.

Chapter 2: Biofilter Design and Performance – This chapter outlines the design and operation of the GAC biofilters utilised in this study. Additionally, this chapter investigates the effect biofilter column size has on biofilter performance against chemical and biological contaminants.

Chapter 3: Optimisation of Molecular Methods – This chapter outlines the optimisation of the molecular methods utilised in an attempt to limit as far as possible experimental bias and demonstrate a robust methodology generating data to inform the work in this thesis.

Chapter 4: Spatial and Temporal Investigation of Biofilter Microbial Communities – This chapter investigates the changes in the microbial communities of the filter bed over time and spatially through the depth of the filter bed. This chapter also investigates the effect of column size on the microbial community of the filter bed after 23 weeks of operation.

Chapter 5: Effect of Column Size on Effluent Microbial Communities - This chapter investigates the effect of column size on the biological composition of the effluent water and how it may be change over time.

Chapter 6: Characterisation of Patescibacteria in GAC Biofilters - This chapter investigates the Patescibacteria community found in the biofilters of this study by examining the phylogeny and abundance of Patescibacteria found in the influent water, effluent water and filter bed.

Chapter 7: Conclusions and Recommendations – This chapter outlines the main findings of this thesis and provides recommendations for future areas of research.

1.2. Slow Sand Filtration

1.2.1. History of Slow Sand Filtration

While there is evidence that suggests sand has been used as a filtration media as far back as 2000 BC, the first usage of slow sand filtration for treatment of a public water supply was recorded in 1804 in Paisley, Scotland. Seeking to improve the quality of water for use in his bleachery, John Gibb developed a primitive sand filtration system to treat the heavily polluted waters of the River Cart. This early filter depended on the lateral movement of water from a settling basin filtering through coarse gravel and finer sand to a clean water basin. The surplus filtered water was then sold and transported via cart to the people of Paisley (Baker 1949).

The Paisley filter, while undoubtedly a pioneer in public water treatment, was not the design on which modern slow sand filters have been modelled. In 1829, James Simpson, working for the Chelsea Water Company in London, designed the first piped slow sand filter for a public water supply. Occupying an acre of land, the London filter took its raw water from the River Thames and filtered it downward through layers of sand and gravel. The treated water was then piped to consumers in the city of London. Several design parameters refined by Simpson are implemented in modern filters to this day (Barrett et al. 1991).

When the health benefits of filtered water became apparent, the remaining five commercial water suppliers in London began filtering their water in 1839. The practice spread to continental Europe in the 1850's and the United States by 1872 (Barrett et al. 1991; Baker 1949). A famous example of the health benefits of slow sand filtration was illustrated during the Hamburg cholera epidemic of 1892. Hamburg drew its water from the River Elbe, and in 1892, 16,000 cases of cholera were reported leading to over 8000 deaths. In contrast, Altona, located downstream of Hamburg suffered only a few hundred cases as it had adopted the practice of slow sand filtration, providing a treated water source to the city (Hamlin 1990).

1.2.2. Components of a Slow Sand Filter

The basic design of a slow sand filter is relatively simple. The filter is usually contained within a concrete tank, though other materials can be utilised. A head of supernatant water lies upon a bed of filter media supported by gravel (Huisman and Wood 1974). Over time the

influent drains through the filter media and suspended particles are removed through a variety of physical and biological interactions. The filtered water is collected in an under-drainage system and removed via an outlet hose.

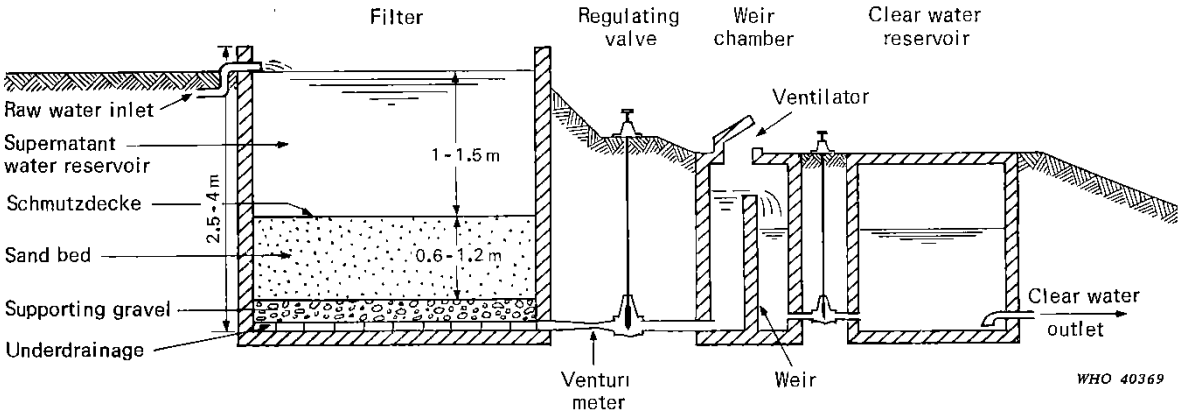


***Figure 1.2**. Diagram of a slow sand filter (Huisman and Wood 1974).*

Typically, slow sand filters operate with a flow rate between 0.1 and 0.4 M3/m2/Ł/h (Visscher 1990). The head of water will ideally provide enough pressure to drive the water through the filter bed by gravitational means while still ensuring a long enough retention time for the biological and physical interactions of filtration to take place (Guchi 2015). A head of between 1.0 and 2.0m is sufficient for most slow sand filters. Head loss can occur due to clogging or microbial overgrowth, signalling the need to clean the filter (Huisman and Wood 1974).

1.2.3. Drinking Water Contaminants

The supernatant water is slowly and continually introduced to the filter causing a head of water to rise above the filter bed. This water can be introduced to the filter after some pre-treatment or can be taken directly from ground or surface sources. All naturally occurring surface or ground water contain some level of contaminants that require removing by slow sand filtration (Ritter 2002). Various organic and inorganic compounds can be introduced through the geological strata or through pollution delivered by human activity (Fawell 2003). Common contaminants of drinking water can usually be classed as one of four types, physical, chemical, biological and radiological (Fawell 2003).

Physical contaminants are anything that alters the physical properties of the water, such as colour, odour, taste and/or turbidity. Water colour is affected by turbidity, dye pollution, algal blooms and/or a high concentration of metals such as iron or manganese (Sharma and Bhattacharya 2017). Water odour can often be attributed to microbiological contaminants, such as cyanobacteria or actinomycetes (Watson et al. 2008; Zaitlin and Watson 2006). The decomposition or metabolic products from such microbes can result in the release of odour causing compounds such as geosmin and 2-methyl isoborneol (Bruder et al. 2014). Other contaminants can also alter the odour and taste of drinking water. For example, high metal or sodium concentration can lead to metallic or brackish tasting water (Burlingame et al. 2007). While physical contaminants may pose less risk to human health, discoloured or foul-tasting water can be distressing to the consumer and must be considered when assessing the efficiency of the filtration process.

Chemical contaminants can include any naturally occurring or man-made element or compound. Naturally occurring contaminants can be introduced via the geological strata through which groundwater flows (Ritter 2002). Metals such as iron and manganese can be introduced and may subsequently be oxidized to compounds of low solubility, influencing the colour and taste of the water (Fawell 2003). Arsenic, a component of the earth's crust, can also be found in ground water at high concentrations in some parts of the world (Petrusevski et al. 2007). Inorganic arsenic as found in water sources is highly toxic and is a known carcinogen when consumed in drinking water (Rahman et al. 2009; Naidu 2009). Human activity can also introduce chemical contaminants to source water. Generally, these contaminants are introduced through "point" or "diffuse" sources (Fawell 2003). Point sources are usually identifiable and more easily measurable, for example discharge from an industrial or sewage plant. Diffuse sources can be more difficult to isolate, such as agricultural pesticides or fertiliser bleeding through to the water table (Wirmvem et al. 2017; Xiaoyan 2005). Many chemicals of natural or human origin can lead to detrimental health effects, for example high levels of nitrate in drinking water have been associated with methemoglobinemia occurrence (Fan and Steinberg 1996). Chemical contaminants can also encourage the growth of microorganisms. For example, the run-off from a sewage plant may provide an influx of nutrients encouraging microbial growth (Harry et al. 2016).

Biological contamination consists of organisms which can be found in the source water, particularly those which pose a risk to human health, including bacteria, viruses, protozoa and parasitic organisms (Cabral 2010). The majority of bacteria which naturally inhabit the

source water are not typically considered pathogenic. However some varieties such as *Pseudomonas* can be a danger to immunocompromised individuals (Mena and Gerba 2009). Intestinal bacteria which reside in both animals and humans present a much greater risk to human health (Viswanathan et al. 2009; Ashbolt 2004). Numerous diseases are associated with enteric bacteria that can be introduced to surface water via animal defecation, poor sanitation and poor sewage management (Cabral 2010; Pandey et al. 2014). The ingestion of pathogenic bacteria such as *Shigella*, *Legionella* and *Vibrio cholera* through drinking water can result in the outbreak of gastrointestinal illness and diarrhoeal diseases resulting in human fatalities, particularly in low to middle income countries (Inamori and Fujimoto 2010; Bain et al. 2014; Fischer-Walker et al. 2012). Other pathogens such as *Clostridium perfringens* can present a danger of infection when exposed to tissues, say through open wounds when bathing (Brook 2008). Due to the variety and culturing difficulties these pathogens present, indicator coliforms such as *Escherichia coli* are often used to determine if the source water is contaminated with enteric bacteria (Motlagh and Yang 2019).

While bacterial pathogens are often thought to be the main contributor to water borne illnesses, viral pathogens must also be considered when examining the cleanliness of drinking water. Viruses which may be introduced to source water via human wastewater include, adenoviruses, hepatitis A and E, polioviruses, enteroviruses and rotavirus (Gall et al. 2015). These viruses can cause a range of severe illnesses such as encephalitis, meningitis, myocarditis and polio and are also associated with gastroenteritis (Gibson 2014). Viral pathogens are more difficult to detect than larger bacterial or protozoan detection due to their small particle size and the lack of indicator organisms (Bosch et al. 2008). Identifying viral pathogens also involves costly and laborious molecular techniques that are not always feasible to carry out at all facilities. Due to the small size of viruses, filtration techniques may prove less effective than against larger organisms (Gall et al. 2015).

Protozoan and parasitical organisms are also a pressing concern when introduced to a water supply. Balantidiasis is an intestinal disease caused by the ciliated protozoa *Balantidium coli*, thought to be commonly introduced through pig waste (Schuster and Ramirez-Avila 2008). Amoebic dysentery caused by the parasite *Entamoeba histolytica* causes nausea, fever, ulcers and diarrhea and is common in developing countries (Ralston and Petri 2011). Giardiasis caused by the parasite *Giardia lamblia* is also a water borne risk to human health, made more problematic by its resistance to chlorine disinfection (Jarroll, et al. 1981). Another protozoan parasite which leads to gastroenteritis when consumed via contaminated drinking water is

Cryptosporidium (Betancourt and Rose 2004). Like Giardia, Cryptosporidium is resistant to traditional water treatment methods such as coagulation, filtration and disinfection by chlorine. This is due to both Giardia and Cryptosporidium being able to form oocysts, hardy and thick-walled cysts which contain the protozoan zygote (King and Monis 2007). These oocysts are expelled in the faeces of infected organisms and offer protection from environmental stresses (Betancourt and Rose 2004).

Radiological contaminants include elements which may undergo radioactive decay such as uranium, radium and potassium. These can be produced naturally and introduced to groundwater through the geological strata or can be introduced through human activity (Lytle et al. 2014). For instance, uranium has been shown to be introduced to ground water through the use of phosphate fertilisers (Liesch et al. 2015). While there is evidence to suggest that exposure to drinking water contaminated with radionuclides can increase the risk of certain cancers above 100mSv (WHO 2006), research is lacking concerning prolonged exposure at lower doses. It is assumed however, that a linear relationship exists between dosage and potential health concerns and so any exposure to radiological contaminants carries with it an inherent risk (WHO 2006).

1.2.4. Filter Bed Media

Sand is usually the media of choice in a slow sand filter due to it being cheap, durable and readily available, valuable attributes considering the large area covered by industrial slow sand filters. The grain of sand is usually kept quite fine and uniform at between 0.15-0.3mm and a uniformity coefficient of less than 3 (Huisman and Wood 1974). Finer sands will contribute to more thoroughly filtered effluent but may increase initial head loss (Verma et al. 2017). Courser sands may extend the biological activity to greater depths within the filter bed as nutrients can penetrate the filter bed more easily extending the reach of the biofilm. However, physical straining becomes less efficient and the risk of pathogens breaking through the filter bed becomes greater when using courser sand (Jenkins et al. 2011). Research conducted by Jenkins et al in 2011, concluded that sand size and retention times were the most significant factors in bacterial and viral removal, smaller grain size paired with longer retention times being the most efficient of the parameters tested (Jenkins et al.2011).

The size, grade, shape and chemical composition of sand is highly variable. Some examples include mixed silicate, carbonate sand, a mixture of inorganic and organic sand grains, black sand composed of volcanic rocks or heavy minerals and biogenic sand composed of shells and skeletons of marine organisms (Pettijohn et al. 2012). Different types of sand may have different adsorption properties as well as differing surface areas and charge for bacterial adhesion, therefore the physical and chemical properties of the sand may heavily influence the efficiency of a filter (Rolland et al. 2009). For instance, a study comparing three types of Moroccan sand determined that river sand made a more efficient filter bed than beach or desert sand for the removal of total suspended solids, chemical oxygen demand and faecal coliforms. This was attributed to the smaller particle size and increased concentration of aluminium and iron oxides and the organic matter constituting the make-up of the sand (Yettefti et al. 2013). While sand has been shown to adsorb some contaminants such as inorganics and dyes (Bello et al. 2013; Rodgers et al. 2005) its capacity for adsorption is lower and not as versatile as other media types such as granular activated carbon (Paredes et al. 2016). Lower adsorption rates mean that sand biofilters are more dependent on physical and biological methods of filtration though are less susceptible to media saturation and may potentially have a longer service life (Xu et al. 2021). This lack of saturation allows sand to be dried and reused during re-sanding potentially lowering the cost of filter maintenance.

An often-used alternative media is granular activated carbon (GAC). GAC is produced from high carbon organic materials such as coal, wood and coconut shell (McDougall 1991). These raw materials are thermally or chemically activated to produce a porous, granular, carbonaceous substance with a large surface area and high adsorption capacity for organic material (McDougall 1991). This means that organic particles are drawn to the surface of the GAC and held by Vann der Waals forces, removing them from the water (Jung et al. 2001). The manufacturing method and initial raw materials used heavily influences the properties of the GAC produced, particularly its porosity and adsorptive qualities. A particle of GAC consists of larger macropores, often linked to the outer surface of the GAC and micropores found deeper within the particle (Lu et al. 2020; Velten et al. 2007). Pores which are over 50nm in diameter are described as macropores, those with a diameter of between 2nm and 50nm are described as mesopores and micropores have a diameter of less than 2nm (Mahajan et al. 1980). These are linked by transitionary pores ensuring a large surface area is available for adsorption and bacterial attachment. It is thought that up to 97% of the surface area of GAC is contained within the micropores (Yenkie and Natarajan 1993)

Due to its high adsorption potential GAC is commonly used in water treatment, either as a late treatment chemical adsorption method or as media in a granular biofilter. Often GAC is used as an adsorption method of water treatment often after ozonation and is backwashed frequently to prevent biofilm formation (Corwin and Summers 2011). An advantage of using GAC, as a late-stage adsorption medium, is that it can be used to remove disinfection by products (DBP's) produced after chlorination (Kim and Kang 2008; Erdem et al. 2020). Strong oxidising agents such as chlorine and chloramine can react with organic matter present in the water to form DBP's such as haloacetic acids (HAA's) and trihalomethanes (THM's) which have been linked to certain cancers (Villanueva et al. 2006). The major issue with using GAC in this fashion is saturation. Over time organic compounds bind to the surface of the GAC until all adsorption sites are occupied, allowing contaminants to breakthrough (Kennedy et al. 2015). When this occurs, the GAC may be rendered useless for adsorption and must be replaced or regenerated. GAC can be regenerated and reused through chemical or thermal means, but regeneration may be costly and laborious (Lambert et al. 2002; Larasati et al. 2021).

Alternatively, biofilm can be allowed to develop on the GAC at which point it becomes biologically active carbon (BAC). The advantage of BAC is that even after a saturation point is reached the media continues to function, though the mechanism of removal is through biological activity rather than adsorption (Liang et al. 2007). BAC filters generally undergo three phases during their service time. During phase 1, most of the organic removal can be attributed to adsorption as the biofilm has not yet been fully established. With time adsorption decreases as adsorption sites are used up while biological activity increases as microorganisms replicate and acclimatise. Phase 2 is characterised by both mechanisms working in parallel. Finally, during phase 3 or the steady state phase all the carbon has been saturated and a fully established biofilm is now responsible for the majority of contaminant removal (Velten et al. 2011).

Several studies have shown biologically active carbon consistently outperforming filters of other media such as sand and anthracite (Reaume 2012; Reaume et al. 2015; Emelko et al. 2006; LeChevallier et al. 1992; Cochran, Lalor, and Barron 2010; Paredes et al. 2016). For example, a study by Cochran et al 2010, compared steady state BAC to a dual media sand/anthracite filter and a ceramic media filter. This study found that there was little difference in TOC removal between the sand/anthracite and the ceramic filter, while the removal levels of the BAC was markedly higher (Cochran et al. 2010). Another study,

conducted by Emelko et al 2006, also demonstrated higher TOC removal by BAC when compared to anthracite, particularly at lower temperatures (Emelko et al. 2006). BAC has also been shown to outperform sand in DOC and genotoxin removal from secondary municipal wastewater. Poorly biodegradable organic compounds were also shown to be better removed by BAC (Reaume 2012; Reaume et al. 2015). Concurringly, research conducted by Paredes et al 2016, demonstrated that BAC significantly outperformed sand when removing organic micropollutants from secondary effluent, particularly against recalcitrant compounds such as diazepam or diclofenac (Paredes et al. 2016).

There are currently two schools of thought as to why steady state BAC offers superior organics removal to sand or anthracite despite fulfilling its adsorption potential. The first is the density of biomass that can be supported by GAC, which has been shown to be higher than sand or anthracite (Wang et al. 1995; Reaume et al. 2015; Shimp and Pfaender 1982). The irregular shape and porosity of GAC is thought to offer protected areas and crevices which prevent recently attached bacteria from being sheared off the surface due to backwashing or fluid forces (Shimp and Pfaender 1982). As bacteria have been shown to populate irregularities in the topography of sand grains at higher densities, (Weise and Rheinheimer 1977) it stands to reason that the more numerous pores and fissures in the surface of GAC would serve to encourage bacterial growth at a higher density to sand which is typically smoother. The pores and irregularities in GAC also serve to increase the surface area available for bacterial attachment. The importance of the topography of GAC regarding bacterial adhesion was demonstrated by Wang et al 1995, in a study comparing three types of GAC (bituminous, wood and lignite coal based) to sand and anthracite filters. All three GAC filters held over three times more biomass than either the sand or anthracite filters, however the lignite-coal based GAC held considerably more than the bituminous or wood-based GAC. The bituminous GAC, while having the largest surface area was microporous, preventing bacteria from penetrating further than the surface of the grain. The wood-based GAC was microporous, however had the largest surface area of the three. The mesoporous lignite-coal based GAC, was thought to have offered the best conditions for bacterial attachment consisting of a balance in porosity and surface area. Reducing the grain size, thereby increasing the effective surface area of GAC was also shown to lead to an increase in biomass density in this study. Although it should be noted that surface tension and charge may have also contributed to the difference in bacterial density (Wang et al. 1995). The surface charge

of activated carbon has been shown to be altered by a biofilm through changes in local pH, potentially increasing its adsorption potential to heavy metals (Rivera-Utrilla et al. 2001).

Another attribute of GAC, which may lend itself to increasing biomass density, is its adsorption capacity. Biodegradable organic compounds are readily adsorbed to the surface of the GAC, presenting a dense bed of substrate to sustain bacterial growth (Li and DiGiano 1983; Walker and Weatherley 1999). Interestingly, research conducted by Li and DiGiano, 1983, seemed to indicate that surface area and texture was less important in terms of increasing biological activity compared to the availability of adsorbed materials (Li and DiGiano 1983). This is dependent on desorption of materials from within the micropores of the GAC. While bacteria are typically too large to penetrate the micropores of GAC, smaller organic molecules may pass through and occupy adsorption sites within (Yongmei et al. 2020; Ocampo et al. 2013). It is thought that these molecules may eventually become desorbed based on a concentration gradient of the external environment (Morley et al. 2006). Once desorbed these materials migrate from the interior of the GAC and are free to be utilised by the bacteria on the surface (Olmstead and Weber 1991). This may offer insight as to why decreasing the grain size seems to increase the biological load of the GAC despite the entirety of the surface area not being utilised by bacteria (Wang et al. 1995; Shimp and Pfaender 1982). When reducing the grain size the effective surface area increases while the diffusion distance between the micropores and the external environment decreases, allowing bacteria easier access to internally adsorbed particles (Li and DiGiano 1983).

The availability of adsorbed compounds gives rise to another mechanism that is thought to improve the removal capacity of BAC over non-adsorbing media, known as bioregeneration. Bioregeneration is defined as the renewal of the adsorption capacity of activated carbon by biological activity (El Gamal et al. 2018). Bioregeneration is thought to be a major factor in the removal of poorly biodegradable organic matter and the extension of the service life of activated carbon (Aktaş and Çeçen 2007). Desorption of organic molecules from active carbon has been shown to be a prerequisite for bioregeneration (De Jonge et al 1996; Walker and Weatherley 1997). Thus, materials which irreversibly adsorb are not thought to be candidates for bioregeneration (Walker and Weatherley 1997). Two models have been proposed for the mechanisms tanking place during the bioregeneration of active carbon. The first is bioregeneration by the action of exoenzymes and the second is via desorption of materials influenced by a concentration gradient (Aktaş and Çeçen 2007).

It has been hypothesised that exoenzymes released by bacteria penetrate the micropores of the GAC and act upon the adsorbed organics within (Sirotkin,et al 2001). The action of these enzymes alters the substrate resulting in hydrolytic degradation or simply desorbed and made available for biodegradation (Sirotkin,et al 2001). The validity of this hypothesis has been called into question however, by suggesting that exoenzymes released by the biofilm would themselves be adsorbed before acting upon their substrate (Li and DiGiano 1983). Furthermore, it has been suggested that the small size of the micropores would not facilitate the entrance of the enzymes or would severely inhibit their action if they managed to achieve access (Xiaojian,et al. 1991; Aktaş and Çeçen 2007). However, this would not exclude the action of exoenzymes in macropores and there is some evidence of enzymatic action occurring in mesopores (Aktaş and Çeçen 2006).

The competing hypothesis suggests that desorption of organic materials is facilitated by a concentration gradient which are then biodegraded as they diffuse from the carbon. The metabolism of the biofilm on the surface of the carbon causes a reduction of organic matter in the liquid phase in turn causing a concentration gradient between the micropores of the GAC and the bulk liquid (Morley et al. 2006). Readily desorbable materials react to this gradient and diffuse from the micropores where they can be accessed and biodegraded by the bacteria on the surface of the GAC (Kim et al. 1986; Schultz and Keinath 1984). Bioregeneration in this manner is thought to explain the higher removal efficiency of recalcitrant and poorly biodegradable compounds when compared to sand or other media. The removal of readily desorbable organics through biodegradation frees adsorption sites that would otherwise be occupied. Recalcitrant compounds are then able to occupy these sites, removing them from the supernatant water (Olmstead and Weber 1991).

1.2.5. Effluent Quality and Optimisation

The current model of slow sand filtration delivers effluent water of a reasonable quality through a combination of physical/chemical mechanisms of filtration and biological action (Huisman and Wood 1974). While slow sand filtration has shown to be highly effective at removing several contaminants (Table 1.1) improvement is needed to reach the standard of drinking water required by water companies and the regulator.

***Table 1.1**. Average removal efficiency achieved by slow sand filtration for a range of parameters.*

Parameter	Removal efficiencies	References
Turbidity	<1NTU	(Lambert and Graham 1995a; Guchi 2015; Ellis and Wood 1985; Poynter and Slade 1978; Aslan and Cakici 2007; Lambert and Graham 1995b; Ellis and Aydin 1995; Bagundol, Awa, and Enguito 2013; Haig et al. 2011; Ellis 1987)
Coliform bacteria	>99%	
Enteric bacteria	90-99.9%	
Enteric Viruses	99-99.9%	
True colour	25-40%	
Total organic carbon (TOC)	<15-25%	
Dissolved organic carbon (DOC)	5-40%	
Biodegradable dissolved organic carbon (BDOC)	46-75%	
Assimilable organic carbon (AOC)	14-40%	
Nitrate	95%	
UV absorbance	5-35%	
Trihalomethane (THM)	<25%	
Iron and Manganese	30-90%	
Pesticides	0-100%	

Without optimisation slow sand filtration generates relatively clean effluent, however may fall short of the parameters set out by the European Drinking Water Directive (Petrescu-Mag and Petrescu 2014). Slow sand filtration may currently be sufficient for the removal of some contaminants such as nitrate or iron, 50mg/l and 200µl/l permitted respectively, depending on the initial concentration present in the source water. Other parameters such as enteric bacteria and viruses must be completely removed from the water before it is deemed safe to consume (Petrescu-Mag and Petrescu 2014). Slow sand filtration offers some advantages over more energy intensive means of treatment. The simplicity of design and manufacture in theory should allow communities to build and maintain these filters using materials that are cheap and likely to be sourced with ease. A good quality of effluent can be achieved with little investment in cost or energy and require little supervision or maintenance to run, ideal for small rural communities. Traditionally, the main drawbacks of slow sand filtration have been

the large land footprint required and the undefined nature of the biological mechanisms at play. However, research into the biological workings of a slow sand filter may reveal opportunities for optimization, allowing a great reduction in size while still providing excellent water quality. It is likely that even after optimisation a final polishing step would be required for a point of use biofilter to meet the exacting standards set out in the European directive. However, optimisation and improvement of current slow sand filtration efficiency may reduce the severity of this final polishing step in a low cost and low energy manner. In order to successfully optimise the filtration process, the mechanisms responsible for contaminant removal in the filter bed must first be understood.

1.2.6. Physical/Chemical Mechanisms of Filtration

A proportion of the contaminants removed from the source water during slow sand filtration are not attributed to biological action. Many contaminants are removed through chemical interactions with the media and other particles are physically prevented from passing through the filter bed. These mechanisms can be described as transport mechanisms, those which bring the contaminant particles into contact with the filter media, and attachment mechanisms, those which hold the contaminants in place (Huisman and Wood 1974).

The simplest of these non-biotic transport mechanisms is straining or screening. Particles that are larger than the spaces between the grains of the media are excluded from passing through and rest on the top of the bed (Verma et al. 2017). The efficiency of straining is related to media grain size, with smaller, more numerous grains being more effective than larger grains. As time passes, straining becomes more effective as the growth of a biofilm and the collective build-up of particles further reduces the space between the grains, allowing smaller particles to be removed in this fashion (Weber-Shirk and Dick 1997). Other transport mechanisms are sedimentation and hydrodynamic action. Sedimentation occurs when particles of higher density than the surrounding water settle on the surface of the media (Ellis and Wood 1985). Sedimentation can occur throughout the filter bed as any upward surface of the media grains offers a potential site for particles to settle. The efficiency of sedimentation is related to the porosity and the flow rate of the filter (Ellis and Wood 1985). Hydrodynamic forces may act on particles with a higher specific gravity to that of water, the inertia of such particles causing them to leave the flow path and impact the grains of the media. Such

particles may then themselves act as interceptors, reducing the space between the grains and catching other particles as the travel through the bed (Guchi 2015).

Other mechanisms can be described as both transport and attachment mechanisms, these include Van der Waals and electrostatic forces. The attraction of oppositely charged particles may cause contaminants to be transported towards the media grains and held (Guchi 2015). Alternatively, oppositely charged particles may bind to intercepting particles already attached to the media. Although weaker, Van der Waals forces may also attract and hold particles within the bed providing no other forces, such as electrostatic repulsion, override it (Huisman and Wood 1974).

An attachment mechanism of great importance when using GAC as a medium is adsorption. Adsorption is the attachment of particles to a solid surface through various means of bonding, such as Van der Waals, covalent or electrostatic (Jung et al. 2001). This type of bonding is dependent on the chemical properties of both the media and the adsorbing material. Particles which are adsorbed to the media can again act as interceptors catching further particles or can act as substrate for bacterial growth. Particles can also become attached to the extracellular polymeric substances (EPS) extruded from the biofilm (Weber-Shirk and Dick 1997). As a biofilm develops, EPS are secreted, forming a matrix which aids surface attachment and protects the integrity of the biofilm (Czaczyk and Myszka, 2007.). Particles which are transported to the EPS layer can become bound within it. Assimilable compounds may eventually be utilised by the bacteria within the biofilm for growth or division, while inert compounds may remain within the EPS matrix until the media is cleaned or replaced (Weber-Shirk and Dick 1997; Huisman and Wood 1974).

1.2.7. Biological Mechanisms of Filtration

While it is known that biological activity plays a pivotal role in the workings of slow sand filtration, the exact mechanisms are not entirely understood. The literature generally agrees that the majority of biological activity occurs in the uppermost 400mm of the filter bed and the schmutzdecke, though biological activity has been shown to occur throughout the depth of the bed, albeit to a lesser extent (Ellis and Wood 1985; Guchi 2015; Huisman and Wood 1974; Aslan and Cakici 2007). The schmutzdecke or “dirt cover” is a slime layer of highly diverse and active organisms that develops at the sand-water interface. As raw water is fed

into the filter, particulate organic matter is transported to the top of the filter media where it builds a thick layer of substrate. Bacteria and other organisms, initially seeded from the source water, selectively multiply using these organics to fuel their metabolism (Ellis and Wood 1985). The schmutzdecke consists of a wide variety of organisms, such as algae, bacteria, archaea, fungi, protozoa, amoeba, various eukaryotes and invertebrates (Wakelin et al. 2011a). These organisms are thought to contribute to the purification process through various mechanisms, such as the assimilation of biodegradable materials and predatory grazing, both within the schmutzdecke and deeper in the filter bed (Unger and Collins 2008; Maurya et al. 2020; Fitriani et al. 2020; Guchi 2015). Thus, it is the combination of the biological utilisation of organics and other compounds for cellular metabolism and growth, the interactions between predatory organisms and prey and bio-antagonism (the growth of one organism inhibiting the growth of another) that are thought to be the main biological mechanisms of contaminant removal within biofilters (Guchi 2015).

Bacterial metabolism removes contaminants from raw water through two processes, assimilation and dissimilation. Organic compounds, such as carbohydrates and lipids are taken up by the cell and broken down through oxidation reactions to generate energy, for example, the aerobic respiration of glucose to produce CO_2, H_2O and ATP (Rezvani et al. 2019; Reber 1974). This breaking down of organic substrates is known as dissimilation. Alternatively, bacteria may uptake organic or inorganic compounds to maintain cellular activity, these are assimilation reactions (Jurtshuk 1996; Ali et al. 2020). Bacterial growth is therefore controlled by the concentration of easily assimilable compounds available in the local environment (Tsai et al. 2004). As a slow sand filter matures, the growth rate of bacteria eventually stabilizes with the death rate. As cells perish, the dissimilation products and other organic materials are released, becoming available to organisms within the filter bed (Bayles 2007; Ye et al. 2010). These products are in turn utilised resulting in the gradual transformation of these organic contaminants into more innocuous compounds throughout the depth of the filter bed (Huisman and Wood 1974; Ellis and Wood 1985). As the bio-available compounds are used by bacteria, organic nutrients become scarcer resulting in a reduction of biomass deeper in the filter bed. A gradient of biomass concentration can be expected in the filter bed as a result, with the highest concentration at the top of the bed, reducing with depth (Campos et al. 2002).

One of the most important features of any water treatment system is the removal of pathogenic organisms, achieved in slow sand filtration mainly through biological action.

While it is possible that some pathogens may be removed though adsorption or physical straining, the high removal efficiency achieved by slow sand filtration is thought to be mainly attributed to predation (Weber-Shirk and Dick. 1997.; Haig et al. 2013; Haig et al. 2015; Hijnen et al. 2007). Pathogenic organisms such as enteric bacteria, viruses and protozoan oocysts are removed from the source water as it passes through the biofilter by complex food web interactions composed of predatory bacteria, protozoa and various eukaryotes. The importance of predator action in the removal of pathogens has been demonstrated by many authors. For example, a study conducted by Weber-Shirk et al 1999, examined *E. coli* removal by slow sand filtration in the presence and absence of sodium azide. Sodium azide is a reversible inhibitor of oxidative phosphorylation and can therefore be used to block the metabolism of bacterial and protozoan predators in the filter while leaving the chemical attributes of the biofilm and media unaltered. As the removal of *E. coli* rapidly reduced in the presence of sodium azide the authors concluded that predation was the major contributor of bacterial removal and adsorption was of little significance (Weber-Shirk and Dick. 1997). Further research by Weber-Shirk demonstrated that 99.7% of *E. coli* was removed in the presence of a predatory heterotrophic nanoflagellate which was recovered and from the effluent of a slow sand filter and cultured before being added to a new filter. This was compared to a control filter which was not seeded with the nanoflagellate culture resulting in less than 10% *E.coli* removal in the same timeframe (Weber-Shirk and Dick 1999). Haig et al 2015, further demonstrated through Stable Isotope Probing and subsequent metagenomic analysis that non-specific protozoan grazing was a major factor in slow sand filtration, contributing up to 99% of the total *E. coli* removal. In addition, viral lysis was also found to regulate *E. coli* populations through fluctuations of lysogenic and lytic cycles (Haig et al. 2015). Removal of *Cryptosporidium* oocysts and spores of *C. perfringens* in slow sand filters have also been attributed to predatory action (Hijnen et al. 2007). A study conducted by Elliot et al. 2011, examined the removal potential of viral particles from slow sand filters. The authors concluded that the removal of the viral particles could be attributed to either predatory grazing or the release of proteolytic enzymes from within the biofilm. The authors concluded that further research was required to differentiate the importance of each potential pathway (Elliott et al. 2011).

The action of predators is largely beneficial to the filtration process, however there may be some potential drawbacks. When a pathogen is ingested by a predator it is ideally digested and thus removed from the water system. Some pathogens however, may produce hardy

spores or oocysts or themselves be resistant to digestion (Bichai et al. 2014). In this case, pathogenic organisms can be transported within the predator and protected against further predation or environmental stresses. In time cell lysis may occur or the predator may release the undigested pathogen with its excreted waste allowing it to potentially break through the filter bed (Bichai et al. 2014; Hijnen et al. 2007). King et al 1988, demonstrated that ingested coliforms can be recovered and cultured form protozoan hosts following disinfection by chlorination (King and Shotts 1988). Likewise, Freely et al 1988, demonstrated that *Giardia* cysts which have shown resistance to chlorine at low temperatures, can play host to intracellular bacteria (Jarroll et al. 1981; Feely et al. 1988). The ability of pathogens to 'hide' from chlorine disinfection within other organisms is problematic as chlorine is heavily relied upon in many water treatment systems.

Some organisms have adapted to the presence of predators and evolved parasitic or endosymbiotic relationships. Ingested bacteria are thought to behave in one of three ways when taken into a host cell, assuming digestion does not take place (Brown 1999). Certain coliform species such as *Salmonella typhimurium* survive intracellularly as described above but do not undergo cellular multiplication (King and Shotts 1988). Other species invade the cell and begin to multiply, though do not cause the host cell to lyse. Both *Mycobacterium* and *Vibrio cholerae* have been shown to multiply within amoeba and have been found to survive within amoeba cysts. In this case the amoeba acts as a reservoir to maintain the bacteria and aid their propagation (Yu et al. 2007; Thom et al. 1992). Finally, an organism can invade the cell and multiply causing the host cell to lyse, the most studied pathogen being *Legionella* (Fields et al. 2002; Garduñ 2007). *Legionellae* are aerobic, gram-negative bacteria found in most aquatic environments and multiply within amoeba. When internalised by an amoeba, *Legionella* inhibits phagosome/lysosome bonding thus preventing digestion. The *Legionella* then proceeds to multiply within a phagosome that associates with the rough endoplasmic reticulum. Eventually the *Legionella* lyses the host through the formation of pores in the cellular membrane, freeing the newly divided cells (Garduño 2007). *Legionella* is of importance as certain species of the genus, such as *Legionella pneumophila,* are opportunistic human pathogens (Fields et al. 2002). Legionella, being heavily associated with biofilms and protected by amoeba during multiplication, have also been shown to be enriched by slow sand filtration (Calvo-Bado et al. 2003).

Bio-antagonism is a term used by some authors to describe several biological mechanisms which remove pathogens from raw water during slow sand filtration. These are

environmental or biological factors which inhibit the growth of pathogens within the filter bed (Guchi 2015). One such factor is competition between the established, indigenous bacteria which were seeded by the source water and introduced pathogens. The environment in a slow sand filter can be described as oligotrophic, or nutrient deprived, therefore favouring the growth of oligotrophic organisms (Guchi 2015). Enteric pathogens often introduced through human or animal waste differ in their requirements to environmental pathogens such as *Mycobacterium* or *Legionella* found naturally in the source water (Pacheco and Sperandio 2015). Environmental pathogens are often opportunistic causing illness only when the immune system is in some way compromised (Rusin et al. 1997). Enteric pathogens, often favour high nutrient environments and have higher specific growth rates thus may be outcompeted by indigenous bacteria which prefer a nutrient scarce environment and have lower specific growth rates (Coveney and Wetzel 1995; Hendricks 1972). Similarly, the temperature of the water within the filter may lend a competitive advantage to the indigenous community. The optimum temperature of many enteric pathogens is 30-37ºC (Motsi et al. 2002). At lower temperatures, their metabolism and growth rate will begin to slow (Abberton et al. 2016). Organisms with a lower optimum temperature will grow at a higher rate, again outcompeting enteric pathogens. Although it should be noted that at lower temperatures predatory protozoa may also have a lower metabolism reducing removal through predation (Huisman and Wood 1974). The optimum temperature range for the biological removal of contaminants in a slow sand filter is generally accepted as between 15-25°C (Stewart-Wade 2011; Gimbel et al. 2006).

1.2.8. Microbial Community Structure and Activity

The microbial community is responsible for the biological filtration taking place within a slow sand filter and its importance cannot be overstated. In order to optimise this biofiltration, the organisms and their processes must be identified so desirable activity can be enhanced and directed. The microbial community within a slow sand filter can be highly variable due to the many factors which can affect its composition. Nevertheless, several authors have investigated the microbial composition of slow sand filters (Bai et al. 2013a; Li et al. 2017; Wakelin et al. 2011; Pfannes et al. 2015; Haig, Quince, et al. 2015; Haig et al. 2014; Chen et al. 2021; Fitriani et al. 2020). The composition of the microbial community observed may be attributed to many factors. For example, temperature or nutrient availability

may lend a competitive advantage to some species more than others, allowing the advantaged organisms to propagate at the expense of the disadvantaged organisms (Hibbing et al. 2010). Also, as slow sand filters are thought to be seeded by the source water, differing populations may be introduced to the filter via differing influent (Li et al. 2017). This makes comparison of communities between filters difficult when considering optimisation of biological activity and may explain the variety evidenced between the authors cited (Haig et al. 2014; Pfannes et al. 2015; Wakelin et al. 2011). The microbial community can also vary within a filter. It is generally accepted that biomass is greatest at the top of the filter bed and so the microbial community will change with depth where the nutrient availability also changes (Pfannes et al. 2015). Filter maturity can also dictate the composition of the microbial community as certain organisms become more established with time (Haig et al. 2014; Li et al. 2017).

Despite the high variability of slow sand filtration community compositions, several phyla have been shown to be consistently predominant. Proteobacteria, for example, have been shown to be the predominant phylum present in the filter (Wakelin et al. 2011; Li et al. 2017; Haig et al. 2014; Pfannes et al. 2015). While the ratio varies between studies, the dominant classes of proteobacteria appear to be the Alpha, Beta and Gamma classes (Wakelin et al. 2011; Haig et al. 2014; Pfannes et al. 2015). The proteobacteria within slow sand filters are richly varied and no one family appears to dominate (Wakelin et al. 2011). Actinobacteria is another phylum which appears in high abundance in a slow sand filter, though their abundance varies between studies more so than the proteobacteria (Wakelin et al. 2011; Haig et al. 2014; Pfannes et al. 2015; Li et al. 2017). Dominant families of Actinobacteria as described by Wakelin et al 2011, include the *Micrococcaceae*, *Micromonosporacea*e, and *Mycobacteriaceae* (Wakelin et al. 2011). Other notable phylum include, Firmicutes, Planctomycete, and Bacteroidetes. The vast majority of the biomass present in a slow sand filter is bacterial with eukaryotes and archaea only constituting a small fraction in comparison (Bai et al. 2013). The majority of eukaryotic organisms appear to be predatory protozoa, thought to be enriched due to the high volume of food available in the schmutzdecke (Wakelin et al. 2011; Pfannes et al. 2015).

The predominant phylum observed may not be entirely unexpected as they are ubiquitous and usually predominant in fresh water sources, particularly Betaproteobacteria and Actinobacteria (Newton et al. 2011). As the filter bed is populated by organisms seeded from the raw water initially it stands to reason that the most numerous organisms in the source water would be found to be most predominant in the filter bed. As the filter matures however,

selective pressures may encourage the proliferation of less predominant species altering the community composition and evenness in the filter bed from the source water (Haig et al. 2014). Research conducted by Haig 2014, compared the microbial community of a filter which had been initially sterilised before being allowed to grow a biofilm with a non-sterile laboratory filter and with the community found in an industrial filter. In the first three weeks of the filters run, both laboratory filters communities were heavily predominated by proteobacteria, much like the influent water, and lacked the diversity of the industrial filter, particularly in the case of the sterilized filter. By week ten, the percentage of proteobacteria had reduced and the diversity of both laboratory filters was comparable to the industrial filter. This demonstrates the change in community composition as a filter matures and that laboratory filters can indeed mimic the community composition found in industrial filters within a relatively short timeframe (Haig et al. 2014). Further research by Haig offered concurring evidence that both diversity and community evenness increased with filter maturity. Increasing community evenness was also positively correlated with improving effluent quality and thus filter performance (Haig et al. 2015).

When looking to optimise the biological processes of filtration taking place within the filter bed, the microbial community composition is of importance, as it is the microbial community which is ultimately responsible for a great deal of the removal efficiency achieved by slow sand filtration. It is important to consider however, that many organisms may be present but inactive, thus not contributing biologically to contaminant removal. Metagenomic analysis or 16S rRNA amplicon sequencing of the microbial community as performed by many authors are valuable in determining the structure of the community but are limited in determining which species or genes are active and which processes are taking place (Wakelin et al. 2011; Haig et al. 2014; Haig et al. 2015; Pfannes et al. 2015). As metagenomic analysis encompasses the entirety of the community's genetic material, genes which may potentially be expressed and contribute to contaminant removal can be identified. However, such analysis cannot determine which of these genes are being actively expressed. A study of transcribed genes through RNA analysis would offer great insight into the microbial workings of a slow sand filter. Currently research into the actively transcribed genes of a slow sand filter community is lacking. Therefore, a full transcriptomic profile throughout the depth of the filter bed would be invaluable when considering optimisation and a promising avenue of research. Similarly, biological activity could be investigated through analysis of the

proteome. Determining which proteins are being expressed would give a strong indication of the processes occurring within the filter bed.

Previous research has attempted to shed some light on the biological processes which occur within the filter bed, the most studied being the fate of inorganic nitrogenous compounds and the metals iron and manganese (Nakhla and Farooq 2003; Murphy, McBean, and Farahbakhsh 2010; Bai et al. 2013; Manav Demir 2016). The removal of ammonia, nitrite and nitrate occurs through the process of nitrification. Nitrification is driven by microorganisms, such as *Nitrosomonas,* which firstly oxidise ammonia to nitrite, and *Nitrobacter,* which then oxidise nitrite to nitrate. Ammonia oxidising bacteria (AOB) have been shown to be the main contributors to ammonia oxidisation during nitrification when compared to ammonia oxidising archaea (AOA), particularly *Nitrosomonas* and *Nitrosospira* (Bai et al. 2013). More recently, organisms capable of the complete oxidisation of ammonia to nitrate, known as Comammox *Nitrospira* have also been shown to be present and active in sand filters (Fowler et al. 2018). These pathways require an aerobic environment and so are thought to occur more frequently towards the top of the filter bed where oxygen is more readily available (Murphy et al., 2010). Nitrate, which is produced as a result of nitrification, or indeed already present in the source water is removed through the process of denitrification. Microbes present in the filter bed reduce nitrate to nitrite which is then further reduced to nitrogen gas (Barnard et al. 2005). This process requires a low oxygen to anoxic environment and is thought to occur deeper within the filter bed (Murphy et al., 2010). When raw water is first introduced to the filter, the high oxygen levels at the top of the filter bed allow nitrification to take place. As the water passes through the filter bed, its oxygen is depleted creating anoxic areas within the pore spaces allowing denitrification to occur (Murphy et al., 2010). Research conducted by Nakhla and Farooq 2003, demonstrated that nitrification and denitrification occur simultaneously within a slow sand filter, contributing to an overall reduction of total nitrogen (Nakhla and Farooq 2003). This study also demonstrated that both processes were enhanced using finer sand grains over courser grains, which the authors contributed to higher biomass occupying the greater surface area of the fine sand grains. Interestingly, the process of denitrification appeared to be the more stable of the two when the parameters of the filter grain size and flow rate was altered, also bed depth appeared to have no effect on either process (Nakhla and Farooq 2003). Understanding the limitations of these processes is of importance when considering optimisation. For example, optimisation of the nitrification process could involve the introduction of oxygen within the

filter bed, encouraging nitrification deeper in the filter. However, the addition of oxygen may be detrimental to the denitrification process and a deeper filter bed may be required to compensate.

The removal of heavy metals from potable water is fundamental to water treatment. Therefore, it is important that the biological processes, that facilitates metal removal during slow sand filtration are well understood. Iron and manganese are common metal contaminants and so the organisms and their processes responsible for the removal of these metals have been extensively studied (Manav Demir 2016 ; Emerson et al., 2010; Hedrich et al., 2011; Bai et al. 2013). A metagenomic analysis of a slow sand filter community conducted by Bai et al 2013, identified sequences associated with ferric reductase which suggests that reduction of ferric iron may have been taking place in the filter. This study also identified genes for the transport of manganese, though interestingly found no sequences associated with manganese oxidation (Bai et al. 2013). A further study conducted by Manav Demir attributed iron and magnesium removal to several organisms shown to be found in the schmutzdecke and filter bed of slow sand filters. The authors of this study concluded that organisms of the genus *Gallionella* contributed to iron removal, while *Leptothrix* and *Crenothrix* contributed to the removal of both iron and manganese (Manav Demir 2016). The betaproteobacteria *Gallionella*, of which *Gallionella ferruginea* is the most studied, are known iron oxidisers. They are described as lithoautotrophic (Kelly 1971), meaning they derive energy through the reduction of mineral compounds, in this case the reduction of Fe(II) to Fe(III). *Gallionella* form twisted stalks consisting of a fibril matrix embedded with iron oxyhydroxides as a result of this process (Hedrich et al., 2011). The presence of *Gallionella* in the schmutzdecke and filter bed is a strong indicator that the biological removal of iron is occurring (De Vet et al. 2011). Another betaproteobacteria genus, *Leptothrix*, identified in the study by Manav Demir is known as an oxidiser of both iron and manganese. The main distinguishing feature of *Leptothrix* is the formation of a polysaccharide sheath, consisting of fibrils held by disulphide bonds (Emerson et al., 2010; Emerson and Ghiorse 1993). The mechanism by which oxidisation takes place is thought to involve extracellular iron and manganese oxidising enzymes that are associated with the sheath. Iron and manganese oxides are then deposited onto the sheath, thought to protect the cell from environmental stresses and protozoan grazing (Emerson and Ghiorse 1993).

Further research has investigated the biological processes responsible for the removal of other contaminants. For example, the metagenomic analysis carried out by Bai et al 2013,

demonstrated the presence of arsenite oxidase genes and to a lesser extent arsenite reductase genes, suggesting the capability of the slow sand filter community to transform arsenic. These sequences were attributed to *Proteobacteria, Nitrospirae, Chloroflexi, Chlorbi* and an archaea, *Crenarchaeota* (Bai et al. 2013). This study also identified several genes associated with the degradation of aromatic compounds as well as a diverse range of aromatic oxygenase and dehydrogenases. The most abundant genes for aromatic degradation targeted easily biodegradable compounds, while genes targeting more persistent compounds were less common in the community (Bai et al. 2013). The extracellular enzymes phosphatase and β-glucosidase have also been detected in slow sand filters. Both enzymes were reduced in concentration in the sand filter as compared to the source water, however β-glucosidase concentrations reduced with filter bed depth while phosphatase levels remained relatively stable (Hendel et al. 2001). The reduction in β-glucosidase concentration was attributed to reduced biomass and substrate availability with filter bed depth.

1.2.9. Optimisation

The literature thus far may offer insight into potential areas of optimisation that could be exploited to improve the biological activity of the filter community. Parameters which can be easily controlled offer the simplest mechanisms for optimisation. Temperature, for example, can be adjusted in a closed system by a heating or refrigeration unit. It is known that biological activity increases with temperature so it would be safe to assume that heating the water would be more useful in terms of optimisation than cooling it (Emelko et al. 2006). The temperature of the filters could be maintained at a temperature which is favourable to indigenous organisms, while still being below the optimum for enteric bacteria.

Another method which could be used to direct the microbial community towards desirable outcomes could involve the addition of nutrients to the filter to increase initial biomass and select which organisms flourish within the filter. For example, the addition of Fe(II) to the media before the filter is run may select for iron oxidising bacteria early on in the filters lifespan. This could prove advantageous to treat waters which are high in iron as such bacteria may gain a stronger foothold at the beginning of the filter run when there is less competition for space and nutrients. This could potentially be applied to any problematic contaminant such as adding ammonia to increase nitrifying bacteria to treat waters high in inorganic nitrogen. Though it should be noted that the addition of such materials may inhibit the growth of other desirable organisms, reducing the removal efficiency of a different

contaminant. The addition of nutrients may also increase biomass and microbial activity, quickening the ripening period and increasing the overall efficiency of filtration. Research conducted by Lauderdale et al., 2012 demonstrated that nutrient and peroxide supplementation increased removal of 2-methylisoborneol, DOC and manganese in a GAC/sand dual media filter. Interestingly, nutrient supplementation also decreased EPS formation leading to later clogging of the biofilter (Lauderdale et al. 2012). Nutrient supplementation could be easily implemented when using GAC as a media due to its high adsorption capacity. The GAC could be allowed to soak in the desired nutrients until saturated before being used in the filter, although said nutrients would need to be readily able to desorb for the nutrients to be available to the biofilm (Aktaş and Çeçen 2006).

A potential area of investigation may study the fate of low nucleic acid (LNA) bacteria during slow sand filtration. LNA bacteria are bacteria of a small particle size, typically isolated using a 0.2µm filter membrane (Proctor et al. 2018). These bacteria are ubiquitous in source water and have been shown to be more resistant to disinfection than larger species of bacteria (Ramseier et al. 2011). While most identifiable bacteria found in surface water fall into the HNA category, little is known of LNA bacteria comparatively. The species, viability and potential pathogenicity of LNA bacteria currently remains unclear (Ramseier et al. 2011). Following slow sand filtration, LNA bacteria have been shown to be more predominant in the effluent than high nucleic acid (HNA) bacteria (Vital et al. 2012). This could potentially be due to the small size of LNA bacteria allowing them easier access through the filter bed and into the effluent. Alternatively, the oligotrophic environment supplied by slow sand filtration favours LNA bacteria (Lautenschlager et al. 2014). It has also been suggested that higher predation of HNA bacteria may lead to LNA predominance in the effluent (Boenigk et al. 2004).

Again, research which attempts to unravel the biological activity and the organisms responsible is essential before optimisation of the slow sand filtration process becomes a possibility. Therefore, a transcriptomic or indeed proteomic investigation throughout the depth of the filter bed would provide valuable insight into the metabolic activity of the biology of a slow sand filter and potentially reveal factors which could be altered to increase filtration efficiency. Only through adding to the body of knowledge concerning the biological mechanisms of slow sand filtration does the prospect of a highly efficient, point of use filter become possible.

Chapter 2

Biofilter Design and Performance

Contributions

Biofilter design and construction – Dominic Quinn lead

GAC preparation – Dominic Quinn

Biofilter operation – Dominic Quinn, Marta Vignola, Jeanine Lenselink, Steven Joyce

Water collection – Dominic Quinn, Steven Joyce

Total coliform and *E. coli* counts – Dominic Quinn

Legionella, total and intact cell counts – Marta Vignola

DOC, iron, manganese, ammonia, nitrite, nitrate monitoring – Jeanine Lenselink

Water filtration for molecular analysis – Dominic Quinn

Biofilter deconstruction – Dominic Quinn lead

2.1. Introduction

The main focus of this research is to investigate the changes in biofilter community with an eye towards eventual optimisation of the biological mechanisms of contaminant removal and increasing filter efficiency for development of a scaled down point of use system. Potential optimisation solutions may lie in engineered changes to the biofilter design, driving the community composition of the filter bed in desirable directions (Lauderdale et al. 2012; Ma, LaPara, and Hozalski 2020; Kirisits, Emelko, and Pinto 2019). A fundamental design parameter which has thus far been under studied is the effect of biofilter media depth on the biological communities of biofiltration. In order for this to be properly investigated, an experiment must be designed which allows a direct comparison of biofilter media depths and access to the communities involved. This chapter outlines the design and operation of a series of laboratory scale biofilters to investigate the biological communities that develop and how they are affected by media depth over time, and how depth impacts biofilter performance. Lab scale filters have been shown to mimic the communities found in full scale slow sand filters and allow for much greater control of the experiment design (Haig et al. 2014). Aside from eliminating factors such as weather and seasonal changes, operating lab scale filters allows for wider scope in terms of sampling, media selection and influent water choice. Furthermore, initial community changes can be investigated in the early weeks of a filters set-up which may not be possible when sampling from filters already in operation.

Many studies have shown that biomass and biological activity is highest in the top section of the filter bed and decreases with depth. As such it has been largely accepted that the majority of contaminant removal by biological means occurs within the top 40cm of the filter bed (Chen et al. 2021; Matuzahroh et al. 2020; Hijnen et al. 2021; Bellamy et al. 1985). As it is thought the community at the top of the filter bed is responsible for the majority of contaminant removal, it may potentially be targeted when investigating optimisation strategies. Thus, any changes that occur within the community and factors which drive these changes are of particular interest.

In an operational biofilter, changes in community structure mainly occur along two axes, the length of time the filter has been in operation and through the depth of the filter bed (Chen et al. 2021; Haig et al. 2015; Wakelin et al. 2011; Campos et al. 2002). Early in a biofilters lifespan, biomass begins to accumulate on the media of the filter bed. Over time selection

pressures within the bed of the filter assert themselves leading to changes in the microbial community (Campos et al. 2002). Similarly, differing nutrient and oxygen gradients throughout the depth of the filter bed act as selective pressures leading to community changes with depth (Calvo-Bado et al. 2003). As biomass accumulates at the top of the filter bed over time, metabolites produced or freed by cell lysis may alter nutrient gradients again leading to community changes throughout the depth of the filter bed and over the length of the filters' lifespan (Boon et al. 2011).

In terms of biological contaminants, the current literature suggests that increasing filter columns size has little effect on removal (Freitas et al. 2021; Verma et al. 2019). Due to predation in the top layer and the filter environment being hostile to most pathogens, a shorter filter bed size appears to be sufficient for biological removal (Freitas et al. 2021). In contrast, filter bed size has been shown to be of more importance when considering chemical contaminants. Longer filter bed sizes have been shown to be more effective at removing specific chemical contaminants, particularly recalcitrant compounds (Jun et al. 2002; Hoang et al. 2008; De Vera et al. 2019).

While studies exist comparing the removal capacities of different filter bed sizes, little is known if filter bed depth has an influence on the community within the bed itself. Oxygen and nutrient gradients are known to drive differences in community composition and may differ between different filter bed sizes (Boon et al. 2011; Mitri et al. 2016). For example, nutrients and oxygen in the bottom layers of a shorter bed may be less depleted than in the bottom layers of a longer filter, potentially leading to differences in communities as they are forced to adapt to differing conditions (Boon et al. 2011; Fenchel and Finlay 2008). Similarly, no studies have investigated a direct comparison of filter bed depth in relation to the community composition of the effluent water. When considering media depth, the community of the effluent water as compared to that of the filter bed and influent over time is of great value to investigate. As the overall aim is safe, biologically stable water, the effluent community and how it is influenced by media depth may be of great importance. Furthermore, building a community profile of taxa entering and leaving the filters over time, compared to the taxa found in the filter beds will help to determine which organisms are being removed, which organisms are being retained in the filter and which organisms are escaping into the effluent. This may also determine the origin of the effluent community, whether it is being seeded from the filter bed community or indeed passing through from the

influent. This community profile, from influent through treatment to effluent over time, will provide valuable data which may shed some light on how the communities in biofiltration are assembled.

To this end we designed and ran an experiment to explore the effects of filter bed length on biofilter performance over time and characterise the microbial communities within the filter bed and the influent and effluent waters and how these communities may change with time.

Increasing media depth increases the overall adsorption capacity of GAC, empty bed contact time between the influent water and the media and opportunities for particulate contaminants to be captured through physical straining. Furthermore, longer bed depths may facilitate nutrient and oxygen gradients which may select for different communities than shallower bed depths. For these reasons we can hypothesise that media depth will have an effect on biofilter performance and that the longest filter bed will achieve the highest removal efficiency for the contaminants tested.

In this chapter, the design, operation and efficiency of the biofilters to remove DOC, Fe, Mn, microbial cells and pathogens is described. The sample collection of GAC through depth and time, along with influent and effluent water collection is described. This forms the experimental basis for chapters 4 and 5. Comparing the microbial communities of the filter bed over time and mature communities of the three filter bed lengths at week 23 (Chapter 4). Also comparing the effect filter bed length has on the microbial communities of the effluent water over time (Chapter 5).

2.2. Materials and Methods

2.2.1. Biofilter Construction

Short (30cm), Medium (60cm) and Long (90cm) laboratory scale biofilters were constructed from 32mm PE80 and MDPE pipe fittings (Figure 2.2), using Norbit Granular Activated Carbon (GAC) as the filter bed. Four sets of 90cm biofilters were constructed in triplicate, resulting in twelve individual 90cm biofilters. Each set of triplicate filters was deconstructed for depth profile analysis at different timepoints

(weeks 5, 9, 12 and 23) during the experiment run. A set of biofilters (n=3) were constructed with a 60cm GAC bed and a further set (n=3) constructed with a 30cm GAC bed to operate over 23 weeks and deconstructed for depth profile analysis at the end of the experiment run. This resulted in a total of 18 individual biofilters. A further set of 90cm biofilters (n=3) were constructed for mathematical modelling, this data is reported elsewhere.

2.2.2. GAC Preparation

Cabot Norit GAC 1240 W (Cabot Corporation) was used as a media for the filter beds (Table 2.1). This GAC was selected as it is routinely used by Scottish Water.

Table 2.1. *Characteristics of the Cabot Norit GAC 1240 W.*

Particle size > 12 mesh (1.70 mm)	**Min. 10**	**mass-%**
Particle size < 40 mesh (0.425 mm)	Max. 5	mass-%
Moisture (as packed)	Max. 5	mass-%
Iodine number	975	-
Methylene blue adsorption	20	g/100g
Total surface area (B.E.T.)	1100	m^2/g
Apparent density	500	kg/m^3
Density backwashed and drained	445	kg/m^3
Ball-pan hardness	97	-
Effective Size D10	0.6 - 0.7	Mm
Uniformity coefficient	1.7	-
Ash content	12	mass-%
Water soluble Ash	0.1	mass-%
pH	Alkaline	-
Dechlorination halving value	2.5	cm

For each biofilter, 260g DW of GAC was weighed and added to a 1L glass bottle. The GAC was rinsed twice by adding 260ml of Milli-Q water and shaking by hand, then emptying the Milli-Q water using a steel sieve to keep the GAC in the bottle. 260ml of Milli-Q water was added a third time and shaken and the lid of the bottle loosened to release trapped air. The GAC in Milli-Q water was then sonicated for 10 minutes in an ultrasonic bath. The GAC was left to settle in the Milli-Q water for 48 hours.

2.2.3. Experiment Design

Biofilters were run for 23 weeks. The overall experimental design is outlined in Figure 2.1.

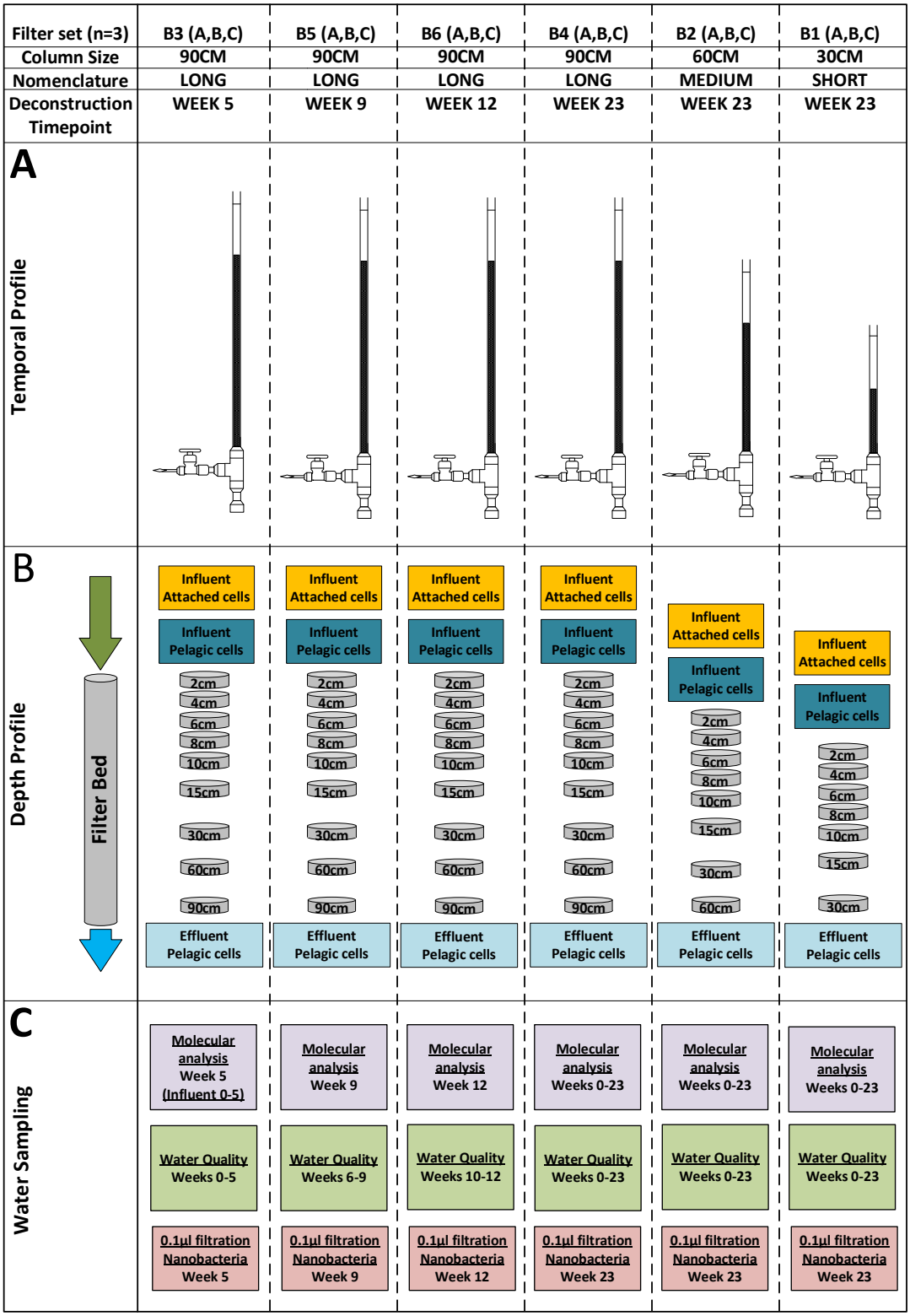


Figure 2.1. A *– Illustration of the constructed biofilters with assigned nomenclature, column size, and deconstruction timepoint. Each filter set consisted of triplicate biofilters (A, B, C).* ***B****- Samples taken for molecular analysis from each filter set at its deconstruction timepoint.* ***C*** *– Weeks in which the influent and effluent water was sampled from each filter set for molecular analysis and water quality testing.*

Individual biofilter construction is illustrated in Figure 2.2. Each filter was constructed with 32mm PE80 piping and MDPE fittings. The main length of each filter was cut to the desired length of the filter bed plus an additional 370mm. A 32mm MDPE pipe insert was wrapped in stainless steel mesh with an aperture of 75µm and inserted into one end of the PE80 pipe. This end of pipe was attached to a 32mm x 25mm x 32mm MDPE reducing T, following the manufacturer's instructions. A 180mm length of PE80 pipe and a MDPE terminal end cap were added to the open 32mm side of the reducing T to act as a trap for any particles of GAC which may bypass the stainless-steel mesh. A 160mm length of 25mm PE80 pipe was added to the open 25mm end of the reducing T and a 15mm x 25mm reducing stop cock was attached following the manufacturer's instructions. A 15mm pipe inset was added to a 200mm length of BRAND® special laboratory tubing and added to the stop cock. A 14mm tube pinch valve was added to the other end of this tubing. An 8mm hole was drilled 50mm down from the top of the filter to later fit an overflow pipe. The filter was inverted and vigorously tapped after drilling to remove any plastic particles which may have resulted from drilling. The filters were then attached to a plywood frame using 32mm pipe clips.

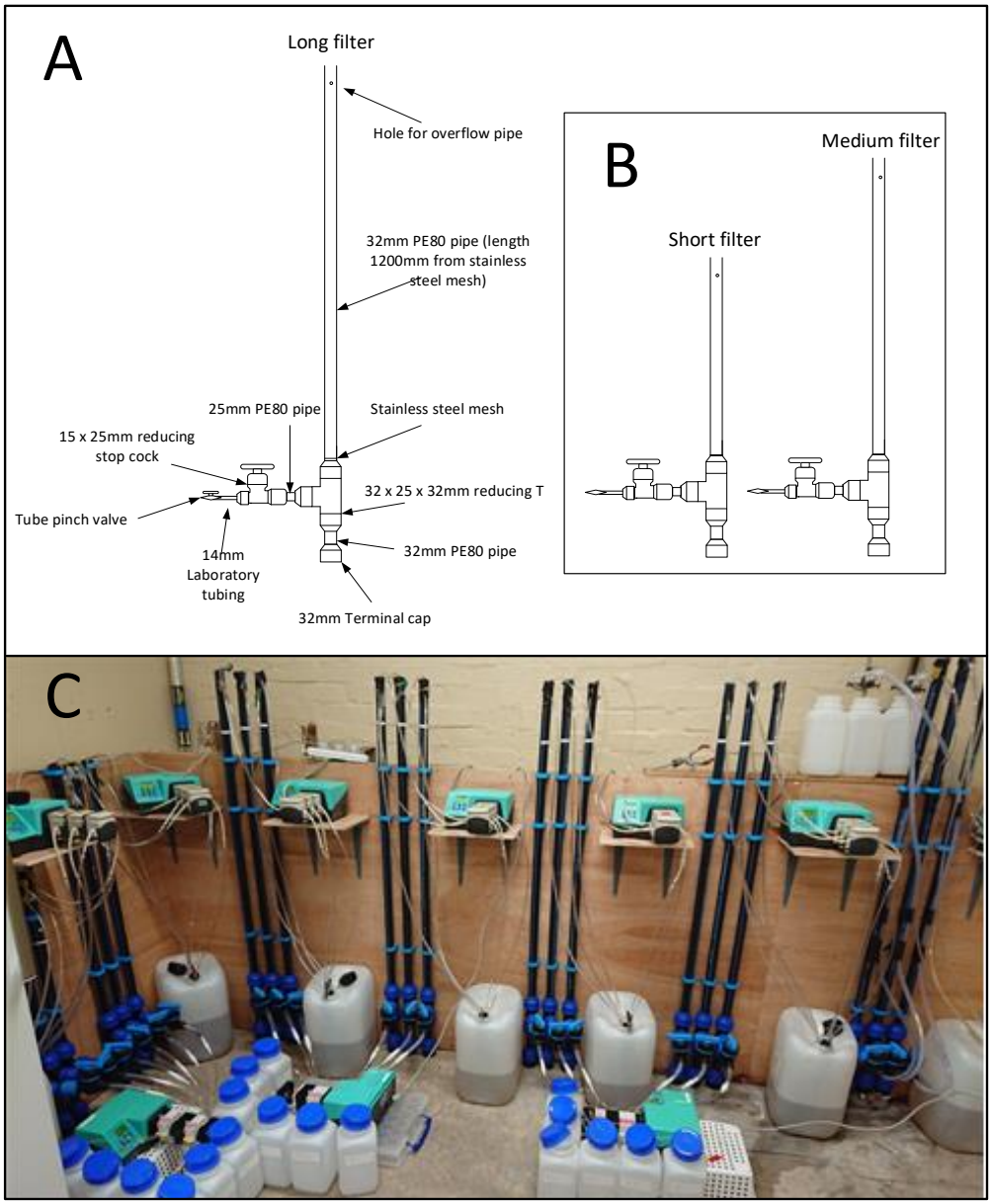


Figure 2.2. A *- Diagram of the constituent parts of the long 90cm bed filter.* ***B*** *- The 30cm and 60cm bed filters were constructed identically, though with shorter lengths of PE80 pipe above the stainless-steel mesh. The 60cm filters had a length of 900mm from the stainless-steel mesh, and the 30cm filters had a length of 600mm.* ***C*** *– The constructed biofilters in-situ and operational during the experiment run.*

2.2.4. Filter bed packing

All biofilters were held upright to the plywood frame (Figure 2.2. C) and the stop cocks were closed to prevent the passage of water as the GAC was added to each biofilter. A packing tool was constructed, consisting of a circular plastic disk attached to a 1M stainless steel pole. The plastic disk was filed to a diameter of 28mm, so as to fit the inner diameter of the 32mm PE80 pipe and allow the escape of water as the GAC was pushed into the filter.

GAC was added to each filter in small batches and pushed down with the packing tool until resistance was felt. This was achieved by shaking the bottle containing the GAC and pouring Milli-Q water through a funnel into the neck of the biofilter. Milli-Q water was added to the GAC as needed and the GAC was pushed into the filter using the packing tool. After each small batch of GAC was added, the distance from the bed of GAC to the top of the filter bed was measured. When the filter bed reached 300mm from the top of the filter, no more GAC was added. Approximately 230g dry weight GAC was added to each of the 90cm filters and 54g GAC was added to the 30cm filters. Once the filter beds were at the correct height for each filter, Milli-Q water was added until it poured from the hole drilled from the overflow, resulting in a 232ml head of water for each filter.

2.2.5. Biofilter set up

In order to ensure the GAC filter beds had fully settled the filters were initially run with Milli-Q water for two weeks. A Watson Marlow 300 series peristaltic pump with three attached 313 OEM pump heads were used to supply water to each filter set. A 250mm length of marprene tubing was cut for each filter and attached to a peristaltic pump head, 6mm straight tubing connectors were added to each end of the marprene tubing. A 900mm length of 6mm hydrolysis resistant tubing was inserted into the top of the biofilter and held in place with a crocodile clip. The other end of this length of tubing was attached to the marprene tubing via the straight tubing connectors at the outlet side of the pump head. A second 900mm length of 6mm hydrolysis resistant tubing was added to the inlet side of the marprene tubing to be added to the influent container.

A 1200mm length of 8mm hydrolysis resistant tubing was cut for each filter and one end was inserted into the 8mm hole drilled 50mm down from the top of the filter to act as an overflow. The 8mm tube was adjusted so only around 5mm of tube was present inside the

filter. Self-amalgamating tape was wrapped around the filter at the entrance of the overflow to prevent leakage.

An autoclaved 1L Nalgene bottle was filled with Milli-Q water and the free end of the inlet tubing attached to the peristaltic pump was fed into the bottle. The free end of the overflow tubing was also fed into the bottle and the peristaltic pump was switched on at 70RPM. This rate allowed water to be added to the filter at the same rate as it escaped via the overflow, maintaining a constant head of water for all filters. The stop cock of each filter was then opened fully, and the tube pinch valves were opened slightly, measuring and adjusting the flow rate of each filter to 1ml/min. Once the correct flow rate was achieved by all filters, the Nalgene bottle feeding the filter was placed under the valve to catch the effluent as it left the filter. The filters were run in this self-cycling fashion for two weeks, the Milli-Q water being changed after 3, 5 and 7 days. After running the filters for two weeks it became apparent that the tube pinch valves were unsuitable for maintaining a consistent flow rate across all filters and so were replaced with more sensitive needle valves.

2.2.6. Influent collection

Influent water for treatment using our experimental biofilters was untreated surface water collected from the reservoir at Patsehill treatment works run by Scottish Water. The influent water was chosen due to the proximity of the treatment works and the high levels of organics and metals in the water.

Table 2.3. *Average and standard deviations of raw water quality parameters of surface water from Patsehill treatment works as measured by Scottish Water from August to November 2017*

	Average	**SD**	**Unit**
Colour	168	19.22	mg/l Pt/Co
Hydrogen ion	7.1	0.16	pH value
TOC	18	2.16	mgC/l
Ammonium	<0.02	0.00	mgNH4/l
Turbidity	1.5	0.27	NTU
Aluminium	264	91.93	µgAl/l
Iron	647	65.58	µgFe/l
Manganese	43	19.16	µgMn/l
Presumptive *E. coli*	7	4.86	CFU in 100ml
Presumptive coliforms	22	11.29	CFU in 100ml

Approximately 500L of raw water was collected every two weeks in 20L jerrycans. The water was prefiltered on site using a submersible pump and a 10µm filter (Spectrum® 10inch cartridge filter 10 micron). The influent water was then stored at room temperature until utilised.

2.2.7. Beginning filter run

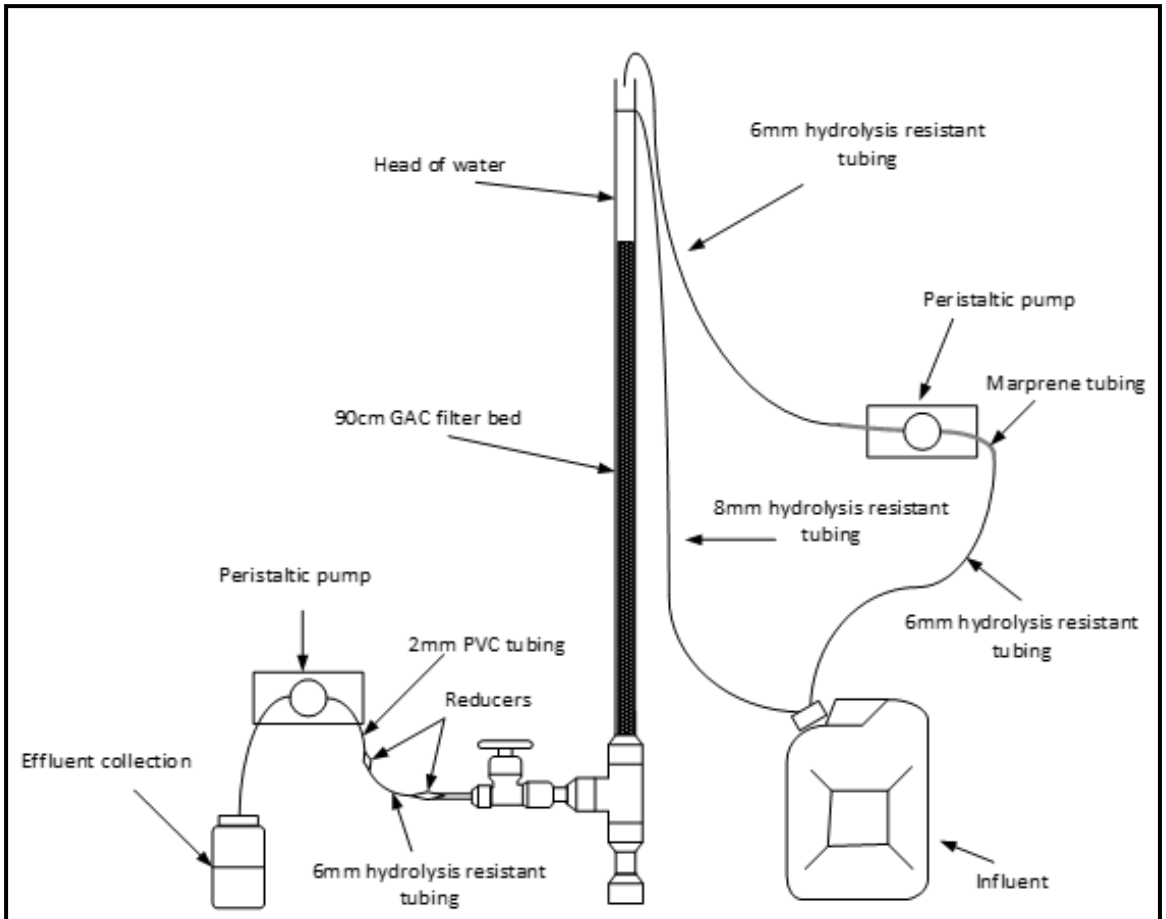
After being run with Milli-Q water for two weeks, the pumps to all filters were switched off and the distance from the top of the filter bed to the top of the PE80 pipe was measured to determine the size of the GAC filter bed. More prepared GAC was added to any filters which had a smaller filter bed than desired, caused by the settling of the GAC during the two-week run. Once all GAC beds were measured at the correct size, the raw 10µm pre-filtered water collected from Patsehill was introduced to the filters. The influent inlet tubing and the overflow of each set of filters were added to a 20L jerrycan, so one jerrycan was supplying all three replicate filters of each set. Effluent water continued to be collected in the 1L Nalgene bottles. The filters were run at 70RPM and left overnight.

After 15 hours, samples of GAC were taken from the top of the filter bed of all filters and added to a sterile 100ml sample tub. The samples were homogenised and aliquoted aseptically into sterile 2ml cryogenic tubes, 0.5g of GAC being added to each tube. The aliquots were then stored at -80°C for subsequent molecular analysis as initial starting point samples.

All biofilters were then run continuously for 24 weeks or until their deconstruction timepoint (Figure 2.1). Influent water was replenished as needed by replacing the emptied jerrycans with a full one. Effluent water was collected in Nalgene bottles and discarded unless water sampling was taking place.

2.2.8. Valve replacement

After two weeks, it became apparent that maintaining a consistent flow rate across all filters was not possible while using a valve system. As a result, the valve system was replaced by a peristaltic pump system which permitted much greater control of the flow rate between filter sets. This was achieved by removing the valves and adding a 6mm – 15mm straight reducer to the 14mm laboratory tubing attached to the stop cock of each filter. A small length of 6mm hydrolysis resistant tubing was added to the reducer and a 2mm – 5mm reducer was added to this tubing. Finally, 2mm tagged PVC pump tubing was added to the 2mm end of the reducer. This tubing was attached to a peristaltic pump set to 7rpm, providing a flow rate of 1ml/min. A Watson Marlow 300 series peristaltic pump and a 5 channel microcassette pump head was used per filter set. The final filter set up is shown in Figure 2.3.



Biofilter	Long	Medium	Short
Influent water	Surface water – prefiltered (10µm)	Surface water – prefiltered (10µm)	Surface water – prefiltered (10µm)
Influent pH	7.9 ± 0.16	7.9 ± 0.16	7.9 ± 0.16
Influent conductivity	117.5 ± 4.7	117.5 ± 4.7	117.5 ± 4.7
Temperature	21°C - 23°C	21°C - 23°C	21°C - 23°C
Length	90cm	60cm	30cm
Diameter	2.6cm	2.6cm	2.6cm
Area	5.31cm²	5.31cm	5.31cm
Flow rate	63.7ml/h	63.7ml/h	63.7ml/h
Volume	477.9cm³	318.6cm³	159.3cm³
HLR	0.12m/h	0.12m/h	0.12m/h
EBCT	7.5h	5h	2.5h
GAC			
Packed bed density (WW)	4.5 ± 0.1 Kg/cm2	4.5 ± 0.1 Kg/cm3	4.5 ± 0.1 Kg/cm4
Dry weight (APPROX)	230g	154g	77g
Uniformity coefficient	1.7	1.7	1.7
Particle size > 1.7mm	Min 10 mass-%	Min 10 mass-%	Min 10 mass-%
Particle size < 0.425mm	Max 5 mass-%	Max 5 mass-%	Max 5 mass-%

Figure 2.3. *Final design of biofilter set up from beginning of week 3 onwards and the parameters of the three column sizes. Influent is introduced to the biofilter via the 6mm hydrolysis resistant tubing and effluent is caught in a 1L autoclaved Nalgene bottle. Again, the short and medium filters were identical in set up, though with shorter columns and filter bed sizes.*

2.2.9. Water Quality Monitoring

Influent and Effluent water was sampled weekly for a range of biological and chemical water quality parameters to compare the performance of the three column sizes over time. Water quality parameters included DOC, ammonia, nitrite, nitrate, iron, manganese, total coliform, *Escherichia coli* and *Legionella pneumophila* counts, total and intact cell counts by flow cytometry, pH and conductivity. A full 20L of influent water was provided for each filter set and run overnight before sampling took place.

2.2.10. Chemical analysis

Work carried out by Jeanine Lenselink

Influent and effluent samples for DOC analysis were collected in 10ml borosilicate vials (Fisherbrand™), 8-10ml was collected per sample. The water was prefiltered through 0.2mm polyether sulfone membrane filters (Whatman Puradisc). DOC was analysed by combustion (TOC-L, Shimadzu, Japan). The samples were analysed in duplicate or triplicate when the standard deviation exceeded 0.1. Samples for metal analysis were collected in 15ml centrifuge tubes (Fisher Scientific™), 10-15ml of water influent or effluent water was collected per tube. Samples were filtered through 0.2mm Chromacol nylon syringe filters (Thermo Scientific™) and two drops of pure nitric acid was added for preservation. Samples were then sent to the University of Strathclyde for analysis by inductively coupled mass spectrometry (ICP-MS).

2.2.11. Total Coliform and *Escherichia coli* Counts

Work carried out by Dominic Quinn

Oxoid MLGA agar was made following the manufacturer's instructions and autoclaved at 120°C for 15 minutes. The molten the agar was poured into 50mm sterile petri dishes at between 8 – 10g of agar per plate and the plates were then left to dry for 24 hours.

100ml of influent or effluent water was collected in 200ml sterile corning cup and filtered onto a 0.45µm Sartorius gridded sterile cellulose nitrate membrane filter using a Pall filtering manifold and vacuum pump. The Pall filling bells and filter support were autoclaved at

120°C for 15 mins prior to use. Between samples, 70% ethanol followed by a rinse of autoclaved MilliQ water was flushed through the system. The filling bells were also sprayed with 70% ethanol which was allowed to evaporate. After filtration the membrane filter was removed aseptically from the filter support and placed on the top of the MGLA agar in a 50mm plate. The plate was then wrapped in parafilm and inverted inside an incubator at 30°C for 4 hours and then 37°C for a minimum of 14 hours. After incubation the colonies visible on the membrane filter were counted, yellow colonies identified as coliform bacteria and green colonies signified *Escherichia coli.*

2.2.12. Legionella pneumophila counts

Work carried out by Marta Vignola

Legionella pneumophila counts were conducted using the Legiolert test kit. 50ml of influent or effluent water was sampled in a sterile vessel and left for 30mins to acclimatise to room temperature. 50ml of autoclaved DI water and the contents of a Legiolert blister pack was added to each sample vessel, 100ml of autoclaved DI water was also processed to act as a negative control. The samples were shaken until the contents of the blister pack had dissolved and then added to a Legiolert tray. The trays were then sealed in a IDEXX Tray Sealer PLUS as per the operational instructions. The trays were then incubated for 7 days at 39°C.

2.2.13. Total and Intact cell counts

Work carried out by Marta Vignola (Hammes et al. 2008)

3ml of influent or effluent water was collected in a sterile 15ml centrifuge tube. The sample was fixed with 3ml of 1% v/v glutaraldehyde in DI water, resulting in a final glutaraldehyde concentration of 0.5%; samples were stored at 4 °C in the dark and measured within 24 h by flow cytometry. For the staining procedure, 1ml of the fixed sample was transferred to autoclaved 1.5 ml centrifuge tubes. For total cell counts, 10µl of SYBR Green I 10,000 × in DMSO (1:100 dilution in Tris-EDTA buffer solution, pH 8.0) was added to 1ml of the fixed sample. For intact cell counts, 1ml of sample was stained with 10µl of SYBR Green I and Propidium Iodide stain; the stain solution was prepared by combining 10µl of SYBR Green I 10,000 × in DMSO to 400 µl propidium iodide solution (1mg/mL) and 600 µl of filtered (0.22 µm) Tris-EDTA buffer solution. The samples were vortexed for 5s and incubated in the

dark at 37°C for 13 minutes. The samples were then analysed on a BD AccuryTM C6 Plus flow cytometer equipped with a laser emitting at 488 nm (100 µl/min flowrate; 50 µl sample analysed). Samples of DI water; influent and effluent water filtered through 0.22 µm filters were run to design the electronic gating and distinguish total and intact cells from the background.

2.2.14. Water Filtration for Molecular analysis

Work carried out by Dominic Quinn

In addition, for the molecular analysis of microbial communities (chapters 4-6), influent and effluent water was sampled for 16S rRNA sequencing every second week and at the deconstruction timepoints (Figure 2.1. C).

2L of influent water was sampled per filter set and 2L of effluent water was sampled per replicate filter of each set. Influent water was sampled in two 1L Nalgene bottles which had been rinsed with Milli-Q water and autoclaved at 120°C for 15 minutes. The influent tubing was removed from the filters and the influent pumped directly into the sterile Nalgene bottles, 1L in each bottle. The bottles were labelled and stored at 4°C overnight.

Effluent water was collected 18 hours after the influent water to allow the newly changed influent water to begin passing through the filter. Effluent water was collected in two 1L Nalgene bottles which had been rinsed with Milli-Q water and autoclaved at 120°C for 15 minutes. The bottles were set in polystyrene containers with ice packs at the bottom of the filter. The effluent tubing was wiped with 70% ethanol and clipped to the top of the bottles, allowing the effluent to drip in. The lids of the bottles were sat over the top of the tubing to prevent contamination. Effluent was collected over 34 hours, changing the bottles and ice packs when required. Full bottles were stored at 4°C overnight before filtration. A further 100ml of effluent water was collected in a 200ml sterile corning tub to be used for presumptive coliform and *E. coli* counts.

2.2.15. Influent Filtration

Influent water feeding filter sets B1, B2 and B4 was filtered weekly to week 12 and then every second week. Influent feeding filter set B3 was filtered until week 6, feeding B5 from weeks 5 to 9 and influent feeding B6 from weeks 9 to 12.

2L of influent water was size fractionated by inline filtration through a FisherBrand MF200 glass microfibre filter (retention 1.2µm) and then through a 0.22µm Sterivex filter. The glass microfibre filter was inserted in a 47mm Swinnex filter holder, a barbed ¼ NPT pipe adapter and a male luer lock were then attached to the applicable ends. A 200mm length of 6mm Marprene tubing was attached to the barbed and of the NTP adapter and a further 200mm of 6mm silicone tubing was connected to the end of the Marprene tubing with a straight tubing connector. A separate 200mm length of silicone tubing was connected to the male luer lock which was attached to the Swinnex filter holder, a second male luer lock was attached to the opposite end of the tubing. All connected tubing was then placed in an autoclave bag and autoclaved for 15 minutes at 120°C.

Once autoclaved a sterile Sterivex filter was attached to the free male luer lock and the connected tubing were attached to a peristaltic pump with the Marprene tubing at the pump head, ensuring the Sterivex filter was at the outlet end. The free silicone tubing was inserted into a the 1L Nalgene bottle of collected influent and the pump was switched on at 100rpm allowing the influent to pass through the glass microfibre and Sterivex filters. The influent water bottle was replaced by the second 1L bottle of collected once empty and the filtrate was collected in a beaker and discarded at the weekly timepoints. At the deconstruction timepoints the filtrate was collected in 2 autoclaved Nalgene bottles and stored at 4°C for 2 hours.

Once all water had been filtered, the glass microfibre filter was removed from the Swinnex filter holder with flame sterilized tweezers and placed in a 50mm sterile petri dish. The petri dish was then labelled and wrapped in Parafilm before being added to long term storage at -80°C. The Sterivex filter was removed from the tubing and inverted and shaken to remove any excess water that may be contained in the filter housing. The Sterivex filter was then labelled and again added to long term storage at -80°C.

2.2.16. Effluent Filtration

Effluent water collected from all replicates of filter sets B1, B2 and B4 was filtered weekly to week 13 and then every second week to week 24. Effluent water from filter set B3 was filtered until week 6, B5 from weeks 6 to 10 and B6 from weeks 10 to 13.

2L of effluent water was filtered directly onto a Sterivex filter without size fractionation through a glass microfibre filter. The tubing was set up similarly to the influent water, however the Marprene tubing was connected to the silicone tubing at the outlet end with a straight tubing connector as opposed to via the Swinnex filter holder. Again, all tubing was autoclaved at 120°C for 15 minutes before filtration.

After autoclaving, the Sterivex filter was attached to the luer lock and the connected tubing attached to a peristaltic pump at the Marprene tubing. The silicone tubing at the inlet end was placed in to the 1L Nalgene bottle of collected effluent and the pump was switched on at 100rpm. The speed of the pump was reduced as needed during filtration. Filtrate was collected in a beaker and discarded at the weekly timepoints and collected in 2 autoclaved Nalgene bottles at the deconstruction timepoints, being stored at 4°C for 2 hours.

After filtration excess water was removed from the Sterivex housing before the filter was labelled, wrapped in Parafilm and added to long term storage at -80°C.

2.2.17. 0.1µm Filtration

Previous studies have shown an increase in low nucleic acid bacteria (LNA bacteria) in the effluent water following slow sand filtration, suggesting enrichment by the filtration process (Liu et al. 2013; Vital et al. 2012; Chan et al. 2018). A characteristic of LNA bacteria is small cell size (Ghuneim et al. 2018). As such, it is possible that these small bacteria may pass through the 0.22µm pores of the Sterivex filter. In order to sequence and characterise these bacteria, the filtrate from the Sterivex filters was then passed through a 0.1µm filter membrane, aiming to sequester bacteria between 0.1µm and 0.22µm in size for molecular analysis.

At weeks 5, 9, 12 and 23, the filtrate obtained from the influent water was further filtered onto a 0.1µm PDVF membrane filter to capture the nano bacterial fraction. 1L of the influent filtrate from filter sets B1, B2 and B3 were passed through a single 0.1µm filter, loading a

total of 3L combined influent biomass per filter. The filtrate obtained from the effluent water was also combined at 1L from each replicate from each filter set.

The tubing was set up similarly to the weekly influent filtration, though without the Sterivex filter. A 47mm Swinnex filter holder was used to house the 0.1µm PDVF filter. All connected tubing was again autoclaved at 120°C for 15 minutes. After autoclaving the tubing was connected to a peristaltic pump at the Marprene tubing and the silicone tubing at the inlet end placed in the 1L Nalgene bottle of influent or effluent filtrate previously collected. The pump was switched on at 20rpm and the filtrate was collected in a beaker and discarded. Once 3L of influent or effluent filtrate had passed through the filter, the 0.1µm filter was removed from the Swinnex filter holder with flame sterilised tweezers and placed in a 50mm sterile petri dish. The petri dish was then labelled, wrapped in parafilm and added to long term storage at -80°C.

2.2.18. Biofilter Deconstruction

Dominic Quinn lead

At specific timepoints throughout the run (Figure 2.1. A), sets of 90cm biofilters were sacrificially deconstructed to analyse the community of the GAC filter bed. In addition, pore water samples were taken for fluorescence excitation emission matrix (FEEM) and anion and cation analysis (data reported elsewhere).

For each deconstruction the peristaltic pump was switched off and the tubing supplying the influent and the overflow was removed from the filter. The stop cock was then closed and the tubing after the 6 – 15mm straight reducer was removed. The stopcock was then reopened and the biofilter allowed to drain, the remaining water in the filter was collected in a 1L Nalgene bottle and its volume measured. Once drained the biofilter was removed from the plywood backing and held in place with a burette clamp for deconstruction. A tape measure was wiped with 70% ethanol and used to measure the distance from the top of the GAC bed to the top of the filter neck. This distance was marked on the outside of the filter and cut horizontally using 32mm pipe cutters which had been sprayed and wiped with 70% ethanol, exposing the top of the filter bed.

2cm was measured down from the top of the GAC filter bed and cut horizontally with the 32mm pipe cutters which had been cleaned with 70% ethanol. The GAC from the 2cm

section was collected in a sterile 200ml Corning tub and weighed. This process was repeated down the length of the filter bed sampling sections shown in Figure 2.4.

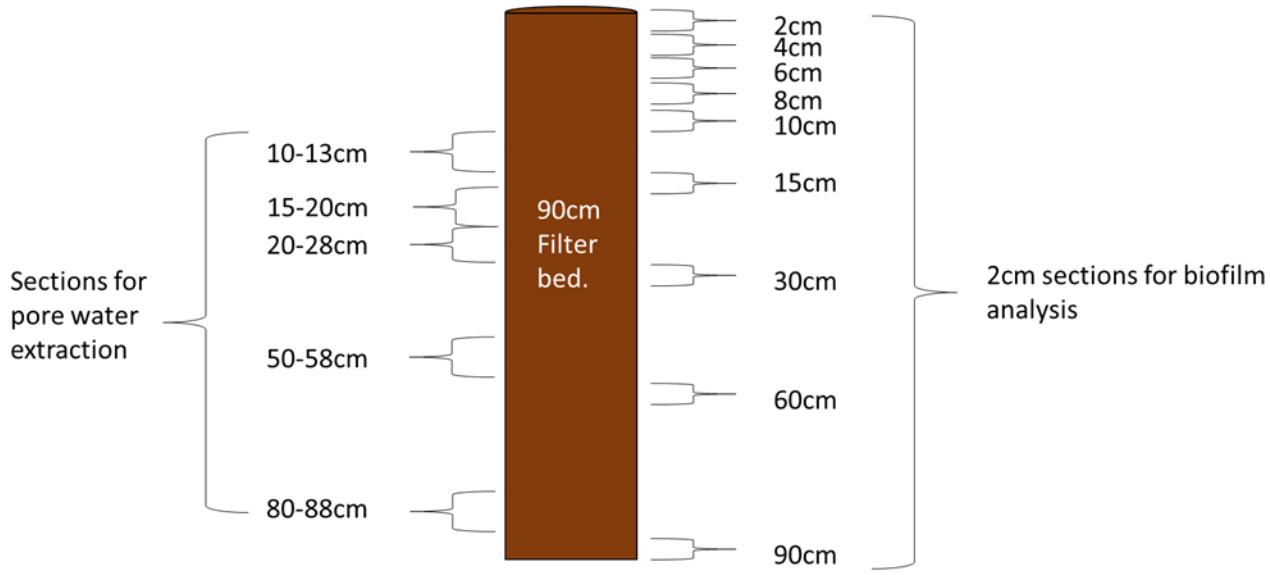


Figure 2.4*. The sections sampled from the GAC bed of a 90cm filter. Each 2cm section was taken for community analysis and additional samples were taken to ensure sufficient volume was available for pore water analysis. The 30cm and 60cm filters were deconstructed identically up to 30cm and 60cm respectively.*

From each 2cm section around 9g of GAC was obtained and homogenised. From this, 5g was taken for subsequent molecular analysis (chapters 4, 5 and 6) and ATP measurements. The remaining GAC was combined for pore water analysis. Around 25g of GAC was left for pore water analysis for sections 2- 10cm and around 39.7g was left for pore water analysis from sections 15 – 90cm.

From the 5g, 400mg was taken for ATP analysis (chapter 4) which was carried out immediately. 500mg was fixed in 1% glutaraldehyde for total and intact cell counts by flow cytometry. The remaining 4.1g was aliquoted aseptically at 0.5g into 2ml cryotubes and stored at -80°C until further processing.

2.2.19. Pore water sampling

Work carried out by Jeanine Lenselink

Wet GAC was added to 50ml centrifuge tubes and centrifuged for 5 minutes at 5000rpm. The dry GAC at the top of the tube was removed from the tube and collected in a separate container. The remaining wet GAC was transferred into a 15ml syringe. This was then placed in a clean 50ml centrifuge tube and centrifuged at 1000rpm for 5 minutes. The dry GAC in the syringe was removed and added to the container with the GAC that was previously collected. The water was transferred to a 15ml centrifuge tube and centrifuged for 5 minutes at 5000rpm. The supernatant was collected in an 8ml glass vial and stored in the dark at 4°C until analysis.

2.2.20. ATP analysis

Work carried out by Marta Vignola (Hammes et al. 2010; Velten et al. 2011)

Promega ATP reagent mix was prepared following the manufacturer's instructions and aliquoted in sterile Eppendorf tubes at 350µl. For each replicate, 200mg x 2 of wet weight GAC was added to sterile Eppendorf tubes with 100µl of phosphate buffer. Both the ATP reagent and GAC was incubated at 30°C for a minimum of 3 minutes. After incubation the GAC was vortexed and 300µl of reagent was transferred to the GAC, vortexed for 5s and incubated in the dark for 1.5 min, the sample was briefly vortexed every 30. At the end of the incubation period, 200µl of supernatant was transferred into a clean autoclaved Eppendorf and measured on a Promega Glo/max luminometer using a 10 second integration measurement. A calibration curve was prepared following the same protocol using standards supplied with the ATP kit and deactivated GAC. After a cycle of gentle washing with Phosphate Buffer, the GAC was deactivated by placing the GAC in a water bath at 60C for a minimum of 21h.

2.3. Results

2.3.1. Biofilter Run

All 18 biofilters constructed for this study were operational for the entirety of the 24-week run. After the valves were replaced with a peristaltic pump (week 3) the flow rate was maintained consistently at 60ml/h across all filters for the entirety of the run. As the influent water was prefiltered, no issues with filter bed clogging were observed. Minor leaks were observed at the overflow during operation which were rectified by reapplication of the self-amalgamating tape sealing the join of the overflow to the filter column. Twice during operation, air bubbles in the overflow pipes of individual filters prevented the passage of influent causing the head of water to rise and overspill the filter column. However, as these issues occurred at the top of the filter column, they were deemed unlikely to impact the biofilter communities or its performance. Furthermore, as the filters were monitored daily, any issues were quickly addressed further reducing the likelihood of impacting the results.

2.3.2. Chemical Removal

Alongside the data gathered on biological removal, the three filter bed sizes were monitored weekly for the removal of dissolved organic carbon (DOC) and every four weeks for removal of the metals iron and manganese. Ammonia, nitrite and nitrate were also measured weekly; however, these were found in very low levels in the influent water and the majority of effluent water samples were below the limit of quantification. The results for the removal of DOC, iron and manganese display clear trends over time and clear differences between the three filter bed sizes, as shown in Figure 2.5.

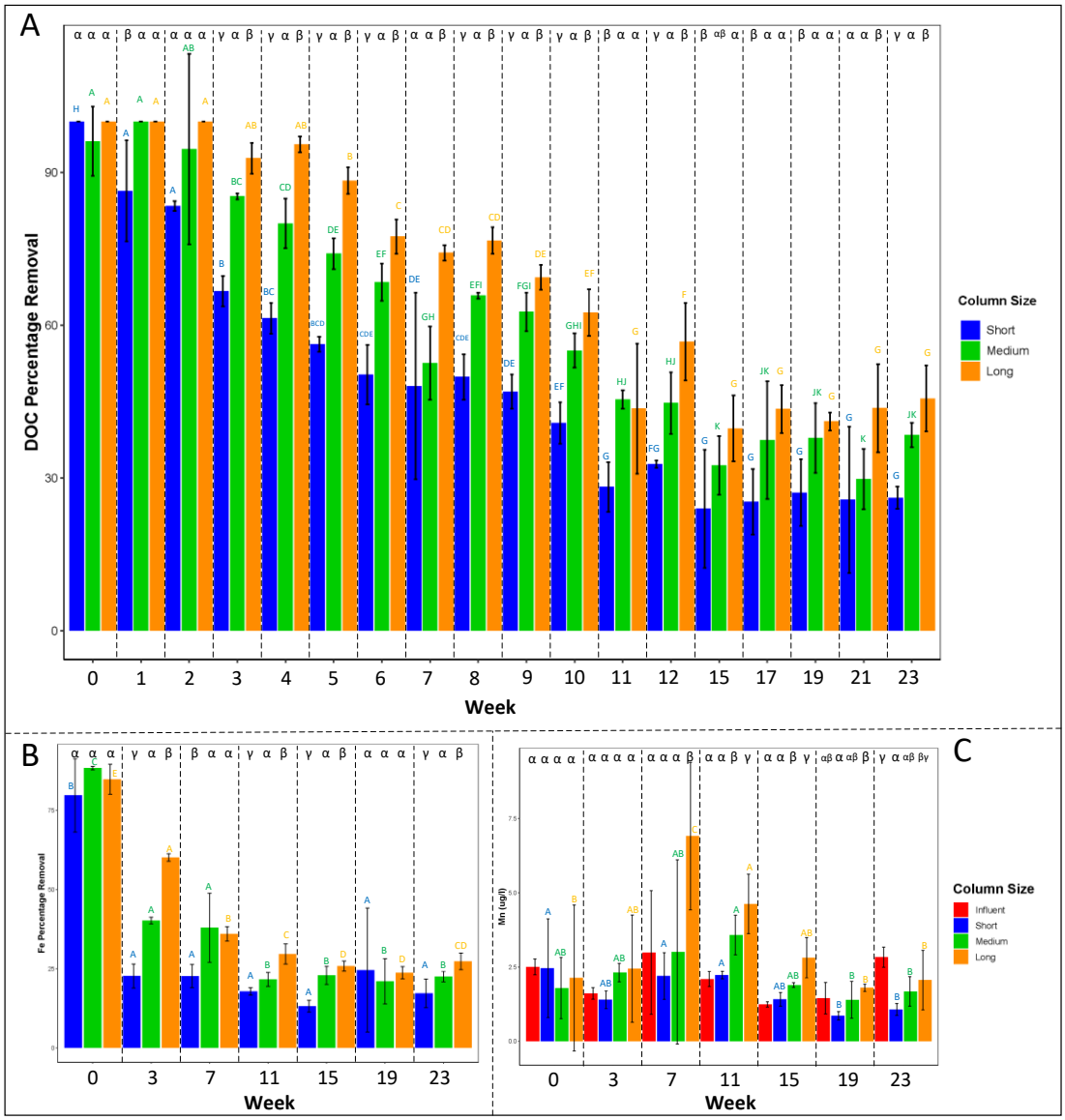


Figure 2.5. A *– Percentage removal of DOC of the short, medium and long filters over the 23 weeks.* ***B*** *- Percentage removal of iron of the short, medium and long filters over the 24 weeks.* ***C*** *– Total manganese found in the influent water and the effluents of the short medium and long filters.* ***A, B, C*** *- Letters of significance (A-K) generated by one-way ANOVA and Tukey HSD. Variance in percentage removal of DOC (**A**) and iron (**B**) and total manganese (**C**) was calculated over time for each individual column size. Letters are coloured to signify the column to which they refer (red – influent, blue – short, green – medium, orange – long). Greek letters of significance (α, β, γ) generated by one-way ANOVA and Tukey HSD between the three column sizes at individual weeks. Variance in percentage removal of DOC (**A**) and iron (**B**) and total manganese (**C**) was calculated between column sizes at each individual week. Analysis for each week separated by vertical dotted lines.*

Initial DOC removal by all three column sizes was high with both the long and short filters at 100% removal and the medium at 96% ± 3.4%. The long and medium filter maintained their removal efficiencies between weeks 0 and 2, while the short filter had began to decline by week 1 (86.4% ± 4% at week 1 from 100% at week 0). All filter bed depths show a clear reduction in the removal of DOC over time (Figure 2.5. A.) as well as significant differences between the three filter bed sizes. All three filter bed sizes display a similar trend over time, with removal efficiencies beginning high at over 95% before decreasing until week 15. From week 15 the removal capacity had plateaued for all three column sizes, with the long filter showing highest DOC removal at 42.7% ± 3.4% between weeks 15 and 23. The short filter plateaued at the lowest DOC removal of 25.7% ± 4.1% between weeks 15 and 23. The DOC removal of the medium filter plateaued between the long and short at 35.2% ± 4.7% between weeks 15 and 23. At week 23 the percentage removal of the short filter had dropped to 26.1% ± 0.6% from 100% at week 1, the medium filter had dropped to 38.4% ± 0.4% from 96% ± 3.4% and the long filter to 45.6% ± 1.7% from 100% at week 1. Of the three filter bed sizes, the long filter bed consistently maintained the highest removal efficiency and the short filter bed the lowest beyond week 2. From weeks 0 to 2 there was little difference in the removal efficiencies of the three bed sizes, however a significant difference between the three bed sizes became apparent from week 2. The short and long filters were significantly different at all timepoints beyond week 2. While the medium filter was significantly different from the short and long filter beds at the majority of timepoints beyond week 2, it displayed similar removal efficiencies to the short filter at weeks 7 and 23, and to the long at weeks 11, 17 and 19, also being similar to both at week 15. The removal efficiencies of all filters eventually pataued at different removal rates, the long filter demonstrating the highest removal efficiency and the short filter the lowest. After platuing the differences in removal efficiencies did not significantly change over time. The short filter plateaued the earliest at week 11 and the medium and long filters plateaued at week 15.

The removal of iron (Figure 2.5. B.) follows a similar trend to that of DOC, with the removal efficiency being highest at week 0 (long filter – 79.8% ± 5.8%, medium filter – 88.4% ± 0.3%, short filter – 84.8% ± 2.4% at week 0) and dropping over time (long filter – 27.3% ± 1.3%, medium filter – 22.5% ± 0.8%, short filter – 17.3% ± 2.3% at week 23). All three filter bed sizes followed the same trend of highest removal iron at week 0 before decreasing and stabalising at later weeks. Interestingly, filter bed size appeared to have an effect on the length of time before iron removal plateaued. The removal efficiency for the long filter

appeared to plateau at week 16, displaying no significant difference in percentage removal between weeks 15 to 23. The medium filter plateaued at week 11, again showing no significant difference in removal from weeks 11 to 23. The short filter plateaued earliest, showing no significant difference in removal after week 0. Concurring with DOC removal, the long filters had the highest percentage removal of iron over the 23 weeks and the short filter the lowest. The three filter bed sizes performed differently for the majority of timepoints measured after week 0 in which the removal efficiencies were similar. At weeks 7 and 19 the removal efficiency of the medium filter was comparable to the long. At week 19 all three filter bed sizes performed similarly, although the high standard deviation of the short filter at this timepoint points to a potential outlier in the data obtained for this sample.

The removal of DOC and iron follow a similar trend, with the long filter outperforming the medium and short over the 24 weeks. The removal of manganese however, follows a different trend. At the initial two timepoints, there is no significant difference between the concentration of manganese in the influent water and the effluent water of any of the three filter bed sizes. This suggests little to no manganese is being removed up to 3 weeks. Between weeks 7 and 15, manganese was detected at higher concentrations in the effluent water of the long and medium filters compared to the influent water. This trend was not mirrored by the short filters which maintained a similar concentration of manganese to the influent water until week 24. This would suggest that the filtration through the longer filter beds was contributing to an increase in manganese in the effluent water. The increase in manganese was highest in the long filter at week 7 and decreased over time. By week 23, the short and medium filters were showing a significantly lower level of manganese in the effluent water, while the long filter maintained a similar concentration to the influent. In the case of manganese removal the short filters outperformed the medium and long filters by not contributing to an increase in manganese at any timepoint and displaying the highest removal efficiency at week 23.

2.3.3. Biological Removal

Throughout operation the effluent water of all three filter bed sizes was monitored weekly for removal of microorganisms. Culture methods were used to monitor the removal of presumptive coliforms, *Escherichia coli* and *Legionella pneumophila,* which are all known or potential pathogens (Figure 2.6. A, B). Furthermore, the number of total and intact cells of the three filter bed sizes was measured weekly by flow cytometry (Figure 2.6. C, D). Biological removal was found to be consistent over all weeks and not significantly different between the three media depths tested.

The data obtained from the *Escherichia coli* counts was insufficient to draw any significant conclusions. This was due to the low number of *Escherichia coli* cells found in the influent water. *Escherichia coli* was detected in the influent water of only 7 of the 23 weeks the filters were in operation and was detected in very low cell numbers. The highest count of *Escherichia coli* found at week 8 was only 3 cells. This low sample size would cast doubt on comparisons which could be drawn across the three filter bed sizes and indeed over the 23 weeks. The results from the presumptive coliforms and *Legionella pneumophila* along with the total and intact cells counts over the 23 weeks are found in Figure 2.6.

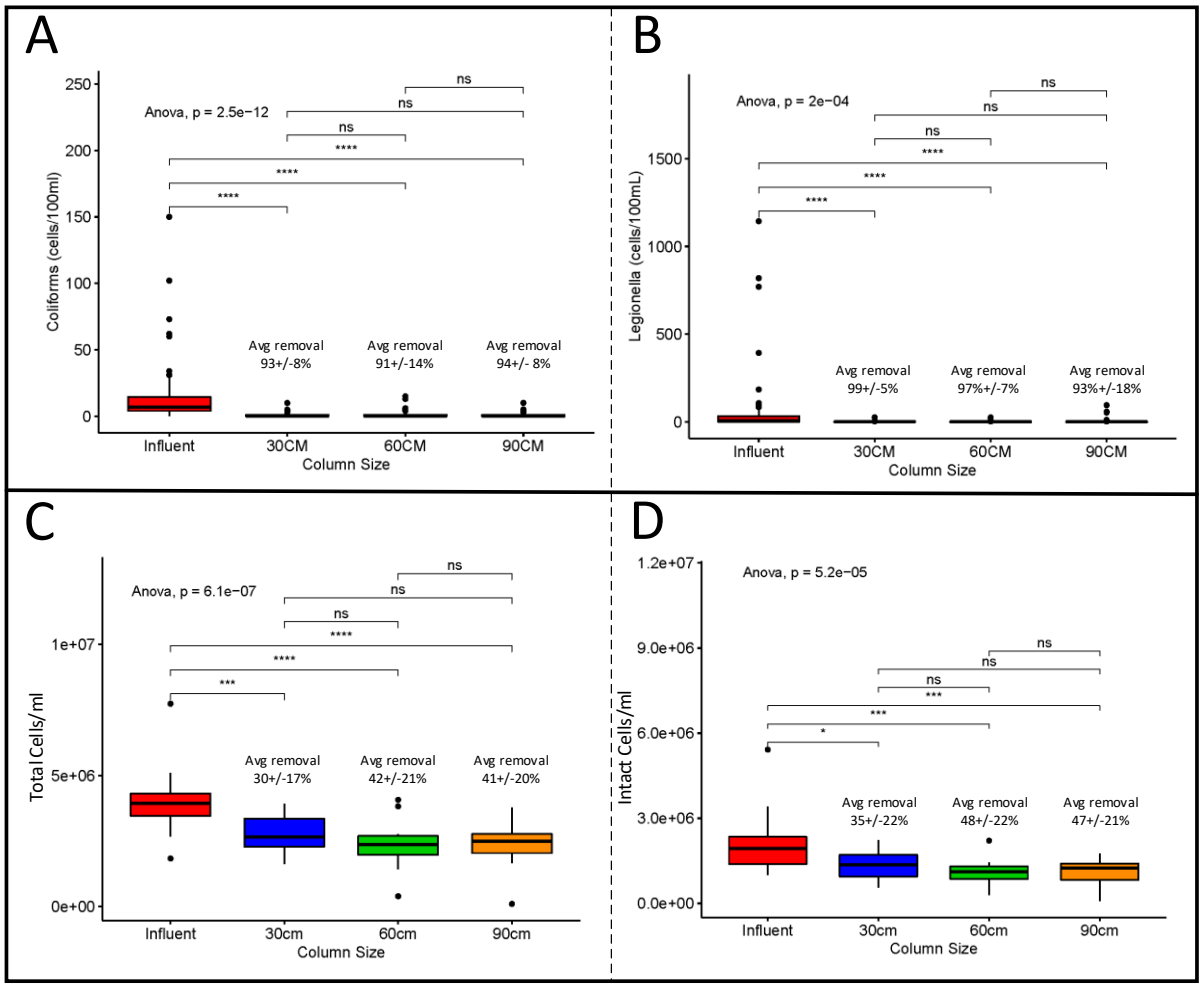


Figure 2.6. *Total coliforms (A) and Legionella pneumophila (B) counts by culture methods and total (C) and intact (D) cell counts measure by flow cytometry over the combined 23 weeks. Significance bars generated by one-way ANOVA.*

In terms of biological removal, the three filter bed sizes performed similarly over the 23 weeks of operation. The cell counts of the effluent water was significantly different to that of the influent water consistently across all three filter bed sizes for both potential pathogens and total/intact cell counts. No statistically significant difference was observed between filter bed sizes during any of the analysis. The average percentage removal of presumptive coliforms (Figure 2.6. A.) and *Legionella pneumophila* (Figure 2.6. B.) was relatively high, ranging from 91-94% for presumptive coliforms and 93-99% for *Legionella pneumophila.* The average percentage removal of total (Figure 2.6. C.) and intact cells (Figure 2.6. D.) was found to be lower, ranging from 30-41% of total cells and 35-48% removal of intact cells.

While a statistically significant difference was observed between the total and intact cell counts of the influent and the three filter bed sizes, the difference was less significant for the short 30cm filters than the medium and long filters. Similarly, the lowest percentage removal is found in the short filters for total and intact cell counts. This suggests that the shorter filter bed may be slightly less effective in terms of overall biological removal, though not by so much as to become significantly different to the longer bed sizes. Seeming to reinforce this, the total and intact cell counts of the short filters were shown to more strongly correlated with the influent water, shown in Figure 2.7.

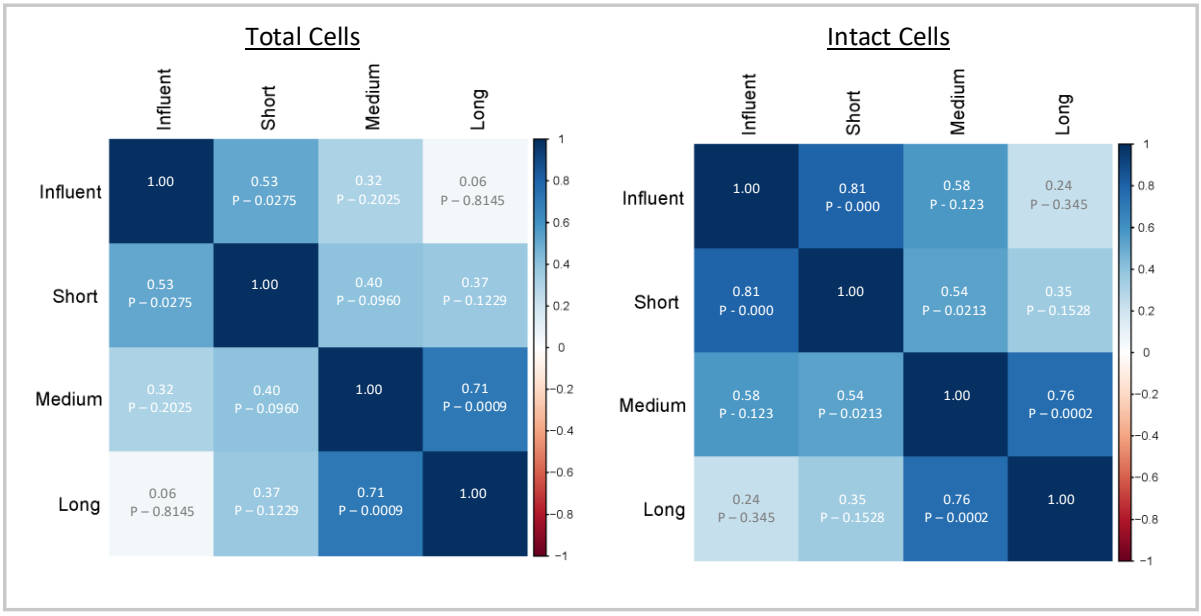


Figure 2.7*. Pearson correlation calculated with associated P value of total and intact cell numbers measure by flow cytometry over the combined 23 weeks.*

A moderate correlation coefficient of 0.53 was found between the influent and short filter when considering total cells and was found to be statistically significant. A weaker correlation was found in the medium filter and no real correlation between the long filter and the influent was observed. When considering intact cells, the correlation becomes stronger across all filter bed sizes. Again, the strongest correlation is found in the short filter with the influent water at a statistically significant coefficient of 0.81. Similar to the total cells, the long filter displays the weakest correlation with the medium filter in between, though neither correlation is shown to be significant. Between the filter bed sizes the strongest correlation is

observed between the medium and long filters. No correlation was found between the influent or any of the filter bed sizes when considering coliforms or *Legionella pneumophila*. Despite the differences in correlation and removal between total and intact cells, the percentage of total cells which are intact remain unchanged across the three filter bed types, shown in Figure 2.8.

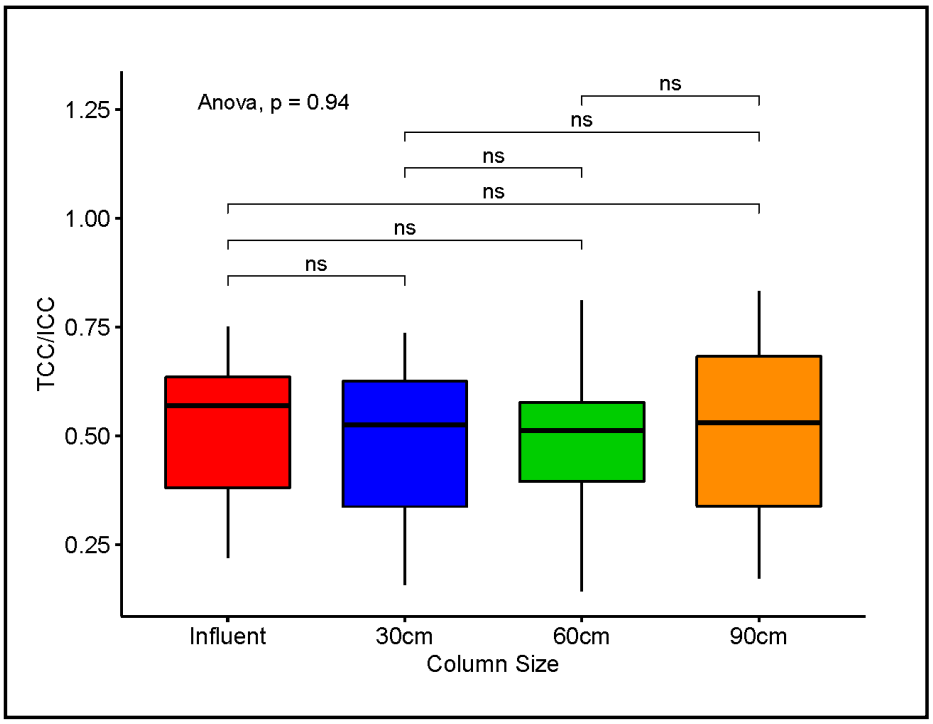


***Figure 2.8**. Proportion of total cells found to be intact over the combined 23 weeks. Significance bars generated by one-way ANOVA.*

Again, there is no significant difference between the three filter bed sizes when considering the percentage of total cells that are intact. Interestingly, there was also no significant difference in the percentage of intact cells between the influent water and the effluent water of any of the filter bed sizes. This suggests that the proportion of intact cells in the influent water is not significantly changing despite the filtration process.

Deatiled analysis of the microbial communites within biofilters with depth and over time is dealt with in chapter 4 and analysis of differences between the effluent microbial communities of the three column sizes in chapter 5.

2.4. Discussion

The aim of this chapter was to design operate and monitor biofilter performance over 23 weeks from start up over three different filter bed lengths (short, medium and long). This experiment would be the basis for subsequent chapters examining the development of microbial communities in the filter bed and effluent water over time.

To this end we designed a biofilter experiment which allowed the performance of three different filter bed sizes to be monitored from start up over 23 weeks. The experiment was designed as to allow access to the filter bed at various timpoints (weeks 5, 9, 12 and 23) for molecular analysis of the communities through the depth of the filter bed and their development over time.

2.4.1. Biofilter Design

When designing an experiment involving lab scale biofilters a balance must be struck between data requirements and what can feasibly be achieved in the lab. Operating replicate filters is recommended, not only to act as biological replicates which produces a far more robust data set but to also act as an insurance against filter failure. In the laboratory setting leaks or blockages are likely over a long filter run and so it is beneficial to run replicate filters to prevent loss of data points in case of filter failure. Filter size is also an important consideration as larger filters will occupy more space in the lab and also require more influent water which may be difficult to transport and store. In this study, several design conditions were outlined to produce the data set required by the research questions posed in later chapters while working within the limitations of the laboratory environment.

- Access to the biomass contained within the filter bed was essential, both at varying depths and at various timepoints during the filter's lifespan.
- The filters were to be as reproducible as possible. Replicate filters were to be fed the same influent water, run the same length of time and maintain the same flow rate.
- Frequent sampling of the influent and effluent water was required, for both chemical and biological analysis.

As the biological community is known to change both through the depth of the filter bed and over time as the filter matures, access to the filter bed media at various depths and timepoints was required (Chen et al. 2021). From a design perspective, this meant being able to extract media from the biofilters while allowing them to continue to run until the end of the experiment, also while minimising any interference which may introduce changes to the community. Two options were considered which would allow the filter bed media to be extracted from various depths and timepoints. The first being sample ports located in the wall of the biofilter and the second being the parallel running of several sacrificial filters to be deconstructed at various timepoints. Both options have advantages and disadvantages, though the option of sacrificial filters was chosen for this study.

Sample ports have been used in previous studies investigating the biological communities of filter beds and have the advantage of consistent sampling of the same filter over its lifespan (Calvo-Bado, Pettitt, et al. 2003; Haig et al. 2014; Li et al. 2016). This means that the communities sampled over time have originated from the same filter and have been subjected to identical conditions. Sampling consistently from the same filter theoretically ensures that any differences in community structure observed are the result of changes over time and not due to differences in filter environment. This is opposed to sacrificial filters in which samples taken over time have been extracted from different filters running in parallel. Sample ports also allow the running of fewer filters as the same filter set can be sampled throughout its lifespan. This reduces the amount of space and influent water required during the experiment run. Furthermore, this may allow for a greater resolution over time as extra filters do not need to be operated per sampling timepoint.

While the use of sample ports has some advantages, there are also factors which need to be considered. The first is the potential for interference in the community's development introduced by sampling. When opening sampling ports to extract the media, it is likely to introduce oxygen through air exposure to the filter bed interfering with the natural oxygen gradient which may run through the bed with depth (Yan et al. 2019; De Beer et al. 2018). Furthermore, the extraction of media from the filter bed has the potential to produce air spaces in the media again interfering with oxygen gradients. These sudden injections of oxygen may potentially alter the development of the biological communities beyond what would be expected in a filter with a more consistent oxygen gradient (Yan et al. 2019). Aside from altering the biological community, introduced oxygen could potentially affect filter

performance by facilitating oxygen dependent metabolic pathways in layers of the filter bed where they would normally be limited. The addition sampling ports into the filter bed may also create water shortcuts through removal of media and introduce the possibly of cross contamination between communities.

The addition of sample ports also comes with practical concerns. Sample ports installed in the filter walls could potentially foster leaks and would need to be properly sealed. Over a long filter run the seal over the sampling port could become less effective with exposure to the flow of water and be difficult to maintain with the filters in operation. Related to this, any seal or stopper would likely be in contact with the interior of the filter bed and potentially offer an area for microbial attachment aside from the filter media and column (Hallam et al. 2001). Finally, as the filters would be required to continue running after sampling the volume of media able to be taken would be more limited than with sacrificial filters. A relatively small volume of media would need to be extracted using sampling ports so as to avoid leaving large air spaces and drastically shortening the length of the filter bed. Reducing the volume of media sampled would result in less available biomass and thus DNA available for analysis. This could be mitigated to some degree by increasing the area of the filter column, though this would increase the volume of influent water required throughout the filter run.

An advantage of using sacrificial filters is access to the entirety of the filter bed during deconstruction. This not only allows an increased volume of media to be sampled resulting in a higher volume of biomass and DNA, but also allows for greater resolution when sampling through the depth of the filter bed. As the filter is no longer in use after deconstruction, as much of the media as required can be taken for analysis. Furthermore, as the first sampling of the filter bed is at deconstruction, the filters can be allowed to run without interference. This prevents the unintentional introduction of oxygen at sampling points through the filter bed depth which may occur using sampling ports. Running the filters without the interference of filter bed sampling is more likely to result in a biological community which would be more representative of that in a household filtration unit. A disadvantage of using sacrificial filters is the potentially limited resolution over time that can be achieved in a laboratory setting. Each sampling timepoint requires the operation and subsequent destruction of a filtration unit, so the parallel operation of a larger number of individual filters is required than necessary when using sampling ports. Consequently,

running a larger number of filters involves greater initial expense, larger influent water volumes to be collected and stored and occupies more space. These factors may limit the number of filters able to be run depending on the facilities available and as such limit the number of timepoints which can be sampled. Another potential difficulty considering sacrificial filters is ensuring reproducibility between the individual filters. As the media is sampled from independently operated filters at each timepoint, differences in filter operation could lead to changes in biofilter community, changes which could be wrongly interpreted as temporal changes (Ma et al. 2020). Overall, the benefits of using sacrificial filters were deemed to outweigh the disadvantages. The running of numerous individual filters was thought achievable after considering the resources available for this study and the number of timepoints required for a satisfactory temporal resolution. From a design perspective, the potential to alter the biological communities through interference using sampling ports appeared more difficult to mitigate than the running of reproducible sacrificial filters.

A number of design considerations were taken into account in order to ensure that any observed changes in the microbial community were due to the passage of time and not due to differences between the individual sacrificial filters. Firstly, the sacrificial filters for each timepoint were run in triplicate. This not only provided biological replicates leading to a more robust data set after 16S rRNA sequencing but acted as insurance against aberrations in the environment of an individual biofilter. A method was developed to ensure that the filter beds were kept consistent across the filter sets. This involved the submergence and sonication of the media in MilliQ water to eliminate as far as possible any pockets of air which may be contained within the pores of the media (Krupa and Cannon 1996). The saturated media was then added to the filter columns with water and packed down with a specifically constructed packing tool. The filters were then run with recycled Milli-Q water for two weeks. This allowed the filter beds to fully settle and presented the opportunity to address any leaks or other issues with individual filters before the experiment began. After these two weeks, the filter beds were measured and topped up with media to the desired size if required, thus maintaining consistency of filter bed depths across all filter sets. A consistent head of water was maintained across all filters using an overflow system. An overflow tube was fitted 250mm above each of the filter beds, allowing influent water to leave the filter column when it reached this level. The influent water was then added to the column at the same rate it exited through the overflow, allowing a constant volume of water to be maintained across the filter sets.

The most challenging aspect of using sacrificial filters was found to be maintaining a consistent filtration rate across all filter sets. Filtration rate and by extension contact time of the influent water with the media has been shown to have an effect on the biological community and performance of biofiltration (Moona et al. 2021). Therefore, it is essential that a consistent flow rate can be maintained across all filter sets. The filtration rate selected for this study was 1ml/min (HLR – 0.12m/h) this was selected as it is within the recommended range of full-scale slow sand filter operation, 0.08 – 0.15$m^3/m^2/h$ (Crittenden et al. 2012), and was deemed manageable in terms of the amount of influent water which was required during the filter run. Initially, two forms of flow control were considered for the biofilters constructed for this study. The first method was the use of a valve which could be opened or closed to as required to maintain the selected filtration rate. The second option was a hydraulic head method of flow control, in which a head of effluent water was elevated to maintain flow control (Post and Von Asmuth 2013). The advantage of either of these methods is that changes to the filtration rate during the biofilter run can be easily recognised. For example, if the filters begin to clog the filtration rate will decline and require adjustment. Furthermore, changes in flow rate may be an early indicator of filter failure (Le Bihan and Lessard 2000). Unfortunately, neither of these methods were able to produce a consistent filtration rate across all of the filter sets. A solution was found using a peristaltic pump which was able to draw the effluent at the selected filtration rate consistently across the filter sets. The disadvantage of using a pump is that filter clogging may not be as apparent, however it was deemed more important to maintain a consistent flow rate across all filters. To reduce the chance of filter clogging and predation of bacteria from higher organisms, the influent water was prefiltered through a 10-micron aperture which removed any large particles likely to contribute to clogging.

The media selected was granular activated carbon (GAC) which has been shown to harbour a greater density of biomass and diversity than sand and have been shown to outperform sand filters in terms of contaminant removal (Emelko et al. 2006; Reaume et al. 2015). This is likely due to the large surface area and porous structure of GAC allowing more space for bacterial attachment, and its high adsorption capacity. GAC has a high affinity for organic adsorption and is often used as a biologically independent form of water treatment (Jung et al. 2001). However, once the adsorption sites are saturated, the biofilm colonising the GAC becomes the main mechanism for water treatment, at this point it is known as biologically active carbon (BAC) (Lu et al. 2020). From a design perspective, the adsorption capacity of

GAC may be advantageous to a home filtration unit as it would allow some form of water treatment to take place in the early days of a filter lifespan before the biofilm is fully developed. However, from an experimental perspective it may prove difficult to decouple adsorption from biological mechanisms of water treatment. Again, as the main overall objective is the design of a household point of use filtration system, the potential benefits of GAC were deemed more valuable than avoiding experimental complications.

2.4.2. Biofilter Performance

The aim of this experiment was to assess the performance of the biofilters of different media depths in the removal of a range of biological and chemical contaminants. In the case of chemical contaminants (DOC, iron and manganese), media depth was shown to influence biofilter performance. The long filter consistently demonstrated superior DOC and iron removal to the medium and short filters over time, while the short filter demonstrated the poorest performance. In the case of biological removal (total coliform, *Escherichia coli, Legionella pneumophila* and total/intact cell counts), media depth was found to have no significant impact on filter performance.

The biofilters operated in this study demonstrated a clear capacity for removal of both biological and chemical contaminants, similar to those achieved by full scale slow sand filters in the case of DOC and coliform removal. Full scale filters have been shown to remove up to 40% DOC and between 90 and 99% of enteric bacteria (Guchi 2015). The long 90cm filters in this study, the most comparable in size to a full-scale slow sand filter, plateaued at around 43% removal of DOC and 94% removal of coliforms after 23 weeks. The biofilters in this study also displayed clear trends over time and differences between the three filter bed sizes when considering the removal of chemical contaminants. All three filter bed sizes demonstrated highest removal efficiencies for DOC and iron at week 1. These efficiencies then decreased before eventually plateauing.

The removal of DOC achieved by all three filter bed sizes was found to decline from the first timepoint before plateauing between weeks 11 and 15. This decline is likely due to the adsorption capacity of GAC (Kennedy et al. 2015; Paredes et al. 2016). At early timepoints the majority of organics are adsorbed onto the GAC, removal by adsorption decreases over time as adsorption sites on the surface of the GAC become occupied, reducing the number of

sites available for further adsorption. As the GAC approaches saturation, the biological community becomes responsible for the majority of organic removal (Velten et al. 2011; Vignola et al. 2018). As the removal capacity of the filters in this study plateaus, it is possible that the adsorption capacity of the GAC had become exhausted and the biological community is mainly responsible for organic removal. This further explains the short filter plateauing earliest, as the increased volume of GAC in the longer filters provides a higher number of adsorption sites and would take longer to become exhausted. However, a meta-analysis of GAC filters in wastewater treatment found that between 800 and 28000 bed volumes of influent water were required to have passed through the filter before saturation (Benstoem et al. 2017). While the short filter fell into the low end of this range at 1008 bed volumes, the medium and long filters had encountered only 504 and 336 bed volumes of influent water by week 15 when all three column sizes had plateaued. Thus, it seems unlikely that the GAC had become fully exhausted. Alternatively, the plateauing in DOC removal observed may be due to a reduction in adsorption rate as opposed to halting. Adsorption of compounds to GAC is thought to occur over two stages. Initially, adsorption occurs on the surface of the GAC particles, followed by a slower phase of adsorption as particles diffuse into the pores of the GAC (Wang et al. 2020; Ocampo et al. 2013). Between weeks, 1 and 15 the higher removal of DOC may be due to this initial surface adsorption and from week 15 onwards, adsorption had entered this slower phase.

Overall, the long filter performed the best in terms of DOC removal over the 23 weeks, even after biological removal was thought the main contributor to removal. One possibility is the longer empty bed contact time led to increased removal in the long filters. Empty bed contact time (EBCT) defined as the average amount of time the influent water would take to travel through an empty column (Fundneider et al. 2021). Factors which determine EBCT are the volume of the filter bed and the filtration velocity. Thus, EBCT can be adjusted by increasing or decreasing the depth of the filter bed or indeed increasing or decreasing the flow rate of the water passing through the filter. Several authors have demonstrated the importance of a sufficient EBCT, particularly in the removal of organics and recalcitrant compounds such as certain pharmaceuticals (Fundneider et al. 2021; Nemani et al. 2016; Moona et al. 2021). Research conducted by Fundneider et al demonstrated that a minimum EBCT of between 20 and 30 minutes was required to efficiently remove organic micropollutants (Fundneider et al. 2021). Further research by Nugroho et al demonstrated that increasing EBCT resulted in more efficient removal of the pharmaceutical Gabapentin in BAC filters. The short filters in

this study had an EBCT of 2.5 hours, the medium 5 hours and the long 7.5 hours. Thus, the influent water was in contact with the filter media for three times longer in the long 90cm filters than the short filters, providing more opportunity for the biological uptake of organics. Alternatively, differing nutrient gradients in the depths of the long filter could potentially lead to a more oligotrophic environment forcing the biological community to adapt to more recalcitrant organics which have not been utilised by the community at shallower bed depths (Egli 2010).

The removal capacity of iron and manganese achieved by the filters in this study was found to be poorer than expected based on published literature. Many studies have shown that biofiltration can achieve removal efficiencies of over 90% for iron and manganese removal (Tobiason et al. 2016; Manav Demir 2016; Ismail et al. 2017; Tekerlekopoulou et al. 2013). In the case of iron removal, the biofilters displayed a similar trend to that of DOC removal. Again, the highest removal was found at week 0, before declining and plateauing at a lower removal efficiency across all three filter bed sizes. As this trend mirrors that of the DOC and iron has been shown to adsorb to filter media (Sharma et al. 2001; Sharma et al 1999) it is likely that the high removal efficiency at week 1 is due to adsorption. At later timepoints the removal efficiency plateaus, at which point it is thought that the main mechanisms for iron removal are biological. The long filters again outperformed the short filters, potentially due to increased EBCT or differing oxygen gradients found in the long filters (Sobolev and Roden 2001). In the case of manganese removal, the long filters performed poorest due to the seeming introduction of manganese from the filter bed at week 7. This may be due to the desorption of manganese from the filter media (Gabelich et al. 2005). As the long filter contains more adsorption sites and therefore likely more adsorbed manganese, it would be capable of releasing more manganese into the influent than the short filters as it desorbs. The biofilters in this study did not show any manganese removal until week 23, during which the short filter had the highest removal efficiency. However, it should be noted that the levels of manganese found in the effluent water of all filters were well below the 50µg/l allowance permitted by Scottish Water.

Overall, it is clear that the size of the filter bed has an effect on the removal of chemical contaminants. The long filter bed was shown to perform the best overall and the short filter performed poorest. The superior chemical removal achieved by the long filter may be attributed to an increased adsorption capacity or longer EBCT. However, it is possible that

differences in microbial communities between the three filter bed depths may lead to differences in chemical removal through biological mechanisms.

The removal of total coliforms achieved by the biofilters in this study was greater than 90% for all three filter bed depths and comparable to removal capacities found in published studies (Hijnen et al. 2021; Yogafanny et al. 2014; Matuzahroh et al. 2020). These studies were conducted in full and lab scale sand filters with bed depths of 1.2m and 1.5m (Hijnen et al. 2021; Yogafanny et al. 2014) Between the three column sizes there was no significant difference in coliform removal. This is in agreement with Verma et al, who found no significant difference in coliform removal between media depths of 22cm, 32cm and 42cm in the treatment of wastewater by slow sand filtration (Verma et al. 2019). Similarly, Freitas et al found that household slow sand filters with media depths of 25cm and 50cm performed comparably in the removal of coliforms (Freitas et al. 2021). The removal of *Legionella pneumophila* was found to be in a slightly higher than that of coliforms, the highest being 99% removal in the short filter, though no significant difference was found between the three filter bed depths. A study by Molloy et al reported a similarly high removal efficiency for *Legionella pneumophila* from point of use carbon filters in domestic water systems (Molloy et al. 2008). However, this study also reported increasing *Legionella pneumophila* populations on the filter media and effluent over time. This was attributed to colonization of the filter media by *Legionella pneumophila* followed by the eventual sloughing off into the effluent. Several other studies have also reported colonization of filter media by *Legionella* species leading to an increase in effluent water concentration (Wang et al. 2013; Huo et al. 2021; Vignola et al. 2018). Potential colonisation of the filter media by *Legionella pneumophila* would have serious implications on the design of a point of use system and is thus in need of further research. Despite relatively high removal efficiencies, if *Legionella pneumophila* is found to be colonising the filter bed the potential for breakout into the effluent water over time may increase. This would pose a serious risk to human health and would be important to consider in the design of a point of use system. In terms of total biological removal, the biofilters in this study did not perform as well as have been reported in other studies in which removal ranged from 54-66% in sand and GAC biofilters (Vital et al. 2012; Vignola et al. 2018). There was no significant difference observed between the three filter bed sizes investigated for total or intact cell removal. However, the difference between the influent water and the short filter was less significant than between the influent and the medium and long filters. Similarly, the strongest correlation was found between the short

filter and influent water, particularly for intact cells. This suggests that the short filter may be slightly less effective in biological removal than the longer filter beds, though not by so much as to cause a significant difference. Physical straining has been suggested as an important factor in biological removal and may explain the slightly poorer performance of the short filter bed as the fewer media particles would present fewer opportunities to catch cells during filtration (Hijnen et al. 2021). Studies have shown a difference in the ratio of high nucleic acid (HNA) and low nucleic acid (LNA) bacteria after slow sand filtration, with the LNA increasing in proportion in the effluent water (Liu et al. 2013; Vital et al. 2012; Chan et al. 2018). Due to the larger size of the HNA bacteria making them more susceptible to capture between media particles, this would appear to support physical straining as a major mechanism of biological removal. However, it does not take into account other biological mechanisms which may result in an increased proportion of LNA in the effluent. For example, LNA bacteria have been shown to be less susceptible to predatory grazing (Bernard et al. 2000). It is also a possibility that LNA bacteria are better suited to survive the low nutrient environment of the filter bed (Wang et al. 2009).

The results from this study suggest that filter bed depth has little effect on the removal of biological contaminants. However, the true effect of filter bed depth on biological removal cannot be known without analysing the biological composition of the effluent water. For example, if physical straining is indeed a major mechanism for biological removal it would be expected to find a higher abundance of HNA bacteria in the effluent of the short filter. It would also be of great interest to determine if filter bed size has an effect on potential pathogens in the effluent water. Examination of the biological composition of the filter bed (chapter 4) will also help to identify the origin of the cells in the influent water (chapter 5) and inform future experiments on potential routes to optimisation.

2.5. Conclusions

- This work demonstrates the successful design and operation of several lab scale GAC biofilter columns. Sacrificial filters allowed access to the filter bed at 4 timepoints and several bed depths to build a comprehensive profile of the biological communities associated with biofiltration over time and through the depth of the filter bed.
- The biofilter design allowed for the effluent water of three different bed sizes to be compared over time for biological and chemical removal. The biological community of the effluent water can also be compared between the three bed sizes, as well as the filter beds at week 23.
- For chemical removal the long 90cm biofilter performed the best with comparable removal efficiencies in the case of DOC and to a lesser extent iron to full scale slow sand filters. The short 30cm filter performed poorest.
- For biological removal there was no significant difference observed between any of the three filter bed sizes for pathogen or total biological removal. However, the total and intact cell counts of the short filter correlated strongest with the influent water.
- Filtration process did not significantly alter the percentage of total cells which were intact from the influent water. However, there was around 37% reduction in total and 43% intact cell numbers.

Chapter 3

Optimisation of Molecular Methods

3.1. Introduction

Investigating the microbial community composition of environmental samples such as those associated with biofiltration can be a challenging enterprise. This is because environmental samples consist of very diverse, mixed populations, with the vast majority of members unculturable by traditional methods (Bodor et al. 2020; De Mandal and Panda 2015) within complex environmental matrices. High throughput sequencing analysis has become a widely used tool to investigate the community composition of environmental samples (Sanschagrin and Yergeau 2014). 16S rRNA amplicon sequencing as used in this study is capable of parallel analysis of a high number of samples, and coverage of the high level of diversity contained within environmental samples. Comparing the variable regions of the 16S rRNA gene between taxa allows members to be identified at Genus level regardless of their cultivability while also providing data on their relative abundance (Sinclair et al. 2015). However, the use of 16S rRNA amplicon sequencing is not without its challenges.

In an ideal scenario the 16S rRNA sequencing data would be a perfect reflection of the starting community, correctly identifying every community member in their true relative abundance. In reality however, numerous factors, such as primer bias, DNA extraction efficiency and environmental inhibitors can influence the complex molecular mechanisms during library preparation, introducing biases which may skew the sequencing data from the true composition of the starting community (Kennedy et al. 2014; Seguin-Orlando et al. 2013). Biases introduced at the library preparation stage can therefore lead to sequencing data misrepresenting the starting community, leading to erroneous conclusions after data analysis (Kircher et al, 2011). Thus, it is of importance to consider and minimise as far as possible the introduction of experimental bias during library preparation to ensure the data is as representative of the true starting community as possible and best protect the integrity of final conclusions.

Each stage of library preparation, from sample collection to final library sequencing, can introduce the opportunity for experimental bias which can influence the data downstream. The workflow used in this study follows that of the Earth Microbiome Project protocol for 16S rRNA Illumina sequencing (Caporaso et al. 2000). The recommended primers F515 and R926 contain Illumina adapters and a Golay barcode on the forward primer for sample identification within the library pool. This allows for a single PCR step after DNA extraction to amplify the 16S rRNA genes of the community while adding the required adapters and

indexes for sequencing. A basic outline of the workflow is shown in Figure 3.1. The initial starting communities were extracted from GAC, glass fibre membrane and Sterivex filters obtained from the biofilter experiment as outlined in Chapter 2.

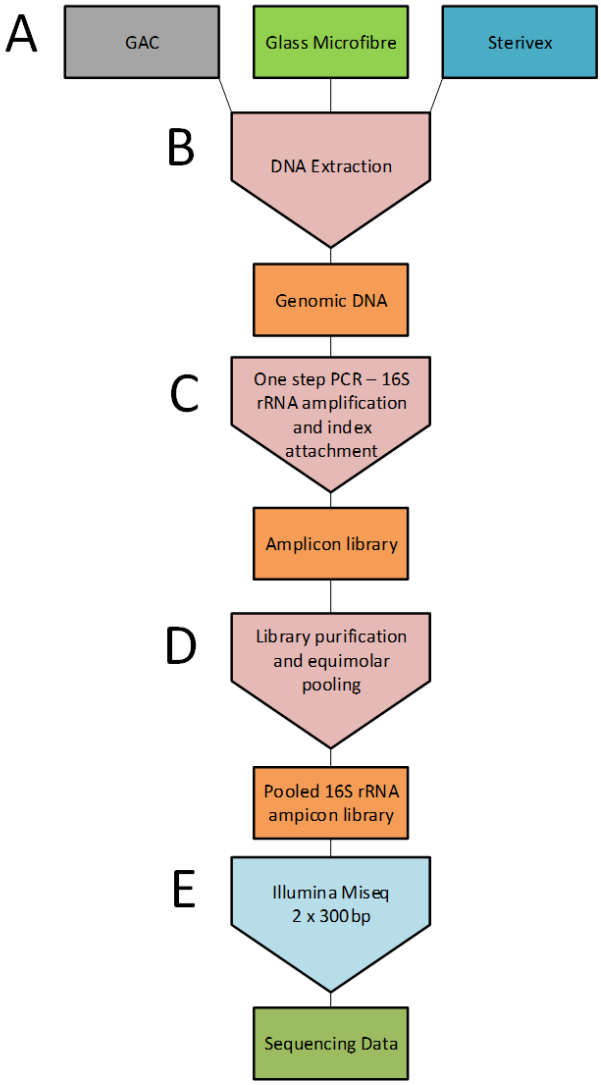


Figure 3.1. *A – The initial starting samples were obtained from the media bed (GAC) and influent (Glass fibre & Sterivex) and effluent water (Sterivex) as outlined in Chapter 2. B – DNA was extracted from each individual sample. C – 1ng of gDNA was PCR amplified for each sample adding Illumina adapters and index barcodes. D – Each individual sample was purified using AMpure magnetic beads and quantified by Qubit. All samples were then pooled equimolarly. E – The pooled library was sent for Illumina Miseq sequencing (Earlham Institute, Norwich).*

The aim of this workflow was to produce sequencing data as representative of the starting communities as possible, ensuring observed community differences over time and depth were as accurate as can be. Again, each stage of this workflow presents the chance to introduce bias which can influence downstream sequencing data (Sinclair et al. 2015). Thus, it is of importance to consider and minimise the introduction of bias when selecting and optimising the molecular methods utilised to build a 16S rRNA library.

3.1.1. DNA Extraction

After sampling, the first stage to consider is the extraction of DNA from the starting community. Ideally, DNA extraction would ensure that every cell of the community is lysed equally, and the entirety of their DNA made available for amplification. In reality, different cellular properties among community members may confer different levels of resistance to lysis (Starke et al. 2019). As such, easily lysed cells may be overrepresented after sequencing while taxa which are more resistant to lysis may be underrepresented in the starting community. This issue is compounded when comparing communities on different support material as each material may offer differing degrees of protection against lysis (Frostegård et al. 1999; Moré et al. 1994).

Two approaches are widely accepted when extracting DNA from environmental samples. The first method is to separate the cells from the support material before lysis takes place (Robe et al. 2003; Bakken and Lindahl 1995). While this method minimises the co-extraction of extracellular DNA and inhibitory particles, bias may be introduced based on the proportion of the community which are separated from the support material (Roose-Amsaleg et al. 2001). Again, different cellular properties within the community allow some members to adhere more strongly to the support material than others (Liao et al. 2015; Zhao et al. 2014). Thus, those members which detach more easily may be overrepresented and the more difficult to remove members underrepresented. The second approach involves the in-situ lysis of the community with the support material (Robe et al. 2003; Ogram et al. 1987). While this approach offers access to the entire starting community, there are potential biases which require consideration. As the community is lysed with the support material, there is potential for newly freed DNA to adsorb to particles of the support material and thus not be included in analysis (Robe et al. 2003; Cai et al. 2006). Again, this issue is compounded when comparing communities from different sources as different support materials may offer a higher affinity

for the adsorption of DNA than others. Furthermore, extracellular DNA may have previously become adsorbed to the support material leading to an overestimation of live cells after analysis (Frostegård et al. 1999; Pathan et al. 2021). Another consideration is the co-extraction of inhibitory substances such as humic acids which may have a detrimental effect on amplification downstream (Lakay et al. 2007).

3.1.2. PCR Amplification

Amplification by Polymerase Chain Reaction (PCR) is an essential component of 16S rRNA library preparation for Illumina sequencing of environmental samples. The 16S rRNA gene consists of highly conserved regions and nine hypervariable regions which can be utilised to identify taxa at genus or species level (Clarridge 2004). Primers designed to target the conserved regions common to all taxa span these hypervariable regions, allowing for taxa to be identified by comparing the sequences of these regions to an existing database (Roy 2014). However, the complex nature of multi-template environmental samples can lead to biases which need to be considered (Kanagawa 2003).

Perfect amplification of a multi-template community would ensure that the target region of the 16S rRNA gene amplifies equally for all community members while preserving their true relative abundances. However, variations at the molecular level can lead to unequal amplification across the target community leading to skewed or misleading sequencing data (Acinas et al. 2005; Kalle et al. 2014). One consideration is the specificity and coverage of the primers chosen. Highly specific primers will have a high binding affinity with their target region, reducing the chance of non-target amplification affecting PCR efficiency (Baker et al. 2003; Wilcox et al. 2013). However, slight variations in the target sequence between community members may lead to differences in primer binding efficiency between taxa (Polz and Cavanaugh 1998; Sipos et al. 2010). These differences may lead to preferential amplification of sequences with higher primer specificity resulting in an overestimation in the abundances of associated taxa. Similarly, the fidelity of *Taq* polymerase can be affected by variations in sequences between taxa. For example, Nichols et al found that choice of polymerase can lead to preferential amplification based on G-C content (Nichols et al. 2018). Preferential amplification can also be exacerbated by high cycle numbers due to the exponential nature of PCR (Acinas et al. 2005). Another factor which could alter the efficiency of PCR is the presence of inhibitors. A wide range of inhibitory substances such as

polyphenols, metal ions and humic acids can interfere with molecular mechanisms during the PCR reaction (Schrader et al. 2012). These interactions can reduce the fidelity of *Taq* polymerase, bind to template DNA or reduce the availability of co-factors (Schrader et al. 2012). Varying levels of PCR inhibitors will lead to varying PCR efficiencies between samples, thus biasing the resulting data.

3.1.3. Challenges for this study

The work in this thesis aims to compare the microbial communities from three different environments. These are the communities found through the depth of the filter bed and influent and effluent water of biofilter columns (Chapter 2). Thus, a robust methodology must be developed to extract DNA and generate amplicons as efficiently as possible across the three starting materials (GAC, glass fibre membrane, and Sterivex filter).

Biomass estimation based on ATP analysis conducted on the day of deconstruction (Chapter 4) suggested low cell numbers in the bottom sections of the filter bed. To ensure enough DNA would be available for sequencing, selecting an extraction method which would produce the maximum yield of amplifiable DNA across all sample types was the first challenge.

The properties of GAC can make it a difficult matrix to extract DNA from. Firstly, its porous nature may allow colonisation within the GAC particles (Velten et al. 2007; Walker and Weatherley 1998). GAC consists of macro, meso and micropores and it has been suggested that bacteria may be able to colonise the larger macropores (Stoeckli et al. 2002; Velten et al. 2007). As such, these would likely prove difficult to successfully separate from the media. This means that an extraction method must be chosen which extracts from cells in-situ across the three sample types. Furthermore, to ensure access to any microbes colonising these pores the structure of the GAC must be broken down. For this reason, optimisation of the extraction method focuses on lysis by bead beating to ensure the destruction of the GAC particles.

A further complication which may arise when extracting genomic DNA from GAC follows from its adsorption capacity. GAC is known for being a highly adsorbent material and may sequester DNA from lysed cells reducing the yield (Kennedy and Summers 2015; Kirtane et al. 2020). To combat this, this study tests the use of AMPLIQON G2 beads developed to increase yields from adsorbent materials (Gobbi et al. 2019; Jacobsen et al. 2018). G2 beads

are coated with un-amplifiable salmon sperm DNA which are thought to occupy adsorption sites before cell lysis, thus leaving fewer adsorption sites available to DNA freed by cell lysis.

The second challenge to overcome is derived from potential differences in PCR efficiency when amplifying DNA extracted from the three sample types. To focus optimisation on biases that might arise due to differences in sample type, the primers and *Taq* polymerase were kept consistent throughout this study. The primers used were recommended by the Earth Microbiome Project Illumina 16S rRNA protocol (Caporaso et al. 2000.). These primers, 515F and 926R cover the V4/V5 region of the 16SrRNA gene and have been widely used in studies focussing on biofiltration (De Vera and Wert 2019; Gerrity et al. 2018; Haig et al. 2014; Vera et al. 2018). When compared to primers covering other variable regions, this primer set has been shown to provide good coverage and accurately reflect the expected abundances and phylogenies of the target community (Claesson et al. 2010; Fadeev et al. 2021). Constructed with attached Illumina adapters and 12bp indexes, these primers follow a single PCR step protocol, desirable due to the large number of samples investigated in this study. The *Taq* polymerase used in this study was Qiagen Hotstart DNA polymerase. This *Taq* polymerase boasts high specificity with minimum optimisation. A feature of Hotstart *Taq* polymerase is an inhibitor attached to the enzyme which renders it inactive at room temperature, preventing binding taking place before the samples are placed in the cycler (Dahiya et al. 1995).

A difficulty with in-situ extraction is the co-extraction of PCR inhibitors with the DNA (Nair et al. 2014). This is further complicated when extracting from different sample types. Different starting materials may contain different concentrations of PCR inhibitors leading to varying efficiencies during amplification. Dilution of the template DNA is an often-used technique to lessen the effect of inhibitors (Eckhart et al. 2000; Monteiro et al. 1997; Scipioni et al. 2008). However, the nature of the samples to be compared in this work limits the use of this method. To normalise between communities derived from liquid and solid media environments the volume of template added to each PCR reaction is to be consistent across all samples. As such the template DNA requires dilution to bring all samples to the desired concentration of DNA. Due to a wide range of cell numbers between samples (Chapter 4) there will be a range of dilution factors dependent on the yield of each sample. The challenge facing this study is to optimise a PCR method which amplifies across all sample types as equally as possible despite variations in PCR inhibitor concentrations.

Overall, this chapter aims to develop a robust methodology for DNA extraction and subsequent PCR of samples derived from biofilter media and associated water in the creation of a 16S rRNA sequencing library. This methodology aims to minimise bias when comparing different starting samples and will be utilised to process all samples generating sequencing data which will inform Chapters 4, 5 and 6.

3.2. Materials and Methods

Five different extraction methods were tested on the GAC, Sterivex and glass microfibre samples. Each extraction method utilised bead beating for cell lysis. Two commercial kits, the Powerwater DNA extraction kit (Qaigen) and the FastDNA Spin Kit for Soil (MPBio) were tested, as was a phenol/chloroform DNA/RNA co-extraction adapted from Griffiths et al, 2000. The phenol/chloroform method was used with either the Lysis matrix E beads (MPBio) or G2 beads (Ampliqon). Table 1 outlines a summary of the extraction methods tested against each sample. A range of GAC volumes was tested (0.1g – 0.5g) when extracting using the FastDNA spin kit for soil.

Table 3.1. Extraction methods tested using GAC, Sterivex and Glass microfibre samples.

Extraction Method	GAC	Sterivex	Glass microfibre
Qaigen PowerWater DNA extraction kit	N	N	Y×
Phenol/chloroform (LME) (Griffiths et al. 2000)	Y×	Y×	Y×
Phenol/chloroform (G2) (Griffiths et al. 2000)	Y×	Y✓	Y✓
MPBio FastDNA spin kit for soil (5ml tubes)	Y×	N	N
MPBio FastDNA spin kit for soil (15ml tubes)	Y✓	Y✓	Y✓

Legend: Y – method tested on sample, N – method not tested on sample, ✓– method performed well on sample, × -method performed poorly on sample.

3.2.1. Sample Preparation

For each extraction method 0.5g of GAC obtained from the 30-50cm section of filter B6C deconstructed on week 12 was used. The GAC, previously aliquoted in 0.5g sections and stored at -80°C, was thawed on ice and transferred aseptically in duplicate to the bead beating tube required for each method.

For water, 2L of influent water (collected on the 16/05/19) was filtered through a Fisherbrand MF200 glass microfibre filter (retention 1.2µm) and then through a 0.22µm Millipore® Sterivex™ filter in duplicate. Filtration was carried out via the method outlined in Chapter 2 (section 2.2.17)

The glass microfibre (GF) filters were placed in a sterile 50mm petri dish and taped and the Sterivex filters were labelled and wrapped in parafilm. Both were stored at -80°C. For each extraction method the GF filters were thawed on ice and then cut into pieces using sterile tweezers and added to the required bead beating tube. The Sterivex filters were thawed on ice

before being pried open and the filters transferred in pieces to the required bead beating tubes aseptically.

3.2.2. Extraction Methods

The **Qiagen DNeasy PowerWate**r was used to extract DNA from the GF filters following the manufacturer's instructions.

The **MPBIO FastDNA spin kit for soil** extraction methods were carried out following the manufacturer's instructions, with an extended centrifugation of 15 minutes at step 5 of the FastDNA protocol. Different volumes of GAC added to the lysis matrix E tube was tested, ranging from 0.1 – 0.5g. The Sterivex and glass microfibre filters were added thawed on ice and added into the lysis matrix E tube as described in section 3.2.1.

Different tube sizes were also compared at step 7 of the FastDNA spin kit for soil protocol. 0.4g of GAC of all filter bed depths from filter B3A deconstructed at week 6 were added to lysis matrix E tubes and extracted following the manufacturer's instructions. At step 7 the recommended 15ml tubes were replaced with LoBind 5ml tubes and the extraction continued as per the manufacturers protocol. All extractions carried out with the FastDNA spin kit were eluted in 50µl DES water as supplied with the kit.

A phenol/chloroform extraction based on Griffith et al 2000 was carried out on all sample types using **Lysis Matrix E tubes** and **Ampliqon G2 beads** (0.1mm) (Gobbi et al. 2019). The samples were added to either the lysis matrix E or G2 bead tubes as above. 0.5ml of 5% CTAB buffer and 0.5ml liquified Phenol:Chloroform:Isoamyl alcohol (24:1) was added and the cells lysed using the MPBIO FastPrep machine at speed 6 for 40 seconds. The tubes were then centrifuged for 20 minutes at 12000 g at 4°C. The top aqueous layer was transferred to a new 1.5ml microcentrifuge tube and 0.5ml Chloroform:Isoamyl alcohol added. The tubes were inverted several times creating an emulsion and then centrifuged at 16000g for 5 minutes. The top aqueous layer was transferred to a new microcentrifuge tube and two volumes of 30% PEG added and left on ice for two hours before centrifugation at 16000g for 30 minutes. The supernatant was then gently poured away, and the pellet washed with 1ml ice cold 70% ethanol and centrifuged at 16000g for 30 minutes at 4°C. Ethanol was removed and the pellet allowed to air dry before being resuspended in 20µl ultrapure water.

The DNA was quantified by Qubit 2.0™ using the Invitrogen™ Qubit™ dsDNA HS Assay Kit according to the manufactures instructions and visualised on a 1% agarose gel. The gel was prepared by adding 0.4g of agarose to 40ml of TAE buffer and boiled allowing the agarose to melt. The liquid agarose was allowed to cool for 5 minutes and 4µl SYBR safe was added. The liquid agarose was then poured into a running tray with the appropriate well comb and allowed to set for 30 minutes.

3.2.3. Quantitative PCR

16S rRNA Q-PCR was carried out in 96 well plates on a Quantstudio 3.0 Real time PCR system using Biorad iTaq universal probe reagents. Each 20µl reaction contained 10µl iTaq universal probes supermix, 0.4µl 1389 Probe 10µm (CTTGTACACACCGCCCGTC), 1.8µl 1369F 10µm (CGGTGAATACGTTCYCGG), 1.8µl 1496R 10µm (GGWTACCTTGTTACGACTT) (Suzuki et al, 2000) 5µl ultrapure PCR water and 1µl of template DNA. Premade standards (Smith et al. 2006) were run at 5-fold dilutions from 130,000,000 to 1664 copy numbers in triplicate with a no template control in triplicate. The QPCR was run for an initial hold stage of 95°C for 10 minutes and then 40 cycles of 95°C for 10 seconds and 60°C for 30 seconds.

3.2.4. End Point PCR

End point PCR was carried out using Qiagen Hotstart reagents. Primers F515 and R926, targeting the V4/V5 region of the 16S rRNA gene were taken from the Earth Microbiome Project 16S rRNA Illumina protocol (Caporaso et al. 2012.)The primer constructs are outlined in Table 1 and the final optimised reaction mixture is outlined in Table 2. The cycling conditions were an initial hold stage at 90°C for 15 minutes, followed by 25 amplification cycles of 95°C for 30 seconds, 55°C for 30 seconds and 72°C for 30 seconds. The final elongation stage was at 72°C for 10 minutes. PCR products were visualised on a 1% SYBR safe agarose gel run at 90v for 40 minutes.

Table 3.2. *Primer constructs used in preparation of 16S rRNA amplicon library. Golay barcodes of all forward primers found in Appendix (A.1).*

Forward Primer				
Illumina 5' Adapter	**Golay Barcode**	**Forward Primer Pad**	**Forward Primer Linker**	**515F Forward Primer**
AATGATACGGCGACCACCGAGATCTACACGCT	XXXXXXXXXXXX	TATGGTAATT	GT	GTGYCAGCMGCCGCGGTAA
Reverse Primer				
RC of 3' Illumina Adapter	**Golay Barcode**	**Reverse Primer Pad**	**Reverse Primer Linker**	**926R Reverse Primer**
CAAGCAGAAGACGGCATACGAGAT		AGTCAGCCAG	GG	CCGYCAATTYMTTTRAGTTT

Table 3.3. *Un-optimised PCR protocol per reaction as recommended by the Qiagen Hotstart protocol for a 25µl reaction.*

Component	**Volume/reaction (25µl)**	**Final Concentration**
PCR Water	Variable	-
X10 PCR Buffer	2.5µl	x1
dNTP mix (10mM of each)	0.5µl	200µM of each dNTP
Forward primer 515(10µM)	Variable	0.1-0.5µM
Reverse primer 926 (10µM)	Variable	0.1-0.5µM
***Taq* polymerase**	0.125µl	2.5 units
Template (0.5ng/µl gDNA)	Variable	≤1µg

Table 3.4. *Optimised PCR protocol per 25µl reaction.*

Component	Volume/reaction (25µl)	Final Concentration
PCR Water	14.75µl	-
X10 PCR Buffer	2.5µl	x1
dNTP mix (10mM of each)	0.5µl	200µM of each dNTP
Forward primer 515(10µM)	1.5µl	0.6µM
Reverse primer 926 (10µM)	1.5µl	0.6µM
MgCl+ (25mM)	2µl	2mM
***Taq* polymerase**	0.25µl	5 units
Template (0.5ng/µl gDNA)	2µl	0.04ng/µl

3.3. Results

3.3.1. DNA extraction method

Initial testing using the Qiagen PowerWater kit deemed it unsuitable for the GF/C filters and thus discounted as a potential extraction method. This was due to the lack of separation of the glass fibre filters and supernatant after cell lysis and centrifugation. Subsequently, three extraction methods were compared, a phenol/chloroform extraction with lysis matrix E and another with G2 beads and the FastDNA spin kit for soil, on GAC, Glass Fibre filters and Sterivex filters (Figure 3.2.A). The MPBIO FastDNA spin kit for soil was deemed the best suited when considering all sample types.

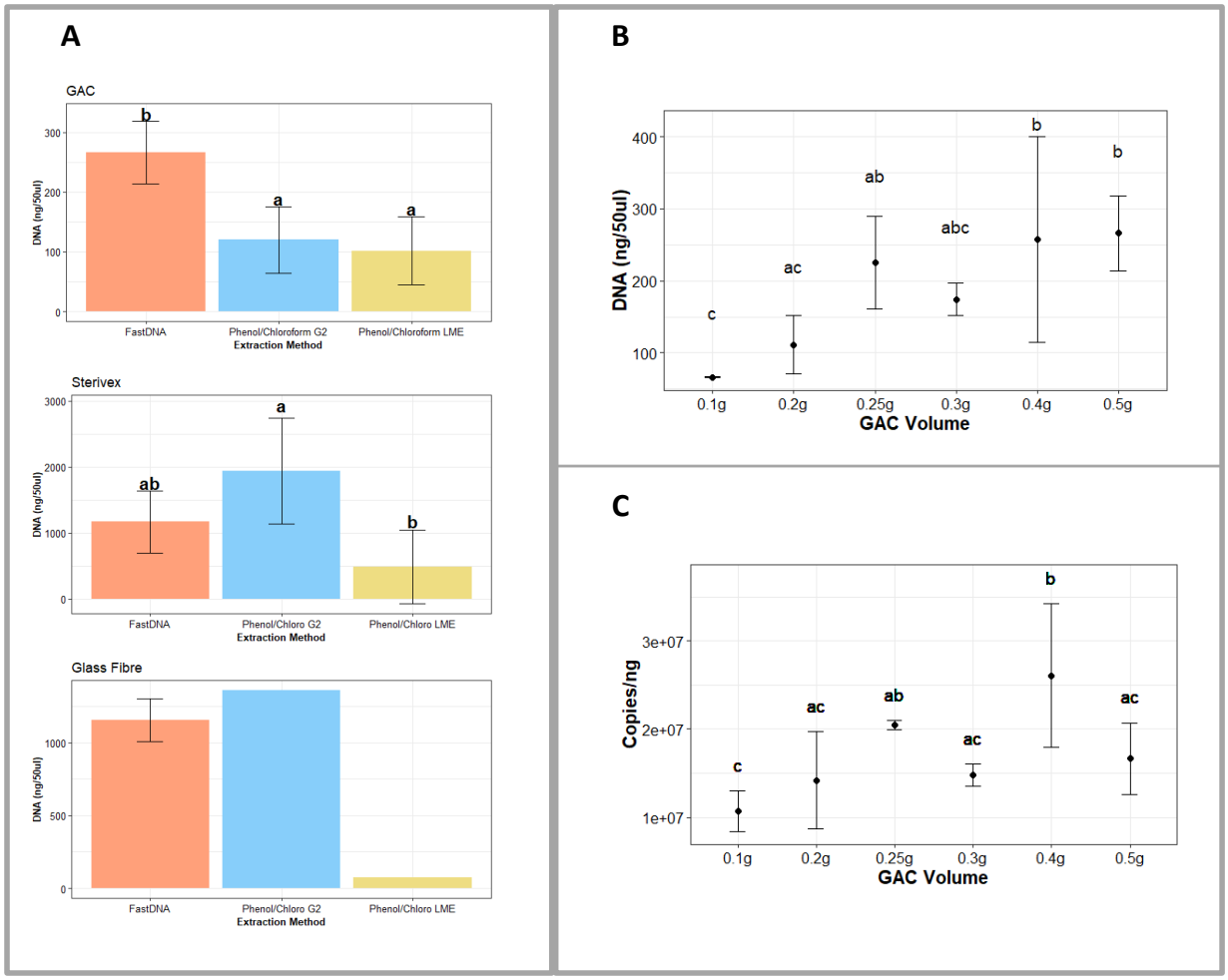


Figure 3.2. *A - Comparison of the three extraction methods, MPBIO FastDNA Spin Kit for soil (FastDNA), the phenol/chloroform method with G2 beads and (phenol/chloroform G2) and the phenol/chloroform method with Lysis Matrix E (LME) beads. The three extractions methods were tested against all three sample types, GAC (n=2), Sterivex (n=2) and Glass fibre filters (n=1). Significance letters generated by one way ANOVA and Tukey HSD. B – DNA yield per extraction eluted in 50µl DES water of different volumes of GAC using the Fast DNA Spin Kit for Soil. Significant letters generated by one way ANOVA and Tukey HSD. C – Quantitative PCR of the extractions of the different GAC volumes using the FastDNA Spin Kit normalised to copies per nanogram. Significance letters generated by one way ANOVA and Tukey HSD.*

The extraction method which resulted in the lowest yield across all samples was the phenol/chloroform extraction using lysis matrix E beads (Figure 3.2. A). Replacing the lysis matrix E tubes with the G2 beads resulted in a significant increase in yield for both the glass fibre and Sterivex filtered samples. However, there was no significant difference between the two bead types when tested with the GAC samples. The FastDNA spin kit resulted in a comparable yield to the phenol/chloroform method with G2 beads for both the glass fibre and Sterivex samples and was not significantly different from the phenol/chloroform method with lysis matrix E beads against the Sterivex sample. The FastDNA spin kit performed significantly better against the GAC samples than either of the phenol/chloroform methods. As the FastDNA spin kit performed comparatively with the phenol/chloroform methods against both water samples with G2 beads and significantly better against the GAC, it was selected as the DNA extraction method for this study.

Then, extraction of DNA from a range of GAC volumes from 0.1 – 5g was tested using the FastDNA spin kit to determine the optimum volume to deliver the highest yield of DNA (Figure 3.2. B). When considering the volume of DNA obtained per extraction, it would appear that increasing the volume of GAC past 0.25g has little effect. Unsurprisingly, the lowest yield was obtained from 0.1g of GAC while the highest was obtained from 0.4g and 0.5g, though the yields obtained from 0.25g and 0.3g were similar. While the yield of DNA per extraction appeared similar from 0.25g to 0.4g the amount of amplifiable DNA investigated by real-time PCR revealed a slightly different trend (Figure 3.2. C). Again, the fewest copies per gram was obtained from 0.1g of GAC which was not significantly different from the 0.2g, 0.3g and 0.5g samples. The highest number of copies per gram of GAC was obtained from the 0.4g extraction, which was statistically distinct from all other samples with the exception of the 0.25g sample. Due to the 0.4g extraction producing one of the highest yields of DNA per extraction and demonstrating the greatest volume of amplifiable DNA, it was chosen as the optimum volume of GAC from which to extract.

The FastDNA spin kit for soil recommends the use of 15ml tubes when binding the nucleic acids to the silica matrix. The difficulty with using 15ml tubes at this stage is that it risks cross contamination between samples as, due to the length of the tube, the shaft of the pipette may come in contact with the inner side when transferring the binding matrix to the spin column. To combat this shorter LoBind 5ml tubes were tested against the 15ml tubes and the yields compared (Figure 3.3).

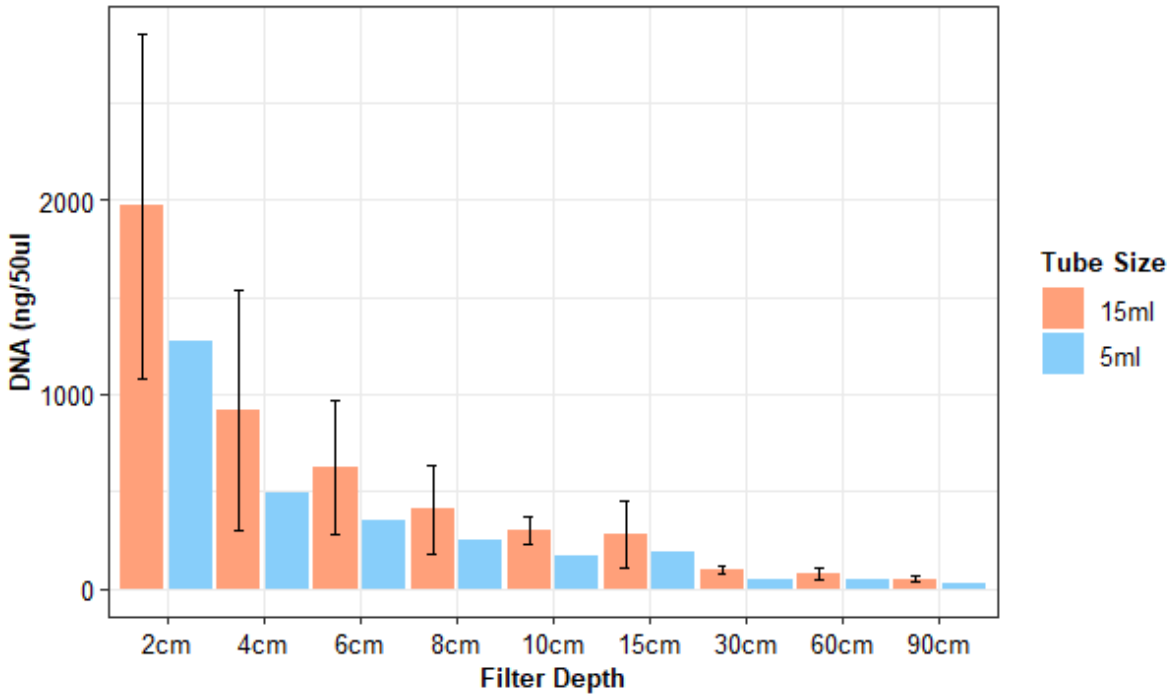


Figure 3.3. *DNA yields obtained per extraction eluted in 50µl DES water using the FastDNA Spin kit for soil against 15ml (n=2) conical centrifuge tubes and 5ml (n=1) LoBind tubes.*

When tested against all bed depths of filter B3A, the 15ml tubes consistently outperformed the 5ml tubes, producing a higher yield of DNA per extraction. As a result, it was determined that the recommended 15ml tubes were to be used and another solution to the cross-contamination issue was found. This solution involved the use of extended pipette tips and an autoclaved cutting of a 10ml pipette tip being placed in the top of the 15ml tube to act as a sterile barrier between the side of the tube and the shaft of the pipette (Figure 3.4).

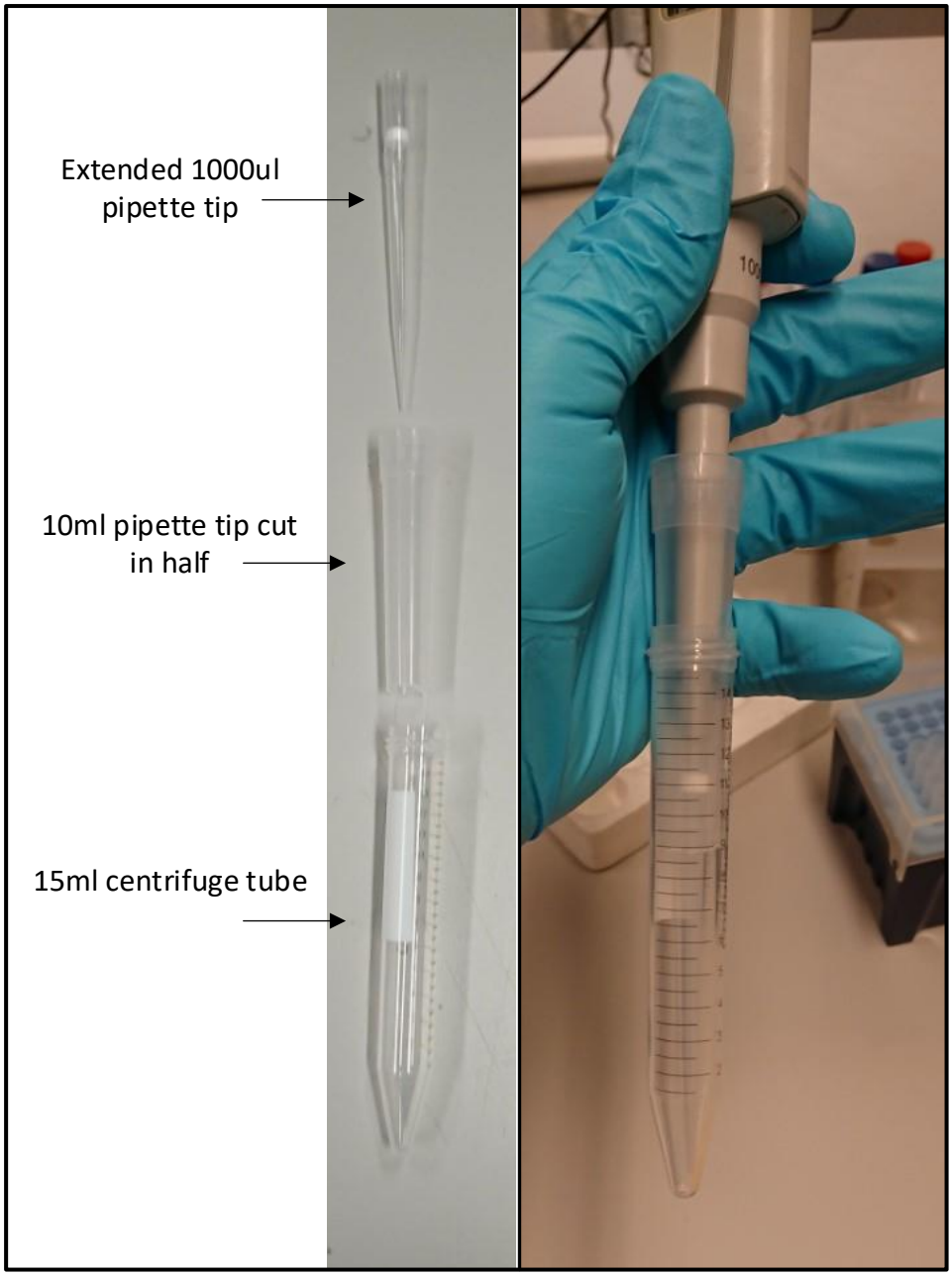


Figure 3.4. *Photograph of the solution to the potential cross contamination issue of using the 15ml tubes. An autoclaved cutting of a 10ml pipette tube was used as a sterile barrier preventing the shaft of the pipette from coming into contact with the side of the 15ml tube during extraction.*

3.3.2. 16S rRNA PCR Optimisation

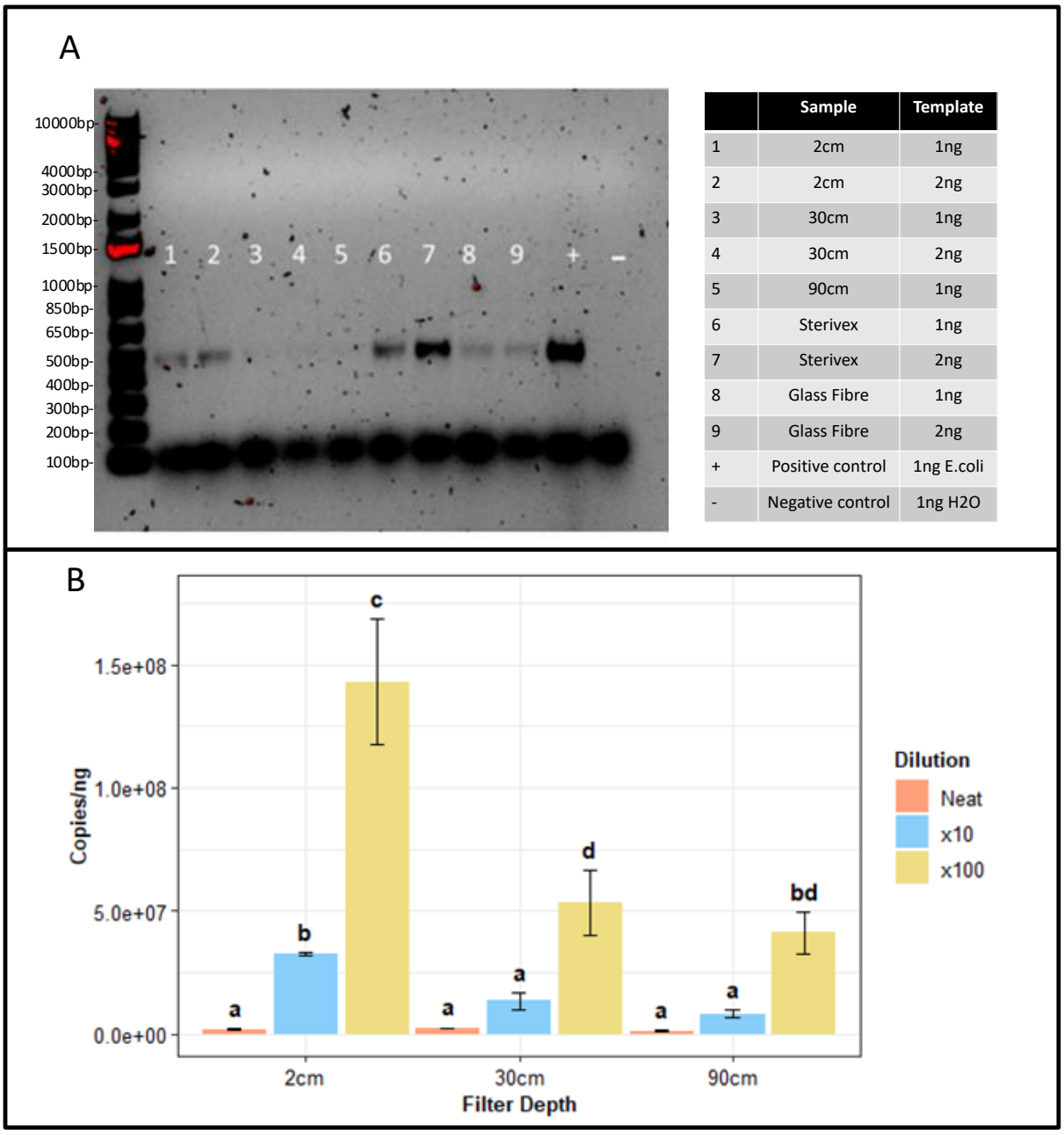
3.3.2.1. Optimal Annealing Temperature

The optimal annealing temperature was determined using a temperature gradient PCR from 50°C to 59° with PCR conditions as recommended by the Qiagen Hotstart *Taq* protocol (Table 3) and PCR cycling conditions

Initial testing based on the recommendations provided by the Qiagen Hotstart *Taq* protocol and an annealing temperature 55°C provided the optimum cycling conditions for 16S rRNA PCR, found in the methods section, these were used throughout the optimisation process.

3.3.2.2. 16S rRNA gene amplification from filter bed depth

DNA extracted from bed depths 2cm, 30cm and 90cm of filter B3A (Figure 3.5. A.) were amplified using primers F515 and R926 (Table 2) alongside extractions from Sterivex and GF filters loaded with 2L of influent water collected on the 16/05/19. Template DNA was diluted to 1ng or 2ng/µl and 10% of PCR product was run on a 1% agarose gel. The expected product size was 503bp.



	Sample	Template
1	2cm	1ng
2	2cm	2ng
3	30cm	1ng
4	30cm	2ng
5	90cm	1ng
6	Sterivex	1ng
7	Sterivex	2ng
8	Glass Fibre	1ng
9	Glass Fibre	2ng
+	Positive control	1ng E.coli
-	Negative control	1ng H2O

Figure 3.5. *A – PCR product from GAC samples 2cm, 30cm and 90cm of filter B3A along with PCR product from 2L of influent water filtered through glass fibre and Sterivex filters with 1ng and 2ng of template added. Positive control – 1µl of 1µl/ng Escherichia coli DNA. Negative control - 1 µl PCR water.2.5µl of PCR product and 0.5µl loading dye in each well of a 1% agarose run at 90V for 40 minutes with a 1kb ladder. B – Quantitative PCR of DNA extracted from GAC samples 2cm, 30cm and 90cm from filter B3A. 1µl of template DNA was added neat, diluted 1:10 and 1:100. Significance letters generated by two-way ANOVA and Tukey HSD.*

The brightest band on the agarose gel was found in the DNA extracted from the Sterivex filter using 2ng/µl of template (Figure 3.5 A), indicating the water had the fewest inhibitors. While amplification was observed for both the GF filter and the 2cm GAC sample at both 1ng and 2ng/µl, the GAC samples from 30cm and 90cm were below gel detection limits despite the same amount of template added. Next samples were amplified by real-time PCR to determine if inhibitors were causing the apparent lack of amplification in the 30 and 90 cm depths.

A 16S rRNA QPCR on neat, 10^{-1} and 10^{-2} dilutions of the GAC DNA template demonstrated a significant increase in copies/ng of DNA when all samples were diluted at 1:100 (2cm – $1.97 \times 10^6 \pm 9.4 \times 10^4$ to $1.4 \times 10^8 \pm 1.3 \times 10^7$, 30cm – $2.4 \times 10^6 \pm 1.2 \times 10^5$ to $5.3 \times 10^7 \pm 6.7 \times 10^6$, 90cm - $1.4 \times 10^6 \pm 1.6 \times 10^5$ to $8.3 \times 10^6 \pm 7 \times 10^5$ p-values range 0 to 0.0000011) (Figure 3.5 B). A significant increase was also observed in the 2cm sample ($1.97 \times 10^6 \pm 9.4 \times 10^4$ to $3.2 \times 10^7 \pm 2.9 \times 10^5$) at a 1:10 dilution (p-value 0.0002). An increase in copy number per nanogram was also observed in the 30cm ($2.4 \times 10^6 \pm 1.2 \times 10^5$ to $1.4 \times 10^7 \pm 1.7 \times 10^6$) and 90cm ($1.4 \times 10^6 \pm 1.6 \times 10^5$ to $4.1 \times 10^7 \pm 4.1 \times 10^6$) samples however the difference was not significant. All samples demonstrated an increase in copy number per nanogram with dilution, with the largest increase being in the 2cm and decreasing with depth. This strongly suggests the presence of PCR inhibitors and that the inhibitors are at a higher concentration towards the top of the filter bed (Lance and Guan 2020). While the concentration of inhibitors may be higher in samples from the top of the filter bed, the results from the end point PCR demonstrate that the samples from deeper were failing to amplify. This can likely be attributed to the higher dilution factor required to bring the 2cm samples to 1ng or 2ng/µl (needed for 16S rRNA amplicon sequencing libraries to normalise amount of DNA per sample) reducing the influence of the inhibitors on amplification. As the samples from deeper in the filter bed proved more difficult to amplify, further optimisation focused on the samples obtained from the 90cm bed depth at week 24.

3.3.2.3. Optimisation of 90cm samples

The 90cm section from filter B4A, deconstructed at week 23 was selected to optimise PCR amplification from the lower depths. This section was chosen as it was thought to possibly have a higher concentration of inhibitors than the same section at earlier weeks. If the inhibitors were originally adsorbed to the GAC, samples from the latest timepoint would have

the longest contact time to collect inhibitors by adsorption. As biomass in the filter bed is known to increase over time (Chen et al. 2021; Haig et al. 2015) then samples taken at week 23 would also have higher biomass load in case the biomass was the origin of the inhibitors. To test if the volume of template was adequate for the lower samples, a gradient of template concentration was also tested.

A template DNA concentration gradient from 1ng to 4ng was investigated alongside a positive control of 1ng *E. coli* DNA following the master mix recipe recommended by the Hotstart PCR kit (Table 3). When run on a 1% agarose gel, amplification was only observed in the positive control suggesting that increasing the template concentration was not sufficient to overcome the amplification issues in the 90cm samples. A template concentration gradient from 0.25ng to 3ng was then tested with an increased concentration of *Taq* polymerase from the recommended 2.5units to 5 units added to the reaction.

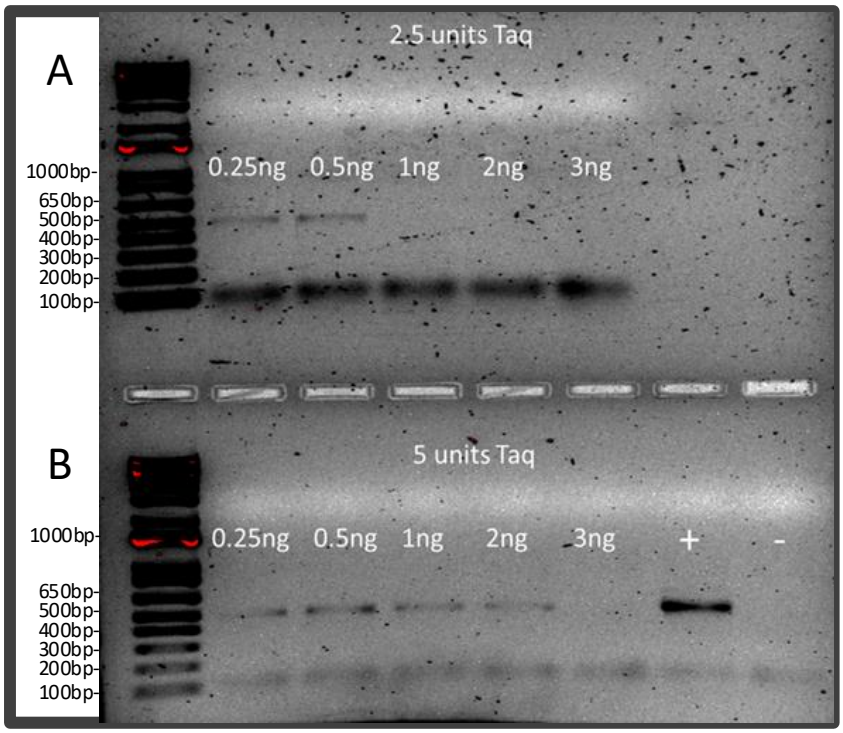


Figure 3.6. A *- PCR product from a range of template concentrations (0.25ng – 3ng) of the 90cm filter bed depth of filter B4A with 2.5 and* ***B*** *5 units of Qiagen Hotstart Taq polymerase. Positive control – 1µl of 1µl/ng Escherichia coli DNA. Negative control - 1 µl PCR water.2.5µl of PCR product and 0.5µl loading dye in each well of a 1% agarose run at 90V for 40 minutes with a 1kb ladder.*

As previously, no amplification was observed at the recommended 2.5 units of *Taq* polymerase at 1ng to 3ng of template. However, there was amplification at 0.25ng and 0.5ng of template using 2.5 units of *Taq*. As the initial DNA concentration of this sample was 0.9ng/µl, the 0.25ng and 0.5ng samples required dilution which may have reduced the influence of inhibitors in these reactions. At 5 units of *Taq* polymerase, amplification was observed up to 2ng of template. This demonstrates that increasing the *Taq* polymerase results in successful amplification of both diluted and undiluted template. This is of importance when considering samples from earlier timepoints and deeper in the filter bed, in which the lower yields prevent dilution and so must be added neat, with associated inhibitors. An attempt to use the PCR additive BSA was made to reduce the effect of the inhibitors, however this was found not to be effective (data not shown). Therefore, further optimisation was conducted using the increased units of *Taq* polymerase. 0.5ng of template appeared to deliver sufficient product with and without increased *Taq* Polymerase and so it was selected as the optimum template concentration moving forward.

As magnesium is a co-factor of *Taq* polymerase (Cline, Braman, and Hogrefe 1996), a MgCl+ gradient was tested with the increased volume of 5 units *Taq*. This showed that increasing the $MgCl^{+}$ concentration in the reaction to 2mM improves amplification with 5 units of Taq polymerase (Figure 3.7).

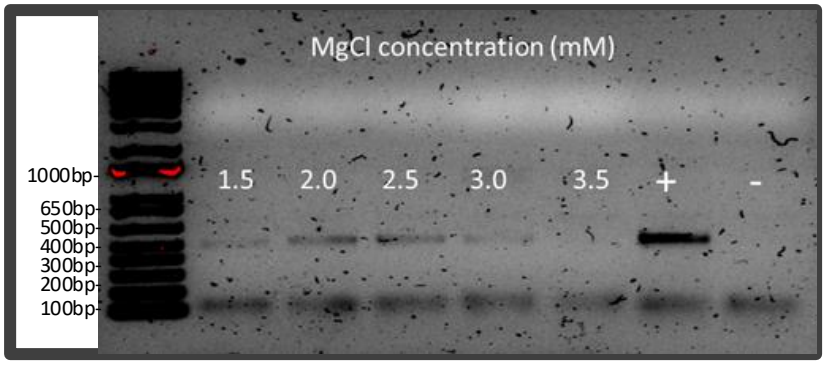


Figure 3.7. *0.5ng of 90cm template from filter B4A amplified with a $MgCl^{+}$ concentration gradient of 1.5 to 3.5mM. Positive control – 1µl of 1µl/ng Escherichia coli DNA. Negative control - 1 µl PCR water.2.5µl of PCR product and 0.5µl loading dye in each well of a 1% agarose run at 90V for 40 minutes with a 1kb ladder.*

The optimised PCR reaction using 0.5 ng template, 2mM $MgCl^{+}$ and 5 units of *Taq*, was used successfully to amplify 16S rRNA genes from the 2 cm to 90cm depth at week 23. However, when used with samples earlier timepoints (weeks 5-9) it became apparent that further optimisation was required as no samples from some filter replicates were amplified

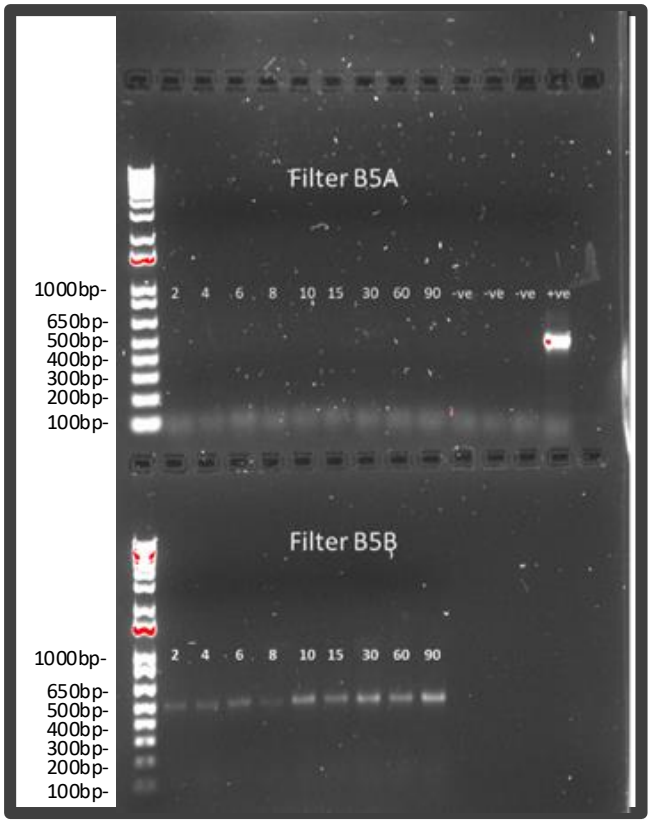


Figure 3.8*. PCR product of template from all filter bed depths from filters B5A and B5B, 0.5ng template amplified with increased Taq polymerase (5 units) and a $MgCl^{+}$ concentration of 2nM. Positive control – 1µl of 1µl/ng Escherichia coli DNA. Negative control - 1 µl PCR water.2.5µl of PCR product and 0.5µl loading dye in each well of a 1% agarose run at 90V for 40 minutes with a 1kb ladder.*

Interestingly, when applied to some filters the newly optimised PCR method failed to deliver a product. It is unclear why individual filters failed in their entirety when as part of experimental biological replicates, they were fed the same influent water and exposed to the same conditions, clearly there are complex unknown interactions involved in the formulation of inhibitors. Furthermore, as the filters were amplified from a single master-mix of PCR

reagents meaning human error was unlikely to be the cause of the failure, and that it was variation in the DNA extracts causing it. As some filters (B3B, B5A, B5C) were proving more difficult to amplify, further optimisation focused on these. An increase in both primer and template concentration was then applied in an attempt to improve the amplification in these difficult to amplify filters.

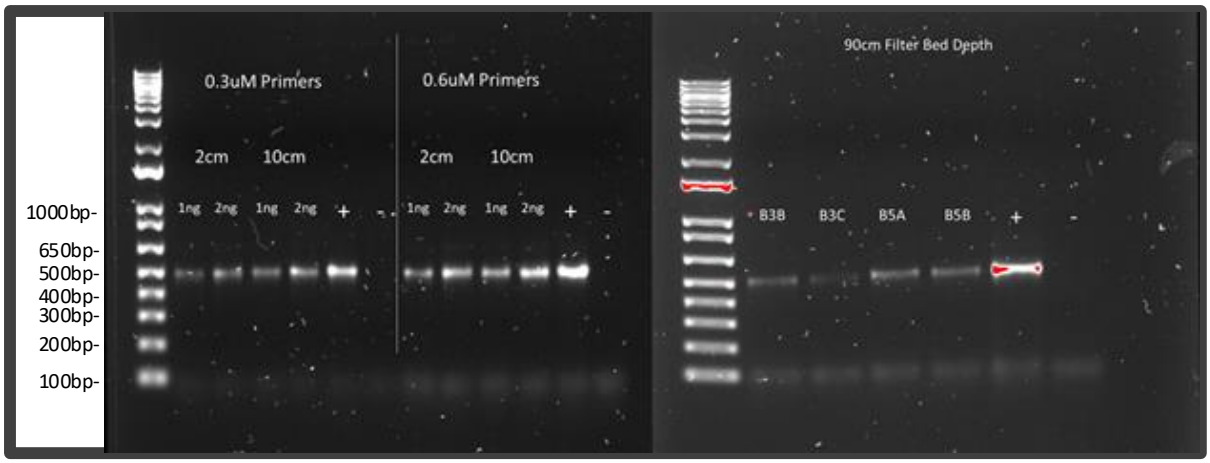


Figure 3.9. *PCR product with 1ng and 2ng of template from 2cm and 10cm of filter B3B with primer concentrations of 0.3 and 0.6µM. and product from 1ng template of 90cm of filters B3B, B3C, B5A and B5B with a primer concentration of 0.6µM. Positive control – 1µl of 1µl/ng Escherichia coli DNA. Negative control - 1 µl PCR water.2.5µl of PCR product and 0.5µl loading dye in each well of a 1% agarose run at 90V for 40 minutes with a 1kb ladder.*

Sections from 2cm and 10cm of filter B3B were amplified using increased template and primer concentration. Increasing the volume of DNA in the reaction resulted in product at both primer concentrations at these depths. Increasing the primer concentration of the reaction increased the amount of product for both 1ng and 2ng of template. While both concentrations of template worked, 1ng was deemed more suitable for this project due to the low yield obtained from sections deeper in the filter bed fuelling concerns of being short of DNA for further studies. The increased primer concentration was then tested against the 90cm depths of filters which had previously failed to amplify, alongside replicates which had previously amplified successfully. The increased primer concentration amplified successfully at 1ng of template for all four filters tested at 90cm sections.

The newly optimised PCR method (Table 3) was tested against bed depth 10cm and the bottom section of each filter along with several water samples and all amplified successfully against all samples tested. With successful amplification of all sample types, the optimised Hotstart PCR protocol was utilised in the construction of 16S rRNA libraries for this thesis.

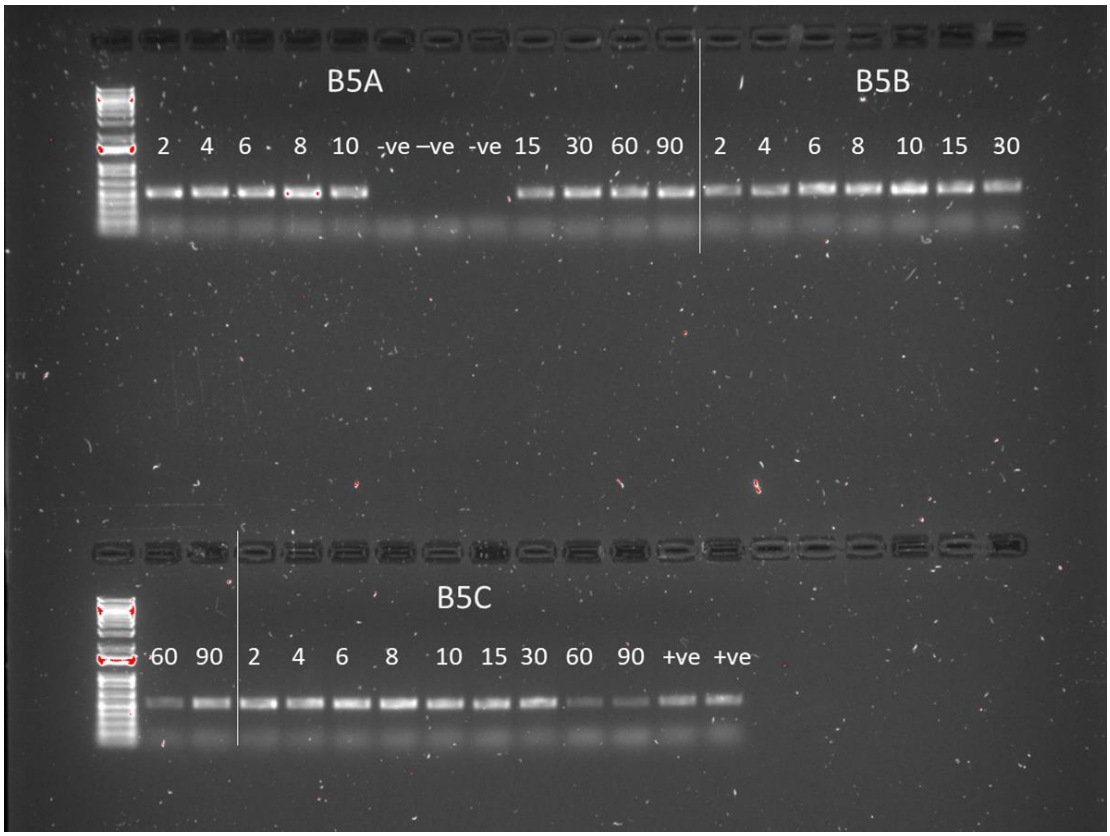


Figure 3.10. *PCR amplification using optimised protocol from a range of bed depths and time. PCR product from all filter bed depths from filters B5A, B5B and B5C using the newly optimised protocol. Positive control – 1µl of 1µl/ng Escherichia coli DNA. Negative control - 1 µl PCR water.2.5µl of PCR product and 0.5µl loading dye in each well of a 1% agarose run at 90V for 40 minutes with a 1kb ladder.*

The optimised Hotstart PCR protocol was shown to give consistent product across all sample types during construction of the 16S rRNA amplicon library. Increasing the primer and *Taq* polymerase concentration with added magnesium chloride was found to improve the product consistency through all filter bed depths when compared to the recommended protocol without the need to increase amplification cycles. Furthermore, the optimised method

achieved consistent product after normalisation to 1ng DNA despite the varying levels of PCR inhibitors thought to be present in the samples.

3.4. Discussion

The work carried out in this chapter serves to highlight the difficulties in working with environmental samples and demonstrate the importance of optimisation of laboratory methods to reduce as far as possible amplification bias that would impact downstream analysis and interpretation of the results.

When selecting a DNA extraction method, it is important to consider the nature of the starting material. In this study, three different starting materials GAC, glass microfibre membrane and Sterivex filters were used, each with unique properties which may influence the yield obtained. Thus, it was of importance to find an extraction method which worked consistently across all sample types, delivering the highest possible yield of amplifiable DNA. While several commercial DNA extraction kits are available, these tend to be optimised towards one sample type and may work inconsistently when applied to different starting materials (Ffler et al. 1997.; Karaaslan et al. 2014.). For example, the Qiagen PowerWater extraction kit tested proved ineffective at extracting from the glass microfibre filter. This was due to the fibrous nature of the glass microfibre filter becoming entangled in the bead matrix after beating, resulting in difficulty withdrawing the supernatant for further processing.

Of the three sample types used in this study, the most difficulty was expected to be encountered extracting from the GAC samples, this was due to several factors. Firstly, the amount of biomass contained on the GAC was expected to be considerably lower than that of the water samples. While the filtration through the glass fibre and Sterivex filters was expected to capture the majority of attached and free-living cells in 2L of water, the biomass in the GAC samples consisted only of those colonising the GAC. This was of concern with samples collected at the earliest timepoints and from the deeper sections of the filter bed which were expected to harbour the least biomass according to ATP analysis on the day of deconstruction (Chapter 2). Another potential issue was due to the high adsorption capacity of GAC (Jung et al. 2001b; Ghosh et al. 1999). It was thought likely that DNA released from freshly lysed cells would adsorb to the GAC and thus not contribute to the yield obtained. While this issue could theoretically be overcome by first removing the cells from the GAC

before lysis, the porous structure of GAC removed this as a viable solution. Any smaller organisms residing in the macropores would likely prove very difficult to remove from the GAC before lysis and fail to be included in the final yield. In order to reveal the truest representation of the biological community, it was determined the structure of the GAC was to be destroyed by bead beating, ensuring access to any cells which may be contained in pores. The bead beating method utilised by the FastDNA spin kit was found to successfully destroy the GAC structure.

Of the three methods tested, the highest GAC yield was obtained from the FastDNA spin kit for soil. Interestingly, the use of G2 beads with the phenol/chloroform extraction method made no significant difference to the yield obtained. This was surprising as the addition of G2 beads have been shown to increase the yield obtained from recalcitrant soils (Gobbi et al. 2019). Furthermore, the addition of G2 beads significantly increased the yield from both the glass fibre and Sterivex water samples. This was surprising as it was expected that the G2 beads would have the greatest influence on yield obtained from the GAC samples. Due to the high adsorption capacity of GAC seeming likely to sequester DNA following lysis, it was expected that the salmon sperm DNA coating the G2 beads would fill available adsorption sites resulting in an increased yield of DNA (Jacobsen et al. 2018). One explanation is that the adsorption sites on the GAC were exhausted, meaning the non-amplifiable DNA coating the G2 beads was not necessary to occupy adsorption sites in place of the DNA released from the newly lysed cells and thus had a negligible effect on the yield obtained. Another possibility is the difference in bead size affecting the yield of DNA obtained (De Boer et al. 2010). The G2 beads tested were a uniform 0.1mm, while the lysis matrix E beads consisted of a mix of 1.4mm and 0.1mm beads including a 4mm glass bead. Potentially the mixed beads were more efficient at cell lysis in the GAC samples, compensating for any DNA lost to adsorption (Gobbi et al. 2019). In the case of the Sterivex and glass microfibre samples, the G2 beads may have been more or as efficient at cell lysis while reducing the influence of DNA lost to any free adsorbents in the samples. At the time of this study, AMPLIQON did not offer a mixed set of G2 beads so direct comparison of bead size with the Lysis matrix E tube was not possible. A more robust comparison would have compared the G2 beads with uniform 0.1mm silica beads. However, as the FastDNA extraction kit had already demonstrated a superior yield for the GAC samples and considering the cost and length of the protocols it was determined this was not worth pursuing. It may also have been beneficial to test the G2 beads with the FastDNA extraction kit, though the combined cost of both the G2

beads and the extraction kits meant that this was not a viable option for this study and again was not pursued.

3.4.1. Optimisation of the FastDNA spin kit for soil method

With the FastDNA spin kit for soil chosen as the optimum method, further optimisation on the protocol itself was conducted. Again, the optimisation focused primarily on the GAC samples as these were the samples from which the lowest yield was obtained. Firstly, the volume of GAC added to the lysis matrix E tube was tested to determine which volume maximised the yield of amplifiable DNA. Unsurprisingly the lowest yields were obtained from 0.1g of GAC, due to the fewer GAC particles leading to fewer cells from which to extract. Increased yield was obtained by increasing the volume of GAC, though the trend was not as linear as might be expected. GAC volumes of 0.2g and 0.3g were not shown to be statistically different from 0.1g, and there was no statistical difference observed between the 0.25g to 0.5g volumes. This suggests that the number of cells present is not the only factor which influences the yield obtained. Potential factors influencing yield may be the amount of air space in the tube altering the freedom of movement for the GAC particles and beads during beating or differences in the cell to buffer ratio (Khosravinia et al., 2007). While the FastDNA spin kit recommends the use of up to 0.5g of soil, and this presented with one of the highest yields, 0.4g was chosen for this study. After amplifying each of the extraction by 16S QPCR, 0.4g was shown to provide the highest yield of amplifiable DNA. As the ultimate goal was to maximise the yield of amplifiable DNA, 0.4g was deemed the most appropriate volume to use.

At step 7 of the FastDNA spin kit for soil protocol, the use of 15ml tubes is recommended to ensure sufficient mixing and binding of nucleic acids to the silica binding matrix. Due to the length of the 15ml tubes, it was difficult to extract the entirety of the binding matrix without the shaft of the pipette having to enter the tube. This potentially could lead to cross contamination between samples, as the shaft of the pipette may come into contact with the inside of the tube, particularly when processing a high number of samples. An obvious solution to this would be the use of smaller tubes which would allow the entirety of the binding matrix to be extracted without the shaft of the pipette entering the tube. However, the use of smaller tubes may result in less efficient mixing of the DNA and the binding matrix reducing the final yield. To test this, the yield obtained from using 5ml LoBind tubes was

compared to that using 15ml tubes, as recommended by the FastDNA spin kit protocol, against samples from all bed depths of filter B3A (Figure 3.3). Across all samples the 15ml tubes outperformed the 5ml tubes, demonstrating that the larger tubes were indeed necessary to maximise yield. Therefore, another solution to the cross-contamination issue had to be found. This was achieved by using 10ml pipette tips which were cut in half and autoclaved. The top half of the 10ml pipette tips were inserted into the 15ml tubes when the binding matrix was to be extracted. This created a sterile barrier between the side of the 15ml tube and the shaft of the pipette, allowing the transfer of the binding matrix while vastly reducing the chance of cross-contamination.

3.4.2. 16S rRNA PCR Optimisation

For the eventual sequencing data to be the truest reflection of the starting community, the amplification step of library preparation should faithfully amplify all template while maintaining the relative abundances found in the environment. However, interactions at a molecular level can introduce biases which can skew the sequencing data from the starting community (Kalle et al. 2014). For example, differences in the binding efficiency of primers or *Taq* polymerase between community members can lead to preferential amplification (Wilcox et al. 2013; Nichols et al. 2018). Sequences with which the primers or polymerase bind to most efficiently will be amplified at a greater rate leading to an artificially inflated abundance of community members associated with those sequences. Preferential amplification can be exacerbated by increasing cycle number as bias is increased exponentially with each new cycle (Acinas et al. 2005). Further bias can be introduced by the presence of PCR inhibitors which can detrimentally affect PCR efficiency through interactions with the template, primers or *Taq* polymerase (Schrader et al. 2012). PCR inhibitors are a known concern when extracting from environmental samples (Robe et al. 2003; Nair et al. 2014). When looking to compare communities from different environments, the presence of PCR inhibitors can become more problematic. Different environments may contain different concentrations of inhibitors, thus affecting PCR efficiency to different degrees (Hedman and Rådström 2013). This was found to be a challenging aspect to overcome in this study.

The presence of PCR inhibitors was verified by 16S rRNA QPCR and demonstrated that the highest concentration of inhibitors was found at the top of the filter bed, decreasing with

depth. One possibility is that the inhibitors may have been adsorbed onto the GAC, as the inhibitors would initially fill adsorption sites at the top of the filter bed as these would be encountered first (Duan et al. 2003). Alternatively, the inhibitors may have been derived from the biomass which was shown to be largest at the top of the bed, decreasing with depth (Schrader et al. 2012). While the highest concentration of inhibitors was found to be at the top of the filter bed, in practice the most difficult samples to amplify were from the bottom of the filter bed. This was likely due to the dilution factors of the template DNA being higher for samples taken from the top of the filter bed. Reducing the concentration of inhibitors by dilution of the template is a known method of combating PCR inhibitors (McKee, et al. 2015; Wang et al. 2017). However, the need to normalise the template to 1ng/ul between the three sample types meant that there was a range of dilution factors based on the DNA yield obtained after extraction. As there was a higher yield obtained from samples taken from the top of the bed, the dilution factor required to normalise these samples was considerably higher than those at the bottom of the filter bed. As the dilution factors were set based on the recovered yield of DNA, another method to combat the effects of inhibitors had to be found. While increasing the number of cycles may have increased the product yield of the difficult to amplify samples, it may also have increased unknown selective biases introduced by the primer or polymerase choice. Due to the exponential nature of PCR amplification, any biases are likely to be amplified with each additional cycle (Polz and Cavanaugh 1998). For this reason, the number of cycles was limited to 25. Another option to increase product yield would be to perform multiple PCR reactions which could then be combined and concentrated at the clean-up stage. This is a wiser approach to reduce PCR amplification bias but would not address the effects of PCR inhibitors that appeared to be varying between the samples.

A method to reduce the concentration of inhibitors within the samples is the addition of a DNA clean up step such as AMPure magnetic beads. However, any cleaning method comes with an inherent loss of DNA (Sagova-Mareckova et al. 2008). Due to the already low yield obtained from the 90cm samples, this method was deemed unsuitable. Other methods which are known to combat PCR inhibitors include PCR additives and increasing the concentration of *Taq* polymerase, both of which were tested (Schrader et al. 2012). The addition of BSA was tested as it is known to reduce the effects of humic acids, a common PCR inhibitor in water samples. While the addition of BSA was shown to slightly improve amplification, it also resulted in unexplained smearing in the agarose gel. Increasing the concentration of *Taq* polymerase improved amplification up to 2ng of template added. This was important as it

demonstrated that increasing *Taq* polymerase improved product yield against both diluted and non-diluted template. The low DNA yield obtained from the 90cm samples meant that they could not be diluted, thus any optimisation had to be able to amplify from un-diluted template. As magnesium is a co-factor of *Taq* polymerase (Cline et al.1996), it was necessary to test an increased $MgCl^+$ concentration with the increased *Taq* polymerase. This was done using a $MgCl^+$ gradient as excess magnesium has been shown to decrease the fidelity of *Taq* polymerase (Ignatov et al. 2003). A $MgCl^+$ concentration of 2mM was found to be optimal with the increased concentration of *Taq* polymerase.

The increased concentration of *Taq* polymerase and $MgCl^+$ was shown to improve product yield on the 90cm samples from filter B4A, thought likely the most difficult to amplify. The rationale being that samples from week 24 would have the highest concentration of inhibitors adsorbed to the GAC and the highest biomass if the inhibitors were derived from there (Velten et al. 2011). When tested against samples from higher in the filter bed of filter B4 and against the Sterivex and Glass fibre samples, this method resulted in sufficient amplification of all sample types. At this stage optimisation was thought complete and work began on processing the samples from the earlier GAC samples. However, when processing these samples, amplification of samples from some filters failed in their entirety. It is not known why certain filters failed while others within the same set of biological replicates amplified without issue. The filters in each set were fed the same influent water and subjected to the same conditions for the same amount of time. The filters in each set of replicates were also amplified simultaneously using the same PCR master mix so it seems unlikely to be caused by PCR error. Further optimisation focusing on the filters which had failed to amplify was required. It was found that increasing the template from 0.5ng to 1ng per reaction and doubling the primer concentration allowed these difficult filter sets to amplify. To test the newly optimised method, samples from bed depths 10cm and 90cm from each of the filters were amplified. Once it was demonstrated that the newly optimised method was effective against these samples from all the filters, processing the samples for library preparation could begin. The newly optimised PCR method amplified consistently across all filters at all bed depths as well as across the Sterivex and glass fibre sample types.

3.5. Conclusions and Recommendations

The work in this chapter serves to highlight some of the difficulties which may be encountered when constructing a 16S rRNA gene amplicon sequencing library from environmental samples. Particularly when sequencing data is to be compared across multiple sample types. Experimental bias introduced in the workflow can influence downstream sequencing data casting any comparisons or conclusions into doubt. Thus, it is highly beneficial to take steps to minimise these biases before sample processing begins.

When designing an experiment, the author recommends that extra samples are procured out with those used in the final library if possible. These samples should be representative of all sample types used in the final library and can be used to optimise the workflow before processing of the final samples begins. When selecting a DNA extraction method, it is important to consider the nature of the starting material. There are various DNA extraction methods and commercial kits available, and the optimum is often sample dependant. Thus, it is important to test different extraction methods against all sample types which are included in the final sequencing library. Furthermore, the selected extraction method may require an altered protocol to optimise for all sample types.

At the PCR stage it is beneficial to initially test a subset of samples which are representative of all sample types to be included in the library. It may also be beneficial to run a QPCR assay to test for PCR inhibitors in each of the sample types. If PCR inhibitors are causing difficulty in certain samples increasing primer, *Taq* polymerase or template concentration or indeed PCR additives can be tested before increasing cycle number. Optimisation should initially focus on the samples which are amplifying poorly. When beginning to process the samples for the Illumina library, the author recommends starting with a subset of samples which are representative of the entire set to ensure that the optimised PCR protocol is effective across all sample types.

Each stage of preparing a 16S rRNA library for Illumina sequencing can potentially introduce biases which can influence the final interpretation of data and indeed any conclusions that may be drawn. The work carried out in this chapter demonstrates the steps taken to minimise as far as possible the introduction of bias during library preparation and to best ensure that the data presented in the remainder of this thesis is the truest possible reflection of the microbiological communities investigated and provided an optimised approach that was applied to all samples used in the following chapters.

Chapter 4

Spatial and Temporal Investigation of Biofilter Microbial Communities

4.1. Introduction

The removal of contaminants by biofiltration in general is intrinsically linked to the biological community contained within the filter. Over time microbial biomass accumulates on the filter media leading to a diverse population which drives the removal of biological and chemical contaminants (Haig, Quince, et al. 2015a). Members of this community utilise organic or inorganic contaminants as an energy source depending on their associated metabolic pathways while biological contaminants can be preyed upon by predatory organisms within the filter bed (Fdz-Polanco et al. 2000; Guchi 2015; Weber-Shirk and Dick, 1997.). Thus, it is this bacterial consortium which largely dictates the removal efficiency achieved by biofiltration.

The growing availability of high throughput sequencing techniques has meant that the communities involved in biofiltration can be investigated in greater detail than ever before (Hou et al. 2018; Haig et al. 2015; Zhao et al. 2019; Lautenschlager et al. 2014). Despite this, the relationship between community composition and biofilter performance remains poorly understood. This is largely due to the myriad of factors which can influence the composition of communities involved in biofiltration. Influent water source, pre-treatment of the influent water, media choice and engineering parameters such as temperature and EBCT have all been demonstrated to influence biofilter communities (Ma et al. 2020; Vera et al. 2018; Vignola et al. 2018; Nemani et al. 2018; Moll et al. 1999). These factors mean that it is difficult to compare community composition between studies. Many studies have investigated the communities inhabiting biofilters in full scale drinking water treatment plants, however these filters are often part of varied treatment trains and the communities subjected to pre-treatments likely to influence community composition (Hou et al. 2018; Gerrity et al. 2018; Lautenschlager et al. 2014). Similarly, the varied biofilter design parameters used in lab scale studies make comparisons between studies difficult (Delgado-Gardea et al. 2019; Haig et al. 2014; Crognale et al. 2019). While attempts have been made to link biological community to contaminant removal, these studies tend to focus on specific taxa and their associated contaminant, such as nitrifiers or iron oxidisers (Fdz-Polanco et al. 2000; Gülay et al. 2018; Yapsakli et al. 2010).

Changes in biofilter communities are known to occur both through the depth over the filter bed and over time as the filter matures (Velten et al. 2011). Initial colonisation of the filter bed occurs as microorganisms are introduced to the filter media via the influent water and

begin to attach to the particles of the media. Over time biomass and biological activity increases until a steady state is reached and removal efficiency plateaus (Velten et al. 2011). At this steady state the biofilter is thought to be mature and operating at maximum removal efficiency until clogging, head loss, or dropping removal efficiency signify filter failure (Xiang et al. 2013; Liao et al. 2015). Along with changes over time, the biological community has been shown to differ through the depth of the filter bed (Haig et al. 2015; Vera et al. 2018). Biomass and activity have consistently been shown to be highest at the top of the filter bed decreasing with depth, as such it is though the majority of biological contaminant removal occurs in the top section of the filter bed (Velten et al. 2011; Zhang et al. 2018; Chen et al. 2021; Matuzahroh et al. 2020). However, little is currently known of the contribution made by communities colonising deeper layers of the filter bed. Studies have shown that the length of filter bed can have an effect on contaminant removal (Jun et al. 2002; Hoang et al. 2008; De Vera et al. 2019). This might suggest that the communities deeper in the filter bed are important to achieve maximum contaminant removal. Furthermore, nutrient gradients which develop through the filter bed may force the communities residing deeper in the filter bed to adapt to available carbon sources which may differ from those available at the top of the filter bed (Boon et al. 2011). If so, the communities which develop deeper in the filter bed may be important for the removal of specific contaminants.

While depth has been shown to be a factor which influences community development, the greater biomass and activity at the top of the filter bed suggest this is where the majority of contaminant removal takes place. Current research suggests that increasing media depth can improve the removal efficiency for chemical contaminants (Jun et al. 2002; Hoang et al. 2008; De Vera et al. 2019). In the case of biological contaminants filter media depth appears to be of less importance (Verma et al. 2019; Freitas et al. 2021). The results from the filters in this study, outlined in Chapter 2, seem to concur with the published literature. Increasing filter bed depth improved the removal efficiency of DOC and iron removal though had no significant effect on the removal of total or intact cells or indeed the pathogens monitored.

However, it is currently poorly understood how overall media depth might affect the communities which develop within the filter bed. Filters run identically and fed influent water from the same source might be expected to develop similar communities in the top section of the filter bed. The communities in biofiltration are thought to be assembled by both stochastic and deterministic forces (Vignola et al. 2018). Nutrient and oxygen availability may act as

selective pressures driving community development. As fresh influent water is continually added to the top of the filter bed, it is likely these areas will be nutrient and oxygen rich, forming a decreasing gradient with depth. As such, it would be likely that the top layer of the filter bed would have similar environmental conditions regardless of bed length. However, as bed length increases it may lead to broader nutrient and oxygen gradients leading to oligotrophic or anoxic conditions not able to be reproduced within a shorter filter bed (Song et al. 2017; Fraleigh and Bungay, 1986). This would create different selective pressures to the top section of the filter bed and potentially select for taxa incapable of colonising a shorted bed length (Boon et al. 2011). However, this assumes nutrient and oxygen gradients are generated from the top down. It is also possible that proximity to the bottom of the filter bed introduces selective pressures which may affect the microbial community. For example, if the community in the bottom section of the filter can claim nutrients or oxygen from the reservoir of effluent water to which it is in contact. Currently there are no studies which offer a direct comparison of established biofilter communities between different filter media depths.

The development of the microbial community of the filter bed is an essential component linked to filter performance. Both time and bed depth have been shown to be influential factors as the community within the filter bed becomes established (Velten et al. 2011; Haig et al. 2015). For advancements in the field of biofiltration to occur, a deeper understanding of the changes in community composition influenced by time and bed depth would be highly beneficial. This study aims to utilise lab scale biofiltration columns, designed to allow access to the filter bed at various depths and timepoints, to monitor changes in the microbial communities of the filter bed over time and depth at a higher resolution than has been previously reported.

Furthermore, the effect of filter bed length on the development of microbial communities remains poorly understood and may have implications concerning filter performance related to bed length. Specifically, this study aims to compare the established microbial communities of three different bed lengths, short, medium and long, at the top, middle and bottom sections of each biofilter. To this end, the microbial community developing within long (90cm filters) was monitored over 23 weeks, with depth resolved sections taken at weeks, 5, 9, 12 and 23; In addition, the influent and effluent water at each time point was examined. Short (30cm) and medium (60cm) filters were operated alongside, and deconstructed at the end of 23 weeks, with matching depth resolved community analysis. This was to compare the microbial

community development between the three column sizes. in addition to examining the effects filter length had, if any, on the microbial community of the final effluent water.

4.2. Materials and Methods

4.2.1. DNA extraction

DNA extractions were carried out using the FastDNA spin kit for soil (MPBIO) 0.4g aliquots of GAC collected from filter deconstruction at weeks 5, 9, 12 and 23 and depths 2cm, 4cm, 6cm, 8cm 10cm, 15cm, 30cm, 60cm and 90cm were removed from the -80°C freezer and allowed to thaw on ice and transferred aseptically to the lysis matrix E tubes supplied with the FastDNA kit. For matching time points influent and effluent water samples filtered onto GF-C and subsequently 0.22µm Sterivex filters were removed from the -80C. G-FC filters were removed from the -80°C and allowed to thaw in ice. Once thawed the glass fibre membrane filters were cut in half and split between two lysis matrix E tubes, a separate extraction being carried out on each. The glass fibre membrane filters were cut into small pieces using a sterile scalpel and tweezers and transferred into the lysis matrix E tubes. For corresponding 0.22µm Sterivex filters once thawed on ice, the plastic casing of the Sterivex filter was pried open using a pair of wire cutters which had been strenuously cleaned with 70% ethanol. The filter was removed from the plastic casing and cut from its support using sterile tweezers and added to the lysis matrix E tubes in small pieces. Extractions were carried out following the instructions provided with the FastDNA spin kit for soil with an extended centrifuge time of 15 minutes at step 7. Final DNA was eluted in 50µl DNase free water supplied with the kit. The two extractions carried out from the glass fibre filters were combined in one 2ml catch tube supplied with the kit. 5µl of DNA was then visualised on a 1% agarose gel at 90V for 40 minutes and quantified by Qubit using the Thermofisher HS dsDNA Qubit kit (Invitrogen™) according to the manufacturer's instructions.

4.2.2. 16S rRNA Gene Library Preparation

Genomic DNA from all samples were diluted to 0.5ng/µl and used as template DNA for end point PCR. A one step PCR was carried out using primers F515 and R926 as recommended by the EarthMicrobiome Project covering the V4/V5 region of the 16S rRNA gene (Parada, Needham, and Fuhrman 2016; Quince et al. 2011). These primers contained Illumina adapters and a 12bp Golay barcode on the forward primer. The general primer constructs are detailed in Table 3.2. Each sample individual sample for the pooled library used a forward primer with a different Golay barcode (See Appendix Table A.1) to distinguish between unique samples after sequencing. PCR was carried out in sterile 0.2ml strip tubes and using the Qiagen Hotstart kit, the components of each reaction are outlined in Table 3.4.PCR cycles were carried out on Applied Biosystems 2720 thermal cycler. Each reaction was held at 95°C for 15 minutes followed by 25 cycles of 95°C for 30 seconds, 55°C for 30 seconds and 72°C for 30 seconds with a final elongation stage of 72°C for 10 minutes. PCR product was visualised on a 1% agarose gel for 40 minutes at 90V and stored at -20°C until further processing.

4.2.3. Purification and Pooling

PCR products were removed from the -20°C freezer and allowed to thaw on ice. Once thawed product was transferred into sterile 96 well plates. AMpure magnetic beads (Agencourt) were shaken and added to each sample in a 0.7:1 bead ratio. The product and bead mixture were mixed by pipetting up and down 10 times and then left at room temperature to incubate for 10 minutes. The plate was then added to the magnetic rack for two minutes separating the beads from the solution. The solution was removed, and the beads washed twice with 200µl 70% molecular grade ethanol, incubated at room temperature for 30 seconds with each wash. The beads were then left to dry for 1 minute before being removed from the magnetic rack and resuspended in 20µl DNase free water. The plate was then put back on the rack to again separate the beads from solution. The purified PCR product in solution was then transferred to a new 0.2ml sterile tube. The purified product was then quantified by Qubit using the Thermofisher HS dsDNA kit. The product from each sample was then pooled equimolarly and concentrated by a second clean up as above eluting in 1/3 volume of DNase free water.

4.2.4. Data Analysis

The pooled library was sent to Earlham Institute for Illumina Miseq 300 x 300bp sequencing. Two sequencing runs were required due to the number of samples and the raw sequencing data from both was combined before processing. Amplicon sequence variants (ASVs) were constructed by processing raw sequencing data using the Qiime2 pipeline and DADA2 algorithim (Callahan et al. 2016). The full workflow can be found at https://github.com/umerijaz/tutorials/blob/master/qiime2_tutorial.md. Reads were demultiplexed and visualised following Qiime2 and DADA2 workflow before being quality trimmed. Forward reads were trimmed by 240 and the reverse 200bp. Forward and reverse reads were merged to construct ASVs and dereplicated to generate abundance data. ASVs were aligned using Maftt (Katoh et al. 2009) and rooted phylogenetic tree was constructed using FastTree (Price et al. 2010). Taxonomy was assigned with the Bayesian lowest common ancestor algorithm against the Silva v138 database. Abundance, taxonomy and the phylogenetic tree were combined in a Biom file for further analysis. Statistical analysis was carried out on R studio Version 1.4.1717. Singletons, taxa unassigned at phylum level and chloroplast and mitochondrial contaminants were removed before analysis. Alpha and beta diversity was calculated using the Vegan R package, these scripts and for generating taxa plots are available at http://userweb.eng.gla.ac.uk/umer.ijaz/bioinformatics/ecological.html. Richness was calculated using the "rarefy" function of the vegan package. Pielou's evenness was calculated using the "diversity" and "specnumber" functions of the Vegan package, dividing the diversity out by the log of specnumber output. R scripts for richness and Pielou's evenness can be found at https://rdrr.io/github/umerijaz/microbiomeSeq/src/R/alpha_div.R. R scripts for generating heat tree cohorts are available at https://grunwaldlab.github.io/metacoder_documentation/example.html. Taxa assigned to the core microbiome were selected at a minimum prevalence of 0.9, scripts at https://microbiome.github.io/tutorials/Core.html. Core microbiomes were compared through Venn diagrams constructed using the VennDiagram package in R. P-values were generated through PERMANOVA, one and two-way ANOVA and Tukey HSD post hoc test at 0.95 confidence intervals.

4.2.5. ATP Analysis and 16S rRNA QPCR

ATP analysis was performed following the protocol outlined in Chapter 2. 16S rRNA qPCR was performed using the standards and protocol outlined in Chapter 3. DNA template for QPCR was diluted with ultrapure water at 1:50.

4.3. Results

4.3.1. Spatial and temporal investigation of the Prokaryote communities of 90cm filter columns

Differences were observed in community composition both through the depth of the filter bed and over time. Biomass and diversity were shown to be highest at the top of the filter bed and at week 23 ($8.47 \times 10^{10} \pm 2.05 \times 10^{10}$ 16S rRNA gene copies/g GAC, Richness – 731.1 ± 83.2). Figures 4.1 and 4.2 show a summary of differences found on the abundance and taxonomy of bacteria as determined across several analytical methods grouped for 0 to 2cm, 2 to 30cm, 30 to 60 and 60 to 90cm intervals throughout the depth of the long filter columns from 5 to 23 weeks.

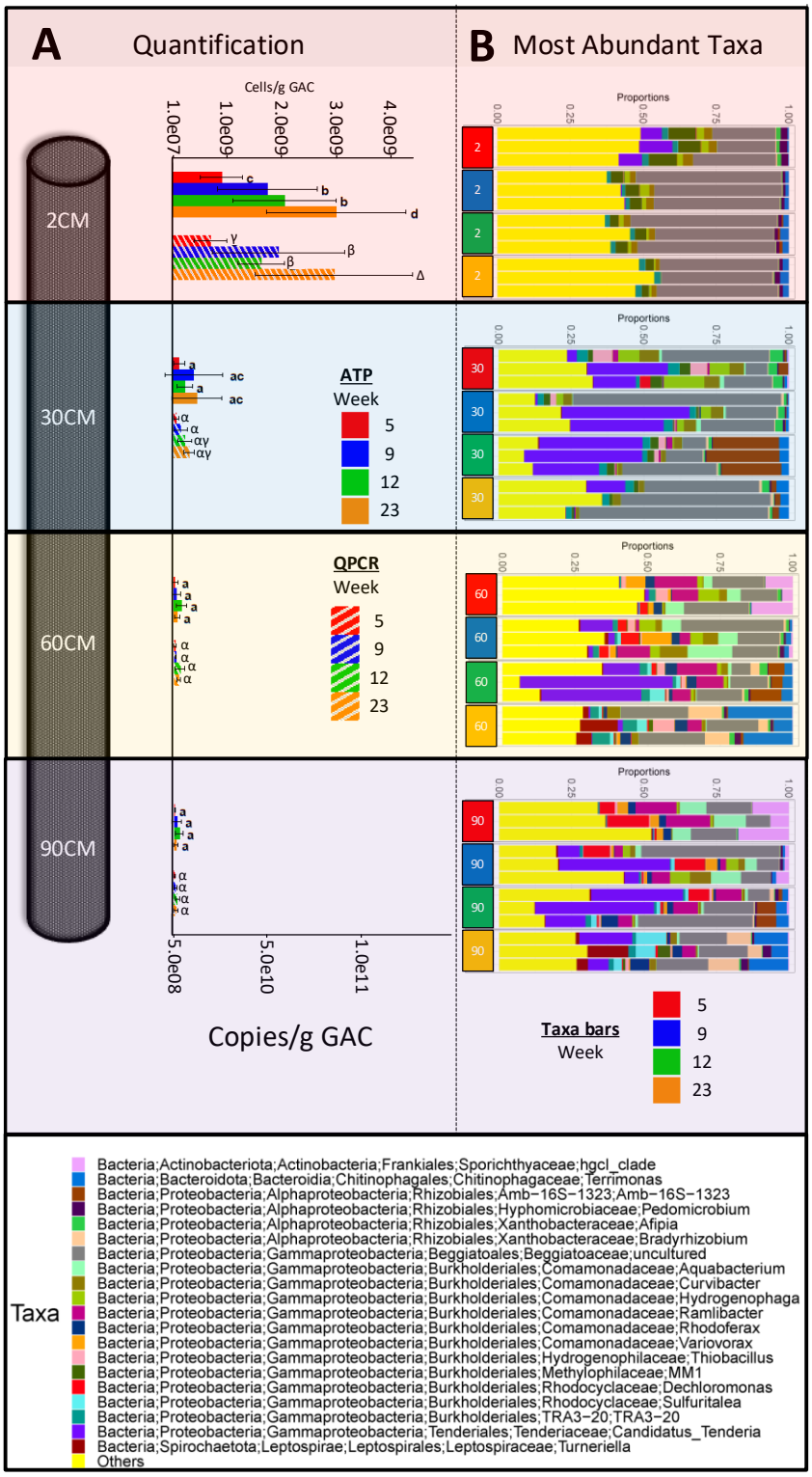


Figure 4.1. A *– 16S rRNA Copies per gram of GAC as measured by QPCR (stripped blocks) and cells per gram of GAC estimated by ATP analysis (solid boxes). Significance letters generated by one-way ANOVA and Tukey HSD.* ***B*** *– Relative abundances of the 20 most abundant taxa at genus level in the 2cm, 30cm, 60cm and 90cm sections of the filter bed over time.*

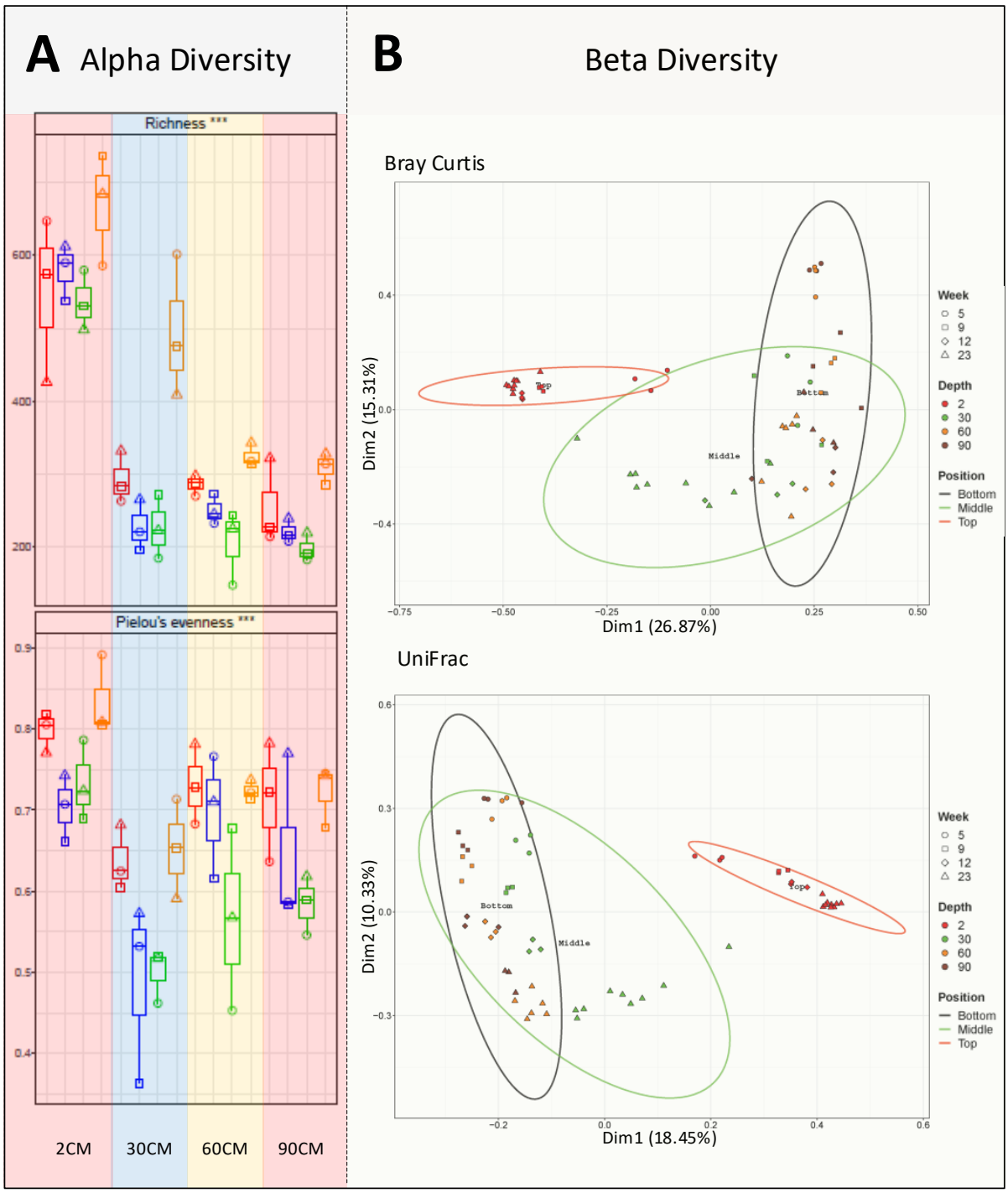


Figure 4.2. A *– Community richness and evenness values of the 2cm, 30cm, 60cm and 90cm sections of the filter bed over time.* ***B*** *– PCOA of Bray-Curtis dissimilarity and UniFrac distance between the communities of the 2cm, 30cm, 60cm, and 90cm sections of the filter bed over time. Ellipses based on standard deviation of filter sections.*

Quantification by 16S QPCR and ATP analysis (Figure 4.1. A) show that biomass is significantly higher in the top 2cm section of the filter bed than in any of the deeper sections. Over time biomass, as measured by qPCR, increases in this top section (from 2 x 10^{10} ± 4.29 x10^{9} 16S rRNA gene copies/g GAC at week 5) to its highest at week 23 (8.47 x 10^{10} ± 2.05 x 10^{10} 16S rRNA gene copies/g). ATP was used as a proxy for biological activity and followed the same trend (8.78 x 10^{8} ± 1.91 x 10^{8} cells/g GAC in 2cm section at week 5, 2.93 x 10^{9} ± 6.24 x 10^{8} cells/g GAC by week 23. At the deeper 60cm and 90cm sections biomass remains comparatively low (1.66 x10^{9} ± 6.24 x10^{8} copies/g GAC at week 23 at 90cm depth) and shows no significant change over time ($P > 0.05$).

The 20 most abundant taxa in the top 2cm section of the filter bed (Figure 4.1. B) remain stable over time, with *Beggiatoaceae* being the most relatively abundant taxa (Grey bar) mean over all weeks - 27% ± 16%)). In the top layer at genus level 44% ± 5% were other taxa, not among the 20 most abundant. At deeper layers the top 20 taxa show considerably more variation with the passage of time. At a depth of 30cm there was an increasing abundance of *Tenderiaceae*, from a relative abundance of 15% ± 12% at week 5 to 33% ± 9% at week 12. However, *Tenderiaceae* dropped to 4% ± 7% by week 23. In deeper sections of the filter (60cm & 90cm) a number of taxa were found in higher relative abundance than the top with *Tenderiaceae* among them (mean 11% ± 15% at bottom, 2% ± 4% at top). The bottom sections of the filter bed also saw increased relative abundances of *Comamonadaceae* (2% ± 1% at bottom, 0.06% ± 0.05% at top), *Sporichthyaceae* (3% ± 5% at bottom, absent from top 2cm) and *Rhodocyclaceae* (2% ± 2% at bottom, 0.025% ± 0.04% at top).

Richness is highest in the top 2cm section (Figure 4.2. A) increasing over time from 613.5 ± 129.5 to 731 ± 83.2 from weeks 5 to 23. Richness is lowest in the bottom 90cm section however is also shown to have increased by week 23 from 274.9 ± 69 to 328.8 ± 18.9. Evenness is again highest at the top of the filter bed increasing from 0.79 ± 0.02 to 0.83 ± 0.05 by week 23. Lowest evenness is interestingly found in the middle 30cm section of the filter bed (0.65 ± 0.06 at week 23). While the bottom of the filter was 0.72 ± 0.01 (60cm) and 0.71 ± 0.04 (90cm) at week 23. All four sections of the filter bed show an increase in community evenness by week 23. Beta diversity analysis by Bray Curtis dissimilarity and UniFrac (Figure 4.2. B) show the samples clearly clustering by week and filter bed depth. The top 2cm is a more distinct community than the other bed depths and demonstrates tightest clustering showing least variation over time. The bottom and middle sections cluster clearly by depth and week though show greater variation over time. For both Bray Curtis and

UniFrac depth was shown to explain the majority of variance by PERMANOVA with an R^2 of 0.299 and 0.224 respectively ($P < 0.01$).

At 30cm intervals the results from this study appear to concur with current literature, with the top 2cm section containing the most biomass and highest diversity increasing to week 23 (Velten et al. 2011; Zhang et al. 2018; Chen et al. 2021; Matuzahroh et al. 2020). While the bottom sections of the filter bed harbour the least biomass and richness, though display a more even community than that of 30cm. Despite the higher diversity and biomass at the top of the filter bed, this community shows less variation over time than the deeper sections of the filter bed. These findings are further confirmed when the full suite of samples is taken into consideration, exploring the finer scale variation in depth profiles (Figures 4.3 and 4.5). Based on community composition and depth, the samples of the filter bed can be divided into defined top, middle and bottom layers. The top layer in this study has been defined as the first 10cm of the filter bed (samples taken from 2cm, 4cm, 6cm 8cm and 10cm). The middle section has been defined as the 15cm and 30cm samples and the bottom includes the 60cm and 90cm samples. Quantification by 16S rRNA QPCR of the biomass found in these layers are shown in Figure 4.3.

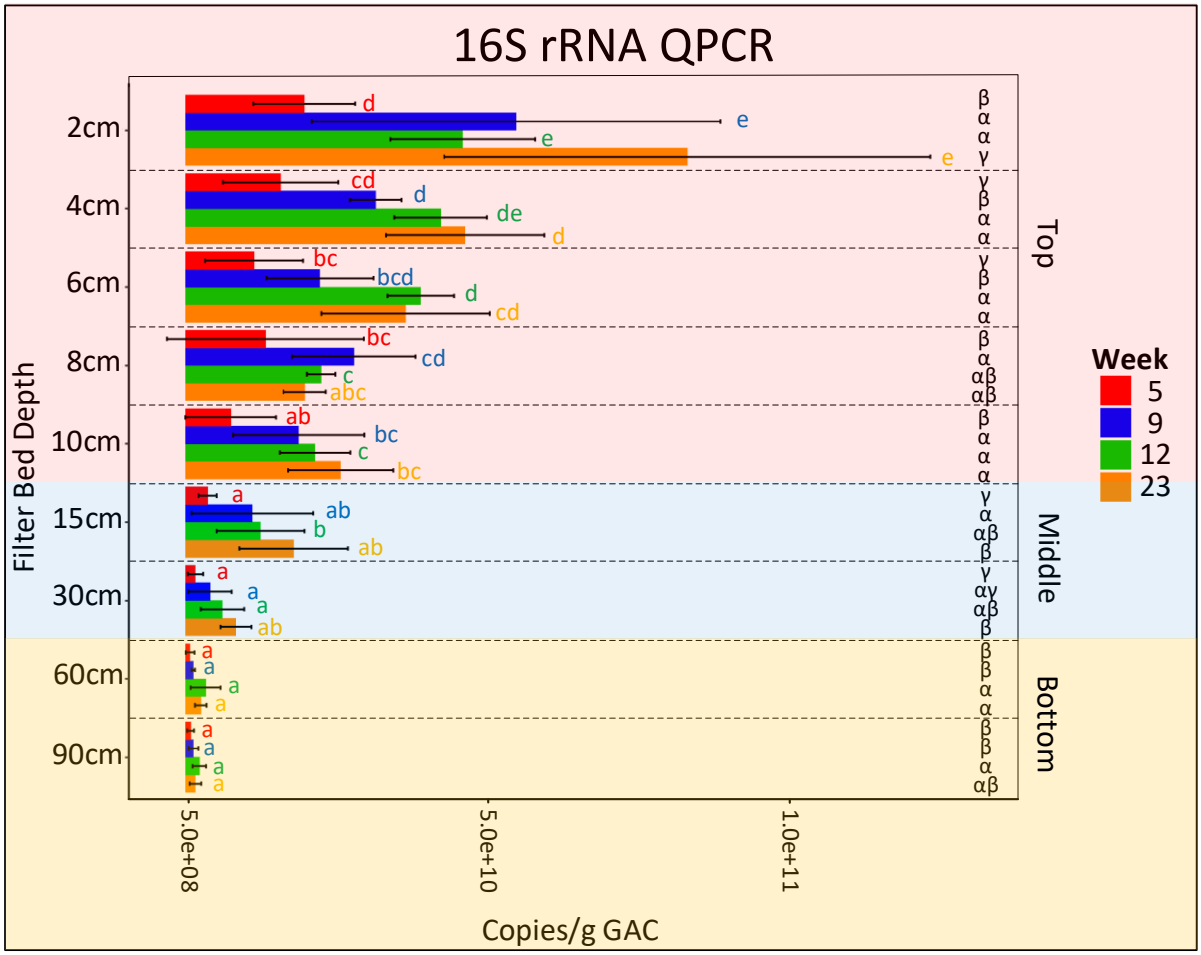


Figure 4.3. *16S rRNA copies per gram of GAC of the top, middle and bottom sections of the filter bed at weeks 5, 9, 12 and 23. Letters of significance (a-d) generated by one-way ANOVA and Tukey HSD. Variance in copies/g of GAC was calculated by depth for each week. Letters are coloured to signify the week to which they refer (red –week 5, blue – week 9, green – week 12, orange – week 23). Greek letters of significance (α, β, γ) generated by one-way ANOVA and Tukey HSD. Variance in copies/g of GAC was calculated between weeks for each bed depth. Analysis for each bed depth separated by horizontal dotted lines.*

Biomass is highest in the top section for each week and increases over time of the filter bed and at week 23, decreasing steadily through the top 10cm from 8.47 x 10^{10} ± 2.05 x 10^{10} to 2.61 x 10^{10} ± 4.4 x 10^{9} gene copies/g GAC. Over time, the 2cm section saw the greatest increase in biomass being significantly different from the other timepoints at week 23 (p values < 0.01). However, the 4cm and 6cm sections saw no significant increase between weeks 12 and 23 (4cm - 4.31 x 10^{10} ± 3.91 x 10^{9} to 4.72 x 10^{10} ± 6.67 x 10^{9} gene copies/g GAC, 6cm – 3.97 x 10^{10} ± 2.8 x 10^{9} to 3.71 x 10^{10} ± 7.12 x 10^{10} gene copies/g GAC) and

sections 8cm and 10cm no significant change between 9 and 23. All sections of the top layer displayed a significant increase in biomass between weeks 5 and 9 (p values < 0.01). Changes in biomass in the middle and bottom layers appeared less significant both through depth and over time. At week 5 there was no significant change in biomass from 15cm to 90cm of the filter bed and no significant change from 30cm to 90cm from week 9 onwards. There was no significant change in biomass between 60cm and 90cm at any of the timepoints, here gene copy numbers were ranged from $7.38 \times 10^8 \pm 3.63 \times 10^8$ to $8.5 \times 10^9 \pm 1.32 \times 10^9$). In the bottom section of the filter bed there was no change in biomass between weeks 5 and 9 or between 12 and 23. Overall biomass is shown to be highest in the top layer of the filter bed, which is the layer in which the majority of changes occur both over time and its depth. At deeper layers of the filter bed, changes in biomass through depth and over time become less significant. ATP analysis followed a similar trend and correlated with QPCR data (Pearsons correlation coefficient 0.809). The use of ATP data as a proxy for biological activity suggests that the majority of biological activity occurs in the top section of the filter bed and decreases with depth. ATP analysis also suggests that biological activity increases over time in the top section of the filter bed in agreement with the trends shown in the QPCR data, with the highest volume of activity occurring at week 23. QPCR analysis quantifies total DNA and is unable to distinguish between live and dead cells or extracellular DNA. As such an increase in biomass as shown by QPCR does not necessarily mean a sustained growth of cells. ATP analysis by Marta Vignola demonstrates that cell growth occurred most rapidly between weeks 5 and 9 (Figure 4.4).

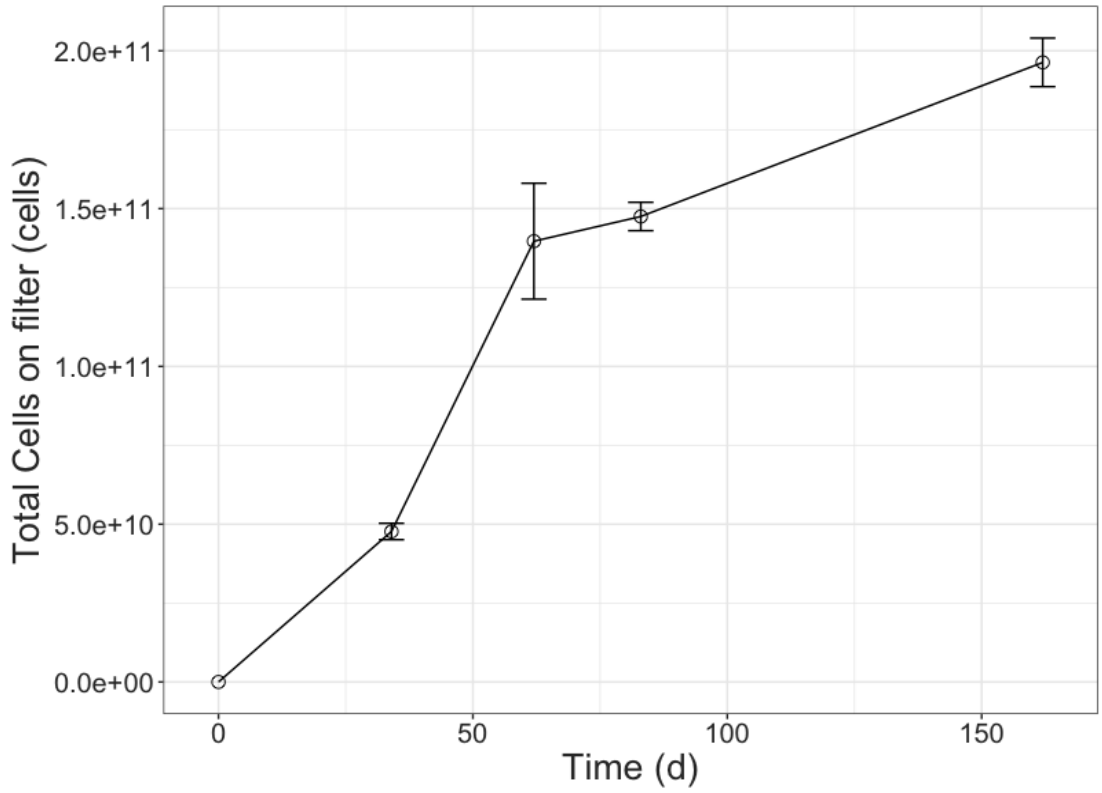


***Figure 4.4**. Data analysis and figure by Marta Vignola. Estimated total cell number in the long filter bed at the deconstruction timepoints. ATP/g was calculated and divided by cell number/g GAC provided by flow cytometry for an estimated ATP content per cell. This was multiplied by the grams of GAC in each section of the filter bed for an estimated cell number through the filter bed. Each datapoint represents a deconstruction timepoint (weeks 5, 9, 12 and 23).*

The estimated number of cells through the filter increased most rapidly between weeks 5 and 9, following a period of slower growth up to week 5. From weeks 9 to 23 an increase in cells was observed however the increase was not as steep. This suggests that while biomass is increasing until week 23, the growth rate of cells has slowed after week 9.

In contrast to the quantitative data, the fewest changes in the most abundant taxa occur in the top layer of the filter bed. Of the 20 most abundant taxa throughout the filter bed the majority consisted of alpha and gamma proteobacteria. Proteobacteria were the most abundant phylum throughout the filter bed at 75% ± 11% (mean). The mean relative abundances of the top 20 phyla detected in the top, middle and bottom section are outlined in table 4.3.

Table 4.3*. The mean relative abundance and standard deviation of the 20 most abundant phyla found in the top, middle and bottom sections of the filter bed.*

Phylum	**Mean relative abundance %**		
	Top	**Middle**	**Bottom**
Proteobacteria	72 ± 11	83 ± 7	76 ± 12
Acidobacteriota	8 ± 5	2 ± 3	0.4 ± 0.5
Actinobacteriota	0.4 ± 0.3	0.3 ± 0.2	4 ± 6
Armatimonadota	0.4 ± 0.2	0.3 ± 0.3	0.06 ± 0.1
Bacteroidota	5 ± 2	6 ± 3	10 ± 6
Bdellovibrionota	0.6 ± 0.2	0.6 ± 0.4	0.4 ± 0.3
Chloroflexi	1.4 ± 1.2	0.25 ± 0.3	0.16 ± 0.2
Cyanobacteria	0.13 ± 0.14	0.4 ± 0.5	1 ± 0.9
Dependentiae	0.14 ± 0.09	0.2 ± 0.15	0.4 ± 0.3
Desulfobacterota	0.35 ± 0.2	0.08 ± 0.07	0.06 ± 0.1
Elusimicrobiota	0.32 ± 0.1	0.15 ± 0.1	0.05 ± 0.07
Gemmatimonadota	0.4 ± 0.2	0.14 ± 0.1	0.06 ± 0.06
Hydrogenedentes	0.3 ± 0.2	0.15 ± 0.1	0.04 ± 0.08
Myxococcota	0.6 ± 0.3	0.3 ± 0.2	0.2 ± 0.2
Nitrospirota	0.8 ± 1	0.23 ± 0.5	0
Patescibacteria	0.9 ± 0.9	1.2 ± 1.5	0.7 ± 0.9
Planctomycetota	5 ± 2	2.5 ± 1	3 ± 3
RCP2−54	0.3 ± 0.3	0.06 ± 0.1	0
Spirochaetota	0.02 ± 0.04	0.1 ± 0.1	2 ± 4
Verrucomicrobiota	1.5 ± 0.6	1 ± 0.7	1 ± 0.5
Others	0.9 ± 0.6	0.4 ± 0.4	0.2 ± 0.2

Acidobacteriota were found to be the second most abundant phylum after Proteobacteria and were found in highest abundance in the top section of the filter bed (8 ± 5% at top, 2 ± 3% in the middle and 0.4 ± 0.5% at the bottom) Why the majority of taxa were distributed fairly equally through the filter bed at phylum level, several showed some variance with depth. Nitrospirota and RCP2−54 were present in the top and middle sections of the filter bed while were entirely absent from the bottom sections. Bacteroidota and Spirochaetota were found in

higher average relative abundances in the bottom section of the filter bed (Spirochaetota 0.02 ± 0.04% at top, 2 ± 4% at bottom, Bacteroidota 5 ± 2% at top, 10 ± 6% at bottom). However, the standard deviation at the bottom for these taxa were high suggesting a lot of variability in the relative abundances of these taxa between replicates in the bottom section of the filter. At genus level, there was little change over time in the top section of the filter bed (Figure 4.5).

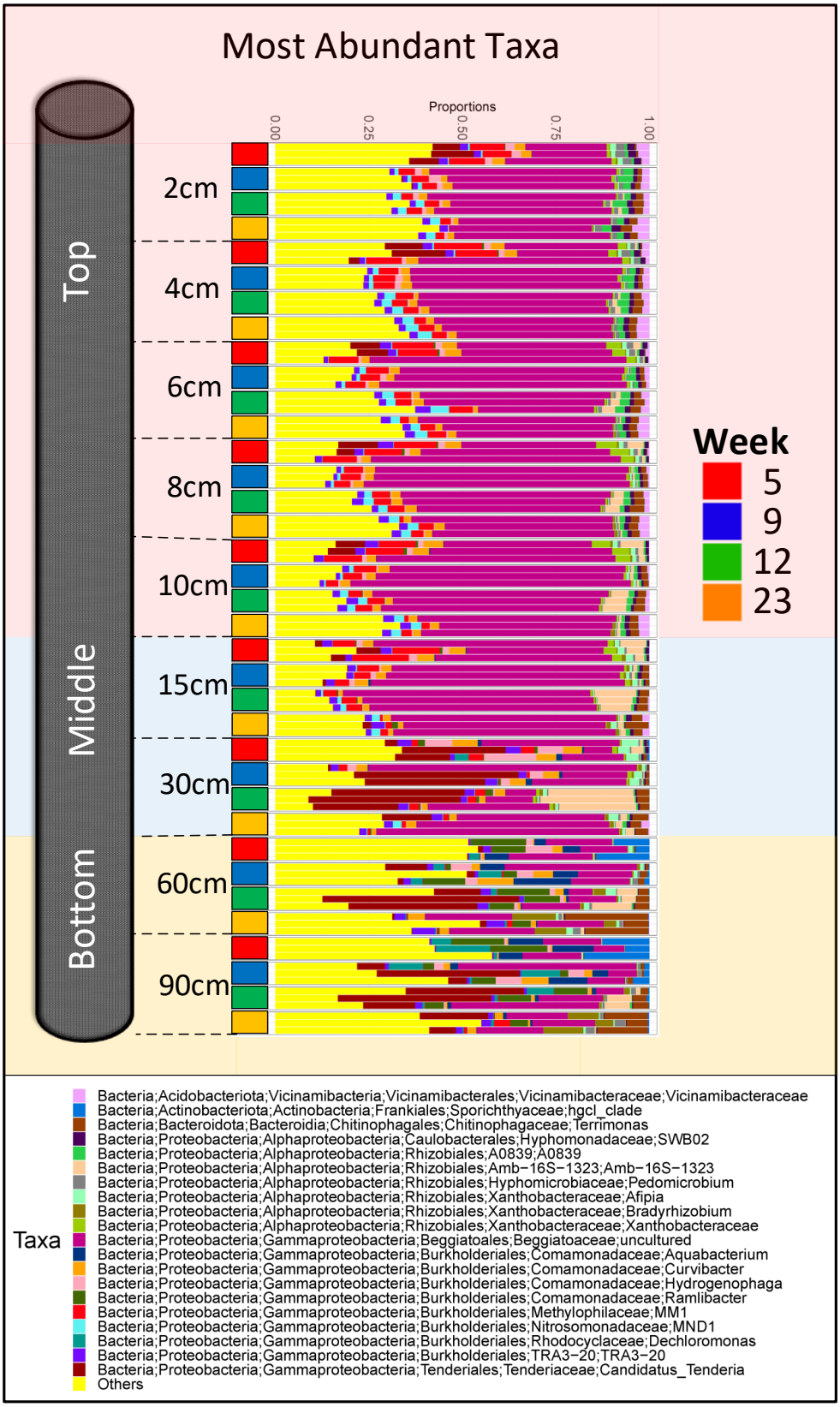


Figure 4.5*. Top 20 most abundant taxa (relative abundance) at genus level through the depth of the filter bed and over time. Others (yellow bar) is the remainder of the taxa in each sample not among the top 20 most abundant.*

Over time there is comparatively little change in the most abundant taxa in the top 15cm of the filter bed (Figure 4.5). In this layer, taxa of the family *Beggiatoaceae* are the most abundant in relative terms (mean – 51% ± 12%) while in the bottom layer become far less abundant (mean – 18% ± 10). Sequences assigned as *Beggiatoaceae* have previously been isolated from groundwater sources following a nucleotide search on NCBI Blast. *Nitrosomonadaceae* and *Methylophilaceae* are also notable for being present in higher relative abundance in the top 15cm of the filter bed (mean *Nitrosomonadaceae* – 1.5% ± 1.2%, mean *Methylophilaceae* – 5% ± 3%). The most abundant taxa in the bottom layer of the filter bed shows far more variation over time. The most abundant taxa in the bottom section of the filter bed particularly between weeks 10 and 13 is identified in the Silva database as of the family *Tenderiaceae* (mean – 12% ± 15%). Two sequences were identified as *Tenderiaceae*. Following a nucleotide search on NCBI Blast, the closest match at 99.7% similarity was previously isolated from drinking water systems, specifically from biofilm on copper exposed to cold tap water (sequence ID - MT462138). Information on the metabolism of *Tenderiaceae* was lacking in current literature. However, when the phylogeny of *Tenderiaceae* was investigated (see Appendix) it's closest relatives were *Thioalkalispiraceae*, *Acidiferrobacteraceae* and *Beggiatoaceae* all known oxidisers of sulphur (Flood et al. 2021; Issotta et al. 2018; Mori et al. 2011). The high abundance of *Beggiatoaceae* at the top of the filter and *Tenderiaceae* in the deeper sections may suggest that sulphur oxidation may be a driving factor for the development of the filter bed community. The top section of the middle of the filter bed (15cm) was found to be similar to the top section throughout all weeks, while the 30cm section contained a higher relative abundance of *Tenderiaceae* between weeks 5 and 12 (mean – 19% ± 16%). Interestingly, by week 23 the 30cm section was found to be more similar to the top section of the filter with *Beggiatoaceae* being the most dominant taxa 52% ± 13%).

Overall, while biomass and activity measured by ATP analysis increase with time in the top section of the filter bed, the relative abundance of the 20 most abundant taxa remain fairly constant with the family *Beggiatoaceae* by far the most dominant. In contrast, little change is observed in terms of biomass and activity with time in the bottom section of the filter bed, while the relative abundance of the most abundant taxa shows more variability over time.

While the proportions of the most abundant taxa show little change over time in the top section of the filter bed, community diversity was found to be highest in this section and at week 24. In terms of diversity between communities, depth was shown to be an important

factor explaining variance. Alpha and beta diversity of the various filter bed depths and influent and effluent water at the deconstruction timepoints are shown in Figure 4.6.

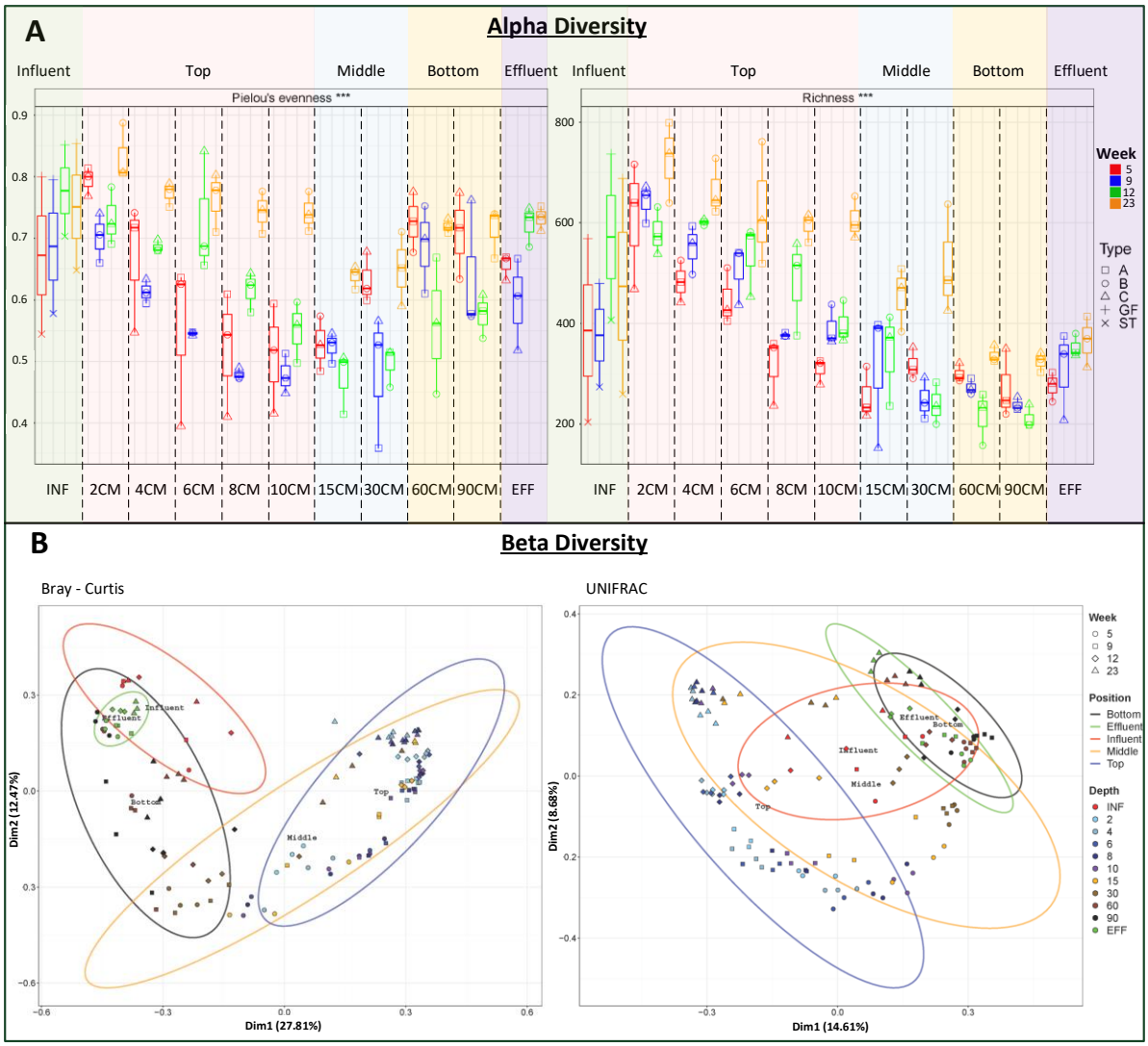


Figure 4.6. A *– Richness and Pielou's evenness values of the influent, effluent, top, middle and bottom sections of the filter bed by sampling depth at weeks 5, 9, 12 and 23.* ***B*** *– PCOA's of Bray-Curtis dissimilarity and UniFrac distance of the influent, effluent, top middle and bottom sections of the filter bed by sampling depth and at weeks 5, 9, 12 and 23. Alpha diversity P-values generated by ANOVA are available in Appendix tables A.2. and A.3.*

Community richness (Figure 4.6. A. right) was found to be highest in the top 2cm of the filter and decreased with bed depth (from 731.3 ± 83.3 to 328.9 ± 18.5 at week 23). In the top section of the filter bed richness increased over time to its highest (731.3 ± 83.3) at week 23, although this increase was not statistically significant between weeks 5 and 12. For the

majority of depths in the top 15cm of the filter bed, the increase between weeks 5 and 23 was significant (p values < 0.05) with the exception of the 2cm and 6cm depths. In the bottom 30cm of the filter bed, richness appears to initially decrease between weeks 5 and 12 before increasing at week 23. In the bottom section the increase between weeks 12 and 23 was significant (p values < 0.05). Community evenness (Figure 4.6. A. left) is again highest (0.83 ± 0.04) in the top section of the filter bed and at week 23. The top 10cm of the filter bed follows a similar trend to community richness, increasing to week 23. However, the bottom section of the filter bed had higher evenness than the middle sections at all timepoints. This suggests that community evenness initially decreases in the top section of the filter bed before increasing at deeper sections. The evenness of the effluent water (range - 0.52 to 0.75) is similar to that of the bottom sections of the filter bed (range - 0.45 to 0.78) and again higher than the middle sections of the filter. At week 23 the richness of the effluent water is not significantly different to the bottom section of the filter bed and demonstrates a significant decrease compared to the community in the top of the filter bed. Of the two influent water communities investigated, the presumed attached cells (G/FC) were consistently more diverse (mean evenness – 0.83 ± 0.03, mean richness – 618.1 ± 116.3) (than the pelagic cells (ST 0.22μm) (mean evenness – 0.62 ± 0.07, mean richness – 285.9 ± 86). Interestingly, for both richness and evenness the attached cells fell into ranges more similar to the top section of the filter bed while the pelagic cells were more similar to the bottom section.

Principal component analysis of Bray-Curtis dissimilarity and UniFrac distance (Figure 4.6. B.) displayed clear clustering by both week and depth. PERMANOVA analysis of Bray-Curtis dissimilarity identified depth as explaining the majority of variance with an R^2 of 0.41 or 41% of variance (p value < 0.01). The interaction between week and depth explained 22% of variance and week the least at 15%. PERMANOVA of UniFrac distance identified the interaction between week and depth as the factor which explained the majority of variance at 25%. Depth explained 24% of variance and again week the least at 15%. For both UniFrac and Bray-Curtis all three factors were statistically significant (p values < 0.01). These findings point to bed depth as the more important factor for explaining variance between communities than the time the filter has been in operation. Another interesting observation is that the effluent water is found to cluster closely with both the influent water and bottom section of the filter bed. This may have implications concerning the origins of the community found in the effluent water. The effluent community being similar to the bottom section of the filter bed may suggest seeding of the effluent by taxa colonising the filter bed. Also, the

similarity to the influent community may suggest a proportion of taxa in the influent community is simply passing through the filter and into the effluent.

4.3.2. Effect of Column Size on Biofilter and Effluent Microbial Communities

While differences in diversity and taxa were observed through the depth of the filter bed, media depth appears to have had little effect on community diversity or most abundant taxa at week 23. Figure 4.7 outlines a comparison of alpha and beta diversity along with the top 20 most abundant taxa of the three filter columns (short, medium and long).

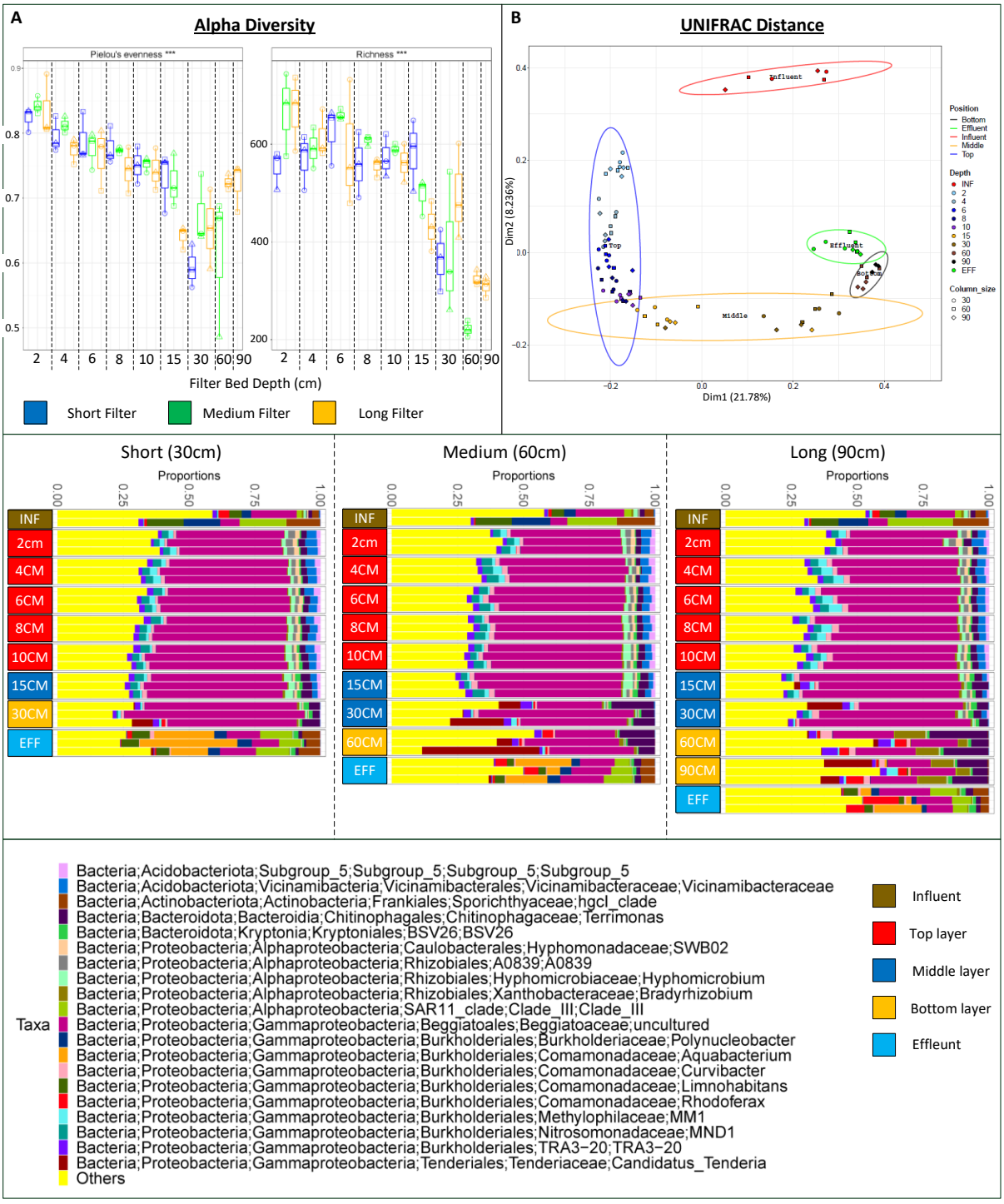


Figure 4.7. A *– Richness and Pielou's evenness values compared between the short, medium and long filters at all sampling depths including influent and effluent at week 23.* ***B*** *– PCOA of UniFrac distance between the influent, effluent and all sampling depths of the short, medium and long filters.* ***C*** *– Relative abundances of the 20 most abundant taxa at all sampling depths of the short, medium and long filters.*

For both community evenness and richness (Figure 4.7. A.) there is no significant difference observed between the three column sizes for the majority of bed depths (Evenness p value- 0.378, richness p value – 0.221). At a bed depth of 15cm the long filter displayed significantly lower evenness than the short and medium filters (0.7 ± 0.05, p values, short – 0.036, long- 0.03) and significantly lower richness than the short filter (540 ± 89, p-value 0.044). At a bed depth of 60cm, the medium and long filters were significantly different for both richness and evenness with the medium filter showing the lowest diversity. At bed depths 4cm and 8cm the medium and long filters were found to be significantly different for evenness and richness respectively. Despite the differences in some individual bed depths between the column sizes, it would appear that the media depth of the biofilter columns has a minimal effect on alpha diversity overall.

Similarly, column media depth was found to have little effect on beta diversity with sampling depth being the main driver of variance between communities (Figure 4.7. B). A principal component analysis of UniFrac distance displayed clear clustering by sampling depth and no real separation between the short, medium and long filter columns. PERMANOVA identified sampling depth as responsible for 45% of variance (R^2 0.44687, p-value < 0.01) compared to just 3% of variance identified as column size (R^2 0.03848, p-value < 0.01). The interaction between sampling depth and column size was not found to be statistically significant (p-value – 0.07).

The most abundant taxa in the top 15cm of the filter bed were very similar across the three column sizes, with *Beggiatoaceae* the most abundant taxa (mean abundance short – 48% ± 4%, medium – 47% ± 5%, long – 48% ± 6%) (Figure 4.7. C.). Differences between the three column sizes begin at a sampling depth of 30cm. The short filter, in which the 30cm sampling depth is the very bottom of the filter, is similar in composition to the top 15cm of the filter bed, albeit with some taxa at reduced relative abundances. Specifically, taxa of families *Vicinamibacteraceae* (0.2%), *Hyphomicrobiaceae* (0.6% ± 0.3%), *Methylophilaceae* (0.9% ± 0.2%) and *Nitrosomonadaceae* (0.3% ± 0.2%) were found in lower relative abundance. Interestingly, the most abundant taxa in the 30cm section of the long and short filters were more similar to each other than to that of the 30cm section of the medium filter. The 30cm section of the medium filter showed a higher relative abundance of *Chitinophagaceae* (9.7% ± 6%) and *Tenderiaceae* (9.6% ± 10%) that the same section of the short and long filters. An increased relative abundance of *Chitinophagaceae* was also observed in the bottom section of the medium (60cm section – mean 10% ± 4%) and long filters (60cm and 90cm sections

mean 13% ± 6%). The bottom sections of the long filter displayed differences in the abundance of some taxa when compared to the other two columns, mainly taxa of Genus *Bradyrhizobium* (8% ± 3%) and *Rhodoferax* (3% ± 2%).

Taxa of the family *Sporichthyaceae hgcl clade* (mean influent – 8% ± 6%, effluent – 5% ± 2%) and an uncultured *Alphaproteobacteria clade III* (mean influent – 11% ± 10%, effluent – 8% ± 3%) were found to be amongst the most abundant taxa in both the influent and effluent water of all three column sizes. While in relatively high abundance in the influent and effluent, these taxa were far less abundant in the filter bed (filter bed, mean *Clade III* 0.03% ± 0.09%, *hgcl clade* – 0.02% ± 0.07%) suggesting these taxa may be passing directly through the filter. The effluent water of all three column sizes contained *Aquabacterium* which was found in much lower abundance in the influent water and only in the bottom section of the medium and long filters (influent – 0.02% ± 0.04%, filter bed – 0.2% ± 0.3%). The effluent water of the short filter had the highest relative abundance of *Aquabacterium* (Effluent short – 25% ± 11%, medium – 14% ± 8%, long – 8% ± 9%) suggesting that media depth may influence the abundance of *Aquabacterium* released into the effluent.

Overall media depth appears to have little effect on the communities residing in the top section of the filter bed. Figures 4.8, 4.9 and 4.10 display heat tree cohorts illustrating taxa which are found to be in higher abundance in the top (green) and bottom (brown) sections of the three filter bed depths.

Short Filter

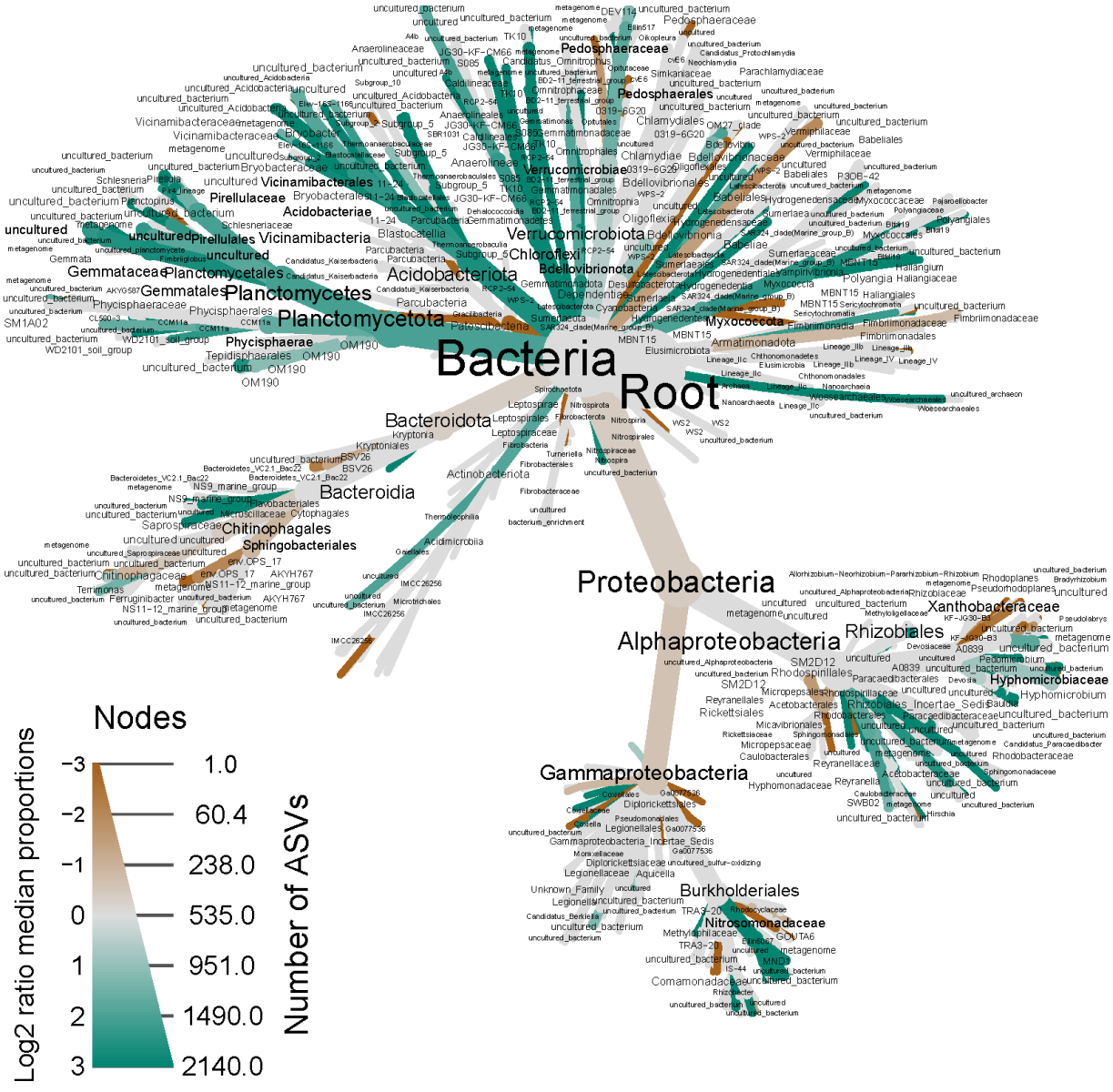


Figure 4.8. *Heat tree cohorts comparing the log2 fold proportional ratios of the top and bottom sections of the short filter. Taxa found in higher abundance in the top of the filter are shaded green and taxa found in higher abundance in the bottom of the filter are shaded brown.*

Medium Filter

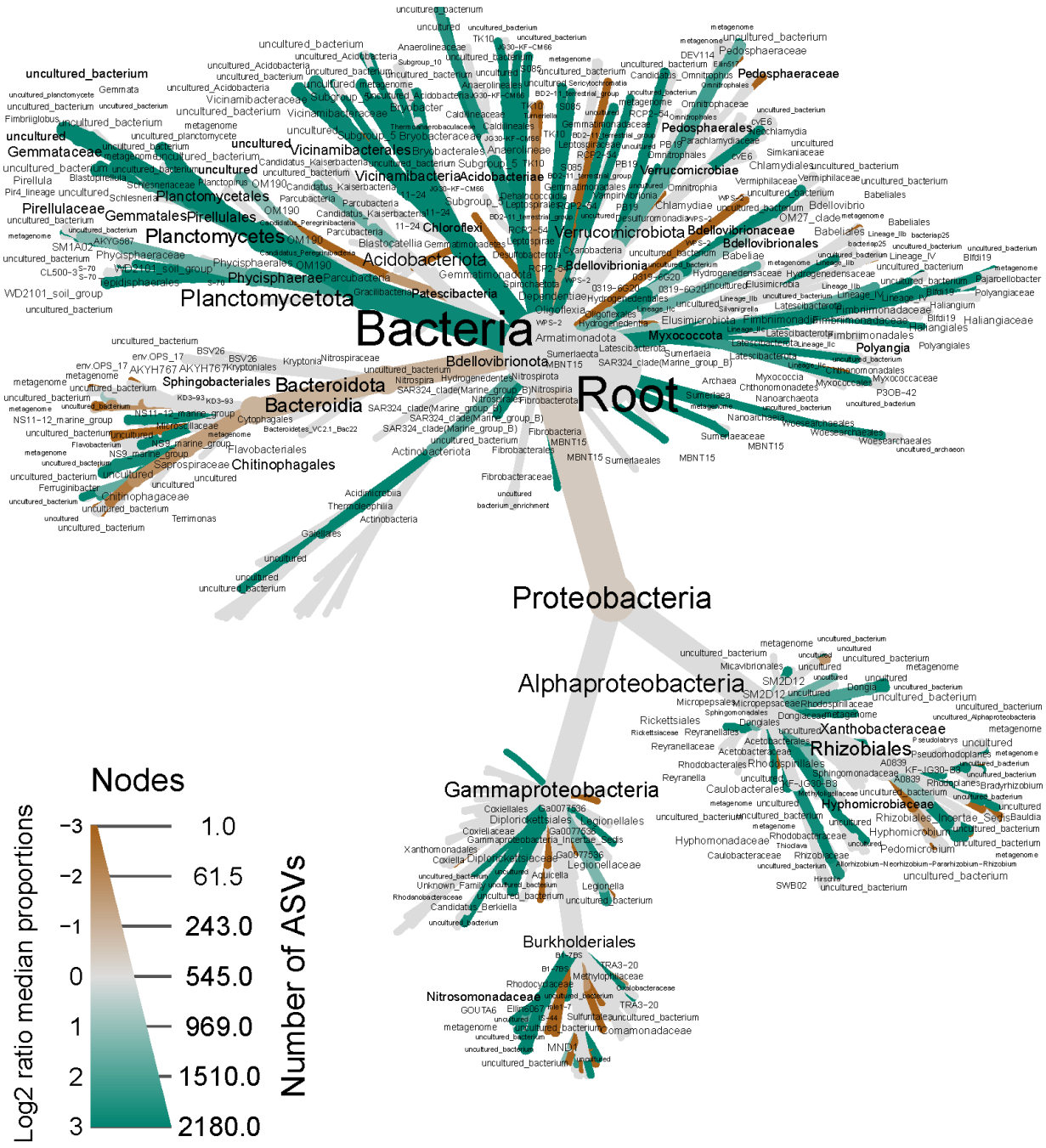


Figure 4.9. *Heat tree cohorts comparing the log2 fold proportional ratios of the top and bottom sections of the medium filter. Taxa found in higher abundance in the top of the filter are shaded green and taxa found in higher abundance in the bottom of the filter are shaded brown.*

Long Filter

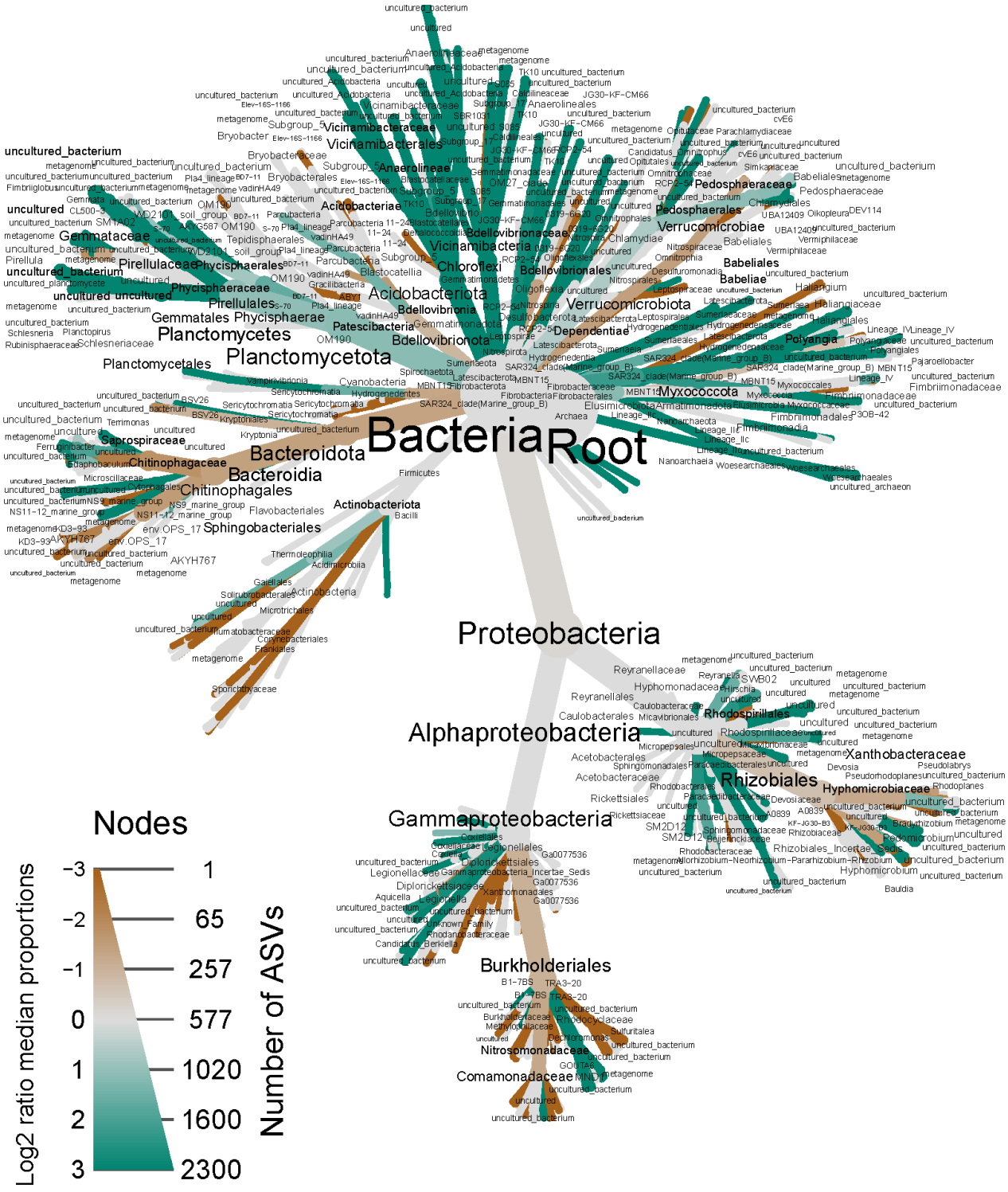


Figure 4.10. *Heat tree cohorts comparing the log2 fold proportional ratios of the top and bottom sections of the long filter. Taxa found in higher abundance in the top of the filter are shaded green and taxa found in higher abundance in the bottom of the filter are shaded brown.*

Taxa found in higher abundance in the top section of the filter bed appear relatively consistent across the three filter bed sizes. *Planctomycetota*, *Acidobacteriota*, *Verrucomicrobiota* and several species of Alpha and Gamma *Proteobacteria* are amongst taxa which are consistently at higher abundance in the top section of the filter bed. While the abundance of taxa in the top section of the filter bed is consistent between column sizes, differences in the bottom sections are more apparent. Several taxa are found in increased abundance in the bottom of the long filter when compared to the short and medium filters including *Burkholderiales*, *Rhizobiales* and *Actinobacteria*. Again, this points to media depth having a greater effect on deeper sections of the filter bed.

This was reinforced by investigating shared taxa between the three filter columns in the top and bottom 2cm of the filter bed. Venn diagrams of theses sections, highlighting that the majority of taxa in the very top section of the filter bed is shared between all three column sizes (Figure 4.11).

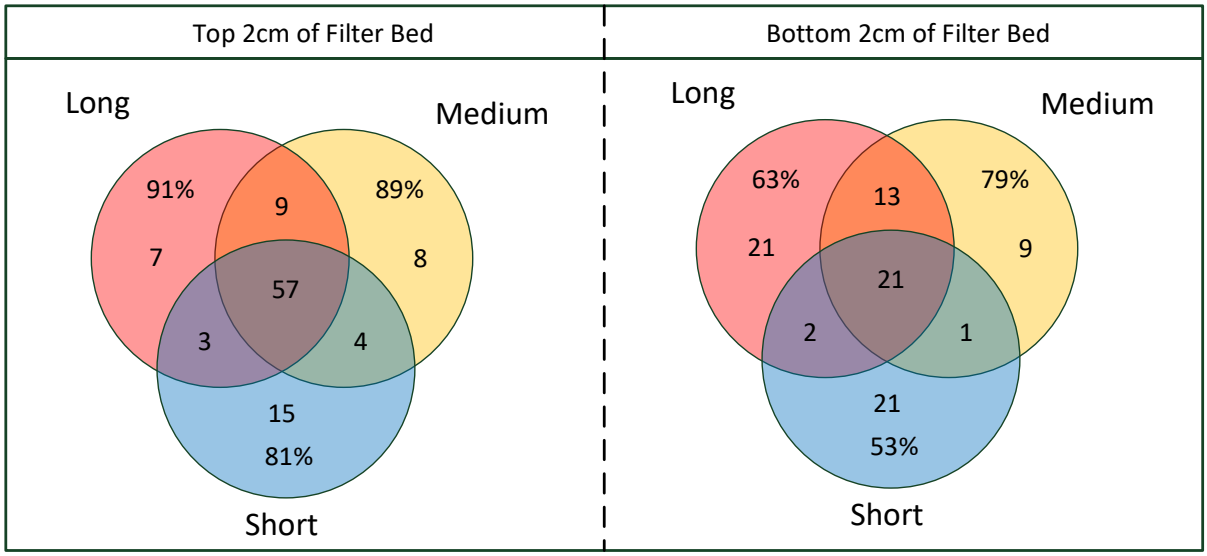


Figure 4.11. *Venn diagrams of shared taxa between the top 2cm and bottom 2cm of the short medium and long filters. Percentages signify the percentage of the filters taxa which is shared with one or both of the other two column sizes. Shared taxa include ASV's identified in 90% of the sampling depths compared.*

In the top 2cm of the filter bed 91% of taxa of the long filter is shared with one or both of the medium and short filters. The medium filter shares 89% and the short 81% of taxa with one

or both of the other column sizes. When the very bottom sections of all column sizes are compared, the percentage of shared taxa drops to 57% for the short filter, 63% for the long filter and 79% for the medium filter. Thus, the bottom sections of each filter are more different to each other than the top section.

However, when the bottom 30cm section of the short filter is compared to the corresponding 30cm sections in the medium and long filters, the percentage of shared taxa increases (Figure 4.12.)

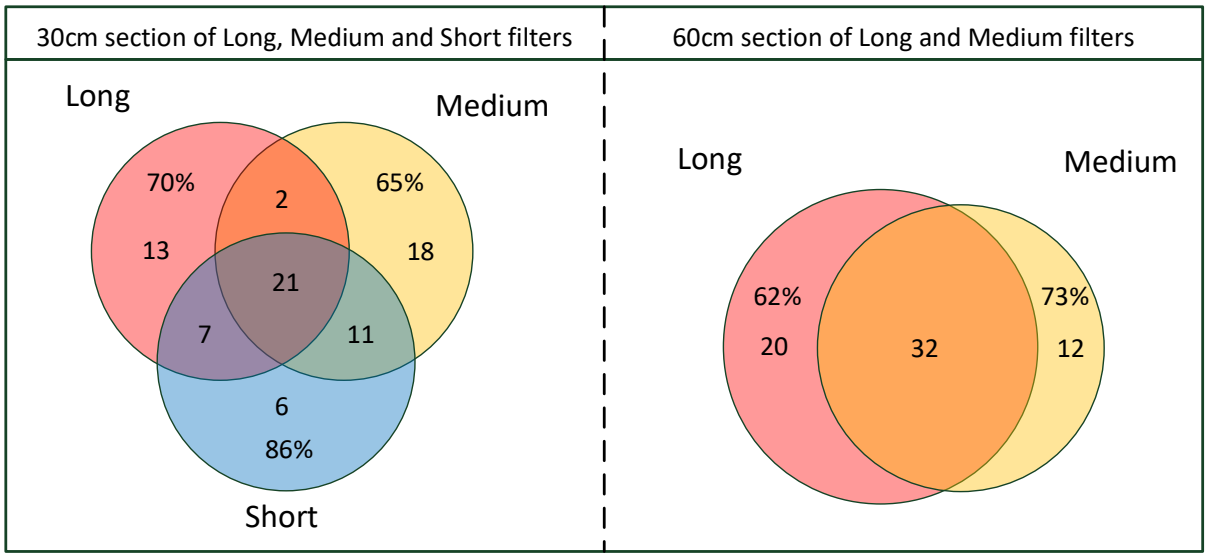


Figure 4.12*. Venn diagrams of shared taxa between the sampling depth of 30cm of the short medium and long filters and the 60cm sampling depth between the medium and long filters. Percentages signify the percentage of the filters taxa which is shared with one or both of the other two column sizes. Shared taxa include ASV's identified in 90% of the sampling depths compared.*

The percentage of ASVs shared between the bottom of the short filter and the 30cm sections of the medium and long filters increases to 86% with the number of unique ASVs in the short filter reduced to 6. Similarly, the bottom 60cm section of the medium filter shares 73% of taxa with the 60cm section of the long filter. Indeed, when the 60cm and 90cm sections of the long filter were compared, 79% of taxa in the 60cm section was shared with the 90cm section. These findings suggest that it is proximity to the top of the filter bed which is a greater influence for community selection than proximity to the bottom of the filter. This concurs with the beta diversity analysis which identified bed depth as responsible for the

majority of variance. Comparing the effluent water of the three filter sizes it was found that the majority of taxa was shared between the three column sizes (Figure 4.13).

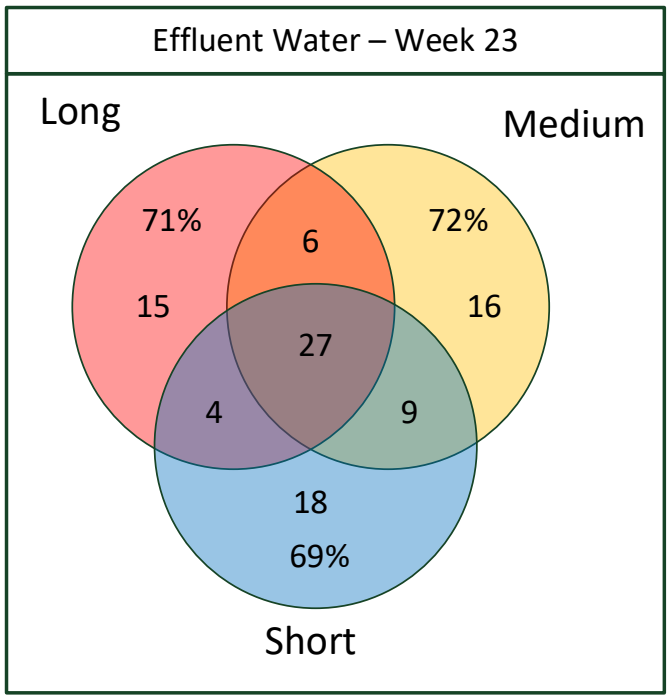


Figure 4.13*. Venn diagram of shared taxa between the effluent waters of the short medium and long filters. Percentages signify the percentage of the filters taxa which is shared with one or both of the other two column sizes. Shared taxa include ASV's identified in 90% of the sampling depths compared.*

The effluent water from the long filter was found to share 71% of taxa with the effluent water from one or both of the other two column sizes. The medium filter was found to share 72% and the short 69% of taxa. The effluent water was found to share most taxa with the bottom section of each filter. The bottom section of the long filter shared 43% of taxa with its effluent water, the bottom section of the medium filter shared 25% of taxa with its effluent water and the short filter shared 57% of taxa with its effluent water.

4.4. Discussion

The aim of this chapter was to investigate the microbial communities of biofiltration through the depth of the filter bed and how they develop over time. Furthermore, to investigate if column length has an impact on the microbial communities which develop in the filter bed. The results from this study show changes in biomass and diversity over time and through the depth of the filter bed, with bed depth being the more important factor explaining differences in community diversity. Biofilter column length was found to have little influence on the microbial communities in the top section of the filter bed. Indeed, the top 30cm was found to be highly reproducible, measuring little difference between the three column sizes in terms of diversity and abundant taxa. Differences were observed in the abundance of specific taxa when comparing the bottom sections of each filter column. In particular, specific taxa were found in higher abundance in the bottom section of the long filter column such as *Tenderiaceae* and Xanthobacteraceae.

4.4.1. Spatial and Temporal Investigation of Biofilter Communities

The results from this study largely concur with the majority of published literature in terms of biomass and activity (Ma, et al 2020; Haig et al. 2014; Velten et al. 2011). Over time biomass increased as the filter matured and was highest at the top of the filter bed decreasing with depth (Ma et al. 2020). Over time biomass was observed to increase from its lowest at week 5 (ranging from 2×10^{10} to 7.4×10^{8}) to its highest at week 23 (8.5×10^{10} to 1.7×10^{9} gene copies/g GAC). This was similar to 16S gene copy number reported in other GAC and sand biofilter studies which ranged from 10^{7} to 10^{11} 16S rRNA gene copies per gram of media (Haig et al. 2014; Ma et al. 2020a; Ma et al. 2020b; Xu et al. 2020; Bai et al. 2013). This was more apparent in the top 30cm of the filter bed in which a significant increase in biomass was observed between weeks 5 and 23 (p-values < 0.05). While an increase in biomass was observed in the bottom sections (sampling depths 60cm and 90cm) between weeks 5 and 23, it was not found to be statistically significant (p-values > 0.05). The 16S rRNA QPCR data suggests that there is an increase of biomass over time. However, this does not give an indication of biological activity. DNA from dead or damaged cells, inactive cells and the extracellular environment will be amplified alongside DNA from biologically active cells. As such, an increase in 16S rRNA gene copy number does not necessarily mean a direct increase

in biological activity. As biological activity is linked to contaminant removal (Xiang et al. 2013; Keon et al. 2021), ATP analysis was carried out to give an indication of activity through the depth of the filter bed and over time. ATP analysis was found to follow the same trend as the QPCR data, although cell number was estimated to be an order of magnitude lower. This is likely due to differences in the analytical methods. While 16S rRNA QPCR, may amplify from dead cells and extracellular DNA, the main drawback with ATP analysis is that cell number is calculated based on an estimated ATP content per cell (Pharand et al. 2014). As the ATP content per cell can vary between community members, it can be difficult to determine a "true" cell count using either technique. However, the ATP analysis was found to correlate with the QPCR data (correlation coefficient – 0.809) and followed much the same trend. The two analyses in unison, strongly suggest that both biomass and biological activity are highest at the top of the filter bed, decreasing with depth and increasing over time. Again, this is in agreement with current literature (Pharand et al. 2014; Velten et al. 2011; Magic-Knezev and Van der Kooij 2004). However, estimated cell numbers through the filter bed (Figure 4.4) suggests that while biomass was shown to increase up to week 23, the growth rate had slowed after week 9. This may indicate the filters were reaching a steady state by week 23, where cell death and growth occur in equal measure and biomass accumulation effectively halts (Velten et al. 2011).

Alpha diversity followed a similar trend to those of biomass and activity, with communities being most diverse in the top 2cm of the filter bed and at week 23. Community richness was found to decrease with bed depth, the least diverse communities found in the bottom of the filter bed. Richness ranged from 152 to 799 in through the filter bed samples collected in this study. This is comparable to other studies investigating the diversity of slow sand and GAC filter beds, shown to range from 117 to 1649 (Gerrity et al. 2018; Xu et al. 2020; Haig et al. 2015; Lautenschlager et al. 2014; Chen et al. 2021). Community evenness was also found to initially decrease in the top 15cm of the filter. However, in the deeper 30cm, 60cm and 90cm sections evenness was found to increase. The bed depths which had the lowest evenness were the 10cm and 15cm sections. Evenness was found to range from 0.36 to 0.89 through the filter bed samples of this study, similar to evenness values reported in other studies (Haig et al. 2015; Gerrity et al. 2018; Feng et al. 2012) Haig et al, demonstrated that increased community evenness was linked to superior filter performance so this this increase of evenness in the bottom section of the filter may potentially have some importance when selecting media depth (Haig et al. 2015).

Bed depth was found to be the most important factor in explaining variance in beta diversity explaining 41% of variance in a Bray-Curtis dissimilarity measure and 24% of the variance of UniFrac distance matrix. While depth was identified as the most important factor in explaining variance, time was also shown to be significant explaining 15% of variance for both Bray-Curtis and UniFrac. This serves to demonstrate that changes in microbial communities occur both through the depth of the filter bed and over time as the filter matures. PCOA (Figure 4.6. B.) shows distinct communities clustering by week and depth. The top section of the filter bed was found to be least similar to the influent and effluent water. Interestingly, the communities of the influent and effluent water were found to be similar, clustering together and distinctly from the top and middle sections on the filter bed. The bottom section of the filter bed was also found to cluster with the influent and effluent water. The community of influent water having an influence on that of the effluent water has been previously reported. Vignola et al found that influent water had a greater impact on the effluent community than the choice of filter media (Vignola et al. 2018). An unpublished meta-analysis currently in preparation by Cholet et al has also identified the influent water as the main driver of community composition of the filter bed and effluent water over several biofilter studies, suggesting a global pattern. Similarly, Ma et al reported little change in the community composition from influent water to effluent following filtration through GAC-sand and GAC-anthracite filters. Ma also found more similarities between the effluent water and filter bed community than between the influent and filter bed communities, suggesting the filter bed community has some influence on that of the effluent (Ma et al. 2020). Another study by Lautenschlager et al found that the effluent water community was reflective of that of the filter bed in a full-scale drinking water treatment plant, again suggesting the biofilter community has some influence on the effluent community (Lautenschlager et al. 2014). The findings reported by these authors and the results from this study suggest the effluent water community is influenced by the community of the effluent and the filter bed. Similarities between the influent and effluent communities may suggest a proportion of taxa entering the filter is simply passing through into the effluent, remaining unaffected by the filtration environment. Several studies have found an increased abundance of LNA bacteria in the effluent water, which may be due to these cells passing through the filter due to their reduced cells size (Lautenschlager et al. 2014; Vital et al. 2012; Chan et al. 2018). The fate of small cell sized bacteria is investigated in detail in Chapter 6 of this thesis.

The influence of the filter bed community may be due to taxa detaching from the biofilm within the filter bed. This may explain the similarity observed between the bottom section of the filter bed and the effluent water, as biofilm which detached from the media would have fewer opportunities to be recaptured by the filter media before entering the effluent.

The most abundant taxa throughout the depth of the filter bed were Proteobacteria, specifically *α-Proteobacteria* and *γ-Proteobacteria*. This was unsurprising due to the ubiquity of *Proteobacteria* in freshwater and drinking water systems (Vaz-Moreira et al. 2017). Furthermore, numerous studies have found Proteobacteria to be the dominant phylum found in biofilter media samples (Gerrity et al. 2018; Nemani et al. 2018). Proteobacteria were by far the most dominant phylum at 75 ± 11% relative abundance through the filter bed. Acidobacteriota were the next most abundant in the top section of the filter at 8 ± 5%, though were at much lower abundances at the bottom at 0.4 ± 0.5%. Bacteroidota and Spirochaetoa were found at in the highest relative abundances in the bottom section of the filter 10 ± 6% and 2 ± 4%, although the high standard deviations suggest high variability between the filter replicates. The majority of the 20 most abundant phyla were at similar relative abundances throughout the filter bed. However, Nitrospirota and RCP2-54 were entirely absent from the bottom section of the filter bed. While the metabolism of RCP2-54 is unknown, Nitrospirota contain genera which oxidise nitrite (Potgieter et al., 2020). As this is an oxygen dependent process, their absence in the bottom of the filter may be due to insufficient oxygen levels to fuel their metabolism.

In the top section 15cm of the filter bed there was little change in the most abundant taxa over time despite increasing biomass and richness. There was also little change through in most abundant taxa through the depth of the filter bed in this top section, again despite a decrease in biomass and richness. An uncultured *γ-Proteobacteria* of the family *Beggiatoaceae* was by far the most dominant taxa through the top 15cm of the filter bed (Figure 4.5). The metabolism of *Beggiatoaceae* is often chemolithoautotrophic, dependent on the oxidation of reduced sulphur species and have been known to thrive in sediments and microbial mats (Teske and Salman 2014). Although some strains, particularly from freshwater are capable of heterotrophic growth supplemented by sulphur oxidation (Teske and Salman 2014; Flood et al. 2021; Patritskaya et al. 2001), Further strains have been shown to be capable of denitrification and dissimilatory reduction of nitrate to ammonium (Schutte et al. 2018). A notable trait of *Beggiatoaceae* is the ability to store polysulphide compounds in intracellular vacuoles as a potential electron reserve (Berg et al. 2014). Few studies on biofilter

communities have identified this family as abundant. However, one study identified *Beggiatoaceae* as among the most abundant taxa in effluent water following carbon filtration in a full scale drinking water treatment plant (Bruno et al. 2021). *Beggiatoaceae* was also identified in BAC filters in a full scale drinking water treatment plant utilising ozonation, though at lower relative abundances than in this study (<10%) (Li et al. 2021). *Beggiatoa* have also been identified in biofilter systems associated with aquaculture and wastewater treatment (De Sanctis et al. 2013; Nikiforov-Nikishin et al. 2020). While few biofilter studies have identified *Beggiatoaceae* as a dominant family, it should be noted that the majority of studies report microbial taxonomy at phylum or order level. Thus, it is unclear if the high abundance of *Beggiatoaceae* is unique to this study or simply masked behind the wider classification of *Proteobacteria* reported by other authors.

While few changes in most abundant taxa occurred over time in the top 15cm of the filter bed, the 30cm, 60cm and 90cm sections showed more variation. Unlike the sections above, *Beggiatoaceae* were not found to dominate the 30cm section of the filter bed between weeks 5 and 12. An increase in the abundance of *γ-Proteobacteria Tenderiaceae* was observed from week 5 to 12, becoming the most abundant taxa in this section. While information on the metabolism of *Tenderiaceae* is lacking, examination of their phylogeny (Appendix Figure A.1) revealed their closest relatives to be *Thioalkalispiraceae*, *Acidiferrobacteraceae* and *Beggiatoaceae.* These taxa are all known oxidisers of sulphur (Flood et al. 2021; Issotta et al. 2018; Mori et al. 2011). This suggests that *Tenderiaceae* are themselves capable of sulphur oxidation. The high abundance of *Beggiatoaceae* at the top of the filter and *Tenderiaceae* at the bottom may indicate that availability of reduced sulphur compounds may be a deterministic factor for community selection. Interestingly, *Tenderiaceae* were found at higher abundance in the top of the filter bed at week 5, while the relative abundance of *Beggiatoaceae* was lowest. This might suggest some competition for reduced sulphur compounds between the two taxa.

At week 12 in the 30cm section there was also a notable rise in abundance of the *α-Proteobacteria Rhizobiales* to 23 ± 2%. *Rhizobiales* are commonly form symbiotic relationships with plant life, aiding in nutrient supplementation and nitrogen fixation (Erlacher et al. 2015). The uncultured genus found in this study has previously been identified in ground and reservoir freshwater sources and hypoxic environments (Humbert et al. 2009; Kotik and Faměrová 2012; Yergaliyev et al. 2020). However, by week 23 these taxa had significantly reduced in abundance and the 30cm section saw an increase in the

abundance of *Beggiatoaceae* to 52 ± 13%, again becoming the most dominant taxa. Thus, by week 23 the most abundant taxa of the entire top 30cm of the filter bed was similarly composed between the short, medium and long column lengths. This extension of the most abundant community from the top of the filter may have interesting implications for the development of biofilter communities. It would seem that time and in turn, EBCT is responsible for this extension of the top community. Unfortunately, the design of this experiment does not allow for a direct comparison of filter bed communities based on EBCT across the three column sizes. However, an interesting avenue of research may be to investigate the factors behind this time or EBCT dependent extension of the top community to deeper layers. A possibility is altering nutrient gradients with increasing biomass. As biomass increases easily assimilable nutrients may be released from dying cells altering nutrient availability further down the filter creating a more favourable environment for specific taxa (Kollu and Örmeci 2015; Hansen et al.1986). A potential example of this may found between *Beggiatoaceae* and *Tenderiaceae. Beggiatoaceae* form vacuoles containing polysulphide compounds which can utilised for oxidation. It is possible that lysis of *Beggiatoaceae* cells is releasing these compounds which are then being utilised deeper in the filter bed by *Tenderiaceae*. Nutrient availability may also be affected by the adsorption capacity of GAC (Kennedy and Summers 2015). Saturation of the GAC is likely to happen from the top down as organics encounter the GAC particles at the top of the bed first (Fu et al. 2017). It is unclear to what extent the microbial community is able to utilise adsorbed carbon (Li and DiGiano 1983; Aktaş and Çeçen 2007). It is possible that between weeks 5 and 12 the microbial community was competing for nutrients supplied by the influent with adsorption on to the GAC. DOC removal seemed to plateau between weeks 12 and 15 (84 – 105 days) which might suggest that the GAC had become saturated, or the rate of adsorption had slowed. Adsorption of GAC is thought to occur it two stages, initial adsorption to the surface of the GAC and then a more gradual adsorption within the pores of the GAC particles (Wang et al. 2020; Ocampo et al. 2013). This slower adsorption phase may potentially allow the microbial community to make use of more nutrients before they are sequestered through adsorption.

The bottom section of the filter bed (60cm and 90cm) showed more variation over time than the top section. For example, at week 5 *Sporichthyaceae* and *Ramlibacter* were found in relatively high abundance though had reduced by the following weeks (*Sporichthyaceae* - 10 ± 5% at week 5 and 0.2 ± 0.1% by week 23, *Ramlibacter* – 8 ± 7% at week 5, 2 ± 1.7% by

week 23). *Tenderiaceae* was found to be relatively abundant at weeks 9 and 12 (20 ± 12%), though had again reduced by week 23 (4 ±7%). At week 23 *Chitinophagaceae* (12 ± 6%) and *Xanthobacteraceae* were found to be amongst the most dominant taxa, while being less abundant in earlier weeks. Overall, the bottom section of the filter showed far more variation over time than the top section of the filter bed. However, given the eventual uniformity of the top 30cm by week 23, it would have been interesting to see if the replication of the top layer had continued deeper into the filter bed if given more time.

4.4.2. Effect of Column Length on Biofilter and Effluent Communities

The results from this study seem to indicate that the media depth of the biofilter has little impact on the communities that develop at the top of the filter bed. However, a longer filter bed may select for specific taxa in the bottom section. Furthermore, column size appears to have an influence on the abundance of certain taxa in the effluent water, however the communities remain largely similar.

In terms of alpha and beta diversity there was very little difference between the three column lengths. The short and medium filters mirrored the trends of the long filter for evenness and richness, exhibiting highest diversity at the top of the filter bed (Figure 4.5. A) Generally, there was no significant difference between the column sizes with the exception of the 60cm sections of the medium and long filter. The bottom section of the medium filter (60cm) was found to have lower diversity than the 60cm section of the long filter. Considering UniFrac distance there was again little differences between the column sizes. Clear clustering by sample depth was observed on the PCOA (Figure 4.5. B.) with no real separation between the column sizes. While column size was identified as a statistically significant factor, it explained only 3% of variance after PERMANOVA. Depth was found to explain the majority of variance at 45%. The effluent water again clustered with the bottom section of the filter bed, although the bottom section of the short filter was more separate from the effluent cluster than the bottom sections of the other two columns. This suggests a greater phylogenetic difference between the communities of the effluent water and the bottom of the short filter than the bottom of the medium and long filters. While the effluent of the short filter was more distinct from the bottom of its filter, the effluent water of the different column sizes was found to cluster together.

The similarity between the communities of the effluent water from the three column sizes was reinforced when examining the most abundant taxa. The effluent water from each column size was largely similar, with each displaying relatively high abundances of *Sporichthyaceae, Beggiatoaceae, Burkholderiaceae, Aquabacterium* and a SAR11 clade *α-proteobacteria*. Interestingly, these taxa with the exception of *Aquabacterium* were found to be the most abundant in the influent water. This similarity is further evidence of the influence the community of the influent water has on the eventual biological composition of the effluent water as reported in other studies (Lautenschlager et al. 2014; Vignola et al. 2018). While largely similar, there were differences in the abundances of certain taxa between the column sizes. The effluent of the short filter contained the highest abundance of *Aquabacterium*, the lowest in the effluent from the long filter. As this taxon was not found at high abundance in any section of the filter bed, it is likely passing through the filter from the influent water. As such, it would appear that the deeper media depth afforded by the long filter is more efficient in the removal of *Aquabacterium*. A *Comamonadaceae* of the genus *Rhodoferax* was found in higher relative abundance in the effluent water of the medium and long filters. As this taxon was also found in higher abundance in the bottom sections of these filters it is likely an example of taxa breaking free of the biofilm and entering the effluent water as postulated in earlier studies (Lautenschlager et al. 2014).

The most abundant taxa in the top 15cm of all three column sizes were very similar at week 23, with *Beggiatoaceae* the most abundant taxa across all three filter beds (Figure 4.7 C.). The 30cm section was also very similar across all three column sizes, though an increase in *Chitinophagaceae* was observed in the medium filter. *Chitinophagaceae* were also found to be in higher abundance in the 60cm section of the medium filter and both the 60cm and 90cm sections of the long filter. Members of *Chitinophagaceae* have been shown capable of chitin degradation and the hydrolysis of cellulose and have been found in aerobic and anoxic bioreactors (Rosenberg 2014; Szabó et al. 2017).

The bottom section of the long filter was the most different of the three, seeing increased relative abundances of *Xanthobacteraceae, Rhodoferax* and *Burkholderiales*. This may suggest that the deeper layers of the long filter bed are providing an environment which selects for these taxa. This is further evidenced when the log2 fold ratios are taken into consideration (Figures 4.8, 4.9, 4.10.).

The log2 fold ratios between the top and bottom layers of the filter bed show that taxa which are found in increased abundance in the top section are largely uniform across the three column sizes. This suggests that selective pressures in the top section of the filter bed are similar regardless of media depth. In contrast, certain taxa are found in higher abundance in the bottom sections of the long filter than in the bottom sections of the short and middle. For example, *Bacteroidota* were found to be in slightly higher abundance in the bottom section of the short filter. In the medium filter the abundance of *Bacteroidota* was found to be higher and in the long filter higher still. Other taxa such as *Burkholderiales* and *Rhizobiales* were found in increased abundance only in the bottom section of the long filter. This suggests that a deeper filter bed may be required to select for specific taxa.

It would appear that some taxa are being selected for by the deeper layers of the filter bed, however the mechanisms of selection currently remain unclear. It has been reported that nutrient gradients within GAC biofilters drive community development (Boon et al. 2011). As such the deeper media depth of the long filter may facilitate a broader nutrient gradient which favours certain taxa. Alternatively, the longer filter bed may also contain a broader oxygen gradient selecting for certain taxa (Adrados et al. 2014). As oxygen contained in the influent water is sequestered by the biofilm at the top of the filter bed it may lead to the deeper sections of the bed being more oxygen deprived. Interestingly, the genus *Rhodoferex* found in highest abundance in the bottom section of the long filter bed has been found to thrive in anoxic conditions (Salka et al. 2011). Similarly, the nitrogen fixing genera *Bradyrhizobium* found in highest abundance in the bottom layer of the long filter, has been shown to display sensitivity to oxygen (Torres et al. 2017; Tsoy et al. 2016; Masloboeva et al. 2012). *Bradyrhizobium* has also been found in high abundance in other GAC biofilter studies (Gerrity et al. 2018; Oh, Hammes, and Liu 2018). The increased abundance of these taxa in the bottom section of the long filter may suggest oxygen concentration is a selective factor. The percentage of ASVs shared between each section of the filter bed suggest that proximity to the top of the filter has more effect on the development of the community than proximity to the bottom, which may indicate some importance on nutrient or oxygen gradients. While a measure of oxygen concentration through the depth of the filter bed would have been highly beneficial to this study, it proved too difficult to implement in practice.

In the top 2cm of the filter, the percentage of shared ASVs was very high (long – 91%, medium – 89%, short – 81%). This further reinforces the reproducible nature of the top section of the filter bed. When the bottom sections were compared the percentage of shared

taxa dropped (long – 63%, medium – 79%, short – 53%). However, when the 30cm section was compared across the three column sizes the percentage of shared taxa increased, with the short filter sharing 86% of taxa with one or both of the other two column sizes. Similarly, the bottom section of the medium filter (60cm) shared 73% of ASVs with the 60cm section of the long filter. The high percentage of shared ASVs between equal depths of the filter bed suggest that sampling depth from the top of the filter highly influences community composition regardless of overall media depth. Again, this supports the PERMANOVA of Unfrac distance which identified sampling depth as responsible for 45% of variance between communities.

The effluent water was also found to be similar between the three column sizes with the long filter sharing 71% of its ASVs, the medium 72% and the short 69%. The most abundant taxa were also found to be similar, with the exception of increased abundances of *Aquabacterium* and *Rhodoferax* in the effluent of the short and long filters respectively. This initial data suggests that media depth has little influence on the biological communities of the effluent. Total cell counts by flow cytometry reported in Chapter 2 showed no significant difference in cell removal between the three column sizes. These combined results seem to indicate that filter media depth has little effect on biological removal. However, a deeper investigation on the effect of media depth on effluent communities and their relationship to the influent water communities will be conducted in Chapter 5.

Overall, the results from this study indicate that column length has little effect on biological communities at the top of the filter bed, though a longer filter may select for certain taxa at its deepest layers. The importance of these taxa selected in deeper layers is currently unknown. It was reported in Chapter 2 that the longer filter bed achieved the highest percentage removal of DOC. However, this superior performance cannot be attributed to these taxa at this stage. Other factors such as increased GAC adsorption sites offered by the deeper bed or longer contact time of the influent water could have influence on the filter's performance. Further research investigating the function of the communities through the depth of the bed at RNA level may provide more insight, however this was beyond the scope of this study.

4.5. Conclusions

- Over time biomass, alpha diversity and activity were found to increase to highest levels at week 23.
- Biomass, alpha diversity and activity was found to be highest in the top section of the filter bed, decreasing with depth.
- The most abundant taxa in the top 15cm of the filter bed showed little change over time and depth despite increasing biomass and diversity. *Beggiatoaceae* was consistently the most abundant taxa in this section.
- More variation in the most abundant taxa was observed in the bottom section of the filters over time and depth. *Tenderiaceae, Xanthobacteraceae* and *Sporichthyaceae* were found in higher abundances in this section.
- Bray-Curtis and UniFrac beta diversity showed distinct communities clustering by time and depth. Sampling depth was identified as the factor responsible for the majority of variance.
- Little difference was observed in alpha or beta diversity between column sizes. Column size was found to explain only 3% of variance between communites.
- The most abundant taxa in the top 30cm of the filter bed of all three columns were similar. Most differences were found in the bottom section of the long filter where *Rhodoferex* and *Bradyrhizobium* were found in higher abundances.
- Most abundant taxa in the influent water found to be present in the effluent water at similar abundances.
- Most abundant taxa largely similar in the effluent waters of the three column sizes. Abundance of *Aquabacterium* highest in the effluent of the short filter.
- Taxa found in increased abundances in the top section of the filter were found to be fairly uniform across the three column sizes. Again, differences were observed in the bottom section of the long filter, suggesting certain taxa are being selected for in this location.
- High percentage of taxa shared between the same sampling depth across the three column sizes. Proximity to the top of the filter bed has more influence on communities than proximity to the bottom or indeed overall media depth.

- High percentage of taxa shared in the effluent waters of the three column sizes. Media depth appears to have little effect on the microbial communities of the effluent water.
- Overall, filter bed depth appears to have little influence on the communities which develop in the top section of the filter bed. However, increasing media depth may have a selective effect on specific taxa, leading to differences in community composition in the bottom sections of the filter bed. Though it is currently unknown if these deeper communities alter biofilter performance.

Chapter 5

Effect of Column Size on Effluent Microbial Communities

5.1. Introduction

The aim of any drinking water treatment system is the production of safe and biologically stable potable water. Water is thought biologically stable when it no longer supports the conditions necessary for microbiological growth (Prest et al. 2016). While the presence of microorganisms in drinking water is usually unavoidable and largely harmless, excessive growth in drinking water systems can lead to health, aesthetic and operational concerns (Chowdhury 2012; Berry et al. 2006). Furthermore, opportunistic pathogens have been found to grow in low nutrient conditions and drinking water distribution systems (Wingender et al. 2011; September et al. 2007; Idi et al. 2010). The growth of other organisms may also facilitate the growth of opportunistic pathogens by acting as hosts for their safe reproduction such as *Legionella pneumophila* invading *Amoeba* (Escoll et al. 2014). Microbial growth can also lead to issues with colour and odour. Increases in turbidity and odour causing compounds would be unacceptable to the consumer often accompany microbial growth (Gauthier et al. 2003; Zhou et al. 2017). Operational systems such as distribution networks can also be negatively affected by excessive growth through biocorrosion (Galarce et al. 2020). Factors which can influence biological stability include the nutrient availability of the effluent water, disinfectant measures in place and the composition of the community present (Prest et al. 2016; Melo and Bott 1997; Berry et al. 2006; Hallam et al. 2001).

This last has implications for the process of biofiltration and is the focus of this study. The community composition of the effluent water is important for biological stability. A factor which influences biostability is the type of cells contained in the effluent, be they free living or capable of forming a biofilm (Prest et al. 2016). Biofilm formation in the effluent water is undesirable as it may lead to taste and turbidity issues or act as a reservoir for opportunistic pathogens (Liu et al. 2016; Wingender et al. 2011). In biofiltration, the community of the effluent water has been shown to be influenced by the community of the influent water (Lautenschlager et al. 2014; Ma et al. 2020; Vignola et al. 2018). As such, it would be helpful to remove biofilm forming cells from the influent water during the filtration process to prevent subsequent biofilm formation in the effluent water. Surface water is known to consist of free living, pelagic organisms and cells attached to particulate matter (Kathol et al. 2011; Woodhouse et al. 2018). As attachment is characteristic of biofilm formation (Petrova and Sauer 2012) it may be beneficial to reduce as far as possible the abundance of particle associated bacteria in the effluent water.

As the effluent community composition may affect biological stability, it is of importance to investigate changes in composition which may occur due to the operational conditions of the biofilter. The greatest microbiological risk posed comes from the presence of pathogens in the effluent water. As such, many studies investigating the biological nature of the effluent water focus on known pathogens and indicator organisms (Dai et al. 2018; Matuzahroh et al. 2020; Maurya et al.2020; Xing et al. 2018; Li et al. 2012). Biofiltration has been shown to be effective in the removal of potential pathogens and indicator organisms (Li et al. 2012; Matuzahroh et al. 2020; Maurya et al. 2020). However, Dai et al (2018) found an increase in the abundance of *Legionella pneumophila* in hot water plumbing systems following biofiltration when compared to unfiltered water (Dai et al. 2018)

Other studies investigating biofilter effluent communities tend to focus on full scale DWTPs, involving long treatment trains including ozonation or filter backwashing which may influence the community of the effluent water (Oh et al. 2018; Lautenschlager et al. 2014; Gerrity et al. 2018). Thus, it can be difficult to decouple these factors from biofilter design parameters which may be influencing the effluent community. The biological communities of effluent water have been shown to differ from those in the filter bed (Gerrity et al. 2018). However, the communities of the filter bed and influent water are thought to have an influence on the effluent water following biofiltration (Lautenschlager et al. 2014)

Some design parameters have been directly investigated such as temperature and media (Moll et al. 1999; Vignola et al. 2018; Ma et al. 2020). However, an important design parameter which remains poorly understood is column size.

The biofilters in this study demonstrated that column size has no significant effect on the removal of total cells or of the pathogens investigated over the six-month experiment (Chapter 2). The filters in this study removed 30 ± 17% to 42 ± 21% of total cells from the influent water. However, ANOVA revealed there was no statistically significant difference between the column sizes suggesting column size had no effect on overall removal. Previous studies have indicated that increasing column size does not increase the removal of coliform and *Escherichia coli* (Bagundol et al. 2013; Freitas et al. 2021; Aris et al. 2021) However, what is unclear is the effect media depth may have on the community composition of the effluent as a whole. The composition of biofilter effluent has been shown to be largely influenced by the community of the influent water (Lautenschlager et al. 2014; Ma et al.

2020; Vignola et al. 2018). This suggests that there is a proportion of taxa simply passing through the filter bed and forming part of the effluent community. The community of the filter bed has also been shown to influence the community of the effluent water albeit to a lesser degree than the influent water (Lautenschlager et al. 2014). This suggests that taxa may be breaking free of the filter media and seeding the effluent water during the filtration process.

These factors influencing the effluent community may be affected by media depth. If taxa are passing through from the influent, a longer filter bed may offer more opportunity to capture cells through physical straining or predatory grazing (Bomo et al. 2004). Similarly, taxa breaking free of the biofilm would potentially have more opportunities to be recaptured by the filter media in a longer filter bed. Also, nutrient or oxygen gradient variations between media depths may also effect taxa entering the effluent from the filter community (Adrados et al. 2014; Boon et al. 2011). A longer media depth may have broader gradients and thus select for taxa unable to colonise a shorter bed depth. If these taxa break free from the bed, it may result in a different community composition between bed sizes.

It has been suggested that the majority of biological removal occurs in the top section of the filter bed (Matuzahroh et al. 2020; Velten et al. 2011). If this is the case, increasing the length of filter bed may have a negligible effect on the community of the effluent water. Indeed, the effluent communities of the biofilters in this study were found to be largely similar by week 23, sharing around 70% of taxa (Chapter 4). Despite this, there were some differences between the most abundant taxa of the different media depths. For example, the abundances of *Aquabacterium* and *Rhodoferax* were found to be influenced by media depth. However, these observations were made at only one timepoint during the filter run. Furthermore, the communities of the influent and effluent were compared only at week 23. Thus, it is unclear how media depth might affect the effluent communities over time.

The community of the filter bed is known to develop over time, increasing in biomass and diversity until reaching a steady state (Velten et al. 2011). As the community in the filter bed changes, it is conceivable that this may affect the community composition of the effluent water. Changes in the community composition of the filter bed may lead to variations in cell removal from the influent water or different taxa breaking free and seeding the effluent water over time (Pinto et al. 2012). If the development of the filter bed community has an influence on effluent communities, then it may be expected for the influent and effluent communities to

become more distinct from each other over time. Due to differences in the filter bed communities facilitated by media depths, differences in effluent communities may also differ by bed depth over time. Alternatively, if the effluent community remains reflective of the influent water over time, it may suggest that the influent water is the main driver of the effluent community despite any development of the filter bed community.

Overall, the aim of this chapter is to investigate the effect that column size has on the biological community of the effluent water. This will inform the overall column length of our enhanced biofilter. By comparing the community of the influent water to the effluents from three bed sizes (short, medium and long (Chapter 2)) weekly for the six-month experiment, we determine what effect the length of filter bed has on the community of the effluent water.

Additionally, the community of the influent water was size fractionated into likely attached cells and pelagic cells by filtration. The rationale behind this was to determine which fraction of the influent community has the stronger influence on the community of the effluent water. This is of importance in terms of biological stability. Cells attached to particulate matter may be more likely to form biofilms and thus important to remove from the effluent water (Petrova and Sauer 2012). As adhesion is often required for biofilm formation (Petrova and Sauer 2012) and cells attached to particulate matter may be more susceptible to physical straining, we hypothesise that cells attached to particulate matter will have a weaker influence on the community of the effluent than the pelagic fraction which may be more likely to pass through the filter and into the effluent.

The results from chapter 4 showed that the community of the effluent water was more similar to the bottom section of the filter bed than the top or middle. As the most abundant taxa differed in the bottom sections of the three column sizes, we hypothesise that this will be reflected in the effluent water.

5.2. Materials and Methods

The methods used in this study have been previously outlined in earlier chapters. Influent and effluent sampling and filtration were outlined in Chapter 2. A summary of samples investigated in this chapter is shown in Figure 5.1. DNA extraction, end point PCR and library preparation were outlined in Chapter 4. Sequencing data processing and statistical analysis were also outlined in Chapter 4. Species contribution to beta diversity (Figure 5.4) calculated using the adespatial package in R and the Hellinger transformation method and 999 permutations (Ijaz et al. 2018; Legendre and De Cáceres 2013).

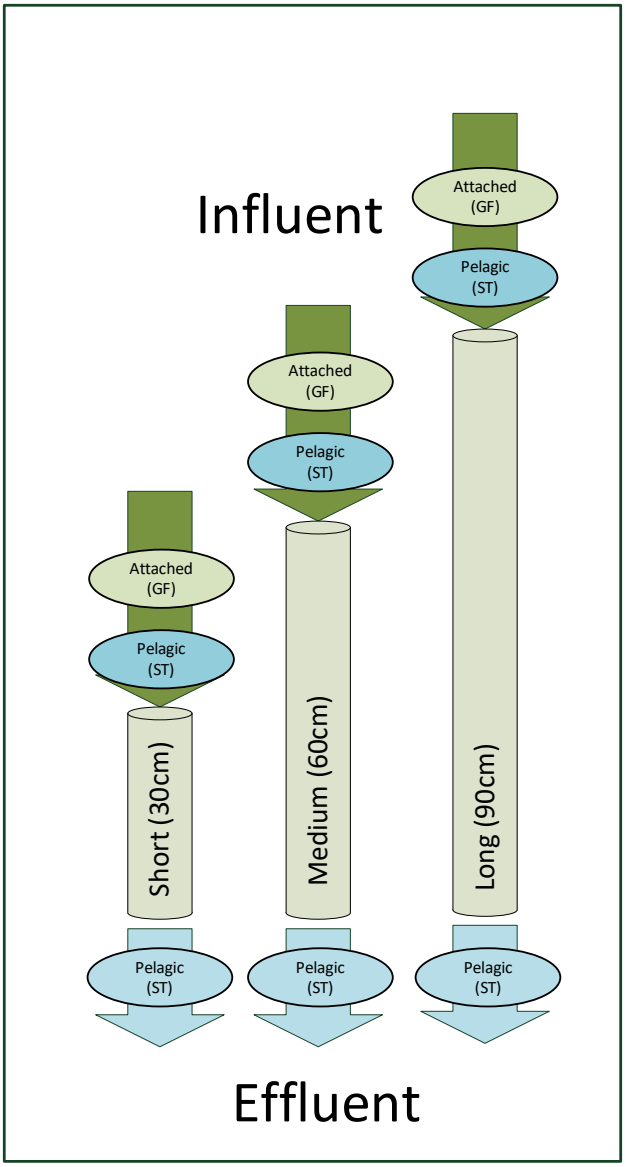


Figure 5.1*. Summary of samples investigated in this chapter. Influent and effluent samples were filtered every second week throughout the run. Influent was size fractionated on glass microfibre (GF) and 0.22µm Sterivex (ST) filters to separate attached and pelagic fractions. Effluent community was captured on a 0.22µm Sterivex filter.*

5.3. Results

Quantitative data was gathered through flow cytometry was reported in Chapter 2. Total cells in the influent water ranged from 1.8 x 10^6 to 7.7 x 10^6 cells/ml with an average of 4 x 10^6 ± 1.2 x 10^6 cells/ml. The short filter saw a significant 30 ± 17% reduction in cells to 2.8 x 10^6 ± 6.7 x 10^5 cells/ml (p-value 0.025). The medium filter was significantly reduced by 42 ± 21% to 2.3 x 10^6 ± 8.2 x 10^5 cells/ml (p-value < 0.001). The long filter was significantly reduced by 41 ± 20% to 2.4 x 10^6 ± 8.2 x 10^5 cells/ml (p-value < 0.001). No significant difference was observed between the three column sizes by way of ANOVA (p-values 0.99, 0.51, 0.43).

5.3.1. Alpha and Beta Diversity

In terms of alpha diversity there was little difference between the effluents of the three column sizes. Richness and evenness of the influent and effluent water from the three column sizes over time are shown in Figure 5.2. The influent water was size fractionated during filtration (Chapter 2) to separate cells attached to particulate or in aggregate from pelagic cells, forming the two influent communities plotted in Figure 5.2.

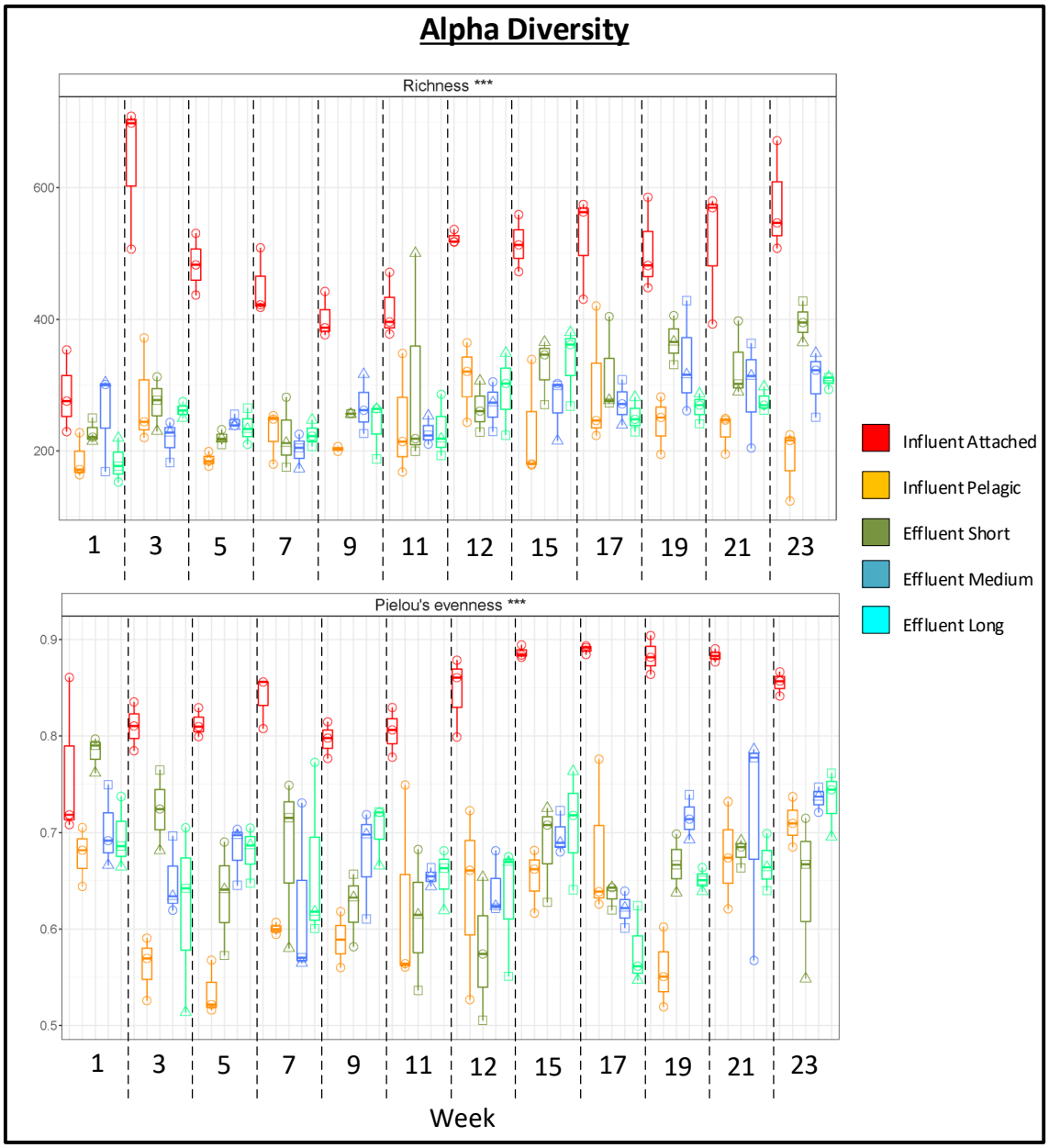


Figure 5.2. *Richness and Pielou's evenness values of the Attached and Pelagic fraction of the influent water and the effluent from the short, medium, and long filters as measured every second week of the 23-week biofilter experiment. P-values generated by ANOVA are available in Appendix tables B.1 and B.2.*

The highest diversity over the 23 weeks was in the attached cells from the influent water. For both richness and evenness (richness range- 229 to 709, evenness - 0.7 to 0.9), the diversity of this community was significantly higher than the effluent water and the pelagic community of the effluent water (p-values > 0.05). Diversity between the three column sizes of the

effluent water was not significantly different for each timepoint. The pelagic community was also similar to the effluent water showing no significant difference for the majority of timepoints (mean influent pelagic richness – 236.7 ± 67, evenness 0.6 ± 0.07, mean effluent richness – 272 ± 63, evenness – 0.67 ± 0.06). This might suggest that the pelagic fraction of the influent water is passing through the filter bed while the attached fraction is either being removed or colonising the bed. While there was little difference at each timepoint between the effluent of the three column sizes, there was an increase in richness from week 1 to 23. By week 23 the effluent with highest richness was that of the short filter, which was significantly higher than the effluent of the long filter and the pelagic fraction of the influent water.

Beta diversity somewhat mirrored alpha diversity with little difference between the effluent waters of the three column sizes. Furthermore, the pelagic fraction of the effluent water was found to be more similar to the effluent water than the attached fraction. PCOAs of Bray-Curtis dissimilarity and UniFrac distance measures are plotted in Figure 5.3.

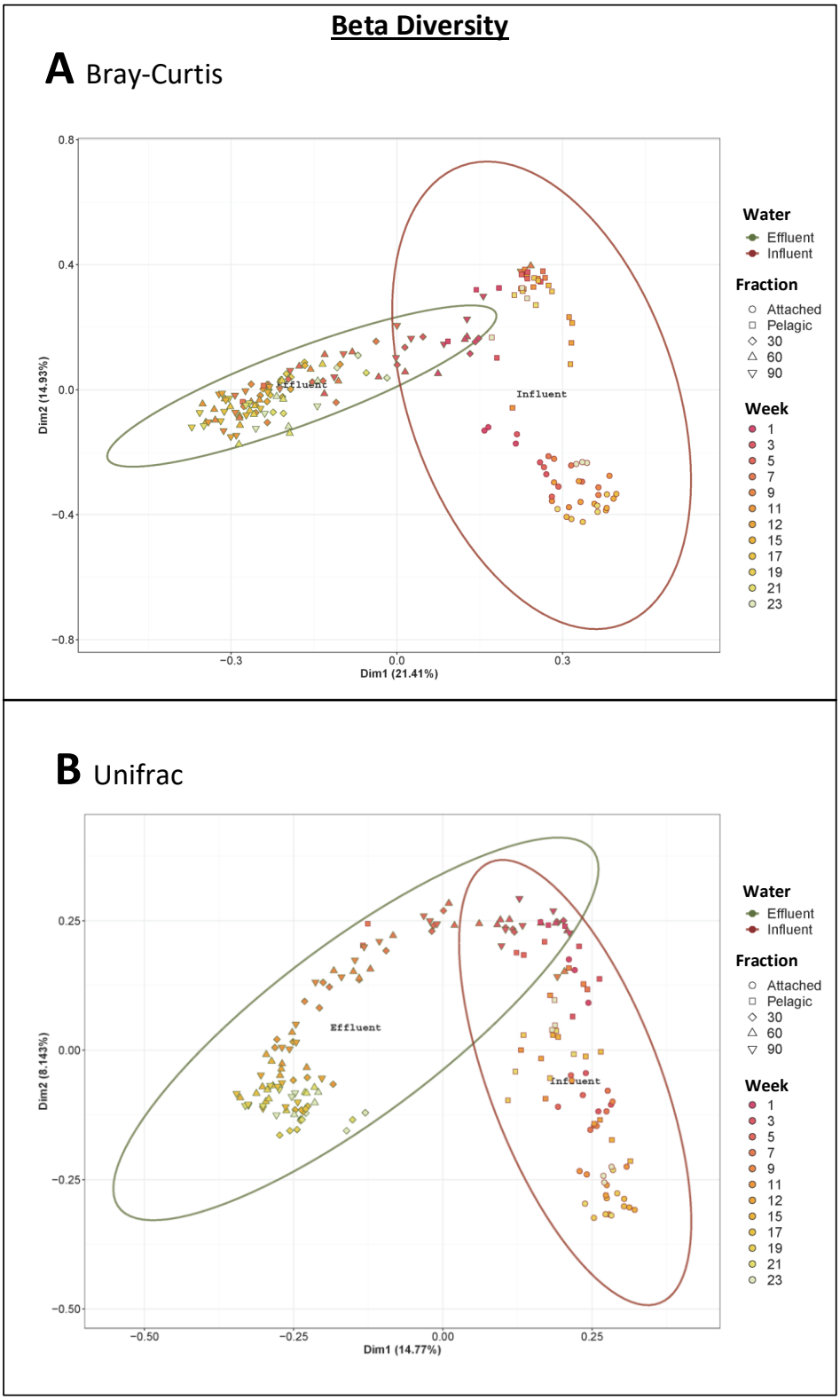


Figure 5.3. *PCOA's of Bray-Curtis dissimilarity (A) and UniFrac distance (B) of the Attached and Pelagic fractions of the influent water and the effluents from the short (30), medium (60) and long (90) filter columns over the 23-week run. Ellipses generated with standard deviation of water type.*

Clustering on the PCOAs show clear separation between the communities of the influent and effluent water. Further separation can be observed between the attached and pelagic fractions of the influent water with the pelagic fraction being closer to the effluent community. There is also clear variation over time, with the communities of both the influent and effluent clustering by timepoint. Interestingly, at week 1 the communities of the influent and effluent water were the most similar and diverged with the passage of time. This suggests that the community developing in the filter bed has an effect on the effluent water, possibly through seeding or cell removal from the influent. Between the effluents of the three media depths there was found to be little separation suggesting these communities are similar. PERMANOVA identified media depth as a significant factor though only explained a small percentage of variance (Bray-Curtis – R^2 0.08, UniFrac - R^2 0.04) This was compared to time which explained the majority of variance (Bray-Curtis – R^2 0.39, UniFrac - R^2 0.4). The interaction between week and media depth was also found to be significant (Bray-Curtis – R^2 0.18, UniFrac - R^2 0.17). PERMANOVA between influent and effluent communities again found time (Bray-Curtis – R^2 0.19, UniFrac - R^2 0.29) to explain more variance than between influent and effluent type (Bray-Curtis – R^2 0.16, UniFrac - R^2 0.1).

While water type was significant, the results from the both UniFrac and Bray Curtis dissimilarity point to time being the most important factor in explaining variance between the communities. This suggests that the development of the community of the filter bed has an influence on the community of the effluent water, whether through removal of cells via the filtration process or through seeding of the effluent from the biofilm. When the influent water was analysed separately, time was again found to be a significant factor explaining variance (Bray-Curtis – R^2 0.19, UniFrac - R^2 0.29). However, the divergence between the influent and effluent communities from week 1 to 23 (Figure 5.3. A & B) suggest that the filtration process has an influence on the effluent community. Again, the pelagic fraction of the influent water was found to be most similar to the effluent water which might indicate that this fraction is driving the community composition of the effluent water at a greater degree than the attached cells. It may be that pelagic cells are more likely to pass through the filter bed thus having more influence on the effluent community or perhaps attached cells are more affected by the filtration process and being removed with more efficiency. Media depth was found to explain only a small percentage of the variance between the effluent communities. Thus, any differences which may occur between the filter bed communities as a result of

media depth (Chapter 4) do not seem to translate significantly to differences in effluent water diversity.

Species contribution to beta diversity identified 50 taxa which contributed the most to variance within UniFrac distance. Figure 5.4 shows the taxa which were found to have the highest contribution to beta diversity and the relative abundance of each in the attached and pelagic influent fractions and effluent water of the three column sizes at genus level over the 23 weeks.

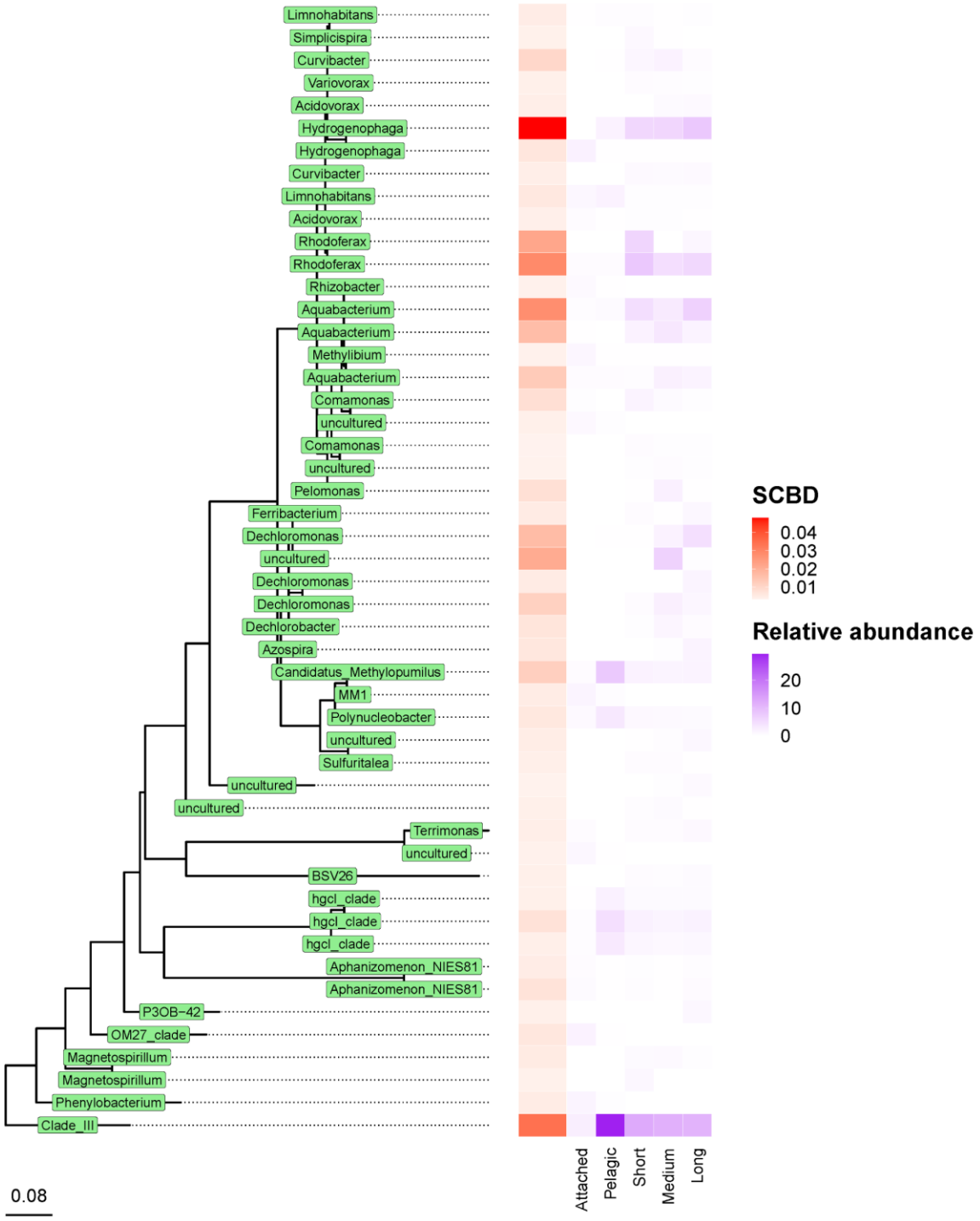


Figure 5.4. *Top 50 genera with the highest contribution to beta diversity measure by UniFrac distance and their relative abundances in the Attached and Pelagic influent fractions and effluents of the short medium and long filters over 23 weeks. SCBC = Heatmap of species contribution to beta diversity calculated with Hellinger method (Legendre and De Cáceres 2013).*

Taxa which had the highest contribution to beta diversity include *Hydrogenophaga, Rhodoferax, Aquabacterium, Dechloromonas, Candidatus Methylopumilus* and *Clade_III Alphaproteobacteria.* The taxa which had the highest contribution was *Hydrogenophaga* and was found in higher abundance in the effluent water of all three column sizes than the influent water. *Rhodoferax* and *Aquabacterium* were also found in higher abundance in the effluent water of all three column sizes than in the influent water. This suggests these taxa are being enriched in some way by the filtration process. Generally, taxa which were found in highest abundance in the attached fraction of the influent water were not abundant in the effluent water. Conversely, taxa found in high abundance in the pelagic fraction were found to be more abundant in the effluent water. The clearest example of this is *Clade III Alphaproteobacteria.* Which was found in highest abundance in the pelagic fraction of the influent water and high abundance in the effluent water of all three column sizes. This supports the greater influence on the effluent community being applied by the pelagic fraction of the influent water.

Between column sizes there were differences observed in the abundance of certain taxa. *Dechloromonas*, *Pelomonas*, and *Ferribacterium* were found at higher abundance in the effluent of the medium and long filters, while *Comamonas* and *Rhodoferax* were found in highest abundance in the effluent of the short filter. The results from chapter 4 identified *Aquabacterium* as being more abundant in the effluent of the short filter at week 23. However, across all weeks *Aquabacterium* appears in similar abundances between the effluents of the three bed sizes. Interestingly, the taxa most effected by media depth were found in very low abundance in the influent water. This may suggest that that these taxa originate from the filter bed community. If so, different communities developing between bed sizes may explain the differences in abundance of these taxa in the effluent water.

5.3.2. Most Abundant Taxa

Differences in the most abundant taxa were observed between the influent water and the effluent water of the three column sizes over time. Figure 5.5 shows the relative abundances of the 20 most abundant taxa in the attached and pelagic fractions of the influent water and effluent water of the three column sizes over 23 weeks.

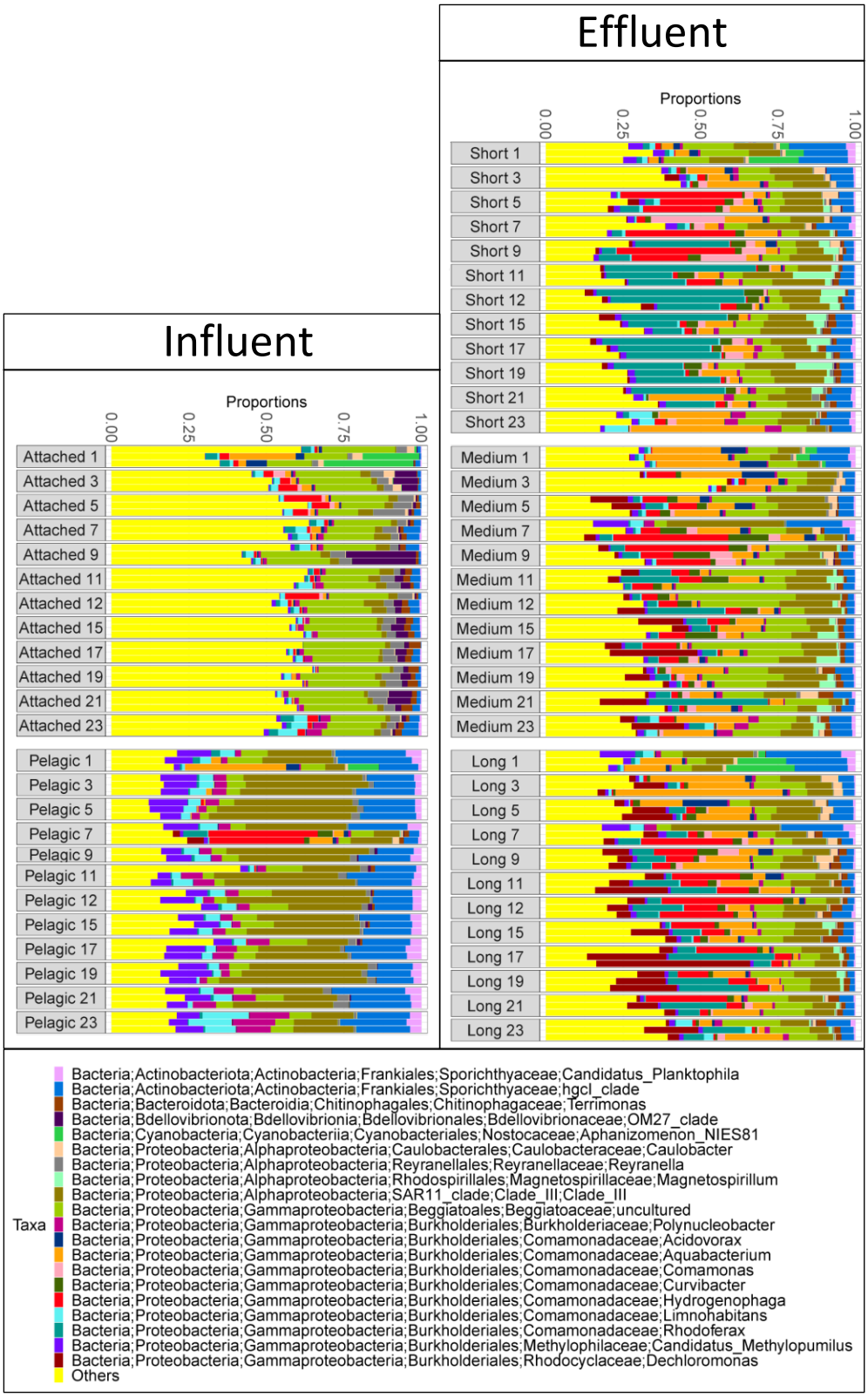


Figure 5.4. *Relative abundances of the top 20 most abundant taxa at genus level in the Attached and Pelagic fractions of the influent water and the effluents of the short medium and long filter columns over the 23 weeks. Boxes are labelled with the sample type and week (1 – 23).*

Over time the most abundant taxa in the influent water were quite stable. Differences in abundances were observed between the attached and pelagic fractions of the influent water. *Beggiatoaceae, Bdellovibrionaceae, Terrimonas* and *Reyranellaceae* were found in higher abundance in the attached fraction while *Sporichthyaceae hgcl clade, Clade_III Alphaproteobacteria, Polynucleobacter* and *Methylophilaceae* were found in higher abundance in the pelagic fraction of the influent water. The most abundant taxon in the Attached fraction was *Beggiatoaceae* (20 ± 3%) while *Clade_III Alphaproteobacteria* dominated the pelagic fraction (16 ± 16%). Again, the most abundant taxa of both fractions of the influent water were relatively stable over time, with the exception of week 7, which saw a large increase in the proportion of *Hydrogenophaga* to 11 ± 16% in the pelagic fraction and week 9 which saw an increase in *Bdellovibrionaceae* in the attached fraction 9 ± 11%. Though the increase of these taxa were only observed in two of the filter replicates. The general stability of the influent water means that changes in most abundant taxa in the effluent water cannot simply be attributed to fluctuations in the community composition of the influent water over time.

Generally, the most abundant taxa of the effluent water more closely resembled the pelagic fraction of the influent water than the attached. The two most abundant taxa in the pelagic fraction of the influent water, *Sporichthyaceae hgcl* clade and *Clade_III Alphaproteobacteria* were found in all effluent water over the 23 weeks. The most abundant taxa of the attached fraction *Beggiatoaceae* was also found in the effluent water of all column sizes over the 23 weeks. It should be noted that *Beggiatoaceae* was also found to be a highly abundant coloniser of the filter bed (Chapter 4), so it is unclear if the *Beggiatoaceae* found in the effluent originates directly from the influent water or is being seeded by the filter bed. There were several taxa found in high abundance in the pelagic fraction which were at lower abundance or not amongst the 20 most abundant taxa of the effluent water including *Methylophilaceae* (influent - 4 ± 4%, effluent – 0.18 ±0.17%), *Limnohabitans* (influent - 3 ± 3%, effluent - 1 ± 1%) and *Polynucleobacter* (influent – 3 ± 3%, effluent – 1 ± 1%). Several taxa were also found to be more abundant in the effluent water than in the pelagic fraction of the influent, including *Hydrogenophaga* (influent - 2 ± 5%, effluent – 7 ± 9%) *Aquabacterium* (influent - 1 ± 4%, effluent – 9 ± 8%) and *Rhodoferax* (influent – 1 ± 1%, effluent – 9 ± 11%). This means that the pelagic fraction of the influent water is not simply passing through the filter unaltered, and the filtration process has influence on the community composition of the effluent.

The most abundant taxa of the effluent water showed more variation than the influent water, both over time and between the three filter sizes. Over time the relative abundances of the effluents of the medium and long filters appeared to fluctuate from week to week. Despite this, some taxa were generally found in higher abundances in these filters such as *Dechloromonas* (long and medium - 3 ± 7%, short – 0.6 ± 1%) and *Hydrogenophaga* (long and medium 5 ± 8%, short – 4 ± 9%) The effluent of the short filter showed the most interesting changes with time. Between weeks 5 and 9 the abundance of *Hydrogenophaga* was high in the effluent of the short filter before dropping considerably in relative abundance (12 ± 17% at week 9, 3 ± 4% by week 11). Similarly, between weeks 11 and 19 there was a large increase in the relative abundance of *Rhodoferax* (17 ± 16%) and a smaller increase of *Magnetospirillum* (3 ± 4%). However, by week 23 the relative abundances of these taxa had fallen considerably (Rhodoferax – 2 ± 2%), *Magnetospirillum* – 0.1 ± 0.2%) and *Aquabacterium* was the dominant taxa in the effluent of the short filter (16 ± 18%). These results suggest that the most abundant taxa in the effluent water is affected by column length at least while the filter bed community is in development. It should be noted that the composition of the most abundant taxa between the three column sizes were similar at week 23, albeit with increased relative abundance of *Aquabacterium* (16 ± 18%) in the short filter and *Dechloromonas* (4 ± 7%) in the medium and long. It is unclear whether this represents an eventual stabilising of effluent communities between column sizes or if the communities would continue to fluctuate had the biofilters continued to run.

5.3.3. Differences in the abundance of taxa between the influent and effluent of the short medium and long filters.

The most abundant taxa between the effluents of the three column sizes showed differences between the bed sizes relating to certain taxa, particularly as the filter bed develops. When the abundances of all taxa were compared between bed sizes, the majority appeared not to be affected by media depth. However, there were differences in the abundances of certain taxa between the bed sizes. Figure 5.6 displays a heat tree matrix highlighting taxa at increased abundances between the attached and pelagic fractions of the influent water and between the effluents of the three column sizes.

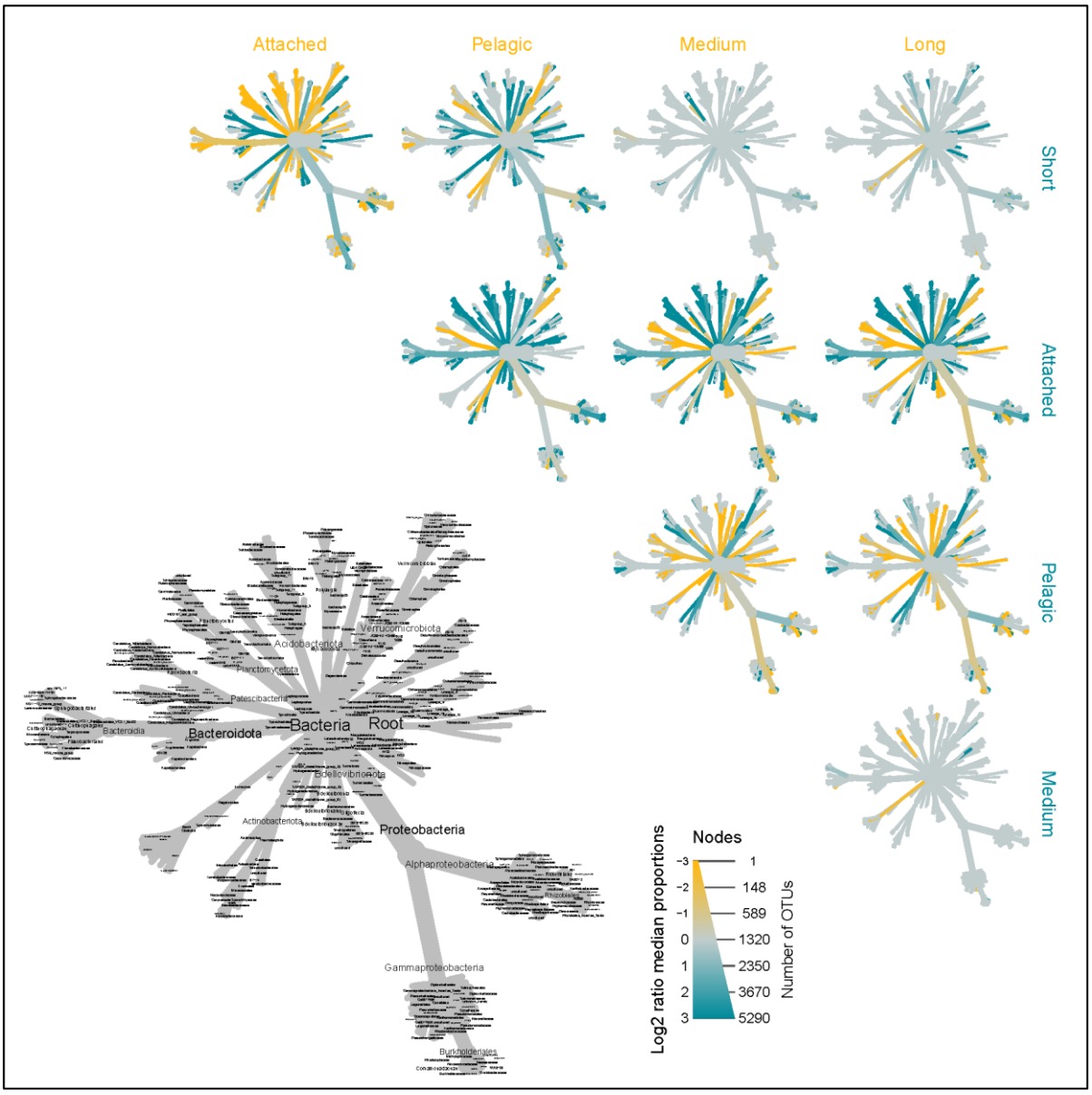


Figure 5.6. *Heat tree matrix comparing the abundances of taxa by log2 ratios in the Attached and Pelagic fractions of the influent to the effluent of the short medium and long filters averaged over 23 weeks.*

Between the attached and pelagic fractions of the influent water, the vast majority of taxa was found in higher abundance in the attached fraction. Only *Patescibacteria, Actinobacteriota* and certain families of *Verrucomicrobiota* were in higher abundance in the pelagic fraction. Similarly, when the attached fraction of the influent water was compared to the effluent of the three bed sizes, the majority of shared taxa were in higher abundance in the influent water. The heat trees comparing the abundance of taxa in the attached fraction of the influent to the

effluent of the different column sizes were largely similar. *Planctomycetota, Acidobacteriota, Myxococcota* and *Rhizobiales* were in higher abundance in the influent, while *Patescibacteria, Nanoarchaeota, Firmicutes* and *Burkholderiales* were consistently found in higher abundance in the effluent. When the pelagic fraction was compared to the effluent, again the heat trees were similar between column sizes. In contrast to the attached fraction, fewer taxa were found in higher abundance in the influent than the effluent, mainly *Planctomycetota, Verrucomicrobiota* and *Actinobacteriota*.

Between the effluents of the three column sizes there was no change in the abundance of the majority of taxa, however there were some changes in the relative abundances. The largest differences were found between the effluents of the long and short filters. In the short filter effluent, *Verrucomicrobiaceae, Fimbriimonadaceae, Nitrospiraceae* and *Acidobacteriae* increased in relative abundance. While in the effluent of the long filter *Firmicutes* and *Sericytochromatia* were in higher abundance. Fewer differences were observed between the effluent of the medium filter and the other two column sizes. *Firmicutes* and *Sericytochromatia* were again at higher abundance in the effluent of the long filter compared to the medium, while *Sphingobacteriales* and *Desulfovibrionales* were more abundant in the effluent of the medium filter. *Sphingobacteriales* and *Sericytochromatia* were more abundant in the effluent of the medium than the short and *Caenarcaniphilales* and *Margulisbacteria* were more abundant in the effluent of the short filter. Aside from a few specific families, the relative abundance of the vast majority of taxa did not change between the effluents of the three column lengths. This suggests that the composition of the effluent water is not strongly affected by media depth when all timepoints are taken into consideration.

5.3.4. Core Microbiome between attached and pelagic influent water fractions and effluent of the three column sizes

Taxa which were present in each community (influent attached, influent pelagic, short, medium and long effluent) throughout the entirety of the 23-week run were identified as the core microbiome of each community. Taxa which were present in 90% of samples from each community were deemed part of this core microbiome. Analysis of the core microbiomes of the effluent water showed the majority of taxa was shared with the core microbiome of the

influent water. Furthermore, the majority of taxa found in the effluent core microbiome was shared between the effluents of the three column sizes. Figure 5.7 displays the core microbiome of the attached and pelagic fractions of the influent water at genus level and Figure 5.8 displays the core microbiome of the three effluent waters, alongside Venn diagrams showing the number of taxa shared with the core community of the influent.

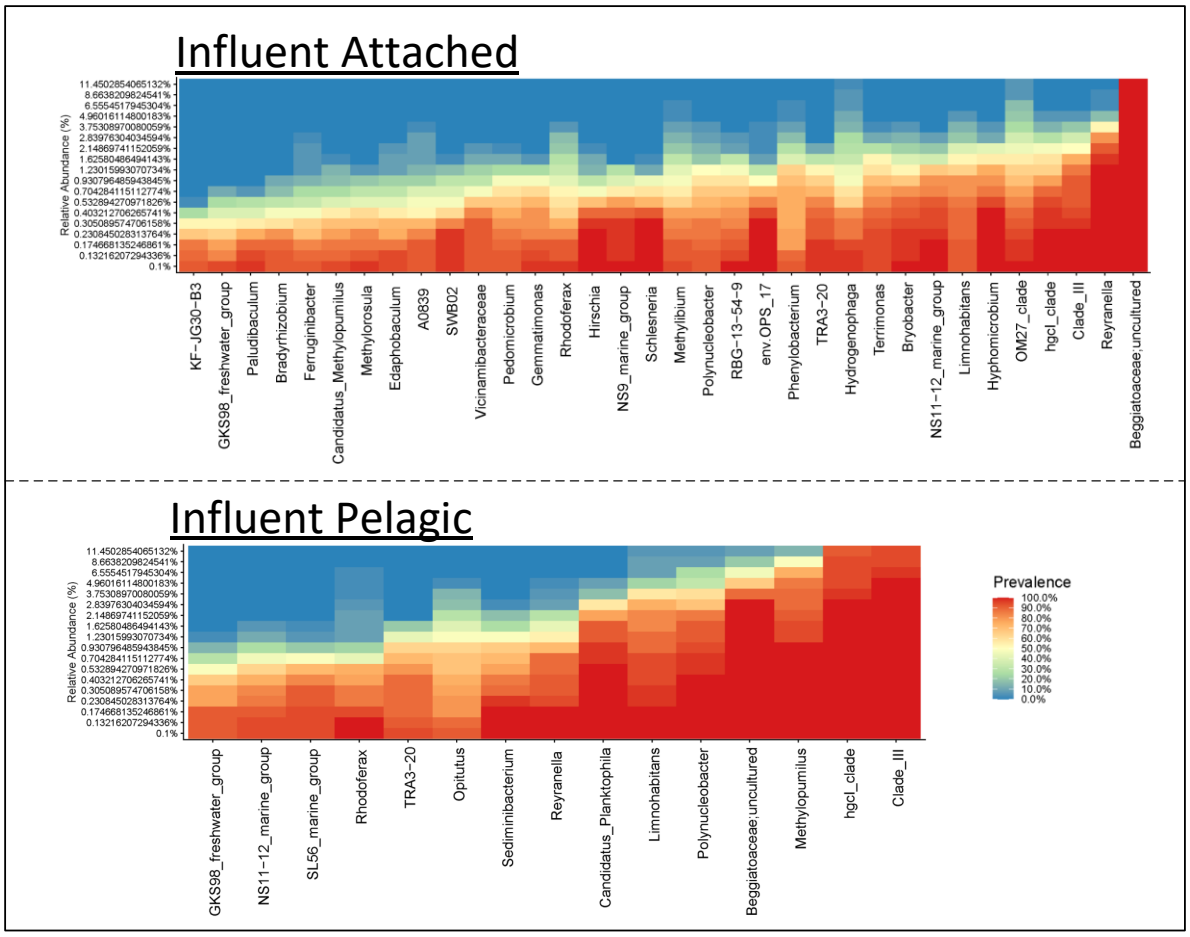


Figure 5.7. *Heatmap of the relative abundances and prevalence of the core microbiome of the Attached and Pelagic fractions of the influent water over the 23 weeks. Minimum prevalence for addition to the core microbiome was 90%.*

The core community of the attached fraction of influent water was found to have more members than the pelagic fraction. The most abundant genera were *Beggiatoaceae* and *Reyranella.* The core microbiome of the pelagic fraction contained fewer members and the majority were shared with the core microbiome of the attached fraction. Only four genera of

sixteen, *Methylopumilus, Sediminibacterium, Opitutus* and *SL56_marine_group* were unique to the pelagic fraction, compared to 23 unique genera in the attached fraction. The most abundant genera in the pelagic fraction were *Clade_III* and *hgcI_clade*. The effluent water shared a high proportion of genera with the influent water (Figure 5.8.).

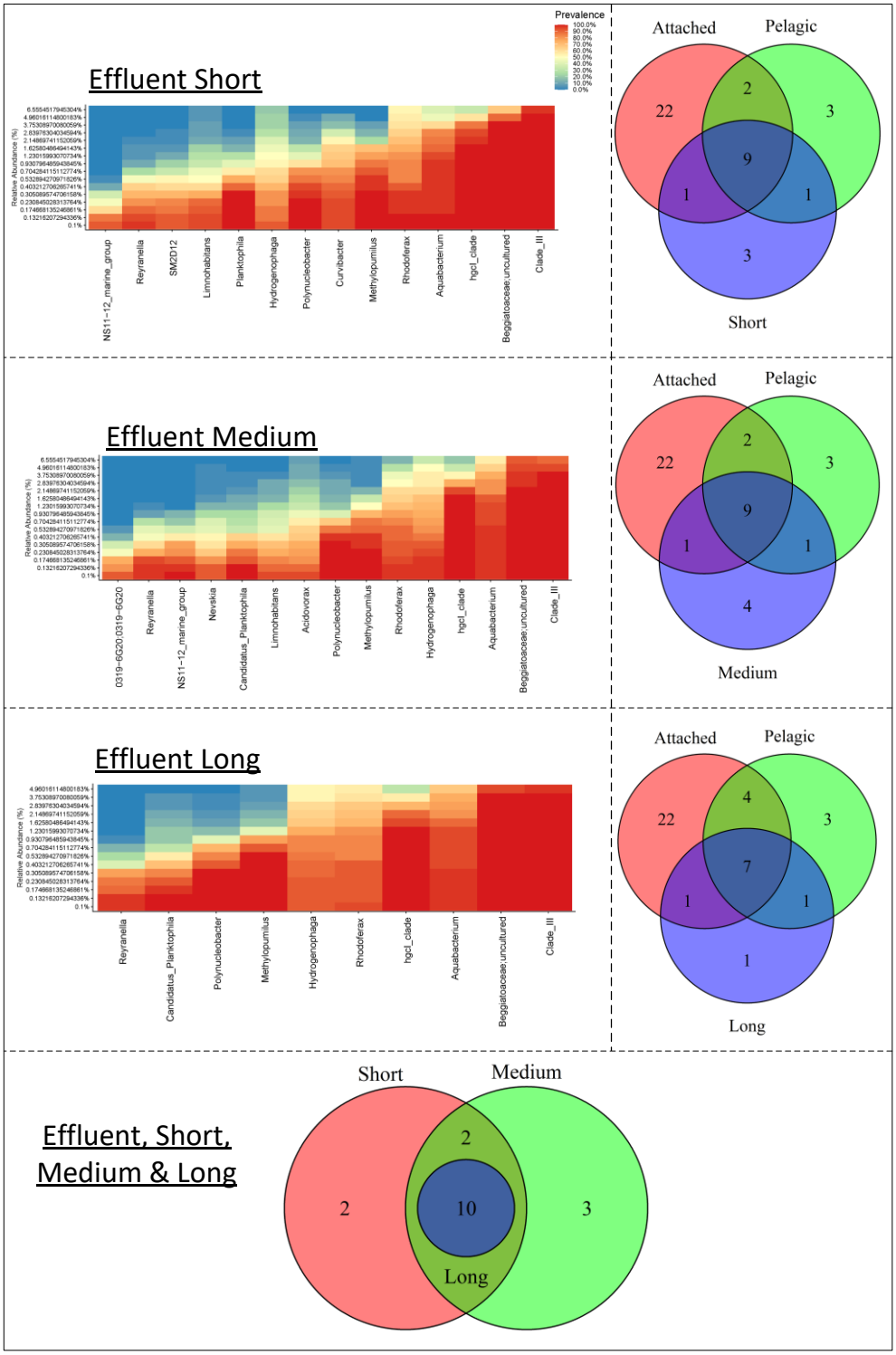


Figure 5.8. *Heatmaps of the relative abundances and prevalence of the core microbiome of the effluent water from the short, medium and long filter columns over the 23 weeks at genus level. Minimum prevalence for addition to the core microbiome was 90%. Heatmaps are accompanied by Venn diagrams displaying shared taxa between the influent fractions and effluent of each filter column and shared taxa between the effluents of the short, medium and long filters.*

The core microbiomes of the effluent water from all three column sizes shared the majority of taxa with the core microbiome of the influent. The effluent of the short filter contained only three genera which were absent in the influent water, *Curvibacter, SM2D12* and *Aquabacterium*. The core microbiome of the effluent of the medium filter contained the most genera absent in the influent water, *Nevskia, Acidovorax, 0319-6G20-0319-6G20* and *Aquabacterium*. The effluent of the long filter contained only one genus which was not in the core microbiome of the influent water which was *Aquabacterium*. 26 genera from the attached fraction and 7 genera from the pelagic fraction of the influent water was not found in any of the core microbiomes of the effluent water. Between the effluents of the three column sizes the core microbiomes were similar. All members of the core microbiome of the long filter effluent were found in the effluents from both the short and medium filters. Two genera were found to be shared between only the medium and short filter effluents, *NS11-12* marine group and *Limnohabitans.* Three genera were unique to the effluent of the medium filter, *Nevskia, Acidovorax* and *0319-6G20-0319-6G20,* and two were unique to the effluent of the short, *Curvibacter* and *SM2D12*. These genera unique to the short and medium filters were not present in the core microbiome of either influent water community. Similarly, *Aquabacterium* was not found in the core microbiome of the influent water despite being present in relatively high abundance in all the effluent communities. The most abundant genera in all three effluent core microbiomes were *Clade_III* and *Beggiatoaceae.* These were found to also be of highest abundance in the core communities of the influent water.

These results show the similarities between the core microbiome of the influent water and effluent. Proportionally, the pelagic fraction of the influent water shared more taxa with the effluent at 73% compared to 32% of the attached fraction, shared with the effluent of the medium and short. The core microbiome of the long filter shared the highest proportion of genera with the effluent water at 90%. The short filter shared 71% of its core microbiome with the medium and long, and the medium filter shared 66%. The high proportion of shared taxa suggests that the core microbiome is not strongly affected by media depth.

5.4. Discussion

The results from this study suggest that biofilter media depth does not have a strong influence on the community of the effluent water in terms of diversity and microbial composition. Time and thus the development of the filter bed community appear to have a greater influence on the community of the effluent water than overall media depth. Despite this, media depth does appear to have an effect on the abundances of certain taxa found in the effluent water such as *Dechloromonas* and *Hydrogenophaga*. The pelagic fraction of the influent water was found to be more similar to the effluent water than the attached fraction, sharing more abundant and core taxa and more similar to the effluent in terms of alpha and beta diversity.

5.4.1. Effluent mirrors pelagic but not attached community

The influent water in this study was size fractionated by filtration through a 1.2µm glass microfibre membrane (Whatman GF-C) followed by a 0.22µm Sterivex filter (Chapter 2 Section 2.2.17). This was necessary from an experimental standpoint to ensure a large enough volume of influent water could be processed to capture sufficient biomass on the 0.22µm filter. Furthermore, bacterial association with particulate matter in surface water has been previously documented and show to differ in composition to the free living fraction (Characklis et al. 2005; Cantwell and Hofmann 2008; Li et al. 2009; Lynch et al. 2014). We hypothesised that the bacteria associated with particulate matter in the influent water, were likely to be biofilm formers and would more readily colonise the biofilter than the free- living bacteria. As such it was important to include explore this fractionation of the community during analysis of the influent water.

Filtration of cells for molecular analysis generally utilises membranes of around 0.22µm to ensure the majority of the community is captured on the filter (Boström et al. 2004; Djurhuus et al. 2017; Putri et al. 2021). The larger 1.2µm aperture of the glass microfibre membrane was thought more likely to capture cells attached to particulate matter or in aggregate while free living single cells would be more likely to pass through to be captured on the 0.22µm Sterivex filter. As such, the majority of the community extracted from the glass fibre membrane were assumed to be cells capable of attachment to particulate matter or in aggregates. The majority of the community extracted from the Sterivex filter was assumed to be singular or pelagic cells. Obviously, this method of assigning the fractions of cells is not perfect. It is possible that cells attached to particulate may escape capture on the glass fibre

membrane and be caught on the Sterivex filter. Likewise, it is possible for pelagic cells to become caught in the glass fibre filter and analysed as part of the attached community. Further research would be required to more accurately assign the fractions of cells in the influent water. However, for the purposes of this study, cells extracted from the glass fibre membrane were thought to be representative of cells attached to particulate or in aggregate, while cells extracted from the Sterivex filter were thought to represent cells which are pelagic in nature. It also possible that very small bacteria such as Patescibacteria may pass through the 0.22µm filter (Ghuneim et al. 2018). This was investigated by subsequent filtration of the influent and effluent filtrate through a 0.1µm filter membrane at the deconstruction timepoints. These results are explored in Chapter 6.

Of the two fractions of influent water, the attached community was found to be the most diverse. Richness and evenness were found to be highest in the attached community and its core microbiome contained more taxa. These studies are consistent with other studies which report higher alpha diversity in the particle associated fraction than the pelagic in freshwater lakes and other aquatic environments (Zhao et al. 2017; Yao Zhang et al. 2016; Mohit et al. 2014) Diversity of the attached fraction alone was greater than the overall diversity of the effluent water. The pelagic fraction was more similar in terms of composition to the effluent water than the attached fraction. Richness and evenness of the pelagic community was generally within similar ranges to the community of the effluent water (Figure 5.2). The pelagic community was also found to cluster more closely to the effluent through Bray-Curtis and UniFrac distance (Figure 5.3). Taxa which were identified as having the highest contribution to beta diversity, such as *Clade III* and *Hydrogenophaga*, were also found to be in higher abundance in the pelagic fraction than the attached (Figure 5.4).

While the physical process of filtration may explain why the pelagic community is more similar to the effluent water than the attached fraction, it does not explain the entirety of the effluent water composition. The process of biofiltration involves many complex mechanisms which are not replicated through mechanical size fractionation. Factors such as predation and nutrient availability may eliminate or lower the abundance of certain taxa as the pass through the filter bed (Lauderdale et al. 2012; Bomo et al. 2004; Oh et al. 2018). Furthermore, taxa which colonise the filter bed may break free from the media and enter the effluent water (Lautenschlager et al. 2014). These factors will likely have an influence on the community composition of the effluent water.

This study identified time as the factor which explained the majority of variance between communities by PERMANOVA of Bray-Curtis and UniFrac distances (Bray-Curtis – R2 0.39, UniFrac - R2 0.4). It is well documented that development of the microbial community within the filter bed is a time dependent process (Velten et al. 2011; Haig et al. 2015; Ramsay et al. 2018). Indeed, the microbial community of the filter beds in this study were shown to increase in diversity, biomass and activity over time (Chapter 4). As the community of the filter bed develops, it may introduce changes to the conditions of filtration altering the community composition of the filter bed community. For example, increasing biomass and EPS structures may decrease space between media particles increasing the efficiency of physical straining (Lauderdale et al. 2012). Increasing biomass may also lead to differing oxygen or nutrient gradients over time which may increase or decrease the survivability of certain taxa (Adrados et al. 2014; Boon et al. 2011). Organisms favoured by the filter environment may also proliferate within the filter bed before separating and entering the effluent water (Lautenschlager et al. 2014). As such, it is likely that the variance between communities over time is caused by the development of the community within the filter bed. This is further evidenced by examination of the PCOA plots in figure 5.3. At week 1, the influent and effluent communities were most similar before diverging over time. This divergence suggests that the development of the filter bed community is having an influence on the microbial composition of the effluent water.

The influence of biological filtration can be observed through investigation of the most abundant taxa (Figure 5.5). While the most abundant taxa in the influent water, namely *Hgcl Clade, Clade III* and *Beggiatoaceae. Beggiatoaceae* were found consistently among the most abundant in the effluent water and in the filter bed. Several taxa were absent or in reduced abundance in the effluent. *Polynucleobacter, Limnohabitans* and *Candidatus Methylopumlis* were consistently of high relative abundance in the pelagic fraction of the influent water while found at much lower relative abundances in the effluent. These taxa may be reducing in number through biological removal mechanisms or alternatively the filtration process may be increasing the abundance of other taxa thus lowering the relative abundance of these organisms. Several taxa were indeed found in increased relative abundance in the effluent water as compared to the influent. *Hydrogenophaga, Rhodoferax, Dechloromonas* and *Aquabacterium* were found to be highly abundant in the effluent water while being largely absent from the most abundant taxa in the influent water. Interestingly, these taxa were found to be the among the most abundant taxa colonising the filter bed and at higher relative

abundances in the bottom sections (Chapter 4). It is likely that these taxa are breaking free of the filter bed and seeding the effluent water. *Patescibacteria* and *Nanoarchaeota* were also found in increased abundance in the effluent water (Figure 5.6). These taxa are characterised by small cell sizes and may be less susceptible to removal by mechanical filtration (Tian et al. 2020; Huber et al. 2003). Further taxa found to be more abundant in the effluent include *Firmicutes, Babeliae* and *Oligoflexia.*

While the effluent water differed from the influent in the abundances of several taxa, there were still similarities between the communities, particularly between the effluent and pelagic fraction. Several studies have identified the influent water as being a driving force in the development of effluent water composition (Lautenschlager et al. 2014; Vignola et al. 2018). In this study, PERMANOVA of water type again found time to be the factor which explains the majority of variance (Bray-Curtis – R^2 0.19, UniFrac - R^2 0.2). While the effect of water type was significant ($P < 0.001$), it explained less of the variance between communities than time (Bray-Curtis – R^2 0.16, UniFrac - R^2 0.1). Therefore, changes in community over time through natural variations in the influent water or the influence of the filter bed on the effluent water have more effect on variation between communities than the biofiltration process. The core microbiome of the effluent water of all three column sizes were also found to share a majority of taxa with the influent water. Lautenschlager et al (2014) found that while the effluent water was weakly influenced by the community of the filter bed, the influent water had the strongest influence on the community of the effluent water (Lautenschlager et al. 2014). Vignola et al (2018) found that the community of the influent water had a greater impact on the community of the effluent water than the material of the filter bed media (Vignola et al. 2018). An unpublished meta-analysis by Cholet et al (in prep) has also identified the community of the influent water to be the main driver of the community composition of the filter bed. The findings of this study and that of these other authors strongly suggests that influent water source may be the most important predictor of the community composition of the final effluent water.

The effluent of the short filter was found to share 79% of taxa with the influent, the medium filter 73% and the long 90%. The core microbiome of the pelagic fraction of the influent water was found to share the highest proportion of taxa with the effluent. Five genera out of 15 were found in the core microbiome of the pelagic fraction while being absent from the core microbiome of the effluent, *SL56 Marine group, TRA3-20, Sediminibacterium, Opitutis* and *GKS98 freshwater group*. In contrast, 24 of 34 genera were found in the core microbiome

of the attached influent but not in the effluent water. 10 genera were found in the core microbiome of the effluent, however 9 were also found in the pelagic fraction of the influent. Only *Hydrogenophaga* was found as part of the attached core microbiome of the effluent water and attached fraction while not part of the pelagic core microbiome. This suggests that the majority of the attached core microbiome is being removed by the filtration process while the majority of the pelagic fraction is being retained in the effluent water. While the majority of genera were shared between the core microbiome of the influent water and effluent, the relative abundances of these genera were generally lower in the effluent water.

These results suggest that while biofiltration has an influence on the community composition and abundances of certain taxa in the effluent water, there is an undeniable link between the starting composition of the influent community and the community of the effluent water. The overall greater similarity of the pelagic fraction of the influent to the effluent suggest that it is this community exerting the greater influence on the composition of the effluent water.

5.4.2. Effect of Column Size on Effluent Community Composition

The results from this study indicate that column size has a minimal effect on the composition of the effluent water in terms of overall diversity. However, the abundances of specific taxa found in the effluent may be affected by column size.

Generally, there was little significant difference found between the effluents of the three column sizes for richness or evenness over the 23 weeks (Figure 5.2). PCOAs of Bray-Curtis and UniFrac distance also found no distinct separation between the effluents of the three column sizes (Figure 5.3). PERMANOVA of these beta diversity measures found column size to be a significant factor explaining variance ($P > 0.001$). However, column size was found to explain only a small percentage of variance between communities (Bray-Curtis – R^2 0.08, UniFrac - R^2 0.04). This suggests that in terms of overall diversity, media depth has little effect on the composition of the effluent water. In addition, the core microbiomes of the effluent from the three column sizes shared the vast majority of taxa, with the core microbiome of the long filter sharing 100% of its members with the medium and short.

While overall diversity is not strongly influenced, the abundances of certain taxa appear to differ with media depth. The effluent water of the long and medium filters generally had

higher relative abundances of *Hydrogenophaga* and *Dechloromonas* than the effluent water of the short filter (Figure 5.5). While the short filter had higher abundances of *Fimbriimonadaceae* and *Nitrospiraceae* (Figure 5.6). *Fimbriimonadaceae* and *Nitrospiraceae* were found in increased abundance in the attached fraction of the influent water while *Hydrogenophaga* and *Dechloromonas* were found at highest abundance in the effluent. This may suggest that *Fimbriimonadaceae* and *Nitrospiraceae* originate from the influent water and are removed less efficiently with a shorter filter bed. In contrast, *Hydrogenophaga* and *Dechloromonas* appear to be seeded from the filter bed in higher abundance from the long filter. In terms of the impact of the biological composition of the effluent water, *Hydrogenophaga* and *Dechloromonas* were found at higher abundance and contributed more to UniFrac distance (Figure 5.4). Thus, it appears increasing the length of the filter column has a greater effect on the composition of the effluent water than shortening it.

Differences in the most abundant taxa were also observed over time between the three column sizes. *Aquabacterium* for example, was more abundant in the effluent of the long and medium filters in weeks 1 and 3, though by week 23 was most abundant in the effluent of the short filter. The short filter showed the most interesting trends over time in terms of its most abundant taxa. For instance, *Hydrogenophaga* was of high relative abundance between weeks 5 and 9 before reducing severely for the rest of the filter run. Similarly, *Rhodoferax* was at a high relative abundance between weeks 9 and 21, however was again reduced by week 23. In the effluents of the long and medium filters, the relative abundances of *Hydrogenophaga* and *Rhodoferax* appeared to fluctuate from week to week. These taxa, *Hydrogenophaga, Dechloromonas, Aquabacterium* and *Rhodoferax,* appear to be most affected by media depth. They were found to be among the biggest contributors to beta diversity over the 23 weeks (Figure 5.4). All four taxa were found in higher abundance in the effluent water when compared to the influent. *Dechloromonas* and *Hydrogenophaga* were found to be most abundant in the effluent of the long filter, while *Rhodoferax* was found to be more abundant in the effluent of the short. Interestingly, these taxa most affected by media depth were found to be colonisers of the filter bed which were at highest abundance in the bottom section of the long filter (Chapter 4). The consistent presence of these taxa in the effluent at higher abundances than the influent suggest that they are being seeded from the bottom section of the filter bed.As these taxa were found in highest abundance in the bottom section of the long

filter, it would appear that increasing media depth may increase the likelihood of these taxa being found in the effluent.

While the abundances of *Hydrogenophaga, Dechloromonas, Aquabacterium* and *Rhodoferax* appear to be influenced by media depth, what this means for the biological stability of the effluent water remains unclear. The likelihood of the continued growth of these organisms in the effluent water may be linked to selective pressures associated with deeper sections of the filter bed. Nutrient gradients have been shown to act as a selective pressure through bed depth (Boon et al. 2011). As fresh nutrients are added to the filter via the influent water, organisms at the top of the filter bed are able to utilise easily biodegradable compounds as an energy source (Velten et al. 2011). However, as bed depth increases easily biodegradable compounds may become more scarce forcing community members to adapt to utilising more recalcitrant compounds (Hasan et al. 2021). Taxa which have adapted to utilising these compounds may find an energy source in the effluent water unavailable to other community members, encouraging their growth. As *Hydrogenophaga, Dechloromonas, Aquabacterium* and *Rhodoferax* were found to be colonising the deeper section of the filter bed, they may be capable of utilising recalcitrant compounds, potentially lowering the biological stability of the effluent water. Indeed, species of *Hydrogenophaga,* and *Dechloromonas* have previously been shown to degrade recalcitrant aromatic compounds (Fan et al. 2019; Gan et al. 2017; Pieper et al. 2004).

Another potential selector is the availability of oxygen in the deeper sections of the filter bed. As oxygen in the influent water is utilised by biomass in the top section of the bed, its availability may decrease before reaching deeper sections (Andrus et al. 2014). Thus, taxa which colonise the deeper sections may have adaptations to survive in an anoxic environment. Species of both Dechloromonas and Rhodoferax have previously shown a capacity to grow in low oxygen conditions (Salinero et al. 2009; Salka et al. 2011). The influence of oxygen gradients may be further evidenced by the increased abundance of *Firmicutes* in the effluent of the long filter (Figure 5.6), many species of which are known to be facultative anaerobes (Kampmann et al. 2012; McBride and Turnbull 1998). *Sericytochromatia* a non-photosythesising cyanobacteria was also found in increased abundance in the effluent of the long filter and is thought to survive in low oxygen conditions (Monchamp et al. 2019). If taxa colonising the bottom section of the filter bed have adapted to anoxic conditions their growth may be limited if the effluent water become reoxygenated.

If the growth of these taxa is limited the biological stability of the effluent water may not be affected by media depth.

5.5. Conclusions

- Size fractionation of the influent water identified two distinct communities. Of these communities the pelagic fraction was found to be more similar to the effluent water in terms of diversity, most abundant and core taxa.
- Time and thus development of the filter bed community was found to be the most significant variable explaining variance between communities, against both water type and column size.
- Overall diversity and the core microbiome of the effluent water was minimally affected by column size. However, the relative abundance of specific taxa increased in the effluent water of the long filter as compared to the short and medium.
- *Hydrogenophaga*, *Dechloromonas, Aquabacterium* and *Rhodoferax* were found amongst the highest contributors to beta diversity and varied in relative abundance between the effluents of the three column sizes. These taxa were found in highest abundance in the effluent water suggesting they originate from the filter bed community. As these taxa were found in highest abundance in the bottom section of the long filter bed, it would appear that lengthening the filter bed has a greater effect on the biological composition of the effluent water than shortening it.
- While media depth was found to influence the abundance of specific taxa in the effluent water, the implications for biological stability remain unclear. However, this study has identified key taxa which are most influenced by media depth and their likely origins. Future research into the biological stability of effluent water following biofiltration can investigate in greater detail the communities found in deeper sections of the filter bed and their implications for biological stability.

Chapter 6

Characterisation of Patescibacteria in GAC Biofilters

6.1. Introduction

The advancement of metagenomic and single cell sequencing techniques in recent years, in microbial ecology, led to an enormous expansion of the tree of life (Castelle and Banfield 2018). This expansion of the tree of life is largely due to the discovery of the candidate phyla radiation (CPR bacteria). CPR bacteria were identified as a large and diverse evolutionary radiation characterised by small cell and genome size and predicted reduced metabolic capabilities (Hug et al. 2016). This reduced cell size has often led to this group being termed nanobacteria, ultramicrobacteria (UBM) or low nucleic acid (LNA) bacteria when identified by flow cytometry (Tian et al. 2020; Herrmann et al. 2019; Vigneron et al. 2020; Proctor et al. 2018). CPR bacteria are non-culturable and thus only identified through molecular analysis (He et al. 2021; Tian et al. 2020). As such, this group has been excluded from many earlier environmental studies before molecular techniques became widespread (Bruno et al. 2017). Furthermore, the small cell size of this group may prevent capture by widely used filtration methods, such as through a 0.22µm pore size, leading to this group being underrepresented after analysis of sequencing data (Ghuneim et al. 2018). Due to these factors, this group remains poorly understood. This is despite CPR bacteria accounting for an estimated 15% to 50% of bacterial diversity and over 70 phyla (Anantharaman et al. 2016; Brown et al. 2015; Hug et al. 2016; Méheust et al. 2019). Recent work by Parks et al, suggested the reclassification of CPR bacteria to under the single phylum Patescibacteria (Parks et al. 2018).

Patescibacteria have been detected in a range of environments including, marine water, marine sediment, soil, fresh-water lakes and ground water (Lannes et al. 2019; Orsi, Richards, and Francis 2018; Starr et al. 2018; Tian et al. 2020; Vigneron et al. 2020; Herrmann et al. 2019; Cabello-Yeves et al. 2020). The presence of this understudied phylum in freshwater environments may have implications for drinking water treatment. Patescibacteria have been found to be particularly abundant in ground water, including sources utilised as a drinking water supply (Chaudhari et al. 2021; He et al. 2021). Patescibacteria have also been identified in drinking water treatment plants (DWTP) and present at each stage of the treatment process including purified effluent water (Bruno et al. 2017; 2018). Indeed, Bruno et al found Patescibacteria to be the second most abundant phyla (41%) after Proteobacteria (43%) in water samples collected at various treatment points through a DWTP (Bruno et al. 2018). Despite the prevalence of this phylum in freshwater

and drinking water systems, Patescibacteria have so far been largely overlooked in biofilter studies.

Patescibacteria have certain traits which may allow them to thrive in the biofilter environment or that of treated effluent water. Firstly, a small cell size allows for a higher surface to volume ratio facilitating efficient uptake of nutrients from the oligotrophic environment associated with biofiltration (Sowell et al. 2009). This small cell size may also offer protection from predatory grazers such as heterotrophic protists, which have previously been shown a preference for medium sized bacteria (Batani et al. 2016; Corno et al. 2008; Glücksman et al. 2010). Patescibacteria are also thought to have reduced genomes, maintaining metabolic systems for simple metabolites such as glucose and apparatus for gene expression and replication while sacrificing motility, outer membrane function, biosynthesis and polysaccharide production (Tian et al. 2020). It has been suggested that bacteria with small genomes are dependent on intermediate metabolites produced by more complex organisms (Morris et al. 2012.; Méheust et al. 2019; Tian et al. 2020). As such the diverse microbial community within the filter bed may act as a continual reservoir of these required metabolites. Simplified membrane structures may also provide protection from phage invasion by lacking phage target proteins (Tian et al. 2020). The simplicity of Patescibacteria metabolism may also mean they are less susceptible to environmental changes which would be detrimental to more complex organisms with more stringent needs (Liu et al. 2018).

The environment provided by biofiltration may therefore be more advantageous to Patescibacteria than to more complex organisms. Indeed, several studies have found an increase in the ratio of low nucleic acid (LNA) bacteria to high nucleic acid (HNA) bacteria in the effluent water following biofiltration (Lautenschlager et al. 2014; Vital et al. 2012; Chan et al. 2018). Thus, these studies suggest that LNA bacteria such as Patescibacteria are being enriched in the effluent water by the filtration process. The mechanism of this enrichment remains unclear. It has been suggested that the smaller cell size of the LNA bacteria means they are less susceptible to removal by physical straining than the larger HNA bacteria (Lautenschlager et al. 2014). Alternatively, it has been suggested that size selective predatory grazing may preferentially target HNA bacteria (Boenigk et al. 2004). In any case, this enrichment of LNA bacteria in the effluent water may have unknown implications for the future of biofiltration research.

The recent discovery and lack of cultured isolates of Patescibacteria means that little is currently known of the phylum in general. As such, little is known of the effect these organisms may have on the biological stability of the effluent water or indeed interactions with the community of the filter bed. One issue is that Patescibacteria associated with biofiltration remain poorly characterised. Many studies have identified LNA bacteria by flow cytometry (Lautenschlager et al. 2014; Vital et al. 2012; Chan et al. 2018) while others attempting more in depth molecular detail and characterisation have focused on DWTP's involving long treatment trains potentially introducing factors which effect the diversity of Patescibacteria (Liu et al. 2018; Bruno et al. 2018; 2021).

The aim of this chapter is to offer the first in-depth characterision of the phylogeny of Patescibacteria found in the influent, effluent and the filter bed of lab-scale GAC biofilters. By utilising 0.1µm filtration following filtration through the 0.22µm Sterivex filters we aim to capture the smaller Patescibacteria able to pass through 0.22µm pores in the water samples. The relative abundance of Patescibacteria in this 0.1µm filtered fraction and the GAC filter bed samples will be used to investigate the hypothesis that biofiltration enriches nanobacteria in the effluent water.

6.2. Materials and Methods

6.2.1. Sampling

GAC sampling was carried out during filter deconstruction outlined in chapter 2 (2.1.7). Filtration through the 0.1µm membrane filter was carried out on the same weeks as deconstruction (5, 9, 12 and 23, Table 1) following methods outlined in chapter 2 (2.1.6), filters were stored at -80C until molecular analysis. 3L of water was filtered onto each 0.1µm membrane, consisting of 1L from the influent and effluent of each filter replicate. Then 0.1µm filter were thawed on ice, cut into pieces and added to a lysis matrix E tube using sterile tweezers and scalpel. DNA extraction was carried out using the FastDNA spin kit for soil (MPBio) and following the methods outlined in chapter 4 (4.2.1). 16S rRNA library preparation and subsequent sequencing data processing were conducted following methods outlined in chapter 4 (sections 4.2.2 – 4.2.4).

Table 6.1. *Influent and Effluent water samples and GAC samples available at each deconstruction timepoint.*

0.1µm Filtration (water samples)		
Sample	**Sample number/week**	**Total samples sequenced**
Influent	2	8
Effluent Short	1	4
Effluent Medium	1	4
Effluent Long	2	8
GAC samples (filter bed)		
Top (2cm -10cm)	15	60
Middle (30cm & 60cm)	6	24
Bottom (60cm & 90cm)	6	24

6.2.2. Statistical Analysis

The abundance and taxonomy tables were extracted from the biom file following sequencing data processing. Sequences identified as Patescibacteria were extracted and written into a FASTA file which was used to align the phylogenetic tree. The tree was visualised using the treeio and ggtree packages in R studio v1.4.1717. Relative abundances of all taxa present in the samples were calculated and added to a table. The table was trimmed to include only Patescibacteria ASV's found in the tree. Taxa which had a relative abundance of 0 across all sample types were removed. Means, standard deviations and ranges of abundance of the GAC and water were calculated including only taxa which were found present in at least one of the samples at the sampling timepoints. Venn diagrams were created by creating separate tables of taxa with an average relative abundance of >0 across the sample types, filter bed, influent and effluent and visualised using the VennDiagram package in R.

6.3. Results

This study (influent, effluent and biofilter samples) identified 318 ASV's corresponding to the phylum Patescibacteria in the Silva v138 database. These ASV's were divisible into 6 classes, Parcubacteria, Saccharimonadia, Microgenomatia, Gracilibacteria, ABY1, WWE3, and 33 identified families. The relative abundance of Patescibacteria in the 0.1µm filtered fractions were generally found to be higher in the effluent water than the influent. The phylogeny and relative abundance of Patescibacteria in the influent and effluent water is shown in figure 6.1.

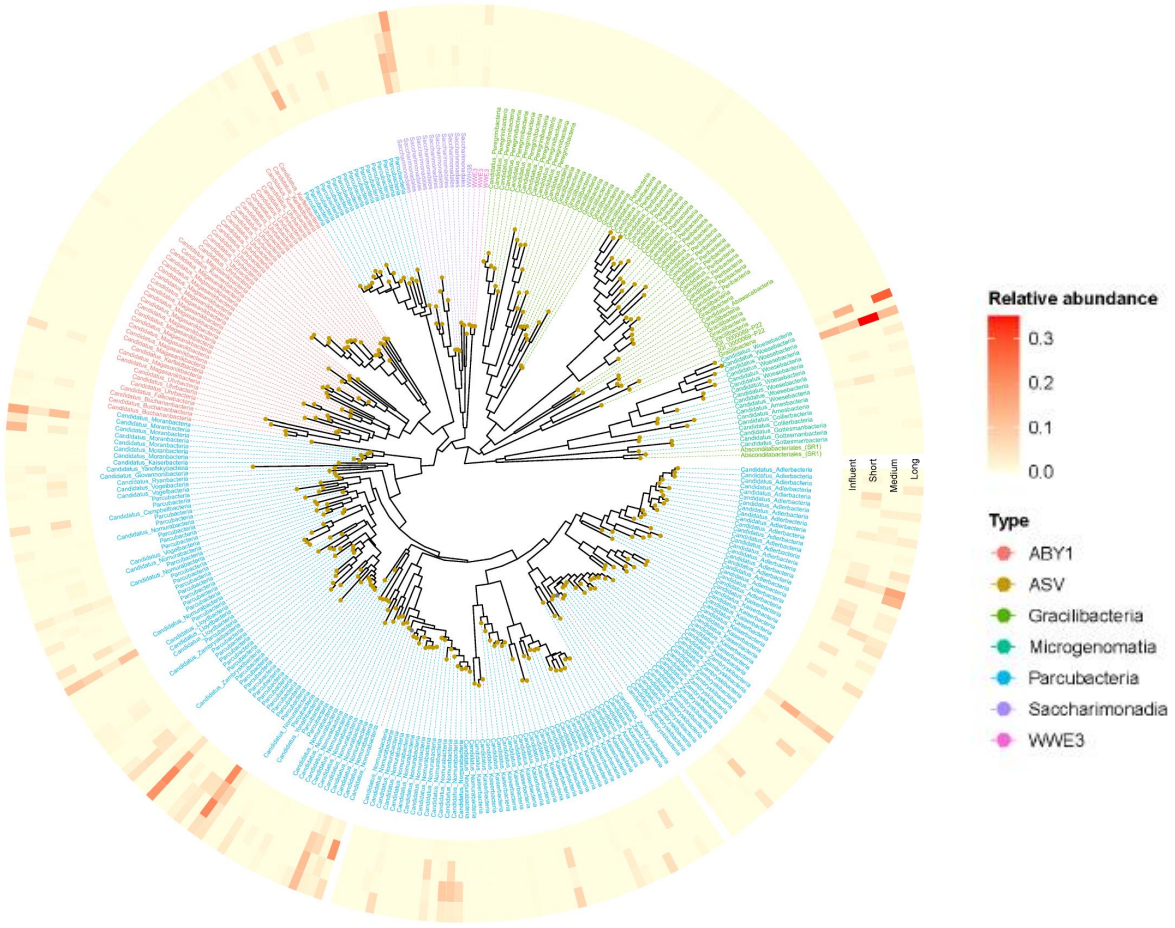


Figure 6.1. *Phylogenetic tree (unrooted) of all sequences assigned to Patescibacteria from full 16S rRNA biofilter data set. Heatmap shows the relative abundances of taxa found in the 0.1µm filtered fraction of the influent water and effluent from the short, medium and long filter columns.*

Of the 6 classes of Patescibacteria identified, Parcubacteria was found to contain the highest number of ASV's. 183 ASV's were identified as Parcubacteria with 13 named families within. 53 ASV's within Parcubacteria were not resolved at a lower taxonomic level than class. The class Gracilibacteria consisted of 58 ASV's and 6 families with 19 ASV's resolved at only class level. The class ABY1 consisted of 45 ASV's and 6 families. Microgenomatia contained 18 ASV's and 4 families. Saccharimonadia contained 13 ASV's and 2 named families, Saccharimonadales and WWH38.

The average relative abundance of Patescibacteria was found to be higher in the effluent water than the influent (influent – 0.005 ± 0.02%, short – 0.015 ± 0.03%, medium – 0.01 ± 0.04%, long – 0.017 ± 0.036%). In the 0.1µm filtered fraction at the deconstruction timepoints, 42 ASV's were found to be present in the influent water while 127 ASVs were present in the effluent water. There were some small differences between the different filter lengths, with only 16 ASV's found in the influent water at higher relative abundance than the short filter; 27 higher in the influent than the medium filter and 17 higher in the influent water than the short filter. In contrast, 78 ASV's were found at higher relative abundance in the effluent of the short filter than the influent and 68 higher in the effluent of the long filter than the influent. The medium filter had the fewest ASV's at higher relative abundance than the influent at 39. 276 ASV's were absent from the 0.1µm filtered fraction of the influent water and 193 absent from the effluent water.

The class which contained taxa with the highest relative abundance was Microgenomatia which averaged 0.014 ± 0.039% in the influent water and 0.03 ± 0.06% in the effluent. Relative abundances of taxa within this class ranged from 0 to 0.21% in the influent, 0 to 0.14% in the short effluent, 0 to 0.35% in the medium effluent and 0 to 0.27% in the effluent of the long filter. A taxon of the family *Candidatus* Woesebacteria were found in highest abundance within Microgenomatia, found at 0.14 ± 0.24% in the influent water, 0.11 ± 0.22% in the short effluent, 0.35 ± 0.7% in the medium effluent and 0.12 ± 0.23% in the long filter effluent. A closely related *Candidatus* Woesebacteria was also found in high relative abundance in the effluent water of the short (0.14 ± 0.16%) and long (0.28 ± 0.5%) filter. This taxon was found in lower abundance in the influent water (0.013 ± 0.04%) and was absent from the effluent water of the medium filter. The class Parcubacteria averaged relative abundances of 0.005 ± 0.02% in the influent water and 0.018 ± 0.03% in the effluent. Relative abundances ranged from 0 to 0.21% in the influent water, 0 to 0.19% in the effluent of the short filter, 0 to 0.21% in the effluent of the medium filter and 0 to 0.19% in the

effluent of the long filter. The class Saccharimonadia averaged relative abundances of 0.016 ± 0.03% in the influent and 0.021 ± 0.041% in the effluent water. Relative abundances ranged from 0 to 0.1% in the influent, 0 to 0.12% in the effluent of the short filter, 0 to 0.94% in the effluent of the medium filter and 0 to 0.16% in the effluent of the long filter. The classes ABY1 and Gracilibacteria were found in lower relative abundances in the 0.1µm filtered fraction. ABY1 averaged only 0.00003 ± 0.0002% in the influent water and 0.0025 ± 0.005% in the effluent. Relative abundances ranged from 0 to 0.0008% in the influent, 0 to 0.02% in the effluent of the short filter, 0 to 0.004% in the effluent of the medium filter and 0 to 0.04% in the effluent of the long filter. Gracilibacteria were entirely absent from the influent water and averaged a relative abundance of 0.0002 ± 0.001% in the effluent water. Relative abundances ranged from 0 to 0.004% in the effluent of the short filter, 0 to 0.009% in the effluent of the long filter and 0.005% in the effluent of the long filter. Only one ASV from the class WWE3 was present in the influent and effluent water at a relative abundance of 0.013% in the influent, 0.004% in the effluent of the short filter and 0.028% in the effluent of the long filter. This class was entirely absent from the effluent water of the medium filter.

These results show the diverse phylogeny of Patescibacteria associated with the biofilters in this study. In general, Patescibacteria were found in higher relative abundance in the effluent water then in the influent in the 0.1µm filtered fraction at the timepoints measured (weeks 5, 9, 12 and 23). The classes Microgenomatia, Saccharimonadia and Parcubacteria were found in highest abundance in the water samples while Gracilibacteria and ABY1 were found at lower relative abundances. The higher abundances in the effluent water suggest that the filtration process may be enriching Patescibacteria in the effluent water. Potentially this may mean that Patescibacteria are colonising the filter bed and being shed into the effluent water. As such, the relative abundances of Patescibacteria in the top, middle and bottom sections of the filter bed were also investigated (Figure 6.2).

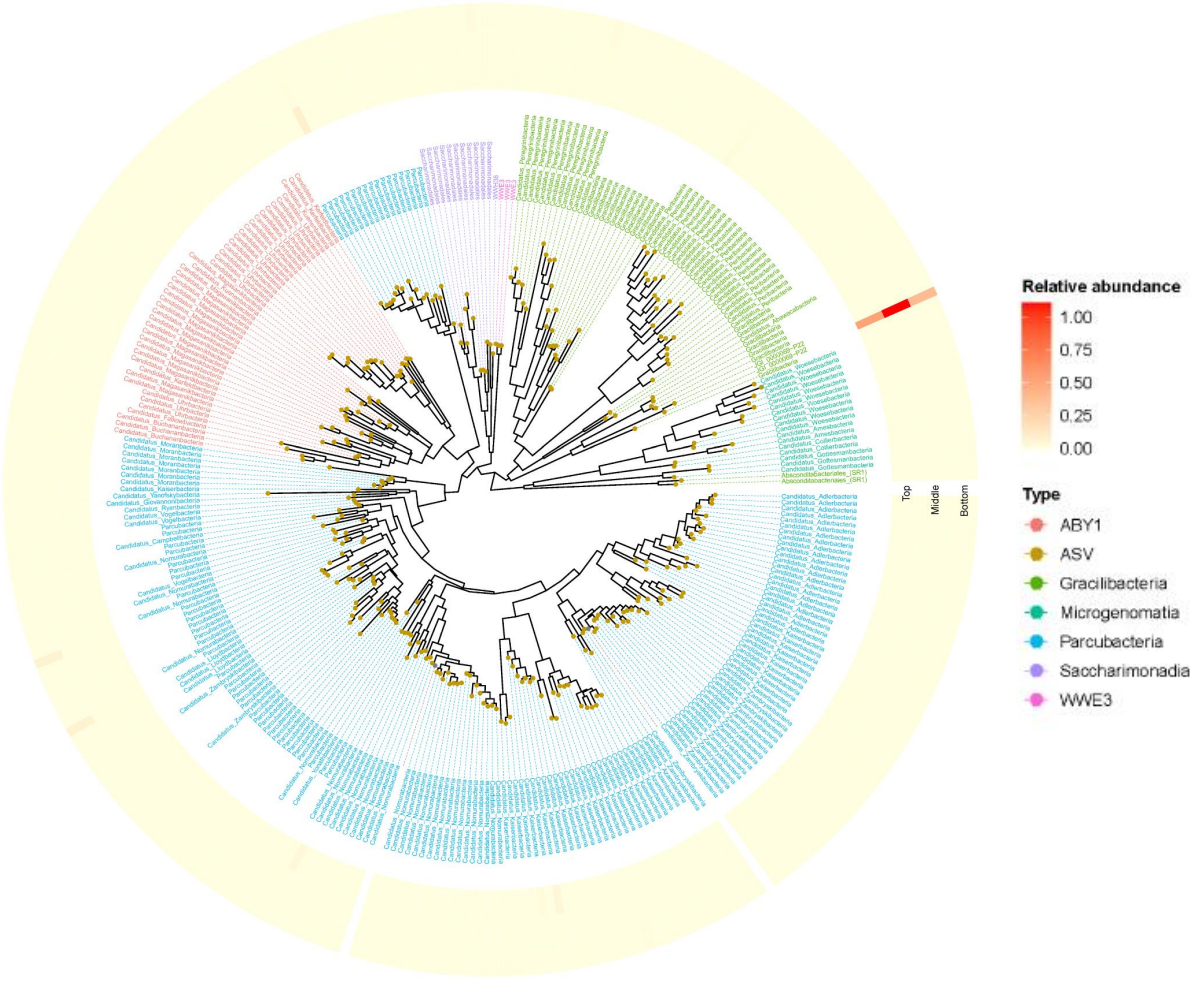


Figure 6.2. *Phylogenetic tree (unrooted) of all sequences assigned to Patescibacteria from full 16S rRNA biofilter data set. Heatmap shows the relative abundances of taxa found in the GAC samples taken from the filter bed. GAC samples were grouped by depth, at the top 2-10cm, middle 15cm & 30cm, and bottom 60cm & 90cm.*

The average relative abundance of Patescibacteria in the filter bed was lower than in the effluent water. The top section averaged at 0.004 ± 0.037%, the middle at 0.006 ± 0.08% and the bottom at 0.003 ± 0.03%. Compared to 0.015 ± 0.03% in the effluent of the short filter, 0.01 ± 0.04% in the effluent of the medium and 0.017 ± 0.036% in the effluent of the long. While 143 taxa were present in the filter bed, 228 were absent from the top section of the filter, 258 absent from the middle section and 252 absent from the bottom suggesting distribution of Patescibacteria is not uniform throughout the filter bed. One taxon of the class Gracilibacteria was found to be at much higher relative abundance through the filter bed than

any other taxa. A taxon assigned the family JGI0000069-P22 was found at relative abundances of 0.53 ± 0.68% in the top section of the filter, 1.1 ± 1.4% in the middle section and 0.39 ± 0.52% in the bottom section of the filter. This taxon was found at much higher relative abundance than any other. Interestingly, this taxon was entirely absent from the effluent water in the 0.1µm filtered fraction at the deconstruction timepoints.

The class Parcubacteria ranged from 0 to 0.087% in the top section, 0 to 0.01% in the middle and 0.068% in the bottom sections. Microgenomatia ranged from 0 to 0.0001% in the top section, 0 to 0.0004% in the middle and 0 to 0.0036% in the bottom section. Saccharimonadia ranged from 0 to 0.004% in the top, 0 to 0.0025% in the middle and 0 to 0.021% in the bottom section. ABY1 ranged from 0 to 0.0034% in the top, 0 to 0.0022% in the middle and 0 to 0.0064% in the bottom section. The class WWE3 was found to be entirely absent from any section of the filter bed. The differences in abundances between classes may suggest a degree of stratification between the classes through the depth of the filter bed. Microgenomatia, Saccharimonadia and ABY1 appear to increase in relative abundance with bed depth, while Parcubacteria was highest at the top and bottom section of the filter bed.

While Patescibacteria were found to be present in varying abundances in the influent and effluent water and in all sections of the filter bed, the origin of Patescibacteria in the effluent water remains unclear. As such, the taxa shared between the effluent water, the influent water and filter bed was investigated (Figure 6.3).

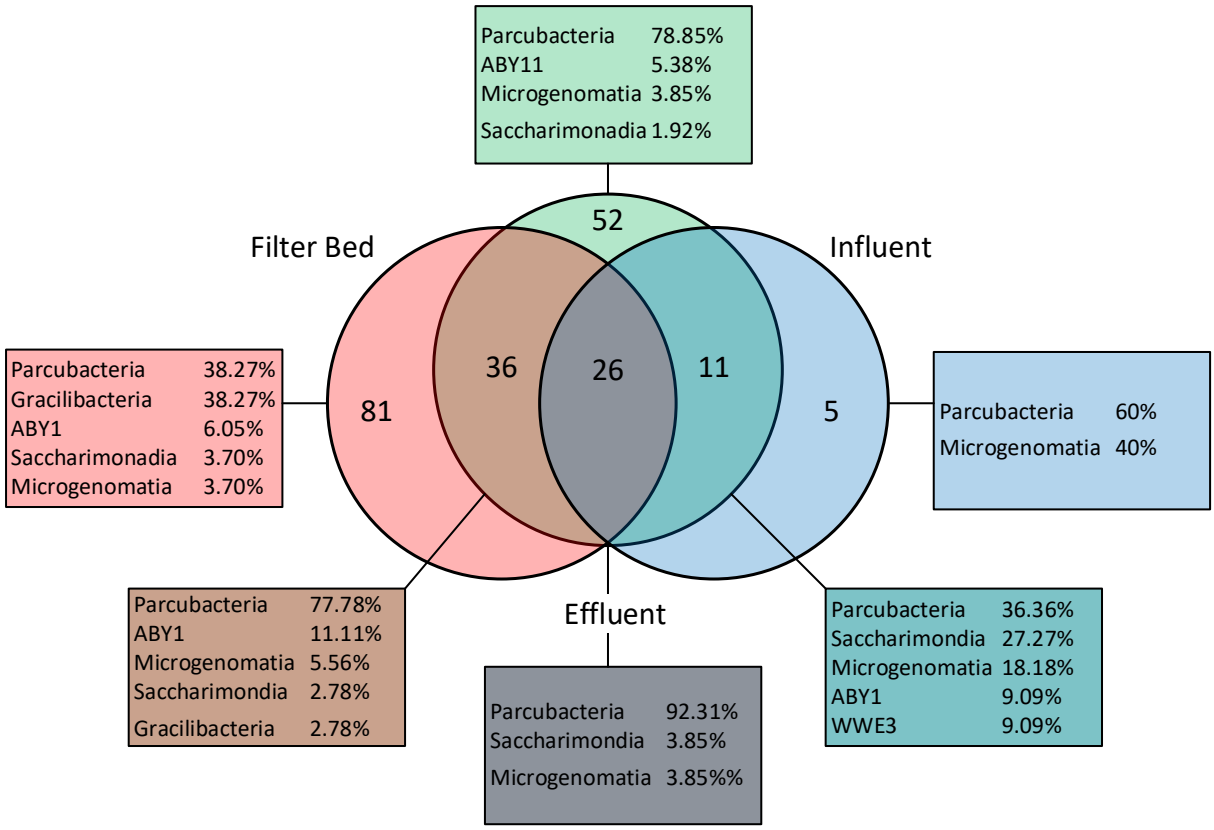


Figure 6.3. *Venn diagram of shared taxa between the 0.1µm filtered fractions of the influent and effluent water and GAC samples from the filter bed. The boxes show a breakdown of classes found by percentage.*

In total 26 ASV's were found to be shared between the influent, effluent and filter bed. These were largely dominated by Parcubacteria at 92.31%. Saccharimonadia and Microgenomatia were the only other two classes found in all three environments at a smaller percentage of ASV's at 3.85% for both. 81 ASV's were found to be unique to the filter bed, of these Parcubacteria and Gracilibacteria shared the highest percentage of ASV's at 38.27%. Gracilibacteria were found in both the filter bed and effluent, however, were absent from the influent water. This may suggest that the source of Gracilibacteria in the effluent water may be the filter bed community. In total 36 ASV's were found in both the filter bed and effluent water while being absent from the influent from classes ABY1, Microgenomatia, Saccarimondia, Gracilibacteria and Parcubacteria. The highest percentage of ASV's were of class Parcubacteria at 77.78%. Only 5 ASV's were found to be unique to the influent water, 3 Parcubacteria and 2 Microgenomatia. As the majority of ASV's in the influent water were

shared with the effluent or effluent and filter bed, it may be indicative of a low removal of Patescibacteria through biofiltration. 11 ASV's were found to be shared between the influent and effluent water while absent from the filter bed, of classes Parcubacteria, Saccharimonadia Microgenomatia, ABY1 and WWE3. The class WWE3 was only found shared between the influent and effluent water, suggesting it may be simply passing through the filter. 52 ASV's were found to be unique to the effluent water. As these taxa were not present in the filter bed or influent water their origins remain unexplained. Of these taxa Parcubacteria was found to have the highest percentage of ASV's at 78.85%. ASV's of classes Saccharimonadia Microgenomatia and ABY1 were also found to be unique to the effluent water.

6.4. Discussion

The results from this study illustrate the diverse phylogeny of Patescibacteria found in the filter bed and influent and effluent water of GAC biofilters. The Patescibacteria associated with the biofilters in this study consisted of 318 ASV's, covering 6 classes and 31 families. In general, Patescibacteria were found in higher relative abundance in the effluent water than the influent, suggesting that the process of biofiltration may enrich Patescibacteria in the effluent water.

Due to their recent discovery and chronic under-sampling, the role of Patescibacteria in the environment and their interaction with other community members remains poorly understood. It is largely assumed that Patescibacteria live a symbotic lifestyle, dependant on metabolites produced by more complex organisms (Tian et al. 2020; Nelson and Stegen 2015). Parcubacteria, the most diverse class identified in this study containing 183 ASV's over 13 families, have been shown to lack genes for the biosynthesis of many amino acids, vitamins and lipids (Castelle et al. 2017). Similarly, genomic analysis of taxa from the classes Saccharimonadia, Microgenomatia and Gracilibacteria have shown little metabolic potential, suggesting a symbiotic lifestyle (Lemos et al. 2019; Sieber et al. 2019.; Kadnikov et al. 2020). Patescibacteria are also generally thought to thrive in oligotrophic environments. Small cell size allows for a larger surface to volume ratio facilitating efficient uptake of nutrients (Sowell et al. 2009). Furthermore, the simplified metabolism of Patescibacteria may reduce the need of complex metabolites for growth (Tian et al. 2020). Indeed, Patescibacteria have been shown to thrive in oligotrophic freshwater following mobilisation from soils (Herrmann et al. 2019). These traits may mean that Patescibacteria are particularly suited to

the environment provided through biofiltration. The diverse microbial community of the filter bed may offer a continual supply of metabolites required for the survival of Patescibacteria (Ponomarova and Patil 2015). Furthermore, their small cell size and simplified metabolism may allow Patescibacteria to thrive in nutrient limited effluent water (Bruno et al. 2018; Herrmann et al. 2019). Indeed, the Patescibacteria identified in this study appeared to be enriched through the filtration process, resulting in higher abundances in the effluent water than the influent.

The effluent water in this study presented a higher average relative abundance of Patescibacteria (influent – 0.005 ± 0.02%, short – 0.015 ± 0.03%, medium – 0.01 ± 0.04%, long – 0.017 ± 0.036%) and a higher number of ASV's present in the 0.1μm filtered fraction (effluent – 125, influent – 42). Previous studies have shown LNA bacteria in higher abundance in the effluent water following biofiltration (Lautenschlager et al. 2014; Vital et al. 2012; Chan et al. 2018). Interestingly, quantification of nanobacteria by flow cytometry (work carried out by Marta Vignola) on the influent and effluent water from the biofilters in this study showed no significant difference between the influent or effluent of the three filter bed sizes for total or intact cells (ANOVA P-values range, total – 0.299 to 0.999, intact – 0.331 to 0.996). While no significant difference was observed by flow cytometry, it is unknown which proportion of these nano-organisms are Patescibacteria. A proportion of cells observed through flow cytometery may be similarly sized organisms such as Nanoarchaeota or different phyla with reduced cells size due to stresses such as nutrient deprivation (Ghuneim et al. 2018).

Enrichment of Patescibacteria was further evidenced when looking at shared taxa between the influent and effluent water. Of the 42 ASV's present in the influent water, only 5 were found absent in the effluent water. As the majority of ASV's were accounted for in the effluent, this may suggest that Patescibacteria show a degree of resistance to removal by biofiltration. This may be due to the small cell size of Patescibacteria being more resistant to removal by physical straining than larger organisms (Lautenschlager et al. 2014). Alternatively, Patescibacteria may be more likely to be overlooked by grazing protists which have previously shown a preference for medium sized bacteria (Batani et al. 2016; Corno et al. 2008). More interestingly, of the 37 ASV's present in both the influent and effluent water, 28 were found in higher relative abundance in the effluent water than the influent (mean influent – 0.026 ± 0.44%, mean effluent – 0.057 ± 0.49%). This may suggest that Patescibacteria are not only failing to be removed but also thriving in the biofilter environment. Thus, it may be

a combination of factors leading to an enrichment of Patescibacteria in the effluent water. Their small cell size may provide some protection from physical and biological mechanisms of removal, while their metabolic simplicity allows them to survive in an oligotrophic environment while being supplied necessary metabolites from the filter bed community.

The relative abundance of Patescibacteria was also higher in the effluent water than in the filter bed (mean effluent – 0.014 ± 0.3%, filter bed – 0.004 ± 0.05%). However, it is difficult to compare relative abundances between the GAC and water samples. The water samples were prefiltered through 0.22µm Sterivex filters thereby removing the majority of larger cells and increasing the proportional abundance of Patescibacteria. As there was no way to separate Patescibacteria from larger cells on the GAC particles, the relative abundance was calculated alongside all other community members. As such, it may be expected that the relative abundance of Patescibacteria would be found lower in the filter bed. In total 143 ASV's were present in the GAC samples. Of these 81, were found to be unique to the filter bed and absent from the influent and effluent water.

Little is currently known of the interaction of Patescibacteria and communities in biofilm. Given their reduced genome and metabolic capabilities, it seems unlikely that Patescibacteria are capable of forming complex biofilms independent of other organisms (Karatan and Watnick 2009). Indeed, the average relative abundance of Patescibacteria in the filter bed was low (0.004 ± 0.05%), suggesting they are not colonising the filter bed to the same degree as other more complex organisms such as Proteobacteria. Despite this, 81 ASV's were found to be unique to the GAC samples, suggesting some form of retention of Patescibacteria in the filter bed. One mechanism of retention may be within the pores of the GAC. Particles of GAC contain macropores, mesopores and micropores which may be capable of harbouring very small bacteria (Velten et al. 2007). It has been hypothesised that Patescibacteria may be found as small as 0.009 ± 0.002 µm3 (Luef et al. 2015; Nakai 2020) and as such, may be capable of entering the pores of GAC. Patescibacteria capable of regularly entering the pores of GAC may also be small enough to pass through the 0.1µm filter. This would explain the absence of the 81 ASV's found unique to the filter bed in the influent and effluent samples. Another mechanism of retention could be through interactions with the filter bed community. Some Patescibacteria have been shown to have episymbiotic (surface attached) relationships with host cells (He et al. 2021; Nie et al. 2022). Thus, Patescibacteria could potentially be retained in the filter bed through attachment to members of the resident biofilm. An interesting case is the class Gracilibacteria which constituted 38.27% of Patescibacteria

unique to the filter bed. This class was found only in the filter bed and effluent water and has been previously suspected of episymbiosis with host cells (Sieber et al. 2019; Wrighton et al. 2012). One taxon of Gracilibacteria was found to be considerably more abundant in the filter bed than any other. JGI0000069-P22 was found at relative abundances of 0.53 ± 0.68% in the top section of the filter, 1.1 ± 1.4% in the middle section and 0.39 ± 0.52%. Despite its comparatively high abundance in the filter bed, this taxon was entirely absent from the influent or effluent water. Interestingly, this taxon was found in the 0.22µm fraction of the effluent at the deconstruction timepoints (mean – 0.2 ± 0.2) but absent from the influent. A potential explanation is that this taxon may form an endosymbiotic relationship with other taxa. Patescibacteria have been shown to form endosymbiotic relationships with larger cells (Beam et al. 2020). As such, it is possible that this taxon enters and proliferates within larger community members of the filter bed which are sequestered on the 0.22µm filtered fraction, explaining its absence in the 0.1µm filtered fraction. The results from this study may also suggest that the abundance of Patescibacteria may be affected by bed depth, with classes Saccharimonadia, Microgenomatia and ABY1 being more abundant in the bottom section of the filter. Patescibacteria have previously been thought to rely on fermentation and may thrive in anoxic environments (Nelson and Stegen 2015; Vigneron et al. 2020; Cabello-Yeves et al. 2020; Castelle et al. 2017). Therefore, distribution of Patescibacteria may be driven by oxygen gradients through the depth of the filter bed. Alternatively, the distribution of Patescibacteria may be driven by the distribution of host cells through which episymbiotic or endosymbiotic relationships are maintained (Beam et al. 2020; Sieber et al. 2019; Wrighton et al. 2012).

The results from this study also identified 52 ASV's which were present in the effluent water while absent from the influent and filter bed samples, ranging from relative abundances of 0.0008% to 0.043%. The source of these taxa in the effluent water is currently unclear. One possibility is that they are retained in the pore water of the filter bed and being released into the effluent during the filter run. As the biofilters were drained before deconstruction and subsequent sampling of the GAC, taxa residing in the pore water between media particles may well have been lost and thus not found in the GAC samples. However, this is largely speculative and further research would be required to test this hypothesis.

While this chapter serves to highlight the diverse phylogeny of the often-overlooked phylum Patescibacteria and offers some evidence that Patescibacteria are enriched by the process of biofiltration, it is important to note the limitations of this study. In total 318 ASV's assigned

to Patescibacteria were recovered from 16S rRNA sequencing data. However, only 211 ASV's were found to be present in the 0.1µm filtered fraction or indeed the samples from the filter bed. The 107 ASV's not present in these samples were likely found in the 0.22µm or even glass fibre filtered fractions. While Patescibacteria are thought small enough to pass through a 0.22µm pore size (Ghuneim et al. 2018), it is possible a proportion may be retained on the larger pore sized filters. This may be due to filter clogging with biomass or particulate matter reducing the size of the pores. Alternatively, Patescibacteria associated with larger cells or particulate matter may be retained on a filter of larger pore size (Herrmann et al. 2019). Therefore, it is likely an unknown proportion of Patescibacteria collected on the 0.1µm filter is lost at the earlier filtration steps. In order to ensure enough biomass was available for DNA extraction and PCR, the filtrate of the 0.22µm water fractions were combined before subsequent filtration through the 0.1µm. As such, there were no directly linked 0.22µm and 0.1µm samples between filter replicates. For the sake of consistency, this study chose to focus on only the 0.1µm filtered fraction of the water samples as it was assumed the majority of Patescibacteria was sequestered on this filter. Another limitation of this study lies in the number of data points available. Due to time constraints in the collection and subsequent filtration of the effluent water 0.1µm filtration was only carried out at the deconstruction timepoints. The combination of the filtrate of the three filter replicates also limited the data points available at each time point. As a result, there was only a single data point for the short and medium filters at weeks 5, 9, 12 and 23. This meant that a robust comparison could not be made between column sizes or over time.

The results reported in this chapter suggest that Patescibacteria are enriched in the effluent water through the process of biofiltration. What implications this may have for filter efficiency and effluent water quality remains unclear. A substantial knowledge gap exists concerning the metabolism of Patescibacteria, while some have been implicated in hydrogen, sulphur and carbon cycling (Rahlff et al. 2020; Wrighton et al. 2012). Little is also known of the interactions between Patescibacteria and the wider microbial community, and thus their importance in the process of biofiltration. Further research is necessary to elucidate the role this phylum may have in the treatment of drinking water by biofiltration.

6.5. Conclusions

- A diverse phylogeny of Patescibacteria associated with biofiltration through GAC filters. 318 ASV's were identified encompassing 6 classes and 33 families.
- A majority of ASV's were found to be in higher relative abundance in the effluent water than the influent, suggesting enrichment by the biofiltration process.
- 81 ASV's were found present in filter bed but absent from the influent and effluent water, suggesting a degree of retention of Patescibacteria in the filter bed.
- Only 5 ASV's were found to be unique to the influent water suggesting that Patescibacteria may be somewhat resistant to removal by biofiltration.
- 52 ASV's were found to be unique to the effluent water though their source is currently unknown.
- While Patescibacteria were shown to be present and likely enriched through filtration, the implications this may have for effluent water quality remains unclear. Further research investigating the metabolism of Patescibacteria and their interactions with the wider biological community of biofiltration would be beneficial.

Chapter 7

Conclusions and Recommendations

7.1. Thesis Objectives

The work undertaken in this thesis aimed to utilise a series of lab-scale GAC biofilter columns to address several research objectives outlined below.

- To investigate the changes in the microbial community of the filter bed over time and spatially through the depth of the filter bed.
- To investigate the effect of column length on effluent water quality against key biological and chemical contaminants.
- To investigate the effect of column length on the biological composition of the effluent water and how it may change with time.
- To investigate and characterise the community of Patescibacteria found in the influent water, effluent water and filter bed.

These objectives were set out to gain insight into the fundamental yet under-researched design parameter of filter bed length and its effect on filter performance and biological communities of the filter bed and effluent water. This work was carried out with an eye towards optimisation of the biofiltration process and the eventual design of a point-of-entry drinking water treatment system for deployment in rural areas.

7.2. Main Findings

The biofilters designed in this study were found to be comparable to full scale biofilters in operation for drinking water treatment. Typical removal capacities for full scale slow sand filters as reported by Guchi 2015, were 5-40% removal of DOC, 30-90% of iron and up to 90-99% removal of enterobacteria and coliforms (Guchi 2015). The biofilters in this study achieved 26.1% ± 0.6% to 45.6% ± 1.7% DOC removal and 17.3% ± 2.3% to 27.3% ± 1.3% iron removal at week 23. They were also found to remove 91 ± 14% to 94 ± 8% of coliform bacteria and 93 ± 18% to 99 ± 5% *Legionella pneumophila* over the 23 weeks of operation. Between column sizes (short-medium-long), no significant difference was observed for biological removal of pathogens or total and intact cells. The long filter showed a higher removal efficiency for chemical contaminants however it is unclear what proportion of removal can be attributed to biological removal or the adsorption capacity of GAC. As reported in many other studies, biomass and species richness was highest at the top of the

filter bed and decreased with bed depth (Velten et al. 2011; Zhang et al. 2018; Chen et al. 2021; Matuzahroh et al. 2020). ATP analysis also indicated that biological activity was likewise highest at the top of the filter bed, again decreasing with depth. An increase in diversity, biomass and biological activity was also observed over time again concurring with many previous studies (Chen et al. 2021; Haig et al. 2015; Wakelin et al. 2011; Campos et al. 2002; Ramsay et al. 2018).

At genus level the most abundant taxa found in the top section of the filter bed showed little variance over time and was dominated by *Beggiatoaceae*. Members of this genus are known sulphur oxidisers with a chemolithoautotrophic metabolism, capable of intracellular storage of polysulphide molecules in vacuoles (Teske and Salman 2014; Berg et al. 2014). However, some have been shown to be capable of heterotrophic growth and denitrification (Schutte et al. 2018). This genus was found at a high relative abundance in the top section of the filter bed at 51 ± 12%, but only at 18 ± 10% in the deeper sections of the bed. Interestingly, one of the most abundant taxa found in the deeper sections was of the family *Tenderiaceae*. While little is known of this family's metabolism, an examination of its phylogeny shows its closest relatives as *Thioalkalispiraceae*, *Acidiferrobacteraceae* and *Beggiatoaceae*, again all known sulphur oxidisers (Flood et al. 2021; Issotta et al. 2018; Mori et al. 2011). This points to *Tenderiaceae* being capable of sulphur oxidation. The high abundance of *Beggiatoaceae* at the top of the filter and *Tenderiaceae* at the bottom might suggest that availability of reduced sulphur is a deterministic factor selecting for these taxa. *Tenderiaceae* was generally found at higher abundance in areas of the filter where *Beggiatoaceae* was at lower abundance. This may indicate *Tenderiaceae* being outcompeted by *Beggiatoaceae* for reduced sulphur contained in the influent (Nadell et al. 2016). Alternatively, the presence of *Tenderiaceae* may be encouraged due to the presence *Beggiatoaceae*. The ability of *Beggiatoaceae* to store intracellular sulphur may act as a supply of reduced sulphur compounds which are released during cell lysis (Berg et al. 2014). At present, the metabolism of *Tenderiaceae* is largely unknown, so this is highly speculative. However, it may be indicative of the potential web of complex interactions between community members of the filter bed, highlighting the need for further research.

Between columns sizes the top section of the filter bed was highly reproducible, sharing 87% of core taxa. By week 23, the top 30cm of all column sizes were very similar in terms of most abundant taxa. At earlier timepoints, the 30cm section of the long filter set varied from the top 15cm, containing a higher relative abundance of *Rhizobiales* and *Tenderiaceae* which had

reduced by week 23. It would appear that the community composition of the top 15cm had extended to 30cm over time and related EBCT. The design of the biofilter experiment in this study did not allow for a clear comparison of filter bed community at similar EBCT, however this may be an interesting area to research in future experiments. While the top section of the filter bed was largely similar in terms of the biological community, the bottom sections of the filter bed showed more variation between column sizes. Several taxa were in higher abundance in the bottom section of the long filter including, *Rhodoferex*, *Burkholderiales*, *Rhizobiales* and Actinobacteria. This might suggest selective pressures exist in the deeper section of the filter which favour these taxa, for example broader nutrient or oxygen gradients (Cohen 2001; Boon et al. 2011). As such, it would appear that increasing column length has little effect on the community at the top of the filter bed but may select for certain taxa at the bottom.

The taxa found in the deeper section of the long filter also had an impact on the biological composition of the effluent water. PCOA plots of Bray-Curtis and UniFrac distance of the filter bed community and influent and effluent waters (Figure 4.3B) showed the effluent water to cluster with the influent water and bottom section of the filter bed. SCBD analysis between the influent and effluent of the three column sizes also identified *Hydrogenophaga*, *Rhodoferax* and *Aquabacterium*, as being strong contributors to beta diversity (Figure 5.4). These taxa were found in highest abundance in the bottom section of the long filter. Several studies have identified influent water as the main driver of the biological composition of the effluent water (Lautenschlager et al. 2014; Ma et al. 2020; Vignola et al. 2018). Indeed, in this study 80% of the core microbiome of the effluent water was shared with the influent. This is likely due to a proportion of taxa simply passing through the filter bed and into the effluent water (Lautenschlager et al. 2014). Studies have also shown that the biological community of the filter bed has an impact on the biological composition of the effluent water, albeit to a lesser extent than the influent water (Lautenschlager et al. 2014; Vignola et al. 2018). This is likely due to taxa shedding from the filter bed community and entering the effluent water. The results from this study seem to suggest that taxa selected in the deeper sections of the long filter bed are being shed more readily and having the greater influence on the biological composition of the effluent water. Despite this, the biological composition of the effluent water from the three column sizes were largely similar, displaying no significant difference in alpha diversity and accounting for only 4% of variance in the UniFrac distance analysis.

Several studies have reported an increase in the abundance of LNA bacteria in effluent water following biofiltration, thus suggesting an enrichment of small cell bacteria by the filtration process (Lautenschlager et al. 2014; Vital et al. 2012; Chan et al. 2018). This was investigated through the characterisation of Patescibacteria in the influent, effluent water and filter bed of the GAC biofilters of this study. This study found a diverse phylogeny of Patescibacteria consisting of 318 ASV's over 6 classes. A majority of Patescibacteria ASV's were found to be in higher relative abundance in the effluent water than the influent, seeming to confirm a mechanism of enrichment by the filtration process. Only 5 ASV's were found to be unique to the influent water suggesting that Patescibacteria show some resistance to removal. Previous authors have hypothesised that the small cell sized bacteria are more resistant to removal by physical straining or are overlooked by predatory protists (Tian et al. 2020; Batani et al. 2016). As such, the enrichment of LNA bacteria observed is the result of a reduction of HNA bacteria as opposed to an increase in LNA numbers (Lautenschlager et al. 2014). However, in this study 81 ASV's were found in the filter bed but not in the influent or effluent water. This may suggest a degree of retention of Patescibacteria within the filter bed. At this time the mechanism of retention is not clear. One possibility is that very small cell bacteria are able to reside within the pores of the GAC but pass through the 0.1µm filter membrane utilised for influent and effluent sampling. Another possibility is episymbiotic or endosymbiotic interactions with other community members of the filter bed (He et al. 2021; Nie et al., 2022.; Beam et al. 2020). In any case the interactions of this poorly understood phylum would benefit from further research.

7.3. Challenges and Future Research

The results presented in this study give some indication of the effect column size has on effluent water quality and the biological communities of the filter bed and effluent water. However, there are still unknown factors which have implications for the design of a point of entry water treatment method. This study implies that increasing filter bed length selects for certain taxa at higher abundance in the deeper sections of the long filter bed. However, the importance of these taxa for the removal of contaminants is currently unknown. In this study, the long filter achieved higher removal of DOC and iron at week 23. While this may suggest that the longer filter bed facilitated higher biological removal, it is difficult to decouple adsorption and the longer EBCT afforded by the long filter from biological mechanisms of

contaminant removal. The larger bed volume of the long filter will provide more adsorption sites and likely take longer to saturate than the short and medium filter (Xing et al. 2008). As such, the proportion of removal attributed to adsorption may be higher in the long filters. Future experiments could combat this by utilising an inert filter media such as glass beads to investigate the effect of column length on biological removal without being complicated by the high adsorption capacity of GAC.

The long filter also had three times as high an EBCT as the short filter. As such, the influent water is in contact with the filter media and thus the biological community longer. This may offer more time for the removal of contaminants, particularly recalcitrant compounds which are slower to biodegrade (Fundneider et al. 2021; Nemani et al. 2016; Moona et al. 2021). Future experiments could compare filter lengths while factoring in EBCT. This could be achieved by running each filter set to a certain EBCT volume as opposed to week 23 as in this study. This would mean staggering the deconstruction of the filter sets. It would be interesting to compare the removal capacity of the three filter sets once all had reached the same EBCT volumes. This would also provide insight into the effect of EBCT on the development of the microbial communities. For example, it would be interesting to see if the extension of the top community continued further down the filter bed than 30cm in the medium and long filters.

As the communities found at the bottom section of the long filter were the most different between column sizes and seemed to be seeding the effluent water, it would be interesting to gain a better understanding of their metabolism and selective factors driving their abundance in this area. Metagenomic or transcriptomic analysis of these communities would be beneficial to elucidate their metabolic capabilities and potential importance in contaminant removal. It would also be beneficial to include a measure of oxygen throughout the depth of the filter bed to determine if oxygen gradients may be a selective factor for taxa at deeper depths (Cohen 2001). However, it is unclear how this may be implemented. As column size was shown to have some effect on the biological composition of the effluent water it would be beneficial to test the biological stability of the effluent water.

This study also investigated Patescibacteria in the effluent water and filter bed. However, due to experimental limitations a weakness of this study was the number of samples available for analysis. Future experiments could filter the effluent water on 0.1µm more frequently building a more robust profile of Patescibacteria and their abundance in the influent and

effluent water, potentially between column sizes and over time. As these bacteria were shown to be enriched by the filter process, metagenomic analysis of this community would also be beneficial to reveal their metabolic potential and possibly determine the implications of their enrichment.

One of the most challenging aspects of developing a point of entry method of water treatment utilising biofiltration is the effect of influent water on the filter bed and effluent community. Several studies have identified the influent water as the main driver of the community composition of the filter bed and effluent water (Lautenschlager et al. 2014; Ma et al. 2020; Vignola et al. 2018), including a meta-analysis of biofilter communities currently in preparation by Cholet et al (in prep) suggesting a global pattern. Indeed, in this study the influent and effluent water were shown to be of similar biological composition. A point of entry system introduced across rural Scotland would use a variety of different influent water sources and thus have a variety of biological community compositions on the filter bed. As such it may be difficult to design a "fits all" method of optimisation of the filter bed community. Ultimately, this might not be an issue if there is a degree of functional redundancy between taxa. However, it may be of interest to run a series of biofilters using influent water from different sources in an attempt to identify a core community on which optimisation could be targeted. Identification of a core community or the specific biological mechanisms to enhance may help reveal strategies to optimise the biological removal achieved by biofiltration, increasing the viability of the technology for a point of entry water treatment system.

Another avenue of research may be to investigate the assembly processes which drive the community composition. Biofilter communities have been shown to be determined through both stochastic and deterministic forces (Vignola et al. 2018). Analysis of the assembly processes potentially through neutral modelling (Sloan et al. 2006) may identify which taxa are advantaged by the filter environment and help to identify the deterministic factors in community selection. Identifying these factors may reveal engineering strategies designed to drive the community in desirable directions.

A limitation of this study was that the microbial communities were identified based on total DNA. As such there is no way to distinguish between active and inactive cells or indeed extracellular DNA. At this stage the functioning of the filter bed community remains a mystery. The high abundance of sulphur oxidisers suggests that sulphur oxidation is

occurring within the filter bed. This could be tested through quantification of functional genes associated with sulphur oxidation. However, the community members responsible for carbon removal remain unknown. A more thorough understanding of the community functions of the filter bed would be beneficial to the optimisation process and should be considered as an important avenue of research. Linking microbial community structure, through 16S rRNA sequencing, to environmentally transcribed genes, through mRNA analysis, would provide an excellent starting point for considering how to optimise the removal capacity of a slow sand filter. Information gleaned from such a study could identify the organisms which are most active, and which contribute the most to contaminant removal. The environmental conditions of the filter could then be engineered to cater towards desirable organisms giving them a competitive advantage and increasing their number within the filter. The filter could also potentially be engineered to be more hostile to undesirable organisms like pathogens or organisms which are inactive, freeing up space and nutrients for desirable organisms.

Appendix A – Chapter 4

Table A.1 *All forward primer constructs utilised for 16S rRNA sequencing (Parada, Needham, and Fuhrman 2016; Quince et al. 2011)*

Name	Illumina 5' Adapter	Golay Barcode	Forward Primer Pad	Forward Primer Linker	515F Forward Primer (Parada)
515rcbc1	AATGATACGGCGACCACCGAGATCTACACGCT	TCCATACCGGAA	TATGGTAATT	GT	GTGYCAGCMGCCGCGGTAA
515rcbc2	AATGATACGGCGACCACCGAGATCTACACGCT	AGCCCTGCTACA	TATGGTAATT	GT	GTGYCAGCMGCCGCGGTAA
515rcbc3	AATGATACGGCGACCACCGAGATCTACACGCT	CCTAACGGTCCA	TATGGTAATT	GT	GTGYCAGCMGCCGCGGTAA
515rcbc4	AATGATACGGCGACCACCGAGATCTACACGCT	CGCGCCTTAAAC	TATGGTAATT	GT	GTGYCAGCMGCCGCGGTAA
515rcbc5	AATGATACGGCGACCACCGAGATCTACACGCT	TATGGTACCCAG	TATGGTAATT	GT	GTGYCAGCMGCCGCGGTAA
515rcbc6	AATGATACGGCGACCACCGAGATCTACACGCT	TACAATATCTGT	TATGGTAATT	GT	GTGYCAGCMGCCGCGGTAA
515rcbc7	AATGATACGGCGACCACCGAGATCTACACGCT	AATTTAGGTAGG	TATGGTAATT	GT	GTGYCAGCMGCCGCGGTAA
515rcbc8	AATGATACGGCGACCACCGAGATCTACACGCT	GACTCAACCAGT	TATGGTAATT	GT	GTGYCAGCMGCCGCGGTAA
515rcbc9	AATGATACGGCGACCACCGAGATCTACACGCT	GCCTCTACGTCG	TATGGTAATT	GT	GTGYCAGCMGCCGCGGTAA
515rcbc10	AATGATACGGCGACCACCGAGATCTACACGCT	ACTACTGAGGAT	TATGGTAATT	GT	GTGYCAGCMGCCGCGGTAA
515rcbc11	AATGATACGGCGACCACCGAGATCTACACGCT	AATTCACCTCCT	TATGGTAATT	GT	GTGYCAGCMGCCGCGGTAA
515rcbc12	AATGATACGGCGACCACCGAGATCTACACGCT	CGTATAAATGCG	TATGGTAATT	GT	GTGYCAGCMGCCGCGGTAA
515rcbc13	AATGATACGGCGACCACCGAGATCTACACGCT	ATGCTGCAACAC	TATGGTAATT	GT	GTGYCAGCMGCCGCGGTAA
515rcbc14	AATGATACGGCGACCACCGAGATCTACACGCT	ACTCGCTCGCTG	TATGGTAATT	GT	GTGYCAGCMGCCGCGGTAA
515rcbc15	AATGATACGGCGACCACCGAGATCTACACGCT	TTCCTTAGTAGT	TATGGTAATT	GT	GTGYCAGCMGCCGCGGTAA
515rcbc16	AATGATACGGCGACCACCGAGATCTACACGCT	CGTCCGTATGAA	TATGGTAATT	GT	GTGYCAGCMGCCGCGGTAA
515rcbc17	AATGATACGGCGACCACCGAGATCTACACGCT	ACGTGAGGAACG	TATGGTAATT	GT	GTGYCAGCMGCCGCGGTAA
515rcbc18	AATGATACGGCGACCACCGAGATCTACACGCT	GGTTGCCCTGTA	TATGGTAATT	GT	GTGYCAGCMGCCGCGGTAA
515rcbc19	AATGATACGGCGACCACCGAGATCTACACGCT	CATATAGCCCGA	TATGGTAATT	GT	GTGYCAGCMGCCGCGGTAA
515rcbc20	AATGATACGGCGACCACCGAGATCTACACGCT	GCCTATGAGATC	TATGGTAATT	GT	GTGYCAGCMGCCGCGGTAA
515rcbc21	AATGATACGGCGACCACCGAGATCTACACGCT	CAAGTGAAGGGA	TATGGTAATT	GT	GTGYCAGCMGCCGCGGTAA
515rcbc22	AATGATACGGCGACCACCGAGATCTACACGCT	CACGTTTATTCC	TATGGTAATT	GT	GTGYCAGCMGCCGCGGTAA
515rcbc23	AATGATACGGCGACCACCGAGATCTACACGCT	TAATCGGTGCCA	TATGGTAATT	GT	GTGYCAGCMGCCGCGGTAA
515rcbc24	AATGATACGGCGACCACCGAGATCTACACGCT	TGACTAATGGCC	TATGGTAATT	GT	GTGYCAGCMGCCGCGGTAA
515rcbc25	AATGATACGGCGACCACCGAGATCTACACGCT	CGGGACACCCGA	TATGGTAATT	GT	GTGYCAGCMGCCGCGGTAA
515rcbc26	AATGATACGGCGACCACCGAGATCTACACGCT	CTGTCTATACTA	TATGGTAATT	GT	GTGYCAGCMGCCGCGGTAA
515rcbc27	AATGATACGGCGACCACCGAGATCTACACGCT	TATGCCAGAGAT	TATGGTAATT	GT	GTGYCAGCMGCCGCGGTAA
515rcbc28	AATGATACGGCGACCACCGAGATCTACACGCT	CGTTTGGAATGA	TATGGTAATT	GT	GTGYCAGCMGCCGCGGTAA
515rcbc29	AATGATACGGCGACCACCGAGATCTACACGCT	AAGAACTCATGA	TATGGTAATT	GT	GTGYCAGCMGCCGCGGTAA
515rcbc30	AATGATACGGCGACCACCGAGATCTACACGCT	TGATATCGTCTT	TATGGTAATT	GT	GTGYCAGCMGCCGCGGTAA
515rcbc31	AATGATACGGCGACCACCGAGATCTACACGCT	CGGTGACCTACT	TATGGTAATT	GT	GTGYCAGCMGCCGCGGTAA
515rcbc32	AATGATACGGCGACCACCGAGATCTACACGCT	AATGCGCGTATA	TATGGTAATT	GT	GTGYCAGCMGCCGCGGTAA
515rcbc33	AATGATACGGCGACCACCGAGATCTACACGCT	CTTGATTCTTGA	TATGGTAATT	GT	GTGYCAGCMGCCGCGGTAA
515rcbc34	AATGATACGGCGACCACCGAGATCTACACGCT	GAAATCTTGAAG	TATGGTAATT	GT	GTGYCAGCMGCCGCGGTAA
515rcbc35	AATGATACGGCGACCACCGAGATCTACACGCT	GAGATACAGTTC	TATGGTAATT	GT	GTGYCAGCMGCCGCGGTAA
515rcbc36	AATGATACGGCGACCACCGAGATCTACACGCT	GTGGAGTCTCAT	TATGGTAATT	GT	GTGYCAGCMGCCGCGGTAA
515rcbc37	AATGATACGGCGACCACCGAGATCTACACGCT	ACCTTACACCTT	TATGGTAATT	GT	GTGYCAGCMGCCGCGGTAA
515rcbc38	AATGATACGGCGACCACCGAGATCTACACGCT	TAATCTCGCCGG	TATGGTAATT	GT	GTGYCAGCMGCCGCGGTAA

515rcbc39	AATGATACGGCGACCACCGAGATCTACACGCT	ATCTAGTGGCAA	TATGGTAATT	GT	GTGYCAGCMGCCGCGGTAA
515rcbc40	AATGATACGGCGACCACCGAGATCTACACGCT	ACGCTTAACGAC	TATGGTAATT	GT	GTGYCAGCMGCCGCGGTAA
515rcbc41	AATGATACGGCGACCACCGAGATCTACACGCT	TACGGATTATGG	TATGGTAATT	GT	GTGYCAGCMGCCGCGGTAA
515rcbc42	AATGATACGGCGACCACCGAGATCTACACGCT	ATACATGCAAGA	TATGGTAATT	GT	GTGYCAGCMGCCGCGGTAA
515rcbc43	AATGATACGGCGACCACCGAGATCTACACGCT	CTTAGTGCAGAA	TATGGTAATT	GT	GTGYCAGCMGCCGCGGTAA
515rcbc44	AATGATACGGCGACCACCGAGATCTACACGCT	AATCTTGCGCCG	TATGGTAATT	GT	GTGYCAGCMGCCGCGGTAA
515rcbc45	AATGATACGGCGACCACCGAGATCTACACGCT	AGGATCAGGGAA	TATGGTAATT	GT	GTGYCAGCMGCCGCGGTAA
515rcbc46	AATGATACGGCGACCACCGAGATCTACACGCT	AATAACTAGGGT	TATGGTAATT	GT	GTGYCAGCMGCCGCGGTAA
515rcbc47	AATGATACGGCGACCACCGAGATCTACACGCT	TATTGCAGCAGC	TATGGTAATT	GT	GTGYCAGCMGCCGCGGTAA
515rcbc48	AATGATACGGCGACCACCGAGATCTACACGCT	TGATGTGCTAAG	TATGGTAATT	GT	GTGYCAGCMGCCGCGGTAA
515rcbc49	AATGATACGGCGACCACCGAGATCTACACGCT	GTAGTAGACCAT	TATGGTAATT	GT	GTGYCAGCMGCCGCGGTAA
515rcbc50	AATGATACGGCGACCACCGAGATCTACACGCT	AGTAAAGATCGT	TATGGTAATT	GT	GTGYCAGCMGCCGCGGTAA
515rcbc51	AATGATACGGCGACCACCGAGATCTACACGCT	CTCGCCCTCGCC	TATGGTAATT	GT	GTGYCAGCMGCCGCGGTAA
515rcbc52	AATGATACGGCGACCACCGAGATCTACACGCT	TCTCTTTCGACA	TATGGTAATT	GT	GTGYCAGCMGCCGCGGTAA
515rcbc53	AATGATACGGCGACCACCGAGATCTACACGCT	ACATACTGAGCA	TATGGTAATT	GT	GTGYCAGCMGCCGCGGTAA
515rcbc54	AATGATACGGCGACCACCGAGATCTACACGCT	GTTGATACGATG	TATGGTAATT	GT	GTGYCAGCMGCCGCGGTAA
515rcbc55	AATGATACGGCGACCACCGAGATCTACACGCT	GTCAACGCTGTC	TATGGTAATT	GT	GTGYCAGCMGCCGCGGTAA
515rcbc56	AATGATACGGCGACCACCGAGATCTACACGCT	TGAGACCCTACA	TATGGTAATT	GT	GTGYCAGCMGCCGCGGTAA
515rcbc57	AATGATACGGCGACCACCGAGATCTACACGCT	ACTTGGTGTAAG	TATGGTAATT	GT	GTGYCAGCMGCCGCGGTAA
515rcbc58	AATGATACGGCGACCACCGAGATCTACACGCT	ATTACGTATCAT	TATGGTAATT	GT	GTGYCAGCMGCCGCGGTAA
515rcbc59	AATGATACGGCGACCACCGAGATCTACACGCT	CACGCAGTCTAC	TATGGTAATT	GT	GTGYCAGCMGCCGCGGTAA
515rcbc60	AATGATACGGCGACCACCGAGATCTACACGCT	TGTGCACGCCAT	TATGGTAATT	GT	GTGYCAGCMGCCGCGGTAA
515rcbc61	AATGATACGGCGACCACCGAGATCTACACGCT	CCGGACAAGAAG	TATGGTAATT	GT	GTGYCAGCMGCCGCGGTAA
515rcbc62	AATGATACGGCGACCACCGAGATCTACACGCT	TTGCTGGACGCT	TATGGTAATT	GT	GTGYCAGCMGCCGCGGTAA
515rcbc63	AATGATACGGCGACCACCGAGATCTACACGCT	TACTAACGCGGT	TATGGTAATT	GT	GTGYCAGCMGCCGCGGTAA
515rcbc64	AATGATACGGCGACCACCGAGATCTACACGCT	GCGATCACACCT	TATGGTAATT	GT	GTGYCAGCMGCCGCGGTAA
515rcbc65	AATGATACGGCGACCACCGAGATCTACACGCT	CAAACGCACTAA	TATGGTAATT	GT	GTGYCAGCMGCCGCGGTAA
515rcbc66	AATGATACGGCGACCACCGAGATCTACACGCT	GAAGAGGGTTGA	TATGGTAATT	GT	GTGYCAGCMGCCGCGGTAA
515rcbc67	AATGATACGGCGACCACCGAGATCTACACGCT	TGAGTGGTCTGT	TATGGTAATT	GT	GTGYCAGCMGCCGCGGTAA
515rcbc68	AATGATACGGCGACCACCGAGATCTACACGCT	TTACACAAAGGC	TATGGTAATT	GT	GTGYCAGCMGCCGCGGTAA
515rcbc69	AATGATACGGCGACCACCGAGATCTACACGCT	ACGACGCATTTG	TATGGTAATT	GT	GTGYCAGCMGCCGCGGTAA
515rcbc70	AATGATACGGCGACCACCGAGATCTACACGCT	TATCCAAGCGCA	TATGGTAATT	GT	GTGYCAGCMGCCGCGGTAA
515rcbc71	AATGATACGGCGACCACCGAGATCTACACGCT	AGAGCCAAGAGC	TATGGTAATT	GT	GTGYCAGCMGCCGCGGTAA
515rcbc74	AATGATACGGCGACCACCGAGATCTACACGCT	TTGCGGACCCTA	TATGGTAATT	GT	GTGYCAGCMGCCGCGGTAA
515rcbc75	AATGATACGGCGACCACCGAGATCTACACGCT	GTCGTCCAAATG	TATGGTAATT	GT	GTGYCAGCMGCCGCGGTAA
515rcbc76	AATGATACGGCGACCACCGAGATCTACACGCT	TGCACAGTCGCT	TATGGTAATT	GT	GTGYCAGCMGCCGCGGTAA
515rcbc77	AATGATACGGCGACCACCGAGATCTACACGCT	TTACTGTGGCCG	TATGGTAATT	GT	GTGYCAGCMGCCGCGGTAA
515rcbc78	AATGATACGGCGACCACCGAGATCTACACGCT	GGTTCATGAACA	TATGGTAATT	GT	GTGYCAGCMGCCGCGGTAA
515rcbc79	AATGATACGGCGACCACCGAGATCTACACGCT	TAACAATAATTC	TATGGTAATT	GT	GTGYCAGCMGCCGCGGTAA
515rcbc80	AATGATACGGCGACCACCGAGATCTACACGCT	CTTATTAAACGT	TATGGTAATT	GT	GTGYCAGCMGCCGCGGTAA
515rcbc81	AATGATACGGCGACCACCGAGATCTACACGCT	GCTCGAAGATTC	TATGGTAATT	GT	GTGYCAGCMGCCGCGGTAA
515rcbc82	AATGATACGGCGACCACCGAGATCTACACGCT	TATTTGATTGGT	TATGGTAATT	GT	GTGYCAGCMGCCGCGGTAA
515rcbc83	AATGATACGGCGACCACCGAGATCTACACGCT	TGTCAAAGTGAC	TATGGTAATT	GT	GTGYCAGCMGCCGCGGTAA
515rcbc84	AATGATACGGCGACCACCGAGATCTACACGCT	CTATGTATTAGT	TATGGTAATT	GT	GTGYCAGCMGCCGCGGTAA
515rcbc85	AATGATACGGCGACCACCGAGATCTACACGCT	ACTCCCGTGTGA	TATGGTAATT	GT	GTGYCAGCMGCCGCGGTAA
515rcbc86	AATGATACGGCGACCACCGAGATCTACACGCT	CGGTATAGCAAT	TATGGTAATT	GT	GTGYCAGCMGCCGCGGTAA

515rcbc87	AATGATACGGCGACCACCGAGATCTACACGCT	GACTCTGCTCAG	TATGGTAATT	GT	GTGYCAGCMGCCGCGGTAA
515rcbc88	AATGATACGGCGACCACCGAGATCTACACGCT	GTCATGCTCCAG	TATGGTAATT	GT	GTGYCAGCMGCCGCGGTAA
515rcbc89	AATGATACGGCGACCACCGAGATCTACACGCT	TACCGAAGGTAT	TATGGTAATT	GT	GTGYCAGCMGCCGCGGTAA
515rcbc90	AATGATACGGCGACCACCGAGATCTACACGCT	TGAGTATGAGTA	TATGGTAATT	GT	GTGYCAGCMGCCGCGGTAA
515rcbc91	AATGATACGGCGACCACCGAGATCTACACGCT	AATGGTTCAGCA	TATGGTAATT	GT	GTGYCAGCMGCCGCGGTAA
515rcbc92	AATGATACGGCGACCACCGAGATCTACACGCT	GAACCAGTACTC	TATGGTAATT	GT	GTGYCAGCMGCCGCGGTAA
515rcbc93	AATGATACGGCGACCACCGAGATCTACACGCT	CGCACCCATACA	TATGGTAATT	GT	GTGYCAGCMGCCGCGGTAA
515rcbc94	AATGATACGGCGACCACCGAGATCTACACGCT	GTGCCATAATCG	TATGGTAATT	GT	GTGYCAGCMGCCGCGGTAA
515rcbc95	AATGATACGGCGACCACCGAGATCTACACGCT	ACTCTTACTTAG	TATGGTAATT	GT	GTGYCAGCMGCCGCGGTAA
515rcbc96	AATGATACGGCGACCACCGAGATCTACACGCT	CTACAGGGTCTC	TATGGTAATT	GT	GTGYCAGCMGCCGCGGTAA
515rcbc97	AATGATACGGCGACCACCGAGATCTACACGCT	CTTGGAGGCTTA	TATGGTAATT	GT	GTGYCAGCMGCCGCGGTAA
515rcbc98	AATGATACGGCGACCACCGAGATCTACACGCT	TATCATATTACG	TATGGTAATT	GT	GTGYCAGCMGCCGCGGTAA
515rcbc99	AATGATACGGCGACCACCGAGATCTACACGCT	CTATATTATCCG	TATGGTAATT	GT	GTGYCAGCMGCCGCGGTAA
515rcbc100	AATGATACGGCGACCACCGAGATCTACACGCT	ACCGAACAATCC	TATGGTAATT	GT	GTGYCAGCMGCCGCGGTAA
515rcbc101	AATGATACGGCGACCACCGAGATCTACACGCT	ACGGTACCCTAC	TATGGTAATT	GT	GTGYCAGCMGCCGCGGTAA
515rcbc102	AATGATACGGCGACCACCGAGATCTACACGCT	TGAGTCATTGAG	TATGGTAATT	GT	GTGYCAGCMGCCGCGGTAA
515rcbc103	AATGATACGGCGACCACCGAGATCTACACGCT	ACCTACTTGTCT	TATGGTAATT	GT	GTGYCAGCMGCCGCGGTAA
515rcbc104	AATGATACGGCGACCACCGAGATCTACACGCT	ACTGTGACGTCC	TATGGTAATT	GT	GTGYCAGCMGCCGCGGTAA
515rcbc105	AATGATACGGCGACCACCGAGATCTACACGCT	CTCTGAGGTAAC	TATGGTAATT	GT	GTGYCAGCMGCCGCGGTAA
515rcbc106	AATGATACGGCGACCACCGAGATCTACACGCT	CATGTCTTCCAT	TATGGTAATT	GT	GTGYCAGCMGCCGCGGTAA
515rcbc107	AATGATACGGCGACCACCGAGATCTACACGCT	AACAGTAAACAA	TATGGTAATT	GT	GTGYCAGCMGCCGCGGTAA
515rcbc108	AATGATACGGCGACCACCGAGATCTACACGCT	GTTCATTAAACT	TATGGTAATT	GT	GTGYCAGCMGCCGCGGTAA
515rcbc109	AATGATACGGCGACCACCGAGATCTACACGCT	GTGCCGGCCGAC	TATGGTAATT	GT	GTGYCAGCMGCCGCGGTAA
515rcbc110	AATGATACGGCGACCACCGAGATCTACACGCT	CCTTGACCGATG	TATGGTAATT	GT	GTGYCAGCMGCCGCGGTAA
515rcbc111	AATGATACGGCGACCACCGAGATCTACACGCT	CAAACTGCGTTG	TATGGTAATT	GT	GTGYCAGCMGCCGCGGTAA
515rcbc112	AATGATACGGCGACCACCGAGATCTACACGCT	TCGAGAGTTTGC	TATGGTAATT	GT	GTGYCAGCMGCCGCGGTAA
515rcbc113	AATGATACGGCGACCACCGAGATCTACACGCT	CGACACGGAGAA	TATGGTAATT	GT	GTGYCAGCMGCCGCGGTAA
515rcbc114	AATGATACGGCGACCACCGAGATCTACACGCT	TCCACAGGGTTC	TATGGTAATT	GT	GTGYCAGCMGCCGCGGTAA
515rcbc115	AATGATACGGCGACCACCGAGATCTACACGCT	GGAGAACGACAC	TATGGTAATT	GT	GTGYCAGCMGCCGCGGTAA
515rcbc116	AATGATACGGCGACCACCGAGATCTACACGCT	CCTACCATTGTT	TATGGTAATT	GT	GTGYCAGCMGCCGCGGTAA
515rcbc117	AATGATACGGCGACCACCGAGATCTACACGCT	TCCGGCGGGCAA	TATGGTAATT	GT	GTGYCAGCMGCCGCGGTAA
515rcbc118	AATGATACGGCGACCACCGAGATCTACACGCT	TAATCCATAATC	TATGGTAATT	GT	GTGYCAGCMGCCGCGGTAA
515rcbc119	AATGATACGGCGACCACCGAGATCTACACGCT	CCTCCGTCATGG	TATGGTAATT	GT	GTGYCAGCMGCCGCGGTAA
515rcbc120	AATGATACGGCGACCACCGAGATCTACACGCT	TTCGATGCCGCA	TATGGTAATT	GT	GTGYCAGCMGCCGCGGTAA
515rcbc121	AATGATACGGCGACCACCGAGATCTACACGCT	AGAGGGTGATCG	TATGGTAATT	GT	GTGYCAGCMGCCGCGGTAA
515rcbc122	AATGATACGGCGACCACCGAGATCTACACGCT	AGCTCTAGAAAC	TATGGTAATT	GT	GTGYCAGCMGCCGCGGTAA
515rcbc123	AATGATACGGCGACCACCGAGATCTACACGCT	CTGACACGAATA	TATGGTAATT	GT	GTGYCAGCMGCCGCGGTAA
515rcbc124	AATGATACGGCGACCACCGAGATCTACACGCT	GCTGCCCACCTA	TATGGTAATT	GT	GTGYCAGCMGCCGCGGTAA
515rcbc125	AATGATACGGCGACCACCGAGATCTACACGCT	GCGTTTGCTAGC	TATGGTAATT	GT	GTGYCAGCMGCCGCGGTAA
515rcbc126	AATGATACGGCGACCACCGAGATCTACACGCT	AGATCGTGCCTA	TATGGTAATT	GT	GTGYCAGCMGCCGCGGTAA
515rcbc127	AATGATACGGCGACCACCGAGATCTACACGCT	AATTAATATGTA	TATGGTAATT	GT	GTGYCAGCMGCCGCGGTAA
515rcbc128	AATGATACGGCGACCACCGAGATCTACACGCT	CATTTCGCACTT	TATGGTAATT	GT	GTGYCAGCMGCCGCGGTAA
515rcbc129	AATGATACGGCGACCACCGAGATCTACACGCT	ACATGATATTCT	TATGGTAATT	GT	GTGYCAGCMGCCGCGGTAA
515rcbc130	AATGATACGGCGACCACCGAGATCTACACGCT	GCAACGAACGAG	TATGGTAATT	GT	GTGYCAGCMGCCGCGGTAA
515rcbc131	AATGATACGGCGACCACCGAGATCTACACGCT	AGATGTCCGTCA	TATGGTAATT	GT	GTGYCAGCMGCCGCGGTAA
515rcbc132	AATGATACGGCGACCACCGAGATCTACACGCT	TCGTTATTCAGT	TATGGTAATT	GT	GTGYCAGCMGCCGCGGTAA

515rcbc133	AATGATACGGCGACCACCGAGATCTACACGCT	GGATACTCGCAT	TATGGTAATT	GT	GTGYCAGCMGCCGCGGTAA
515rcbc134	AATGATACGGCGACCACCGAGATCTACACGCT	AATGTTCAACTT	TATGGTAATT	GT	GTGYCAGCMGCCGCGGTAA
515rcbc135	AATGATACGGCGACCACCGAGATCTACACGCT	AGCAGTGCGGTG	TATGGTAATT	GT	GTGYCAGCMGCCGCGGTAA
515rcbc136	AATGATACGGCGACCACCGAGATCTACACGCT	GCATATGCACTG	TATGGTAATT	GT	GTGYCAGCMGCCGCGGTAA
515rcbc137	AATGATACGGCGACCACCGAGATCTACACGCT	CCGGCGACAGAA	TATGGTAATT	GT	GTGYCAGCMGCCGCGGTAA
515rcbc138	AATGATACGGCGACCACCGAGATCTACACGCT	CCTCACTAGCGA	TATGGTAATT	GT	GTGYCAGCMGCCGCGGTAA
515rcbc139	AATGATACGGCGACCACCGAGATCTACACGCT	CTAATCAGAGTG	TATGGTAATT	GT	GTGYCAGCMGCCGCGGTAA
515rcbc140	AATGATACGGCGACCACCGAGATCTACACGCT	CTACTCCACGAG	TATGGTAATT	GT	GTGYCAGCMGCCGCGGTAA
515rcbc141	AATGATACGGCGACCACCGAGATCTACACGCT	TAAGGCATCGCT	TATGGTAATT	GT	GTGYCAGCMGCCGCGGTAA
515rcbc142	AATGATACGGCGACCACCGAGATCTACACGCT	AGCGCGGCGAAT	TATGGTAATT	GT	GTGYCAGCMGCCGCGGTAA
515rcbc143	AATGATACGGCGACCACCGAGATCTACACGCT	TAGCAGTTGCGT	TATGGTAATT	GT	GTGYCAGCMGCCGCGGTAA
515rcbc144	AATGATACGGCGACCACCGAGATCTACACGCT	ACTCTGTAATTA	TATGGTAATT	GT	GTGYCAGCMGCCGCGGTAA
515rcbc145	AATGATACGGCGACCACCGAGATCTACACGCT	TCATGGCCTCCG	TATGGTAATT	GT	GTGYCAGCMGCCGCGGTAA
515rcbc146	AATGATACGGCGACCACCGAGATCTACACGCT	CAATCATAGGTG	TATGGTAATT	GT	GTGYCAGCMGCCGCGGTAA
515rcbc147	AATGATACGGCGACCACCGAGATCTACACGCT	GTTGGACGAAGG	TATGGTAATT	GT	GTGYCAGCMGCCGCGGTAA
515rcbc148	AATGATACGGCGACCACCGAGATCTACACGCT	GTCACTCCGAAC	TATGGTAATT	GT	GTGYCAGCMGCCGCGGTAA
515rcbc149	AATGATACGGCGACCACCGAGATCTACACGCT	CGTTCTGGTGGT	TATGGTAATT	GT	GTGYCAGCMGCCGCGGTAA
515rcbc150	AATGATACGGCGACCACCGAGATCTACACGCT	TAGTTCGGTGAC	TATGGTAATT	GT	GTGYCAGCMGCCGCGGTAA
515rcbc151	AATGATACGGCGACCACCGAGATCTACACGCT	TTAATGGATCGG	TATGGTAATT	GT	GTGYCAGCMGCCGCGGTAA
515rcbc152	AATGATACGGCGACCACCGAGATCTACACGCT	TCAAGTCCGCAC	TATGGTAATT	GT	GTGYCAGCMGCCGCGGTAA
515rcbc153	AATGATACGGCGACCACCGAGATCTACACGCT	CACACAAAGTCA	TATGGTAATT	GT	GTGYCAGCMGCCGCGGTAA
515rcbc154	AATGATACGGCGACCACCGAGATCTACACGCT	GTCAGGTGCGGC	TATGGTAATT	GT	GTGYCAGCMGCCGCGGTAA
515rcbc155	AATGATACGGCGACCACCGAGATCTACACGCT	TTGAACAAGCCA	TATGGTAATT	GT	GTGYCAGCMGCCGCGGTAA
515rcbc156	AATGATACGGCGACCACCGAGATCTACACGCT	ATATGTTCTCAA	TATGGTAATT	GT	GTGYCAGCMGCCGCGGTAA
515rcbc157	AATGATACGGCGACCACCGAGATCTACACGCT	ATGTGCTGCTCG	TATGGTAATT	GT	GTGYCAGCMGCCGCGGTAA
515rcbc159	AATGATACGGCGACCACCGAGATCTACACGCT	CAGGAACCAGGA	TATGGTAATT	GT	GTGYCAGCMGCCGCGGTAA
515rcbc160	AATGATACGGCGACCACCGAGATCTACACGCT	GCATAAACGACT	TATGGTAATT	GT	GTGYCAGCMGCCGCGGTAA
515rcbc161	AATGATACGGCGACCACCGAGATCTACACGCT	ATCGTAGTGGTC	TATGGTAATT	GT	GTGYCAGCMGCCGCGGTAA
515rcbc162	AATGATACGGCGACCACCGAGATCTACACGCT	ACTAAAGCAAAC	TATGGTAATT	GT	GTGYCAGCMGCCGCGGTAA
515rcbc163	AATGATACGGCGACCACCGAGATCTACACGCT	TAGGAACTCACC	TATGGTAATT	GT	GTGYCAGCMGCCGCGGTAA
515rcbc164	AATGATACGGCGACCACCGAGATCTACACGCT	GTCCGTCCTGGT	TATGGTAATT	GT	GTGYCAGCMGCCGCGGTAA
515rcbc165	AATGATACGGCGACCACCGAGATCTACACGCT	CGAGGCGAGTCA	TATGGTAATT	GT	GTGYCAGCMGCCGCGGTAA
515rcbc166	AATGATACGGCGACCACCGAGATCTACACGCT	TTCCAATACTCA	TATGGTAATT	GT	GTGYCAGCMGCCGCGGTAA
515rcbc167	AATGATACGGCGACCACCGAGATCTACACGCT	AACTCAATAGCG	TATGGTAATT	GT	GTGYCAGCMGCCGCGGTAA
515rcbc168	AATGATACGGCGACCACCGAGATCTACACGCT	TCAGACCAACTG	TATGGTAATT	GT	GTGYCAGCMGCCGCGGTAA
515rcbc169	AATGATACGGCGACCACCGAGATCTACACGCT	CCACGAGCAGGC	TATGGTAATT	GT	GTGYCAGCMGCCGCGGTAA
515rcbc170	AATGATACGGCGACCACCGAGATCTACACGCT	GCGTGCCCGGCC	TATGGTAATT	GT	GTGYCAGCMGCCGCGGTAA
515rcbc171	AATGATACGGCGACCACCGAGATCTACACGCT	CAAAGGAGCCCG	TATGGTAATT	GT	GTGYCAGCMGCCGCGGTAA
515rcbc172	AATGATACGGCGACCACCGAGATCTACACGCT	TGCGGCGTCAGG	TATGGTAATT	GT	GTGYCAGCMGCCGCGGTAA
515rcbc173	AATGATACGGCGACCACCGAGATCTACACGCT	CGCTGTGGATTA	TATGGTAATT	GT	GTGYCAGCMGCCGCGGTAA
515rcbc174	AATGATACGGCGACCACCGAGATCTACACGCT	CTTGCTCATAAT	TATGGTAATT	GT	GTGYCAGCMGCCGCGGTAA
515rcbc175	AATGATACGGCGACCACCGAGATCTACACGCT	ACGACAACGGGC	TATGGTAATT	GT	GTGYCAGCMGCCGCGGTAA
515rcbc176	AATGATACGGCGACCACCGAGATCTACACGCT	CTAGCGTGCGTT	TATGGTAATT	GT	GTGYCAGCMGCCGCGGTAA
515rcbc177	AATGATACGGCGACCACCGAGATCTACACGCT	TAGTCTAAGGGT	TATGGTAATT	GT	GTGYCAGCMGCCGCGGTAA
515rcbc178	AATGATACGGCGACCACCGAGATCTACACGCT	GTTTGAAACACG	TATGGTAATT	GT	GTGYCAGCMGCCGCGGTAA
515rcbc179	AATGATACGGCGACCACCGAGATCTACACGCT	ACCTCAGTCAAG	TATGGTAATT	GT	GTGYCAGCMGCCGCGGTAA

515rcbc180	AATGATACGGCGACCACCGAGATCTACACGCT	TCATTAGCGTGG	TATGGTAATT	GT	GTGYCAGCMGCCGCGGTAA
515rcbc181	AATGATACGGCGACCACCGAGATCTACACGCT	CGCCGTACTTGC	TATGGTAATT	GT	GTGYCAGCMGCCGCGGTAA
515rcbc182	AATGATACGGCGACCACCGAGATCTACACGCT	TAAACCTGGACA	TATGGTAATT	GT	GTGYCAGCMGCCGCGGTAA
515rcbc183	AATGATACGGCGACCACCGAGATCTACACGCT	CCAACCCAGATC	TATGGTAATT	GT	GTGYCAGCMGCCGCGGTAA
515rcbc184	AATGATACGGCGACCACCGAGATCTACACGCT	TTAAGTTAAGTT	TATGGTAATT	GT	GTGYCAGCMGCCGCGGTAA
515rcbc185	AATGATACGGCGACCACCGAGATCTACACGCT	AGCCGCGGGTCC	TATGGTAATT	GT	GTGYCAGCMGCCGCGGTAA
515rcbc186	AATGATACGGCGACCACCGAGATCTACACGCT	GGTAGTTCATAG	TATGGTAATT	GT	GTGYCAGCMGCCGCGGTAA
515rcbc187	AATGATACGGCGACCACCGAGATCTACACGCT	CGATGAATATCG	TATGGTAATT	GT	GTGYCAGCMGCCGCGGTAA
515rcbc188	AATGATACGGCGACCACCGAGATCTACACGCT	GTTCTAAGGTGA	TATGGTAATT	GT	GTGYCAGCMGCCGCGGTAA
515rcbc189	AATGATACGGCGACCACCGAGATCTACACGCT	ATGACTAAGATG	TATGGTAATT	GT	GTGYCAGCMGCCGCGGTAA
515rcbc190	AATGATACGGCGACCACCGAGATCTACACGCT	TACAGCGCATAC	TATGGTAATT	GT	GTGYCAGCMGCCGCGGTAA
515rcbc191	AATGATACGGCGACCACCGAGATCTACACGCT	TGACAGAATCCA	TATGGTAATT	GT	GTGYCAGCMGCCGCGGTAA
515rcbc192	AATGATACGGCGACCACCGAGATCTACACGCT	CCTCGCATGACC	TATGGTAATT	GT	GTGYCAGCMGCCGCGGTAA
515rcbc193	AATGATACGGCGACCACCGAGATCTACACGCT	GGCGTAACGGCA	TATGGTAATT	GT	GTGYCAGCMGCCGCGGTAA
515rcbc194	AATGATACGGCGACCACCGAGATCTACACGCT	GCGAGGAAGTCC	TATGGTAATT	GT	GTGYCAGCMGCCGCGGTAA
515rcbc195	AATGATACGGCGACCACCGAGATCTACACGCT	CAAATTCGGGAT	TATGGTAATT	GT	GTGYCAGCMGCCGCGGTAA
515rcbc196	AATGATACGGCGACCACCGAGATCTACACGCT	TTGTGTCTCCCT	TATGGTAATT	GT	GTGYCAGCMGCCGCGGTAA
515rcbc197	AATGATACGGCGACCACCGAGATCTACACGCT	CAATGTAGACAC	TATGGTAATT	GT	GTGYCAGCMGCCGCGGTAA
515rcbc198	AATGATACGGCGACCACCGAGATCTACACGCT	AACCACTAACCG	TATGGTAATT	GT	GTGYCAGCMGCCGCGGTAA
515rcbc199	AATGATACGGCGACCACCGAGATCTACACGCT	AACTTTCAGGAG	TATGGTAATT	GT	GTGYCAGCMGCCGCGGTAA
515rcbc200	AATGATACGGCGACCACCGAGATCTACACGCT	CCAGGACAGGAA	TATGGTAATT	GT	GTGYCAGCMGCCGCGGTAA
515rcbc201	AATGATACGGCGACCACCGAGATCTACACGCT	GCGCGGCGTTGC	TATGGTAATT	GT	GTGYCAGCMGCCGCGGTAA
515rcbc202	AATGATACGGCGACCACCGAGATCTACACGCT	GTCGCTTGCACA	TATGGTAATT	GT	GTGYCAGCMGCCGCGGTAA
515rcbc203	AATGATACGGCGACCACCGAGATCTACACGCT	TCCGCCTAGTCG	TATGGTAATT	GT	GTGYCAGCMGCCGCGGTAA
515rcbc204	AATGATACGGCGACCACCGAGATCTACACGCT	CGCGCAAGTATT	TATGGTAATT	GT	GTGYCAGCMGCCGCGGTAA
515rcbc205	AATGATACGGCGACCACCGAGATCTACACGCT	AATACAGACCTG	TATGGTAATT	GT	GTGYCAGCMGCCGCGGTAA
515rcbc206	AATGATACGGCGACCACCGAGATCTACACGCT	GGACAAGTGCGA	TATGGTAATT	GT	GTGYCAGCMGCCGCGGTAA
515rcbc207	AATGATACGGCGACCACCGAGATCTACACGCT	TACGGTCTGGAT	TATGGTAATT	GT	GTGYCAGCMGCCGCGGTAA
515rcbc208	AATGATACGGCGACCACCGAGATCTACACGCT	TTCAGTTCGTTA	TATGGTAATT	GT	GTGYCAGCMGCCGCGGTAA
515rcbc209	AATGATACGGCGACCACCGAGATCTACACGCT	CCGCGTCTCAAC	TATGGTAATT	GT	GTGYCAGCMGCCGCGGTAA
515rcbc210	AATGATACGGCGACCACCGAGATCTACACGCT	CCGAGGTATAAT	TATGGTAATT	GT	GTGYCAGCMGCCGCGGTAA
515rcbc211	AATGATACGGCGACCACCGAGATCTACACGCT	AGATTCGCTCGA	TATGGTAATT	GT	GTGYCAGCMGCCGCGGTAA
515rcbc212	AATGATACGGCGACCACCGAGATCTACACGCT	TTGCCGCTCTGG	TATGGTAATT	GT	GTGYCAGCMGCCGCGGTAA

***Table A.2**. Significant p-values for species richness by sample grouping. Depth – 2, 4, 6, 8, 10, 15, 30, 60, 90, INF, EFF. Week – 5, 9, 12, 23.*

Richness

from	to	p-values
9 INF	9 2	0.048
9 INF	23 2	0.037
9 INF	23 4	0.046
5 2	5 8	0.025
5 2	9 8	0.034
5 2	5 10	0.016
5 2	9 10	0.048
5 2	5 15	0.011
5 2	12 15	0.042
5 2	5 30	0.018
5 2	9 30	0.0094
5 2	12 30	0.0087
5 2	5 60	0.014
5 2	9 60	0.01
5 2	12 60	0.0078
5 2	23 60	0.021
5 2	5 90	0.016
5 2	9 90	0.0074
5 2	12 90	0.006
5 2	23 90	0.019
5 2	5 EFF	0.011
5 2	9 EFF	0.028
5 2	12 EFF	0.027
5 2	23 EFF	0.037
9 2	5 4	0.0083
9 2	5 6	0.0076
9 2	9 6	0.029
9 2	5 8	0.002
9 2	9 8	0.00028
9 2	5 10	0.00023
9 2	9 10	0.0015
9 2	12 10	0.0018
9 2	5 15	0.0005
9 2	9 15	0.017
9 2	12 15	0.0064
9 2	23 15	0.012
9 2	5 30	0.00035
9 2	9 30	0.00026
9 2	12 30	0.00025
9 2	5 60	0.00016
9 2	9 60	0.00011
9 2	12 60	0.00034
9 2	23 60	0.00023
9 2	5 90	0.0012
9 2	9 90	6.60E-05
9 2	12 90	7.80E-05
9 2	23 90	0.00022
9 2	5 EFF	0.00019
9 2	9 EFF	0.0038
9 2	12 EFF	0.00037
9 2	23 EFF	0.0017
12 2	5 6	0.034
12 2	5 8	0.0054
12 2	9 8	0.0017
12 2	5 10	0.00093
12 2	9 10	0.0062
12 2	12 10	0.0075
12 2	5 15	0.0013
12 2	9 15	0.035
12 2	12 15	0.016
12 2	5 30	0.0013
12 2	9 30	0.00077
12 2	12 30	0.00072
12 2	5 60	0.00066
12 2	9 60	0.00044
12 2	12 60	0.00086
12 2	23 60	0.0011
12 2	5 90	0.003
12 2	9 90	0.00027
12 2	12 90	0.00027
12 2	23 90	0.00097
12 2	5 EFF	0.00068
12 2	9 EFF	0.0091
12 2	12 EFF	0.0017
12 2	23 EFF	0.0058
23 2	5 4	0.0098
23 2	9 4	0.032
23 2	5 6	0.008
23 2	9 6	0.019
23 2	12 6	0.039
23 2	5 8	0.0026
23 2	9 8	0.0017
23 2	12 8	0.028
23 2	5 10	0.001
23 2	9 10	0.003
23 2	12 10	0.0034
23 2	5 15	0.0011
23 2	9 15	0.012
23 2	12 15	0.0056
23 2	23 15	0.01
23 2	5 30	0.0012
23 2	9 30	0.00079
23 2	12 30	0.00075
23 2	5 60	0.00087
23 2	9 60	0.00067
23 2	12 60	0.00078

23 2	23 60	0.0012	9 4	5 60	0.0012	23 4	5 10	0.00056
23 2	5 90	0.0018	9 4	9 60	0.00074	23 4	9 10	0.0024
23 2	9 90	0.00049	9 4	12 60	0.0013	23 4	12 10	0.0028
23 2	12 90	0.00045	9 4	23 60	0.0021	23 4	5 15	0.00076
23 2	23 90	0.0011	9 4	5 90	0.0046	23 4	9 15	0.016
23 2	5 EFF	0.00081	9 4	9 90	0.00044	23 4	12 15	0.0065
23 2	9 EFF	0.0037	9 4	12 90	0.00042	23 4	23 15	0.013
23 2	12 EFF	0.0015	9 4	23 90	0.0018	23 4	5 30	0.00073
23 2	23 EFF	0.0028	9 4	5 EFF	0.0011	23 4	9 30	0.00048
5 4	12 4	0.0084	9 4	9 EFF	0.014	23 4	12 30	0.00045
5 4	23 4	0.011	9 4	12 EFF	0.0033	23 4	5 60	0.00043
5 4	5 8	0.023	9 4	23 EFF	0.011	23 4	9 60	0.00031
5 4	9 8	0.012	12 4	5 6	0.0091	23 4	12 60	0.00053
5 4	23 8	0.02	12 4	5 8	0.0021	23 4	23 60	0.00063
5 4	5 10	0.0035	12 4	9 8	6.10E-07	23 4	5 90	0.0015
5 4	23 10	0.023	12 4	5 10	4.40E-05	23 4	9 90	0.00021
5 4	5 15	0.0041	12 4	9 10	0.00091	23 4	12 90	0.00021
5 4	5 30	0.0054	12 4	12 10	0.0012	23 4	23 90	0.00058
5 4	9 30	0.0022	12 4	5 15	0.00035	23 4	5 EFF	0.00044
5 4	12 30	0.002	12 4	9 15	0.024	23 4	9 EFF	0.004
5 4	5 60	0.0022	12 4	12 15	0.0082	23 4	12 EFF	0.00088
5 4	9 60	0.0012	12 4	23 15	0.017	23 4	23 EFF	0.0024
5 4	12 60	0.0023	12 4	5 30	0.00011	5 6	23 8	0.016
5 4	23 60	0.0049	12 4	9 30	0.00012	5 6	5 10	0.017
5 4	5 90	0.01	12 4	12 30	0.00012	5 6	23 10	0.017
5 4	9 90	0.00063	12 4	5 60	1.20E-05	5 6	5 15	0.012
5 4	12 90	0.0006	12 4	9 60	5.20E-06	5 6	5 30	0.025
5 4	23 90	0.004	12 4	12 60	0.00023	5 6	9 30	0.0076
5 4	5 EFF	0.0021	12 4	23 60	1.50E-05	5 6	12 30	0.0067
5 4	9 EFF	0.035	12 4	5 90	0.0012	5 6	5 60	0.012
5 4	12 EFF	0.0092	12 4	9 90	1.60E-06	5 6	9 60	0.0066
5 4	23 EFF	0.036	12 4	12 90	1.00E-05	5 6	12 60	0.0064
9 4	5 8	0.0087	12 4	23 90	2.10E-05	5 6	23 60	0.031
9 4	9 8	0.0036	12 4	5 EFF	4.70E-05	5 6	5 90	0.027
9 4	5 10	0.0016	12 4	9 EFF	0.0045	5 6	9 90	0.0033
9 4	9 10	0.012	12 4	12 EFF	6.00E-05	5 6	12 90	0.0026
9 4	12 10	0.015	12 4	23 EFF	0.0014	5 6	23 90	0.023
9 4	5 15	0.0021	23 4	5 6	0.0089	5 6	5 EFF	0.0092
9 4	12 15	0.026	23 4	9 6	0.028	9 6	5 8	0.023
9 4	5 30	0.0023	23 4	5 8	0.0024	9 6	9 8	0.02
9 4	9 30	0.0012	23 4	9 8	0.00088	9 6	5 10	0.0061
9 4	12 30	0.0011	23 4	12 8	0.046	9 6	5 15	0.0054

9 6	5 30	0.0083	23 6	5 15	0.0084	12 8	12 60	0.013
9 6	9 30	0.0035	23 6	9 15	0.043	12 8	5 90	0.036
9 6	12 30	0.0031	23 6	12 15	0.032	12 8	9 90	0.012
9 6	5 60	0.0046	23 6	5 30	0.013	12 8	12 90	0.0087
9 6	9 60	0.0028	23 6	9 30	0.0071	12 8	23 90	0.047
9 6	12 60	0.0032	23 6	12 30	0.0066	12 8	5 EFF	0.023
9 6	23 60	0.0091	23 6	5 60	0.01	23 8	5 10	0.00022
9 6	5 90	0.011	23 6	9 60	0.0077	23 8	9 10	0.0022
9 6	9 90	0.0016	23 6	12 60	0.0059	23 8	12 10	0.0028
9 6	12 90	0.0013	23 6	23 60	0.015	23 8	5 15	0.00062
9 6	23 90	0.0074	23 6	5 90	0.012	23 8	9 15	0.027
9 6	5 EFF	0.0038	23 6	9 90	0.0055	23 8	12 15	0.011
9 6	9 EFF	0.032	23 6	12 90	0.0045	23 8	23 15	0.026
9 6	12 EFF	0.014	23 6	23 90	0.013	23 8	5 30	0.00038
9 6	23 EFF	0.036	23 6	5 EFF	0.0085	23 8	9 30	0.00029
12 6	5 8	0.019	23 6	9 EFF	0.021	23 8	12 30	0.00027
12 6	9 8	0.019	23 6	12 EFF	0.019	23 8	5 60	0.00013
12 6	5 10	0.0067	23 6	23 EFF	0.027	23 8	9 60	7.70E-05
12 6	9 10	0.039	5 8	23 8	0.003	23 8	12 60	0.0004
12 6	12 10	0.046	5 8	23 10	0.0034	23 8	23 60	0.0002
12 6	5 15	0.0055	9 8	23 8	0.00022	23 8	5 90	0.0017
12 6	12 15	0.044	9 8	5 10	0.01	23 8	9 90	4.30E-05
12 6	5 30	0.0086	9 8	23 10	0.00072	23 8	12 90	6.10E-05
12 6	9 30	0.0039	9 8	5 15	0.016	23 8	23 90	0.00019
12 6	12 30	0.0035	9 8	5 30	0.032	23 8	5 EFF	0.00018
12 6	5 60	0.0053	9 8	9 30	0.0057	23 8	9 EFF	0.0059
12 6	9 60	0.0035	9 8	12 30	0.0047	23 8	12 EFF	0.00037
12 6	12 60	0.0034	9 8	5 60	0.0022	23 8	23 EFF	0.0025
12 6	23 60	0.0096	9 8	9 60	0.00043	5 10	9 10	0.041
12 6	5 90	0.01	9 8	12 60	0.0061	5 10	12 10	0.036
12 6	9 90	0.0022	9 8	23 60	0.018	5 10	23 10	0.00047
12 6	12 90	0.0018	9 8	9 90	6.30E-05	5 10	23 15	0.021
12 6	23 90	0.008	9 8	12 90	0.00028	5 10	23 30	0.032
12 6	5 EFF	0.0044	9 8	23 90	0.011	5 10	9 90	0.014
12 6	9 EFF	0.025	9 8	5 EFF	0.004	5 10	12 90	0.0088
12 6	12 EFF	0.014	12 8	5 10	0.037	9 10	23 10	0.0031
12 6	23 EFF	0.029	12 8	5 15	0.022	9 10	5 15	0.024
23 6	5 8	0.019	12 8	5 30	0.046	9 10	9 30	0.013
23 6	9 8	0.024	12 8	9 30	0.017	9 10	12 30	0.011
23 6	5 10	0.012	12 8	12 30	0.015	9 10	5 60	0.024
23 6	9 10	0.034	12 8	5 60	0.031	9 10	9 60	0.0097
23 6	12 10	0.037	12 8	9 60	0.02	9 10	12 60	0.01

9 10	9 90	0.0035	12 10	23 10	0.0038	12 10	12 30	0.01
9 10	12 90	0.0027	12 10	5 15	0.022	12 10	5 60	0.022
9 10	5 EFF	0.016	12 10	9 30	0.012			

Table A.3*. Significant p-values for Pielou's evenness by sample grouping. Depth – 2, 4, 6, 8, 10, 15, 30, 60, 90, INF, EFF. Week – 5, 9, 12, 23.*

Pielou's evenness

from	to	p-values
12 INF	9 6	0.025
12 INF	9 8	0.013
12 INF	9 10	0.016
12 INF	12 10	0.043
12 INF	5 15	0.031
12 INF	9 15	0.023
12 INF	12 15	0.02
12 INF	12 30	0.019
12 INF	12 90	0.046
23 INF	9 8	0.039
23 INF	9 10	0.043
23 INF	12 15	0.048
5 2	9 2	0.026
5 2	9 4	0.00051
5 2	12 4	0.0019
5 2	5 6	0.039
5 2	9 6	5.40E-05
5 2	5 8	0.011
5 2	9 8	2.70E-05
5 2	12 8	0.0015
5 2	5 10	0.006
5 2	9 10	0.00017
5 2	12 10	0.0016
5 2	5 15	0.00081
5 2	9 15	0.00017
5 2	12 15	0.00058
5 2	23 15	0.00087
5 2	5 30	0.004
5 2	9 30	0.0087
5 2	12 30	0.00021
5 2	23 30	0.018
5 2	12 60	0.023
5 2	23 60	0.0078
5 2	12 90	0.00091
5 2	23 90	0.043
5 2	5 EFF	0.0016
5 2	9 EFF	0.012
5 2	12 EFF	0.038
5 2	23 EFF	0.027
9 2	23 2	0.021
9 2	9 4	0.027
9 2	9 6	0.0026
9 2	5 8	0.046
9 2	9 8	0.00074
9 2	12 8	0.045
9 2	5 10	0.027
9 2	9 10	0.0017
9 2	12 10	0.015
9 2	5 15	0.0076
9 2	9 15	0.0029
9 2	12 15	0.0037
9 2	9 30	0.032
9 2	12 30	0.0023
9 2	12 90	0.016
12 2	9 4	0.015
12 2	9 6	0.0023
12 2	5 8	0.031
12 2	9 8	0.00079
12 2	12 8	0.024
12 2	5 10	0.019
12 2	9 10	0.0015
12 2	12 10	0.01
12 2	5 15	0.0055
12 2	9 15	0.0025
12 2	12 15	0.0029
12 2	23 15	0.033
12 2	5 30	0.049
12 2	9 30	0.023
12 2	12 30	0.002
12 2	12 90	0.01
23 2	9 4	0.0017
23 2	12 4	0.0061
23 2	5 6	0.028
23 2	9 6	0.00045
23 2	5 8	0.0085
23 2	9 8	0.00021
23 2	12 8	0.0027
23 2	5 10	0.0052
23 2	9 10	0.00042
23 2	12 10	0.0021
23 2	5 15	0.0012
23 2	9 15	0.00055

23 2	12 15	0.00085	12 4	5 10	0.027	9 6	5 30	0.022
23 2	23 15	0.0026	12 4	9 10	0.00046	9 6	23 30	0.039
23 2	5 30	0.005	12 4	12 10	0.01	9 6	5 60	0.0031
23 2	9 30	0.0071	12 4	23 10	0.047	9 6	9 60	0.027
23 2	12 30	0.00052	12 4	5 15	0.004	9 6	23 60	1.50E-05
23 2	23 30	0.014	12 4	9 15	0.00049	9 6	5 90	0.016
23 2	9 60	0.042	12 4	12 15	0.0021	9 6	23 90	0.0021
23 2	12 60	0.017	12 4	23 15	0.018	9 6	5 EFF	0.00084
23 2	23 60	0.015	12 4	9 30	0.034	9 6	12 EFF	0.00075
23 2	9 90	0.045	12 4	12 30	0.00065	9 6	23 EFF	9.00E-05
23 2	12 90	0.0017	12 4	23 60	0.021	12 6	9 8	0.012
23 2	23 90	0.03	12 4	12 90	0.0069	12 6	5 10	0.047
23 2	5 EFF	0.004	12 4	23 EFF	0.023	12 6	9 10	0.014
23 2	9 EFF	0.0099	23 4	9 6	4.10E-05	12 6	5 15	0.034
23 2	12 EFF	0.029	23 4	5 8	0.014	12 6	9 15	0.026
23 2	23 EFF	0.027	23 4	9 8	2.10E-05	12 6	12 15	0.017
5 4	9 8	0.037	23 4	12 8	0.002	12 6	9 30	0.046
5 4	9 10	0.041	23 4	5 10	0.0076	12 6	12 30	0.018
5 4	12 15	0.045	23 4	9 10	0.00018	23 6	5 8	0.02
9 4	12 4	0.0045	23 4	12 10	0.002	23 6	9 8	0.00054
9 4	23 4	0.00058	23 4	5 15	0.00099	23 6	12 8	0.011
9 4	9 6	0.004	23 4	9 15	0.00018	23 6	5 10	0.012
9 4	23 6	0.0073	23 4	12 15	0.00069	23 6	9 10	0.001
9 4	9 8	0.00042	23 4	23 15	0.0011	23 6	12 10	0.006
9 4	23 8	0.0047	23 4	5 30	0.0058	23 6	5 15	0.0034
9 4	9 10	0.0035	23 4	9 30	0.011	23 6	9 15	0.0016
9 4	23 10	0.0042	23 4	12 30	0.00023	23 6	12 15	0.002
9 4	5 15	0.04	23 4	23 30	0.029	23 6	23 15	0.014
9 4	9 15	0.0083	23 4	12 60	0.03	23 6	5 30	0.022
9 4	12 15	0.011	23 4	23 60	0.016	23 6	9 30	0.016
9 4	12 30	0.0059	23 4	12 90	0.0011	23 6	12 30	0.0013
9 4	5 60	0.021	23 4	5 EFF	0.0022	23 6	12 60	0.043
9 4	23 60	0.0013	23 4	9 EFF	0.017	23 6	12 90	0.0056
9 4	23 90	0.018	9 6	12 6	0.033	23 6	5 EFF	0.024
9 4	12 EFF	0.0078	9 6	23 6	0.0014	23 6	9 EFF	0.032
9 4	23 EFF	0.0018	9 6	9 8	0.00029	5 8	23 8	0.023
12 4	23 4	0.0025	9 6	12 8	0.02	5 8	23 10	0.023
12 4	9 6	2.00E-05	9 6	23 8	0.00058	5 8	5 60	0.034
12 4	5 8	0.049	9 6	9 10	0.023	5 8	23 60	0.028
12 4	9 8	1.30E-05	9 6	23 10	0.00048	5 8	23 90	0.038
12 4	12 8	0.023	9 6	23 15	0.0011	5 8	12 EFF	0.031

5 8	23 EFF	0.024	5 10	5 60	0.021	5 15	9 60	0.031
9 8	12 8	0.0022	5 10	23 60	0.016	5 15	23 60	0.002
9 8	23 8	0.00021	5 10	5 90	0.039	5 15	5 90	0.02
9 8	23 10	0.00018	5 10	23 90	0.023	5 15	23 90	0.0061
9 8	9 15	0.043	5 10	12 EFF	0.018	5 15	5 EFF	0.011
9 8	23 15	0.00019	5 10	23 EFF	0.013	5 15	12 EFF	0.0038
9 8	5 30	0.0032	9 10	23 10	0.00058	5 15	23 EFF	0.002
9 8	23 30	0.0082	9 10	23 15	0.0018	9 15	23 15	0.0032
9 8	5 60	0.001	9 10	5 30	0.007	9 15	5 30	0.018
9 8	9 60	0.0076	9 10	23 30	0.012	9 15	23 30	0.028
9 8	23 60	1.00E-05	9 10	5 60	0.0019	9 15	5 60	0.0032
9 8	5 90	0.0051	9 10	9 60	0.01	9 15	9 60	0.021
9 8	12 90	0.01	9 10	23 60	0.00027	9 15	23 60	0.00026
9 8	23 90	0.00063	9 10	5 90	0.0069	9 15	5 90	0.013
9 8	5 EFF	0.00018	9 10	12 90	0.025	9 15	23 90	0.0024
9 8	12 EFF	0.00025	9 10	23 90	0.0015	9 15	5 EFF	0.0022
9 8	23 EFF	3.80E-05	9 10	5 EFF	0.0014	9 15	12 EFF	0.0012
12 8	23 8	0.0096	9 10	12 EFF	0.0008	9 15	23 EFF	0.00036
12 8	9 10	0.0066	9 10	23 EFF	0.00033	12 15	23 15	0.0062
12 8	23 10	0.0089	12 10	23 10	0.0052	12 15	5 30	0.014
12 8	9 15	0.018	12 10	23 15	0.047	12 15	23 30	0.018
12 8	12 15	0.015	12 10	5 60	0.012	12 15	5 60	0.0035
12 8	12 30	0.011	12 10	23 60	0.0048	12 15	9 60	0.014
12 8	5 60	0.031	12 10	5 90	0.035	12 15	23 60	0.0012
12 8	23 60	0.0065	12 10	23 90	0.012	12 15	5 90	0.0095
12 8	23 90	0.031	12 10	5 EFF	0.029	12 15	12 90	0.046
12 8	12 EFF	0.016	12 10	12 EFF	0.0077	12 15	23 90	0.0031
12 8	23 EFF	0.006	12 10	23 EFF	0.0043	12 15	5 EFF	0.0046
23 8	5 10	0.013	23 10	5 15	0.0026	12 15	12 EFF	0.0021
23 8	9 10	0.00064	23 10	9 15	0.00078	12 15	23 EFF	0.0012
23 8	12 10	0.0054	23 10	12 15	0.0015	23 15	12 30	0.0028
23 8	5 15	0.0028	23 10	23 15	0.0089	23 15	5 60	0.045
23 8	9 15	0.00088	23 10	5 30	0.022	23 15	23 60	0.0033
23 8	12 15	0.0016	23 10	9 30	0.017	23 15	23 90	0.044
23 8	23 15	0.0099	23 10	12 30	0.00076	23 15	12 EFF	0.019
23 8	5 30	0.023	23 10	12 90	0.004	23 15	23 EFF	0.0041
23 8	9 30	0.017	23 10	5 EFF	0.018	5 30	12 30	0.011
23 8	12 30	0.00083	23 10	9 EFF	0.037	5 30	23 60	0.024
23 8	12 90	0.0043	5 15	23 15	0.017	5 30	12 EFF	0.04
23 8	5 EFF	0.02	5 15	5 30	0.042	5 30	23 EFF	0.018
23 8	9 EFF	0.038	5 15	23 30	0.048	9 30	5 60	0.025
5 10	23 10	0.013	5 15	5 60	0.0067	9 30	23 60	0.021

9 30	5 90	0.041	12 30	12 90	0.045	12 90	23 90	0.012
9 30	23 90	0.027	12 30	23 90	0.0019	12 90	5 EFF	0.029
9 30	12 EFF	0.023	12 30	5 EFF	0.002	12 90	12 EFF	0.0064
9 30	23 EFF	0.018	12 30	12 EFF	0.0011	12 90	23 EFF	0.0027
12 30	23 30	0.017	12 30	23 EFF	0.00043	5 EFF	12 EFF	0.042
12 30	5 60	0.0025	5 60	12 90	0.013	5 EFF	23 EFF	0.01
12 30	9 60	0.014	23 60	12 90	0.0028	9 EFF	23 EFF	0.039
12 30	23 60	0.00037	23 60	5 EFF	0.011			
12 30	5 90	0.0091	5 90	12 90	0.044			

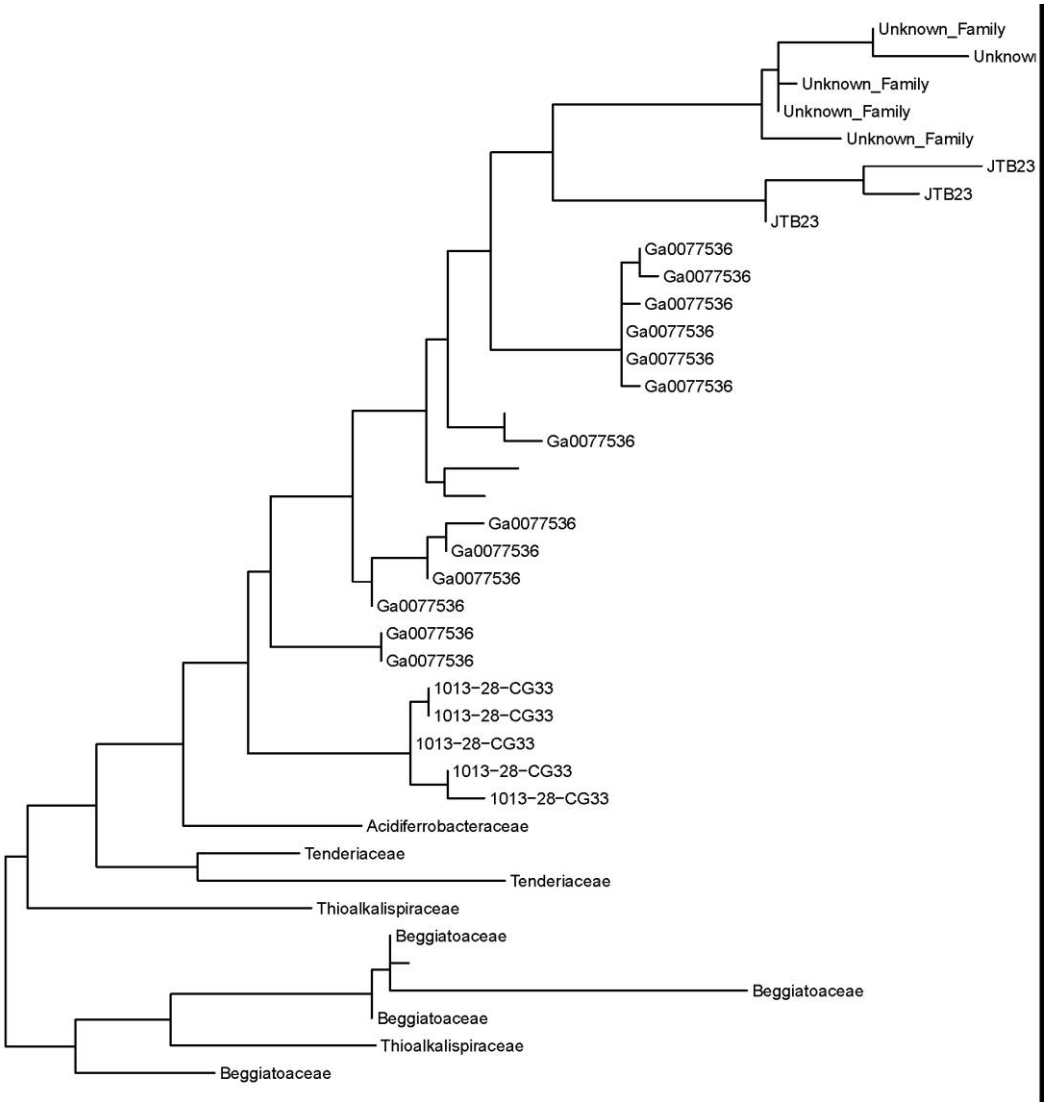


Figure A.1. *Phylogenetic tree featuring Tenderiaceae and Beggiatoaceae.*

Appendix B – Chapter 5

Table B.1. *Significant p-values for species richness by sample grouping. Column size - EFF 30 (short filter effluent), EFF 60 (medium filter effluent), EFF 90 (long filter effluent) INF (influent) Week – 1, 3, 5, 7, 9, 11, 12, 15, 17, 19, 21, 23.*

Richness

from	to	p-values
INF 1	INF 3	0.038
INF 1	INF 12	0.011
INF 1	INF 17	0.029
INF 1	EFF 30 19	0.022
INF 1	EFF 30 23	0.0084
EFF 30 1	INF 12	0.039
EFF 30 1	EFF 30 15	0.033
EFF 30 1	EFF 90 15	0.041
EFF 30 1	EFF 30 19	0.0045
EFF 30 1	EFF 30 21	0.047
EFF 30 1	EFF 90 21	0.036
EFF 30 1	EFF 30 23	0.0014
EFF 30 1	EFF 90 23	0.0035
EFF 60 1	EFF 30 23	0.045
EFF 90 1	EFF 30 3	0.043
EFF 90 1	EFF 90 3	0.019
EFF 90 1	EFF 60 5	0.041
EFF 90 1	EFF 30 9	0.02
EFF 90 1	INF 12	0.017
EFF 90 1	EFF 60 12	0.043
EFF 90 1	EFF 30 15	0.015
EFF 90 1	EFF 90 15	0.018
EFF 90 1	INF 17	0.041
EFF 90 1	EFF 30 17	0.046
EFF 90 1	EFF 60 17	0.032
EFF 90 1	EFF 90 17	0.048
EFF 90 1	EFF 30 19	0.0032
EFF 90 1	EFF 60 19	0.045
EFF 90 1	EFF 90 19	0.025
EFF 90 1	EFF 30 21	0.02
EFF 90 1	EFF 90 21	0.014
EFF 90 1	EFF 30 23	0.0013
EFF 90 1	EFF 60 23	0.024
EFF 90 1	EFF 90 23	0.0039
EFF 30 3	EFF 30 19	0.043
EFF 30 3	EFF 30 23	0.015
EFF 60 3	INF 12	0.033
EFF 60 3	EFF 30 15	0.033
EFF 60 3	EFF 90 15	0.039
EFF 60 3	EFF 30 19	0.0061
EFF 60 3	EFF 30 21	0.044
EFF 60 3	EFF 30 23	0.0023
EFF 60 3	EFF 90 23	0.01
EFF 90 3	EFF 30 5	0.013
EFF 90 3	EFF 60 7	0.023
EFF 90 3	EFF 30 19	0.0098
EFF 90 3	EFF 30 23	0.0024
EFF 90 3	EFF 90 23	0.01
EFF 30 5	EFF 30 9	0.0051
EFF 30 5	INF 12	0.033
EFF 30 5	EFF 30 15	0.023
EFF 30 5	EFF 90 15	0.03
EFF 30 5	EFF 30 19	0.0028
EFF 30 5	EFF 90 19	0.037
EFF 30 5	EFF 30 21	0.034
EFF 30 5	EFF 90 21	0.011
EFF 30 5	EFF 30 23	0.0008
EFF 30 5	EFF 60 23	0.043
EFF 30 5	EFF 90 23	0.00067
EFF 60 5	EFF 30 15	0.048
EFF 60 5	EFF 30 19	0.0052
EFF 60 5	EFF 30 23	0.0013
EFF 60 5	EFF 90 23	0.0019
EFF 90 5	INF 12	0.046
EFF 90 5	EFF 30 19	0.008
EFF 90 5	EFF 30 23	0.0027
EFF 90 5	EFF 90 23	0.015
EFF 30 7	INF 12	0.039
EFF 30 7	EFF 30 19	0.019
EFF 30 7	EFF 30 23	0.0087
EFF 60 7	EFF 30 9	0.022
EFF 60 7	INF 12	0.023
EFF 60 7	EFF 30 15	0.018
EFF 60 7	EFF 90 15	0.023
EFF 60 7	EFF 60 17	0.045
EFF 60 7	EFF 30 19	0.0032
EFF 60 7	EFF 90 19	0.033
EFF 60 7	EFF 30 21	0.026
EFF 60 7	EFF 90 21	0.016
EFF 60 7	EFF 30 23	0.0012
EFF 60 7	EFF 60 23	0.032

EFF 60 7	EFF 90 23	0.0031
EFF 90 7	INF 12	0.037
EFF 90 7	EFF 30 15	0.031
EFF 90 7	EFF 90 15	0.039
EFF 90 7	EFF 30 19	0.0044
EFF 90 7	EFF 30 21	0.044
EFF 90 7	EFF 90 21	0.033
EFF 90 7	EFF 30 23	0.0014
EFF 90 7	EFF 90 23	0.0037
EFF 30 9	EFF 30 19	0.0068
EFF 30 9	EFF 30 23	0.0015
EFF 30 9	EFF 90 23	0.0013
EFF 60 9	EFF 30 19	0.043
EFF 60 9	EFF 30 23	0.016
EFF 90 9	EFF 30 19	0.018
EFF 90 9	EFF 30 23	0.0073
EFF 60 11	INF 12	0.04
EFF 60 11	EFF 30 15	0.036
EFF 60 11	EFF 90 15	0.044
EFF 60 11	EFF 30 19	0.0052
EFF 60 11	EFF 90 21	0.047
EFF 60 11	EFF 30 23	0.0017
EFF 60 11	EFF 90 23	0.0056
EFF 90 11	INF 12	0.046
EFF 90 11	EFF 30 19	0.018
EFF 90 11	EFF 30 23	0.0079
EFF 30 12	EFF 30 19	0.031
EFF 30 12	EFF 30 23	0.011
EFF 60 12	EFF 30 19	0.033
EFF 60 12	EFF 30 23	0.011
EFF 60 15	EFF 30 23	0.022
EFF 60 17	EFF 30 19	0.032
EFF 60 17	EFF 30 23	0.01
EFF 90 17	EFF 30 19	0.012
EFF 90 17	EFF 30 23	0.0038
EFF 90 17	EFF 90 23	0.033
EFF 30 19	EFF 90 19	0.016
EFF 30 19	EFF 90 21	0.02
EFF 90 19	EFF 30 23	0.0044
EFF 90 21	EFF 30 23	0.0048
EFF 30 23	EFF 90 23	0.0091

Table B.2*. Significant p-values for Pielou's evenness by sample grouping. Column size - EFF 30 (short filter effluent), EFF 60 (medium filter effluent), EFF 90 (long filter effluent) INF (influent) Week – 1, 3, 5, 7, 9, 11, 12, 15, 17, 19, 21, 23.*

Pielou's evenness

from	to	p-values
INF 1	EFF 30 12	0.03
INF 1	EFF 90 17	0.019
EFF 30 1	EFF 60 1	0.04
EFF 30 1	EFF 90 1	0.023
EFF 30 1	EFF 60 3	0.0069
EFF 30 1	EFF 90 3	0.047
EFF 30 1	EFF 30 5	0.014
EFF 30 1	EFF 60 5	0.0091
EFF 30 1	EFF 90 5	0.0068
EFF 30 1	EFF 60 7	0.044
EFF 30 1	EFF 30 9	0.003
EFF 30 1	EFF 60 9	0.037
EFF 30 1	EFF 90 9	0.02
EFF 30 1	EFF 30 11	0.017
EFF 30 1	EFF 60 11	0.00045
EFF 30 1	EFF 90 11	0.0039
EFF 30 1	EFF 30 12	0.0098
EFF 30 1	EFF 60 12	0.0032
EFF 30 1	EFF 90 12	0.023
EFF 30 1	EFF 30 15	0.04
EFF 30 1	EFF 60 15	0.0072
EFF 30 1	EFF 30 17	0.00038
EFF 30 1	EFF 60 17	0.00047
EFF 30 1	EFF 90 17	0.0014
EFF 30 1	EFF 30 19	0.0051
EFF 30 1	EFF 60 19	0.017
EFF 30 1	EFF 90 19	0.00053
EFF 30 1	EFF 30 21	0.0017
EFF 30 1	EFF 90 21	0.0048
EFF 30 1	EFF 60 23	0.022
EFF 60 1	EFF 60 17	0.039
EFF 60 1	EFF 90 17	0.022
EFF 90 1	EFF 60 17	0.036
EFF 90 1	EFF 90 17	0.021
EFF 30 3	EFF 30 9	0.038
EFF 30 3	EFF 60 11	0.048
EFF 30 3	EFF 30 12	0.041
EFF 30 3	EFF 30 17	0.025
EFF 30 3	EFF 60 17	0.018
EFF 30 3	EFF 90 17	0.012
EFF 30 3	EFF 90 19	0.045
EFF 60 3	INF 23	0.035
EFF 60 3	EFF 60 23	0.027
EFF 90 3	INF 23	0.032
EFF 30 5	INF 23	0.027
EFF 30 5	EFF 60 23	0.045
EFF 60 5	EFF 60 17	0.046
EFF 60 5	EFF 90 17	0.025
EFF 90 5	EFF 60 17	0.043
EFF 90 5	EFF 90 17	0.025
EFF 90 5	EFF 60 23	0.04
EFF 60 7	INF 23	0.032
EFF 30 9	EFF 60 15	0.046
EFF 30 9	EFF 60 19	0.024
EFF 30 9	INF 23	0.016
EFF 30 9	EFF 60 23	0.0089

EFF 30 9	EFF 90 23	0.021
EFF 90 9	EFF 30 17	0.029
EFF 90 9	EFF 60 17	0.019
EFF 90 9	EFF 90 17	0.014
EFF 30 11	INF 23	0.018
EFF 30 11	EFF 60 23	0.045
EFF 60 11	EFF 60 15	0.038
EFF 60 11	EFF 90 17	0.035
EFF 60 11	EFF 60 19	0.014
EFF 60 11	INF 23	0.033
EFF 60 11	EFF 60 23	0.001
EFF 60 11	EFF 90 23	0.018
EFF 90 11	INF 23	0.037
EFF 90 11	EFF 60 23	0.016
EFF 90 11	EFF 90 23	0.042
EFF 30 12	INF 17	0.037
EFF 30 12	EFF 60 19	0.038
EFF 30 12	INF 21	0.034
EFF 30 12	INF 23	0.0081
EFF 30 12	EFF 60 23	0.023
EFF 30 12	EFF 90 23	0.03
EFF 60 12	EFF 60 19	0.037
EFF 60 12	INF 23	0.026
EFF 60 12	EFF 60 23	0.011
EFF 60 12	EFF 90 23	0.03
EFF 90 12	INF 23	0.029
INF 15	EFF 90 17	0.045
EFF 30 15	EFF 90 17	0.046
EFF 60 15	EFF 30 17	0.015
EFF 60 15	EFF 60 17	0.011
EFF 60 15	EFF 90 17	0.011
EFF 60 15	EFF 90 19	0.036
EFF 90 15	EFF 90 17	0.039
INF 17	EFF 90 17	0.031
EFF 30 17	EFF 60 19	0.0067
EFF 30 17	EFF 30 21	0.018
EFF 30 17	INF 23	0.019
EFF 30 17	EFF 60 23	0.00078
EFF 30 17	EFF 90 23	0.0096
EFF 60 17	EFF 60 19	0.0055
EFF 60 17	EFF 30 21	0.013
EFF 60 17	INF 23	0.013
EFF 60 17	EFF 60 23	0.001
EFF 60 17	EFF 90 23	0.0075
EFF 90 17	EFF 30 19	0.038
EFF 90 17	EFF 60 19	0.0072
EFF 90 17	EFF 90 19	0.041
EFF 90 17	INF 21	0.028
EFF 90 17	EFF 30 21	0.015
EFF 90 17	EFF 90 21	0.037
EFF 90 17	INF 23	0.005
EFF 90 17	EFF 60 23	0.0032
EFF 90 17	EFF 90 23	0.0072
EFF 30 19	EFF 60 23	0.025
EFF 60 19	EFF 90 19	0.014
EFF 90 19	INF 23	0.031
EFF 90 19	EFF 60 23	0.0013
EFF 90 19	EFF 90 23	0.017
EFF 30 21	EFF 60 23	0.0085
EFF 90 21	EFF 60 23	0.023
INF 23	EFF 30 23	0.048

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