THE DEVELOPMENT OF SMALL-SCALE, LOW-COST BIOFILM TREATMENT SYSTEMS FOR THE CONTROL OF PATHOGENS IN FRESH WATER

Joshua Andrew Chalmers Steven BSc. MRes.

A thesis submitted in partial fulfilment of the requirements of the University of the West of England, Bristol for the degree of Doctor of Philosophy

This research programme was carried out in collaboration with Origin Aqua Technologies Ltd

Faculty of Health and Applied Sciences, University of the West of England, Bristol, UK

August 2023

Abstract

Water that is contaminated with pathogenic microorganisms, can cause disease. Despite advances in the provision of safe drinking water, an estimated 580 million people lack consistent access to safe water whilst the consumption of biologically contaminated water leads to approximately 485,000 deaths each year. Well understood chemical and/or physical treatment regimes are commonplace in high income countries but are often not possible or suitable in remote or challenging communities where infrastructure is lacking and where drinking water is collected and managed at source. There is a need for sustainable, scalable, low cost, low energy systems that can be widely distributed and adopted for the provision of safer drinking water.

The aim of this research was to develop biofilm treatment systems (BTS) for the control and/or removal of freshwater contaminants, including but not limited to, common pathogenic bacteria. Laboratory scale and upscaled BTS were designed, developed, and investigated. The BTS utilise naturally occurring microorganisms that attach and form biofilms upon expanded ceramic filter media used in this research. The initial start-up (maturation) and development of the microbial community was investigated using single gene-based sequencing. These BTS were then applied for the control freshwater contaminants including *Escherichia coli*, *Enterococcus faecalis* and *Pseudomonas aeruginosa*.

The single gene-based community analysis demonstrates that under controlled conditions, the formation of an environmental biofilm is reproducible at scale from a singular seed source of environmental microorganisms. Moreover, such BTS operating within a recirculation configuration are able to achieve significant reductions of indicator species within fresh water; *E. coli* (99%), *E. faecalis* (99%), and *P. aeruginosa* (92%) after 24 h, which can be successfully applied in an upscaled configuration. The single gene-based community analysis identified potential antimicrobial producing species of bacteria within the biofilm of the BTS that is the hypothesised method by which the BTS was able to significantly reduce the number of viable pathogens from a source water.

The main findings of this study are that the viability of planktonic bacterial pathogens in fresh water are significantly reduced by functional characteristics inherent to mature environmental biofilms. Through competition sensing, biofilm communities may up-regulate defence mechanisms such as the production of antibiotics, biosurfactants and bactericidal toxins. This has important implications for the control and use of BTS within fresh water that is reproducible at scale. Moreover, this indicates, under controlled conditions, the environmental biofilm community was reproducible at scale; indicating that there is a potential to control biofilm growth within treatment systems based on the operational parameters of a treatment system.

Acknowledgements

I am normally the sort of person who never knows where to start writing, but in this case, it was easy. I wholeheartedly owe my gratitude to my supervisory team and mentors Darren Reynolds, Robin Thorn, Gareth Robinson and Dann Turner. They have all been guiding, supportive, and most importantly, patient throughout my PhD and in the completion of this thesis. I can honestly say, this inspirational team has helped shape me into the man I am today (albeit a little more cynical..!) and taught me that the most important thing at the end of it all, are people. Although most of my PhD involved running experiments in the laboratory, a good proportion was undertaken in the Duck and Willow for the "black op's" meetings or just a general team pint, but they were always there. Darren, I am afraid to say, I still don't have a clue (?).

To my industrial sponsors and to people I can now call friends, Andrew, Jack and to all those at Origin Aqua and Clear Water Revival I have met along the way. Will for his help with the CAD drawings. Andrew and Jack, I don't think I have ever laughed so much... How I made it through this PhD without going "rogue" I will never know. Jack, thank you for all your time and input in this research and for realising that one of the most important elements in robust scientific method is abbreviation... (AFR being my favourite of course). Nothing like a little bit of bonding over a bucket.

To all those at UWE, thank you. To all of the microbiology technicians that have given me so much support. Lee, James, Barbara and Rich thank you so much. I have always enjoyed the chats about science, but also about life and retirement plans, oh, and surfing (Rich)! A special mention to Lee, who without, I would not have the abundance of "character" I do today – thank you for always being there to answer any question, no matter how silly. Alun for all of his support with analytical chemistry. Dave Patton his help with the electron microscopy. I must also acknowledge the people who were very important in the start of my research journey during my undergraduate and MRes degree, Shona Nelson and Elizabeth Anderson. Both amazing, inspiring scientists who supported me as I began my life as a young scientist, your encouragement and time has helped me get to this point, thank you. Thank you to Roy Pemberton for always giving me detailed feedback on my work all the way through this PhD – I always enjoyed the challenge of your questions, and they have prepared me well...! Thank you to all the other academics at UWE who have helped me along the way. Finally, I must give my wholehearted thanks to the Ion Chromatograph for never failing to surprise, annoy me, and break. Thank you for testing my patience the at every step, you will be missed dearly.

Now let us not forget my fellow PhD students, who I would consider my family. We have in whole, or in part, shared this stressful yet *amazing* journey together and I leave this knowing I have made friends I will never forget. My research team old and new, Beth, Gill, Lizzy, Liana (fellow scientist

from Lanson), Rebecca and Alex. To my friend and brother, Dan who has been an absolute legend to live within the final part of this journey, many cups of teas, interior design ideas and plant escapades have helped keep me sane. And to my, slightly older, brother from another mother, Sav, keeping me (in)sane in the lab, it has been an entertaining 4 years sharing a bench with you…. Although I still haven't forgiven you for all my stolen pens. To mention another partner in crime, Sadie, my unofficial MRes, it has been a pleasure to work alongside you on this project, even if it was brief! It has been a wonderful experience to teach and open up doors to my, slight, insanity. Thanks for joining in! But finally, my mob. The back up demons. My best people. Angeliki, Buffy, Marina, and Eva… words cannot explain how wonderful and fortunate I feel to have such amazing, strong, and beautiful people in my life. Your love has taken forms in many ways, whether it be through the many bicycle rides, coffees, walks, debates, dinners, beer, hair cutting antics, or a marathon of The Lord of the Rings. Your presence and love have been the cornerstone of my survival. It has been an absolute joy to grow with you all and I am excited to see what every last one of you go on to do – go and smash it.

Now to mention some of the most important people in my life, my family. They have always been there for me and have supported me throughout the whole of my PhD. To my mother, Caroline, who has always encouraged me and supported me throughout my life even when she doesn't understand why I do what I do. She has always been there for me, with a myriad of ways in which, throughout my life, has actively supported me in my determination to find and realise my potential, and to make this contribution to our world. To my father, Alastair, who never thought we would be having a morning cuppa talking about pumps and filters, not to mention, he has always been there to lend a hand with many tools I have needed during this PhD. Thanks to my sisters, Yasmin, and Lucy for always pestering me to do anything other than my PhD, the breaks did help, I swear… and finally, I guess Fred comes in here too. Thanks for being there for moral support, mate.

Finally, I must mention the awesome, intricate, and diverse microbial world that constantly shapes and sustains the delicate balance of life on our planet, after all, I have spent a large proportion of my life studying it. This invisible world is profoundly influential in every corner of our environment, and a testament to the intricate tapestry of nature's interconnectedness. This has fascinated me every step of the way, yes even the many, many viable counts…

This thesis is not dedicated to anyone in particular, but is a culmination of wonderful, strong and supportive people who all given more than their quota of time and kindness to me, I am forever indebted to you.

Joshua A. C. Steven.

Bristol, UK, 2023.

Table of contents

List of figures

List of tables

List of equations

Abbreviations

ASV	Amplicon Sequence Variant
BHI	Brain Heart Infusion
BTS	Biofilm Treatment Systems
DBPs	Disinfection By-products
CAD	Computer-Aided Design
CFU	Colony Forming Unit
CEC	Contaminants of Emerging Concern
DADA	Divisive Amplicon Denoising Algorithm
DOC	Dissolved Organic Carbon
DWI	Drinking water inspectorate
EBCT	Empty Bed Contact Time
ECAS	Electrochemically Activated Solutions
EDC	Endocrine-Disrupting Compounds
EFC	Empty Filter Column
EPS	Extracellular Polymeric Substances
EU	European Union
FA	Fulvic Acids
sFL16S	16S full-length-based synthetic long-read sequencing
FM	Filter Media
FS	Filter Sterile
GAC	Granular Activated Carbon
GRACE	Gravity Recovery and Climate Experiment
HA	Humic Acids
HAA	Halo Acetic Acids
HIC	High Income Country
HLR	Hydraulic Loading Rate
HMDS	Hexamethyldisilane
HPC	Heterotrophic Plate Count
HS	Humic Substances
IC	Ion Chromatography
IBC	Intermediate Bulk Container
LEfSe	Linear discriminant *analysis* Effect Size
LMIC	Low to Middle Income Country
MDGs	Millennium Development Goals

MDPE	Medium Density Polyethylene
MLGA	Membrane Lactose Glucuronide Agar
MTP	Microbiome Taxonomic Profile
NP	Nonparametric
NA	Nutrient Agar
NB	Nutrient Broth
ORP	Oxidation Reduction Potential
OTU	Operational Taxonomic Unit
PCoA	Principle Coordinate Analysis
PERMANOVA	Permutational Multivariate Analysis of Variance
PPCP	Pharmaceuticals and Personal Care Products
PVC	Polyvinyl Chloride
RLU	Relative Light Units
RPM	Revolutions Per Minute
SAC	Surface Active Compounds
SB	Slantez and Bartley Medium
SDGs	Sustainable Development Goals
SEM	Scanning Electron Microscopy
SFW	Simulated Fresh Water
SUVA	Specific UV Absorbance
TC	Total Carbon
THM	Trihalomethanes
TIC	Total Inorganic Carbon
TOC	Total Organic Carbon
UF	Ultrafiltration
UKCEH	Centre for Hydrology and Ecology
UNDP	United Nations Development Programme
UNICEF	United Nations Children's Fund
UPGMA	Unweighted Pair Group Method with Arithmatic Mean
UV	Ultraviolet
VBNC	Viable But Non-Culturable
WASH	Water Sanitation and Hygiene
WHO	World Health Organisation
WWTP	Wastewater Treatment Plant

Chapter 1 Introduction and Literature Review.

1.1 Thesis overview

The present study was carried out to further understand the development of biofilms within water treatment systems and the potential of such biofilms to control the levels of waterborne pathogenic bacteria in fresh water. The work presented in this thesis is comprised of **7** chapters.

Chapter 1 provides a literature review regarding the global water distribution, the major problems with demand and microbiological contamination of water, current literature on how biofiltration technologies are employed within the water sector and the identification of knowledge gaps. This chapter also outlines the research aims.

Chapter 2 describes materials and experimental methods used in this research, and the development of model systems and techniques.

Chapter 3 investigates the characterisation and maturation of both laboratory and upscaled model biofilm treatment systems used in this research. This chapter also presents the comparison and representation of laboratory scale model treatment systems and up-scaled treatment systems.

Chapter 4 a detailed investigation of the bacterial diversity within the biofilms that establish and develop on the filter media within the biofilm treatment systems.

Chapter 5 demonstrates the use of laboratory scale biofilm treatment systems to reduce the viability of three species of waterborne pathogens within a fresh water of which presents published material (1).

Chapter 6 investigates a trial of up-scaled biofilm treatment systems for the remediation of contaminated environmentally derived fresh water.

Chapter 7 provides a final discussion and summarises the main study findings and their contributions to the knowledge gaps surrounding the role of biofilms for treatment processes within drinking water supplies.

1.2 Water quality and quantity, a global issue

Fresh water is fundamental for all life on earth and an integral part of human existence. Worldwide, only 2.5% of water is fresh water, and of the 0.77% of that which is accessible, only 10% is suitable for human consumption (2,3). Accessible water is not geographically distributed to meet global demand, leaving 580 million people unable to access a clean consistent source of water (4) with a further 1.1 billion people unable to access reliable energy sources (5). Without appropriate access to energy supply, access to treatment methods that require significant energy to operate is hindered. Meeting the global demand for fresh water, in addition to the energy required to achieve this demand, has been identified as a major challenge for society over the coming decades, particularly given that the global population has been estimated to increase to between 9 – 10 billion by 2050 (6–8). The stress on water resources has already been evidenced in Cape Town, South Africa, with the "Day Zero" crisis, when freshwater reservoir levels supplying the city nearly fell below the threshold of 13.5% capacity that would have resulted in the shutdown of the municipal water network (9). Moreover, water scarcity has been most prominent in India, China, Bangladesh, western states of the USA, Pakistan, Nigeria and Mexico, and it is estimated that 4 billion people live under severe water scarcity for at least one month per year (10). Fresh water availability is shifting on a global scale and is significantly driven by anthropological activity. A study conducted by the Gravity Recovery and Climate Experiment (GRACE) quantified 34 trends in terrestrial water storage observed by satellites during 2002–2016 and highlights that the drivers of this change include natural interannual variability in weather patterns (including rainfall), unsustainable groundwater consumption, climate change or combinations thereof (Figure 1.1). The observation-based assessment of the world's water landscape provides a blueprint for evaluating and predicting emerging threats to water and food security (11).

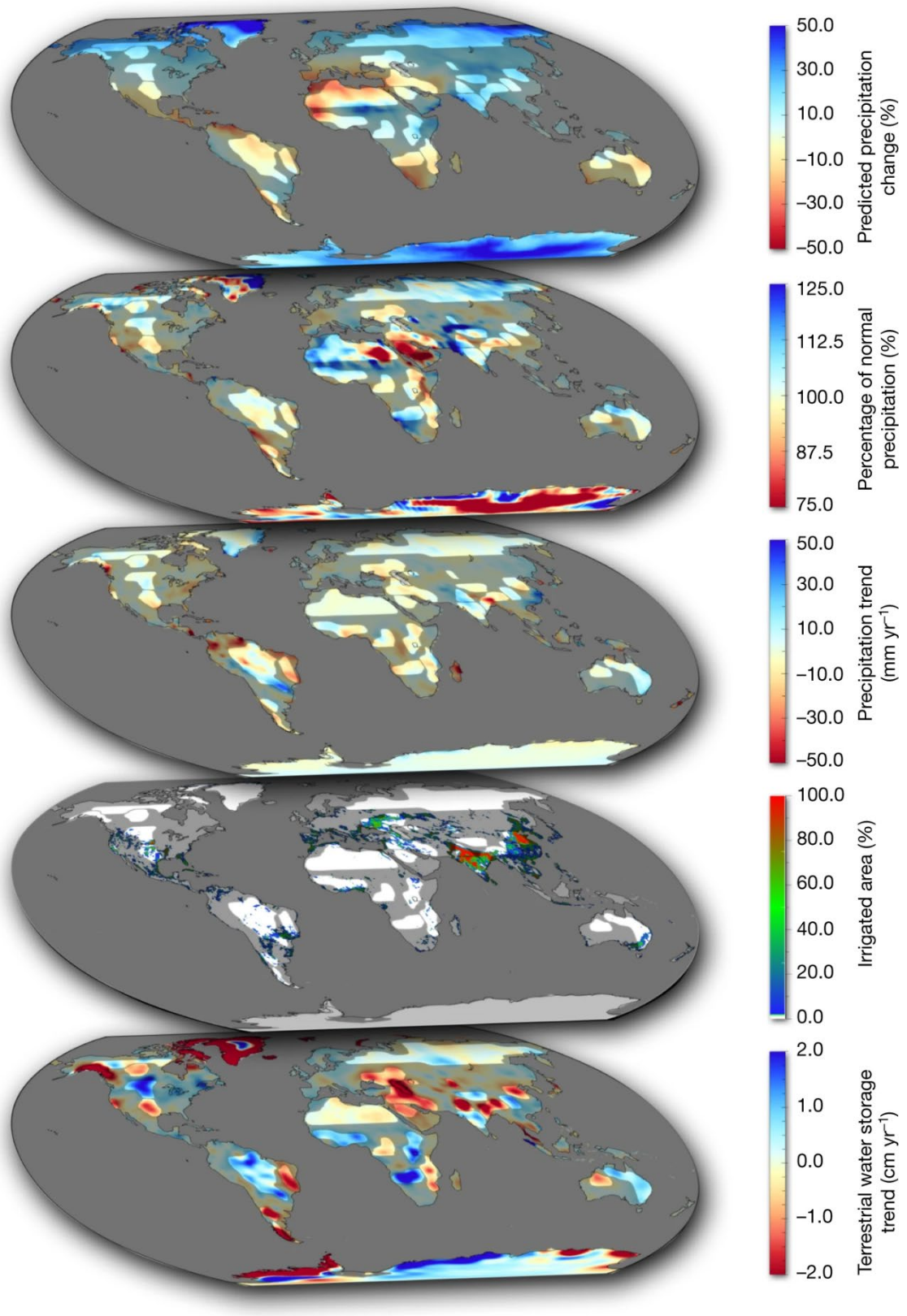


Figure 1.1: Changes in global freshwater availability determined by the Gravity Recovery and Climate Experiment (GRACE) using 34 trends in terrestrial water storage observed by satellites during 2002–2016. Reproduced with permission from Rodell *et al.* (11).

It is evident that the security of global freshwater resources is under threat (Figure 1.1) and to address these global challenges surrounding the access to and availability and security of fresh water the Millennium Development Goals (MDGs) were established in September 2000, and incorporated eight overarching goals set for 2015. Included in these goals was the Water, Sanitation and Hygiene (WASH) strategy (12). WASH is a strategy outlined by the World Health Organisation (WHO) that aims to improve access to and quality of water, sanitation and hygiene provisions and was included as part of MDG 7; to "ensure environmental sustainability". The final summary of the MDG in 2015 reports that 2.1 billion people had gained access to improved sanitation since 2000 and that 91% of the global population had access to improved drinking water sources (13). In 2015, the MDG's were concluded, and replaced with the Sustainable Development Goals (SDGs). The new SDGs are made up of 17 targeted goals to provide a "global blueprint for dignity, peace and prosperity for people and the planet, now and in the future" (14). Within the SDGs there is a specific goal that is set to improve water, sanitation, and hygiene: sustainable development goal number 6 (SDG6). The aim of SDG 6 is to "ensure availability and sustainable management of water and sanitation for all" (14), and is set to be achieved through the specific targets outlined in Table 1.1. SDG 6 encourages better overall management of water in terms of drinking water [SDG 6.1] and sanitation [6.2], reducing pollutants in source water [6.3], becoming more efficient in water usage [6.4 and 6.5] and to better protect and restore water sources [6.6]. These targets are only achievable through international cooperation [6.a] and local engagement [6.b]. There are several conditions in order to determine the safety of a drinking water source by establishing what service level the water is collected from (i.e. safely managed, basic, limited, unimproved or surface water). To be deemed safely managed the water must be from an improved water source (i.e. piped water, borehole, protected spring or delivered water), located on the premises, available when required and biologically and chemically safe (4). Basic drinking water access requires the collection of improved drinking water to be less than a 30-minute round trip, and limited access is collection of water from improved water sources with a greater than 30-minute round trip.

Table 1.1: Sustainable Development Goal 6: clean water and sanitation targets (15).

SDG 6 Targets	
6.1	By 2030, achieve universal and equitable access to safe and affordable drinking water for all.
6.2	By 2030, achieve access to adequate and equitable sanitation and hygiene for all and end open defecation, paying special attention to the needs of women and girls and those in vulnerable situations.
6.3	By 2030, improve water quality by reducing pollution, eliminating dumping, and minimizing release of hazardous chemicals and materials, halving the proportion of untreated wastewater and substantially increasing recycling and safe reuse globally.
6.4	By 2030, substantially increase water-use efficiency across all sectors and ensure sustainable withdrawals and supply of freshwater to address water scarcity and substantially reduce the number of people suffering from water scarcity.
6.5	By 2030, implement integrated water resources management at all levels, including through transboundary cooperation as appropriate.
6.6	By 2020, protect and restore water-related ecosystems, including mountains, forests, wetlands, rivers, aquifers, and lakes.
6.a	By 2030, expand international cooperation and capacity-building support to developing countries in water- and sanitation-related activities and programmes, including water harvesting, desalination, water efficiency, wastewater treatment, recycling, and reuse technologies.
6.b	Support and strengthen the participation of local communities in improving water and sanitation management.

For those without access to adequate water treatment (i.e., availably of either unimproved or surface water sources), the consumption of contaminated water is estimated to cause 485,000 deaths each year (16). A number of diseases resulting from consumption of these unimproved sources are attributed to the presence of pathogenic bacteria, including *Escherichia coli*, enterococci species and *Vibrio cholerae* within untreated water (17–19). The contamination of water sources is not only a problem present within low-middle income countries (LMIC) where the GDP per capita is less than US$ 2581. Intensive agriculture, urbanisation and industrial processes have caused rapid changes in the quality of water sources within high income countries (HIC). This has led to the increased prevalence of emerging contaminants (EC) and pollutants in fresh waters including,

but not limited to, antibiotics (20–24), endocrine-disruptor compounds (EDCs) (25,26), pharmaceuticals and personal care products (PPCPs) (27,28), and microplastics (29) that enter the aquatic environment at significant concentrations through wastewater discharge and ground water runoff. The increased prevalence of such chemicals places serious pressure on the health of aquatic eco-systems and helps contribute to the emergence of novel disinfectant-tolerant and antibiotic-resistant bacteria that do not respond to standard antimicrobial therapies, causing issues in both LMICs and HICs (30–33). This does not only affect water, but also food security whereby arable land that is irrigated with contaminated water can have a negative impact on human health through the consumption of ECs (34). This is a global issue and requires a global effort for the research and development into new approaches for improving the access to potable water, and technologies for the control of emerging contaminants, both of which are crucial steps towards achieving the 2030 Sustainable Development Goals (15). For SDG6 specifically, continued and further development of low energy solutions for the provisioning of potable water that are sustainable, compact and energy efficient is required.

1.3 Solutions; decentralised drinking water management systems

There has been an increase over the last 20 years into the research and development of novel, sustainable, compact water solutions for the production of safe drinking (potable) water (summarised in Table 1.2) (35). Sustainable provision of potable water should provide adequate water quantity and appropriate water quality for a given need, without compromising the future ability to provide this capacity and quality. A few examples of this are aquifers where the demand is no more than the recharge rate, recycled/reclaimed water, and rainwater harvesting. Appropriate treatment methods will also contribute to water sustainability, whereby the maintenance of appropriate water quality can reduce pressure on this resource.

Examples of solutions for the provision of potable water at source incorporate technologies including vapour compression distillation for the removal of physical, chemical and microbiological contaminants (36), ultrafiltration (UF) (37) and the combined use of UF and electrochemically activated solutions (ECAS) (38). However, for the guarantee of potable water in these systems, disinfection is still required. Such disinfection approaches include conventional chlorination (39) ozonation (40) or UV (41). For disinfection using chlorination, residual free chlorine and chloramines are required to ensure microbiological water quality is maintained, providing both stability and safety through the prevention of the growth of pathogenic bacteria (42). The use of chlorine has disadvantages: it is hazardous and produces harmful disinfection by-products (DBPs) upon reaction with organic matter (43). Furthermore, DBPs, including trihalomethanes (THMs) and haloacetic acids (HAA) are known to have negative side effects (44–46) including links between the exposure to DBPs and congenital defects (46), and development and severity of asthma (47).

Not all solutions incorporate filtration and disinfection: other examples of water management include rainwater harvesting (48) where water is stored without any prior treatment or slow sand filtration which involves a physical filtration process with a bioactive layer (49,50). Such solutions are still the most accessible and diffuse method water management strategies due to their reduced cost and simplicity (51). However, these strategies for water management are not always able to safeguard the quality of water (56).

Table 1.2: Summary of decentralized water treatment technologies for controlling waterborne pathogenic bacteria.

Water treatment technology	**Filtration**	**Disinfection**	**Reference**
Chlorine tablets	N/A	Chlorination	Sobsey (52)
Solar disinfection	N/A	Sunlight (UV inactivation)	Carratalà *et al.* (53)
Vapour compression distillation	N/A	Vaporization of water using solar energy	Attisani (36)
Ultrafiltration (UF) coupled with chlorination	UF	Chlorination	Chaidez, C. *et al.* (37)
Ultrafiltration coupled with electrochemically activated solutions (ECAS)	115 µm filter pump, 100 µm reverse flushing filter and 0.0 2 µm UF	Electrochemically activated solution (1% total v/v)	Clayton *et al.* (38)
Biosand filters	Gravel, Sand and Coniferous pinus bark biomass	N/A	Baig *et al.* (54)
Biofilter systems	Foam sleeve/disk incorporating biofilm and carbon filter block.	Chlorination	Wendt *et al.* (55)

There are many solutions available, yet 13% of the population are still unable to access a clean consistent source of water. Therefore, there is a need for low-cost, solutions that can be diffuse, utilise low-cost materials and that do not require specialist expertise to operate. One possible solution is the use of biologically active filters. Biologically active filters are nature-based solutions that utilise naturally occurring bacteria in the form of biofilms for the processing and treatment of water (57,58). This biotechnology can also be configured in small reactor scales, utilising locally sourced materials that can operate in a compact footprint and are energy efficient making them ideal candidates for an affordable technology to improve the accessibility of potable water treatment. However, further research is required into the use of a biologically based treatment

system without the addition of a disinfection treatment step for the control of waterborne pathogens within source water; the cause of the 505 000 deaths each year (59,60).

1.4 Biological filtration

Biological filtration broadly describes the bioremediation (breakdown using microorganisms) of organic matter and pollutants using beneficial microorganisms within biofilms that can be attached to a filter medium and/or be situated in a bioactive layer within slow sand filters, known as the "schmutzdecke" (German for "dirt cover") (61). Within biological filter vessels, filter media can consist of granular activated carbon (GAC), expanded ceramics, sand and/or anthracite, among others (58). These filter vessels can involve large reactors such as those used as a tertiary stage treatment to reduce biodegradable organic matter from raw water entering wastewater treatment plants (WWTPs), reducing the biological oxygen demand of final effluent for decades (62,63) or their incorporation into municipal drinking water treatment plants where they are used for the removal of contaminants, such as organic matter, to reduce the formation of DBPs during disinfection (43,58). Even the earliest form of engineered drinking water treatment involved biological filtration. In 1804, John Gibb designed and built an experimental slow sand filtration system (a form of biological filtration) for his bleachery in Paisley, Scotland (64). The treated water produced from this prototype system was sold and distributed amongst the local community. This went on to be developed by Robert Thorn in 1827 and then, James Simpson later commissioned biological sand filtration within the treatment of drinking water for public distribution by the Chelsea Water Company, London in 1829 (49). However, in the early 20th century, the development of rapid sand filtration soon replaced the demand for slow sand filtration due to the smaller reactor footprints and the ability to tolerate varying water quality (57,58). Nonetheless, the utilisation of microorganisms plays an important role in the access to improved drinking water across the globe in both LMICs and HICs. For example, slow sand filters are commonly used within LMICs (58) and the development of a layer of biomass at the surface of the sand filter facilitates the biodegradation of organic material present in the water. This biomass layer also acts to prevent the physical progression of most pathogenic microorganisms e.g., *E. coli*, coliform bacteria, *Giardia* and *Cryptosporidium* (65–67) through the slow sand filter, commonly achieving between 1 and 4 $\log_{10}$ reductions in pathogen loads. In HICs, biological filters are used to reduce or remove organic matter, a precursor to the formation of harmful disinfection by-products, prior to chlorination (50,58,68). In summary, biological filtration can be configured to have low chemical and energy requirements which therefore makes this a relevant biotechnology that could be applied within small to medium urban, rural, and remote communities.

1.4.1 Biofiltration operation and system modelling

To date, the research and development regarding the use of biological treatment of water has focused on; (i) pre-treatment methods, (ii) process development, filter performance and modelling, and (iii) biological/ecological parameters. To enable this work, appropriate biofilter modelling is key for meaningful study outcomes. However, within the research of biological filtration, there are certain caveats when comparing studies that are the difference in operating conditions, filter design, filter media properties and scale of model systems. When designing model systems, empty bed contact time (EBCT) and hydraulic loading rate (HLR) are important operating parameters that can influence treatment efficiency and can be used to compare biofilter systems (69). The EBCT has been utilised to predict the removal of organic matter in biofilters (70), although there are some inconsistencies which may indicate that other operational parameters still need investigation to help predict performance of biological filters. Early studies have demonstrated that the removal of organic carbon was a measure of biofilter performance, being directly proportional to the EBCT, whereby the removal of total organic carbon using GAC biofilters increased from approximately 30 to 50% when the EBCT was increased from 5 to 20 min (70). Moreover, the removal of organic-by products of ozonation through biological filtration does not significantly increase at an EBCT of 20 minutes (71). However, in some cases the EBCT has had little effect on organic matter removal within a range of between 4 and 20 min (72,73). The EBCT had no effect on removal with two parallel anthracite/sand biofilters with EBCT of 3.2 min and 8.3 min. However, more efficient nitrification performance was observed at longer EBCT (8.3 min) with a 60% increase in removal efficiency compared to 3.2 min (74). Furthermore, the adjustment to the EBCT can be used to help achieve relatively consistent performance. For example, an increase in EBCT has been used to counter the reduction in organic matter removal as a result of a decrease in operating temperatures to achieve steady performance (75). The evidence suggests that the EBCT of a biological treatment system is one of the most significant parameters to consider when investigating biological treatment processes.

HLR is another operational parameter that can affect the process of biological filtration. Several studies have investigated the effect of HLR on the removal of organics; however, the direct impact of HLR on bioremediation is not fully understood. A correlation between the removal of organics and the HLR of biological treatment systems has been demonstrated (76). This study found that as the HLR increased, the removal efficiency of organics (namely, dissolved organic carbon) decreased. It was postulated that the possible that the decreased removal of the dissolved organic carbon at higher HLRs may be due to the reduction of biofilm formation on the filter media suggesting that the biofilm may develop a greater density of biomass at lower HLRs (76). As a

result, biofilms are less able to remove organic carbon as efficiently at the higher HLRs without a sufficient new time period allowed for the biomass to reacclimatise to the new operating conditions (76). Another study found that the removal of oxidation by-products was not directly affected by HLR, but rather by influent concentration and EBCT (71). The varying removal rates was suggested to be attributed to the biomass and bacteria present at higher HLRs, which may prefer more easily biodegradable compounds.

It has been shown that a change in HLR during operation has an impact on bioremediation performance (77). A study by Liu *et al.* (77) investigated biofilter columns that were started on a HLR of 1 m h^{-1} (EBCT = 3 h). After 35 days of operation, the HLR in 5 of the 6 test systems was increased to 1.5 m h^{-1} (EBCT = 120 min), 2 m h^{-1} (EBCT= 90 min), 3 m h^{-1} (EBCT = 58 min), 5 m h^{-1} (EBCT = 35 min) and 8 m h^{-1} (EBCT = 22 min). As the HLRs were increased, a notable decrease in the removal of conventional pollutants (organic carbon, ammonia, total phosphate) was observed. However, this was followed by a trend of increasing removal within 7 days with stabilisation of removal achieved within 14 days for all systems. It has been shown that external mass transfer in the HLR range of 1.5 – 15 m hr^{-1} (measured at the same EBCT) does not constrain the removal of organic carbon, rather utilisation of the substrate at the media surface limits dissolved organic carbon (DOC) biodegradation (68). Thus, the changes in performance observed by Liu *et al.* (77) may have resulted from the changes in EBCT rather than HLR. In summary, it would suggest that EBCT, not HLR, has an impact on the performance of biological filters with respect to chemical contaminants and is the more important parameter to be used for optimisation, given that the EBCT does not conflict with particle removal goals or hydraulic efficiency of the treatment system.

There has been a lot of research into the "mechanical" parameters, however there is a possibility of investigating more detailed biochemical parameters. Such parameters include phosphatase and esterase activity, or the composition of extracellular polymeric substances (EPS) (69,78). Moreover, the composition of the microbial communities has been analysed using single gene-based community analysis (16S rRNA gene, variable region: V4, V5), demonstrating similarities to class level (69). This provides an insight into the biofilms that develop within the filter vessels showing that they are complex systems composed of aggregations of heterogenous cells. Therefore, more research is required to understand the development and function of biofilms within biologically based treatment systems.

1.4.2 Bioremediation of organic matter

Biological treatment of drinking water supplies primarily aims to reduce the presence of organic matter because organic matter can significantly reduce the quality of treated water. For example, the presence of organic matter can form disinfection by-products upon its reaction with the disinfectants (such as chlorine) used in the water treatment process (79). Another example is that organic matter can be used by potentially pathogenic microorganisms for proliferation in treated water (68,77,80). Biological treatment offers an effective approach in reducing the organic content of a water source through its mineralisation by the microorganisms in the biofilm contained within the biological treatment systems (69,73,74,81–83). Research into the removal of organic matter has indicated that removal is dependent on EBCT of biofilters [see section 1.4.1] (68,71,77,84). Temperature has also been shown to impact on the removal efficiencies of organic matter during biological treatment. For example, the removal efficiency of biodegradable organic carbon was reported at 38% operating at 5°C. However, when the operating temperature was increase to 35°C the removal efficiency was also increased to 60% (85).

1.4.3 Bioremediation of contaminants of emerging concern

Contaminants of emerging concern (CEC) are a group of natural and synthetic compounds, and their associated transformation products, occurring in aquatic ecosystems throughout the globe. CECs are not currently monitored in the environment but have the potential to cause environmental damage and are suspected to have negative effects on ecosystems and human health (86–88). A prominent group of CEC are the pharmaceuticals and personal care products (PPCPs). The main release of PPCPs to the environment has been identified in final effluent released from WWTPs that is primarily due to the limited removal efficiency by the conventional wastewater treatment processes, resulting in the contamination of surrounding aquatic ecosystems. A potential solution is the use of biofilters. Biofilters have been shown to achieve the efficient removal of emerging contaminants including pharmaceuticals, EDCs, artificial sweeteners and personal care products (89,90). Sand-anthracite biofilters operating with a 10 min EBCT were capable of reducing nine EDCs and PPCPs by an average of 39% (89). In addition, the operation of a sand-anthracite and GAC biofilter configuration has been shown to effectively remove PPCPs, reducing approximately 53.4% of mass concentration which translated to a reduction in adverse health risks of 79% (90).

1.4.4 Biofilter microbiome

Much of the research involving biofiltration to date has been aimed at the optimisation of mechanical operating conditions that affect the ability of biofilters to remove organic matter under the assumption that the microbiological community will have an overall function; that is to act as

complex sorbent systems. Because of this, research has primarily been aimed at the mechanical function of biofilters rather than their ecological aspects; consequently, they are still considered to be a "black box" technology. Previous research that has attempted to understand the ecological aspect of microbial communities involved with water treatment has been limited to culturable microorganisms using traditional microbiological techniques or focussed on specific biological processes, such as denitrification (91), predation (92) or pathogen removal (93). However, the role of unculturable microorganisms or those in a viable but non-culturable state, which may comprise up to 99% of the environmental community has yet to be determined (94,95). It is becoming increasingly recognised that the microbial community within biological based treatment systems will have a significant impact on the performance of treatment. Single gene-based community analysis of the 16S gene has been used to understand the microbial communities involved in these treatment processes. For example, community profiling has been used on biofilter models to understand the reproducibility of pilot scale biofilters to reflect that of full-scale biofilters (69) and assess the impact of biofilter operation on the microbial community structure (78). For drinking water supplies, studies that have investigated the bacterial communities colonising bench and pilot-scale GAC filters (using the same source water) found that all systems shared the same phyla and class. However, when comparing the order level of the community, the population differed at different operating temperatures, suggesting that some changes in community structure may have occurred with input water variation (78). Differences in the microbial population have been observed between identical pilot- and full-scale biofilters (69). This has provided insights to the process of biological treatment operating as an engineered functional ecosystem and thus, the microbial community involved in treatment will vary based on the input conditions. For example, it has been shown that the removal of organic matter (a function of biological treatment) from a source water was significantly reduced at water temperatures below 5 °C while a change in the microbial composition was observed (75). This highlights the need for more research into the operational function of the microbial communities involved in drinking water supplies to be coupled with the use of genomic tools.

1.5 Biofilms

Microorganisms exist in the natural environment in two forms: planktonic, free-floating cells or in communities, as biofilms. Biofilms are defined as aggregates of microorganisms in which cells are frequently embedded in a self-produced matrix of EPS that are adherent to each other and/or biotic or abiotic surfaces (96). There will be a diverse community of microorganisms within biofilms that exist in the environment. However, under most conditions bacteria are primarily responsible for laying the foundations for biofilm development. Only bacteria, algae and fungi have the ability to actively form biofilms. Biofilms are one of the most widely distributed and successful modes of life

which are essential to the biogeochemical cycling processes on earth (97). Microbial biofilms share common features, they all have three dimensional structures, and their formation is key for the survival of microorganisms in challenging environments (96).

1.5.1 Biofilm formation

The formation of biofilm is thought to enable and/or prolong survival in diverse environmental niches. The formation of a biofilm can be ordered into a defined sequence of events that are summarised in Figure *1.2*. Although differences in microbial community structure and growth rate have been observed between biofilms of different environments (98), the steps of biofilm formation remain relatively consistent (97) and therefore, this applies to the development of biofilms within biological treatment systems. The steps of biofilm formation in more detail are as follows:

(i) **Initial attachment.** Microorganisms reversibly attach to a surface or co-adhere to other microorganisms (99). Planktonic (free-swimming) bacteria come into contact with a surface by gravity or by reducing their motility, then become transiently attached. In some specific bacteria, such as *Pseudomonas aeruginosa* it has been demonstrated that flagella and type IV pili are directly involved in this initial attachment (100).

(ii) **Development.** Cells become irreversibly attached initiating production of extracellular polymeric substances (EPS) (101). The main component of the matrix is water (up to 97%), which contains the structural and functional components of the matrix: soluble, gel-forming polysaccharides, proteins and eDNA (96). The EPS can also contain insoluble components such as amyloids and cellulose (102,103). Attached motile bacteria have been shown to move on the surface forming cell clusters (microcolonies), a phenomenon mediated by the extension of type IV pili (100). This results in cell to cell, and cell to substrate, adhesion.

(iii) **Maturation.** The growth of microcolonies is preponderant and the cell clusters become progressively layered with microorganisms that results in the formation of three-dimensional structures and upregulation of EPS reaching the maximum structure size for the environmental conditions (104).

(iv) **Dispersion.** Bacterial cells can disperse from the surface of the biofilm or from within the biofilm structure and leave the biofilm structure through micropores. Moreover, aggregated sub-populations can also detach from the biofilm. It has been shown that twitching-mediated cell dispersion may be involved in maintaining the structure of biofilm (105). These new daughter cells may rapidly disperse and replicate under the appropriate environmental conditions that can, eventually, then form new biofilm communities.

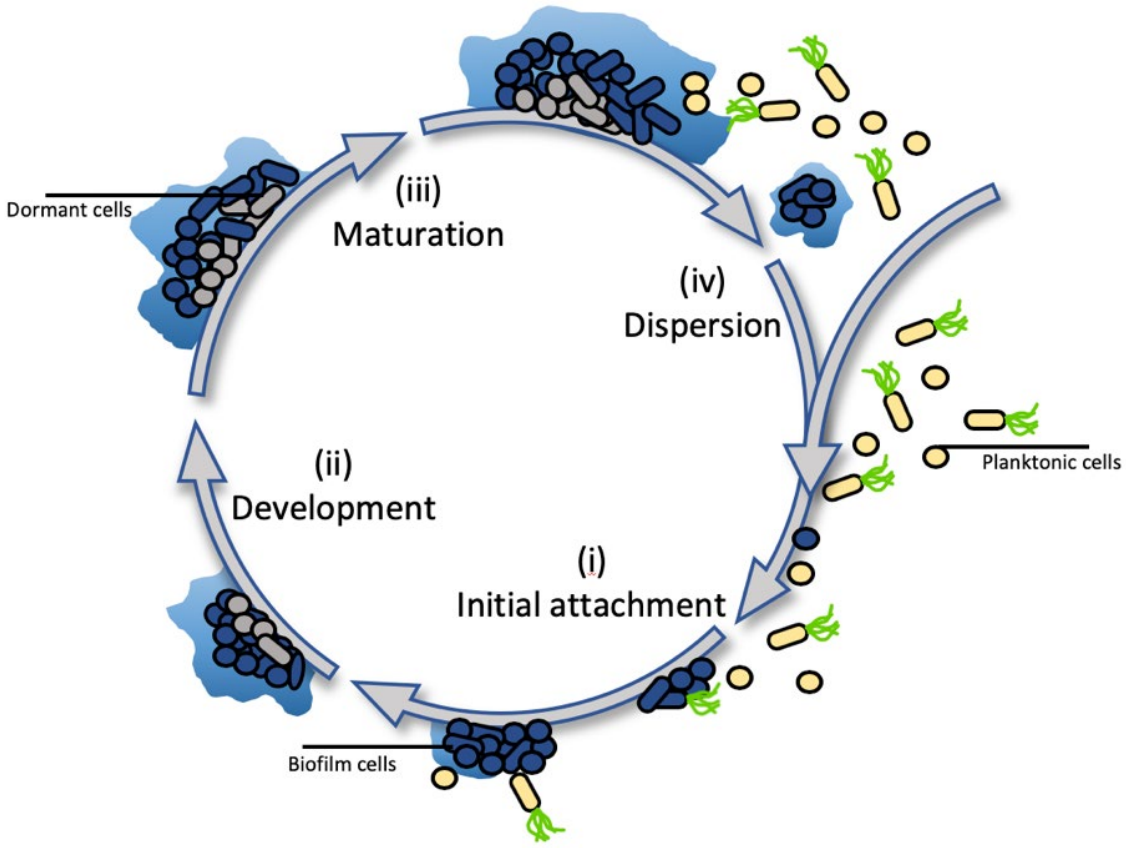


Figure 1.2: Stages of *biofilm formation*. (i) Planktonic cells initially attach to a biotic or abiotic surface (represented by the arrows). Following this, (ii) The development of biofilm begins with a phenotypic change within cells to irreversibly attach to the surface and begin the production of extracellular polymeric substances (EPS). (iii) The biofilm cells proliferate and mature, and the EPS matrix grows. Dormant or slow growing cells also begin to emerge. (iv) When the biofilm has matured, planktonic daughter cells can shed and disseminate from biofilm micropores which may be an ongoing process. Aggregates of biofilm can also shed; defined as sloughing.

1.5.2 Biofilms in biological filters

Biological filtration is a versatile biotechnology that relies on the formation of biofilm on filter media to enable operation. The biofilms that colonise the filter media within biofilters can be utilised in many applications such as the treatment of wastewater and solid waste or as biocatalysts in processes such as the production of bulk and fine chemicals, biofuels and the filtration of drinking water (106–108). Biofilms are essential in the process of biological filtration because they are tenacious, resilient, and adaptable. These properties provide the process of biological filtration with low chemical and energy operation demand. The biofilm community that colonises the biofilter will host a diverse microbial population that will be different between individual biofilters. Differences in the diversity of microbial populations has been observed even between duplicate pilot and bench scale biofilters (69). Nonetheless, the complex community provides biofilters with an overall function; to act as complex sorbent systems. This function and its relationship to community diversity are important for the operation of biological treatment systems, such as

varying sorption mechanisms and binding sites in the cytoplasm and cell walls of the biofilm matrix (96). The formation of the EPS is a highly dynamic natural process that comes with a significant energetic cost. However, this cost is justified by the structural properties of the biofilm matrix that is to facilitate the survival of microbial communities in challenging environments and is important in water treatment processes. Binding sites within these microbial communities include both anionic and cationic exchangers that can remove a wide range of substances and contaminants (109). In contrast, non-biological ionic exchangers utilised in water treatment are often specific to particular compounds, such as the use of activated alumina in the removal of phosphate (110). The sorbent and ionic exchange capacity of biofilms within biofilters can lead to the removal of nutrients, organic load and also chemical contaminants through their accumulation within biofilms (111,112). Substances including nutrients and contaminants that are captured either remain in the biofilm or are metabolised. However, contaminants may be released into the water phase from the matrix, often driven by a steep diffusion gradient into the water (109). Nutrient acquisition is an essential driver for this process, and biofilms have developed a very efficient capture strategy for this. The sorption properties of the EPS matrix enable it to behave like a sponge that influences the exchange of nutrients, gases and other molecules between the water phase and the biofilm (96). Even a wide range of metal ions including but not limited to Cu^{2+} Zn^{2+}, Fe^{2+}, Fe^{3+} and Al^{3+} have been found to accumulate within biofilms demonstrating a large capacity for the capture of contaminants (113). Moreover, a diverse biofilm community will incorporate varying sorption mechanisms and binding sites of biofilm cells and the EPS of the matrix and can apply to both dissolved compounds and suspended solids. These compounds can be trapped by biofilms and incorporated into the matrix, including biodegradable material that can be utilised as a source of nutrients for further microbial growth. The bio-utilisation and degradative capability of biofilms requires significant syntrophic cooperation. For example, in the process of nitrification, ammonia-oxidising bacteria metabolise ammonia into nitrite which is then utilised by nitrite oxidising species of bacteria such as *Nitrospira moscoviensis*. Another example facilitates the survival of anaerobic bacteria, such as those involved in denitrification of nitrates, under aerobic conditions whereby aerobic bacteria consume oxygen which can exceed the rate of diffusion through the biofilm producing an anaerobic niche for anaerobes to survive (114).

The nitrification of ammonium is a key part of the global nitrogen cycle that is driven by microorganisms and occurs under aerobic conditions through the following reaction:

$$2NH_4^+ + 3O_2 \rightleftharpoons NO_2^- + 2H_2O + 4H^+ \rightleftharpoons NO_3^-$$

Equation 1.1: The conversion of ammonium to nitrate.

Further to this, the removal of nitrate (denitrification) occurs under anoxic conditions through the following reaction:

$$2NO_3^- + 10e^- + 12H^+ \rightarrow N_2 + 6H_2O$$

Equation 1.2: The conversion of nitrate to nitrogen gas.

A metabolic interaction that precedes nitrification can occur when nitrite-oxidizing bacteria supply ammonia to ammonia-oxidizers. These interactions rely on the close proximity of cells that exchange metabolites, to enable efficient exchange by diffusion (96).

Although biofilm community behaviour and species interactions are important, the complexity of the underpinning bacterial growth has important implications for their behaviour in biological treatment. Bacterial species that are involved in the formation of environmental biofilms are diverse and adaptable, successfully responding to continuously changing environments that enable their survival. Specifically, when a bacterial population is exposed to an environmental stress, some individual cells enter a state of reduced metabolic activity, becoming dormant, particularly in oligotrophic environments. Stress, including carbon, nitrogen and oxygen limitation, and the presence of antibiotics, may induce dormancy (115,116). Although dormancy is seen as survival strategy, it requires the investment of resources to produce compounds and enzymes for transitioning between dormant and active states (117). Such properties may be vital to the ongoing operation of biological treatment systems in varying nutrient loading and water quality, and this has been observed in times of nutrient starvation (83,118). For example, phosphate dosing increased the removal rate of organic matter during biological treatment; influent to the treatment system (without phosphate dosing) was spiked with 0.1 mg L^{-1} of phosphate. This resulted in a dramatic decrease of organic matter in the effluent from 8.7 mg L^{-1} to 2.2 mg L^{-1} (83). This indicates that under the specific treatment conditions, the potential for the removal efficiencies of organic matter that is present within biological treatment systems could be increased by changing the influent conditions, which would be linked to the intracellular mechanisms that regulate the dormant state in microbial cells.

1.6 Pathogens in the aquatic environment

Waterborne pathogens are disseminated throughout the environment and their ability to propagate within aquatic environments is key for their survival and therefore their ability to cause human infection and disease. These waterborne pathogens can include bacteria, viruses, parasites, and protistans. Disease from water contamination was first reported in the mid-19th Century when John Snow discovered that contaminated water contributed to the spread of cholera in London (119). This discovery contradicted what was originally believed to be miasma, whereby the presence of 'bad air' or contact with a contagion (sick person) was perceived to be the route of transmission. However, it is now understood that the faecal oral route of transmission causes illness; release of faecal matter into water catchments which is then consumed without prior treatment.

Despite our advances in drinking water treatment and disinfection, the consumption of untreated water contaminated by waterborne pathogens still results in significant morbidity and mortality (120). This is most notable through the discharge of faecal matter and waste from hospitals, industry, and agriculture that can release large quantities of pathogens into the environment. Common species responsible for poor water quality resulting from faecal contamination such as *Escherichia coli* and *Enterococcus faecalis* are less adapted for survival in these environments and rely on their host for replication and are less adapted for growth in the aquatic environment, although their growth in aquatic environments has been demonstrated under specific conditions which suggests they may survive in nutrient limited environments (18,121). Other bacterial pathogenic species that are more adapted for growth within these aquatic environments include *Pseudomonas aeruginosa* and some *Mycobacteria* species, for example (122). This also includes viruses, and protozoa. Enteric viruses are related to diarrheal diseases such as Rotavirus, Astrovirus, Adenovirus, Norwalk virus, Picobirnavirus, and Enterovirus, to name a few (123). Protozoa include G*iardia duodenalis, Blastocystis hominis, Entamoeba histolytica, Cryptosporidium* spp., and *Dientamoeba fragilis* that have all been associated with waterborne disease (124).

There is limited knowledge about the ecological niches and survival of aquatic pathogens under the conditions they encounter in the environment. Regardless of the environmental niches these pathogens might occupy during their life cycle outside the host, water plays an important role in the transmission of human infection and disease (125,126). Therefore, the control and eradication of pathogens is an essential part of providing safe drinking water (127). When monitoring microbiological quality of water quality, the use of indicator species that include the bacteria *E. coli*, enterococci, and total coliforms are seen as the standard, although there is some discrepancy on how representative these indicator organisms are in the natural aquatic environment (128). The WHO and Drinking Water Inspectorate (DWI, UK) recommends zero colony forming units (CFU)

per 100 mL for indicator organisms in treated water because their presence infers the contamination of water with potential pathogens.

1.7 Bioremediation of microbiological contaminants

The resilience of biofilm growth and effective sorptive capacity, even under oligotrophic conditions, demonstrates the potential utility of these communities for treating a range of water sources (129). The use of biofilters to control aquatic pathogens for drinking water treatment has not been extensively reported in the literature. One previous study demonstrated that biofilters were capable of reducing viable *E. coli* by 56% through treating contaminated environmental water with gravity fed, open rock biofilter systems (130). In addition, a biofilter system that utilised foam to establish a biofilm has been shown to significantly reduce the non-pathogenic bacterial species, *Raoultella terrigena,* in comparison to a sterile control system (55). This was achieved using a complex multi-step filtration system with the role of the biological component in the removal of *R. terrigena* remaining unclear. Therefore, there is limited knowledge of the direct effect of biofilm within small-scale biological based treatment systems (i.e. in the absence of other treatment interventions), to control the numbers of pathogenic bacteria in drinking water supplies.

A possible mechanism for the bioremediation of contaminants is the process of classical competitive exclusion within the biofilter, whereby the challenging test bacteria could have been inhibited by the autochthonous bacteria established within the biofilm present on the biofilter media (131). Different competition strategies target bacterial ability to form a biofilm, whereas others can be less specific, resulting in death or the limitation of growth of the competing bacteria (132). The competition between cells in biofilms can involve inhibitory or cidal mechanisms, such as the production of antibiotics and bacteriocins (133,134) or strategies that compromise growth, such as nutrient depletion (135). Surface-active compounds (SACs) could also be produced by the microbial biofilm community (biosurfactants), which are amphipathic lipid-based molecules that lower interfacial tension and some of these biosurfactants display antimicrobial properties (136). Therefore, production of these biosurfactants could reduce the ability of the waterborne pathogens to accumulate and establish within the biofilm formed on the biofilter. In addition, interference mechanisms might also be upregulated in response to the presence of competition in the surroundings, known as the competition-sensing hypothesis (137). However, there is a need for research into the role of the potential of novel, small-scale biofiltration to control pathogenic bacteria in the production of safe drinking water.

1.8 Summary

The development of drinking water treatment technologies is an active area of research, yet globally there are still 458,000 deaths per year attributed to the consumption of biologically contaminated water (16). Biological treatment has proven to be a useful technology in drinking water treatment systems, demonstrating the capacity to decrease organic matter content that would otherwise enter the water distribution system and contribute to bacterial proliferation. Advanced oxidation preceding biological treatment has demonstrated good correlation with assisting in organics removal in combination with biofiltration (58). There is limited research investigating microbial speciation on biofilm-based treatment systems and the effects of bacterial growth within the biofilter column and associated removal of organics. Finally, there is a lack of research on cold water temperature effects and novel methods to help overcome limitations to compensate for decreased bacterial growth rates under cold water temperatures with drinking water applications. Nutrient supplementation has demonstrated the potential to facilitate indigenous bacterial growth in the biofilter to maximize organics removal and the need to assess incoming water quality to ensure it is suitable for biofiltration applications.

1.9 Research aims

The main aim of this research was to enhance the understanding of biofilm development within biofilm treatment systems and their potential application for the reduction of bacterial pathogens from a contaminated freshwater source. This research also aimed to further understand the development of biofilms and potential control of waterborne pathogens using upscaled treatment systems. The incorporation of biofilm-based treatment systems into small scale low-cost, off-grid technologies that are accessible to the end user is discussed. To achieve this, research has been undertaken to:

(i) Develop and characterise the microbial community within laboratory scale and upscaled biofilm treatment systems using an environmentally-derived surface-water source as a seed culture for biofilm development for the comparison of biofilm development within laboratory and upscaled BTS **(Chapter 3)**.

(ii) Characterise the morphology of filter media and biofilm during development, and to determine the microbial population of biofilms established within the treatment systems and their changes during development **(Chapter 3)**.

(iii) Determine the impact of filter media properties on the efficiency of biofilm development within laboratory scale BTS **(Chapter 3)**.

(iv) Investigate and compare the microbial community structure of the biofilm population established in laboratory and up-scaled biofilm treatment systems during development **(Chapter 4)**.

(v) Implement the characterised laboratory scale biofilm treatment systems to investigate the control of potential pathogenic bacteria using a simulated fresh water **(Chapter 5)**. The following indicator species were chosen for this objective;

1. *P. aeruginosa*, often isolated from soil, plants, and aquatic environments (138) and known to be a source of infections within healthcare settings as a result of contaminated infrastructure (139).
2. *E. coli,* a well-known resident of the gut microbiota of humans and animals, which is known to cause diarrhoeal and gastrointestinal disease from the consumption of contaminated water (140).
3. *Enterococcus faecalis,* which is part of the normal gut microbiota and can cause gastrointestinal and dermatological illnesses that correlate with their concentration in water (19).

(vi) Apply and upscale biofilm treatment systems to investigate the bioremediation of an environmentally-derived water source naturally contaminated with waterborne pathogens for off-grid applications **(Chapter 6)**.

The summary of these objectives can be seen in figure 1.3 to clearly demonstrate which model BTS was used within this research to address each objective.

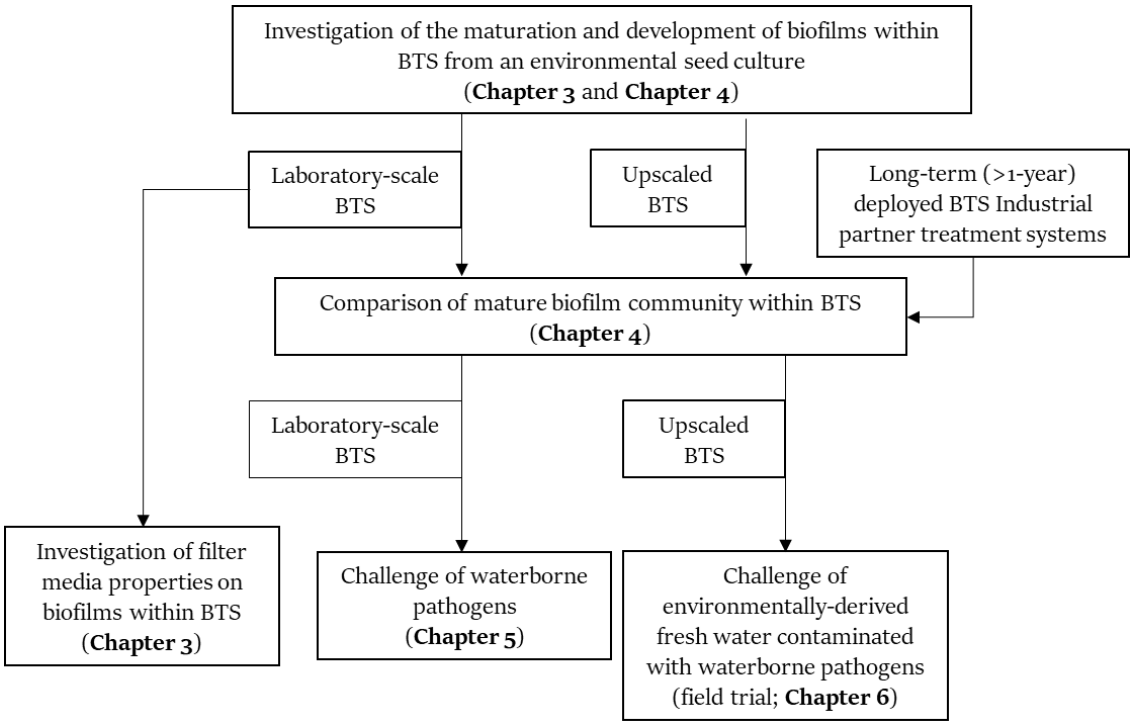


Figure 1.3 Summary flow diagram of the biofilm treatment systems scale and their corresponding objectives.

This research was carried out in collaboration with Origin Aqua Ltd, who kindly provided technical expertise, and materials for building both the laboratory-scale and upscaled biofilm treatment systems. They also provided access to upscaled long-term deployed filtration systems for the single gene-based community analysis in this research.

Chapter 2 Materials and Methods

2.1 Chemicals, reagents, and culture medium

All components of bacterial culture media were obtained from Oxoid™ Ltd. (Basingstoke, UK), unless otherwise stated. Dehydrated culture media were reconstituted and sterilised according to the manufacturer's instructions.

- Nutrient Broth; CM0067 (NB) a nutritious medium suitable for the cultivation of fastidious pathogens and other microorganisms. Used for the culture of *E. coli* and *P. aeruginosa* in this study.
- Brain Heart Infusion Broth; CM1032 (BHI) A highly nutritious liquid medium recommended for the cultivation of fastidious organisms and used for the culture of *E. faecalis* in this study.
- Nutrient Agar; CM0003 (NA) a general purpose medium for maintenance of bacterial strains used in this study.
- R2A agar; CM0906 (R2A) a medium for the culture and quantification of viable bacteria within drinking water.
- Membrane lactose glucuronide agar; CM1031 (MLGA) a medium for the growth, differentiation and enumeration of *Escherichia coli* and other coliforms by membrane filtration.
- Slantez and Bartley agar; CM0377 (SB) a medium for the selective isolation of enterococci.
- *Pseudomonas* CN Selective Agar (CN) comprised of *Pseudomonas* Agar base; CM0559 and CN supplement; SR0102 with glycerol (Fisher, UK) (5 mL per 500 mL), for selective isolation of *Pseudomonas aeruginosa*.

For the laboratory scale pathogen challenge experiments [see section 2.7], a simulated fresh water (SFW) was developed using an adapted method from (141) and is detailed in Table 2.1. The SFW contains low concentrations of nitrate (NO_3^-; 0.3 mg L^{-1}) and no phosphate (PO_4^{3-}; 0 mg L^{-1}) with no addition of excess Carbon. The SFW was prepared by the dissolution of the respective salts into ultrapure water which was then sterilised by autoclaving at 121°C for 15 min. Table 2.1 outlines the concentrations of all ions present within the SFW.

Table 2.1: Anion and cation concentrations of the minimal medium simulated fresh water adapted from Smith *et al.* (141).

Chemical constituents	Final ion concentrations (mg L^{-1})	
	Anions	Cations
$MgCl_2$	1.46	4.25
$CaCl_2$	3.21	5.67
$Ca(NO_3)_2$	0.60	1.86
$CaCO_3$	6.81	10.20
Na_2SO_4	5.29	11.05
$KHCO_3$	0.98	1.53
$NaHCO_3$	0.46	1.22

2.2 Bacterial strains and culture maintenance

Escherichia coli NCTC 10418, *Enterococcus faecalis* NCTC 12679 and *Pseudomonas aeruginosa* NCIMB 8295 were maintained on cryopreservation beads (Microbank, Pro Lab Diagnostics, Canada) at -80°C, and recovered by plating on NA as required. Bioluminescent strains of *E. coli* (nissle 1917 pBBR1MCS-2 lite) and *Pseudomonas aeruginosa* (PAO1 SE1 pBBR1 MCS5-lite) were used within this study and maintained on NA supplemented with kanamycin (10 μg mL^{-1}) and gentamicin (50 μg mL^{-1}) respectively.

2.3 Preparation of test pathogen monocultures

Overnight batch cultures of the test bacterial pathogens (see section 2.2.) were prepared by loop inoculation in NB (10 mL) for *E. coli* and *P. aeruginosa* and BHI broth (10 mL) for *E. faecalis*, and subsequently incubated for 16 – 18 h at 37°C in an orbital shaker (120 rpm). A 2 mL aliquot of overnight culture was then centrifuged at 3000 × *g*, and washed (three times) in SFW (141) to remove any trace nutrients from the NB. This was then adjusted to an optical density of 0.1 OD_{600nm} and further diluted by 100×. Finally, an aliquot of the diluted suspension was added to the sterile SFW to achieve a final density of approximately 1000 CFU 100 mL^{-1}.

2.4 Environmentally derived fresh water

Environmentally derived surface water was used in this study to provide a freshwater medium that would better represent the physiochemical conditions for the upscale applications of BTS. Surface water held in an urban drainage pond located at N 51°29′56″, W 2°32′39″ (Figure 2.1) was used as a source of biologically contaminated fresh water in this study. This urban pond was engineered as part of a multi-step stormwater control system, which is designed to remove pollutants from urban runoff and dampen urban stormwater hydrographs (142). This location was chosen because it receives regular organic and microbiological input from the surrounding environment and animals, and is artificially aerated, providing an oxygen rich environment. Moreover, the use of this

sampling location allowed sample integrity to be preserved due to the proximity to the sampling facilities. The biological quality of the environmental water (Figure 2.1) was recorded a minimum of 3 times per month between the years 2021 – 2022 using two indicator organisms, *Escherichia coli* and enterococci.

Figure 2.1: Sample site for environmentally-derived fresh water. *Surface water was held in and extracted from an urban drainage pond. This on the site of the University of the West of England, Bristol: N 51°29′56″, W 2°32′39″.*

2.5 Humic substance extraction and quantification

2.5.1 Soil preparation

Soil (2.4 kg) was taken from the top 10 cm of the riparian zone surrounding the environmentally-derived surface water source [see section 2.4]. The soil was baked at 100°C for 24 h and was found to have a moisture content of 1.03 L. The dried soil (1.36 kg) was then passed through a 2.0 mm sieve.

2.5.2 Extraction method

Prepared soil was added to NaOH (0.5 M) at a ratio of 100 g to 1 L (1:10) respectively into conical flasks (3 × 1 L) sealed with parafilm. This was allowed to incubate at 30°C for 24 h in an orbital shaker at 100 rpm. Following incubation, the supernatant was extracted into sterile media bottles (3 × 1 L) and centrifuged to remove any remaining suspended solids. This extraction yielded 3 L of humic substances (HS). HS (2 L) were then neutralised using concentrated H_2SO_4 (95%; Fisher Scientific, UK). The pH was equilibrated to the equivalent of the environmental water (pH 7.6). Absorbance (254_{nm}) and Total organic carbon (TOC) were recorded and presented in chapter 3 [see section 3.2.3].

2.5.3 Humic/fulvic acid separation

The remaining HS (1 L) were further separated into two components: Fulvic acids (FA) and Humic acids (HA). HS were acidified using H_2SO_4 to precipitate the Humic acids. This left fulvic acids in solution. Once acidified, the solution was left to settle for 12 – 18 h. The HS were then separated via centrifugation (3000 × *g* for 5 mins) whereby the supernatant contained the FA and the pellet contained HA. The FA and HA were separated, and the pellet was washed with deionised water to remove residual supernatant. Once separated, the FA were neutralised to pH 7.6 using 0.5 M NaOH (Fisher Scientific, UK). The HA were dissolved by the addition of 0.5 M NaOH and left to incubate for a further 24 h at room temperature. After incubation, the HA were then neutralised using $H_2SO_4$to pH 7.6.

2.5.4 Specific Ultraviolet Absorbance (SUVA)

An aliquot (20 mL) of HS, FA, HA and environmentally-derived fresh water was filtered through a syringe filter (0.45 µm) for absorbance and DOC analysis. A doubling dilution series of HS and pond water was used to generate a calibration curve. FA and HA were diluted 1 in 10 before absorbance and DOC analysis.

2.6 Design, setup and the maturation of test biofilm treatment systems

2.6.1 Filter media

The filter media utilised in all experiments comprised of porous, expended ceramic spheres. All filter media that was used in this study was characterised using the following method defined by McKie *et al.*, 2019 that describes the calculation of bed porosities of biological filters:

$$\varepsilon = 1 - \frac{M_p}{\rho_P A L_0}$$

Equation 2.1: Bed porosity calculation for biological filters adapted from McKie *et al.* (69)

The components of the equation correspond to the following system parameters: M_p = total mass of particles in bed (g) ρ_P = particle density (g $m^{3\ -1}$) L_0 = fixed-bed depth (m), A = cross-sectional area of the empty column (m^2). This equation was used to determine the bed porosity of both the laboratory and up-scaled biofilm treatment systems used in this study.

Unless otherwise stated, the ceramic filter media used had a particle size of 20 ± 1 mm and density of 1.16 g mL^{-1} and was sterilised before use (see Table 2.2). To determine the particle density, 16 ± 2 g of surface-dry ceramic filter media was added to water in a 250 mL graduated cylinder such that water displacement could be observed. Ten replicates were performed to calculate an average particle density which was then used to calculate unitless the bed porosity.

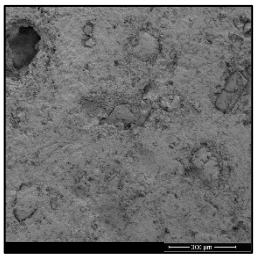
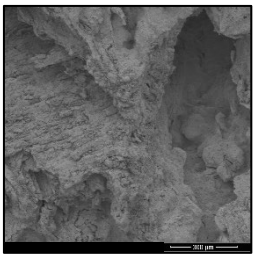
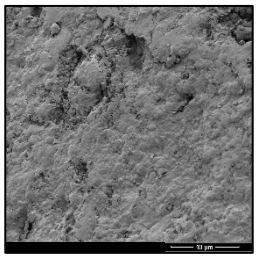
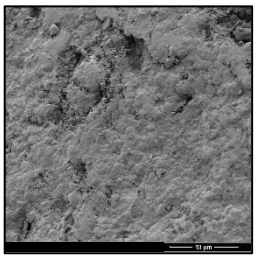
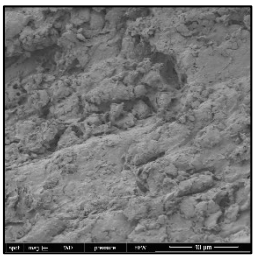
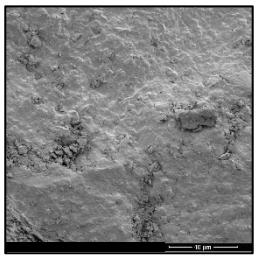
Table 2.2: Physical properties of ceramic substrate and biofilm treatment systems properties developed for the study for establishing environmental biofilms for water treatment.

	Parameter			
	Image	Dimensions (W mm x H mm)	Density (g mL^{-1})	Bed Porosity (69)
Ceramic filter media that was used for all laboratory-scale experiments unless otherwise stated.		20 ± 1 × 20 ± 1	1.42 ± 0.06	0.65 ± 0.02
Ceramic filter media that was used for all upscale experiments.		20 ± 1 × 20 ± 1	1.42 ± 0.06	0.71 ± 0.01

2.6.2 Production of terracotta clay extrusion media

In addition to the ceramic filter media used for the main body of the work (Table 2.2), the production and development of three types of novel ceramic filter media manufactured in-house were investigated (section 3.2.6). This was done to replicate the production of filter media to be used in BTS at source, which could eliminate the need for complex supply chains. All three filter media types were fabricated using terracotta clay/slip with the internal properties determined by the organic burn out material that was incorporated into the clay before firing. The first filter media (FM1) was made using pure terracotta clay, which was well mixed prior to being extruded using a hydraulic extruder. The clay was extruded through a 3D printed die measuring 20 mm wide and then cut into 20 × 20 mm cylinders using a jig. The second filter media (FM2) was produced by mixing sawdust into the clay which was added to the clay at a ratio of 5:3 sawdust to clay and well incorporated, primarily by hand and then using an electric stand mixer. This mixture was allowed to rest over 24 hrs before extrusion. The third filter media (FM3) involved immersing 20 × 20 mm organic cellulose sponge into terracotta clay slip until fully saturated. All pieces were left until entirely dry, before firing in an electric kiln at 1100 °C. During the firing process, the sawdust and organic cellulose sponge is burnt off and the porous ceramic structure remains. The three media types had a mean density of 2.35 g mL^{-1} (FM1), 1.35 g mL^{-1} (FM2) and 1.78 g mL^{-1} (FM3) (See Table 2.3)

Table 2.3: Physical properties of three novel ceramic substrates and the resulting laboratory scale biofilm treatment systems properties developed for the study for establishing environmental biofilms for water treatment.

Parameter	*Media type*		
	FM1	*FM2*	*FM3*
Image			
Scanning electron micrograph (Mag. 100 ×)			
Scanning electron micrograph (Mag. 1000 ×)			
Dimensions (W mm x H mm)	16.7 ± 0.58 × 14 ± 0	17 ± 0 × 14.3 ± 1.53	18.8 ± 1.04 × 13.3 ± 0.58
Density (g mL^{-1})	2.23 ± 0.07	1.35 ± 0.05	1.91 ± 0.04
Bed Porosity (69)	0.58 ± 0.06	0.69 ± 0.01	0.65 ± 0.01

2.6.3 Laboratory scale test biofilm treatment systems

The laboratory test biofilters comprised a 500 mL Polyvinyl chloride (PVC) column (57 mm internal diameter, 160 mm length) containing 250 mL of ceramic filter media with a bed porosity value of 0.65 ± 0.01 [see section 2.6.1]. A total of 9 filter columns containing filter media were connected to individual circulation tanks (25 L) and were allowed to mature over a minimum period of 4 weeks to establish and achieve mature biofilters. Prior to the maturation process, the circulation tanks were filled with mains tap water and flocculated of residual chlorine via aeration for 24 h (termed recirculation water). Water taken from environmentally-derived surface water (250 mL) [see section 2.4] was then added to the tanks with a source of environmental microorganisms to provide a seed community for the formation of biofilm on the filter media and the detailed information of this community is displayed in Table 3.1. At the start of the maturation process, a flow rate of 35 mL min^{-1} through the filter column was maintained via a multi-channel peristaltic pump (Watson-Marlow 505S; Falmouth, UK). This ensured that the entire volume of water in the tank was recirculated through the filter column for every 12 h of operation. To further assist the maturation of the biofilm on the filter media, the recirculation water was supplemented with low levels of nutrients on a weekly basis using HS extracted from soil surrounding the environmental water source (50 mg $week^{-1}$) [see section 2.5] and NH_4Cl (Fischer Scientific, UK; 5 mg $week^{-1}$), over a four-week period.

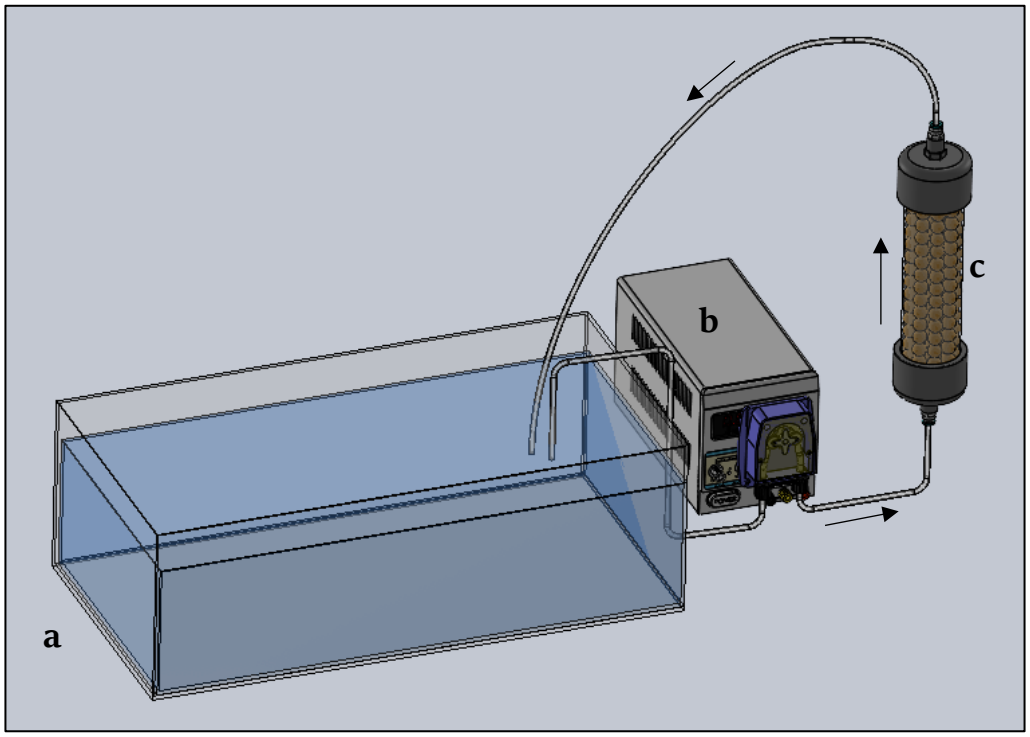


Figure 2.2 Schematic of the laboratory-scale biofilm treatment system using computer-aided design (CAD). The components are labelled (a) 25 L circulation tank (b) peristaltic pump (Watson-Marlow 505S; Falmouth, UK) at 35 mL min-1 and (c) clear polyvinyl chloride (PVC) column (57 mm internal diameter, 160 mm length).

2.6.4 Upscaled test biofilm treatment systems

To best represent the laboratory scale testing, the same materials, operational parameters, and medium were used where possible. The up-scaled biofilm treatment systems were setup in triplicate (n=3) and comprised of 15 L PVC filter vessels containing 10 L of ceramic filter media with a bed porosity value of 0. 70 ± 0.02 [see section 2.6.1] that was sterilised before use. The filter vessels were connected to 1000 L intermediate bulk container (IBC) via 25 mm medium density polyethylene (MDPE) pipe (Figure 2.2 and Figure 2.3). Water was circulated through the filter at a flow rate of 1.38 L min^{-1} maintained by a rotor pump (INTEX); this equates to the total volume of water recirculated through the filter vessel for every 12 h of operation. Prior to the maturation process, the IBCs were filled with mains tap water and flocculated of residual chlorine via aeration for 24 h (termed recirculation water). Water taken from environmentally-derived surface water (10 L) [see section 2.4] was then added to inoculate the tanks with a source of environmental microorganisms to provide a seed community for the formation of biofilm on the filter media. The recirculation water was supplemented with low levels of nutrients on a weekly basis using HS (50 mg $week^{-1}$) and NH_4CL (5 mg $week^{-1}$), over a four-week period.

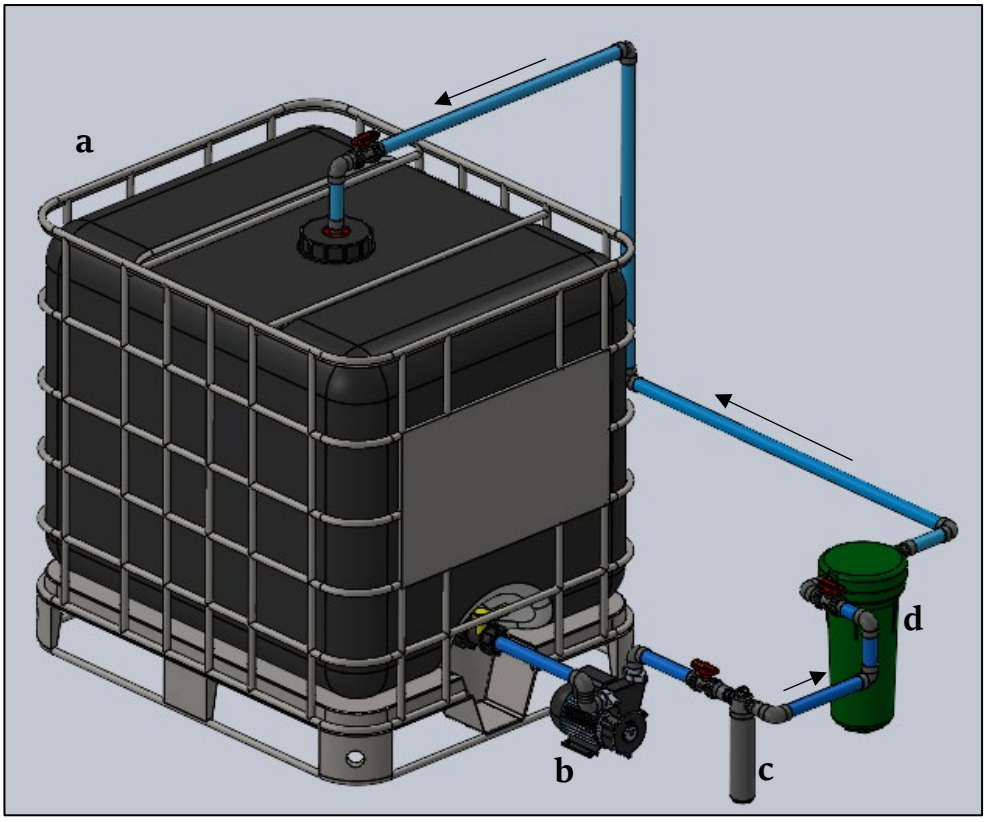


Figure 2.3 Schematic of the upscaled biofilm treatment system using computer-aided design (CAD). The components are labelled (a) intermediate bulk container (IBC) (1000 L), (b) rotor pump, (c) inline water heater and (d) filter vessel (15 L).

Figure 2.4 Image of upscaled biofilm treatment systems deployed on the campus of the university of the West of England.

2.7 Bacterial challenge of laboratory-scale biofilm treatment systems

The biofilm treatment systems were disconnected from the circulation tanks [see section 2.6.3], drained and re-connected to a double ported flask (3 × 1 L) containing the pathogen challenge monoculture [see section 2.3]. For the experimental runs involved in the optimisation of flow rate, *E. coli* was circulated through the biofilm treatment system at flow rates of 35, 69 and 138 mL min^{-1}. For the species investigation, the test pathogens were circulated through the treatment systems at a flow rate of 35 mL min^{-1}. To quantify the reduction of the test pathogens within the SFW, viable counts of *E. coli, E. faecalis and P. aeruginosa* were recorded over a 24 h duration when individually cycled through the biofilm treatment system and two control systems. The biofilm treatment systems were operated in circulation mode to monitor their efficiency to reduce a fixed microbiological load of the test pathogen monoculture and enable the direct comparison between experimental configurations and pathogen species. The SFW was sampled every 2 h for the first 12 h then every 4 h for the remaining 12 h equating to a total 24-hour experimental period; a 10 mL aliquot was taken from the SFW and enumerated by filtration through a Whatman® membrane filter (0.2 µm) and plating onto selective and differential agar [see section 2.1]. A control filter was employed containing sterile ceramic filter media that was pre-soaked with SFW and drained prior to use. An additional control, consisting of an empty 500 mL plastic filter column was used to account for any pathogen reduction resulting from the experimental apparatus. For each of the experimental configurations (empty filter column; column containing sterile filter media; biologically active filter) three separate experiments were undertaken in parallel. This process was then repeated on three independent occasions (9 filters in total). Within each independent

experimental run, all the microbiological sampling (and associated viable counting) was determined in triplicate.

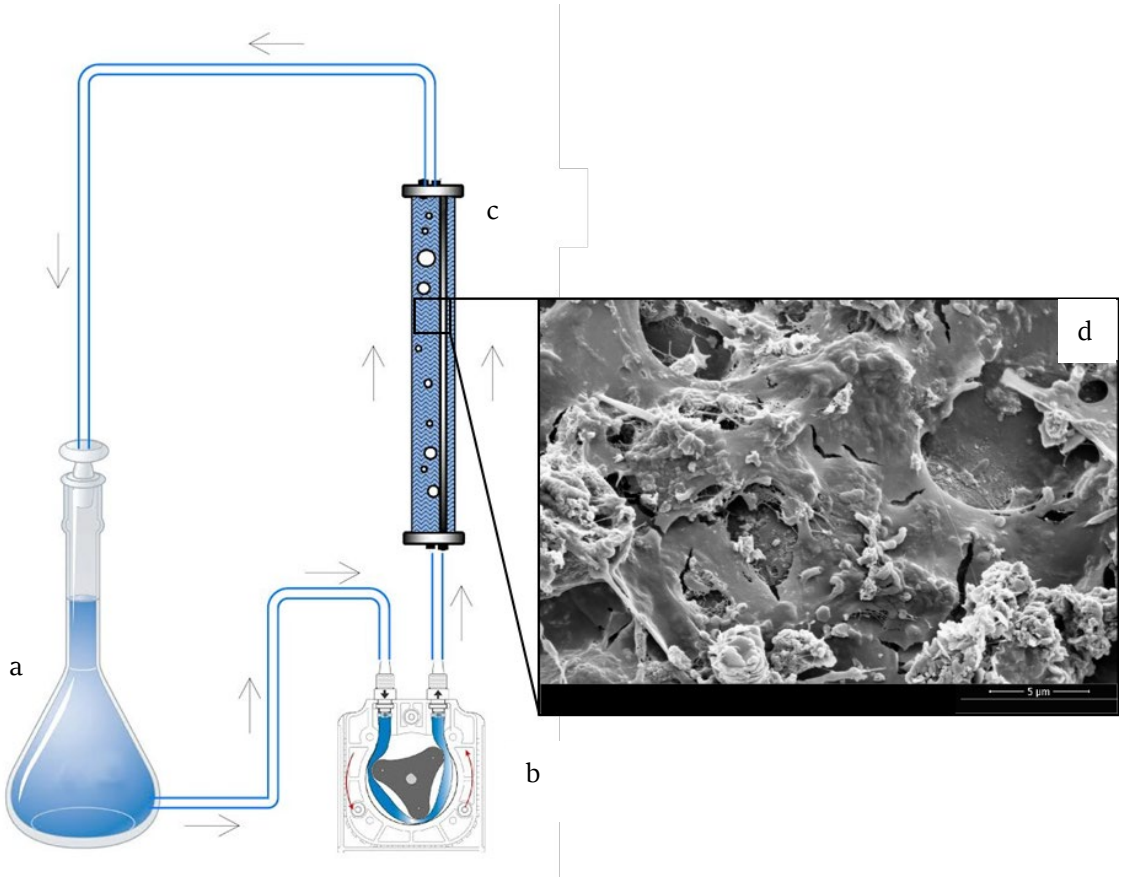


Figure 2.5: Schematic of the laboratory scale biofilm treatment system for monitoring pathogen survival. Arrows refer to water flow direction. (a) Liquid media bottle containing a simulated fresh water (1 L); (b) Peristaltic pump (35 mL min^{-1}); (c) Biofilter column containing the ceramic filter media on which a biofilm was established and (d) a scanning electron micrograph of the environmental biofilm established on the filter media (scale bar 5 µm).

2.8 The presence and viability of pathogens on the filter media

To determine if there was any accumulation or retention of the test pathogens on the biofilter media or sterile filter media following the 24 h circulation, viable counts of *E. coli*, *E. faecalis* and *P. aeruginosa* were taken from the filter media within the filter columns (Figure 2.4c). The filter media was removed from the column and suspended in 200 mL of phosphate buffered saline (PBS; Oxoid™, Basingstoke, UK), sonicated for 30 s and vortexed for 30 s (repeated 3 times). Aliquots of the suspension were enumerated by filtration through a Whatman® membrane filter (0.2 µm) and plated onto selective and differential agar [see section 2.1] to determine the number of test pathogens recovered per filter column.

2.9 Bioluminescence imaging

To further investigate the control of pathogens using biologically active filters, bioluminescent reporter strains of *E. coli* (Nissle 1917 pBBR1MCS-2 lite) and *P. aeruginosa* (PAO1 SE1 pBBR1 MCS5-lite) were used independently to continuously monitor the presence and metabolic activity of these

organisms within the double ported vessel during circulation. Filter-sterilised (0.2 µm) conditioned potable water (1 L) supplemented with NH_4Cl (35 mg L^{-1}), $NH_4H_2PO_4$ (28 µg L^{-1}) and CH_3COONa (7.6 mg L^{-1}), was inoculated with *E. coli* (Nissle 1917 pBBR1MCS-2 lite) at a density of 1 x 10^8 CFU mL^{-1} and circulated through the biofilters at 69 mL min^{-1} for 24 h. The emitted light produced by the test pathogens within the reservoir was continually detected using a low light photon counting camera (iXon EM-CCD, Andor Technologies, UK) controlled by proprietary software (iQ2, Andor Technologies, UK), using a photon integration time of 60 seconds every 15 minutes and a camera EM Gain of 300, over a period of 24 hours.

2.10 Analytical methods

All analytical methods were used on both the laboratory scale and upscaled BTS unless otherwise stated. Ion chromatography and total Carbon analysis were used to assess the concentration of ions in environmental water and circulation tanks.

2.10.1 Ion chromatography

Ion Chromatography (Metro HM, 850 Professional IC Anion) was used to quantify anions and cations of relevant nutrients of interest with regards to water quality and microbial growth; namely phosphate (PO_4^{3-}), nitrite (NO_2^-), nitrate (NO_3^-) and ammonium (NH_4^+). Samples were taken from the circulation tanks of both the laboratory scale and upscale BTS during maturation and the environmental water challenge were filtered through a 0.2 µm filter immediately after collection to prevent any further microbial processing. Aqueous samples were then loaded onto an auto sampler in open top tubes and auto-injected. For anion analysis, a sodium carbonate (3.2 mM L^{-1}) and sodium bicarbonate (1.0 mM L^{-1}) mobile phase was used throughout. Background conductivity of the mobile phase was suppressed by a cation exchanger and regenerated using a dilute sulphuric acid (150 mM L^{-1}) and oxalic acid (100 mM L^{-1}) solution. For cations, a nitric acid (0.7 mM L^{-1}) and dipicolonic acid (1.7 mM L^{-1}) mobile phase was used. IC certified standards (Fisher scientific) were used to generate standard curves from which the water samples were interpolated [see section 2.10.2].

2.10.2 IC standards preparation

Certified standards (Fisher Scientific, Loughborough, UK) were used to make working-standard solutions prepared in ultra-pure water (Sartorius Arium® Comfort) for anions (Phosphate; PO_4^{3-}, Nitrate; NO_3^-, Nitrite; NO_2^-, Sulphate; SO_4^- and Chloride; Cl^-) and for cations (Ammonium; $NH4^+$, Calcium; Ca^{2+}, Potassium; K^+, Sodium; Na^+, Magnesium; Mg^+). A range of standard concentrations for the production of a standard curve were prepared by diluting stock solutions to concentration ranges of 0 – 50 mg L^{-1}.

2.10.3 Sample storage validation

To assess the stability of water samples collected and stored for ion quantification, environmentally-derived water (1 L; Section 2.4) was collected and analysed periodically over the

course of one month. One batch of water was filtered through a 0.2 µm syringe-filter and the other unfiltered. All samples were stored at 4 °C until analysis.

2.10.4 Total carbon

Total carbon analysis (EnviroTOC, Elementar) was used to quantify the level total inorganic carbon (TIC) and total organic carbon (TOC) via a combustion catalytic oxidation method. Samples were collected and immediately filtered through a 0.45 µm millex syringe filter. Aqueous samples were then loaded onto an auto sampler into glass vials (40 mL) capped with foil and auto-injected.

2.10.5 Dissolved oxygen meter

An optical dissolved oxygen meter (HQ10, Hach, CO, USA) was used to record dissolved oxygen of environmental water used to circulate through the biofilters. The meter was calibrated before use using oxygen saturated air.

2.10.6 Scanning electron microscopy

Established biofilms grown on the filter media were visualised using scanning electron microscopy (SEM). Sterile ceramic filter media and ceramic media taken from a matured biofilm treatment system were fixed in 4 % glutaraldehyde for 1 h, followed by three 1 h washes in 0.1 M phosphate buffer. Following fixation, the filter media samples were then dehydrated through a series of washing steps, prepared in a 24-well plate. First, the samples were processed through 9 washing steps in 20, 30, 50, 70, 80, 90 and 100 % (×3) ethanol, each for 5 mins. The samples were then further dehydrated 3 times (× 5 min), in a mix of hexamethyldisilane (HMDS) and 100 % ethanol and finally 3 × 5 min washing steps of 100% HMDS. The samples were then removed from the HMDS and placed in filter paper in a sterile petri dish and allowed to air dry in the safety cabinet. Each individual sample was then mounted onto a stainless-steel stub, gold splutter coated and inserted into a stub holder on the cooler stage. The SEM (FEI Quanta FEG 650) used the Everhart Thornley detector for all imaging. The samples were analysed with an acceleration voltage of 5.00 kV and spot size 3.0.

2.11 Microbial DNA purification

2.11.1 Sample preparation

For environmental water samples, water (250 mL) was filtered through a 0.2 µm Whatman® membrane filter. The filter was then transferred to a sterile petri dish, cut into small squares, and added into a Zymo BashingBead™ lysis tube. For filter media samples, ceramic spheres (×3) were placed in centrifuge tubes (50 mL) and suspended in 20 mL of PBS. The biofilm was extracted from the ceramic spheres using sonication (FB15050; Fisherbrand) for 1 minute and vortexing at maximum speed for 30 seconds, this was repeated 3 times. The supernatant was filtered through a 0.2 µm Whatman® membrane filter. The filter was then transferred to a sterile petri dish and cut into small squares. The small sections of filter paper were then transferred into a BashingBead™ lysis tube (ZymoBIOMICS™; section 2.11.2 and 2.11.3).

2.11.2 ZymoBIOMICS™ miniprep DNA extraction

Samples prepared from the environmental water were extracted using ZymoBIOMICS™ Miniprep D4300 Kit (Cambridge Bioscience Ltd, Cambridge). Lysis Solution (750 µL) was added to the BashingBead™ Lysis Tubes that contained the prepared sample. The tube was then secured in a bead beater fitted (Disrupter Genie®) with a 2 mL tube holder assembly and was processed for 30 min at max speed. The lysis tubes were then centrifuged in a microcentrifuge at 10,000 × *g* for 1 minute. The supernatant (400 µL) was then transferred to a Zymo-Spin™ III-F Filter in a collection tube and centrifuged at 8000 × *g* for 1 minute. The filter was discarded, and DNA Binding Buffer (1.2 mL) was then added to the filtrate in the collection tube. The filtrate was then added to a Zymo-Spin™ IICR Column in a collection tube and centrifuged at 10,000 × *g* for 1 minute. The filtrate was then discarded and the Zymo-Spin™ IICR was transferred to a new collection tube. Then DNA Wash Buffer 1 (400 µL) was added to the Zymo-Spin™ IICR Column and centrifuged at 10,000 × *g* for 1 minute, the filtrate was discarded. DNA Wash Buffer 2 (900 µL) was added to the Zymo-Spin™ IICR Column and centrifuged at 10,000 × *g* for 1 minute and the filtrate was then discarded. The Zymo-Spin™ IICR Column was then transferred to a clean 1.5 mL microcentrifuge tube and DNase/RNase Free Water (100 µL) was added directly to the column matrix and incubated for 1 minute. The Zymo-Spin™ IICR Column was then centrifuged at 10,000 × *g* for 1 minute. The filtrate was then transferred to a Zymo-Spin™ III-HRC Filter (prepared as per manufacturers' instruction) in a clean 1.5 mL microcentrifuge tube and centrifuged 16,000 × *g* for 3 minutes. The extracted and filtered DNA was subsequently utilised for PCR and other downstream applications [see section 2.13.1].

2.11.3 ZymoBIOMICS™ microprep DNA extraction

Samples prepared from the environmental biofilm were extracted using ZymoBIOMICS™ Microprep D4301 Kit (Cambridge Bioscience Ltd, Cambridge). Lysis Solution (750 µL) was added to the BashingBead™ Lysis Tubes that contained the prepared sample. The tube was then secured in a bead beater fitted (Disrupter Genie®) with a 2 mL tube holder assembly and was processed for 30 min at max speed. The lysis tubes were then centrifuged in a microcentrifuge at 10,000 × *g* for 1 minute. The supernatant (400 µL) was then transferred to a Zymo-Spin™ III-F Filter in a collection tube and centrifuged at 8000 × *g* for 1 minute. The filter was discarded and DNA Binding Buffer (1.2 mL) was then added to the filtrate in the collection tube. The filtrate was then added to a Zymo-Spin™ IC-Z Column in a collection tube and centrifuged at 10,000 × *g* for 1 minute. The filtrate was then discarded and the Zymo-Spin™ IC-Z was transferred to a new collection tube. Then DNA Wash Buffer 1 (400 µL) was added to the Zymo-Spin™ IC-Z Column and centrifuged at 10,000 × *g* for 1 minute, the filtrate was discarded. DNA Wash Buffer 2 (900 µL) was added to the Zymo-Spin™ IC-Z Column and centrifuged at 10,000 × *g* for 1 minute and the filtrate was then discarded. The Zymo-Spin™ IC-Z Column was then transferred to a clean 1.5 mL microcentrifuge tube and DNase/RNase Free Water (20 µL) was added directly to the column matrix and incubated

for 1 minute. The Zymo-Spin™ IC-Z Column was then centrifuged at 10,000 × *g* for 1 minute. The filtrate was then transferred to a prepared Zymo-Spin™ II-µHRC Filter (prepared as per manufacturers' instruction) in a clean 1.5 mL microcentrifuge tube and centrifuged 16,000 × *g* for 3 minutes. The extracted and filtered DNA was subsequently utilised for PCR and other downstream applications [see section 2.13.1].

2.12 DNA Quantification

2.12.1 Thermo Scientific™ Nanodrop 1000

The concentration, yield and relative purity of extracted DNA was assessed by ultraviolet spectroscopy. Measurements of absorbance at 230, 260, 280 and 320 nm were performed using a micro-volume spectrophotometer (Nanodrop 1000, Thermo Scientific, UK). The A260/A280 ratio was employed as an estimate of DNA purity. Ratios were calculated post subtraction of the A320 value which represents absorbance caused by non-nucleic acid sample turbidity. A ratio of ≥ 1.8 was considered as acceptable for use in downstream applications.

2.12.2 Qubit™ assay

The quantity of DNA extracted and purified from biofilm samples was assessed using the Invitrogen Qubit broad range assay. This assay utilises target-selective dyes that emit fluorescence when bound to DNA, enabling DNA quantification in the range of 0.2 – 4000 ng μL^{-1}.. A Qubit working solution was prepared as per manufacturers instruction with the addition of a 5 µL sample of purified DNA. This was then vortexed and incubated at room temperature for 2 minutes before analysis. Using generated Qubit broad range standard solutions were used to generate a standard curve from which the samples were interpolated The standards and samples were analysed using the Qubit™ 4 Fluorometer (Thermo Fisher Scientific).

2.13 Single gene-based sequencing

All DNA extracted from the environmental water and biofilm samples was sequenced using a proprietary synthetic full-length based method (Loop Genomics, San Jose, CA, USA). The LoopSeq protocol uses unique molecular barcoding labelling of individual 16S genes. This unique molecular barcode is evenly distributed throughout the gene and leads to fragmentation of the 16S gene. The barcoded 16S gene fragment sequences enable sequencing by short-reads on an Illumina sequencing platform, with subsequent reconstruction of the full-length 16S genes. Therefore, all the hypervariable regions (V1–V9) can be identified and analysed through the sequencing of the entire 16S gene. The libraries were read on an Illumina NovaSeq 6000 sequencer (Illumina, San Diego, CA, USA), using a paired-end 2 × 150 bp reading system. The short-read raw data were collected in real-time on Illumina's BaseSpace, which generates FASTQ file and then were uploaded to the Loop Genomics unique analytic pipeline.

2.13.1 Loop genomics synthetic full-length sequencing

The sequencing raw data (2 × 150 bp PE, NovaSeq, Illumina) were transferred to the Loop Genomics unique barcode identifier cloud. This is a data analysis pipeline that is used for the low-quality base trimming, the unique sample barcode demultiplexing, and synthetic long-read reconstruction. The demultiplexing and synthetic long-read reconstruction is a process that enables the de novo assembly to the full-length 16S long-read data after rearranging the short-leads tagged with the same unique barcode.

2.13.2 Bioinformatics and statistical analysis

FASTQ files were taken and analysed using two different post processing methods. FASTQ files were uploaded to the EzBioCloud database (143) and processed using EzBioCloud data centre generating Microbiome Taxonomic Profiles (MTPs) for comparative and statistical analysis.

A subset of the samples were also processed using the QIIME2 next-generation microbiome bioinformatics pipeline for comparative study (144) and all raw input data were transformed in the form of QIIME2 artifacts (.qza format), which contain information about the data types for inputs into QIIME2 pipeline for further downstream processing. From raw sequences data, the amplicon sequence variants (ASVs) were obtained using the Divisive Amplicon Denoising Algorithm 2 (DADA2) within QIIME 2 plugin, which detects and corrects amplicon errors and filters out the potential base error and chimeric sequences (145). The output FASTQ files were filtered, trimming and dereplicating, and then DADA2 (R 1.14.1v) was applied.

After the sequences had been quality checked and filtered, Alpha and beta diversity analysis plugins were applied, and the remaining sequences were processed within the qiime 2 and EZBioCloud pipeline for analysis. Alpha diversity statistics were performed on selected MTP data sets to record species richness and evenness with $p < 0.05$ regarded as significant. Beta diversity analysis using a permutational multivariate analysis of variance (PERMANOVA). The output data was displayed in principle coordinate analysis plots and Unweighted Pair Group Method with Arithmatic Mean trees. Differences between data sets were determined using Linear discriminant analysis Effect Size (LEfSe) which was able to determine specific bacterial species/genus that were responsible for any significance observed.

2.14 Statistical analysis

Statistical analysis of all data was conducted using GraphPad Prism version 9.2.0 for Windows (GraphPad Software, San Diego, CA, USA). Multiple t-tests and one-way ANOVA with Dunnett's multiple comparisons test were used where appropriate, with $p < 0.05$ regarded as significant. All low light photon count images were analysed post-capture using Image J (132), and results reported as mean Relative Light Units (RLU).

Chapter 3 The Development of Laboratory and Up-Scaled Biofilm Treatment Systems

3.1 Introduction

The function of what is commonly referred to in the literature as "biological filtration" is underpinned by the formation and development of microbial biofilms that can be adhered to a solid phase substrate or "filter media". However, much about how this function that relates to the heterogeneity of the biofilm remains unclear and could still be considered a "black box" in some respects. As such, the use of model treatment systems is required in research to help investigate the mechanisms and operating conditions which could enhance the biological treatment of drinking water supplies. This research has included advanced techniques such as microbial community or metagenomics analyses to characterise the composition and structure of the microbial community (69,78,147). These methods have provided greater insights into microbial communities and their function in drinking water treatment which is discussed in more detail within chapter 4. However, many of these experiments are conducted at a laboratory scale which comes with a compromise. While the best solution for the research on biological treatment systems (referred to in this study as biofilm treatment systems; BTS) is to be performed at scale, upscaled and/or pilot scale treatment systems are often used to acquire detailed information required to design and implement full-scale water treatment systems. However, the use of upscaled systems is not always feasible due to the cost of infrastructure, operation, and maintenance (148). Therefore, smaller, laboratory scale treatment systems are widely utilised. However, there are uncertainties surrounding the reproducibility of phenomena and processes when scaling from small laboratory setups to larger upscaled systems. Therefore, there is still a need for further research into the use of scalable, low-cost means to investigate processes of BTS.

When laboratory scale systems are employed, they typically are designed to match the empty bed contact time (EBCT) of the full-scale process, and the filter media size to best replicate treatment at scale. However, these parameters are not sufficient for laboratory scale BTS to accurately reflect that of upscale BTS performance. McKie *et al.* (69) demonstrates that there is a possibility of investigating more detailed biochemical parameters such as phosphatase and esterase activity, or the composition of EPS between laboratory scale and pilot-scale biofilters. Moreover, the composition of the microbial communities was analysed using 16S sequencing, demonstrating similarities to class level. However, the systems were investigated using identical, pre-treated source water. Moreover, the treatment systems that were investigated had been in operation for 3 years prior to testing and filter media was extracted from the pilot scale filters eliminating the biofilm maturation period. Therefore, a deeper understanding to the impact of treatment system scale on the microbial community remains open for further study in other treatment configurations.

Another important parameter within biological based treatment systems is the material on which the biofilm is established. This has important properties as the material will impact the cost and accessibility of the treatment system and may impact the treatment efficiencies. In this study the use of ceramics manufactured at source was investigated within the BTS. Clay (the raw material of ceramics) is abundant, widespread and inexpensive compared to other raw materials (149). Shale formations, which are mainly clay-size mineral grains, are the most abundant sedimentary rocks in the crust of the Earth (150). Sedimentary rocks make up 5% of the Earth's crust, however they cover about 80% of the surface of the earth and clays and shales form well over 40% of the sedimentary rocks (151). Clay and clay minerals can be modified with a variety of physical, chemical, and thermal treatments which allows for the production of a material with varying physical properties, such as its porosity. There are many different types of clay, and each type has its own properties. For example, the elasticity of clay when wet, enables this material to be shaped, moulded, and mixed with other loose materials easily which in turn, increases its versatility in potential applications. Due to the abundance of clay, it offers an affordable material that can be found all over the world, which is particularly beneficial for communities with low resources. Moreover, clay is a safe material to handle then as opposed to the production of synthetics, such as plastics, due to its non-toxicity, mineral and elemental composition. This means with reasonable measures such as ventilation, a safe working environment could be implemented for end users for the manufacturing of ceramics used within BTS at source and therefore was investigated in this study.

The biofilms that establish within the treatment vessels are complex systems composed of aggregations of heterogenous cells that accumulate on the substrate within the BTS. Biofilm communities, including but not limited to those that inhabit aquatic environments, have unique and emergent properties which are not found in free-living cells, including sustaining biogeochemical cycling processes of most nutrients found in water (96). Some of these emergent properties and the scale of BTS have been investigated such as EPS composition (69). However, during biofilm establishment and maturation, the formation and expression of these emergent properties can be shaped by environmental factors, such as nutrient conditions (152), species competition, and the synthesis and secretion of extracellular material (96). The expression of such emergent properties will be significant in the function of a BTS and requires further research using validated model treatment systems and therefore, the research aims of this chapter were to:

(i) Develop and characterise laboratory scale and up-scaled biofilm treatment systems monitoring biofilm development over 12-weeks using an environmental surface-water source as a seed culture for biofilm development. subpopulations

(ii) Characterise the morphology of filter media and biofilm during development, and to determine the microbial population of biofilms established within the treatment systems and their changes during development.

(iii) Manufacture and investigate the development and functionalisation of biofilms on ceramic media with three different surface properties to be used within laboratory scale biological treatment systems.

3.2 Results

3.2.1 The development of biofilms in biofilm treatment systems

To obtain an insight into the development process of biofilms established on ceramic media within BTS, biofilms were established in triplicate within laboratory and upscaled BTS on two independent occasions. The first BTS were matured in November 2021 (Biofilm treatment systems mark 1: BTS1) and the second in May 2022 (Biofilm treatment systems mark 2: BTS2). On both occasions, the systems were setup in an identical fashion [see section 2.6]. The only factor differentiating the two experimental runs was the time at which the environmentally-derived water, containing the microorganisms used to seed the biofilms, was collected. During the maturation of the environmental biofilms (maturation defined by the complete removal of ammonium from the reservoir over 24 hours), water quality parameters of circulation water were measured on a weekly basis over a period of 4 weeks. An additional 12-week sample was taken within the BTS2. These parameters included heterotrophic counts, cation, anion, and total carbon analysis. All data presented corresponding to the laboratory scale and upscaled biofilm treatment systems, unless otherwise stated, corresponds to BTS2. BTS1 is included within this chapter to observe and discuss the inter-reproducibility of the BTS used within this research.

3.2.2 Environmentally-derived freshwater source and biofilm treatment system start-up

To provide a seed community of microorganisms and aid in the formation of biofilm, environmentally-derived fresh water, that was known to contain environmental microorganisms, was added into the recirculation tanks at the start of biofilm development process. Water quality parameters of the source water (inoculum) were measured and are presented in Table 3.1. The presence of heterotrophic bacteria at a concentration of 4.58 ± 0.27 $Log_{10}CFU\ mL^{-1}$ was sufficient to provide the BTS2 with environmental microorganisms to aid the biofilm formation.

Table 3.1: Water quality parameters of environmentally derived surface water used to provide a source of environmental microorganisms for the maturation of environmental biofilms within the laboratory scale and upscaled biofilm treatment system mark 1 (BTS1) and biofilm treatment system mark 2 (BTS2) n=3 ± s.d.

Parameter	BTS1	BTS2
Presumptive *Escherichia coli* (CFU 100mL^{-1})	50 ± 14.1	170 ± 8.16
Enterococci (CFU 100mL^{-1})	70 ± 23.3	100 ± 57.2
Heterotrophic Plate Count (Log_{10}CFU mL^{-1})	3.99 ± 0.17	4.58 ± 0.27
Total Organic Carbon (mg L^{-1})	0.54 ± 0.08	1.07 ± 0.06
Total Inorganic Carbon (mg L^{-1})	9.83 ± 0.71	7.42 ± 0.66
Ammonium (mg L^{-1})	0.09 ± 0.04	0.04 ± 0.04
Nitrite (mg L^{-1})	0.96 ± 0.01	0.75 ± 0.01
Nitrate (mg L^{-1})	11.8 ± 0.17	6.53 ± 0.56
Phosphate (mg L^{-1})	3.41 ± 0.93	1.67 ± 0.07
ORP (mV)	306 ± 6.63	349.8 ± 9.25
pH	7.84 ± 0.09	8.13 ± 0.05
Conductivity (µS cm^{-1})	712 ± 6.02	674.6 ± 7.76
Total Dissolved Solids (mg L^{-1})	491 ± 8.58	470.8 ± 5.58

3.2.3 Dosing of carbon using humic substances extracted from soil

For much of this study, there was no access to a total carbon analyser. To overcome this issue, specific UV absorbance (SUVA) was used to estimate the concentration of DOC within the water of the biofilm treatment systems. This was achieved through the generation of a calibration curve using water samples that were quantified using DOC analyser at the Centre for Ecology and Hydrology, Wallingford, UK (UKCEH) with their corresponding absorbance at 254 nm. Two sample groups were taken, one using environmental water (section 2.4) and the other using humic substances (HS) (section 2.5). The resulting calibration curve produced positive correlations with the concentration of DOC against SUVA producing an R^2 value of 0.985 and 0.995 for the environmentally derived water and HS respectively (Figure 3.1a and b).

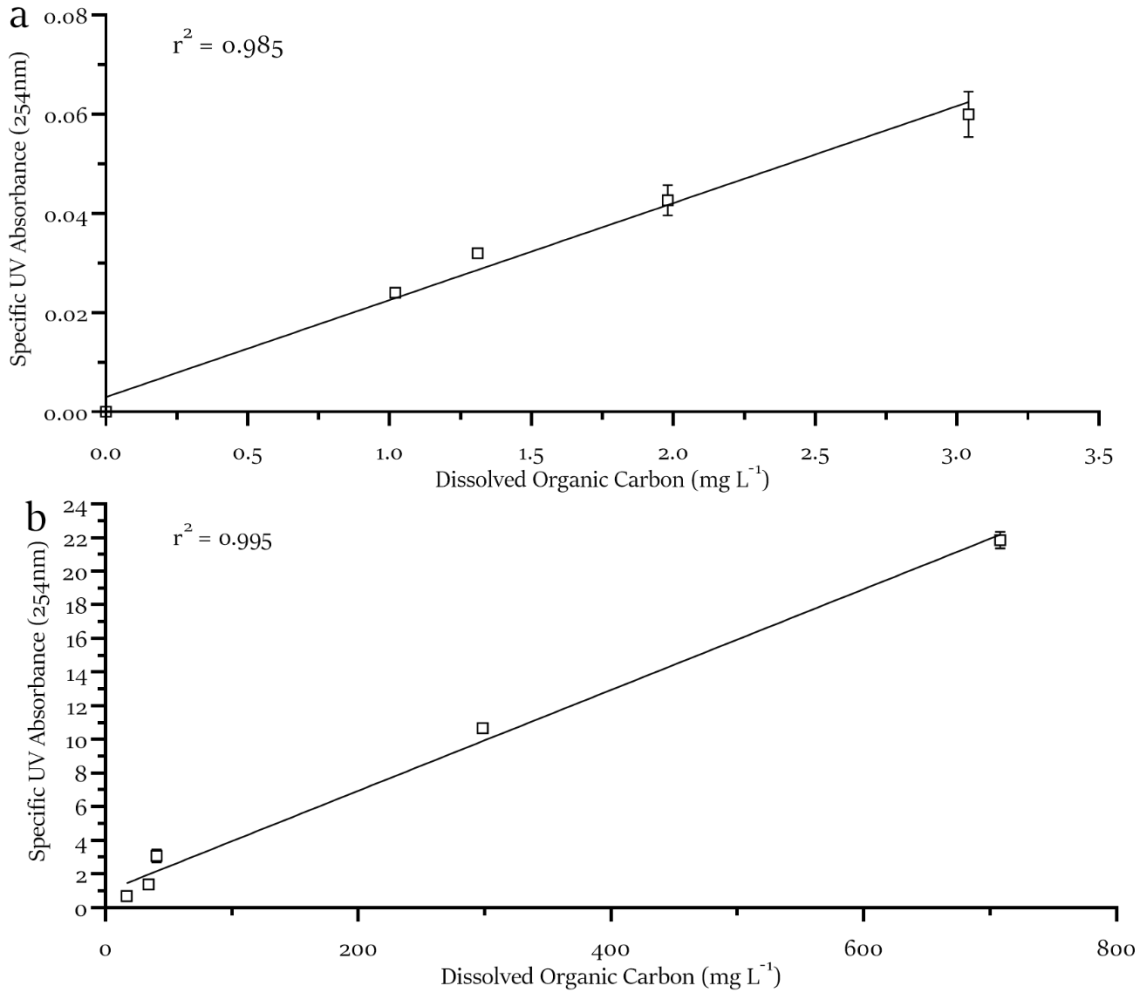


Figure 3.1: The specific ultraviolet absorbance (SUVA; 254nm) of increasing concentrations of Dissolved organic carbon (DOC) in (a) an environmentally-derived water source and (b) humic substances (HS) extracted from hyprian zone of the environmental water body. n=3 ± s.d.

3.2.4 Visualisation of the environmental biofilm

SEM was used to visualise the structure of the sterile ceramic filter media surface (Figure 3.2a and b) and filter media within the BTS to confirm the attachment of microorganisms after 14 days of circulation (Figure 3.2 b and c). Another upscaled BTS that had been in operation for 1 year was also imaged (Figure 3.2e and f) to demonstrate the ongoing successful colonisation of the filter media. Overall, the presence of bacteria within the biofilm was difficult to visualise during this process. Bacterial cells may be hidden within the EPS of the biofilm that is affected by the dehydration process for sample preparation. However, there were suspected fungal hyphae across the surface of the EPS (Figure 3.2f).

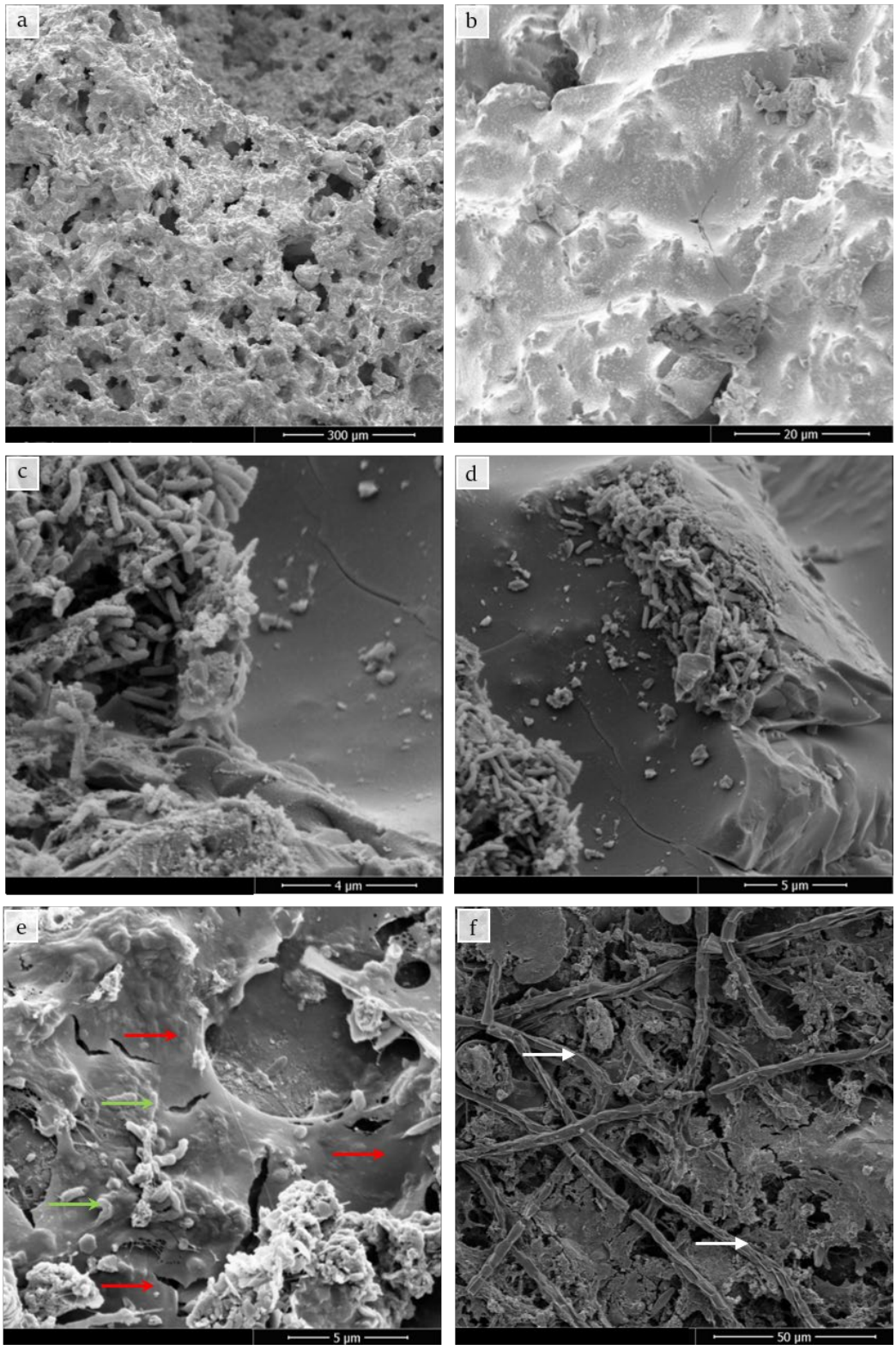


Figure 3.2: Scanning electron micrograph of (a) an overview of the ceramic filter media surface, (b) a closer view of the sterile ceramic surface filter media, (c) a micrograph of the internal structure of an established biofilm within the upscaled biofilm treatment system (BTS) at 14 days, (d) overview of an environmental biofilm attached to the ceramic filter media within the upscaled BTS at 14 days, (e) the suspected layer of extra cellular polymeric substances and (f) overview of a biofilm established on the filter media within an upscaled BTS after 1 year of operation. White arrow indicates potential fungal hyphae across the surface of the biofilm. Red arrows highlight a potential layer of dehydrated extracellular polymeric substances and green arrows represent suspected bacterial cells.

3.2.5 Heterotrophic counts recovered from the biofilm and reservoir of maturing biofilm treatment systems

At the start of the experiment, the addition of environmental water into the circulation tanks resulted in a final density of heterotrophic microorganisms (sampled using heterotrophic plate count (HPC) method) at 4.78 ± 0.22 $Log_{10}CFU\ mL^{-1}$ for the laboratory scale BTS and 4.21 ± 0.30 $Log_{10}CFU\ mL^{-1}$ for the upscaled BTS. The filter media was sterilised before the addition of the seed community to the circulation tanks, hence the density of HPCs was recorded as 0 $Log_{10}CFU\ mL^{-1}$. After 1 week of circulation of the laboratory scale BTS there were 4.19 ± 0.06 $Log_{10}CFU\ g^{-1}$ of heterotrophic microorganisms recovered from the filter media, which was significantly less than the 5.032 ± 0.06 $Log_{10}CFU\ mL^{-1}$ recovered from the circulation tanks ($p < 0.05$; Figure 3.3). Following the HPCs recorded at week 1, there was a significant increase to 5.20 ± 0.48 $Log_{10}CFU\ g^{-1}$ in the HPCs recovered from the filter media of the laboratory scale BTS ($p < 0.01$; Figure 3.3a). A similar trend was observed in the upscaled BTS, whereby a significant increase in the HPCs recovered from the filter media increasing from 3.90 ± 0.17 $Log_{10}CFU\ g^{-1}$ to 4.66 ± 0.14 $Log_{10}CFU\ g^{-1}$ ($p < 0.001$; Figure 3.3b). For the remainder of the sampling period within the laboratory scale BTS, no significant difference was observed between the circulation tanks and the filter media. However, there were significant differences observed in the upscale BTS for the first 4 weeks with a non-significant difference recorded at 12 weeks (Figure 3.3b).

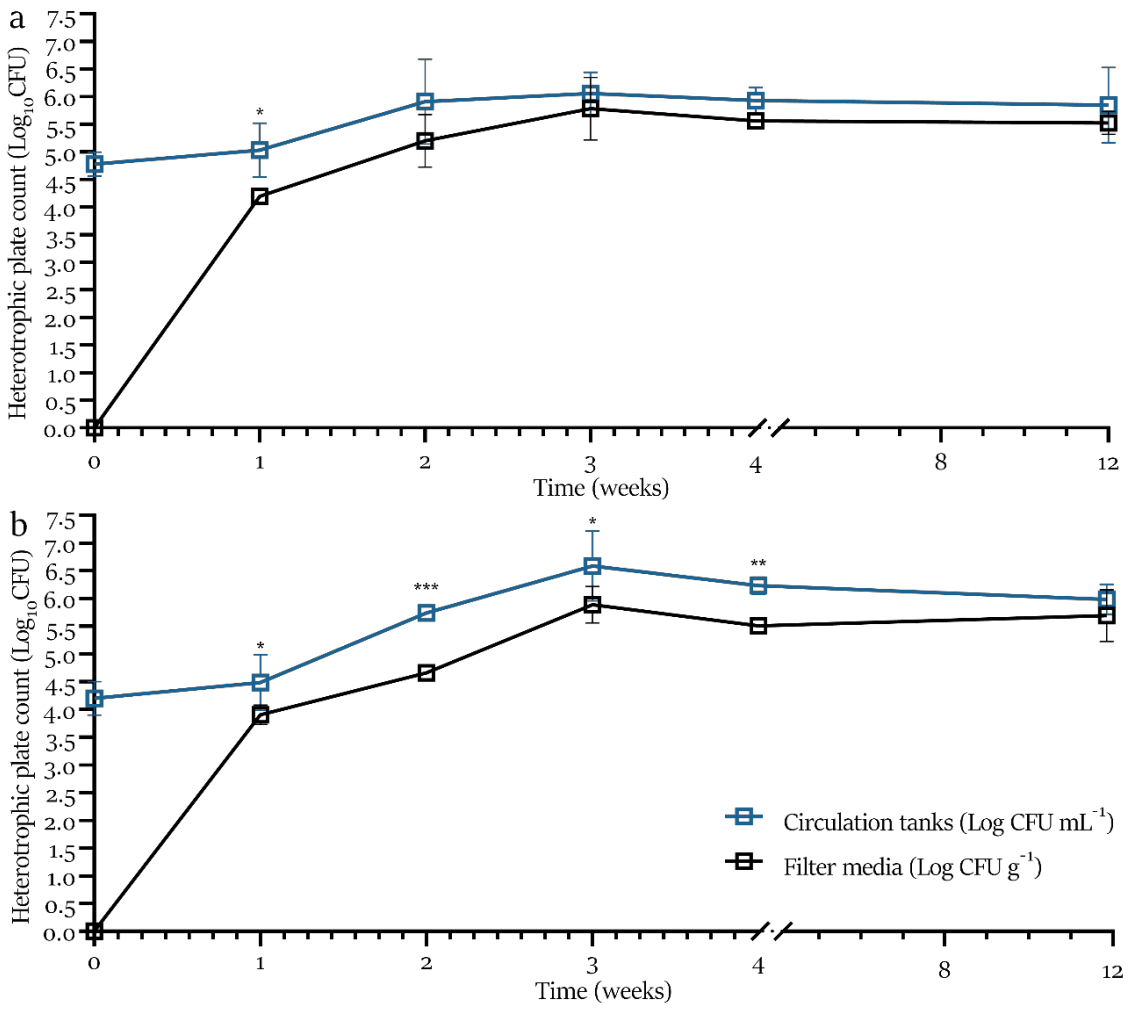


Figure 3.3: Heterotrophic plate counts recovered from the circulation tank and filter media of the (a) laboratory scale and (b) the upscaled biofilm treatment systems over a period of 12 weeks. Samples were taken from the biofilm and reservoir weekly for the first 4 weeks with a final sample at 12 weeks all performed in triplicate n=3 ± s.d.

3.2.6 Development of an environmental biofilm using three types of novel ceramic filter media within laboratory scale biofilm treatment systems

Preliminary work was undertaken to assess the effect of filter media porosity on the biofilm community. The number of viable heterotrophic microorganisms on 3 types of ceramic filter media investigated as a representative population for the comparison of biofilm maturation recorded over 4 weeks of circulation. After 1 week of circulation no significant differences between the three types of filter media were observed, 4.85 ± 0.36 $Log_{10}CFU\ g^{-1}$ was recovered from the FM1, 5.017 ± 0.37 $Log_{10}CFU\ g^{-1}$ for FM2 and 4.67 ± 0.23 $Log_{10}CFU\ g^{-1}$ for FM3 ($p > 0.05$; Figure 3.4). For the remainder of the sampling points over the circulation period, there was no significant difference between HPCs recovered from the three types of filter media of the laboratory scale BTS ($p > 0.05$; Figure 3.4). The laboratory scale systems reaching a final density of 6.85 ± 0.04 $Log_{10}CFU\ g^{-1}$ for FM1, 6.99 ± 0.03 $Log_{10}CFU\ g^{-1}$ for FM2 and 7.05 ± 0.05 $Log_{10}CFU\ g^{-1}$ for FM3 after 4 weeks of circulation ($p > 0.05$; Figure 3.5).

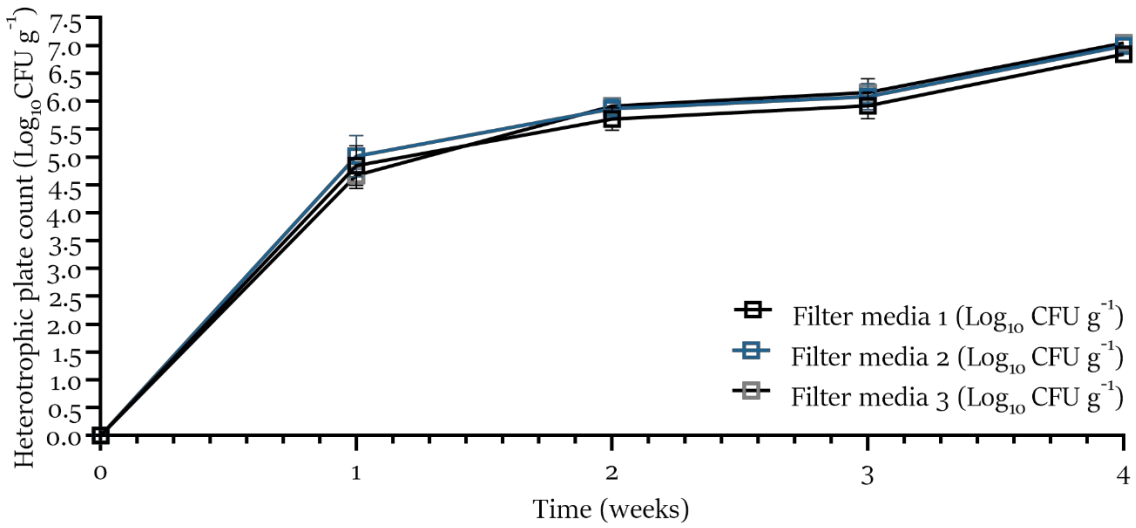


Figure 3.4: Number of heterotrophic plate counts recovered from the biofilm of the laboratory scale biofilm treatment systems containing three novel ceramic filter media (FM1, FM2 and FM3) over a 4-week maturation period n=3 ± s.d.

3.2.7 The comparison of the bacterial biofilm established within the laboratory scale and upscale biofilm treatment systems over a 4-week circulation period.

The number of heterotrophic microorganisms recovered from the filter media was compared between the laboratory scale and upscaled treatment systems over the circulation period. After 1 week of circulation, 4.19 ± 0.06 $Log_{10}CFU\ g^{-1}$ was recovered from the filter media of the laboratory scale BTS which was significantly greater than the upscale BTS with a density of 3.90 ± 0.17 $Log_{10}CFU\ g^{-1}$ ($p < 0.01$; Figure 3.5). However, for the remainder of the sampling points over the circulation period, there was no significant difference between HPCs recovered from the filter media of the laboratory scale BTS and the upscale BTS ($p > 0.05$; Figure 3.5). Reaching a final density of 5.52 ± 0.21 $Log_{10}CFU\ g^{-1}$ for the laboratory scale BTS and 5.69 ± 0.46 $Log_{10}CFU\ g^{-1}$ for the upscaled BTS after 12 weeks of circulation ($p > 0.05$; Figure 3.5).

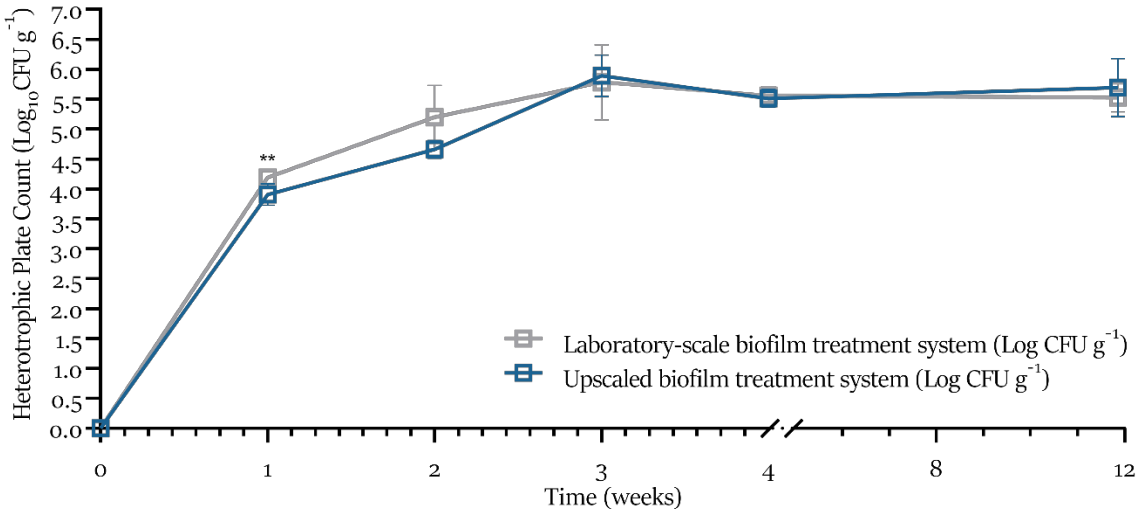


Figure 3.5: Number of heterotrophic plate counts recovered from the biofilm of the laboratory scale and upscale biofilm treatment systems over a 4-week circulation period n=3 ± s.d.

3.2.8 The comparison of bed depth during development of upscaled biofilm treatment systems

During biofilm development within the upscaled BTS, two replicate samples were taken from the top 200 mm (high) and bottom 200 mm (low) of the filter vessel and sampled for heterotrophic microorganisms. The number of heterotrophic bacteria recovered from the filter media at the high and low depths of the filter bed was not significantly different over the whole of the maturation period (Figure 3.6). Therefore, for all subsequent analysis, the samples were pooled to increase the n number per sample point.

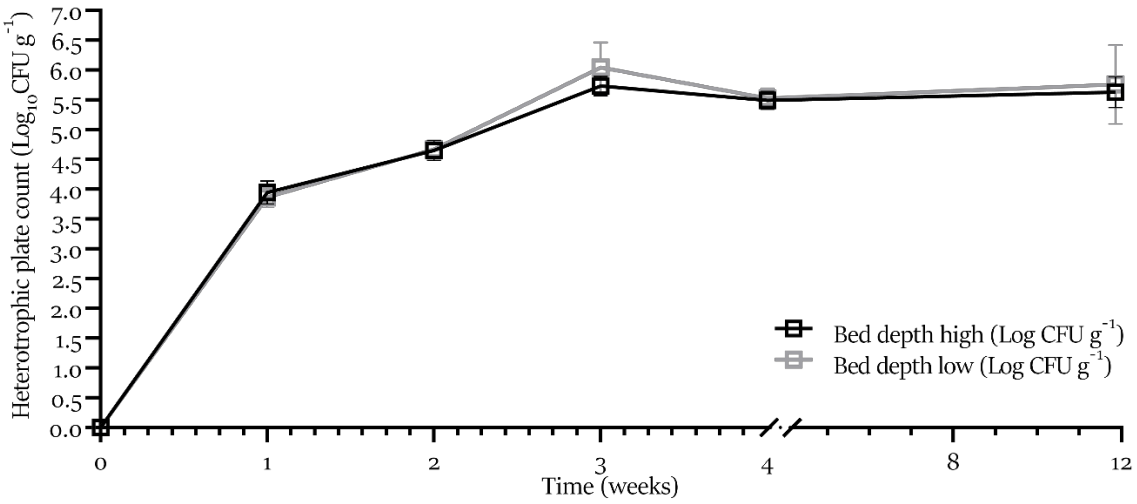


Figure 3.6: Number of viable heterotrophic bacteria recovered from the filter media of the top 200 mm and bottom 200 mm of the filter bed of the upscaled biofilm treatment systems over a 12-week sampling period, n=3 ± s.d.

3.2.9 Reproducibility of biofilm development within BTS

Environmental biofilms were established on the filter media within triplicate identical BTS on two independent occasions; systems were setup in November 2021 (BTS1) and May 2022 (BTS2). Significant differences in the total number of viable HPCs recovered from the biofilm between BTS1 and BTS2 were observed. After the first week of maturation, the number of viable HPCs recovered from the laboratory scale BTS1 was 3.44 ± 0.24 CFU g^{-1} which was significantly lower than 4.19 ± 0.07 CFU g^{-1} recovered from BTS2 (Figure 3.7a; $p < 0.001$). The HPCs for the laboratory scale BTS1 remained significantly lower than BTS2 for the remainder of the maturation period monitored (4 weeks). However, the difference between the laboratory scale BTS1 and BTS2 remained consistent averaging 1.03 ± 0.40 CFU g^{-1} over the 4-week circulation period with a final density of 4.79 ± 0.09 CFU g^{-1} recovered from BTS1 in comparison to 5.56 ± 0.13 from BTS2 (Figure 3.7a; $p < 0.001$). A similar trend was observed for the up-scaled BTS. After the first week of maturation there was 3.00 ± 0.35 CFU g^{-1} recovered from the BTS1 in comparison to BTS2, where 3.90 ± 0.17 CFU g^{-1} was recovered from the filter media (Figure 3.7b; $p < 0.001$) with a consistent difference over the 4 weeks averaging 0.68 ± 0.44 CFU g^{-1}. However, after 3 weeks of maturation there was no significant difference between BTS1 and BTS2. However, the final density of BTS1 was 4.78 ± 0.10 CFU g^{-1} which was significantly less than 5.51 ± 0.15 CFU g^{-1} for BTS2 (Figure 3.7b $p > 0.001$).

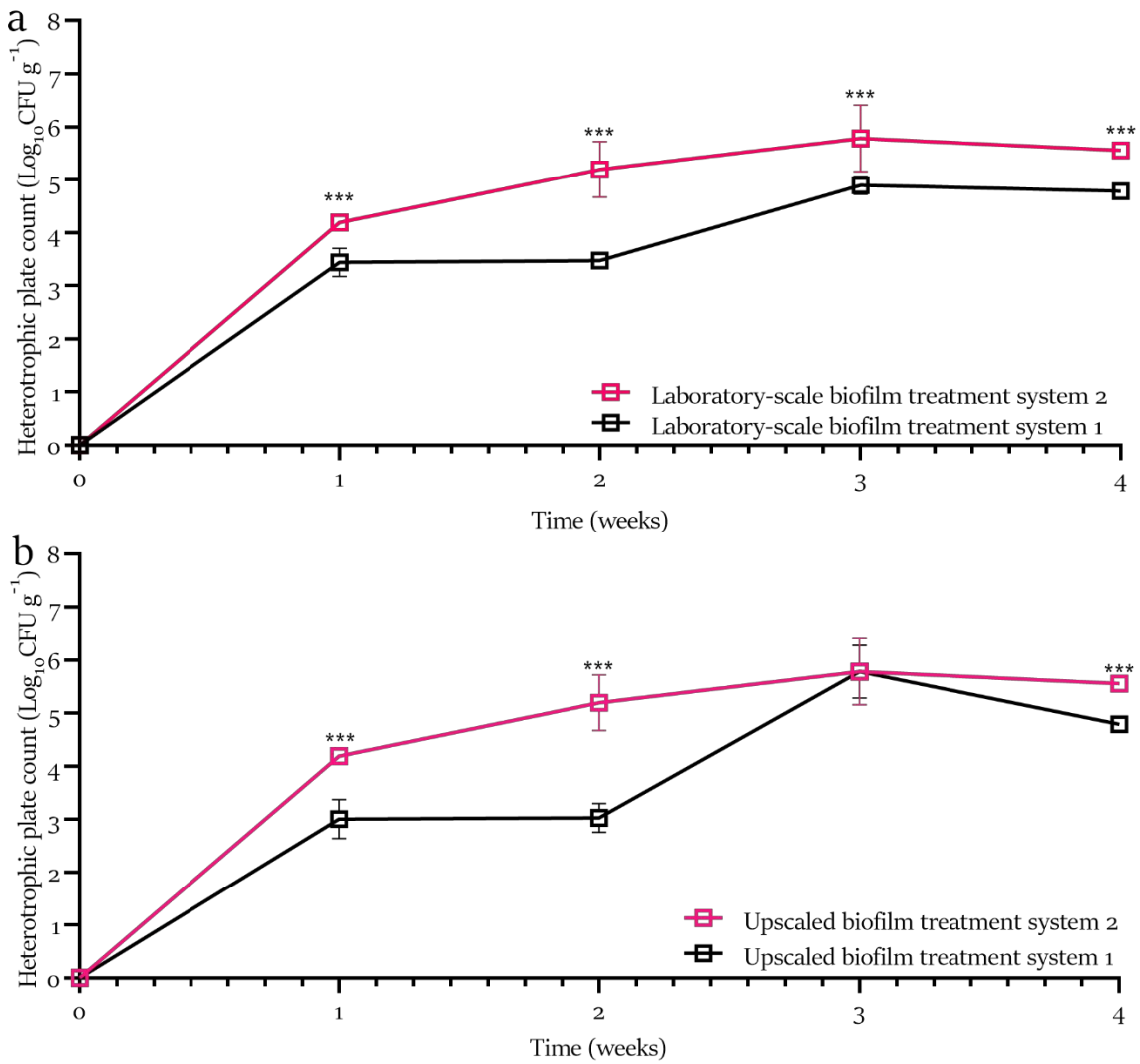


Figure 3.7: Heterotrophic plate counts recovered from filter media of (a) laboratory scale biofilm treatment systems (BTS) and (b) upscaled BTS over 4-weeks of circulation. Two experimental runs were performed on two independent occasions; BTS mark 1 was established in November 2021 (n = 3 ± s.d.) and BTS mark 2 was established in May 2022 n=3 ± s.d (*** = $p < 0.001$).

3.2.10 Nutrient analysis of BTS during maturation

To assess the function of the microbial community establishing within the laboratory scale BTS, in parallel with the density measurements, the degradation of ammonium was recorded on a weekly basis for 4-weeks. Ammoniun was dosed into the circulation tanks on a weekly basis to a concentration of 0.2 mg L^{-1} and the levels of ammonium were recorded every 2 h for 10 h total for the time of dosing with an additional reading at 24 h. There were reductions in ammonium observed from week 0 – week 4, with 100 % reduction obsereved after 4 weeks of circulation (Figure 3.8). The BTS were then deemed "mature" and therefore the maturation period in this study was defined as 4 weeks.

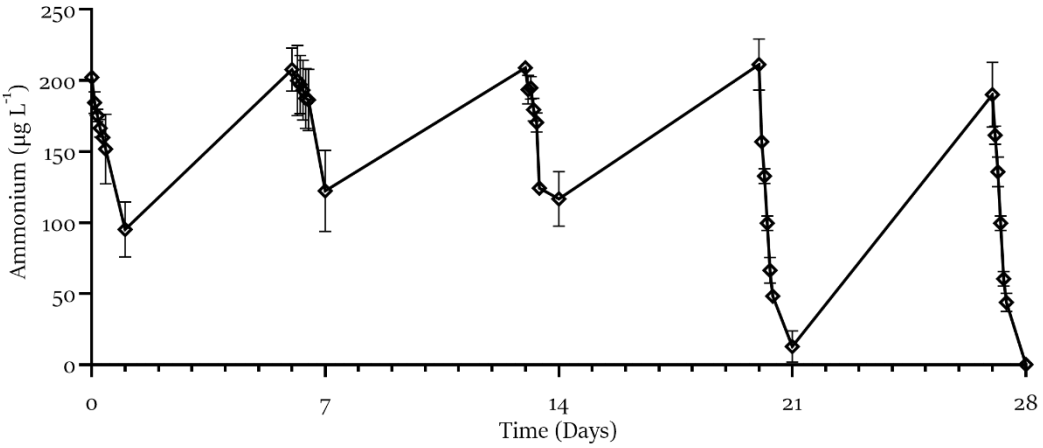


Figure 3.8 Ammonium concentration of the reservoir of the laboratory scale biofilm treatment systems over 4 weeks during circulation n = 3 ± s.d.

3.2.11 Spatial sampling of upscaled biofilm treatment systems currently deployed in long-term operation

To further investigate the characteristics of the biofilm that establishes within BTS, the reservoir and filter media were sampled from 6 unique filtration system within a 50-mile radius of the University of the West of England, Bristol [see Table 3.2]. This long term (> 1 year) deployment of BTS had been successful in achieving good water quality (low nutrient and low pathogen levels). These samples were also subject to single gene-based community analysis, and this is discussed in chapter 4. All the systems in operation had oligotrophic reservoirs with no detectable phosphate in all systems and very low levels of ammonium (0 – 0.016 mg L^{-1}). Very low concentrations of TOC and nitrite within the reservoir were found ranging from 0.497 – 1.53 mg L^{-1} and 0.24 – 0.74 mg L^{-1} respectively. The number of viable HPCs recovered from the biofilm only varied 1 Log_{10}CFU g^{-1} ranging from 6.09 ± 0.24 to 7.04 ± 0.17 Log_{10}CFU g^{-1}. To protect the exact locations of the systems, the coordinates provided are within 1 mile of the system.

Table 3.2: Water quality parameters of long-term up-scaled biofilm treatment systems.

Parameter	Site 1	Site 2	Site 3	Site 4	Site 5	Site 6
Location (GPS coordinates)	51°14'30.3"N 2°21'45.3"W	51°08'22.8"N 2°22'45.3"W	51°20'54.3"N 2°21'51.7"W	51°27'06.0"N 2°21'49.8"W	51°04'22.3"N 2°22'37.7"W	51°35'18.0"N 2°15'35.9"W
UK postal code area	BA11	BA10	BA2	SN14	BA9	GL9
Water reservoir volume (m^3)	90	95	110	90	85	80
Filter media weight (g)	14.9	14.5	17.4	15.3	14.2	12.4
Heterotrophic Plate Count ($Log_{10}CFU\ g^{-1}$)	6.22 ± 0.31	6.33 ± 0.05	7.04 ± 0.17	6.71 ± 0.01	6.09 ± 0.24	6.83 ± 0.42
DNA Yield (µg)	6.35	14.8	16.4	0.23	1.3	3.9
Total Organic Carbon (mg L^{-1})	0.617	0.497	0.715	1.53	0.872	0.761
Total Inorganic Carbon (mg L^{-1})	8.03	6.81	3.49	9.92	4.74	9.38
Phosphate (mg L^{-1})	>0.001	>0.001	>0.001	>0.001	>0.001	>0.001
Nitrite (mg L^{-1})	0.57	0.37	0.29	0.74	0.37	0.24
Nitrate (mg L^{-1})	45.7	11.7	6.97	5.97	21.8	1.76
Ammonium (mg L^{-1})	>0.001	0.008	>0.001	0.016	>0.001	0.016

3.3 Discussion

The results of this study demonstrate that overall, there was no significant difference between the number of viable HPCs recovered from the biofilm of the laboratory and upscaled BTS (Figure 3.5 & Figure 3.7; $p > 0.05$). These findings agree with the methodology outlined by McKie *et al.* (69) that could be used to test BTS performance at a reduced cost and complexity in comparison to those performed at scale. This is a method that further improves the accessibility of research that may otherwise be limited by the cost and infrastructure required for larger scale studies and this could have the potential to reduce costs of future research using laboratory scale BTS. The reduction of costs would result from lowering the maintenance requirements and higher throughput of laboratory scale BTS, with further operational parameters to investigate that would be unfeasible for upscale study. The results collected from laboratory scale BTS could help validate emergent properties of biofilms within BTS, such as the reduction of waterborne pathogens discussed in chapter 5, a phenomenon that was observed at laboratory scale. As such, the data presented here provides validation that laboratory scale BTS used in this study could be used to provide an insight

to the successful upscale of BTS for the development of low-cost systems for the treatment of drinking water supplies.

The start-up process of a BTS is critical because, to achieve functionality, an established microbial community on a substrate within the treatment system is required. Therefore, until this population is established, the process of biological treatment of water supplies is perturbed. The microorganisms that establish within BTS will enter the water from the surrounding environment or already exist naturally in the source water, however, it is also possible to aid this process through the addition of microorganisms into the system. In the case of closed, recirculating systems, the use of seed microbial communities has been used to help establish biofilms for nitrification in brackish water (153) and this was also utilised for the BTS investigated in this study. Moreover, the functionality of BTS may be determined by individual parameters and commonly, nitrification is used for recirculating systems (154–157) and was used in this study and presented in Figure 3.8. A seed community of microorganisms taken from an environmentally-derived fresh water source [see section 2.6] was used to aid in the establishment of the biofilm onto the filter media for both laboratory scale and upscaled BTS1 and BTS2 (Table 3.1). Microbial seed communities are also used at the scale of industrial and municipal water treatment systems. Commercially available inocula or seed cultures are commonly added to reduce the start-up time of BTS by providing the initial bacterial culture (158). The addition of a seed community has been shown to accelerate start-up of nitrifying in recirculating BTS (154). For this study, it was used to simulate the natural consortia that would likely be present at the system start up from an environmentally derived water source. This method was shown to be reproducible in laboratory and upscale BTS (Figure 3.5) however, the seed community did have a significant impact on the early stages of biofilm establishment that can be observed in Figure 3.7.

When comparing the development process of BTS1 and BTS2 with identical experimental setups, the data (presented in Figure 3.7) suggests that the starting density and/or consortia of microorganisms has an impact (at the very least) over the first 4-weeks of biofilm development. The starting density within the reservoir of BTS1 was significantly different from BTS2 and this is reflected by the number of HPCs recovered from the biofilm for each system (Figure 3.7). The decreased density is likely a result of the water temperature of the environmental water source, which was significantly less than BTS2 at the time BTS1 was established. Temperature, among other parameters, is known to impact on the survival of microorganisms and therefore, biofilm maturation (159,160). Nonetheless, both systems were able to establish a biofilm that significantly increased from week 1 to week 4 (Figure 3.7; $p < 0.05$). The temperature of the BTS1 and BTS2 were kept the same utilising inline water heaters, thus the difference in biofilm development is more likely linked to the starting consortia of microorganisms rather than the system conditions.

When comparing the number of heterotrophic bacteria recovered from the two sampling points within the upscaled BTS2, there was no significant difference observed between the top and the bottom of the upscaled BTS2 over the 12 weeks sampling period (Figure 3.6; $p > 0.05$) this suggests the biofilm growth was uniform upon the filter media throughout the whole filter vessel. It does not however, suggest that the microbial populations are the same. Sampling for HPCs is limited through the observation of culturable microorganisms using general purpose culture medium, of which it is thought to accounts for 1% of the sampling population (94). To increase the depth of information regarding a microbial population, particularly in environmental studies, the use of sequencing demonstrates that the number of viable cells does not indicate the level of diversity or indeed the composition of the sampling population. Therefore, to further investigate these differences, single gene-based community analysis was performed in parallel and presented in chapter 4.

The similarities observed between the laboratory and upscaled BTS suggest that the biofilm maturation process is reproducible under the operating conditions investigated, and that a biofilm population will perform to the desired function (defined in this study as ammonium removal) in water treatment processes. Because of this, 6 independent sites with installed recirculating upscaled BTS were sampled to investigate their biofilm and water properties. This spatial sampling of the upscaled BTSs that had been deployed long-term found that the average HPC recovered across all the biofilm samples was 6.54 ± 0.42 CFU g^{-1} (Table 3.2). This was significantly more than the average HPC recovery from the upscaled BTS at 5.69 ± 0.46 Log_{10}CFU g^{-1} after 12 weeks ($p < 0.001$). This suggests that the upscaled BTS used in this study may have the potential for further growth with a longer circulation time. Overall, under the specific parameters tested, the laboratory scale systems are comparable with the upscale systems. This implies that under specific conditions within a circulating BTS, the maturation of an environmental biofilm is predictable.

The substrate material was chosen in this study with chosen with consideration. As previously mentioned, due its porosity and abundance, ceramics have been used within water filtration around the world. Previous research has investigated the efficacy of ceramics for the physical removal of contaminants such as study compared ceramic, granular activated carbon and anthracite media for drinking water and found overall ceramic media has the potential to be an alternative to anthracite when considering biofiltration, especially during cold water conditions (T < 10 °C) (161). A meta regression study comparing different household water treatment methods in low to medium income countries (LMICs) found water filtration systems made from ceramics to be the most effective form in the long term (162). Furthermore, a more recent study which researched biofilm growth on ceramic filtration discs, found ceramic discs used without cleaning grew a layer of biofilm which helped with MS2 virus removal (163). The findings presented in this study demonstrate that biofilms established on ceramic substrates with different porosities did not have

a significant effect on biofilm density during maturation within the laboratory BTS (Figure 3.6; $p > 0.05$). Clay is an abundant and inexpensive resource and is easily modified through a variety of physical, chemical, and thermal treatments. The findings of this study raise the prospect of utilising biofilms as low-cost, locally manufactured systems for the control of pathogens in drinking water supplies using an abundant material (ceramics) at source. This negates the need for specialist materials or operational expertise and removes issues relating to complex supply chains.

Overall, the findings from this study demonstrate that a microbial community was able to establish on the ceramic substrate within both the laboratory scale and upscaled BTS. Furthermore, the analysis of biofilm heterotrophic densities demonstrates that there is no significant difference between the scale of the systems investigated. The microbial communities achieved function after 4 weeks of circulation defined by the complete removal of ammonium over a 24 h circulation period. This study was further investigated with the use of 16S rRNA sequencing to understand, in more detail, the microbial communities that established within the BTSs and is discussed in chapter 4. The systems characterised in this chapter were also used to investigate the potential control of waterborne pathogens in fresh waters presented in chapters 5 & 6.

Chapter 4 The Taxonomic Profiles of Biofilm Treatment Systems

4.1 Introduction

The treatment of water using biofilm treatment systems (BTS) will be influenced by bacterial community diversity and associated metabolic potential (164,165), but it has been demonstrated that different treatment processes and configurations such as pre-treatment methods or varying source water quality can cause changes within the microbial community that is established in biological based water treatment (78,166). For the work presented within this chapter, the microbial communities studied with the experimental BTS were introduced via the environmentally-derived water (seed culture) that was added to the recirculation tanks at the start of the BTS development stage [see section 2.6]. As stated in chapter 3, the BTS were deemed "mature" through the complete removal of ammonium within 24 h of circulation and therefore the maturation period was 4 weeks. The experiment was performed over 12 weeks, collectively referred to as the "circulation period". Biofilm communities that were established within the BTS were analysed using amplicon-based 16S full-length-based synthetic long-read sequencing (sFL16S) technology. Fingerprinting techniques have shown that the 10 – 50 most abundant taxa usually contribute more than 0.1 – 1.0% of the total cell counts; therefore, in agreement with other studies, the bacterial phyla and genera representing more than 1.0% of the total community are usually considered dominant taxa (167–169). Consequently, the microbial composition presented in this chapter is taxa consisting of greater than 1% of the observed community. The sequences in this study were processed using an operational taxonomic unit (OTU) clustering cut-off of 97% sequence similarity through the EzBioCloud pipeline (143) [see section 2.13.2]. Biofilm based treatment systems are still considered to be "black box" systems, whereby there is often limited knowledge about the specific microbial diversity leading to function. In particular, how their development (in terms of community composition) relates to the seed culture, what is the variability upon maturation, and what influence do specific operating parameters have on these processes. This chapter addresses these questions through the following research aims:

(i) Determine the community structure of the environmentally derived-water source used to seed the BTS.
(ii) Investigate the any changes in biofilm community with the BTS during development from sterile filter media.
(iii) Compare the microbial communities of the laboratory scale and upscaled BTS during development.
(iv) Assess the similarity of the microbial communities within the laboratory scale and upscaled BTS with functional independent long term deployed BTS.

4.2 Results

4.2.1 Microbial composition of environmentally-derived fresh water and environmental biofilms

The microbial community from the environmentally-derived water source that was used to seed both the laboratory scale and upscaled BTS was analysed. The observable microbial community of the environmentally-dervied surface water found that the most abundant phylum was Actinobacteria at 31.03 ± 0.21 % , followed closely by the Bacteroidetes at 29.4 ± 0.19 % and Proteobacteria at 29.7 ± 0.37 %. These were not significanly different between biological replicates (Figure 4.1a; $p > 0.05$). Moreover, the number of operational taxonomic units identifed within each of the environmentally-derived water samples exhibited little variation with a count of 1142 for sample 1, 1249 for sample 2, and 1224 for sample 3. The relative abundance of the class composition followed a similar trend with no signifcant differences between the sample replicates was observed between the classes above 1 % (Figure 4.1b; $p > 0.05$). The three most abundent classes were Actinobacteria_c at 26.6 ± 0.23 %, Betaproteobacteria at 15.2 ± 0.21 % and Flavobacteria at 13.4 ± 0.18 % (Figure 4.1b; $p > 0.05$). Alpha diversity nonparametric (np) shannon test found very similar values of 5.35, 5.39 and 5.35 for samples 1, 2 and 3 respectively.

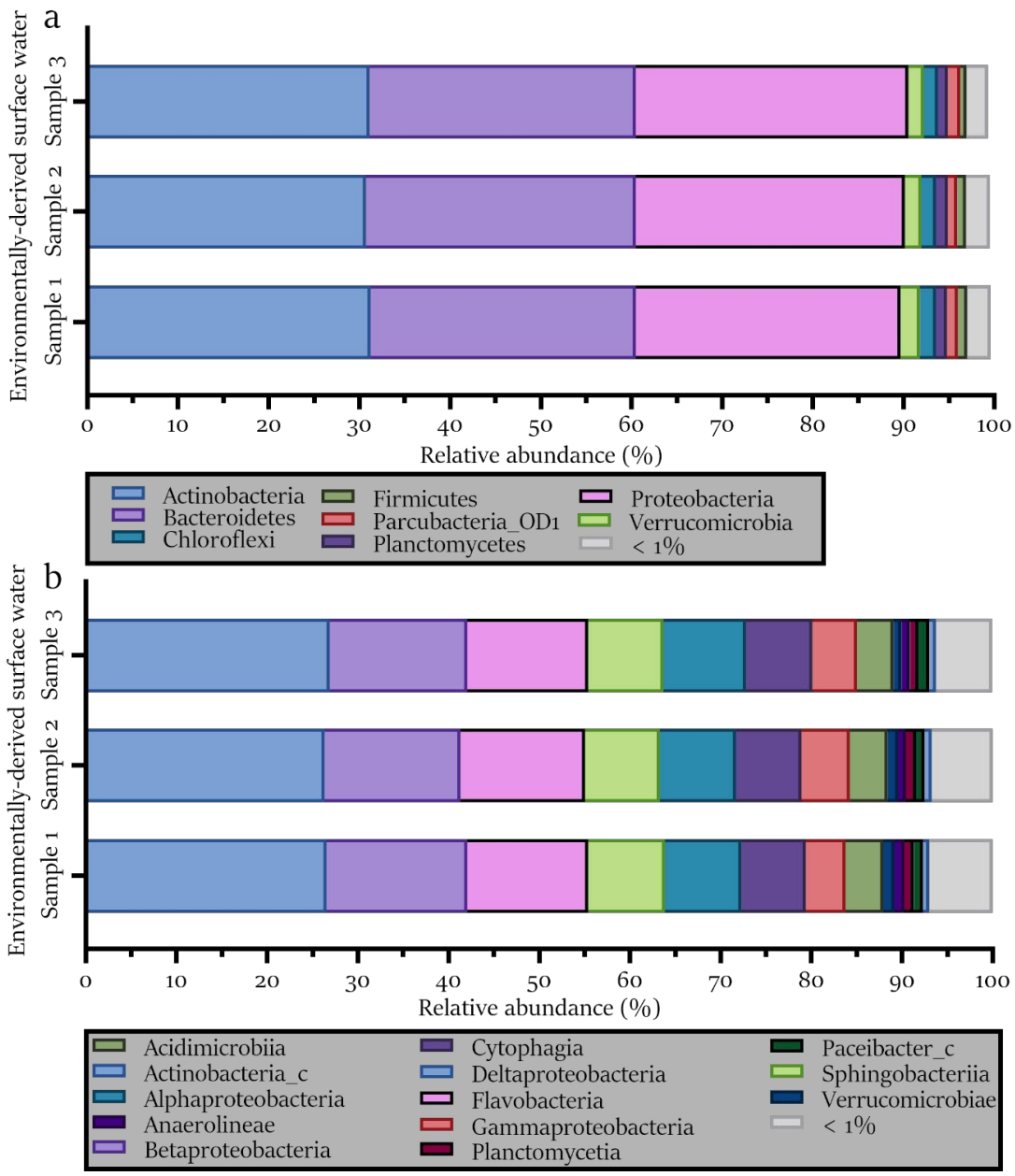


Figure 4.1 Community composition by (a) phylum and (b) class of the environmentally-derived fresh water *used to seed the biofilm treatment systems n=3. Biological replicates (sample 1, 2, 3) are displayed to establish sample variability.*

The microbial community that was analysed within the laboratory scale BTS was performed in triplicate (n=3), however there was an issue with the amplification of BTS 3 at 12 weeks and therefore, for the averaged week 12 samples of the laboratory scale BTS, this is derived from 2 biological replicates (n=2).

The observed microbial community of the developing biofilm within the laboratory scale BTS and the upscaled BTS were analysed for 4 weeks at weekly intervals with a final sample at 12 weeks from the point of inoculating the circulation tanks. For the laboratory scale BTS (Figure 4.2), Proteobacteria was the most abundant phylum within the biofilm accounting for 72.7 ± 1.24 % of

the biofilm sampled after 1 week and remained the most abundant phylum across all of the sample points, albeit decreasing to 55.2 ± 0.63 % after 12 weeks of circulation (Figure 4.2a). After 1 week of maturation, the Bacteroidetes ranked 2nd in the relative abundance within the biofilm of the laboratory scale BTS constituting 25.2 ± 0.87 % of the biofilm; however, the Bacteriodetes decreased after 12 weeks of circulation to a relative abundance of 5.64 ± 0.61 % which ranked 5th of the phyla observed above 1 %. At 12 weeks, the Bacteriodetes were replaced by Actinobacteria which increased from 0.79 ± 0.09 % after 1 week to 9.91 ± 1.88% ranking 1st within the relative abundance after 12 weeks of circulation. Overall, there was a reduction in the relative abundance of the initial colonising phyla which resulted in a more even distribution of the phyla detected above 1 % after 12 weeks of development.

When observing the microbial composition at class level (Figure 4.2b) of the biofilm within the laboratory scale BTS for the first 3 weeks, the most abundant class observed was within Proteobacteria (Gammaproteobacteria at week 1 and Betaproteobacteria at week 2 and 3). This was replaced by Planctomycetia within the phylum Planctomycetota at 4 weeks, replaced by Alphaproteobacteria after 12 weeks of development (Figure 4.2b). However, collectively the phylum Proteobacteria dominated over the circulation period (12 weeks). The class Gammaproteobacteria was the most abundant starting at 40.6 ± 1.73 % after 1 week of circulation which decreased to 7.23 ± 0.01 % (ranking 4th in terms of relative abundance) within the laboratory scale BTS after 12 weeks. After 4 weeks of circulation, this was replaced by Planctomycetia accounting for 22.4 ± 5.64 % of the observable community. Finally, Alphaproteobacteria that accounted for 13.7 ± 12.9 % after the first week of circulation increased to 36.7 ± 2.22 % to rank 1st (in terms of relative abundance) after 12 weeks of circulation (Figure 4.2b).

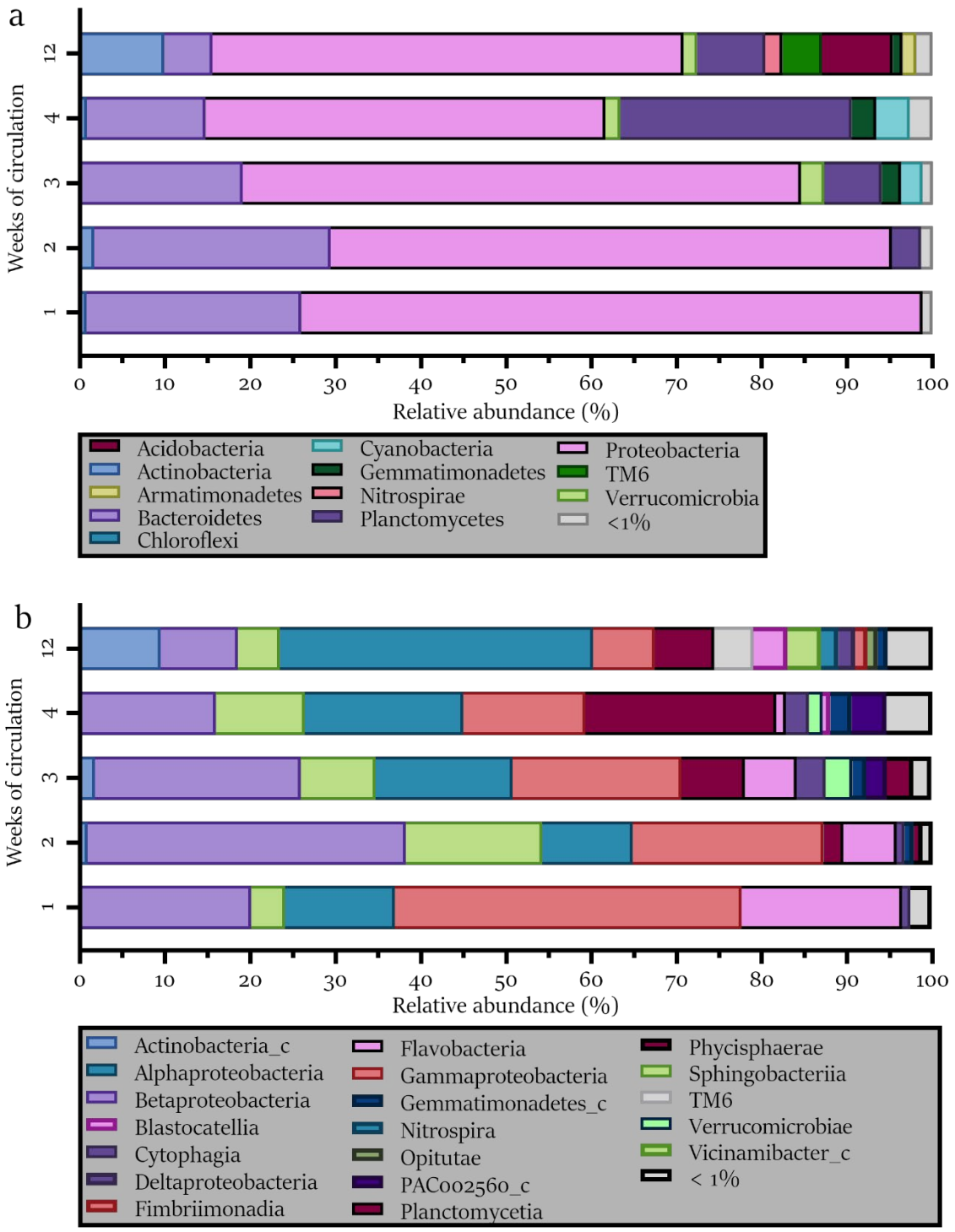


Figure 4.2 The average relative abundance of bacterial (a) phyla and (b) class within an environmental biofilm that established on ceramic substrate within laboratory scale biofilm treatment systems (BTS) were monitored over 12 weeks of circulation. All systems started with sterile substrate using a source water as a seed community of microorganisms to establish within the BTS. Triplicate BTS (n=3) were sampled over 12 weeks of continuous circulation.

Sampling and analysis of the observed microbial composition within the upscaled BTS was experimentally identical to the laboratory scale BTS to facilitate the comparison of both scale systems over time. For the upscaled BTS, Proteobacteria was the most relatively abundant phylum within the biofilm accounting for 70.0 ± 11.3 % after 1 week of circulation. This significantly decreased to 38.7 ± 2.48 % after 12 weeks of circulation; however, in addition to the laboratory scale BTS (Figure 4.2a), Proteobacteria remained the most relatively abundant phylum across all of the sample points within the upscaled BTS (Figure 4.3a). After 1 week of circulation, the Bacteroidetes ranked 2nd in the relative abundance of the biofilm within the laboratory scale BTS constituting 11.4 ± 10.8 % of the biofilm; however, this decreased to 8.72 ± 2.49 % after 12 weeks of circulation which ranked 3rd of the phyla > 1 %. After 12 weeks of circulation, the Bacteroidetes were replaced by Actinobacteria that had increased from 10.6 ± 7.51% at 1 week to 12.7 ± 2.28 % after 12 weeks of circulation (Figure 4.3a).

When observing the microbial composition of the biofilm within the upscaled BTS at class level, the most abundant class was within Proteobacteria over the first 3 weeks of circulation (Gammaproteobacteria for week 1 and 2, and Alphaproteobacteria for week 3). The class Gammaproteobacteria comprised 32.72 ± 15.1 % in terms of relative abundance after 1 week of circulation that decreased to 11.9 ± 2.21 % after 12 weeks. After the 3 weeks of circulation, Gammaproteobacterial was replaced by Alphaproteobacteria, comprised 17.8 ± 2.9 % after the first week of circulation and decreased slightly to 17.5 ± 2.72 % to rank 1st of the relative abundance after 12 weeks of circulation (Figure 4.3b).

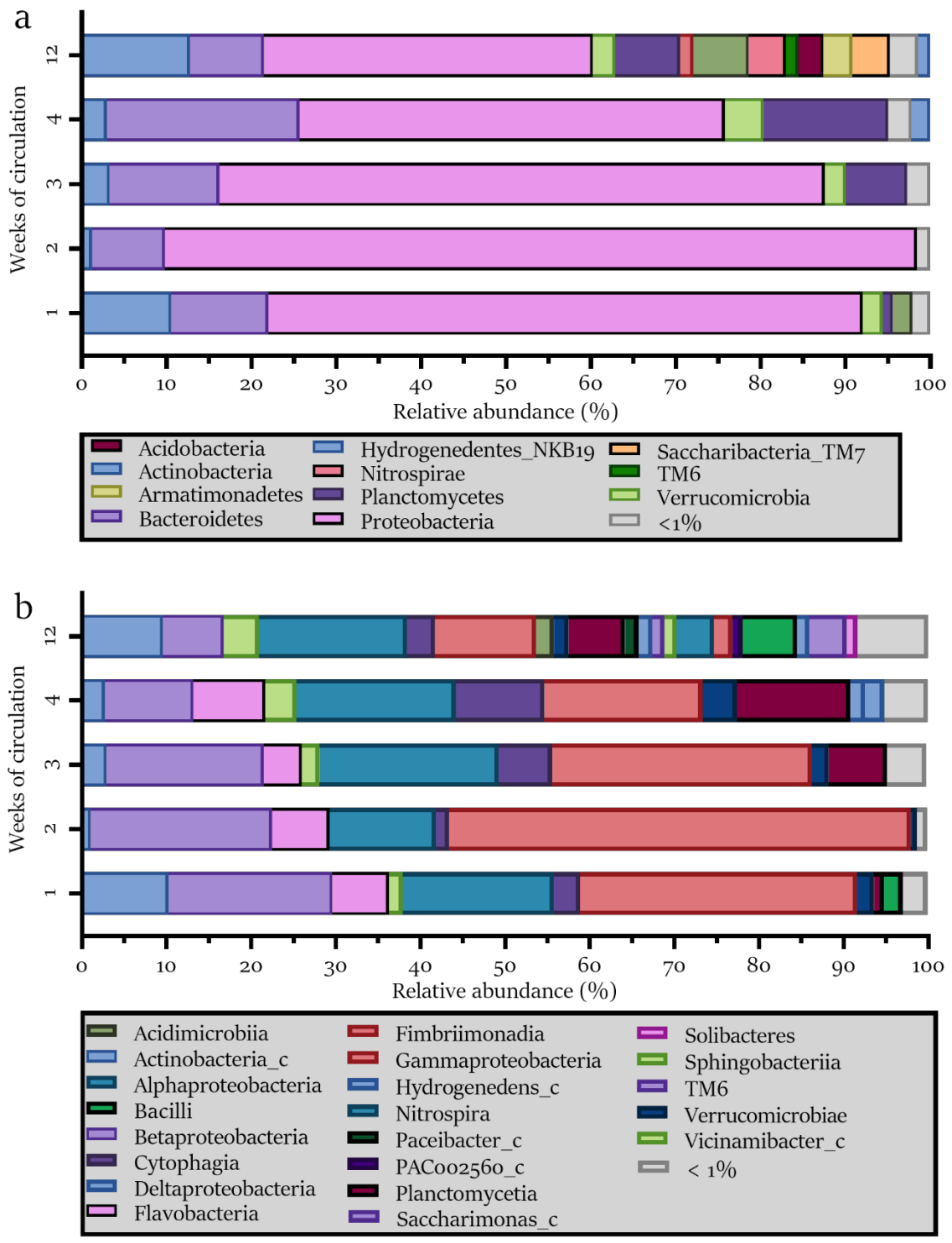


Figure 4.3 The average relative abundance of bacterial (a) phyla and (b) class within an environmental biofilm that established on ceramic substrate within upscaled biofilm treatment systems (BTS) were monitored over 12 weeks of circulation. All systems started with sterile substrate using a source water as a seed community of microorganisms to establish within the BTS. Triplicate BTS (n=3) were sampled over 12 weeks of continuous circulation.

The observable microbial community of the long term deployed BTS shared major phyla across all site samples with proteobacteria making up the largest proportion of the relative abundance within all the long term deployed BTS (Figure 4.4a). Within the most abundant phylum Proteobacteria, the dominant classes were Alphaproteobacterial and Gammaproteobacteria for all of the site samples (Figure 4.4b).

For all systems, one of the major system functions was to control the level of ammonium within the reservoir and therefore, species involved in denitrification processes were of particular interest. For example, selected taxa including Nitrospirae and Nitrosomonas were identified from all of the site samples and the laboratory and upscaled BTS after 12 weeks of circulation.

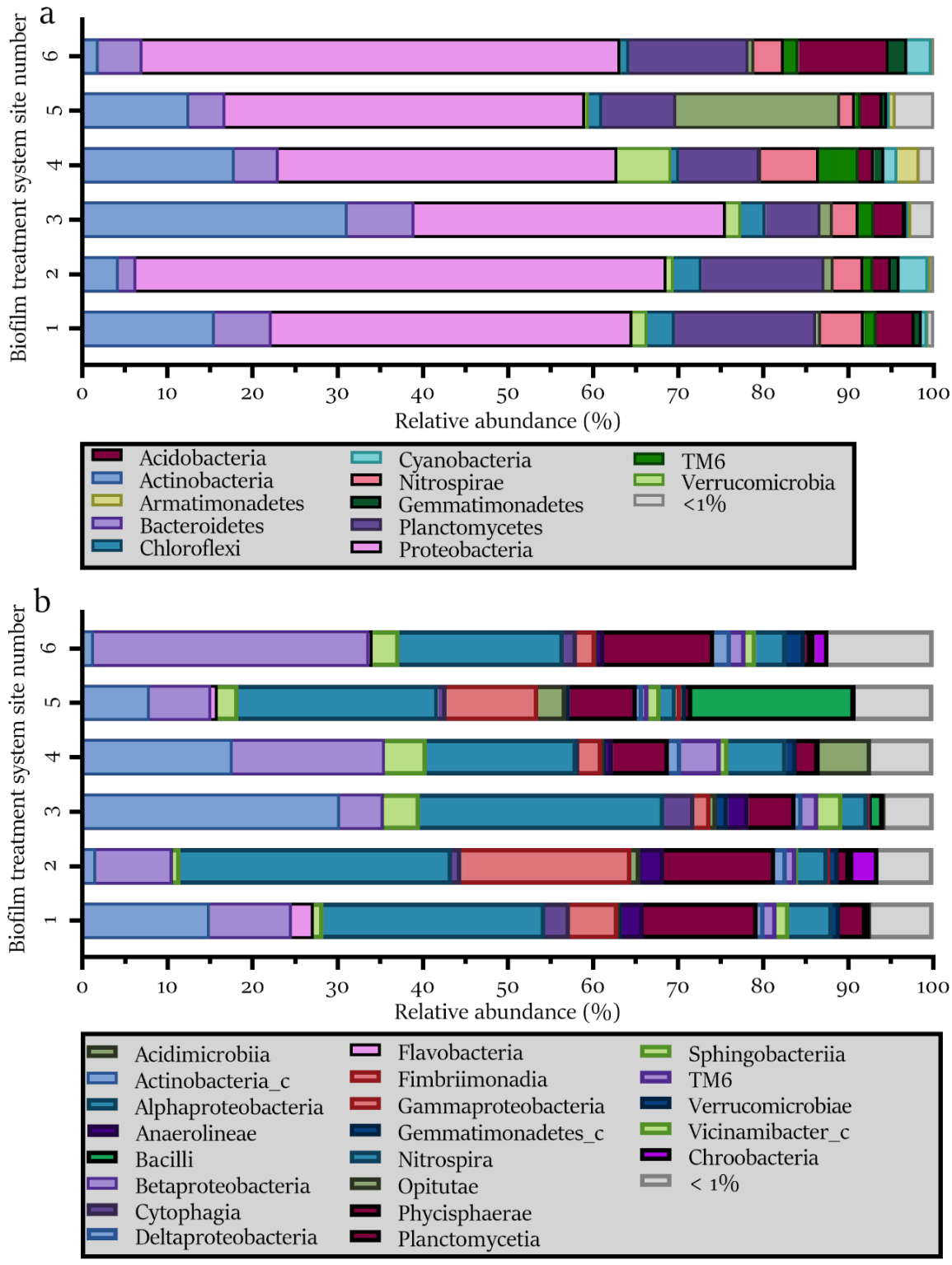


Figure 4.4 The average relative abundance of bacterial (a) phyla and (b) class within an environmental biofilm that established on ceramic substrate taken from 6 independent sites with upscaled BTS in continuous operation for over 1 year. All systems were established using a source water as a seed community of microorganisms to establish within the BTS.

4.2.2 The impact of filter depth on the observed microbial composition of the upscaled biofilm treatment systems

The microbial composition at two filter depths was investigated to understand the distribution of the community within the BTS. The observed microbial composition was very similar when investigating the biofilm community structure at two filter depths of the upscaled BTS averaged over the biofilm development period: the relative abundance of the composition of the observed microbial phyla and class level was not significantly different when the abundance was averaged over the full 12 weeks of circulation (Figure 4.5).

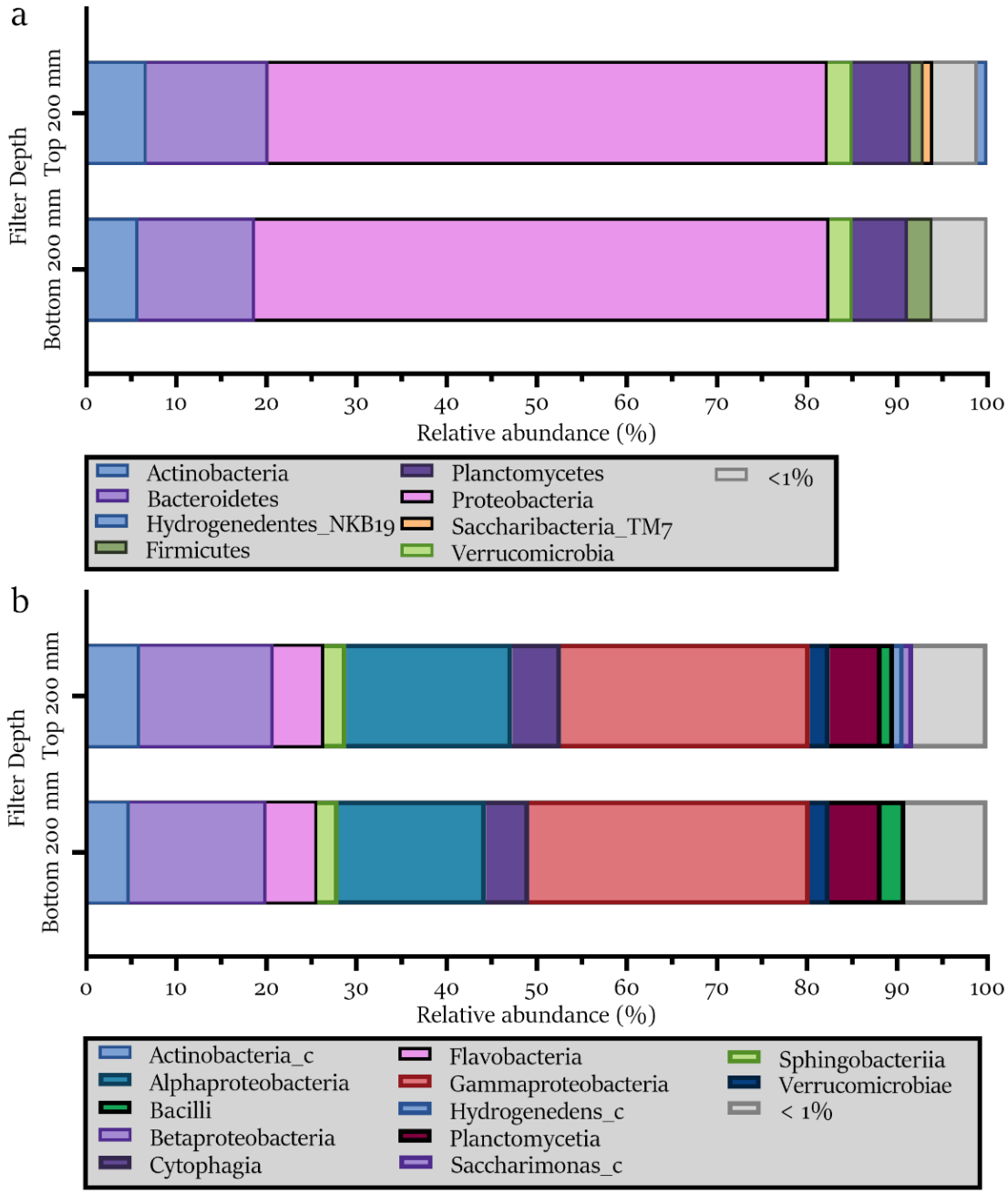


Figure 4.5 The average relative abundance of bacterial (a) phyla and (b) class above 1 % within an environmental biofilm that established on ceramic substrate within the top and bottom of the upscaled biofilm treatment systems (BTS). Triplicate BTS were averaged over the 12-week sampling period and the filter depth (n = 15).

For the alpha diversity analysis, both the species richness and diversity were not significantly different between the top and bottom segments of the upscaled BTS samples (Figure 4.6; $p > 0.05$). Beta diversity analysis using a permutational multivariate analysis of variance (PERMANOVA) also determined that the two data sets (top and bottom, n = 15) were not significantly different from one another ($p > 0.05$). This data suggests that the biofilm composition was evenly distributed across the depth sampled and for subsequent analysis, the upscaled biofilm samples (top and bottom) were grouped to increase the number of replicates per BTS (n = 6 per time point).

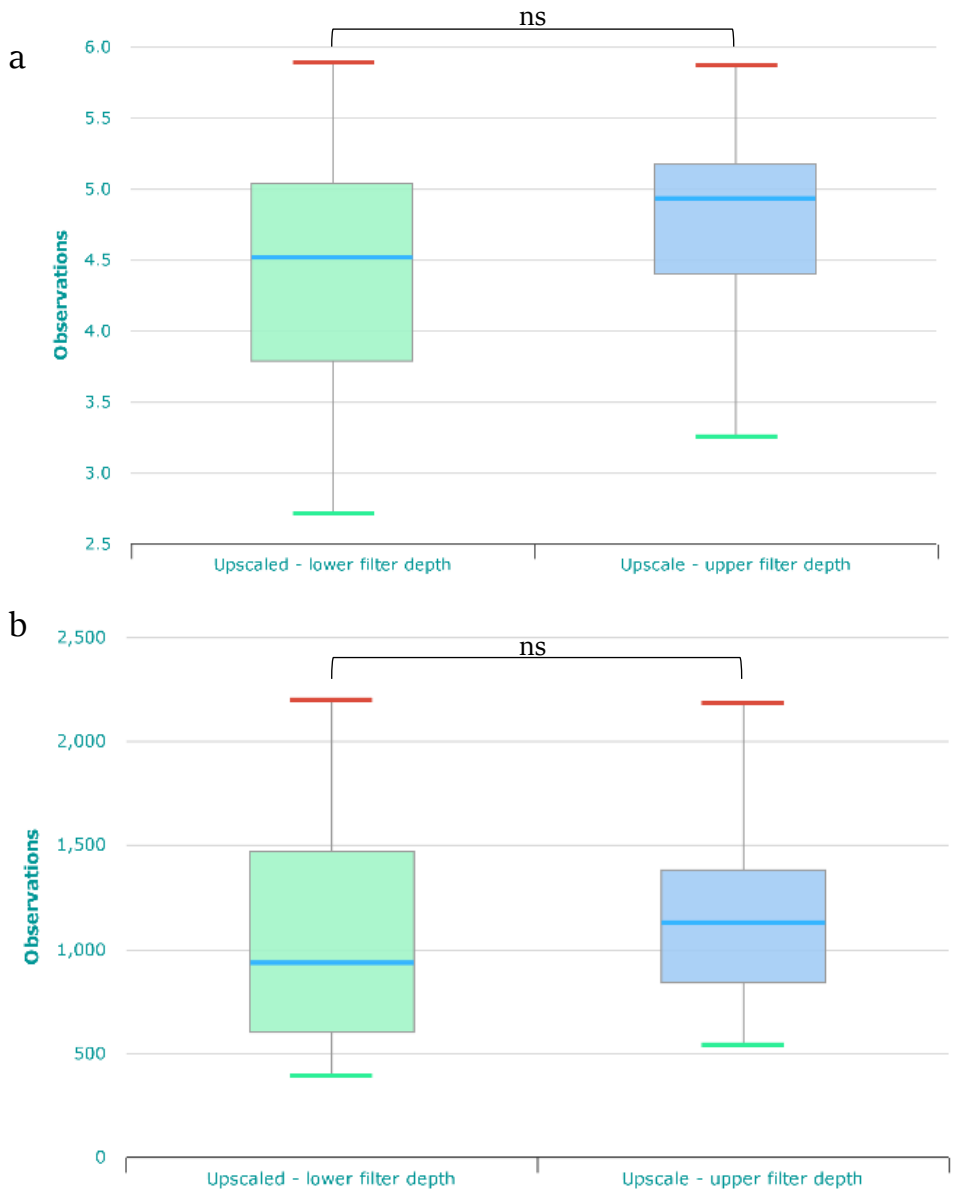


Figure 4.6: Alpha diversity analysis including (a) non-parametric Shannon and (b) Chao1 of upscaled biofilm treatment systems at two filter depths. Triplicate BTS were samples over 12 weeks of continuous operation. The microbial composition was averaged over the entire 12 weeks of circulation to input into the statistical tests (n = 15; ns = p > 0.05).

4.2.3 Alpha diversity of laboratory scale and upscaled biofilm treatment systems

Alpha diversity analysis was performed on the bacterial biofilm community within the laboratory scale BTS and upscaled BTS over the 12 weeks of circulation. For the laboratory scale BTS, the np Shannon diversity index started at 3.73 ± 0.02 after 1 week of circulation which significantly increased after 4 weeks of circulation to 4.47 ± 0.33 (Table 4.1; $p < 0.05$). This did not significantly change for the subsequent 8 weeks of circulation with a np Shannon diversity index of 4.40 ± 0.13 recorded after 12 weeks. Other alpha diversity indexes including Simpson, Phylogenetic diversity and Chao1 all show similar trends over the 12 weeks of circulation.

Table 4.1 Alpha diversity analysis of the laboratory scale biofilm treatment systems over 12 weeks of circulation n=3 ± s.d.

Alpha diversity parameter	Weeks of biofilm development				
	1	2	3	4	12
Library Coverage (%)	98.0 ± 0.10	98.2 ± 0.38	98.3 ± 0.08	98.0 ± 0.22	99.1 ± 0.12
Shannon	3.73 ± 0.02	3.60 ± 0.11	3.86 ± 0.47	4.47 ± 0.33	4.40 ± 0.13
Simpson	0.07 ± 0.01	0.08 ± 0.01	0.08 ± 0.06	0.04 ± 0.01	0.03 ± 0.01
Phylogenetic diversity	849.7 ± 29.5	734 ± 114.0	789.7 ± 113.6	1026 ± 236.6	959.5 ± 113.5
Chao1	902.7 ± 95.3	743.8 ± 156.1	761.3 ± 79.5	910.2 ± 258.1	922.9 ± 167.6

For the upscaled BTS, the np Shannon diversity index significantly decreased from week 1 at 4.58 ± 0.60 to 3.25 ± 0.42 at week 2 of development (Table 4.2; $p < 0.05$). This then subsequently increased over the following week to 4.63 ± 0.54 and remained consistent for the remainder of the circulation period. Other alpha diversity indexes including Simpson, Phylogenetic diversity and Chao1 all show similar trends over the 12 weeks of circulation.

Table 4.2 Alpha diversity analysis of the upscaled biofilm treatment systems over 12 weeks of circulation n=3 ± s.d.

Alpha diversity parameter	Weeks of biofilm development				
	1	2	3	4	12
Library Coverage (%)	97.3 ± 1.30	98.1 ± 0.60	95.2 ± 4.94	96.9 ± 0.58	99.5 ± 0.09
Shannon	4.58 ± 0.60	3.25 ± 0.42	4.63 ± 0.54	5.23 ± 0.40	4.64 ± 0.49
Simpson	0.035 ±0.02	0.16 ± 0.04	0.03 ± 0.01	0.09 ± 0.01	0.02 ± 0.014
Phylogenetic diversity	1171 ± 293	842 ± 257	1172 ± 517	1701 ± 383	776 ± 188
Chao1	1193 ± 415	922 ± 254	1101 ± 552	1648 ± 394	632 ± 159

4.2.4 Beta diversity analysis of Laboratory scale and upscaled biofilm treatment systems

Beta diversity analysis (principal coordinate analysis) of the laboratory scale BTS during the development of the biofilm samples identified two main clusters, that was week 1 and week 12 which was determined to be significant using a permutational multivariate analysis of variance (PERMANOVA). This demonstrated that the OTUs cluster tightly in terms of similarity at the beginning of the biofilm development period, becoming more spread apart for the following weeks from week 2 to week 4, with the final samples (after 12 weeks of recirculation) clustering more closely together (Figure 4.7). The relative similarities are shown in an Unweighted Pair Group Method with Arithmatic Mean (UPGMA) tree (Figure 4.9).

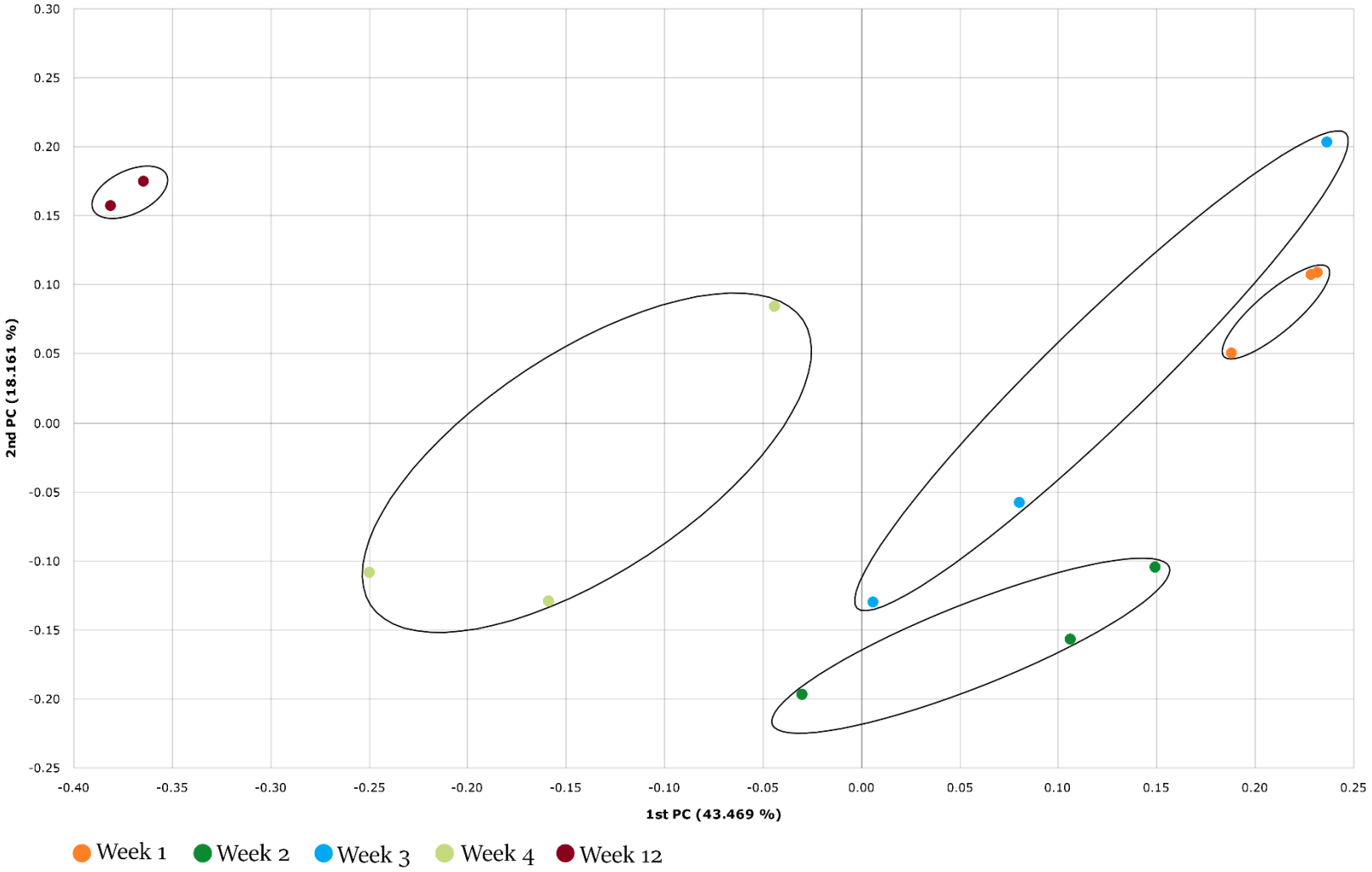


Figure 4.7 Principal Coordinate Analysis (UniFrac) of the biofilm community within laboratory scale biofilm treatment systems over a 12-week development period.

For the upscaled BTS, beta diversity analysis for the 12 weeks of circulation exhibited a similar clustering profile to that of the laboratory scale BTS. A total of 6 replicate samples were taken and analysed per time point, which represented two samples from 3 independent upscaled BTS. There was distinct clustering observed between week 1 – 3 during circulation but two main clusters were observed that were the biofilm at week 4 and week 12 circled in Figure 4.8. The relative similarities are shown and described in an UPGMA tree presented in Figure 4.10.

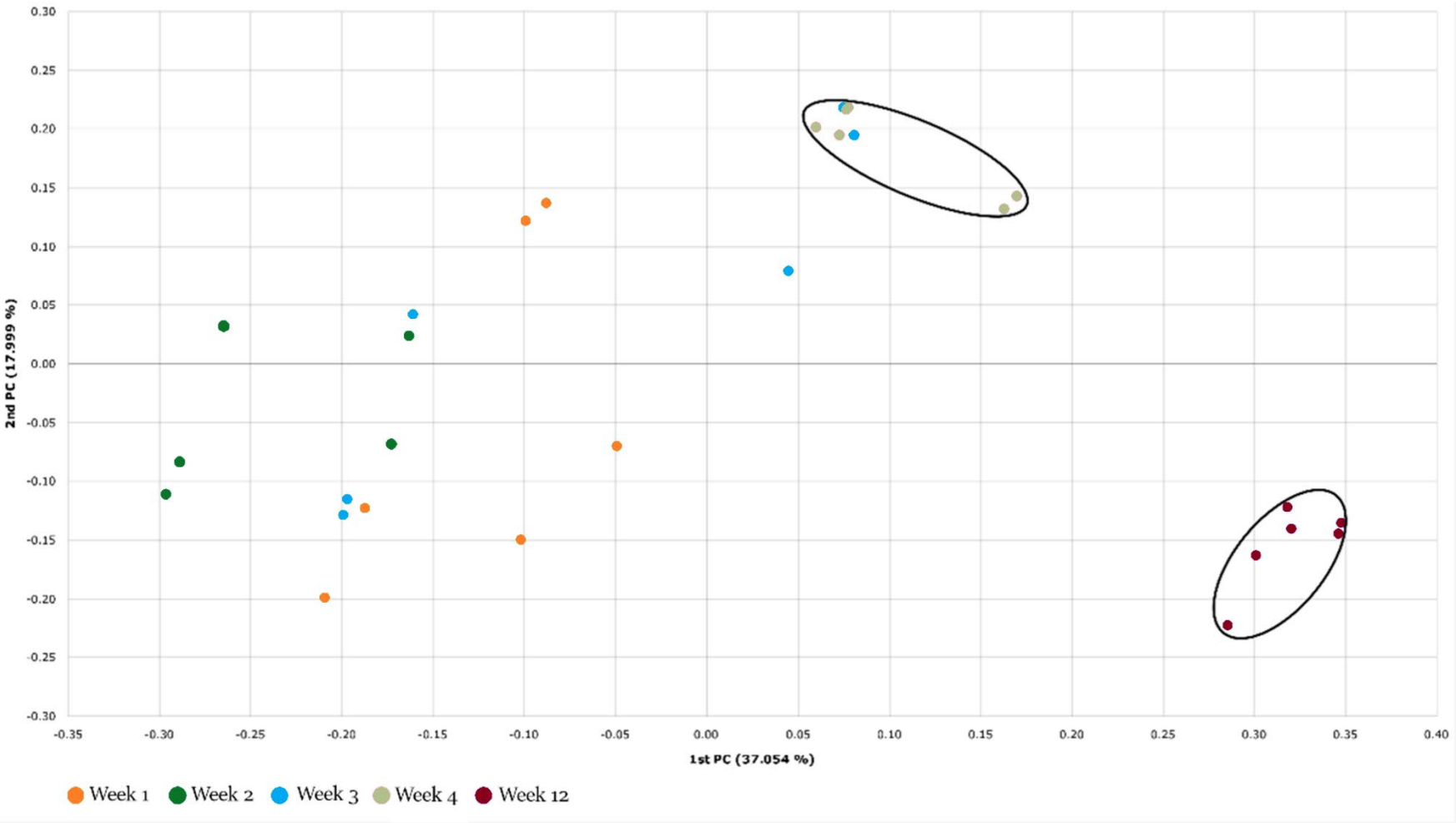


Figure 4.8 Principal Coordinate Analysis (Weighted UniFrac; excluding unclassified operational taxonomic units) of the biofilm community to species level within upscaled biofilm treatment systems over 12 weeks of circulation.

The beta diversity analysis was used to construct a distance *tree* via clustering with the UPGMA clustering during the biofilm development period within the laboratory scale BTS (Figure 4.9) and the upscaled BTS (Figure 4.10). In addition to the biofilm samples, the microbial community sampled from the environmentally-derived fresh water was included in the analysis. For both the laboratory scale and upscaled BTS, this demonstrates that the seed community was more dissimilar from the biofilms that developed within the BTS, and that the biofilm at week 12 formed a distinct cluster away from the bacterial biofilm community present between week 1 – 4 of development.

When observing the biofilm community within the laboratory scale BTS from week 1 – 4, in general there is a pattern of clustering; the microbial communities cluster by time point (Figure 4.9). However, there is less distinct clustering at week 3 and 4. The microbial community at Week 4 within BTS 3 is more representative of week 3 in BTS 3 than with the BTS 1 and 2 at week 4. However, when observing the principle coordinate analysis (PCoA) plot, there is clear grouping as the microbial community within the laboratory scale BTS changes over the 12 weeks of circulation.

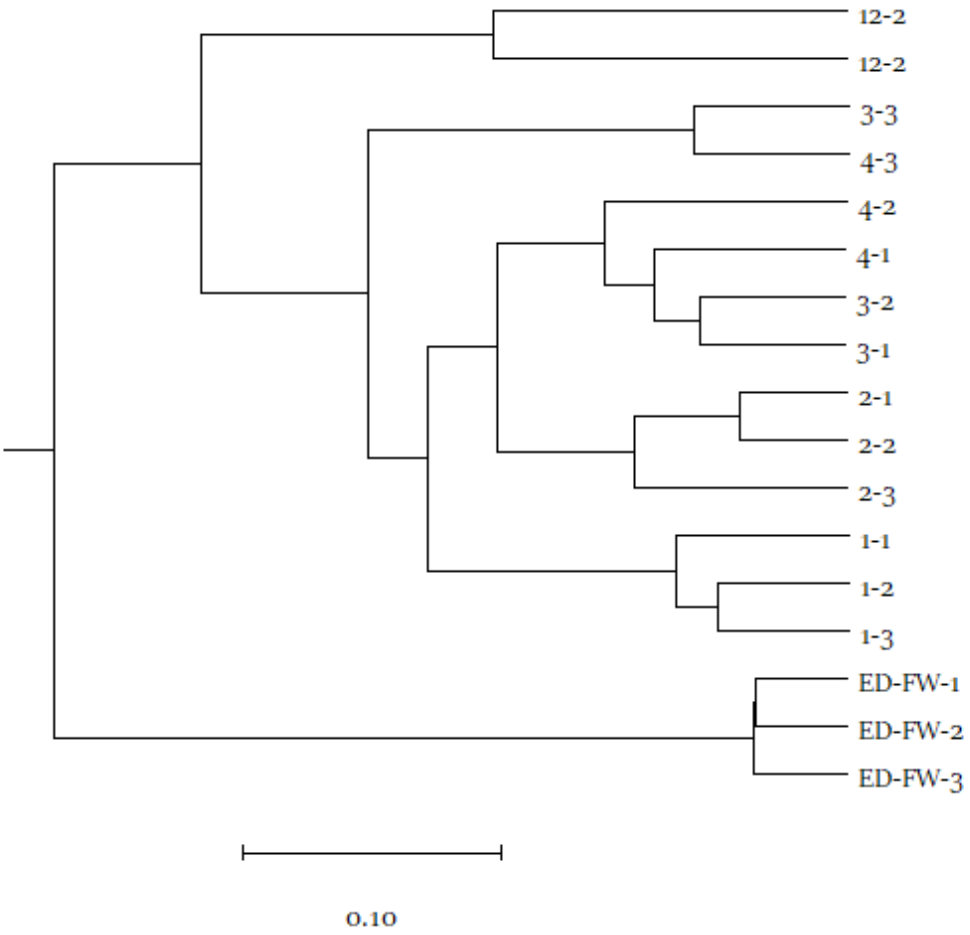


Figure 4.9 UPGMA clustering using weighted unifrac of, the environmentally-dervied fresh water (in figure as: ED-FW-[replicate number]) and the biofilm samples taken from the laboratory scale biofilm treatment systems based on relative abundance of the microbial communtiy to species level. Sample coding: [Week number]-[replicate number]. Scale corresponds to the Euclidean distance between the samples.

The analysis of the upscaled BTS presented in an UPGMA tree demonstrates multiple overlaps in the clustering of the biofilm community between weeks 1 – 4 of circulation (Figure 4.10). However, in general the biofilm community at week 1 clustered at a greater distance from the community after 4 weeks. Moreover, in most cases the biofilm community clustered with the shortest distance of the tree with their respective biological replicates. However, this isn't a complete distinct separation, whereby the biofilm community at week 1 - 4 have some relative overlap. Nonetheless, after 12 weeks of circulation the biofilm community clusters independently from the seed inoculum and the other biofilm samples (Figure 4.10).

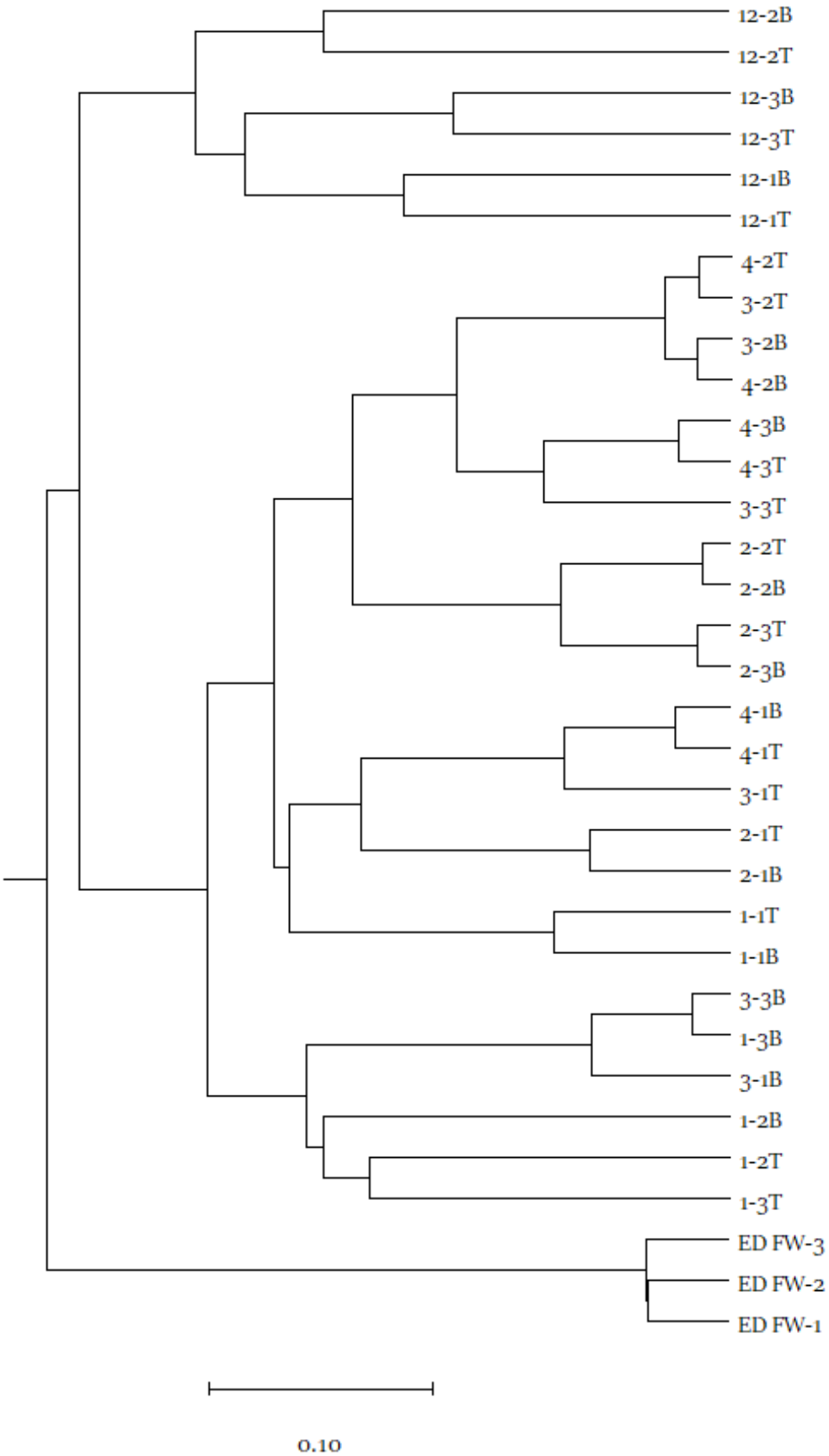


Figure 4.10 UPGMA clustering using weighted unifrac of, the environmentally-dervied fresh water (in figure as: ED-FW-[replicate number]) and the biofilm samples taken from the upscaled biofilm treatment systems based on relative abundance of the microbial communtiy to species level. Sample coding: [week number]-[replicate number/location] T = top, B = bottom. Scale corresponds to the Euclidean distance between the samples.

4.2.5 Comparison of laboratory scale and upscaled biofilm treatment systems

When comparing the observable microbial community within the laboratory scale and upscaled BTS using beta diversity analysis, it was found that the laboratory scale was not significantly different from the upscaled BTS after 3 and 4 weeks of circulation ($p > 0.05$). However, the comparison of the BTS systems after 12 weeks of circulation found that the observed microbial population within the laboratory scale BTS was significantly different from the upscaled BTS ($p < 0.05$). When comparing the alpha diversity index Chao1 (species richness index) and NP Shannon of the Laboratory sale and upscaled BTS after 12 weeks of circulation there was no significant difference observed (Figure 4.11; $p > 0.05$).

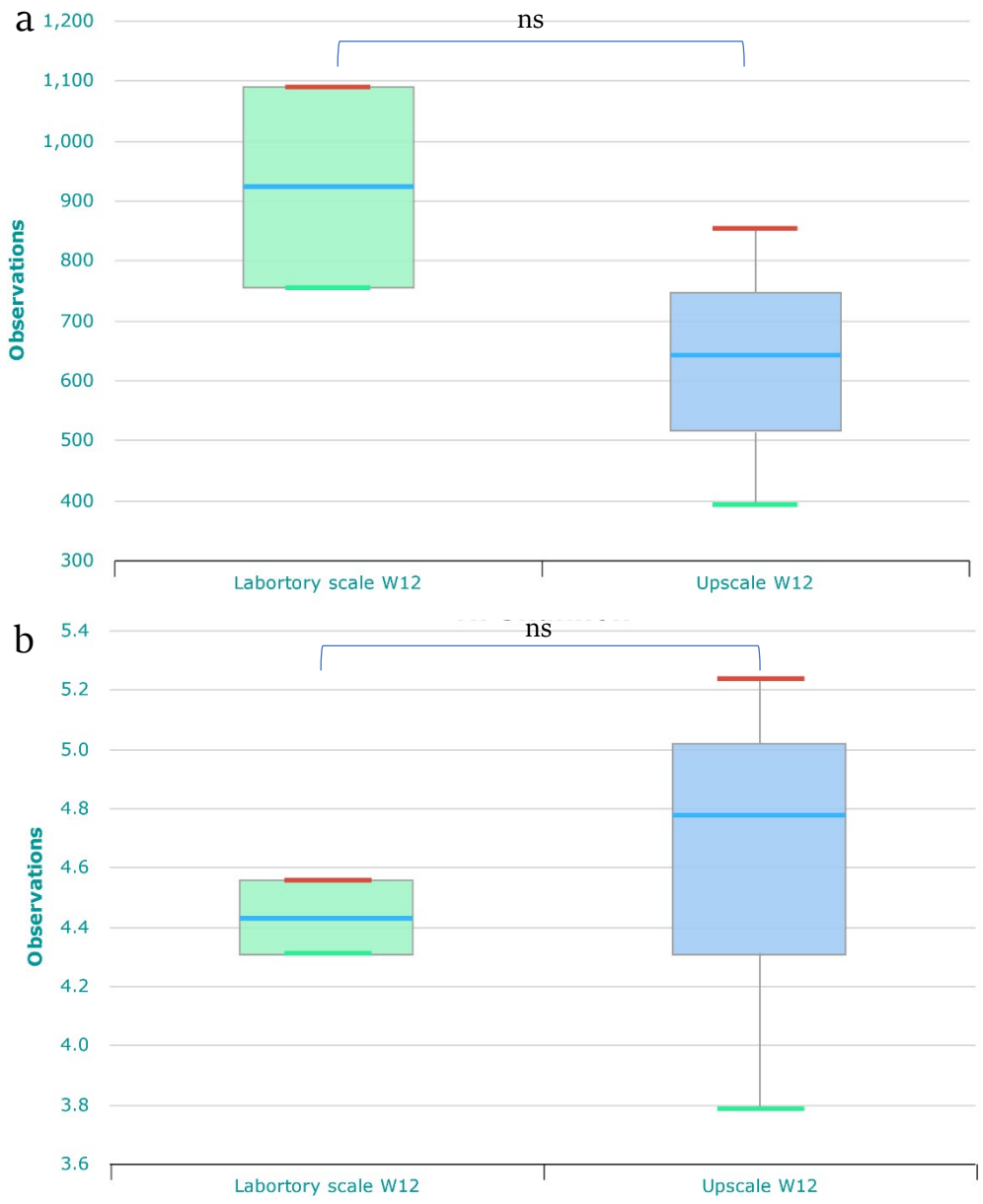


Figure 4.11 (a) Chao1 test and (b) Shannon diversity index of Laboratory scale and Upscaled biofilm treatment systems after 12 weeks of development, ns = non-significant determined using Wilcoxon rank-sum test.

4.2.6 The relationship of the model biofilm treatment systems to the deployed site biofilm treatment systems

The observable biofilm community within the laboratory and upscaled BTS were compared with the biofilm community of the 6 independent long term deployed BTS at different sites tested in this study. The beta diversity analysis, including all the samples taken during biofilm development (week 1 – 12), demonstrates that the observable microbial community of the laboratory scale BTS after 12 weeks of development is more representative of a full-scale BTS that has been deployed long term, than a biofilm after 1 week of development. The observed shift in the PCoA plot demonstrates that after 12 weeks of development, the test (laboratory-scale and upscale) BTS clusters with the site samples, being more closely related to a full scale BTS than the biofilm community within the laboratory scale BTS (Figure 4.12) after 1 week of development. The circled plots contain site samples and 12-week biofilm samples from the laboratory scale BTS and the >1-year long-term deployed BTS.

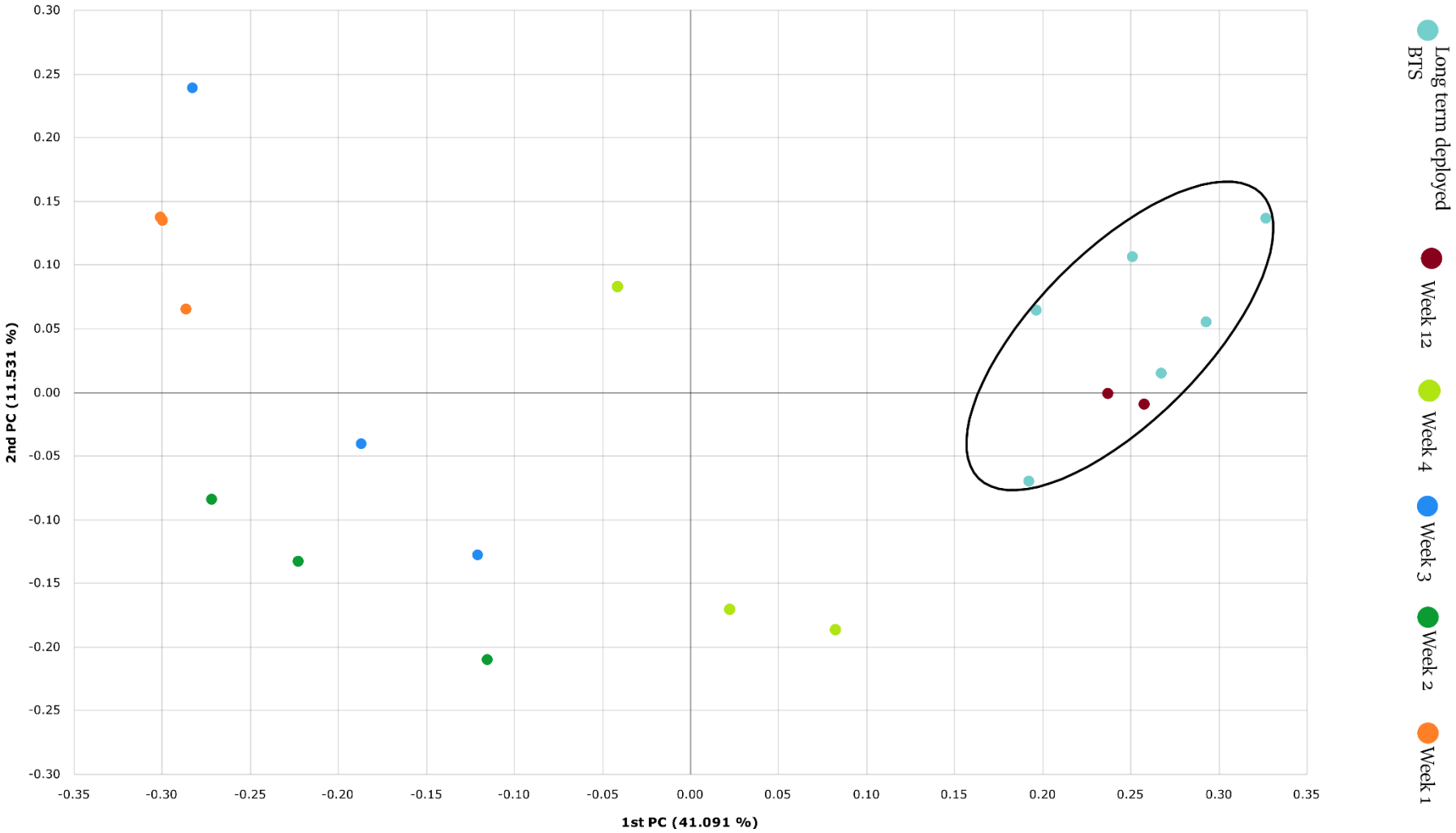


Figure 4.12 Principal Coordinate Analysis (Weighted UniFrac; excluding unclassified operational taxonomic units) of the biofilm community at species level within laboratory scale biofilm treatment systems *over a 12-week development period and biofilm samples from 6 independent BTS deployed for > 1 year*.

For the upscaled BTS, the findings of the beta diversity analysis, including all the samples taken during biofilm development (week 1 – 12), are consistent with the laboratory scale BTS that demonstrates the observable microbial community of the laboratory scale after 12 weeks of development are more closely representative of a full-scale BTS that has been deployed long term, than they are of a biofilm after 1 week of development. The observed shift in the PCoA plot demonstrates that after 12 weeks of development, the test BTS cluster with the site samples from > 1 year, that is more closely related to a full scale BTS than the biofilm community within the upscaled BTS after 1 week of development. The circled plots contain site samples and 12-week biofilm samples from the laboratory scale BTS and the long-term deployed BTS (> 1 year).

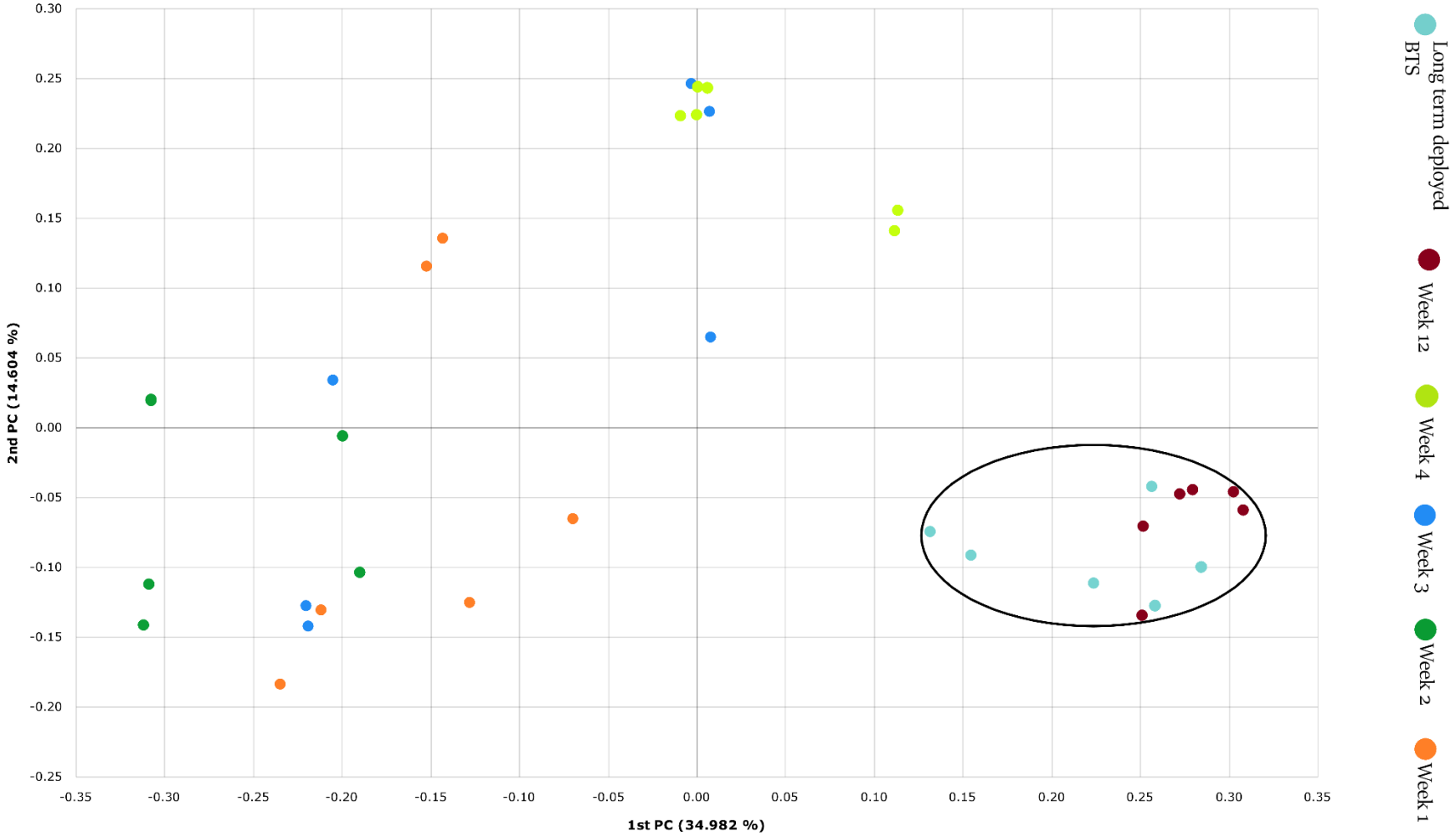


Figure 4.13 Principal Coordinate Analysis (Weighted UniFrac; excluding unclassified operational taxonomic units) of the biofilm community to species level within upscaled biofilm treatment systems over a 12-week development period and biofilm samples from 6 independent BTS deployed for > 1 year.

The UPGMA grouped the site samples separate from the upscaled systems and the laboratory scale BTS with the exception of Site 4 that grouped with the laboratory scale BTS (Figure 4.14). When peforming permanova of three sample groups: laboratory scale BTS, upscaled BTS and site samples, all three groups were signficnatly different from one another ($p < 0.05$) with the greatest signficance between the upscaled systems and site samples (determined using f-values). The signifcance was determined by Linear discriminant *analysis* Effect Size (LEfSe) between the sample groups. The laboratory scale BTS contained, 1 family, 3 genre and 5 species that were not observed in either the upscaled or long term deployed BTS ($p < 0.05$). The family observed was Rhizobiales that comprised 0.048% of the overall composition (n=3). For the long term deployed BTS, the samples contained 7 species, 8 genre, 3 families and 1 order that was unique to these systems that had the most influence on determining the significant difference between the laboratory scale and upscaled systems ($p < 0.01$). For the upscaled BTS there was no unique part of the population, however the difference in the community composition resulted in the significance ($p < 0.05$).

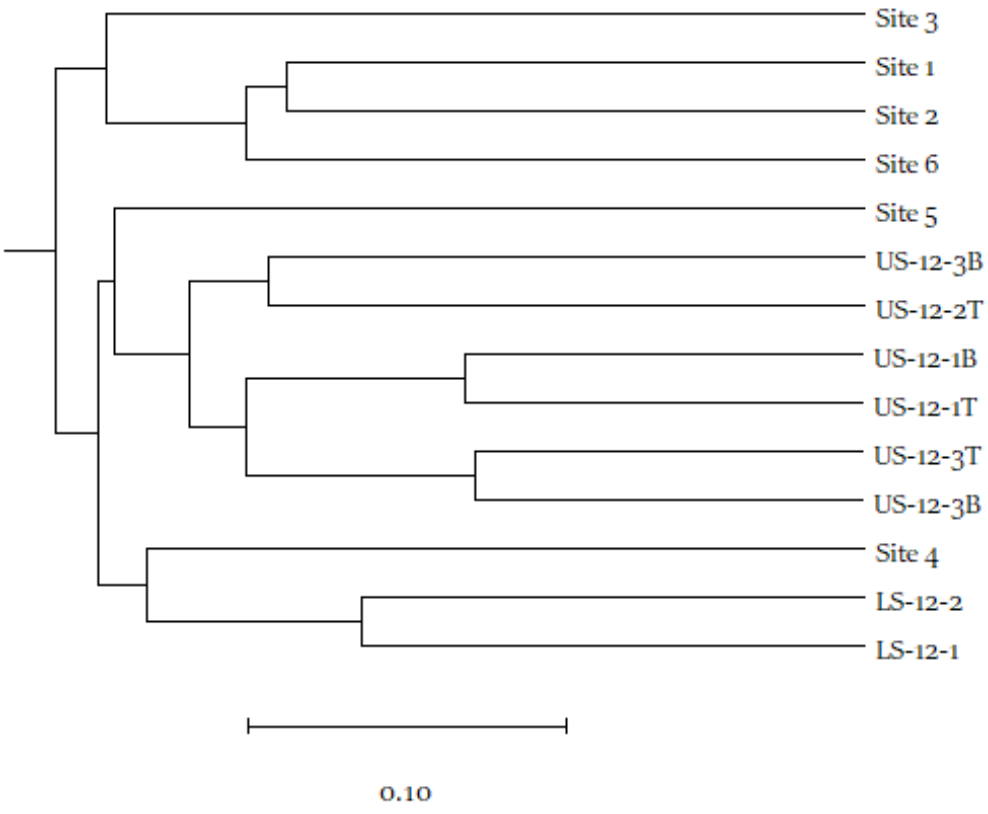


Figure 4.14: UPGMA clustering using weighted unifrac of biofilm samples taken from the laboratory scale, upscaled and the long term deployed biofilm treatment systems (site 1-6) based on relative abundance of the microbial community to species level. Sample coding: [model scale]-[week number]-[replicate number/location]. US =Upscaled BTS, LS =Laboratory Scale BTS, T = top and B = bottom.

4.3 Discussion

The use of the new sFL16S was chosen in this study for the analysis of both the environmentally-derived surface water and the biofilms establishing within the laboratory scale and upscaled BTS. The sFL16S technology is used to read the complete number of variable regions of the 16S rRNA gene (V1–V9) providing high quality base-resolution that accurately classifies bacterial communities by reducing false positives. This technique has been confirmed to have reduced error rates compared to the more traditional taxonomy classification using only a limited variable region (e.g., V3V4) of the 16S rRNA gene (170). Furthermore, this study confirmed that both the calculated richness and evenness score were higher in the sFL16S sequencing method than the V3V4 method. This was also demonstrated when comparing the relative abundance of the bacterial taxonomy classified from two different sequencing methods, it was found that the classification frequency at the species level was higher in sFL16S than in V3V4. Moreover, the V3V4 method was found to have a higher frequency of classified taxa (170). These results indicated that using the V3V4 method, thereby reading partial hypervariable regions, it was difficult to accurately assign the bacterial taxa at the species level. Therefore, sFL16S sequencing was used to analyse the biofilm community that established within the laboratory scale and upscaled BTS.

The BTS used in this study were deemed functional when the complete reduction of ammonium was observed within the circulation tanks over 24 hours characterised in chapter 3. However, independent of this function, the microbial community analysis in Figure 4.2 & Figure 4.3 suggests that the observable microbial community did not stop changing after 4 weeks. The complete removal of ammonium was likely due to the presence of nitrifying bacteria within the observed bacterial community. This analysis identified the presence of Nitrospira, a genus of bacteria commonly associated with the oxidation of nitrite as an energy source (171) [see equation 1.1], being established as part of the microbial community within the BTS. In addition, Nitrosomonas were also detected within the biofilm samples at 12 weeks which may have facilitated the reduction of nitrates within the reservoir of the BTS [see equation 1.2]. However, this function does not indicate that the biofilm was “mature”. The data presented in Figure 4.2 and Figure 4.3 demonstrates that changes in the microbial community were still occurring and that the microbial community at 12 weeks groups tighter with the other replicate systems in contrast to weeks 1 – 4 within both the laboratory scale and upscaled BTS (Figure 4.7 and Figure 4.8). This suggests that a functional BTS is dynamic, and the microbial population can still change and provide function within the water treatment processes.

The alpha diversity indices observed over the 12-week circulation period provide some insight into the changes within the microbial community structure. When observing these indices over the 12 week circulation period, there was a drop in species richness and evenness after the first week of circulation. The Shannon diversity index, an estimator of species richness and evenness (more weight on species richness) indicates that the species diversity was higher after 1 week of circulation which then subsequently decreased over the following week before increasing again for the remaining 10 weeks in both the laboratory scale and upscaled BTS (Table 4.1 & Table 4.2). This indicates that initial stages of biofilm formation within these BTS was from the attachment of planktonic cells on the surface resulting in the higher level of initial diversity observed resulting from the diverse seed community *(*Figure 4.1). This then subsequently decreased when competing microorganisms were growing resulting in a loss of diversity observed but an increase in density observed in Figure 3.5. When observing the changes in Chao1, an estimate of the number of species in a community and importantly, rare species (low abundance) provides the most information about the number of missing species (172). Overall, within the laboratory scale BTS the Chao1 remained consistent across the whole 12 weeks of circulation (Table 4.1). The same trend was observed for the first 4 weeks of circulation for the upscaled BTS, however a significant decrease was observed at the 12-week sampling point (Table 4.2; $p < 0.05$). This indicates that at the 12-week sampling point the community was more evenly distributed with fewer low abundant OTUs.

Beta diversity analysis involved the construction of UPGMA which is a simple approach to construct a phylogenetic tree from a distance matrix. This helps visualise the similarities between the samples taken during development. The data presented in Figure 4.10 demonstrates that the biofilm community does indicate some similarity from weeks 1-4 between replicate BTS. However, there is a lot of overlap between weeks 1,2 and 3 resulting in a mix of ordering within the tree. This is also represented in the PCoA presented in Figure 4.13 where there is overlap of community similarities between weeks 1-4 that can be observed. This is possibly due to lag in the changes of the biofilm community over the sampling period. However, the biofilm samples begin to cluster again after 4 weeks of circulation. This also indicates that the earlier stages of development in this process is less conserved than at 4 – 12 weeks within the upscaled BTS systems used in this study. When observing the UPGMA tree for the laboratory-scale BTS, there are more defined groups by week of circulation in comparison to the upscaled BTS (Figure 4.9). This could be a result of factors coming from the ambient environment affecting the changes in biofilm population during the early stages of development (weeks 1-3). The less defined groups observed in the upscale BTS during week 1-3 could be a result of the outdoor environment having an impact on the communities (Figure 4.10), as opposed to the more regulatory indoor lab environment. Nonetheless both the

laboratory scale and upscale BTS form tight clusters at week 4 and 12 suggesting that the process to a "mature" BTS could have many different pathways but with a similar outcome all other parameters equal.

When comparing the Laboratory scale, upscaled and long term deployed BTS, the signifcance was determined by Linear discriminant *analysis* Effect Size (LEfSe) between the data sets. The laboratory scale BTS contained, 1 family, 3 genre and 5 species that were not observed in either the upscaled or long term deployed BTS ($p < 0.05$). The family observed was Rhizobiales that comprised 0.048% of the overall composition (n=3). For the long term deployed BTS, the samples contained 7 species, 8 genre, 3 families and 1 order that was unique to these systems that had the most influence on determining the significant difference between the laboratory scale and upscaled systems. This was most likely a result from the system age difference and that the long term deployed BTS was established from unique source waters introducing different species into the system. Nonetheless, it is clear from Figure 4.2, & that proteobacteria was the most abundant phylum in all the systems. The domination of abundance of Proteobacteria has been shown in other water treatment systems (166,173). The observed clustering of the > 1-year long-term deployed BTS sites was interesting considering that were setup independently from different source waters. This observation is supported by a finding in the literature where a model drinking water treatment system that was sampled from different sampling positions found that the MTPs clustered together over a 3-year sampling period, indicating a homogeneous community structure (174).

When establishing an environmental biofilm from an environmentally derived water source known to contain indicator species (see Figure 6.2), it was important to ascertain whether the indicator species, present within the seed community, may form a part of the biofilm community. When searching the microbiome taxonomic profiles (MTPs) generated from the environmentally-derived water source, the presence of *E. coli* and *Enterococcus* species were noted. However, when the same search was performed for the laboratory scale and upscaled BTS, there was no *E. coli* or *Enterococcus* observed within the biofilm samples after 12 weeks of development in either the laboratory scale BTS or the upscaled BTS. This suggests that, using *E. coli* as an example, if a BTS is matured from a contaminated water source, not all bacterial species will integrate as part of the biofilm community under the specific conditions tested.

When comparing both the laboratory scale and upscale BTS with the long term deployed BTS, the data presented in Figure 4.12 and Figure 4.13 demonstrate that the microbial communities at 12 weeks are more taxonomically representative of the site samples than the communities after 1 week of development. It was these "mature" systems that were then used in chapter 5 and 6. This supports what is hypothesised in chapter 3; that the laboratory scale systems are representative of

upscaled systems. The findings of this study are in agreement with those presented by McKie *et al.* (69); that laboratory scale BTS (termed bench scale in the cited study) once "mature" are representative of larger scale systems. An interesting observation is that site 4 clusters closely with the laboratory scale BTS at 12 weeks. Site 4 is a long term deployed BTS that is setup indoors. This indoor environment is a variable shared by the laboratory scale BTS and this indicates that the ambient environment could have an impact on the microbial community even if the seed community was from the same source.

Chapter 5 The Control of Waterborne Pathogens Using Laboratory Scale Biofilm Treatment Systems

5.1 Introduction

The control of waterborne pathogenic bacteria in areas with established, centralised water treatment systems is achieved by the combination of physical processes such as screening and filtration, with well understood disinfection methods using chemicals, ozonation and/or UV disinfection (175), all of which require significant energy and resources. Chlorination is preferentially used in drinking water disinfection processes, which require residual free chlorine and chloramines throughout distribution systems to ensure water quality is maintained by preventing the growth of pathogenic bacteria during water distribution (42). However, such disinfection strategies can be hazardous and require complex management strategies which are not always easily implemented in remote or challenging communities where infrastructure is lacking (176). Therefore, there is an unmet need for low energy solutions for the provision of potable water that are sustainable in the long term, i.e. scalable with low energy, maintenance and material requirements. One potential sustainable approach for water treatment is the application of biofiltration, a remediation biotechnology that utilises microbial biofilms adhered to a stationary phase substrate that can be configured within filter columns (58).

Laboratory scale biofilm treatment systems (BTS) have been shown to remove a wide range of chemical substances (69,78,90), however little is known regarding the use of BTS for the control and/or removal of pathogenic bacteria in potable water supplies. For example, biofiltration has been demonstrated to result in a reduction of *E. coli* (up to 58 %) and faecal coliforms (up to 63 %) in urban ponds (130). For potable water production, a combination of biofiltration technology, coagulation, flocculation, filtration and chlorination has been previously demonstrated to be effective for the control of waterborne bacteria (55). However, to date no studies have been reported that demonstrate the direct effect of a biofilm alone to control the levels of pathogenic bacteria in drinking water supplies. The aim of this study was to investigate the control and inhibition of pathogenic bacteria commonly associated with biologically contaminated water supplies using a biofilm treatment system.

The aims of this chapter were to:

(i) Implement the characterised laboratory scale BTS to investigate the control of potential pathogenic bacteria within a simulated fresh water.

(ii) Investigate the effect of flow rate on the control of waterborne pathogens.

(iii) Implement and apply the laboratory scale BTS to investigate the control of indicator species and nutrients within an environmentally-derived surface water.

5.2 Results

5.2.1 Flow rate optimisation of pathogen reduction using Escherichia coli.

Monocultures of *E. coli* suspended in SFW were circulated through the filter columns for 24 hours at three different flow rates. The use of SFW for these experiments facilitates the investigation of a more environmentally representative medium, unlike previous studies which have used growth media with very high baseline nutrient concentrations (e.g. nutrient broth). At the start of all experiments there was no significant difference in the density of *E. coli* between the three different systems or flow rates (Figure *5.1*; $p > 0.05$). The biofilm treatment systems were shown to significantly reduce *E. coli* at all three flow rates tested (Figure *5.1*c, $p < 0.05$). The empty filter column control systems had a starting overall average density of 2.90 ± 0.04 Log_{10}CFU 100 mL^{-1} and there was no significant difference at the start or the end of the experimental period between the three flow rates tested with an average of 2.76 ± 0.07 Log_{10}CFU 100 mL^{-1} remaining after 24 h of circulation for all systems (Figure *5.1*a, $p > 0.05$). For the sterile filter media, there were significant differences observed between the flow rates after 24 h. The average starting density of *E. coli* for the systems containing the sterile filter media was 2.96 ± 0.11 Log_{10}CFU 100 mL^{-1} and after 24 h of circulation, 2.22 ± 0.28 Log_{10}CFU 100 mL^{-1} remained in the reservoir of the system circulated at 138 mL min^{-1} which was significantly lower that the system circulated at 69 mL min^{-1} that had 3.03 ± 0.09 Log_{10}CFU 100 mL^{-1} remaining after 24 h and 35 mL min^{-1} that had 2.48 ± 0.07 Log_{10}CFU 100 mL^{-1} remaining after 24 h (Figure *5.1*b, $p < 0.05$). For the biofilm treatment systems, the average starting density was 2.96 ± 0.08 Log_{10}CFU 100 mL^{-1} that were not significantly different between flow rates (Figure *5.1*c; $p > 0.05$). After 24 h of circulation through the BTS, there were differences observed between the different flow rates. The final *E. coli* density for each flow rate was 0 Log_{10}CFU 100 mL^{-1} for 138 mL min^{-1}, 1.62 ± 0.20 Log_{10}CFU 100 mL^{-1} for 69 mL min^{-1} and 0.20 ± 0.44 Log_{10}CFU 100 mL^{-1} for 35 mL min^{-1}. To compare the rate of reduction through the BTS, the D-value (decimal reduction time) defined by the number of minutes exposure to a defined temperature to reduce viable bacteria by 90% that is a standard method to determine kill kinetics (177), was calculated for each flow rate between 0 – 6, 6 – 12 and 12 – 24 h. These time domains were chosen to best observe the reduction at the beginning, middle and end of the experimental run, and to capture at least 1 log reduction in *E. coli*. The system circulated at 35 mL min^{-1} had the fastest initial reduction with a D-value of 8.25 ± 1.03 h in comparison to the D-values 16.8 ± 1.75 h for 69 mL min^{-1} and 21.4 ± 7.74 h for 138 mL min^{-1}. For 6 – 12 h the systems circulated at 138 mL min^{-1} had the lowest D-value of 6 ± 2.45 when compared to 13 ± 3.04 h for 35 mL min^{-1} and 14 ± 4.55 h for 69 mL min^{-1}. For the final 12 h of circulation the D-values were very similar, 35 mL min^{-1} had a D-value of 2 ± 0.47 h, 69 mL min^{-1} of 3 ± 0.20 h and 138 mL min^{-1} of 3 ± 2.21 h.

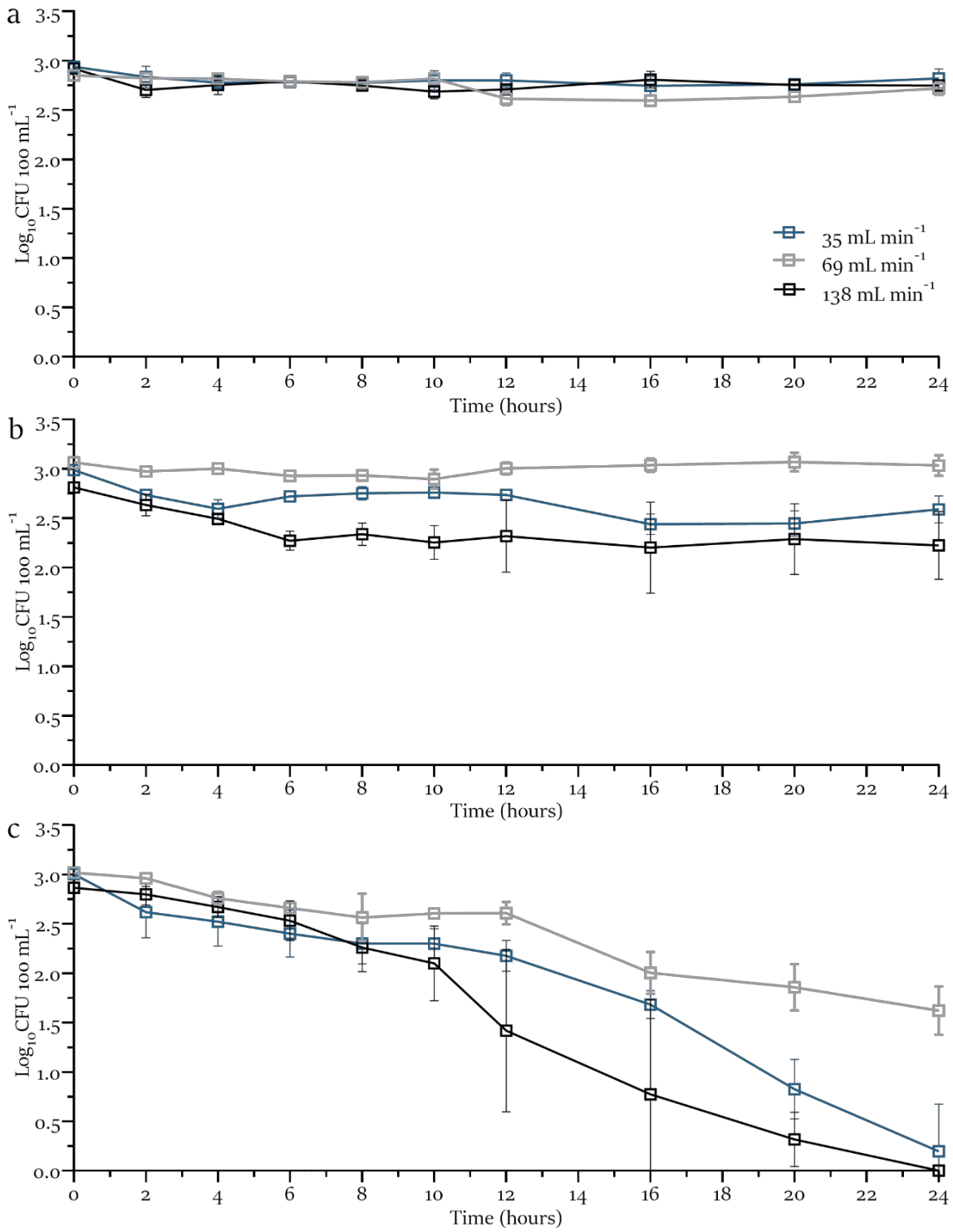


Figure 5.1: Viable counts of Escherichia coli when circulated through laboratory scale systems incorporating (a) an empty filter column, (b) a column containing sterile filter media and (c) an environmental biofilm within simulated freshwater at three flow rates of 35 (blue) 69 (grey) and 138 (black) mL min^{-1} over a period of 24 h, all performed in triplicate n=3 ± s.d.

5.2.2 Viable counts of Escherichia coli, Enterococcus faecalis and Pseudomonas aeruginosa are significantly reduced by biofiltration.

To quantify the reduction of the test bacteria within the SFW, viable counts of *E. coli, E. faecalis and P. aeruginosa* were recorded over a 24 h duration when individually cycled through the BTS and control systems. Based on the experimental data presented in section 5.2.1, the flow rate of 35 mL min^{1} was selected for further experimentation. This flow rate achieved a significant reduction in pathogens and had practical considerations for upscale applications whereby a compromise between energy requirements for operating at a high-volume turnover (upscaling 138 mL min^{-1}) and pathogens reductions were met. For all experiments, there was no significant difference in the starting density of test bacterial species within the simulated freshwater (SFW), between the biofilter, the column containing the sterile filter media and the empty filter column ($p > 0.05$; Figure *5.2*). After 24 h, significant reductions in all three species were observed in the biofilter systems, when compared to the SFW in both the control systems ($p < 0.001$; Figure *5.2*a, b and c). Relative to the initial bacterial load present within the SFW, the biofilter was shown to significantly reduce *E. coli* cell numbers by 99.4 ± 0.60 % (absolute mean reduction of 4.02 ± 0.01 Log_{10}CFU; Figure *5.2*a), *E. faecalis* by 99.9 ± 0.04 % (absolute mean reduction of 4.01 ± 0.22 Log_{10}CFU; Figure *5.2*b), and *P. aeruginosa* by 92.1 ± 10.9 % (absolute mean reduction of 3.93 ± 0.093 Log_{10} CFU; Figure *5.2*c). The control system containing sterile filter media also resulted in significant reductions for *E. coli* and *E. faecalis*, whereby a reduction from 3.97 ± 0.06 Log_{10}CFU to 3.48 ± 0.07 Log_{10}CFU for *E. coli* and 3.91 ± 0.15 Log_{10}CFU to 3.64 ± 0.14 Log_{10}CFU for *E. faecalis* was observed (Figure *5.2*; $p < 0.001$). However, an increase from 4.01 ± 0.04 Log_{10}CFU to 6.55 ± 1.09 Log_{10}CFU was observed for *P. aeruginosa*. For the system with the empty filter column, small non-significant reductions were observed for *E. coli* and *E. faecalis* ($p > 0.05$ Figure *5.2*a and b). There was a reduction from 3.94 ± 0.02 to 3.82 ± 0.08 Log_{10}CFU for *E. coli* and a reduction from 3.94 ± 0.02 to 3.82 ± 0.08 Log_{10}CFU for *E. faecalis*. However, for *P. aeruginosa* there was a significant increase from 3.93 ± 0.13 to 6.69 ± 0.94 Log_{10}CFU after 24 h of circulation in the system with the empty filter column.

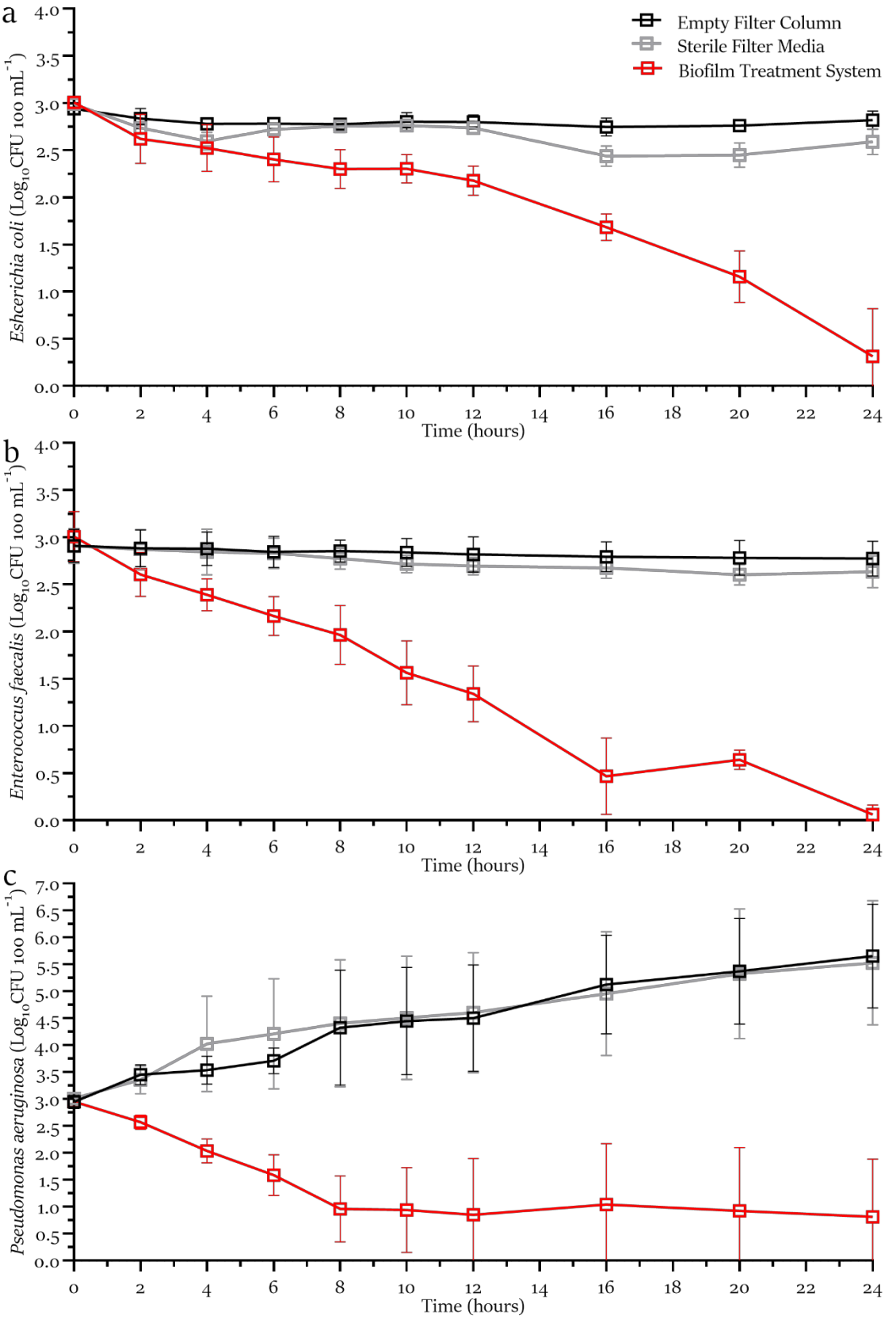


Figure 5.2: Viable counts of three test pathogens: (a) *Escherichia coli*, (b) *Enterococcus faecalis* and (c) *Pseudomonas aeruginosa* within simulated freshwater when circulated through a laboratory scale biofilm treatment system (red) and control systems incorporating an empty filter column (black) and a column containing the sterile filter media (grey) over 24 hours, all performed in triplicate. n=3 ± s.d.

5.2.3 The accumulation of Escherichia coli, Enterococcus faecalis and Pseudomonas aeruginosa on the filter media

To determine if there was any accumulation or retention of the test bacteria on the filter media or sterile filter media following the 24 h circulation (Figure 5.2), viable counts of the test bacteria were taken from the filter media within the filter columns (section 2.8). For *E. coli,* no viable cells were recovered from the biofilter media, whereas 3.03 ± 0.03 Log_{10}CFU $filter^{-1}$ was recovered from the sterile filter media after 24 h of circulation (Table 5.1). For *E. faecalis,* 0.91 ± 0.65 Log_{10}CFU $filter^{-1}$ was recovered from the BTS after 24 h of circulation. This equates to 0.06 % of the starting density that was able to accumulate and survive on the filter media of the BTS after 24 h, although this was significantly less than the 2.46 ± 0.14 Log_{10}CFU $filter^{-1}$ recovered from the sterile filter media ($p < 0.05$; Table 5.1). For *P. aeruginosa*, 1.89 ± 0.99 Log_{10}CFU $filter^{-1}$ (equating to 1.26 % of the starting inoculum) was recovered from the BTS filter media whereas 4.24 ± 0.49 Log_{10}CFU $filter^{-1}$ was recovered from the sterile filter media. Moreover, there was an average increase from 3.93 ± 0.13 Log_{10}CFU to 6.53 ± 1.12 Log_{10}CFU within the system containing sterile filter media in comparison to an average decrease from 3.97 ± 0.04 Log_{10}CFU to 1.89 ± 0.99 Log_{10}CFU for *P. aeruginosa* when cycled through the BTS. To ensure that there was no significant difference between the system containing the sterile filter media and the system containing the empty filter column for all three species of test bacteria after 24 h of circulation, the total number of cells on the sterile filter media and within the SFW were compared against the number of cells in the SFW of the system with the empty filter column and were found to be non-significant for all three test species ($p > 0.05$).

Table 5.1: Total number of viable test bacteria in the simulated freshwater (SFW) recorded in Log_{10}CFU pre and post 24 hours circulation through the filter columns containing sterile filter media (SFM) and biofilm treatment system filter media (BFM). The total number of test bacteria in the system presented in this table is the combination of test bacteria in the SFW and on the filter media. Statistical significance was then determined by comparing the total number of test bacteria in the system of SFM and BFM after 24 h of circulation (***= p < 0.001). All filter media samples and SFW samples were performed in triplicate n=3 ± s.d.

Test pathogen	Filter media	Number of test bacteria pre-circulation in the SFW (Log_{10}CFU)	Number of test bacteria post-circulation in the SFW (Log_{10}CFU)	Number of test bacteria accumulated on Filter media (Log_{10}CFU $filter^{-1}$)	Total number of test bacteria in the system (Log_{10}CFU)
Escherichia coli	SFM	3.97 ± 0.06	3.70 ± 0.05	3.03 ± 0.03	3.78 ± 0.05
	BFM	4.02 ± 0.01	0.63 ± 0.48	0	0.63 ± 0.48***
Enterococcus faecalis	SFM	3.91 ± 0.15	3.64 ± 0.14	2.46 ± 0.14	3.67 ± 0.13
	BFM	4.01 ± 0.22	0.39 ± 0.55	0.91 ± 0.65	0.39 ± 0.55***
Pseudomonas aeruginosa	SFM	4.01 ± 0.04	6.55 ± 1.09	4.24 ± 0.49	6.55 ± 1.08
	BFM	4.00 ± 0.04	1.89 ± 0.99	2.05 ± 0.11	2.53 ± 0.53***

5.2.4 The circulation of environmental water through biofilm treatment systems.

To further investigate the control of potential waterborne pathogens under conditions more representative to that found in the environment, water taken from an environmental water source was circulated through the laboratory scale BTS and a control system consisting of an empty filter column for 24 h. For this experimental run, the column containing sterile filter media was not included, as this was previously demonstrated to not have a significant effect on the viability of the waterborne pathogens equating to a total number of 15 biological replicates (Figure *5.1* and Figure *5.2*). A range of standard biological and physiochemical indicators of water quality (see Table 5.1) were determined before and after 24 h of circulation. After 24 h, significant reductions in the biological indicator species (*E. coli* and enterococci) were observed ($p < 0.05$; Table *5.2*). There was a reduction of 99.8 ± 0.20 % for *E. coli* and 99.4 ± 0.41 % for enterococci. There was no significant change in the heterotrophic plate counts in the environmental water after 24 h circulation through the biofilter, however, there was a significant increase of 403 % (equating to a total increase of 4.98 $Log_{10}CFU\ mL^{-1}$) in heterotrophic plate counts in the environmental water when circulated through the empty filter column.

The concentration of nitrogen species in the environmental water decreased after 24 h of circulation through the BTS. The BTS was able to reduce the concentration of ammonium by 100 %, nitrite by 100 % and nitrate by 30.8 ± 11.3 % of ($p < 0.05$; Table *5.2*). The reduction of ammonium and nitrite by 100 % was recorded as below the detection limit of the IC (concentration < 0.01 mg L^{-1}). Moreover, the reduction of all three nitrogen species were significantly different from the empty filter column at 24 h. The concentration of nitrogen in the environmental water that was circulated through the empty filter column showed an increase in nitrite of 71 % and a small increase in the concentration of ammonium and nitrate ($p > 0.05$; Table *5.2*). The concentration of total inorganic carbon (TIC) increased after 24 h of circulation through the biofilter. There was a significant difference between the biofilter and the empty filter column with the TIC increasing by 5.8 % ($p < 0.05$; Table *5.2*). However, the concentration of total organic carbon (TOC) was not significantly different from the empty filter column ($p < 0.05$; Table *5.2*). When comparing the water quality parameters of the environmental water pre and post circulation, there was a significant reduction in ORP ($p < 0.01$; Table *5.2*) a significant increase in dissolved oxygen (DO) ($p < 0.05$; Table *5.2*) and non-significant changes in conductivity or total dissolved solids (TDS) ($p > 0.05$; Table *5.2*). However, there was no significant difference between the biofilter and the empty filter column post treatment for DO, pH, ORP, conductivity or TDS ($p > 0.05$; Table *5.2*).

Table 5.2: Water quality parameters of environmentally-derived surface water pre and post 24 hours of circulation through biofilm treatment systems and empty filter columns n=3 ± s.d. Significant difference was determined between the Biofilm treatment system (BTS) and the Empty Filter Column (EFC) at 24 h post circulation (* = $p < 0.05$).

Parameter	Pre-circulation	Post-circulation	
		EFC	BTS
Presumptive *Escherichia coli* (CFU 100mL^{-1})	214 ± 31	122 ± 21.7	0.33 ± 0.47*
Enterococci (CFU 100mL^{-1})	302 ± 18	153 ± 6.79	1.67 ± 1.25*
Heterotrophic Plate Counts (Log_{10}CFU mL^{-1})	4.32 ± 0.07	5.06 ± 0.11	4.37 ± 0.03*
Total Organic Carbon (mg L^{-1})	4.87 ± 0.13	7.10 ± 1.06	5.05 ± 0.11
Total Inorganic Carbon (mg L^{-1})	20.78 ± 0.11	20.09 ± 0.11	22.05 ± 0.21*
Ammonium (mg L^{-1})	0.11 ± 0.04	0.2 ± 0.11	<0.01*
Nitrite (mg L^{-1})	0.23 ± 0.08	0.33 ± 0.09	<0.01*
Nitrate (mg L^{-1})	7.49 ± 0.98	7.25 ± 0.26	4.80 ± 0.26*
Dissolved Oxygen (mg L^{-1})	4.86 ± 0.2	8.05 ± 0.06	7.94 ± 0.11
ORP	349.8 ± 9.25	219 ± 8.16	205.3 ± 6.18
pH	8.13 ± 0.05	8.09 ± 0.01	7.75 ± 0.42
Conductivity (µS cm^{-1})	674.6 ± 7.76	679.2 ± 5.23	708.4 ± 8.24
Total Dissolved Solids	470.8 ± 5.58	477 ± 2.83	489.5 ± 1.5

5.2.5 Bioremediation of nutrients within an environmentally-derived water source using biofilm treatment systems.

To ensure that oxygen was not a limiting factor for microbial respiration and some nitrification processes within the treatment systems, DO was recorded [see section 2.10.5] over the experimental period. After the first initial time point, there was a consistent concentration of DO for the remaining 24 h, starting at 7.5 mg L^{-1} after 3 h and finishing at 7.7 mg L^{-1} after 24 h of circulation through the biofilm treatment system.

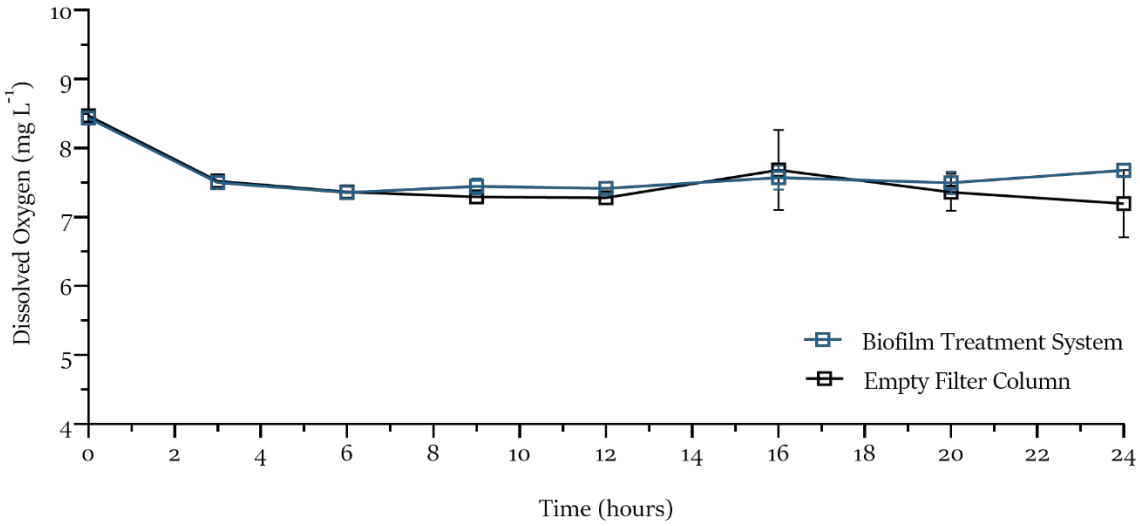


Figure 5.3: The concentration of dissolved oxygen within environmentally-derived water when circulated through a biofilm treatment system and a control system incorporating an empty filter column over 24 hours. n=3 ± s.d.

To further investigate the reduction of nutrient concentrations within the environmentally-derived water observed in the BTS (Table *5.2*), nutrients were monitored at regular intervals over the 24 h circulation period. At the start of the experimental runs there was no significant difference between the BTS and control system for any of the nutrients measured (Figure 5.4 and Figure 5.5; $p > 0.05$). There was a reduction in TOC over the 24 h of circulation through the BTS from 3.69 ± 0.47 mg L^{-1} to 2.88 ± 0.27 mg L^{-1} however, this was not significantly different from the control system after 24 h of circulation. A significant increase over 24 h of circulation was observed in the concentration of TIC from 29.1 ± 0.47 to 31.1 ± 0.39 mg L^{-1} (Figure 5.4; $p < 0.05$) and when comparing the TIC concentration in the BTS and empty filter column, the concentration of TIC was significantly lower than the BTS (Figure 5.4; $p < 0.05$).

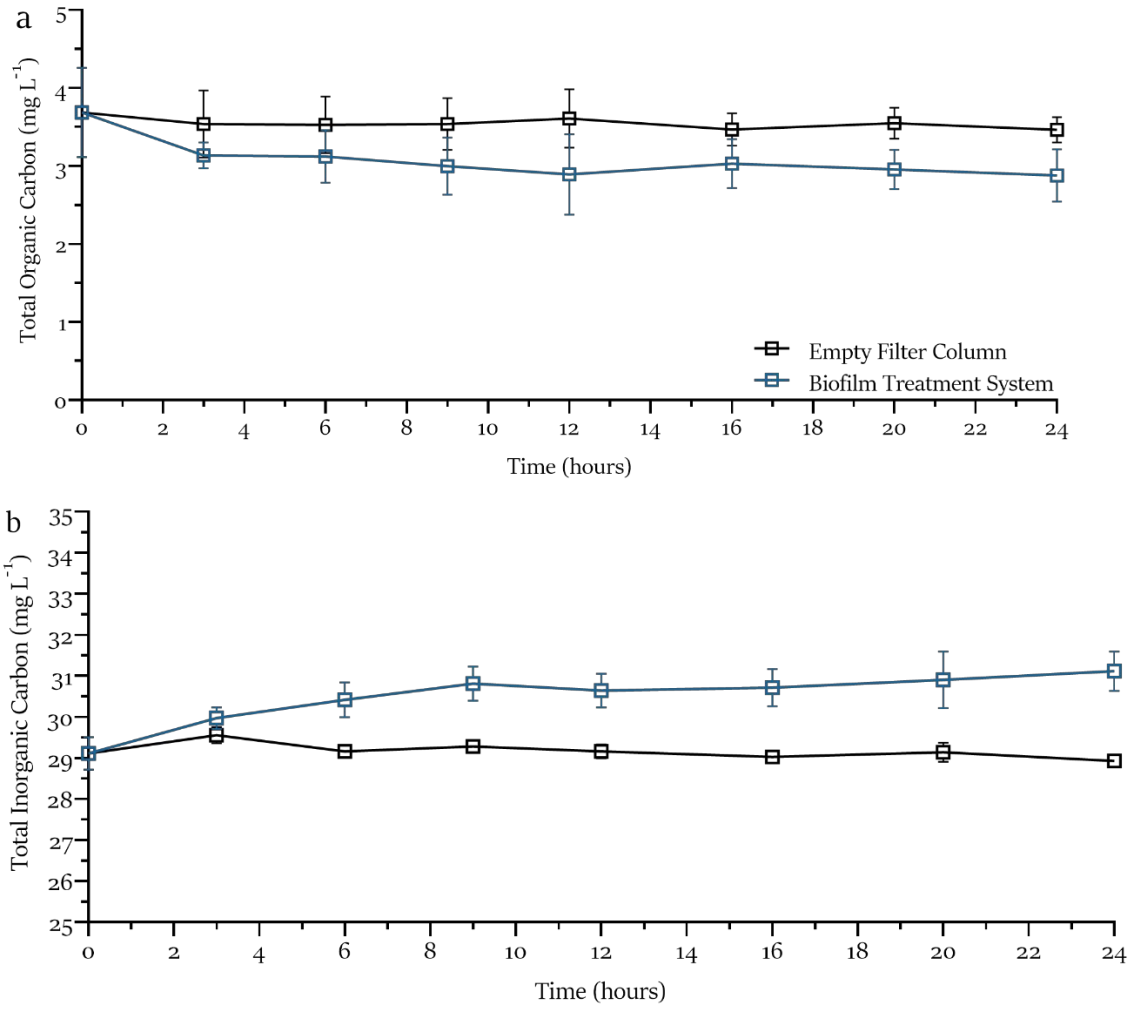


Figure 5.4: Concentration of (a) total organic carbon (TOC) and (b) total inorganic carbon (TIC) within an environmentally-derived surface water source when circulated through laboratory scale systems incorporating a biofilter and an empty filter column for 24 hours. All experiments performed in triplicate. n=3 ± s.d.

For phosphate, there was a significant increase from 1.94 ± 0.56 to 2.81 ± 0.42 mg L^{-1} after 24 h (Figure 5.5; $p < 0.05$). For the nitrogen species, levels of ammonium during experimentation were below the limit of detection (concentration < 0.001 mg L^{-1}) and are therefore not presented. Nitrite concentration did not show any significant change over the 24 h circulation period within the BTS, starting at 0.68 ± 0.04 and ending at 0.72 ± 0.02 mg L^{-1} after 24 h (Figure 5.5). The concentration of nitrate was significantly higher at 12.1 ± 0.40 mg L^{-1} in the biofilm treatment system compared to the 9.47 ± 0.89 mg L^{-1} in the control system after 24 h of circulation (Figure 5.5).

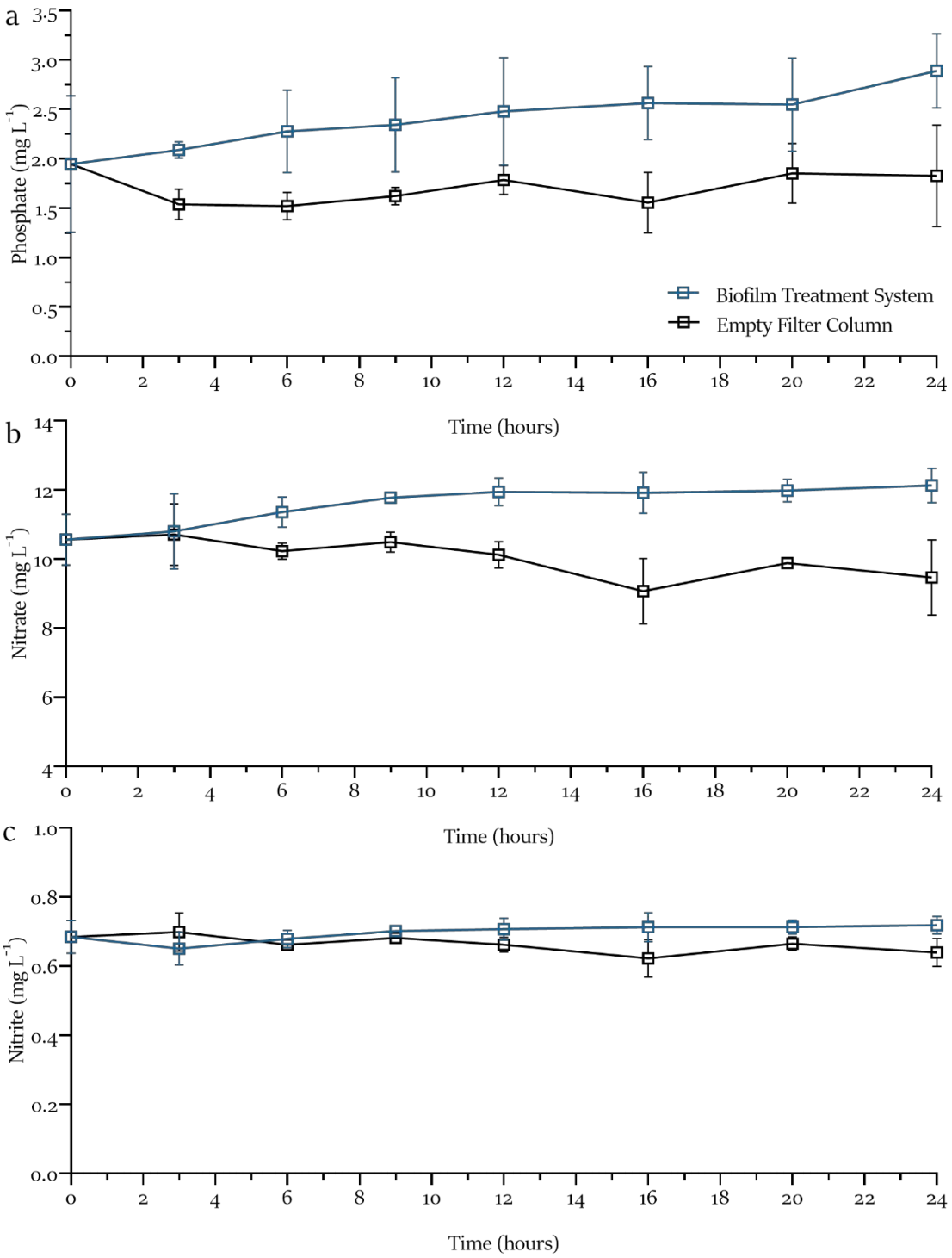


Figure 5.5: Concentration of (a) phosphate (PO_4^{-3}), (b) nitrate (NO_3^-) and (c) nitrite (NO_2^-) within an environmentally-derived surface water source when circulated through laboratory scale biofilm treatment systems and control systems incorporating an empty filter column for 24 hours. All experiments performed in triplicate. n=3 ± s.d.

5.3 Discussion

Previous research that has utilised biofilters in water treatment has been primarily focused on the removal of organic material and contaminants of emerging concern (CEC) (10,78,81,82,89,90,178–180). The use of BTS to control aquatic pathogens for drinking water treatment has not been extensively reported in the literature. One previous study demonstrated that BTS were capable of reducing viable *E. coli* by 56% through treating contaminated environmental water with gravity fed, open rock biofilter systems (130). In addition, a biofilter system that utilised foam to establish a biofilm has been shown to significantly reduce the non-pathogenic bacterial species, *Raoultella terrigena,* in comparison to a sterile control system (55). However, this reduction was achieved using a complex multi-step filtration system and the role of the biofilter component in the removal of *R. terrigena* remains unclear. Therefore, there is limited knowledge of the direct effect of biofilm within small-scale biofilter systems (i.e. in the absence of other treatment interventions), to control the numbers of pathogenic bacteria for potable water.

When initially investigating effect of flow rate on the viability of *E. coli,* significant differences were observed. The greatest reduction was seen by 138 and 35 mL min^{-1} (Figure 5.1) and the flow of 138 mL min^{-1} did have a faster removal rate than 35 mL min^{-1}. One possible reason for this was the shear forces within the filter column at 138 mL min^{-1} resulting in a greater reduction in *E. coli* and that an increased number of 'passes' through the filter had an increased reduction in comparison to 35 mL min^{-1}. The potential effect of the greater shear forces is evidenced by the increased error at 138 mL min^{-1} whereby the standard deviation is larger for both the column containing the sterile filter media and the BTS than 35 mL min^{-1} (Figure 5. 1a & b). Nonetheless, due to the energy input of maintaining a flow rate of 138 ml min^{-1} at scale and that there was no significant difference between 138 and 35 mL min^{-1}, 35 mL min^{-1} was chosen for further experimentation.

The significant reduction in the number of viable test bacteria within the SFW after 24 h of circulation through the BTS (Figure 5.1 & 5.2; $p < 0.001$) may have resulted from cell death that either occurred in part, or in whole, within the SFW or on the biofilter media. To account for any loss of pathogens within the SFW, or through physical filtration effects from the ceramic filter media, control columns consisting of an empty filter column and column containing sterile filter media were employed in parallel with the BTS. Within the systems incorporating the empty filter column and column containing sterile filter media, there was survival of *E. coli* and *E. faecalis* (Figure 5.2a and b) and proliferation of *P. aeruginosa* (Figure 5.2c) within the reservoir. This demonstrates that the SFW was able to support the survival of test pathogens and that there was a minimal physical filtration effect from the ceramic filter media over the 24 h circulation period.

Collectively, this strongly suggests that the biofilm present on the filter media was primarily responsible for the reduction of test pathogens observed (Figure 5.1 & 5.2).

A possible mechanism for this reduction may have been through the direct competition between the planktonic cells and the biofilm established on the filter media. To support this, the accumulation of test bacteria on the filter media was investigated because of the potential for the BTS to become a reservoir for the test bacteria. Previous studies have demonstrated that planktonic bacteria have greater affinity for attachment to mature biofilms, as opposed to sterile surfaces (122,181,182). Moreover, the formation of biofilm is a key survival strategy for microorganisms in challenging environments and therefore, there is a risk that the planktonic test bacteria could survive within the biofilm present on the filter media and contaminate the water supply. Nonetheless, this study demonstrates that viable *E. coli* was unable to accumulate on the filter media within the BTS. This may have resulted from classical competitive exclusion within the biofilm of the BTS, whereby the challenging test bacteria could have been inhibited by the autochthonous bacteria established within the biofilm present on the BTS media (131). For example, competition strategies target the capability of some microorganisms to form a biofilm, resulting in death or the limitation of growth in the competing bacteria (132) The competition between cells in biofilms can involve inhibitory or cidal mechanisms, such as the production of antibiotics and bacteriocins (133,134) or strategies that compromise growth, such as nutrient depletion (135). Surface-active compounds (SACs) could also be being produced by the microbial biofilm community (biosurfactants), which are amphipathic lipid-based molecules that lower interfacial tension and some of these biosurfactants display antimicrobial properties (136). Therefore, production of these biosurfactants could reduce the ability of the test pathogens to accumulate and establish on the biofilm formed within the BTS. In addition, interference mechanisms might also be upregulated in response to the presence of competition in the surroundings, known as the competition-sensing hypothesis (137).

Overall, the BTS resulted in significant reductions of all the test pathogens (Figure *5.2*; $p < 0.001$ and (Table 5.1; $p < 0.05$). However, there were differences in the reductions of test pathogens in the reservoir and on the filter media. Within the reservoir, the total bacterial reductions observed were 99.4 ± 0.60 % for *E. coli*, and 99.9 ± 0.04 % for *E. faecalis* and 92.1 ± 10.9 % for *P. aeruginosa*. This may partially result from the differential tolerance of these microorganisms to persist within the simulated freshwater environment. The lower reduction of *P. aeruginosa* is unsurprising, given it is a ubiquitous environmental bacterium which can survive in oligotrophic environments, including up to 5 years in bottled water (183). On the filter media, some viable *E. faecalis* and *P. aeruginosa* were recovered from the BTS filter media, albeit in very low numbers (Table 5.1). The inability of *E. coli* to colonise the BTS filter media could possibly be related to the lack of an appropriate 'colonising partner', which has been shown within capillary flow cell systems (181). Nonetheless, in the absence of biofilm, all the species tested were able to adhere to and survive upon the sterile filter media in significantly higher numbers (Table 5.1; $p < 0.05$).

Therefore, this provides further evidence of competitive exclusion involving potential inhibitory effects by the biofilm on the test bacteria that accumulated on the filter media within the BTS.

Even though an overall mean reduction of 92 % ± 10.9 % was observed within the SFW (Figure *5.2c*), the action of competitive exclusion within the BTS may also explain why only a small number of *P. aeruginosa* were recovered from the BTS filter media (Table 5.1). It was found that significantly higher numbers of *P. aeruginosa* were able to attach and survive on the sterile filter media (4.24 ± 0.49 Log_{10}CFU $filter^{-1}$) when compared to the BTS filter media (2.05 ± 0.11 Log_{10}CFU $filter^{-1}$) ($p < 0.05$; Table 5.1). Moreover, there was a stark increase of *P. aeruginosa* within the SFW of the systems containing the sterile filter media and the empty filter column to 6.55 ± 1.09 Log_{10}CFU and 6.69 ± 0.94 Log_{10}CFU respectively, in comparison to the mean reduction of *P. aeruginosa* in the BTS to 1.89 ± 0.99 Log_{10}CFU. Collectively, the data presented in this chapter demonstrates that the survival of *P. aeruginosa* is significantly lower within the BTS when compared to the sterile filter media and empty filter column controls ($p < 0.001$; Table 5.1). In this chapter the main findings present that the use of a laboratory scale BTS was able to significantly reduce the number of test pathogens within the SFW. There was a reduction in the survival of *E. coli*, *E. faecalis* and *P. aeruginosa* within biofilms present on the BTS filter media after 24 h of circulation, whereby the introduced test bacteria may have been unable to survive as a result of competitive exclusion by the established biofilm on the filter media.

This study demonstrates that matured biofilms present on a ceramic substrate can reduce the numbers of viable *E. coli*, *E. faecalis* and *P. aeruginosa* within a water source used to challenge these BTS. To further support the reduction of these test bacteria using BTS, the survival of environmental *E. coli* and enterococci present within an environmental water source circulated through the lab-scale BTS was investigated. Biofiltration relies on the processing and metabolism of cells within the biofilm, therefore variability of physiochemical conditions will impact biofiltration in real-world applications. For example, a lower rate of organic biodegradation has been observed at lower temperatures thus affecting filter performance (75,85). Nutrient concentrations within the input water to the BTS have also been shown to have significant impact on performance, whereby the removal of carbon is dependent on the availability of other nutrients such as nitrogen and phosphorous (184). Therefore, it was key to investigate the reduction of waterborne pathogens within a water source that was more applicable to biofilter operation in the real-world. The results of these experiments demonstrated that the BTS was able to reduce the levels of the indicator species within an environmentally-derived water source ($p < 0.05$; Table *5.2*). Moreover, the BTS were able to suppress the growth of heterotrophic bacteria in the environmentally derived water, whereby there was no significant change after 24 h of circulation in contrast to a significant increase of 4.97 ± 0.14 Log_{10}CFU mL^{-1} when circulated through the empty filter column ($p < 0.05$; Table *5.2*). A similar trend was observed when *P. aeruginosa* was used to challenge the biofilter, whereby a reduction was observed when circulated through the biofilter but

an increase of 3.69 ± 0.94 $Log_{10}CFU\ mL^{-1}$ was observed in the SFW when circulated through the empty filter column (Figure *5.2*). *P. aeruginosa* is ubiquitous in the environment and is known for its phenotypic variability and ability to actively grow within aquatic freshwater systems (185). Therefore, it is likely that the *P. aeruginosa* used in this study was able to survive and grow within the environmentally-derived water. Moreover, it is possible that the reduction in *P. aeruginosa* resulted from a phenotypic switch to a viable but non-culturable (VBNC) state where metabolism is downregulated; however, this is more likely when *P. aeruginosa* is established within biofilms rather than in a planktonic state (186). The results from this study indicate that a very small number of *P. aeruginosa* was able to survive on the biofilter, even though there were still significant reductions of viable *P. aeruginosa* in the reservoir and on the filter media when compared to the respective controls. The small sub-population of *P. aeruginosa* that was recovered from the biofilter media after 24 h was significantly less than the sterile filter media. This suggests that the environmental biofilm is exerting some inhibitory effect on planktonic *P. aeruginosa*.

The significant reduction in the concentration of nitrogen sources within the environmental water ($p < 0.05$; Table *5.2*) could influence the survival of waterborne pathogens. The nitrification of ammonium is a key part of the global nitrogen cycle that is driven by microorganisms and occurs under aerobic conditions (Equation 1.1) and anaerobic conditions (Equation 1.2). These interactions rely on the close proximity of cells that exchange metabolites, to enable efficient exchange by diffusion. The reduction of nitrate within this study indicates possible localised low oxygen/anoxic conditions within the biofilm even though the system was vented, therefore it is likely that oxygen diffusion gradients exist within the biofilm attached to the filter media (96). Such localised diffusion gradients of oxygen within biofilms have been previously described, whereby aerobic microbial metabolism can protect anaerobic organisms deeper within the biofilm (114,187). Therefore, it is likely that microorganisms that are able to convert nitrate into nitrogen resulted in the reductions observed (Table *5.2*). this was confirmed but the results presented in chapter 4 whereby Nitrosomonas were detected within the biofilm of the laboratory scale BTS. The effect of nutrients including carbon will impact the growth and survival of planktonic bacteria in water, and this can be demonstrated experimentally (188,189). However, there was no significant reduction in carbon within this study (Table *5.2*; $p > 0.05$), and hence the reduction of viable indicator species observed is more likely to have occurred through direct interactions between the biofilm and the test species. There was no significant difference in TOC between the biofilter and empty filter column control. However, there was a significant difference in TIC after 24 h of circulation. It is possible that the TOC increased from cell death which was then mineralised by the BTS to result in the observed increase in TIC, such cannibalistic behaviour has previously been reported in wastewater treatment processes (190). Moreover, denitrification has been shown to be involved with the precipitation of inorganic carbon (132) that could explain the increase in DIC observed within the circulation tank of the BTS (Table *5.2*; $p < 0.05$).

Chapter 6 Proof of concept; the control of pathogens using up-scaled biofilm treatment systems

6.1 Introduction

There are many technologies that aim to improve the accessibility of clean water. The primary aim of these solutions is to control the levels of waterborne pathogens from a source water. Within low to medium income countries, the research and development of sustainable and compact water treatment systems for improving access to clean water is summarised in Table 1.2 (35). However, as highlighted in chapter 1 these technologies come at substantial cost for the end user. Treatment solutions needed should be sustainable in the long term, i.e., scalable, low cost, with maintenance and material requirements that can be understood and maintained by the end user. The sustainable approach trialled in this chapter is the application of biofilm treatment systems (BTS) consisting of biofilms adherent to ceramic filter media. A ceramic medium was chosen for its low-cost and inert properties (summarised in chapter 3). Ceramics can be manufactured in close proximity to the BTS deployment using clay as an abundant, non-toxic and low impact manufacturing medium. Therefore, this has the potential to be an accessible, low-cost method of water treatment to improve water quality for the 2 billion people that consume untreated water potentially contaminated with faeces. It has been demonstrated in Chapter 5 that BTS can improve water quality through the control of waterborne pathogenic bacteria at laboratory scale. To further demonstrate the utility of this approach requires the development and testing of BTS at scale. Therefore, the aim of this chapter was to:

(i) Develop upscale biofilm treatment systems to investigate the bioremediation of an environmentally-derived water source contaminated with waterborne pathogens for off-grid applications.

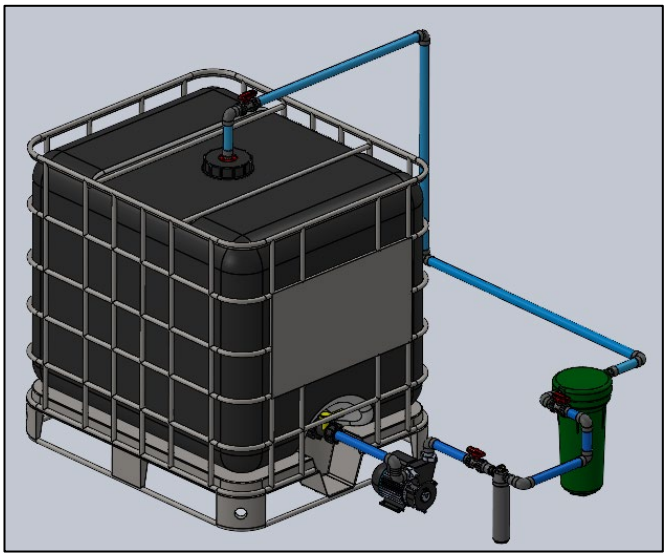


Figure 6.1 Schematic overview of the upscaled biofilm treatment system using computer-aided design (CAD). More detailed schematic presented in section 2.6.4.

6.2 Results

6.2.1 Bacteriological quality of environmentally-derived fresh water

The bacteriological water quality of the environmentally-derived fresh water [see section 2.4] used in this trial was monitored for levels of environmental *E. coli* and enterococci contamination. The detection of *E. coli* (a specific faecal coliform) and enterococci indicates the potential for other pathogen contamination within water (191). Moreover, current regulatory guidelines stipulated by the environment agency use these two organisms to monitor water standards and were therefore monitored with the environmentally-derived fresh water. The levels of indicator species notably increased during between May – October (Figure 6.2), ranging from a mean monthly average of 157.6 to 891.7 CFU 100 mL^{-1} for *E. coli*. The number of viable *E. coli* was markedly lower between November and April with a range of 8 – 133 CFU 100 mL^{-1}. Minimum concentration of *E. coli* was observed in April at 8 ± 1.6 CFU 100 mL^{-1} and the maximum concentration during September, averaging of 891.7 ± 548.1 CFU 100 mL^{-1} (Figure 6.2). The trend in the number of enterococci was similar to that of *E. coli*, ranging from 165.6 – 1223.3 CFU 100 mL^{-1} between May and October, that was significantly higher than that observed between November and April with a range of 3 – 78 CFU 100 mL^{-1} The minimum and maximum concentrations were March averaging 3 ± 10 CFU 100 mL^{-1} and the maximum was observed during September with a concentration of 1223.3 ± 1097.6 CFU 100 mL^{-1} (Figure 6.2).

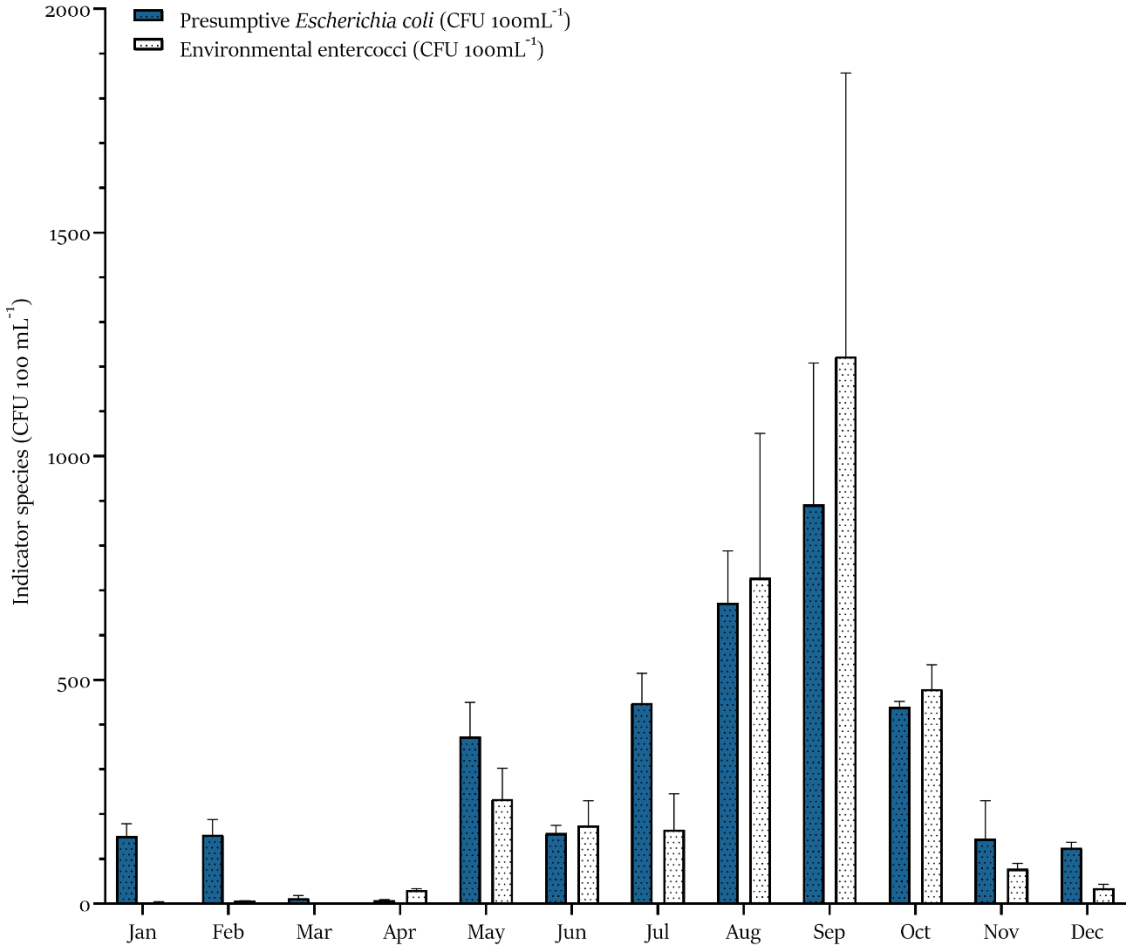


Figure 6.2: Monthly averages of total Escherichia coli and enterococci (CFU 100 mL-1) within the environmentally-derived water body at: N 51°29′56″, W 2°32′39″. Each monthly average is taken from a minimum of 3 samples ± s.e.m.

6.1.1 Viable counts of environmental Escherichia coli and enterococci are significantly reduced using upscaled biofilm treatment systems.

To validate the reduction of waterborne pathogens observed within laboratory scale BTS presented in chapter 5, upscaled BTS (see schematic figure were challenged with an environmentally-derived water source known to be contaminated with *E. coli* and enterococci (Figure 6.2). The experiment was conducted during September when the levels of indicator species were recorded at their highest during the year (Figure 6.2). A control system was employed that consisted of the same experimental setup incorporating an empty filter vessel. At the start of all experimental runs, there was no significant difference in the starting bacterial load of the indicator species tested between the BTS and the control system (Figure 6.3; $p > 0.05$). The starting density for *E. coli* was 3.08 ± 0.10 Log_{10}CFU 100 mL^{-1} and 3.39 ± 0.04 Log_{10}CFU 100 mL^{-1} for enterococci (Figure 6.3). After 24 h of circulation, significant reductions were observed between the upscaled BTS and the control system. The number of viable *E. coli* recovered from the reservoir of the BTS was 2.67 ± 0.13 Log_{10}CFU 100 mL^{-1} in comparison to 3.03 ± 0.04 Log_{10}CFU 100 mL^{-1} recovered from the reservoir of the control system. A significant reduction in the number of viable enterococci was observed. However, significant differences were observed after 48 h of circulation where 3.39 ± 0.04 was significantly reduced to 2.61 ± 0.14 Log_{10}CFU 100 mL^{-1} (Figure 6.3; $p < 0.05$). Further reductions were observed by 7 days. After 7-days there was 1.31 ± 0.28 Log_{10}CFU 100 mL^{-1} of *E. coli* and 1.24 ± 0.21 Log_{10}CFU of enterococci remaining in the reservoir of the BTS, equating to an absolute reduction of 1.73 ± 0.33 Log_{10}CFU for *E. coli* and 2.14 0.23 Log_{10}CFU for enterococci. A reduction in viable counts continued for the remainder of the experimental period resulting in an total reduction (concentration of cells × volume) of *E. coli* and enterococci of 2.77 ± 0.28 and 4.38 ± 2.11 Log_{10}CFU after 28 days of circulation respectively.

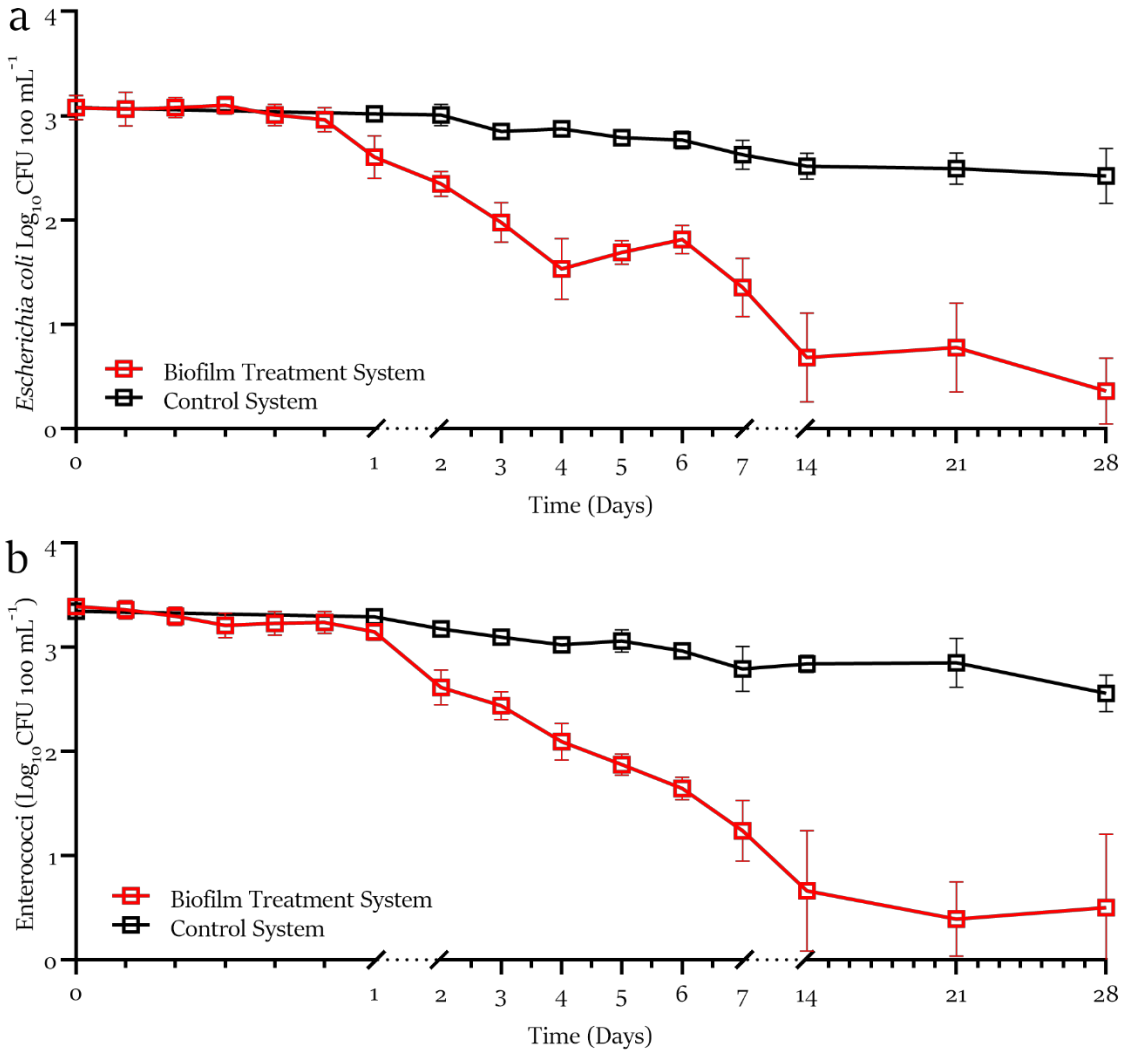


Figure 6.3: Viable counts of (a) Escherichia coli and (b) environmental enterococci within an environmentally derived fresh water when circulated through an upscaled biofilm treatment system and control system for 28 days. All experiments performed in triplicate. n=3 ± s.d.

UK water quality standards are determined by the drinking water inspectorate (DWI) and are presented in Table 6.1. These guidelines were used to provide a benchmark target for the up-scaled BTS. The water quality parameters presented were determined from samples taken at the beginning and end of the 4-week trial. The parameters presented in Table 6.1 show that the circulation water was of DWI standard for the physiochemical parameters tested. However, even at the end of 28 days of circulation the presence of *E. coli* and enterococci (albeit in low numbers) meant that the circulated water failed to meet the microbiological standards required by the DWI.

Table 6.1: Water quality parameters of environmentally-derived surface water over 28 days of circulation through up-scaled biofilm treatment systems (BTS) n=3 ± s.d. Drinking water inspectorate (DWI) standards are also shown for guidance as to the performance of the BTS, therefore only DWI monitored parameters are included in this table (192).

Water quality Parameter	Time (Days)					DWI Standard
	0	**7**	**14**	**21**	**28**	
Escherichia coli (CFU 100 mL^{-1})	1233 ± 300	27 ± 14	7 ± 5	5 ± 5	3 ± 1	*0*
Enterococci (CFU 100 mL^{-1})	2454 ± 260	20 ± 8	7 ± 4	3 ± 2	4 ± 4	0
Ammonium (mg L^{-1})	0.05 ± 0.01	< 0.01	< 0.01	< 0.01	< 0.01	0.5
Nitrite (mg L^{-1})	0.18 ± 0.03	0.24 ± 0.01	0.24 ± 0.01	0.25 ± 0.01	0.16 ± 0.05	0.5
Nitrate (mg L^{-1})	14.7 ± 0.55	13.4 ± 0.26	12.3 ± 0.23	12.0 ± 0.22	10.68 ± 0.38	50
Sodium (mg L^{-1})	57.1 ± 0.30	57.6 ± 1.14	56.0 ± 0.41	54.7 ± 2.06	55.2 ± 1.50	200
Conductivity (µS cm^{-1})	853 ± 4.11	842 ± 2.62	842 ± 5.77	834 ± 6.27	834 ± 2.45	2500 (at 20°C)
pH	7.74 ± 0.08	7.89 ± 0.22	7.62 ± 0.28	8.35 ± 0.39	8.27 ± 0.20	6.5 - 9.5

6.2.2 Nutrient concentrations do not change significantly during treatment

The concentration of nutrients within the environmentally-derived surface water were recorded over the 28-day circulation period. There were significant reductions of TOC within the upscaled BTS, decreasing from 1.96 ± 0.03 mg L^{-1} to 1.21 ± 0.06 after 24 h of circulation and TOC continued to decrease to 0.64 ± 0.05 mg L^{-1} after 28 days of circulation (Figure 6.4a; $p < 0.05$). Little change in the concentration of TIC was observed when circulated through the upscaled BTS for the first 14 days of circulation. TIC then increased over the last 14 days of circulation, increasing from 5.25 ± 0.05 mg L^{-1} to 6.67 ± 0.28 mg L^{-1} (Figure 6.4b; $p < 0.01$), which was significantly greater than the control system (5.40 0.31 mg L^{-1}) after 28 days of circulation (Figure 6.4b; $p < 0.05$).

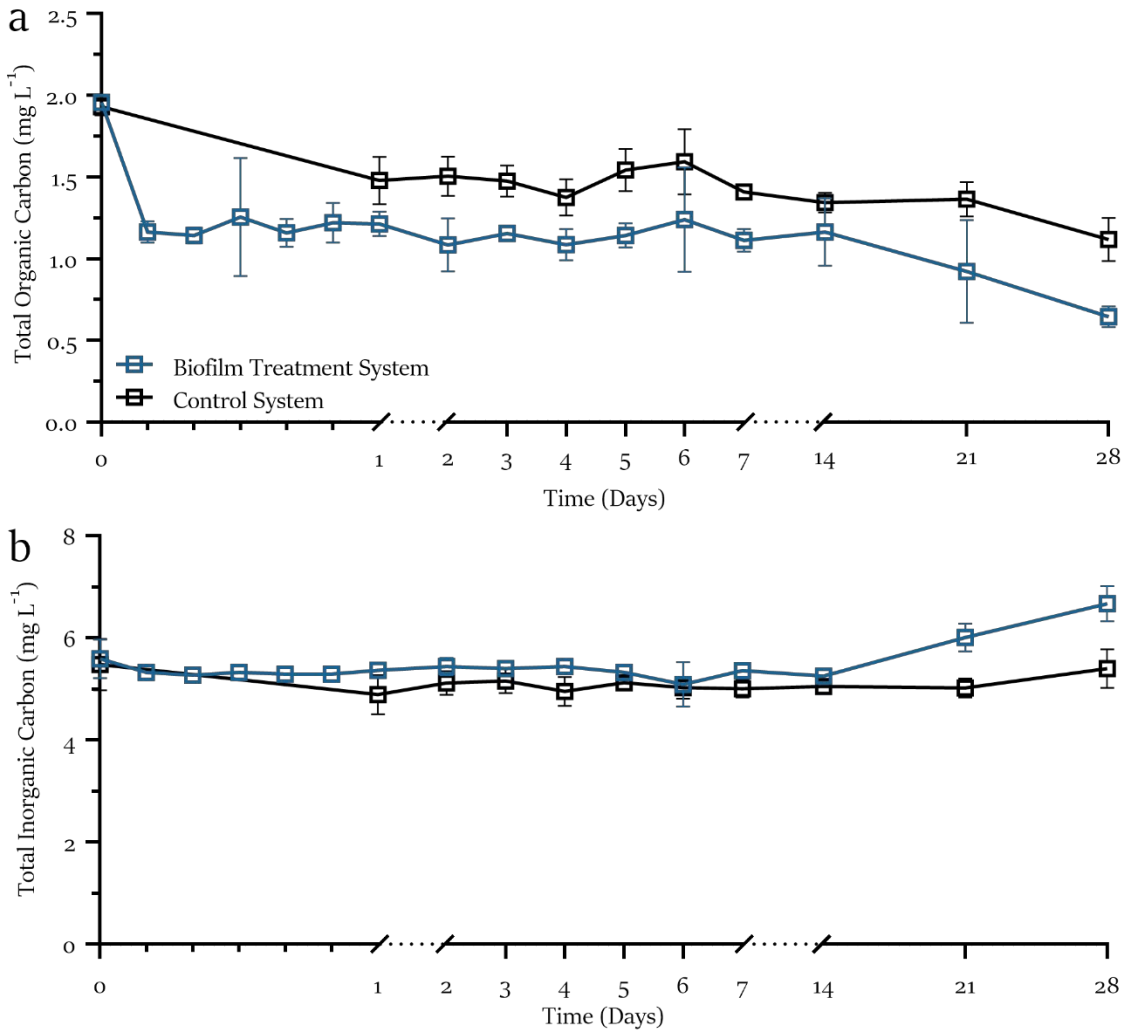


Figure 6.4 the concentration of (a) total organic carbon (TOC) and (b) total inorganic carbon (TIC) within an environmentally-derived surface water source when circulated through upscaled systems incorporating a biofilm treatment system and an empty filter column for 28 days. All experiments performed in triplicate. n=3 ± s.d.

Changes were observed in the concentration of the nitrogen species over the course of 28 days in both the BTS and control systems (Figure 6.5). For ammonium, there were very low concentrations within the environmentally-derived water averaging 0.07 ± 0.05 mg L^{-1} across the BTS and the control systems. After the first 24 h of circulation, 0.006 ± 0.01 mg L^{-1} remained in the BTS in comparison to 0.04 ± 0.03 mg L^{-1} in the control systems (Figure 6.5a). Following a further 24 h of circulation (48 h total) the levels of ammonium dropped below the limit of detection (< 0.01 mg L^{-1}) within the BTS for the remaining 26 days. However, the levels of ammonium within the control system were still detectable at 0.03 ± 0.02 mg L^{-1} after 28 days of circulation. For nitrite, the average starting concentration within the environmental water was 0.34 ± 0.03 mg L^{-1}. These levels remained consistent throughout the 28 days of circulation fluctuating within the BTS from a maximum of 0.38 ± 0.003 mg L^{-1} on day 21 to a minimum of 0.33 ± 0.006 mg L^{-1} on day 28. This was not significantly different from the control systems throughout the 28-day trail (Figure 6.5b $p > 0.05$). There were significant reductions in nitrate observed (Figure 6.5c). At the start of the trial, the concentration of nitrate was 14.7 ± 0.51 mg L^{-1} within the environmental water, which steadily declined within the BTS over the 28 days to reach a final concentration of 11.3 ± 0.65 mg L^{-1} (Figure 6.5c; $p < 0.05$). There were also reductions within the control system, however the final concentration remained higher than the BTS at 13.6 ± 0.53 mg L^{-1} after 28 days of circulation.

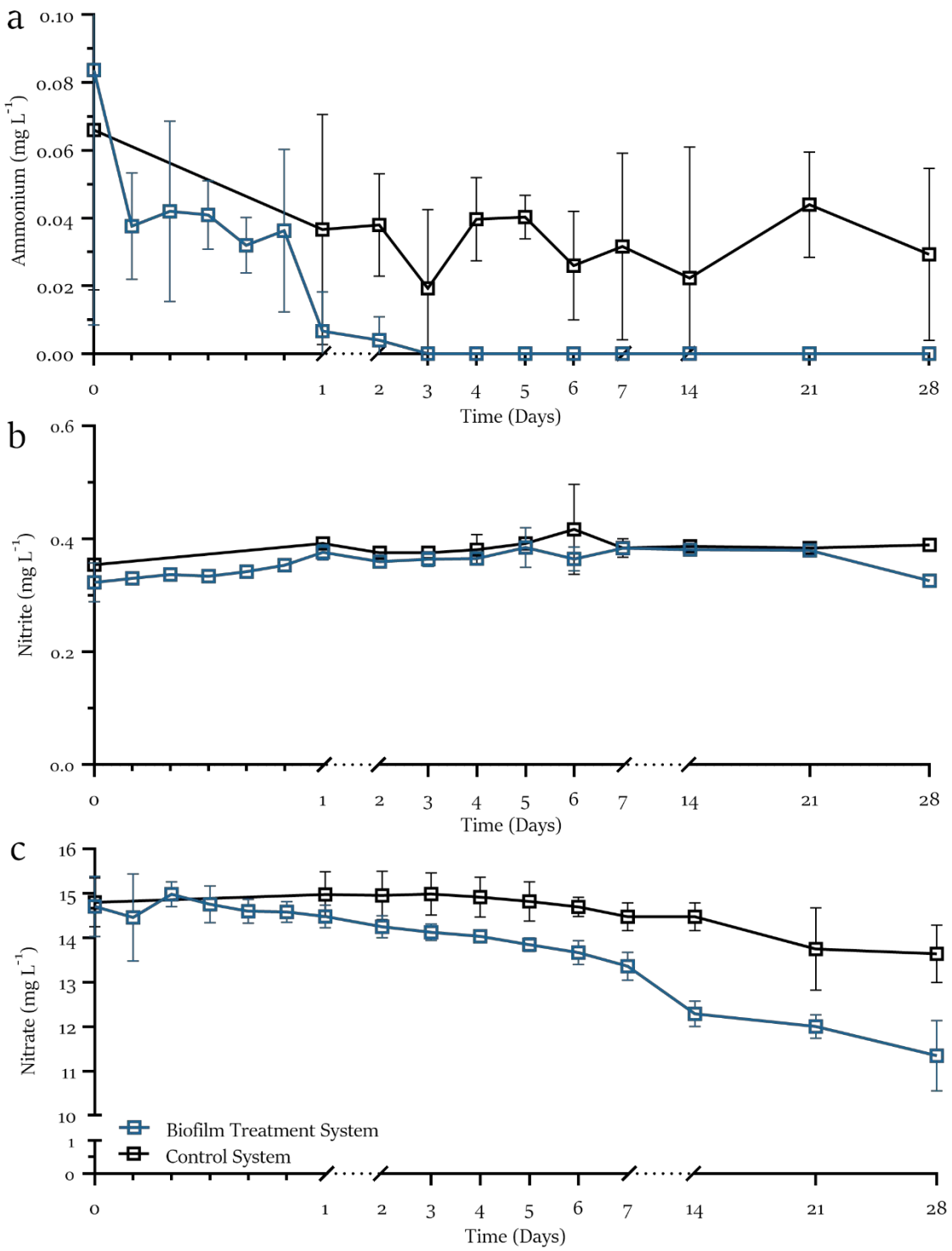


Figure 6.5: The concentration of (a) ammonium (NH_4^+), (b) nitrite (NO_2^-) and (c) nitrate (NO_3^-) within an environmentally-derived surface water source when circulated through upscaled scale biofilm treatment systems and control systems incorporating an empty filter column for 28 days. All experiments performed in triplicate. n=3 ± s.d.

The concentration of phosphate decreased within the BTS over the 28 days of circulation. The starting concentration of phosphate within the environmentally-derived water was 1.89 ± 0.10 mg L^{-1}. This decreased by 0.1 mg L^{-1} within the BTS to 1.79 ± 0.03 mg L^{-1} in comparison to 1.97 ± 0.11

mg L^{-1} in the control system after 24 h of circulation. Further reductions were recorded of 0.27 mg L^{-1} after 14 days of circulation, to a concentration of 1.33 ± 0.03 mg L^{-1} after 28 days of circulation through the BTS. This was significantly less than the 1.89 ± 0.08 mg L^{-1} within the control system after 28 days of circulation (Figure 6.6; $p < 0.05$).

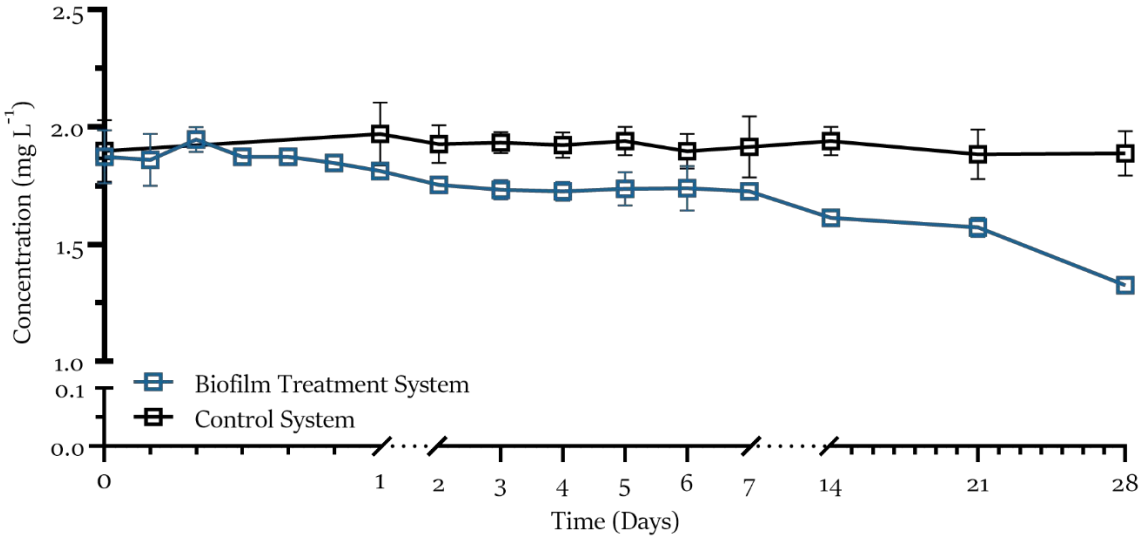


Figure 6.6: The concentration of phosphate (PO_4^{-3}) within an environmentally-derived surface water source when circulated through upscaled biofilm treatment systems and control systems incorporating an empty filter column for 28 days. All experiments performed in triplicate. n=3 ± s.d.

6.2.3 Other physiochemical parameters during recirculation

Standard water quality parameters, DO, temperature, conductivity, pH and TDS were monitored over the 28 days circulation. Overall, the physiochemical parameters within the BTS remained stable over the course of 28 days. For all parameters measured, there was no significant difference between the upscaled BTS and the control systems.

To ensure that the upscale BTS were operating under aerobic conditions DO was monitored over the experimental period. The DO present within the environmentally-derived water at the time of the challenge was 6.30 ± 0.16 mg L^{-1} and this gradually increased in the both the BTS and control system over the 28 days of recirculation significantly increasing to 8.59 ± 0.29 mg L^{-1} within BTS (Figure 6.7; $p < 0.01$). The measurement of DO was not significantly different from the control system throughout all sampling points increasing from 6.36 ± 0.25 to 8.50 ± 0.29 mg L^{-1} (Figure 6.7; $p > 0.05$). This demonstrates that the conditions remained aerobic within the circulation tanks for the duration of the experiment and was not a limiting factor during the challenge of the environmental water.

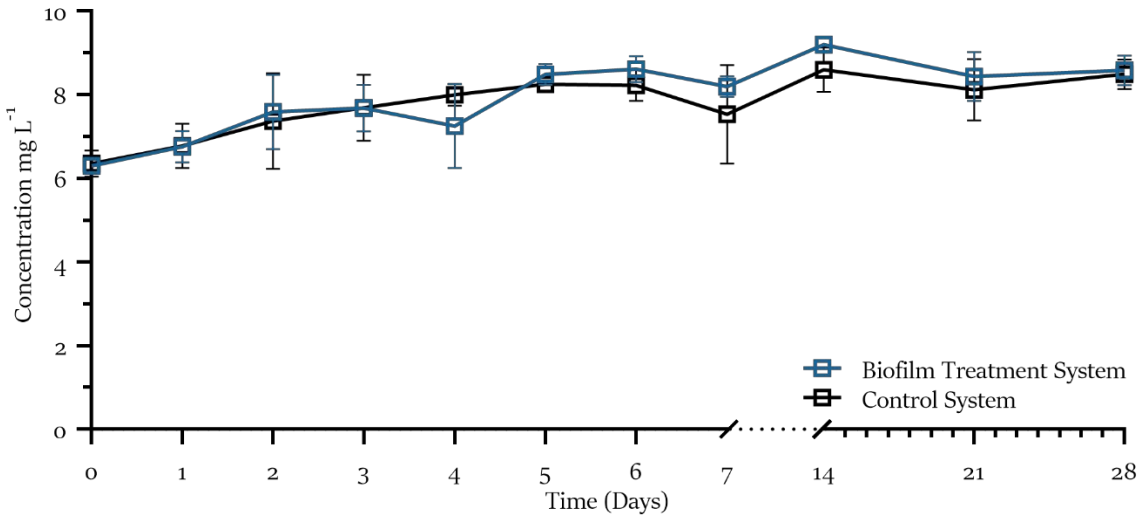


Figure 6.7: Dissolved oxygen concentration within the environmentally-derived surface water taken from the circulation tank of a biofilm treatment system and control system over 28 days n=3 ± s.d.

The temperature of the environmental water was monitored throughout the 28 days circulation due to potential impacts on the microbiological processes involved with BTS. The temperature was controlled through the implementation of inline water heaters and insulation of the reservoir and MDPE pipe, which ranged from 18.5 ± 0.45 to 20.7 ± 0.24°C during experimentation. The BTS was not significantly different from the control system that ranged from 18.3 ± 0.50 to 20.1 ± 0.65°C ($p > 0.05$; Figure 6.8).

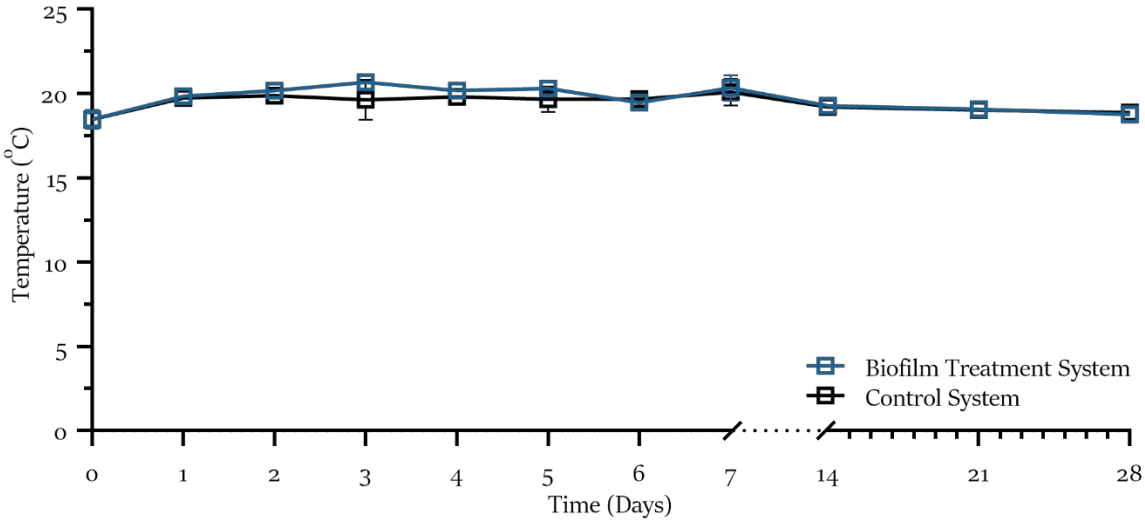


Figure 6.8: Temperature of the environmentally-derived surface water held in recirculation tanks with the biofilm treatment system and control system over 28 days n=3 ± s.d.

Conductivity (a measure of water's capability to pass electrical flow that is directly related to the concentration of ions in the water) demonstrated no significant changes over the 28 days tested, whereby there was also no significant difference between the BTS and control systems (Figure 6.9; $p > 0.05$).

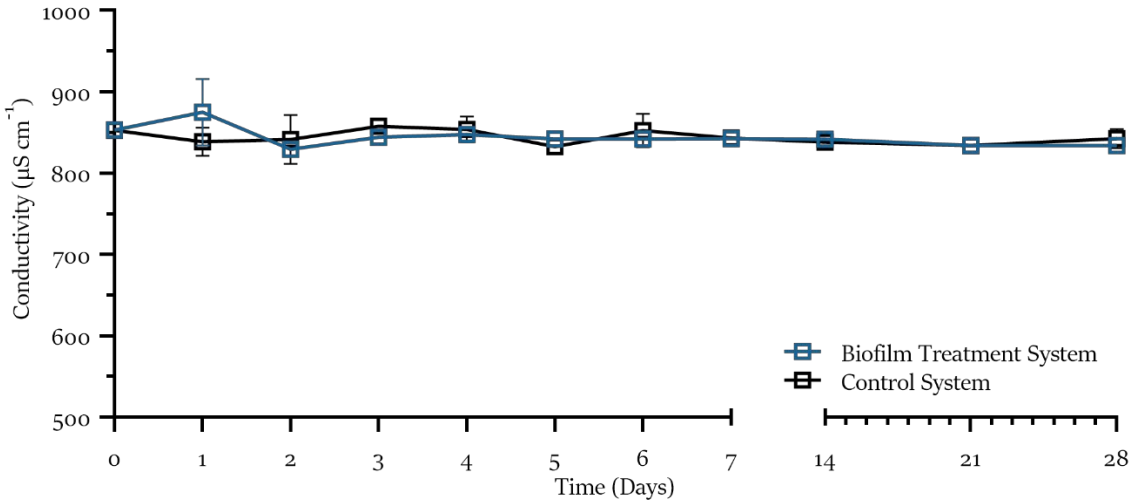


Figure 6.9: Conductivity of environmentally derived surface water within the reservoir of a BTS and control system over 28 days of circulation n=3 ± s.d.

The pH of the reservoir when circulating through both the BTS and control system remained stable across the 28 days. For the BTS the pH averaged 7.74 ± 0.08 increasing to 8.27 ± 0.20 after 28 days of circulation, whereas for the control systems the pH started at 7.74 ± 0.12 and decreased to a pH of 7.52 ± 0.03 after 28 days of circulation (Figure 6.10).

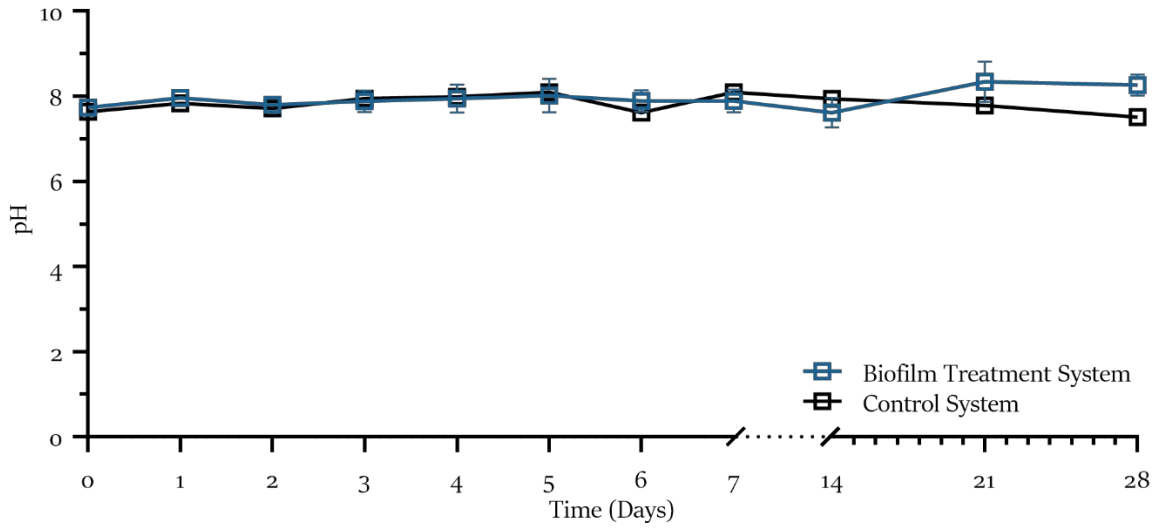


Figure 6.10: pH of the environmentally-derived surface water within the reservoir of a BTS and control systems over 28 days of circulation n=3 ± s.d.

When recording the total dissolved solids (TDS) demonstrated no significant changes over the 28 days tested, whereby there was also no significant difference between the BTS and control systems ($p > 0.05$; Figure 6.11).

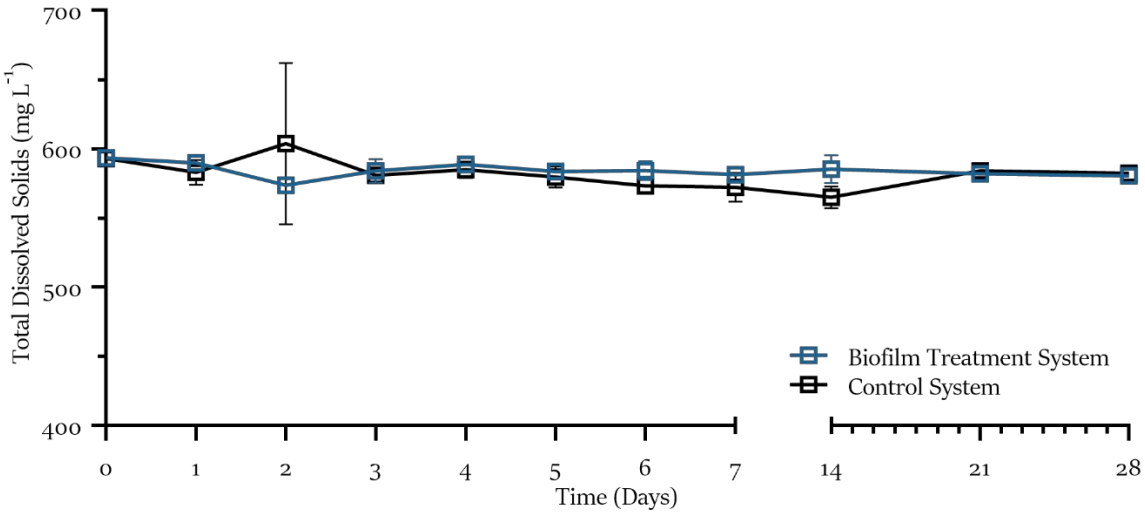


Figure 6.11: The concentration of total dissolved solids (TDS) within the circulation tanks of the biofilm treatment systems and control systems over 28 days of circulation n=3 ± s.d.

6.3 Discussion

To investigate and apply the bioremediation properties observed in chapter 5, upscaled BTS were challenged with an environmentally-derived source water that was known to be contaminated with *E. coli* and enterococci. This trial with the upscaled BTS was conducted with identical parameters that were scaled up for investigating the control of waterborne pathogens using a model that utilises biological interactions within biofilms formed from an environmentally derived population for the control of waterborne planktonic pathogens. The findings in this chapter presented in Figure 6.3 demonstrate the successful up-scale of BTS for the control of waterborne pathogenic bacteria.

The findings in this study demonstrate, supporting the findings presented in chapter 5, that the reduction in pathogens within the environmentally derived source water was through direct contact with the biofilm within the BTS. It is likely that the BTS rely on the processing and metabolism of cells within the biofilm, therefore variability of physiochemical conditions will likely impact on the reduction of pathogens at scale where environmental conditions can fluctuate and impact water treatment. Therefore, it was important to investigate the reduction of waterborne pathogens within a water source that was more applicable to deployment and operation of BTS at scale. In this study, the temperature of the environmental body of water remained consistent over the trial period, 18.5 ± 0.45 to 20.7 ± 0.24°C (Figure 6.8). This was above temperatures that have been reported to significantly reduce the microbial activity ranging from 10 – 5 °C within water treatment processes (193–196), and therefore temperature was not likely to negatively affect or impact the stability of the biofilm consortia within the BTS during experimentation. The other physiochemical parameters measured did not exhibit any statistically significant change over the circulation period, with the exception of DO that increased from 6.30 ± 0.16 mg L^{-1} to 8.59 ± 0.29 mg L^{-1} after 28 days of circulation. However, this was not significantly different from the control system therefore this increase was unlikely to result from the biofilm within the BTS. Overall, this work demonstrated that the upscaled BTS were able to significantly reduce the levels of the indicator species from an environmentally-derived water source ($p < 0.05$; Figure 6.3) which has important implications for communities that have limited access to improved water sources.

This study supports the use of upscaled BTS in a recirculation configuration for the management of water quality of water storage facilities. Although the BTS during this trial failed to meet biological DWI drinking water standards (192), there were still significant reductions of viable of *E. coli* and enterococci (Figure 6.3; $p < 0.05$) within the source water. Currently, in remote communities where access to an improved water source is limited, an estimated 2 billion people are still consuming untreated water contaminated with faeces (4). The management of pathogens

using BTS such as the one used in this study, could significantly reduce the risk of illness associated with contaminated water to the end users. Where water is treated for drinking purposes in remote communities, this is generally achieved using physical filtration as a single step method for providing improved water quality (197). Such methods are capable of reducing levels of organic matter and some bacterial pathogens (15) however, the removal of viable pathogens is likely through the removal of aggregated cells within particulates that remain viable on the surface of the filter that poses a potential risk for further contamination (198). For example, field trials using slow sand filters have demonstrated effective reductions in viable pathogens such as *E. coli*, however recontamination events of treated water were recorded (56,199). Methods of mechanical filtration are not always suitable due to their propensity to biofouling which can significantly affect the rate of treatment soon rendering the physical filter useless until disinfected. The use of recirculating BTS would offer practical solutions to the management of harvested rainwater, stored ground and surface waters. The use of multiple filter vessels configured in series, could significantly scale-up the volumes of water that can be treated. Another practical application of this work is the use of recirculation BTS, in series, to reduce the biological burden of final effluent at the point of discharge through WWTPs that could potentially improve the water quality of designated inland bathing waters or catchments used for drinking water supply.

An overview of current decentralised drinking water treatment technologies was provided in Chapter 1 (Table 1.2). Many of these technologies combine filtration and disinfection steps, that utilise conventional chlorination (37,55,200–202). Many of these solutions are built within the immediate environment around a community, or within a household i.e., sand filter or solar still. The benefit of the BTS investigated in this chapter is that by being self-contained, allows for the potential to be modular and varied output volume. Such self-contained and modular units are beneficial as contamination or wear on equipment is reduced from external factors, such as weather events. Overall, this work clearly demonstrates that environmental biofilms significantly reduce the viability of waterborne pathogenic bacteria; however, the investigation of process parameters, such as contact time and HRT, and their impact on pathogen reduction are critical to further elucidate and optimise this process at scale.

Chapter 7 Final Discussion, Conclusions and Future Work

7.1 Discussion

Overall, the aim of this research has been to characterise, develop and investigate biofilm treatment systems for the control of waterborne pathogens in drinking water supplies. Some 2 billion people are still using water that is contaminated with faeces for drinking and sanitation purposes (4) and chapter 1 highlights the many approaches that aim to address the issue of access to safe water (Table 1.2). The evidence highlighted in chapter 1 indicates that treatment solutions to improve poor water quality are still needed for many people across the globe. Moreover, the Grace study highlights that the unsustainable use of ground water will result in water stressed regions with an increased demand for water treatment and storage thus increasing the demand for water treatment systems for the management of stored water. The development of the BTS used in this research was centred around the establishment of environmental biofilms adhered to an expanded ceramic substrate for the control of potential waterborne pathogens in drinking water supplies that would help address the sustainable development goal number 6 (SDG6) (14).

The data presented in the first results chapter (chapter 3) investigates the viable cell numbers of heterotrophic bacteria within the laboratory scale and upscaled BTS during maturation in parallel. The use of HPC was to assess a representative subpopulation of the microbial community during circulation and in combination with the data presented in chapter 4, to provide an insight into the relationship between cell number and the microbial composition and a greater understanding of the microbial communities within the BTS. The data presented in chapter 3 demonstrate that the biofilm maturation is characterised by an increase in cell number over 4 weeks of circulation which is comparable in both the laboratory scale and upscale BTS (Figure 3.3). Concurrently, the richness of the community decreases from week 1 to week 2 of circulation despite an increase in cell density, a finding which may indicate that initial stages of biofilm formation within these BTS was from the attachment of planktonic cells on the surface of the ceramic substrate resulting in the higher level of initial diversity observed directly from the diverse seed community. This was then changed as a result of competing microbial groups that are selected for in the biofilm during the earlier stages of maturation within the BTS notably within the phylum Proteobacteria within the BTS (Figure 4.2 & Figure 4.3). The data presented in Chapter 4 demonstrate that the development of biofilm is consistent within both the laboratory scale and upscaled BTS using the same seed community within a recirculation configuration. Previous studies have demonstrated that treatment conditions can influence the composition of microbial communities (78) and the comparison of the upscaled BTS with the long term deployed BTS agrees with this finding. However, in many of previous studies, the primary function was for reducing the organic loading of source waters. The function

of the microbial communities developed in this research was for the control of waterborne pathogens within drinking water supplies demonstrated in chapter 5 & 6.

The findings presented in chapter 5 & 6 describe that environmental biofilms were able to directly control the number of viable pathogens within a contaminated source water evidenced by the reductions observed in Figure 5.2 & Figure 6.3. Moreover, the control laboratory scale system incorporating sterile ceramic substrate did not have an overall significant reduction, thus ruling out any physical filtration effects. Therefore, it was likely that the biofilm community (characterised in chapter 4), present on the ceramic substrate, was responsible for the reduction in the tested indicator species. There are some indications in the literature that a biofilm community can impact the number of planktonic pathogens. For example, reductions in the removal of *E. coli* through slow sand filters were impaired at lower temperatures, resulting in a decreased reduction of *E. coli* by 50% when influent temperatures were reduced from 20 to 2°C (203) indicating that the biological activity of the bioactive layer had an impact on *E. coli* removal. Other reductions have been observed that include multistep systems incorporating biological treatment with disinfection (55) and gravity fed rock bed systems incorporating environmental biofilms (130). However, the role of the biofilm within these systems was not fully understood. Within the research presented in this thesis, the hypothesis is that the competition between the biofilm established on the ceramic substrate and the test pathogens, is a key factor for the reduction observed (Figure 5.2 and Table 5.1). Therefore, this research has identified that an emergent property of environmental biofilms within BTS, is the ability to control of waterborne pathogens within fresh waters. To further expand on this hypothesis; during treatment of the environmentally derived fresh water, the EPS matrix enabled the biofilms to act as an external digestive system, which is known for sequestering dissolved and suspended solids for utilisation to meet nutrient and energy requirements (152). During this process, the biofilm matrix retains all the components of lysed cells available, including DNA (204). Moreover, cooperation between the biofilm population is a vital mechanism that enables the removal of substances from the water phase (205). This, in combination with the findings presented in this study, suggests that these biofilm properties were likely responsible for the reductions of planktonic pathogens observed in chapters 5 & 6 and were a result of biological action alone.

The findings in chapter 4 demonstrate that the biofilm within the BTS at all scales are diverse. Further to this, the single-gene community analysis also highlights some key species within the biofilm community that have to the potential to be involved with the hypothesised mechanisms responsible for the reduction of viable pathogens observed in chapter 5 & 6. Such mechanisms are outlined in Figure 7.1. For example, *Bacillus subtilis* was detected within in the biofilm community,

representing a bacterial species recognised for its antimicrobial strategies including the use nanotubes and small molecule toxins (206). These nanotubes act as structures bridging the cytoplasm of neighbouring bacteria and facilitating the direct transport of toxins and other molecules between cells. For small molecules consist of peptides and antibiotics, less than 10 kDa in size, which are released and subsequently disperse toward their intended target cells. Another example detected within the biofilm of the laboratory scale BTS and Upscaled BTS was the genus *Streptomyces* which has the ability to produce bioactive secondary metabolites such as antibiotics which could have resulted in the reduction of viable test pathogens seen in this study (207).

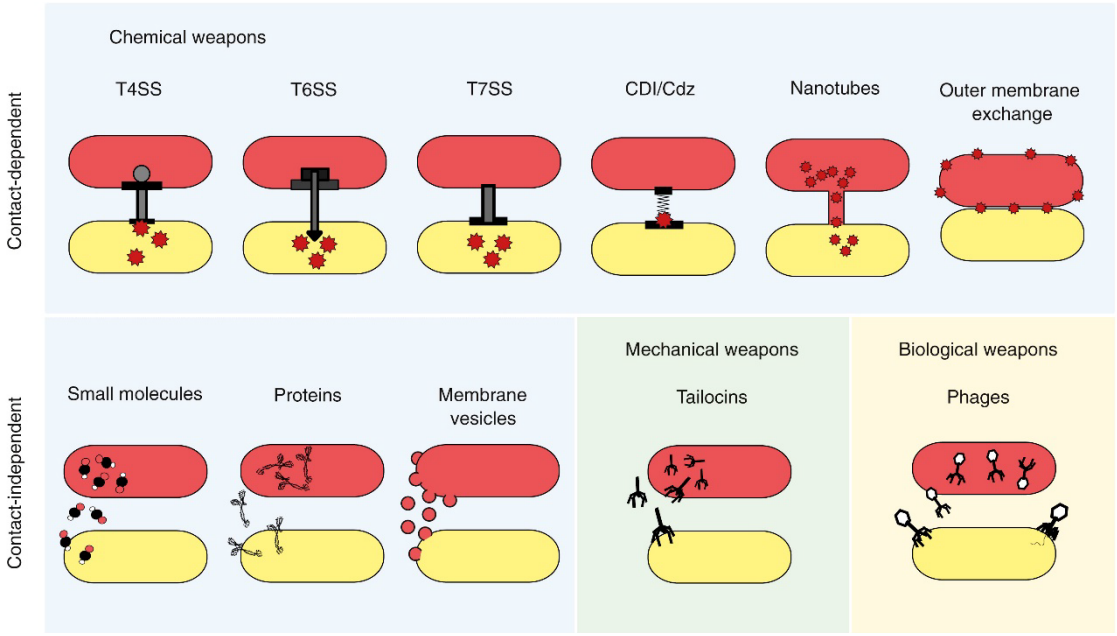


Figure 7.1 Types of antimicrobial mechanisms for competition. Reproduced with permission from Granato *et al.* (207).

Interestingly, *P. aeruginosa*, which has the ability to host and express a diverse range of antimicrobial mechanisms presented in Figure 7.1, was not cultured from the biofilm, even after the bacterial challenge. However, this was not detected within the environmentally-derived fresh water used as a starting seed culture and therefore, still could potentially be able to integrate within the biofilm at the early stages of development if it was present at the start of maturation. Other *Pseudomonads* were detected within the biofilm including but not limited to *Pseudomonas fluorescens, Pseudomonas guineae, Pseudomonas baetica and Pseudomonas helmanticensis*. Known for their strong biofilm forming properties the *Pseudomonas* constitute up to 30 % of the isolated species within the biofilm within the lab scale and upscaled BTS after week 1 and week 2 of circulation, which suggests that they may be of importance for helping lay some of the foundations for the mature biofilm.

The concept of controlling pathogens using BTS without the aid of chemical or UV disinfection is a challenging one. Even so, the data in chapter 5 & 6 demonstrates that both laboratory and upscaled BTS can significantly reduce the levels of three indicator species within a fresh water source. Currently, for water to be biologically safe, the level of indicator species must be 0 CFU 100 mL^{-1}. This study has demonstrated that while the BTS were able to reduce the viability of indicator species to very low levels, this would not be classified as potable water. Nonetheless, the reduction of the indicator microorganisms observed in this study may potentially be significant to reduce the risk of illness. The use of BTS could significantly reduce the load on other treatment methods such as UV water clarifiers that consume low levels of energy to reduce the remainder of the indicator species to potable water standards. Therefore, BTS could be used as the main source of water treatment for low technology solutions for improving access to water free from biological contamination. The global effort into research and development on new approaches of drinking water treatment for developing communities and the remediation of emerging contaminants is crucial to progress towards achieving the 2030 United Nations (UN) Sustainable Development Goals (SDGs) (15). This approach has the potential to work towards the improvement of drinking water supplies and could impact on the delivery of the sustainable development goal number 6: clean water and sanitation for all.

7.2 Study conclusions

The key findings from this research are as follows:

- The maturation of the biofilm in terms of microbial community composition, was similar within both the laboratory scale and upscale treatment systems.
- The laboratory scale BTS used within this study were representative of the upscaled BTS.
- An emergent property of environmental biofilms is their function in the reduction and control of planktonic pathogenic bacteria in fresh water.
- Both the laboratory scale and upscale treatment systems removed potential pathogens from the test water.
- The reduction of planktonic pathogens is through functional characteristics inherent to mature environmental biofilms.
- The pathogens tested were not culturable from the biofilm post treatment.
- The successful application of this phenomenon within upscaled biofilm treatment systems for the reduction of waterborne pathogens within an environmentally derived source water.

7.3 Future work

Overall, these findings demonstrate that environmental biofilms are able to create an unfavourable aquatic environment for the survival of the planktonic test bacteria and environmental indicator species investigated. However, the specific mechanisms that resulted in a reduction of the viability of waterborne bacterial pathogens are not yet known and further research is required. The data presented in this research clearly demonstrates that environmental biofilms significantly reduce the viability of waterborne pathogenic bacteria; however, the following points are critical to further elucidate and optimise this process.

This research has demonstrated the successful application of environmental biofilms adhered to a ceramic substrate for the control of waterborne pathogens within drinking water supplies. However, specifically within the laboratory scale BTS, the indicator species investigated in this study were limited to three laboratory strains. Therefore, further study could be done using strains that were isolated from an environmentally-derived water source that were more representative of potential pathogens that are better adapted for survival in the aquatic environment. The data presented in chapter 6 demonstrates the successful application of upscaled BTS for their application in the control and reduction of indicator species. However, this also comes with limitations. This research was limited to one trial. Further trials investigating other environmentally-derived waters with higher microbial loads could be used to ascertain the maximum limits for the treatment of contaminated fresh waters using the upscaled BTS. Furthermore, the investigation of process parameters including contact time and HRT and their effect on pathogen control would be required to potentially optimise this process.

The primary aim of this research was to assess the function of the BTS for the control of pathogens, however the use of BTS in this configuration for other applications could be investigated such as the remediation of other contaminants including, heavy metals or contaminants of emerging concern (CEC). It is has been previously demonstrated that biological treatment can be utilised to oxidize heavy metals such as Mn(II) or As(III), major pollutants within drinking water supplies in some areas, and remove them from source waters (208,209). The biological oxidation of Mn(II) mainly relies on manganese-oxidizing bacteria (MnOB), which belong to several phyla, including Firmicutes, Actinobacteria, and Proteobacteria (210,211). All of these were identified within the BTS, therefore it is possible that the microbial community function could not just be limited to the control of waterborne pathogens. For As(III), microbial communities from contaminated ground water have been used to seed biological based treatment systems for the oxidation of As(III) (10). This identified several OTUs that are involved with this process many that were included in the phylum Proteobacteria with a comparable community within the BTS used in this study. Therefore,

there is potential that the BTS could have alternate applications. Another group of contaminants that could be investigated within the BTS is the CEC. There is increasing evidence that CECs enter our water systems can have a significant impact on human health where they have been associated with negative impacts on reproduction, increased incidences of breast and testicular cancer (212). The control of CEC is not included in the design of current water treatment infrastructure and therefore can pass through treatment processes into the environment. CEC present in pesticides, metals, food preservatives, pharmaceuticals and personal care products, and antibiotics have become ubiquitous in nature, as a result of increased population growth, industrialisation and mismanagement and therefore require treatment methods that are able to remove them before contaminated water is released into the environment. There has been some research into current infrastructure to remove some types of CEC (90) however on a large, centralised scale. Therefore, the impact of the BTS used in this study on the concentration of these CEC could be investigated.

The microbial communities were characterised over a 12-week circulation period in this study; further sampling could be performed to better understand changes in the microbial community within the BTS. For example, the reservoir of the BTS could be sampled in parallel with the biofilm to understand the relationship between the biofilm and planktonic community during biofilm maturation. Moreover, the data presented in chapter 4 was collected before the BTS were challenged with the environmentally derived water source. Therefore, the microbial community within the BTS could be investigated after treatment.

In relation to the removal of organic matter, the function and stability of BTS has been previously studied through investigating the effect of dosing matured BTS with nutrients, including ammonium (NH_4^+) and phosphate (PO_4^{-3}) (81). These studies concluded that the addition of PO_4^{-3} plays an important role in enhancing the removal of biodegradable dissolved organic carbon. Moreover, it has been shown that in drinking water systems, PO_4^{-3} is the critical rate limiting factor for microbial growth (213) (214). Therefore, the effect of nutrient concentrations on the performance of BTS in relation to the reduction of waterborne pathogens warrants further investigation. Nutrient deprivation within the biofilter could compromise one of the main mechanisms of pathogen control. The relationship between the water quality and environmental conditions for the biofilm within the BTS and the pathogen removal efficiency warrants further investigation.

List of References

1. Steven JAC, Thorn RMS, Robinson GM, Turner D, Lee JE, Reynolds DM. The control of waterborne pathogenic bacteria in fresh water using a biologically active filter. npj Clean Water 2022 5:1 [Internet]. 2022 Jul 12 [cited 2022 Jul 15];5(1):1–10. Available from: https://www.nature.com/articles/s41545-022-00169-y

2. Oki T, Kanae S. Global Hydrological Cycles and World Water Resources. Science (1979). 2006 Aug 25;313(5790):1068–72.

3. Postel SL, Daily GC, Ehrlich PR. Human Appropriation of Renewable Fresh Water. Science (1979). 1996 Feb 9;271(5250):785–8.

4. United Nations Children's Fund (UNICEF) and World Health Organization (WHO). Progress on household drinking water, sanitation and hygiene 2000-2017. Special focus on inequalities. [Internet]. New York; 2019. Available from: https://www.unicef.org/media/55276/file/Progress on drinking water, sanitation and hygiene 2019 .pdf

5. United Nations Development Programme (UNDP). Delivering Sustainable Energy in a Changing Climate. Strategy Note on Sustainable Energy. 2016;1–52.

6. Holland RA, Scott KA, Flörke M, Brown G, Ewers RM, Farmer E, *et al*. Global impacts of energy demand on the freshwater resources of nations. Proc Natl Acad Sci U S A. 2015 Dec 1;112(48):E6707-16.

7. Gleick PH, Palaniappan M. Peak water limits to freshwater withdrawal and use. Proc Natl Acad Sci U S A. 2010 Jun 22;107(25):11155–62.

8. Nations. U. WORLD POPULATION TO 2300, Department of Economic and Social Affairs/Population. New York; 2004.

9. Parks R, McLaren M, Toumi R, Rivett U. Experiences and lessons in managing water from Cape Town. London; 2019.

10. Crognale S, Casentini B, Amalfitano S, Fazi S, Petruccioli M, Rossetti S. Biological As(III) oxidation in biofilters by using native groundwater microorganisms. Sci Total Environ [Internet]. 2019 Feb 15 [cited 2019 Apr 26];651(Pt 1):93–102. Available from: http://www.ncbi.nlm.nih.gov/pubmed/30227294

11. Rodell M, Famiglietti JS, Wiese DN, Reager JT, Beaudoing HK, Landerer FW, *et al*. Emerging trends in global freshwater availability. Nature 2018 557:7707 [Internet].

2018 May 16 [cited 2022 Sep 26];557(7707):651–9. Available from: https://www.nature.com/articles/s41586-018-0123-1

12. World Bank. Water, sanitation and hygiene and the Millennium Development Goals (MDGs). 2003 [cited 2022 Nov 4]; Available from: http://www.measuredhs.com/

13. United Nations. The Millennium Development Goals Report [Internet]. 2015 [cited 2022 Nov 8]. Available from: http://www.undp.org/content/dam/undp/library/MDG/english/UNDP_MDG

14. United Nations (UN). Transforming Our World: The 2030 Agenda For Sustainable Development. [Internet]. 2015 [cited 2020 May 7]. Available from: https://stg-wedocs.unep.org/bitstream/handle/20.500.11822/11125/unepswiosm1inf7sdg.pdf?sequence=1

15. United Nations (UN). Transforming Our World: The 2030 Agenda for Sustainable Development. In: A New Era in Global Health [Internet]. 2018. p. 529–67. Available from: https://stg-wedocs.unep.org/bitstream/handle/20.500.11822/11125/unepswiosm1inf7sdg.pdf?sequence=1

16. Prüss-Ustün A, Wolf J, Bartram J, Clasen T, Cumming O, Freeman MC, *et al*. Burden of disease from inadequate water, sanitation and hygiene for selected adverse health outcomes: An updated analysis with a focus on low- and middle-income countries. Int J Hyg Environ Health [Internet]. 2019 Jun 1 [cited 2022 Jun 28];222(5):765. Available from: /pmc/articles/PMC6593152/

17. Caprioli A, Morabito S, Bruégre H, Oswald E. Enterohaemorrhagic Escherichia coli : emerging issues on virulence and modes of transmission. Vet Res. 2005 May 1;36(3):289–311.

18. Vital M, Fuchslin HP, Hammes F, Egli T. Growth of Vibrio cholerae O1 Ogawa Eltor in freshwater. Microbiology (N Y). 2007 Jul 1;153(7):1993–2001.

19. Agudelo Higuita NI, Huycke MM. Enterococcal Disease, Epidemiology, and Implications for Treatment. In: Enterococci: From Commensals to Leading Causes of Drug Resistant Infection. Boston: Massachusetts Eye and Ear Infirmary; 2014. p. 47–72.

20. Xi C, Zhang Y, Marrs CF, Ye W, Simon C, Foxman B, *et al*. Prevalence of antibiotic resistance in drinking water treatment and distribution systems. Appl Environ Microbiol. 2009 Sep 1;75(17):5714–8.

21. Adler N, Balzer F, Blondzik K, Brauer F, Chorus I, Ebert I, *et al*. Antibiotics and Antibiotic Resistances in the Environment Background, Challenges and Options for Action. 2018.

22. Baquero F, Martínez JL, Cantón R. Antibiotics and antibiotic resistance in water environments. Vol. 19, Current Opinion in Biotechnology. 2008. p. 260–5.

23. Destiani R, Templeton MR. The antibiotic resistance of heterotrophic bacteria in tap waters in london. Water Sci Technol Water Supply. 2019 Feb 1;19(1):179–90.

24. Cooke MD. Antibiotic resistance in coliform and faecal coliform bacteria from natural waters and effluents. N Z J Mar Freshwater Res. 1976;10(3):391–7.

25. Falconer IR, Chapman HF, Moore MR, Ranmuthugala G. Endocrine-disrupting compounds: A review of their challenge to sustainable and safe water supply and water reuse. Vol. 21, Environmental Toxicology. 2006. p. 181–91.

26. Snyder SA, Benotti MJ. Endocrine disruptors and pharmaceuticals: Implications for water sustainability. Water Science and Technology. 2010;61(1):145–54.

27. Caliman FA, Gavrilescu M. Pharmaceuticals, personal care products and endocrine disrupting agents in the environment - A review. Vol. 37, Clean - Soil, Air, Water. 2009. p. 277–303.

28. Ebele AJ, Abou-Elwafa Abdallah M, Harrad S. Pharmaceuticals and personal care products (PPCPs) in the freshwater aquatic environment. Vol. 3, Emerging Contaminants. KeAi Communications Co.; 2017. p. 1–16.

29. Mintenig SM, Löder MGJ, Primpke S, Gerdts G. Low numbers of microplastics detected in drinking water from ground water sources. Science of the Total Environment. 2019 Jan 15;648:631–5.

30. Amarasiri M, Sano D, Suzuki S. Understanding human health risks caused by antibiotic resistant bacteria (ARB) and antibiotic resistance genes (ARG) in water environments: Current knowledge and questions to be answered. Crit Rev Environ Sci Technol. 2019;

31. Kim S, Aga DS. Potential ecological and human health impacts of antibiotics and antibiotic-resistant bacteria from wastewater treatment plants. Vol. 10, Journal of Toxicology and Environmental Health - Part B: Critical Reviews. Taylor & Francis Group ; 2007. p. 559–73.

32. Jones OAH, Voulvoulis N, Lester JN. Potential ecological and human health risks associated with the presence of pharmaceutically active compounds in the aquatic environment. Vol. 34, Critical Reviews in Toxicology. 2004. p. 335–50.

33. Berglund B. Environmental dissemination of antibiotic resistance genes and correlation to anthropogenic contamination with antibiotics. Infect Ecol Epidemiol. 2015 Jan;5(1):28564.

34. Mohy-u-Din N, Farhan M, Wahid A, Ciric L, Sharif F. Human health risk estimation of antibiotics transferred from wastewater and soil to crops. Environmental Science and Pollution Research 2022 [Internet]. 2022 Oct 18 [cited 2022 Nov 10];1–14. Available from: https://link.springer.com/article/10.1007/s11356-022-23412-y

35. Pooi CK, Ng HY. Review of low-cost point-of-use water treatment systems for developing communities. npj Clean Water 2018 1:1. 2018 Aug 6;1(1):11.

36. Attisani M. Can solar technology generate clean water for developing nations? Renewable Energy Focus. 2016 Jul;17(4):138–9.

37. Chaidez C, Ibarra-Rodríguez JR, Valdez-Torres JB, Soto M, Gerba CP, Castro-del Campo N. Point-of-use Unit Based on Gravity Ultrafiltration Removes Waterborne Gastrointestinal Pathogens from Untreated Water Sources in Rural Communities. Wilderness Environ Med [Internet]. 2016 Sep [cited 2019 Jul 15];27(3):379–85. Available from: http://www.ncbi.nlm.nih.gov/pubmed/27591708

38. Clayton GE, Thorn RMS, Reynolds DM. Development of a novel off-grid drinking water production system integrating electrochemically activated solutions and ultrafiltration membranes. Journal of Water Process Engineering. 2017 Aug 1;30:100480.

39. Martínez-Huitle CA, Brillas E. Electrochemical Alternatives for Drinking Water Disinfection. Angewandte Chemie International Edition. 2008 Feb 28;47(11):1998–2005.

40. Zhu J, Fan XJ, Tao Y, Wei DQ, Zhang XH. Study on an integrated process combining ozonation with ceramic ultra-filtration for decentralized supply of drinking water. J Environ Sci Health A Tox Hazard Subst Environ Eng. 2014 Sep 19;49(11):1296–303.

41. Jordan FL, Seaman R, Riley JJ, Yoklic MR. Effective removal of microbial contamination from harvested rainwater using a simple point of use filtration and UV-disinfection device. Urban Water J. 2008 Sep;5(3):209–18.

42. Di Cristo C, Esposito G, Leopardi A. Modelling trihalomethanes formation in water supply systems. Environ Technol. 2013 Jan;34(1):61–70.

43. Richardson SD. Disinfection by-products and other emerging contaminants in drinking water. TrAC Trends in Analytical Chemistry [Internet]. 2003 Nov 1 [cited 2019 Jul 26];22(10):666–84. Available from: https://www.sciencedirect.com/science/article/pii/S0165993603010033

44. Fantuzzi G, Righi E, Predieri G, Giacobazzi P, Mastroianni K, Aggazzotti G, *et al.* Prevalence of Ocular, Respiratory and Cutaneous Symptoms in Indoor Swimming Pool Workers and Exposure to Disinfection By-Products (DBPs). Int J Environ Res Public Health. 2010 Mar 29;7(4):1379–91.

45. Kogevinas M, Villanueva CM, Font-Ribera L, Liviac D, Bustamante M, Espinoza F, *et al.* Genotoxic Effects in Swimmers Exposed to Disinfection By-products in Indoor Swimming Pools. Environ Health Perspect. 2010 Nov;118(11):1531–7.

46. Wright JM, Evans A, Kaufman JA, Rivera-Núñez Z, Narotsky MG. Disinfection By-Product Exposures and the Risk of Specific Cardiac Birth Defects. Environ Health Perspect. 2017;125(2):269–77.

47. Bougault V, Boulet LP. Is there a potential link between indoor chlorinated pool environment and airway remodeling/inflammation in swimmers? Expert Rev Respir Med. 2012 Oct 9;6(5):469–71.

48. Mbilinyi BP, Tumbo SD, Mahoo HF, Senkondo EM, Hatibu N. Indigenous knowledge as decision support tool in rainwater harvesting. Physics and Chemistry of the Earth, Parts A/B/C. 2005 Jan;30(11–16):792–8.

49. Ellis K V., Wood WE. Slow sand filtration. Critical Reviews in Environmental Control. 1985 Jan 1;15(4):315–54.

50. Campos LC, Su MFJ, Graham NJD, Smith SR. Biomass development in slow sand filters. Water Res. 2002 Nov 1;36(18):4543–51.

51. Montgomery AA, Elimelech M. Millions suffer from preventable illnesses and die every year. Environ Sci Technol. 2007;1:17–24.

52. Sobsey MD. Managing Water in the Home: Accelerated Health Gains from Improved Water Supply Water, Sanitation and Health Department of Protection of the Human Environment World Health Organization Geneva. 2002.

53. Carratalà A, Calado AD, Mattle MJ, Meierhofer R, Luzi S, Kohn T. Solar disinfection of viruses in polyethylene terephthalate bottles. Appl Environ Microbiol. 2016;82(1):279–88.

54. Baig SA, Mahmood Q, Nawab B, Shafqat MN, Pervez A. Improvement of drinking water quality by using plant biomass through household biosand filter - A decentralized approach. Ecol Eng. 2011 Nov;37(11):1842–8.

55. Wendt C, Ives R, Hoyt AL, Conrad KE, Longstaff S, Kuennen RW, *et al*. Microbial removals by a novel biofilter water treatment system. Am J Trop Med Hyg [Internet]. 2015 Apr [cited 2019 Feb 13];92(4):765–72. Available from: http://www.ncbi.nlm.nih.gov/pubmed/25758649

56. Liang K, Sobsey M, Stauber CE. Improving Household Drinking Water Quality: Use of Biosand Filter in Cambodia [Internet]. Public Health Faculty Publications School of Public Health; 2010 [cited 2022 Feb 3]. Available from: https://scholarworks.gsu.edu/iph_facpub

57. Haig SJ, Collins G, Davies RL, Dorea CC, Quince C. Biological aspects of slow sand filtration: Past, present and future. Vol. 11, Water Science and Technology: Water Supply. IWA Publishing; 2011. p. 468–72.

58. Basu OD, Dhawan S, Black K. Applications of biofiltration in drinking water treatment - a review. Journal of Chemical Technology & Biotechnology [Internet]. 2016 Mar 1 [cited 2019 Jul 19];91(3):585–95. Available from: http://doi.wiley.com/10.1002/jctb.4860

59. World Health Organization. Burden of disease attributable to unsafe drinking-water, sanitation and hygiene: 2019 update [Internet]. Geneva; 2023 [cited 2023 Jul 14]. Available from: https://www.who.int/publications/i/item/9789240075610

60. Wolf J, Johnston RB, Ambelu A, Arnold BF, Bain R, Brauer M, *et al*. Burden of disease attributable to unsafe drinking water, sanitation, and hygiene in domestic settings: a global analysis for selected adverse health outcomes. The Lancet [Internet]. 2023 Jun 17 [cited 2023 Jul 14];401(10393):2060–71. Available from: http://www.thelancet.com/article/S0140673623004580/fulltext

61. Yildiz BS. Water and wastewater treatment: biological processes. Metropolitan Sustainability: Understanding and Improving the Urban Environment. 2012 Jan 1;406–28.

62. de Vries J. Soil filtration of wastewater effluent and the mechanism of pore clogging. Journal of the Water Pollution Control Federation. 1972;44:565–73.

63. Loh ZZ, Zaidi NS, Syafiuddin A, Yong EL, Boopathy R, Kueh ABH, *et al.* Shifting from Conventional to Organic Filter Media in Wastewater Biofiltration Treatment: A Review. Applied Sciences 2021, Vol 11, Page 8650 [Internet]. 2021 Sep 17 [cited 2022 Feb 15];11(18):8650. Available from: https://www.mdpi.com/2076-3417/11/18/8650/htm

64. Baker MN. The quest for pure water; The History of Water Purification from the Earliest Records to the Twentieth Century, Volume 1. 1st ed. Lancaster: American Water Works Association; 1948.

65. Bellamy WD, Silverman GP, Hendricks DW, Logsdon GS. Removing Giardia Cysts With Slow Sand Filtration. J Am Water Works Assoc. 1985 Feb 1;77(2):52–60.

66. Fogel D, Isaac-Renton J, Guasparini R, Moorehead W OJ. Removing giardia and cryptosporidium by slow sand filtration. JAWWA, Research and Technology. 1993;3:77–84.

67. Hijnen WAM, Schijven JF, Bonné P, Visser A, Medema GJ. Elimination of viruses, bacteria and protozoan oocysts by slow sand filtration. Water Science and Technology. 2004;50(1):147–54.

68. Terry LG, Summers RS. Biodegradable organic matter and rapid-rate biofilter performance: A review. Water Res. 2018 Jan 1;128:234–45.

69. McKie MJ, Ziv-El MC, Taylor-Edmonds L, Andrews RC, Kirisits MJ. Biofilter scaling procedures for organics removal: A potential alternative to piloting. Water Res [Internet]. 2019 Mar 15 [cited 2019 Jan 22];151:87–97. Available from: https://www.sciencedirect.com/science/article/pii/S0043135418310200

70. LeChevallier MW, Becker WC, Schorr P, Lee RG. Evaluating the performance of biologically active rapid filters. J Am Water Works Assoc. 1992;84(4):136–40.

71. Melin ES, Ødegaard H. The effect of biofilter loading rate on the removal of organic ozonation by-products. Water Res. 2000 Dec 15;34(18):4464–76.

72. Hozalski RM, Goel S, Bouwer EJ. TOC removal in biological filters. J Am Water Works Assoc. 1995 Dec 1;87(12):40–54.

73. Yavich AA, Lee KH, Chen KC, Pape L, Masten SJ. Evaluation of biodegradability of NOM after ozonation. Water Res. 2004;38(12):2839–46.

74. Wert EC, Neemann JJ, Rexing DJ, Zegers RE. Biofiltration for removal of BOM and residual ammonia following control of bromate formation. Water Res. 2008 Jan 1;42(1–2):372–8.

75. Hozalski RM, Bouwer EJ, Goel S. Removal of natural organic matter (NOM) from drinking water supplies by ozone-biofiltration. Water Science and Technology. 1999 Jan 1;40(9):157–63.

76. Carlson KH, Amy GL. BOM removal during biofiltration. J Am Water Works Assoc. 1998;90(12):42–52.

77. Liu X, Huck PM, Slawson RM. Factors Affecting Drinking Water Biofiltration. J Am Water Works Assoc [Internet]. 2001 Dec 1 [cited 2022 Nov 12];93(12):90–101. Available from: https://onlinelibrary.wiley.com/doi/full/10.1002/j.1551-8833.2001.tb09358.x

78. Nemani VA, McKie MJ, Taylor-Edmonds L, Andrews RC. Impact of biofilter operation on microbial community structure and performance. Journal of Water Process Engineering [Internet]. 2018 Aug 1 [cited 2019 Feb 19];24:35–41. Available from: https://www.sciencedirect.com/science/article/pii/S2214714418300199

79. Singer PC. Control of disinfection by-products in drinking water. Journal of Environmental Engineering (United States). 1994;120(4):727–44.

80. Escobar IC, Randall AA, Taylor JS. Bacterial growth in distribution systems: Effect of assimilable organic carbon and biodegradable dissolved organic carbon. Environ Sci Technol. 2001 Sep 1;35(17):3442–7.

81. Fu J, Lee WN, Coleman C, Meyer M, Carter J, Nowack K, *et al.* Pilot investigation of two-stage biofiltration for removal of natural organic matter in drinking water treatment. Chemosphere. 2017 Jan 1;166:311–22.

82. Chen F, Peldszus S, Elhadidy AM, Legge RL, Van Dyke MI, Huck PM. Kinetics of natural organic matter (NOM) removal during drinking water biofiltration using different NOM characterization approaches. Water Res. 2016 Nov 1;104:361–70.

83. Nishijima W, Shoto E, Okada M. Improvement of biodegradation of organic substance by addition of phosphorus in biological activated carbon. In: Water Science and Technology. No longer published by Elsevier; 1997. p. 251–7.

84. Laurent P, Prévost M, Cigana J, Niquette P, Servais P. Biodegradable organic matter removal in biological filters: Evaluation of the CHABROL model. Water Res. 1999 Apr 1;33(6):1387–98.

85. Moll DM, Summers RS, Fonseca AC, Matheis W. Impact of temperature on drinking water biofilter performance and microbial community structure. Environ Sci Technol. 1999 Jul 15;33(14):2377–82.

86. Lapworth DJ, Baran N, Stuart ME, Ward RS. Emerging organic contaminants in groundwater: A review of sources, fate and occurrence. Environ Pollut [Internet]. 2012 Apr [cited 2022 Nov 10];163:287–303. Available from: https://pubmed.ncbi.nlm.nih.gov/22306910/

87. Jurado A, Vàzquez-Suñé E, Carrera J, López De Alda M, Pujades E, Barceló D. Emerging organic contaminants in groundwater in Spain: A review of sources, recent occurrence and fate in a European context. Science of the Total Environment, The [Internet]. 2012 [cited 2022 Nov 10];440:82–94. Available from: http://dx.doi.org/10.1016/j.scitotenv.2012.08.029

88. Dulio V, van Bavel B, Brorström-Lundén E, Harmsen J, Hollender J, Schlabach M, *et al*. Emerging pollutants in the EU: 10 years of NORMAN in support of environmental policies and regulations. Environ Sci Eur [Internet]. 2018 Dec 1 [cited 2022 Nov 10];30(1):1–13. Available from: https://enveurope.springeropen.com/articles/10.1186/s12302-018-0135-3

89. McKie MJ, Andrews SA, Andrews RC. Conventional drinking water treatment and direct biofiltration for the removal of pharmaceuticals and artificial sweeteners: A pilot-scale approach. Science of The Total Environment. 2016 Feb 15;544:10–7.

90. Fu J, Lee WN, Coleman C, Nowack K, Carter J, Huang CH. Removal of pharmaceuticals and personal care products by two-stage biofiltration for drinking water treatment. Science of The Total Environment [Internet]. 2019 May 10 [cited 2019 Feb 18];664:240–8. Available from: https://www.sciencedirect.com/science/article/pii/S0048969719305054?via%3Dihub

91. Aslan S, Cakici H. Biological denitrification of drinking water in a slow sand filter. J Hazard Mater. 2007 Sep 5;148(1–2):253–8.

92. Weber-Shirk ML, Dick RI. Bacterivory by a chrysophyte in slow sand filters. Water Res. 1999 Feb 1;33(3):631–8.

93. McConnell LK, Sims RC, Barnett BB. Reovirus removal and inactivation by slow-rate sand filtration. Appl Environ Microbiol. 1984;48(4):818–25.

94. Rappé MS, Giovannoni SJ. The Uncultured Microbial Majority. Annu Rev Microbiol. 2003 Oct 28;57(1):369–94.

95. Huq A, Rivera ING, Colwell RR. Epidemiological Significance of Viable but Nonculturable Microorganisms. In: Nonculturable Microorganisms in the Environment. Springer US; 2000. p. 301–23.

96. Flemming HC, Wingender J, Szewzyk U, Steinberg P, Rice SA, Kjelleberg S. Biofilms: an emergent form of bacterial life. Nat Rev Microbiol [Internet]. 2016 Sep 1 [cited 2019 Mar 4];14(9):563–75. Available from: http://www.nature.com/articles/nrmicro.2016.94

97. Kostakioti M, Hadjifrangiskou M, Hultgren SJ. Bacterial biofilms: Development, dispersal, and therapeutic strategies in the dawn of the postantibiotic era. Cold Spring Harb Perspect Med. 2013 Apr;3(4).

98. Artigas J, Fund K, Kirchen S, Morin S, Obst U, Romaní AM, *et al.* Patterns of biofilm formation in two streams from different bioclimatic regions: Analysis of microbial community structure and metabolism. Hydrobiologia. 2012 Oct 27;695(1):83–96.

99. Eigemann F, Hilt S, Salka I, Grossart HP. Bacterial community composition associated with freshwater algae: Species specificity vs. dependency on environmental conditions and source community. FEMS Microbiol Ecol. 2013;

100. Barken KB, Pamp SJ, Yang L, Gjermansen M, Bertrand JJ, Klausen M, *et al.* Roles of type IV pili, flagellum-mediated motility and extracellular DNA in the formation of mature multicellular structures in *Pseudomonas aeruginosa* biofilms. Environ Microbiol. 2008 Sep 1;10(9):2331–43.

101. Sutherland IW. MINI-REVIEW Biofilm exopolysaccharides : a strong and sticky framework. Vol. 147, Microbiology. 2001.

102. Hobley L, Harkins C, MacPhee CE, Stanley-Wall NR. Giving structure to the biofilm matrix: an overview of individual strategies and emerging common themes. FEMS Microbiol Rev. 2015 Jun 8;39(5):649–69.

103. Serra DO, Richter AM, Hengge R. Cellulose as an architectural element in spatially structured escherichia coli biofilms. J Bacteriol. 2013 Dec;195(24):5540–54.

104. Gómez-Suárez C, Pasma J, van der Borden AJ, Wingender J, Flemming HC, Busscher HJ, *et al.* Influence of extracellular polymeric substances on deposition and redeposition of Pseudomonas aeruginosa to surfaces. Microbiology (N Y). 2002;148(4):1161–9.

105. Kaplan JB. Biofilm Dispersal: Mechanisms, Clinical Implications, and Potential Therapeutic Uses. Vol. 89, Journal of Dental Research. International Association for Dental Research; 2010. p. 205–18.

106. Halan B, Buehler K, Schmid A. Biofilms as living catalysts in continuous chemical syntheses. Vol. 30, Trends in Biotechnology. Elsevier Current Trends; 2012. p. 453–65.

107. Rosche B, Li XZ, Hauer B, Schmid A, Buehler K. Microbial biofilms: a concept for industrial catalysis? Trends Biotechnol. 2009 Nov 1;27(11):636–43.

108. de Carvalho CCCR. Whole cell biocatalysts: essential workers from Nature to the industry. Microbial Biotechnology. 2017.

109. Flemming HC. Biofilms. In: Encyclopedia of Life Sciences. Chichester, UK: John Wiley & Sons, Ltd; 2008.

110. Yee WC. Selective Removal of Mixed Phosphates by Activated Alumina. J Am Water Works Assoc. 1966 Feb 1;58(2):239–47.

111. Métivier R, Bourven I, Labanowski J, Guibaud G. Interaction of erythromycin ethylsuccinate and acetaminophen with protein fraction of extracellular polymeric substances (EPS) from various bacterial aggregates. Environmental Science and Pollution Research. 2013 Oct 7;20(10):7275–85.

112. Writer JH, Barber LB, Ryan JN, Bradley PM. Biodegradation and Attenuation of Steroidal Hormones and Alkylphenols by Stream Biofilms and Sediments. Environ Sci Technol. 2011 May 15;45(10):4370–6.

113. Grumbein S, Opitz M, Lieleg O. Selected metal ions protect Bacillus subtilis biofilms from erosion †. Metallomics. 2014;6:1441.

114. Kragh KN, Hutchison JB, Melaugh G, Rodesney C, Roberts AEL, Irie Y, *et al.* Role of Multicellular Aggregates in Biofilm Formation. mBio. 2016 Mar 22;7(2):e00237.

115. Caño-Muñiz S, Anthony R, Niemann S, Alffenaar JWC. New approaches and therapeutic options for mycobacterium tuberculosis in a dormant state. Vol. 31, Clinical Microbiology Reviews. American Society for Microbiology; 2018.

116. Lewis K. Persister cells, dormancy and infectious disease. Vol. 5, Nature Reviews Microbiology. Nature Publishing Group; 2007. p. 48–56.

117. Van Bodegom P. Microbial maintenance: A critical review on its quantification. Vol. 53, Microbial Ecology. Springer; 2007. p. 513–23.

118. Xin Y, Xiao-jian Z, Xiao-ling L, Xiao-dong Z, Zhan-sheng W. Phosphorus limitation in biofiltration for drinking water treatment. J Environ Sci (China). 2003 Jul 1;15(4):494–9.

119. Snow J. On the mode of communication of cholera. Edinb Med J. 1856;1(7):5–6.

120. World Health Organisation. Drinking-water. Geneva; 2018.

121. Vital M, Hammes F, Egli T. Escherichia coli O157 can grow in natural freshwater at low carbon concentrations. Environ Microbiol. 2008;

122. Nocker A, Burr M, Camper A. Pathogens in Water and Biofilms. In: Microbiology of Waterborne Diseases: Microbiological Aspects and Risks: Second Edition. 2nd ed. Academic Press; 2013. p. 3–32.

123. Liste MB, Natera I, Suarez JA, Pujol FH, Liprandi F, Ludert JE. Enteric Virus Infections and Diarrhea in Healthy and Human Immunodeficiency Virus-Infected Children. J Clin Microbiol [Internet]. 2000 [cited 2023 Jul 13];38(8):2873–7. Available from: https://journals.asm.org/doi/10.1128/jcm.38.8.2873-2877.2000

124. Boughattas S, Behnke JM, Al-Ansari K, Sharma A, Abu-Alainin W, Al-Thani A, *et al.* Molecular analysis of the enteric protozoa associated with acute diarrhea in hospitalized children. Front Cell Infect Microbiol. 2017 Aug 2;7(AUG):265012.

125. Cabral JPS. Water microbiology. Bacterial pathogens and water. Int J Environ Res Public Health. 2010;7(10):3657–703.

126. Pandey PK, Kass PH, Soupir ML, Biswas S, Singh VP. Contamination of water resources by pathogenic bacteria. AMB Express. 2014;4:51.

127. Drinking Water Inspectorate (DWI). Drinking Water Inspectorate Guidance to water companies. 2018;2016(September):1–18.

128. Fewtrell L, Bartram J, Ashbolt NJ, Grabow WOK, Snozzi M. Indicators of microbial water quality. Water Quality: Guidelines, Standards and Health [Internet]. 2001 [cited 2019 Mar 4];1(1):290–318. Available from: http://citeseerx.ist.psu.edu/viewdoc/download;jsessionid=19948C9A549EF851DC69FDB686DA4B7A?doi=10.1.1.375.9839&rep=rep1&type=pdf

129. Battin TJ, Besemer K, Bengtsson MM, Romani AM, Packmann AI. The ecology and biogeochemistry of stream biofilms. Nat Rev Microbiol. 2016 Apr 14;14(4):251–63.

130. Beutel MW, Larson L. Pathogen removal from urban pond outflow using rock biofilters. Ecol Eng. 2014 May 1;78:72–8.

131. Rendueles O, Ghigo JM. Mechanisms of Competition in Biofilm Communities. Microbiol Spectr. 2015 Jun 25;3(3).

132. Rendueles O, Ghigo JM. Mechanisms of Competition in Biofilm Communities. Microbiol Spectr. 2015 Jun 18;3(3).

133. Hibbing ME, Fuqua C, Parsek MR, Peterson SB. Bacterial competition: Surviving and thriving in the microbial jungle. Nat Rev Microbiol. 2010 Jan;8(1):15–25.

134. Aoki SK, Diner EJ, De Roodenbeke CTK, Burgess BR, Poole SJ, Braaten BA, *et al*. A widespread family of polymorphic contact-dependent toxin delivery systems in bacteria. Nature. 2010 Nov 18;468(7322):439–42.

135. MacIntyre DL, Miyata ST, Kitaoka M, Pukatzki S. The Vibrio cholerae type VI secretion system displays antimicrobial properties. Proc Natl Acad Sci U S A [Internet]. 2010 Nov 9 [cited 2019 Mar 4];107(45):19520–4. Available from: http://www.ncbi.nlm.nih.gov/pubmed/20974937

136. Ławniczak Ł, Marecik R, Chrzanowski Ł. Contributions of biosurfactants to natural or induced bioremediation. Appl Microbiol Biotechnol. 2013 Mar;97(6):2327.

137. Cornforth DM, Foster KR. Competition sensing: the social side of bacterial stress responses. Nature Reviews Microbiology 2013 11:4 [Internet]. 2013 Mar 4 [cited 2022 Mar 10];11(4):285–93. Available from: https://www.nature.com/articles/nrmicro2977

138. Driscoll JA, Brody SL, Kollef MH. The Epidemiology, Pathogenesis and Treatment of Pseudomonas aeruginosa Infections. Drugs. 2007;67(3):351–68.

139. Garvey MI, Bradley CW, Tracey J, Oppenheim B. Continued transmission of Pseudomonas aeruginosa from a wash hand basin tap in a critical care unit. Journal of Hospital Infection. 2016 Sep;94(1):8–12.

140. Hunter PR. Drinking water and diarrhoeal disease due to Escherichia coli. J Water Health. 2003 Jun;1(2):65–72.

141. Smith EJ, Davison W, Hamilton-Taylor J. Methods for preparing synthetic freshwaters. Water Res. 2002;36(5):1286–96.

142. Park D, Jang S, Roesner LA. Evaluation of multi-use stormwater detention basins for improved urban watershed management. Hydrol Process [Internet]. 2014 Jan 30 [cited 2022 Oct 9];28(3):1104–13. Available from: https://onlinelibrary.wiley.com/doi/full/10.1002/hyp.9658

143. Yoon SH, Ha SM, Kwon S, Lim J, Kim Y, Seo H, *et al.* Introducing EzBioCloud: a taxonomically united database of 16S rRNA gene sequences and whole-genome assemblies. Int J Syst Evol Microbiol [Internet]. 2017 May 1 [cited 2022 Nov 26];67(5):1613–7. Available from: https://pubmed.ncbi.nlm.nih.gov/28005526/

144. Bolyen E, Rideout JR, Dillon MR, Bokulich NA, Abnet CC, Al-Ghalith GA, *et al.* Reproducible, interactive, scalable and extensible microbiome data science using QIIME 2. Nature Biotechnology 2019 37:8. 2019 Jul 24;37(8):852–7.

145. Callahan BJ, McMurdie PJ, Rosen MJ, Han AW, Johnson AJA, Holmes SP. DADA2: High-resolution sample inference from Illumina amplicon data. Nature Methods 2016 13:7 [Internet]. 2016 May 23 [cited 2022 Oct 11];13(7):581–3. Available from: https://www.nature.com/articles/nmeth.3869

146. Schneider CA, Rasband WS, Eliceiri KW. NIH Image to ImageJ: 25 years of image analysis. Vol. 9, Nature Methods. 2012. p. 671–5.

147. Li Q, Yu S, Li L, Liu G, Gu Z, Liu M, *et al.* Microbial Communities Shaped by Treatment Processes in a Drinking Water Treatment Plant and Their Contribution and Threat to Drinking Water Safety. Front Microbiol [Internet]. 2017 Dec 12 [cited 2019 Feb 19];8:2465. Available from: http://journal.frontiersin.org/article/10.3389/fmicb.2017.02465/full

148. Kawamura S. Integrated Design and Operation of Water Treatment Facilities [Internet]. 2nd ed. New York: John Wiley & Sons, Inc.; 2000 [cited 2022 Oct 18]. 20–23 p. Available from: https://books.google.co.uk/books?id=cOQKKRUNGrwC&printsec=copyright&redir_esc=y#v=onepage&q&f=false

149. Bergaya F, Lagaly G. Chapter 1 General Introduction: Clays, Clay Minerals, and Clay Science. Dev Clay Sci. 2006 Jan 1;1(C):1–18.

150. Oakey J. Fuel Flexible Energy Generation: Solid, Liquid and Gaseous Fuels. [Internet]. Cambridge: Elsevier Science & Technology; 2015. Available from: https://learning.oreilly.com/library/view/fuel-flexible-energy/9781782423997/XHTML/B9781782423782000018/B97817824237820000 18.xhtml

151. al Ani T, Sarapää O. Clay and clay mineralogy. Geological Survey of Finland, Report M19/3232/2008/41, Espoo [Internet]. 2008; Available from: files/77/Al Ani and Sarapää - 2008 - Clay and clay mineralogy.pdf

152. Hall-Stoodley L, Costerton JW, Stoodley P. Bacterial biofilms: from the natural environment to infectious diseases. Nat Rev Microbiol [Internet]. 2004 Feb [cited 2022 Oct 26];2(2):95–108. Available from: https://pubmed.ncbi.nlm.nih.gov/15040259/

153. Navada S, Gaumet F, Tveten AK, Kolarevic J, Vadstein O. Seeding as a start-up strategy for improving the acclimation of freshwater nitrifying bioreactors to salinity stress. Aquaculture. 2021 Jul 15;540:736663.

154. Perfettini J, Bianchi M. The comparison of two simple protocols designed to initiate and stimulate ammonia oxidation in closed aquaculture systems. Aquaculture. 1990 Jul 15;88(2):179–88.

155. Shi K, Zhou W, Zhao H, Zhang Y. Performance of halophilic marine bacteria inocula on nutrient removal from hypersaline wastewater in an intermittently aerated biological filter. Bioresour Technol. 2012 Jun 1;113:280–7.

156. Carmignani GM, Bennett JP. Rapid start-up of a biological filter in a closed aquaculture system. Aquaculture. 1977 May 1;11(1):85–8.

157. Mnyoro MS, Munubi RN, Pedersen LF, Chenyambuga SW. Evaluation of biofilter performance with alternative local biomedia in pilot scale recirculating aquaculture systems. J Clean Prod. 2022 Sep 15;366:132929.

158. Nogueira R, Melo LF, Purkhold U, Wuertz S, Wagner M. Nitrifying and heterotrophic population dynamics in biofilm reactors: effects of hydraulic retention time and the presence of organic carbon. Water Res. 2002 Jan 1;36(2):469–81.

159. Nedwell DB. Effect of low temperature on microbial growth: lowered affinity for substrates limits growth at low temperature. FEMS Microbiol Ecol [Internet]. 1999 Oct 1 [cited 2022 Oct 25];30(2):101–11. Available from: https://academic.oup.com/femsec/article/30/2/101/608571

160. Li H, Yang Q, Li J, Gao H, Li P, Zhou H. The impact of temperature on microbial diversity and AOA activity in the Tengchong Geothermal Field, China. Scientific Reports 2015 5:1 [Internet]. 2015 Nov 26 [cited 2022 Oct 25];5(1):1–12. Available from: https://www.nature.com/articles/srep17056

161. Sharma D, Taylor-Edmonds L, Andrews RC. Comparative assessment of ceramic media for drinking water biofiltration. Water Res. 2018 Jan 1;128:1–9.

162. R. Hunter P. Household Water Treatment in Developing Countries: Comparing Different Intervention Types Using Meta-Regression | Environmental Science &

Technology. 2009; Available from: https://pubs.acs.org/doi/full/10.1021/es9028217

163. Soliman MYM, van Halem D, Medema G. Virus removal by ceramic pot filter disks: Effect of biofilm growth and surface cleaning. Int J Hyg Environ Health [Internet]. 2020;224:113438. Available from: https://www.sciencedirect.com/science/article/pii/S1438463919307151

164. Fonseca AC, Summers RS, Hernandez MT. Comparative measurements of microbial activity in drinking water biofilters. Vol. 35, Wat. Res. 2001.

165. Kim J, Jung H, Lee C. Shifts in bacterial and archaeal community structures during the batch biomethanation of Ulva biomass under mesophilic conditions. Bioresour Technol. 2014 Oct 1;169:502–9.

166. Zeng DN, Fan ZY, Chi L, Wang X, Qu WD, Quan ZX. Analysis of the bacterial communities associated with different drinking water treatment processes. World J Microbiol Biotechnol [Internet]. 2013 Sep 21 [cited 2022 Dec 18];29(9):1573–84. Available from: https://link.springer.com/article/10.1007/s11274-013-1321-5

167. Pedrs-Ali C. The Rare Bacterial Biosphere. https://doi.org/101146/annurev-marine-120710-100948 [Internet]. 2011 Dec 12 [cited 2022 Dec 15];4:449–66. Available from: https://www.annualreviews.org/doi/abs/10.1146/annurev-marine-120710-100948

168. Saunders AM, Albertsen M, Vollertsen J, Nielsen PH. The activated sludge ecosystem contains a core community of abundant organisms. ISME J [Internet]. 2016 Jan 1 [cited 2022 Dec 15];10(1):11–20. Available from: https://pubmed.ncbi.nlm.nih.gov/26262816/

169. Logares R, Audic S, Bass D, Bittner L, Boutte C, Christen R, *et al.* Patterns of rare and abundant marine microbial eukaryotes. Curr Biol [Internet]. 2014 Apr 14 [cited 2022 Dec 15];24(8):813–21. Available from: https://pubmed.ncbi.nlm.nih.gov/24704080/

170. Jeong J, Yun K, Mun S, Chung WH, Choi SY, Nam Y do, *et al.* The effect of taxonomic classification by full-length 16S rRNA sequencing with a synthetic long-read technology. Scientific Reports 2021 11:1 [Internet]. 2021 Jan 18 [cited 2022 Oct 11];11(1):1–12. Available from: https://www.nature.com/articles/s41598-020-80826-9

171. Ehrich S, Behrens D, Lebedeva E, Ludwig W, Bock E. A new obligately chemolithoautotrophic, nitrite-oxidizing bacterium, Nitrospira moscoviensis sp. nov. and its phylogenetic relationship. Arch Microbiol [Internet]. 1995 Jul [cited 2022 Dec 29];164(1):16–23. Available from: https://pubmed.ncbi.nlm.nih.gov/7646315/

172. Chao A. Nonparametric Estimation of the Number of Classes in a Population. Scandinavian Journal of Statistics [Internet]. 1984 [cited 2022 Dec 29];11(4):265–70. Available from: https://www.jstor.org/stable/4615964

173. Guarin TC, Pagilla KR. Microbial community in biofilters for water reuse applications: A critical review. Science of the Total Environment [Internet]. 2021 [cited 2022 Dec 20];773:145655. Available from: https://doi.org/10.1016/j.scitotenv.2021.145655

174. Martiny AC, Jørgensen TM, Albrechtsen HJ, Arvin E, Molin S. Long-Term Succession of Structure and Diversity of a Biofilm Formed in a Model Drinking Water Distribution System. Appl Environ Microbiol [Internet]. 2003 Nov [cited 2022 Dec 29];69(11):6899–907. Available from: https://journals.asm.org/doi/10.1128/AEM.69.11.6899-6907.2003

175. Bennett A. Drinking water: Pathogen removal from water – technologies and techniques. Filtration & Separation [Internet]. 2008 Dec 1 [cited 2019 Apr 18];45(10):14–6. Available from: https://www.sciencedirect.com/science/article/pii/S0015188208704956

176. Li XF, Mitch WA. Drinking Water Disinfection Byproducts (DBPs) and Human Health Effects: Multidisciplinary Challenges and Opportunities. Environ Sci Technol [Internet]. 2018 Feb 20 [cited 2022 Dec 25];52(4):1681–9. Available from: https://pubs.acs.org/doi/full/10.1021/acs.est.7b05440

177. Rutala WA, Weber DJ. Guideline for Disinfection and Sterilization in Healthcare Facilities, 2008.

178. Granger HC, Stoddart AK, Gagnon GA. Direct Biofiltration for Manganese Removal from Surface Water. Journal of Environmental Engineering [Internet]. 2014 Apr [cited 2019 Apr 25];140(4):04014006. Available from: http://ascelibrary.org/doi/10.1061/%28ASCE%29EE.1943-7870.0000819

179. Srivastava NK, Majumder CB. Novel biofiltration methods for the treatment of heavy metals from industrial wastewater. J Hazard Mater [Internet]. 2008 Feb 28 [cited

2019 Apr 25];151(1):1–8. Available from: https://www.sciencedirect.com/science/article/pii/S0304389407013933

180. Fu J, Lee WN, Coleman C, Nowack K, Carter J, Huang CH. Removal of disinfection byproduct (DBP) precursors in water by two-stage biofiltration treatment. Water Res [Internet]. 2017 Oct 15 [cited 2019 Apr 25];123:224–35. Available from: https://www.sciencedirect.com/science/article/pii/S0043135417305493

181. Klayman BJ, Volden PA, Stewart PS, Camper AK. Escherichia coli O157:H7 Requires Colonizing Partner to Adhere and Persist in a Capillary Flow Cell. Environ Sci Technol [Internet]. 2009 Mar 15 [cited 2019 Nov 14];43(6):2105–11. Available from: https://pubs.acs.org/doi/10.1021/es802218q

182. Bauman WJ, Nocker A, Jones WL, Camper AK. Retention of a model pathogen in a porous media biofilm. Biofouling [Internet]. 2009 Feb 10 [cited 2019 Mar 11];25(3):229–40. Available from: http://www.tandfonline.com/doi/abs/10.1080/08927010802688566

183. Legnani P, Leoni E, Rapuano S, Turin D, Valenti C. Survival and growth of Pseudomonas aeruginosa in natural mineral water: a 5-year study. Int J Food Microbiol [Internet]. 1999 Dec 15 [cited 2019 Mar 10];53(2–3):153–8. Available from: https://www.sciencedirect.com/science/article/pii/S0168160599001518

184. Wei J, Imai T, Higuchi T, Arfaritaa N, Yamamoto K, Sekine M, *et al*. Effect of different carbon sources on the biological phosphorus removal by a sequencing batch reactor using pressurized pure oxygen. Biotechnol Biotechnol Equip [Internet]. 2014 May 5 [cited 2022 Dec 9];28(3):471. Available from: /pmc/articles/PMC4434035/

185. Schmidt KD, Tümmler B, Römling U. Comparative genome mapping of Pseudomonas aeruginosa PAO with P. aeruginosa C, which belongs to a major clone in cystic fibrosis patients and aquatic habitats. J Bacteriol [Internet]. 1996 [cited 2022 Feb 24];178(1):85. Available from: /pmc/articles/PMC177624/?report=abstract

186. Nigaud Y, Cosette P, Collet A, Song PCT, Vaudry D, Vaudry H, *et al*. Biofilm-induced modifications in the proteome of Pseudomonas aeruginosa planktonic cells. Biochim Biophys Acta Proteins Proteom. 2010 Apr;1804(4):957–66.

187. von Ohle C, Gieseke A, Nistico L, Decker EM, Debeer D, Stoodley P. Real-Time Microsensor Measurement of Local Metabolic Activities in Ex Vivo Dental Biofilms Exposed to Sucrose and Treated with Chlorhexidine. Appl Environ Microbiol

[Internet]. 2010 Apr [cited 2022 Feb 24];76(7):2326. Available from: /pmc/articles/PMC2849229/

188. Nescerecka A, Juhna T, Hammes F. Identifying the underlying causes of biological instability in a full-scale drinking water supply system. Water Res. 2018 May 15;135:11–21.

189. Prest EI, Hammes F, Kötzsch S, van Loosdrecht MCM, Vrouwenvelder JS. A systematic approach for the assessment of bacterial growth-controlling factors linked to biological stability of drinking water in distribution systems. Water Sci Technol Water Supply. 2016 Aug 1;16(4):865–80.

190. Whitton R, Fane S, Jarvis P, Tupper M, Raffin M, Coulon F, *et al*. Flow cytometry-based evaluation of the bacterial removal efficiency of a blackwater reuse treatment plant and the microbiological changes in the associated non-potable distribution network. Science of the Total Environment. 2018;645:1620–9.

191. Dufour AP. Bacterial Indicators of Recreational Water Quality. Canadian Journal of Public Health [Internet]. 1984 Jan [cited 2022 Nov 21];75(1):49–56. Available from: https://www.jstor.org/stable/41990233

192. Drinking Water Inspectorate (DWI). Drinking Water 2021 [Internet]. London; 2022. Available from: www.dwi.gov.uk

193. Hozalski RM, Bouwer EJ, Goel S. Removal of natural organic matter (nom) from drinking water supplies by ozone-biofiltration. Water Science and Technology [Internet]. 1999 Jan 1 [cited 2022 Nov 18];40(9):157–63. Available from: https://www.academia.edu/23110261/Removal_of_natural_organic_matter_nom_from_drinking_water_supplies_by_ozone_biofiltration

194. Persson F, Heinicke G, Uhl W, Hedberg T, Hermansson M. Performance of direct biofiltration of surface water for reduction of biodegradable organic matter and biofilm formation potential. Environ Technol [Internet]. 2006 [cited 2022 Nov 18];27(9):1037–45. Available from: https://pubmed.ncbi.nlm.nih.gov/17067130/

195. Bellamy WD, Hendricks DW, Logsdon GS. Slow Sand Filtration: Influences of Selected Process Variables. J Am Water Works Assoc [Internet]. 1985 Dec 1 [cited 2022 Nov 18];77(12):62–6. Available from: https://onlinelibrary.wiley.com/doi/full/10.1002/j.1551-8833.1985.tb05659.x

196. Andersson A, Laurent P, Kihn A, Prévost M, Servais P. Impact of temperature on nitrification in biological activated carbon (BAC) filters used for drinking water

treatment. Water Res [Internet]. 2001 [cited 2022 Nov 18];35(12):2923–34. Available from: https://pubmed.ncbi.nlm.nih.gov/11471692/

197. Baig SA, Mahmood Q, Nawab B, Shafqat MN, Pervez A. Improvement of drinking water quality by using plant biomass through household biosand filter - A decentralized approach. Ecol Eng [Internet]. 2011 Nov [cited 2022 Feb 2];37(11):1842–8. Available from: https://www.researchgate.net/publication/251617178_Improvement_of_Drinking_Water_Quality_by_Using_Plant_Biomass_Through_Household_Biosand_Filter_-_a_Decentralized_Approach

198. LeChevallier MW, Evans TM, Seidler RJ. Effect of turbidity on chlorination efficiency and bacterial persistence in drinking water. Appl Environ Microbiol [Internet]. 1981 [cited 2022 Nov 18];42(1):159. Available from: /pmc/articles/PMC243978/?report=abstract

199. Fabiszewski De Aceituno AM, Stauber CE, Walters AR, Meza Sanchez RE, Sobsey MD. A Randomized Controlled Trial of the Plastic-Housing BioSand Filter and Its Impact on Diarrheal Disease in Copan, Honduras. Am J Trop Med Hyg. 2012;86(6):913–21.

200. Zhu J, Fan XJ, Tao Y, Wei DQ, Zhang XH, Tao YI. Journal of Environmental Science and Health, Part A Toxic/Hazardous Substances and Environmental Engineering Study on an integrated process combining ozonation with ceramic ultra-filtration for decentralized supply of drinking water) Study on an integrated process combining ozonation with ceramic ultra-filtration for decentralized supply of drinking water Study on an integrated process combining ozonation with ceramic ultra-filtration for decentralized supply of drinking water. Journal of Environmental Science and Health, Part A [Internet]. 2014 [cited 2022 Feb 2];49(11):1296–303. Available from: https://www.tandfonline.com/action/journalInformation?journalCode=lesa20

201. Sartor M, Schlichter B, Gatjal H, Mavrov V. Demonstration of a new hybrid process for the decentralised drinking and service water production from surface water in Thailand. Desalination. 2008 Mar 1;222(1–3):528–40.

202. Loizidou M, Moustakas K, Malamis D, Rusan M, Haralambous KJ. Development of a decentralized innovative brackish water treatment unit for the production of drinking water. New pub: Balaban [Internet]. 2014 Mar 20 [cited 2022 Nov 22];53(12):3187–98. Available from: https://www.tandfonline.com/doi/abs/10.1080/19443994.2014.933631

203. Huisman L, Wood WE. Slow sand filtration. World Health Organization [Internet]. 1974 [cited 2022 Dec 10]; Available from: https://apps.who.int/iris/handle/10665/38974

204. Flemming HC, Wingender J. The biofilm matrix. Nat Rev Microbiol [Internet]. 2010;8(9):623–33. Available from: http://dx.doi.org/10.1038/nrmicro2415

205. Rendueles O, Ghigo JM. Mechanisms of Competition in Biofilm Communities. Microbiol Spectr [Internet]. 2015 Jun 18 [cited 2022 Jun 28];3(3). Available from: https://journals.asm.org/doi/full/10.1128/microbiolspec.MB-0009-2014

206. Granato ET, Meiller-Legrand TA, Foster KR. The Evolution and Ecology of Bacterial Warfare. Current Biology. 2019 Jun 3;29(11):R521–37.

207. de Lima Procópio RE, da Silva IR, Martins MK, de Azevedo JL, de Araújo JM. Antibiotics produced by Streptomyces. The Brazilian Journal of Infectious Diseases. 2012 Sep 1;16(5):466–71.

208. Vries D, Bertelkamp C, Schoonenberg Kegel F, Hofs B, Dusseldorp J, Bruins JH, *et al.* Iron and manganese removal: Recent advances in modelling treatment efficiency by rapid sand filtration. Water Res [Internet]. 2017 Feb 1 [cited 2022 Dec 30];109:35–45. Available from: https://pubmed.ncbi.nlm.nih.gov/27865171/

209. Vandenabeele J, de Beer D, Germonpré R, Verstraete W. Manganese oxidation by microbial consortia from sand filters. Microb Ecol [Internet]. 1992 Jul [cited 2022 Dec 30];24(1):91–108. Available from: https://link.springer.com/article/10.1007/BF00171973

210. Marcus DN, Pinto A, Anantharaman K, Ruberg SA, Kramer EL, Raskin L, *et al.* Diverse manganese(II)-oxidizing bacteria are prevalent in drinking water systems. Environ Microbiol Rep. 2017;9(2):120–8.

211. Tebo BM, Johnson HA, McCarthy JK, Templeton AS. Geomicrobiology of manganese(II) oxidation. Trends Microbiol [Internet]. 2005 Sep [cited 2022 Dec 30];13(9):421–8. Available from: https://pubmed.ncbi.nlm.nih.gov/16054815/

212. Bergman Å, Heindel JJ, Kasten T, Kidd KA, Jobling S, Neira M, *et al.* The Impact of Endocrine Disruption: A Consensus Statement on the State of the Science. Environ Health Perspect [Internet]. 2013 Apr [cited 2022 Dec 30];121(4):a104. Available from: /pmc/articles/PMC3620733/

213. Miettinen IT, Vartiainen T, Martikainen PJ. Phosphorus and bacterial growth in drinking water. Appl Environ Microbiol [Internet]. 1997 Aug 1 [cited 2019 Feb 13];63(8):3242–5. Available from: http://www.ncbi.nlm.nih.gov/pubmed/9251211

214. Keinänen MM, Korhonen LK, Lehtola MJ, Miettinen IT, Martikainen PJ, Vartiainen T, *et al.* The microbial community structure of drinking water biofilms can be affected by phosphorus availability. Appl Environ Microbiol. 2002;68(1):434–9.

Appendix I Published material

npj | clean water

www.nature.com/npjcleanwater

ARTICLE OPEN

Check for updates

The control of waterborne pathogenic bacteria in fresh water using a biologically active filter

Joshua A. C. Steven[1], Robin M. S. Thorn[1], Gareth M. Robinson[1], Dann Turner[1], Jack E. Lee[2] and Darren M. Reynolds[1]✉

The aim of this study was to investigate the control of three species of bacteria commonly associated with biologically contaminated water, using biofiltration. In this study, a laboratory-scale biofilter system was used to investigate the control of *Escherichia coli*, *Enterococcus faecalis*, and *Pseudomonas aeruginosa* in fresh water. Simulated fresh water was inoculated with the test pathogens at a starting inocula of ~1000 CFU 100 mL ^{1} to challenge the biofilters. Biofilter systems operating within a recirculation configuration demonstrated significant reduction of *E. coli* (99%), *E. faecalis* (99%), and *P. aeruginosa* (92%) after 24 h. Conversely, all sterile control systems did not show any significant reduction in pathogens. Subsequent analysis of the biofilter media after circulation showed that 0% of *E. coli* was recovered from the biofilter, whereas 0.06% and 1.26% of *E. faecalis* and *P. aeruginosa* were recovered respectively. Further investigation demonstrated the reduction of *E. coli* and enterococci from an environmentally-derived surface water of 99.8% and 99.4% respectively. In conclusion, this work demonstrates that biofilter systems can be used to significantly reduce waterborne pathogenic bacteria within fresh water. The potential application of low-cost, energy efficient biofilter systems for the management of waterborne bacterial pathogens in water supplies is discussed.

npj Clean Water (2022)5:30 ; https://doi.org/10.1038/s41545-022-00169-y

INTRODUCTION

Meeting the future demands for fresh water has been identified as a major global challenge over the coming decades[1,2]. Of all water present on Earth, only 2.5% is fresh water. Furthermore, only 0.77% of fresh water is deemed accessible, and of this, only 10% is reported to be suitable for human consumption[3,4]. Since 2000, billions of people have gained access to basic drinking water and sanitation services, however, access to safe water free from chemical and biological contamination (potable water) remains a significant challenge[5]. Water that is contaminated with micro-organisms, can cause disease; the consumption of biologically contaminated water is estimated to cause 485,000 deaths each year[6]. Such diseases can be attributed to the presence of pathogenic bacteria, including, but not limited to, *Escherichia coli*, enterococci species (including *Enterococcus faecalis*) and *Vibrio cholera* within untreated water[7–10]. Nonetheless, biology plays an important role in access to improved drinking water. Slow sand filters are commonly used in low-middle income countries (LMIC). The development of a layer of biomass (schmutzdecke) at the surface of the sand filter facilitates the biodegradation of organic material present in the water. This biomass layer also acts to prevent the physical progression of waterborne pathogens such as *E. coli*, coliform bacteria, Giardia and Cryptosporidium[11–13] through the slow sand filter, commonly achieving between 1 and 4 log reductions in pathogen loads. In high income countries, the biological filters are commonly used to reduce or remove organic matter, a precursor to the formation of harmful disinfection by products, prior to chlorination[14–16]. In addition, biological filtration methods are commonly used for the treatment (tertiary stage) of municipal wastewater[17].

The control of waterborne pathogenic bacteria in areas with established, centralised water treatment systems is achieved by the combination of physical processes such as screening and filtration with well understood disinfection methods using chemicals, ozonation and/or UV disinfection[18], which require significant energy and resources. Moreover, chlorination is used in drinking water disinfection processes, requiring residual free chlorine and chloramines throughout distribution systems to ensure water quality is maintained, preventing the growth of pathogenic bacteria during water distribution[19]. However, such disinfection agents are hazardous, expensive, and not always easily implemented in remote or challenging communities where infrastructure is lacking. Therefore, there is an unmet need for low energy solutions for the provision of potable water that are sustainable in the long term, i.e. scalable with low energy, maintenance and material requirements.

Over the last 20 years, there has been an increase into the research and development of sustainable, compact water treatment systems for the production of drinking water (Table 1)[20]. Sustainable provision of potable water should provide adequate water quantity and appropriate water quality for a given need, without compromising the future ability to provide this capacity and quality. A few examples of this are in ground water stores such as aquifers where the demand is no more than the recharge rate, recycled/reclaimed water, and rainwater harvesting. Appropriate treatment methods will also contribute to water sustainability, whereby the maintenance of appropriate water quality can reduce pressure on this resource. One potential sustainable approach for water treatment is the application of biofiltration, a remediation biotechnology that utilises microbial biofilms adhered to a stationary phase substrate that can be configured within filter columns[15]. Biofilms are complex microbial communities and can be defined as aggregates of microorganisms in which cells are frequently embedded in a self-produced matrix of extracellular polymeric substances (EPS). The production of EPS facilitates the adherence of the microorganisms to each other as well as to biotic and abiotic surfaces[21]. Biofiltration utilises granular filter media upon which biofilms can establish.

[1]Centre for Research in Biosciences, Department of Applied Sciences, University of the West of England, Bristol BS16 1QY, UK. [2]Origin Aqua Technologies, The Old Forge, Wickwar Trading Estate, Station Road, Wotton Under Edge, Wickwar GL12 8NB, UK. ✉email: Darren.Reynolds@uwe.ac.uk

Published in partnership with King Fahd University of Petroleum & Minerals

Table 1. Examples of decentralised water treatment technologies for waterborne pathogen control.

Water treatment technology	Filtration	Disinfection	Reference
Chlorine tablets	N/A	Chlorination	Sobsey[65]
Solar disinfection	N/A	Sunlight (UV inactivation)	Carratalà et al.[66]
Vapour compression distillation	N/A	Vaporization of water using solar energy	Attisani[67]
Ultrafiltration (UF) coupled with chlorination	UF	Chlorination	Chaidez, C. et al.[68]
Ultrafiltration coupled with electrochemically activated solutions (ECAS)	115 µm filter pump, 100 µm reverse flushing filter and 0.02 µm UF	Electrochemically activated solution (1% total v/v)	Clayton et al.[69]
Biosand filters	Gravel, Sand and Coniferous pinus bark biomass	N/A	Baig et al.[70]
Biofilter systems	Foam sleeve/disk incorporating biofilm and carbon filter block.	Chlorination	Wendt et al.[34]

The types of filter media employed are varied and can be comprised of sand, polymer, ceramic, charcoal and granular activated carbon, amongst others[15].

Where biofilters are used in water treatment, the biofilm communities that are established on filter media (biofilter media) are known to be diverse[22–24]. Such biofilters are used to reduce levels of natural organic material in drinking water treatment[22] or the biochemical oxygen demand of wastewaters[25]. These diverse microbial populations exhibit complex sorbent characteristics; whereby binding sites within microbial biofilm communities include both anionic and cationic exchangers that can remove a wide range of substances and contaminants from the water source, even when such contaminants are present at very low concentrations[26–28]. Nutrient acquisition is an essential driver for this process, and biofilms have developed a very efficient capture strategy for this. The sorption properties of the EPS matrix enable it to behave like a sponge that influences the exchange of nutrients, gases and other molecules between the water phase and the biofilm[21]. Moreover, a diverse biofilm community will incorporate varying sorption mechanisms and binding sites of biofilm cells and the EPS of the matrix and can apply to both dissolved compounds and suspended solids. These compounds can be trapped by biofilms and incorporated into the matrix, including biodegradable material that can be utilised as a source of nutrients for further microbial growth. The capability of biofilms to remove a wide range of substances such as nutrients involve cooperation. For example, in the process of nitrification ammonia-oxidising bacteria metabolise ammonia into nitrite which is then utilised by other nitrite oxidising species of bacteria such as *Nitrospira moscoviensis*. Moreover, aerobic bacteria consume oxygen which can exceed the rate of diffusion through the biofilm, facilitating the survival of anaerobic bacteria such as those involved in denitrification of nitrates[29]. Moreover, metal ions including but not limited to Cu^{2+} Zn^{2+}, Fe^{2+}, Fe^{3+} and Al^{3+} have been found to accumulate within biofilms[30]. Although, biofilters have been shown to remove a wide range of chemical substances[24,31,32], little is known regarding the use of biofilters for the control and removal of pathogenic bacteria in potable water supplies. For example, biofiltration has been demonstrated to result in a reduction of *E. coli* (up to 58%) and faecal coliforms (up to 63%) in urban ponds[33]. For potable water production, a combination of biofiltration technology, coagulation, flocculation, filtration and chlorination has been previously demonstrated to be effective for the control of waterborne bacteria[34]. However, to date no studies have been reported that demonstrate the use of biofiltration alone to control the levels of pathogenic bacteria for potable water. The aim of this study was to investigate the control and inhibition of pathogenic bacteria commonly associated with biologically contaminated water supplies using biofiltration.

RESULTS

Viable counts of *Escherichia coli*, *Enterococcus faecalis* and *Pseudomonas aeruginosa* are significantly reduced by biofiltration

At the start of all experiments, there was no significant difference in the starting density of test pathogen monocultures within the simulated freshwater (SFW), between the biofilter, the column containing the sterile filter media and the empty filter column ($p > 0.05$; Fig. 1). After 24 h, significant reductions in all three species were observed in the biofilter systems, when compared to the SFW in both the control systems ($p < 0.001$; Fig. 1a–c). Relative to the initial bacterial load present within the SFW, the biofilter was shown to significantly reduce *E. coli* cell numbers by 99.4 ± 0.60% (absolute mean reduction by 4.02 ± 0.01 Log_{10} CFU; Fig. 1a), *E. faecalis* by 99.9 ± 0.04% (absolute mean reduction by 4.01 ± 0.22 Log_{10} CFU; Fig. 1b), and *P. aeruginosa* by 92.1 ± 10.9% (absolute mean reduction by 3.93 ± 0.093 Log_{10} CFU; Fig. 1c). The control system containing sterile filter media also resulted in significant reductions for *E. coli* and *E. faecalis*, whereby a reduction from 3.97 ± 0.06 Log_{10}CFU to 3.48 ± 0.07 Log_{10}CFU for *E. coli* and 3.91 ± 0.15 Log_{10}CFU to 3.64 ± 0.14 Log_{10}CFU for *E. faecalis* was observed. However, an increase from 4.01 ± 0.04 Log_{10}CFU to 6.55 ± 1.09 Log_{10}CFU was observed for *P. aeruginosa*. For the system with the empty filter column small non-significant reductions were observed for *E. coli* and *E. faecalis* ($p > 0.05$ Fig. 1a, b). There was a reduction from 3.94 ± 0.02 to 3.82 ± 0.08 Log_{10}CFU for *E. coli* and a reduction from 3.91 ± 0.14 to 3.77 ± 0.15 Log_{10}CFU for *E. faecalis*. However, there was increase to from 3.93 ± 0.13 to 6.69 ± 0.94 Log_{10}CFU for *P. aeruginosa* in the system with the empty filter column.

The accumulation of *Escherichia coli*, *Enterococcus faecalis* and *Pseudomonas aeruginosa* on the filter media

For *E. coli*, no viable cells were recovered from the biofilter media, whereas 3.03 ± 0.03 Log_{10}CFU filter ^{1} was recovered from the sterile filter media (Table 2). For *E. faecalis*, 0.91 ± 0.65 Log_{10}CFU filter ^{1} was recovered from the biofilter after the complete 24 h cycle. This equates to 0.06% of the starting density that was able to accumulate and survive on the biofilter media after 24 h, however, this was significantly less than the 2.46 ± 0.14 Log_{10}CFU filter ^{1} recovered from the sterile filter media ($p < 0.05$; Table 2). For *P. aeruginosa*, 1.89 ± 0.99 Log_{10}CFU filter ^{1} (equating to 1.26% of the starting inoculum) was recovered from the biofilter media. Whereas, 4.24 ± 0.49 Log_{10}CFU filter ^{1} was recovered from the sterile filter media. Moreover, there was an average increase from 3.93 ± 0.13 Log_{10}CFU to 6.53 ± 1.12 Log_{10}CFU within the system containing sterile filter media in comparison to an average decrease from 3.97 ± 0.04 Log_{10}CFU to 1.89 ± 0.99 Log_{10}CFU for *P. aeruginosa* when cycled through the biofilter. To ensure that

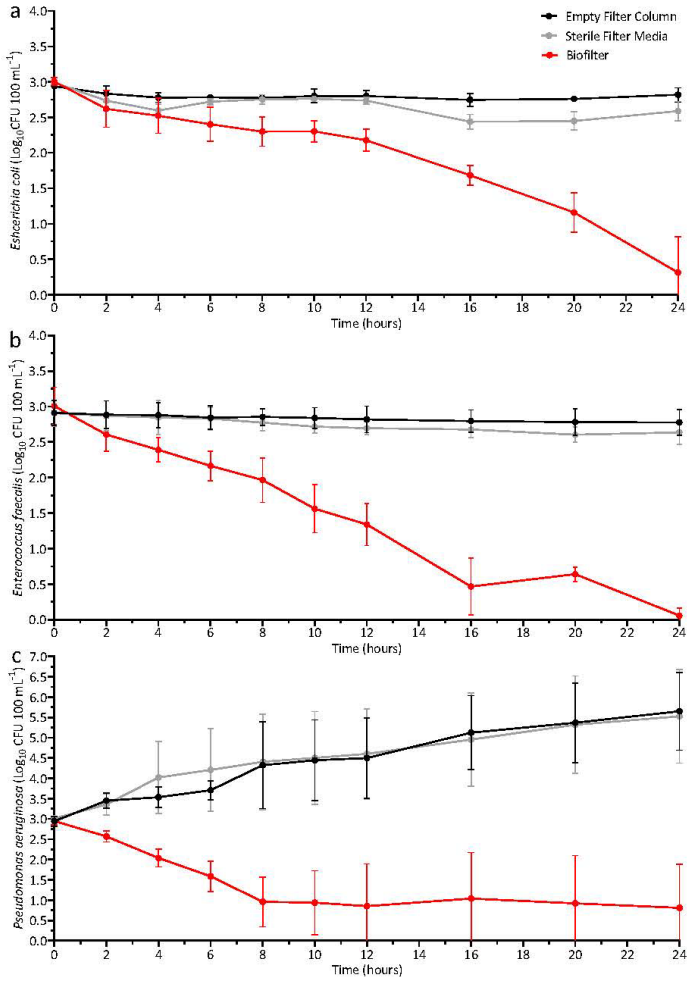


Fig. 1 Viable counts of the test pathogens when cycled through a biofilter. Viable counts of **a** *Escherichia coli*, **b** *Enterococcus faecalis* and **c** *Pseudomonas aeruginosa* within simulated freshwater when circulated through a laboratory-scale system incorporating a biofilter, a column containing the sterile filter media and an empty filter column over 24 h, all performed in triplicate. Statistical significance determined using two-way ANOVA with Tukey's post-hoc test from a minimum of $n = 3 \pm$ s.d. (***=$p < 0.001$).

there was no significant difference between the system containing the sterile filter media and the system containing the empty filter column for all three species of bacteria after 24 h of circulation, the total number of cells on the sterile filter media and in the SFW were compared against the number of cells in the SFW of the system with the empty filter column and were found to be non-significant for all three species ($p > 0.05$).

The circulation of environmental water through biological filters

A range of standard biological and physiochemical indicators of water quality were recorded before and after 24 h of circulation through the test systems. After 24 h, significant reductions in the biological indicator species (*E. coli* and enterococci) were observed. There was a reduction of 99.8 ± 0.20% and 99.4 ± 0.41% for *E. coli* and enterococci respectively. There was no significant change in the heterotrophic plate counts in the environmental water post 24 h circulation through the biofilter, however, there was a significant increase of 403% (equating to a total increase of 4.98 Log_{10}CFU mL ^{1}) in heterotrophic plate counts in the environmental water when circulated through the empty filter column.

The concentration of nitrogen species in the environmental water decreased after 24 h of circulation through the biofilter. There was a significant reduction in the concentrations of 100% of ammonium, 100% of nitrite and 30.8 ± 11.3% of nitrate ($p < 0.05$; Table 3). The 100% reduction of both ammonium and nitrite were

Table 2. Total number of viable test pathogens pre and post 24-hour circulation period.

Test pathogen	Filter media	Number of test bacteria pre-circulation in the SFW ($Log_{10}CFU$)	Number of test bacteria post-circulation in the SFW ($Log_{10}CFU$)	Number of test bacteria accumulated on the filter media ($Log_{10}CFU\ filter^{-1}$)	Total number of test bacteria in the system ($Log_{10}CFU$)
Escherichia coli	SFM	3.97 ± 0.06	3.70 ± 0.05	3.03 ± 0.03	3.78 ± 0.05
	BFM	4.02 ± 0.01	0.63 ± 0.48	0	0.63 ± 0.48***
Enterococcus faecalis	SFM	3.91 ± 0.15	3.64 ± 0.14	2.46 ± 0.14	3.67 ± 0.13
	BFM	4.01 ± 0.22	0.39 ± 0.55	0.91 ± 0.65	0.39 ± 0.55***
Pseudomonas aeruginosa	SFM	4.01 ± 0.04	6.55 ± 1.09	4.24 ± 0.49	6.55 ± 1.08
	BFM	4.00 ± 0.04	1.89 ± 0.99	2.05 ± 0.11	2.53 ± 0.53***

Statistical significance between the total number of test bacteria in the system containing the sterile filter media (SFM) and biofilter (BFM) was determined using a paired *t*-test $n = 3 \pm$ s.d. (***=$p < 0.001$).

recorded as below the detection limit of the ion chromatography. Moreover, the reduction of all three nitrogen species were significantly different from the empty filter column at 24 h. The concentration of nitrogen in the environmental water that was circulated through the empty filter column showed an increase in nitrite of 71% and a small change in the concentration of ammonium and nitrate ($p > 0.05$; Table 3). The concentration of total inorganic carbon (TIC) increased after 24 h of circulation through the biofilter. There was a significant difference between the biofilter and the empty filter column with the TIC increasing by 5.8% ($p < 0.05$; Table 3). However, the concentration of total organic carbon (TOC) was not significantly different from the empty filter column ($p < 0.05$; Table 3). When comparing the water quality parameters of the environmental water pre and post circulation for both the biofilter and empty filter column, there was a significant reduction in ORP ($p < 0.01$; Table 3), a significant increase in dissolved oxygen (DO) ($p < 0.05$; Table 3) and no significant difference in conductivity or total dissolved solids (TDS) ($p > 0.05$; Table 3). However, there was no significant difference between the biofilter and the empty filter column post treatment for DO, pH, ORP, conductivity or TDS ($p > 0.05$; Table 3).

Table 3. Water quality of environmentally-derived surface water pre and post 24-hour circulation period.

Parameter	Pre-circulation	Post-circulation	
		EFC	BF
Presumptive *Escherichia coli* (CFU 100 mL^{-1})	214 ± 31	122 ± 21.7	0.33 ± 0.47*
Enterococci (CFU 100 mL^{-1})	302 ± 18	153 ± 6.79	1.67 ± 1.25*
Heterotrophic Plate Counts ($Log_{10}CFU\ mL^{-1}$)	4.32 ± 0.07	5.06 ± 0.11	4.37 ± 0.03*
Total Organic Carbon (mg L^{-1})	4.87 ± 0.13	7.10 ± 1.06	5.05 ± 0.11
Total Inorganic Carbon (mg L^{-1})	20.78 ± 0.11	20.09 ± 0.11	22.05 ± 0.21*
Ammonium (mg L^{-1})	0.11 ± 0.04	0.2 ± 0.11	<0.01*
Nitrite (mg L^{-1})	0.23 ± 0.08	0.33 ± 0.09	<0.01*
Nitrate (mg L^{-1})	7.49 ± 0.98	7.25 ± 0.26	4.80 ± 0.26*
Dissolved Oxygen (mg L^{-1})	4.86 ± 0.2	8.05 ± 0.06	7.94 ± 0.11
ORP	349.8 ± 9.25	219 ± 8.16	205.3 ± 6.18
pH	8.13 ± 0.05	8.09 ± 0.01	7.75 ± 0.42
Conductivity (µS cm^{-1})	674.6 ± 7.76	679.2 ± 5.23	708.4 ± 8.24
Total Dissolved Solids	470.8 ± 5.58	477 ± 2.83	489.5 ± 1.5

Statistical significance between the Biofilter (BF) and the Empty Filter Column (EFC) at 24 h post circulation was determined using a paired *t*-test $n = 3 \pm$ s.d. (* = $p < 0.05$).

DISCUSSION

Previous research that has utilised biofilters in water treatment has been primarily focused on the removal of organic material and emerging contaminants[22,23,31,32,35–39]. The use of biofilters to control waterborne pathogens for drinking water treatment has not been extensively reported in the literature. One previous study demonstrated that biofilters were capable of reducing viable *E. coli* by 56% through treating contaminated environmental water with gravity fed, open rock biofilter systems[33]. In addition, a biofilter system that utilised foam to establish a biofilm has been shown to significantly reduce the model non-pathogenic bacterial species, *Raoultella terrigena*, in comparison to a sterile control system[34]. Although, this was achieved using a complex multi-step filtration system with the role of the biofilter component in the removal of *R. terrigena* remaining unclear. Therefore there is limited knowledge of the direct effect of biofilm within small-scale biofilter systems (i.e. in the absence of other treatment interventions), to control the numbers of pathogenic bacteria for potable water. In this study, we demonstrate that laboratory-scale biofilters significantly reduced the number of waterborne pathogenic bacteria within a SFW medium and from an environmentally-derived water source.

The significant reduction in the number of viable test pathogens within the SFW after 24 h of circulation through the biofilter systems (Fig. 1; $p < 0.001$) may have resulted from cell death that either occurred in part, or in whole, within the SFW or on the biofilter media. To account for any loss of pathogens within the SFW, or through physical filtration effects from the ceramic filter media, control columns consisting of an empty filter column and column containing sterile filter media were employed in parallel with the biofilter. Within the systems incorporating the empty filter column and column containing sterile filter media, there was survival of *E. coli* and *E. faecalis* (Fig. 1a, b) and proliferation of *P. aeruginosa* (Fig. 1c) within the reservoir. This demonstrates that the SFW was able to support the survival of test pathogens and that there was a minimal physical filtration effect from the ceramic filter media over the 24 hour circulation period. Therefore, this strongly suggests that the biofilm present on the filter media was primarily responsible for the reduction of the three test species observed (Fig. 1). One of the possible mechanisms for this significant reduction may have been through the direct competition between the planktonic cells and the biofilm established on the filter media. To support this, the accumulation of test pathogens on the filter media was investigated because of the potential for the biofilter to become a

reservoir for pathogenic bacteria. Previous studies have demonstrated that planktonic bacteria have greater affinity for attachment to mature biofilms, as opposed to sterile surfaces[40–42]. Moreover, the formation of biofilm is a key survival strategy for microorganisms in challenging environments and therefore, there is a risk that the planktonic pathogens would survive within the biofilm present on the filter media and contaminate the water supply. Nonetheless, this study demonstrates that viable *E. coli* was unable to accumulate on the biofilter media whereas, this has been shown in previous studies[43]. This may have resulted from classical competitive exclusion within the biofilter, whereby the challenging pathogens could have been inhibited by the autochthonous bacteria established within the biofilm present on the biofilter media[44]. Different competition strategies target bacterial ability to form a biofilm, whereas others can be less specific, resulting in death or the limitation of growth in the competing bacteria[44] The competition between cells in biofilms can involve inhibitory or cidal mechanisms, such as the production of antibiotics and bacteriocins[45,46] or strategies that compromise growth, such as nutrient depletion[47]. Surface-active compounds (SACs) could also be produced by the microbial biofilm community (biosurfactants), which are amphipathic lipid-based molecules that lower interfacial tension and some of these biosurfactants display antimicrobial properties[48]. Therefore, production of these biosurfactants could reduce the ability of the test pathogens to accumulate and establish within the biofilm formed on the biofilter. In addition, interference mechanisms might also be upregulated in response to the presence of competition in the surroundings, known as the competition-sensing hypothesis[49].

Overall, there were significant reductions observed for all of the test pathogens (Fig. 1; $p < 0.001$ and Table 2; $p < 0.05$). However, there were differences in the reductions of test pathogens in the reservoir and on the filter media. Within the reservoir, the total bacterial reductions observed were 99.4 ± 0.60% for *E. coli*, and 99.9 ± 0.04% for *E. faecalis* and 92.1 ± 10.9% for *P. aeruginosa*. This may result from the differential tolerance of these microorganisms to persist within the simulated freshwater environment. The lower reduction of *P. aeruginosa* is unsurprising, given it is a ubiquitous environmental bacterium which can survive in oligotrophic environments; including up to 5 years in bottled water[50]. On the filter media, some viable *E. faecalis* and *P. aeruginosa* were recovered from the biofilter media, albeit in very low numbers (Table 2). The inability of *E. coli* to colonise the biofilter media could possibly be related to the lack of an appropriate 'colonising partner', which has been shown within capillary flow cell systems, whereby *P. aeruginosa* was able to form biofilm as a single coloniser[40]. Nonetheless, in the absence of biofilm, all of the test pathogens were able to adhere to and survive upon the sterile filter media in significantly higher numbers (Table 2; $p < 0.05$). Therefore, this provides further evidence of competitive exclusion involving potential inhibitory effects by the biofilm on the test pathogens that accumulated on the filter media within the biofilter systems.

The action of competitive exclusion within the biofilter may also explain why only a small number of *P. aeruginosa* were recovered from the biofilter media (Table 2), even though an overall mean reduction of 92 ± 10.9% was observed within the SFW (Fig. 2c). This indicates that, at the very least, a small sub-population of the *P. aeruginosa* used to challenge the biofilters was able to survive within the system after 24 h. Nonetheless, it was found that significantly higher numbers of *P. aeruginosa* were able to attach and survive on the sterile filter media (4.24 ± 0.49 Log_{10}CFU filter ^{1}) when compared to the biofilter media (2.05 ± 0.11 Log_{10}CFU filter ^{1}) ($p < 0.05$; Table 2). Moreover, there was a stark increase of *P. aeruginosa* within the SFW of the systems containing the sterile filter media and the empty filter column to 6.55 ± 1.09 Log_{10}CFU and 6.69 ± 0.94 Log_{10}CFU respectively, in comparison to the mean reduction of *P. aeruginosa* in the biofilter system to 1.89 ± 0.99 Log_{10}CFU. Collectively, this data demonstrates that the survival of *P. aeruginosa* is significantly lower within the biofilter systems when compared to the sterile filter media and empty filter column controls ($p < 0.001$; Table 2). Overall, there was a reduction in the survival of *E. coli*, *E. faecalis* and *P. aeruginosa* within biofilms present on the biofilter media after 24 h of circulation, whereby the introduced test pathogens may have been unable to survive as a result of competitive exclusion by the established biofilm on the filter media.

This study demonstrates that matured biofilms present on a ceramic substrate can reduce the numbers of viable *E. coli*, *E. faecalis* and *P. aeruginosa* within a water source used to challenge these biofilters. To further support the reduction of these test pathogens using biofilters, the survival of environmental *E. coli* and enterococci present within an environmental water source circulated through the lab-scale biofilters was investigated. Biofiltration relies on the processing and metabolism of cells within the biofilm, therefore variability of physiochemical conditions will impact biofiltration in real-world applications. For example, temperature has been shown to directly affect the biodegradation of organics whereby filters exhibit lower microbiological activity at lower temperatures[51,52]. Nutrient concentrations within the input water to the biofilters have also been shown to have significant impact on performance, whereby the removal of carbon is dependent on the availability of other nutrients such as nitrogen and phosphorous[53]. Therefore, it was key to investigate the reduction of bacterial pathogens within a water source that was more applicable to biofilter operation in the real-world. This demonstrated that the biofilters are able to reduce the levels of faecal indicator species (potential pathogens) from an environmentally-derived water source ($p < 0.05$; Table 3). Moreover, the biofilters were able to suppress the growth of heterotrophic bacteria in the environmentally derived water, whereby there was no significant change after 24 h of circulation. However, a significant increase of 4.97 ± 0.14 Log_{10}CFU mL ^{1} was observed when circulated through the empty filter column ($p < 0.05$; Table 3). A similar trend was observed when *P. aeruginosa* was used to challenge the biofilter, whereby a reduction was observed when circulated through the biofilter but an increase of 3.69 ± 0.94 Log_{10}CFU mL ^{1} was observed in the SFW when circulated through the empty filter column (Fig. 1). *P. aeruginosa* is ubiquitous in the environment, and is known for its phenotypic variability and ability to actively grow within aquatic freshwater systems[53]. Therefore, it is likely that the *P. aeruginosa* used in this study was able to survive and grow within the environmentally-derived water. Moreover, it is possible that the reduction in *P. aeruginosa* resulted from a phenotypic switch to a viable but non-culturable (VBNC) state where metabolism is downregulated; however, this is more likely when *P. aeruginosa* is established within biofilms rather than in a planktonic state[54]. The results from this study indicate that a very small sub-population of *P. aeruginosa* was able to survive on the biofilter, even though there were still significant reductions of viable *P. aeruginosa* in the reservoir and on the filter media when compared to the respective controls. The small sub-population of *P. aeruginosa* that was recovered from the biofilter media after 24 h was significantly less than the sterile filter media. This suggests that the environmental biofilm is exerting some inhibitory effect on planktonic *P. aeruginosa*.

The significant reduction in the concentration of nitrogen sources within the environmental water ($p < 0.05$; Table 3) could influence the survival of waterborne pathogens. The nitrification of ammonium is a key part of the global nitrogen cycle that is driven by microorganisms and occurs under aerobic conditions through the following reaction:

$$NH_4^+ \rightarrow NO_2^- \rightarrow NO_3^- \quad (1)$$

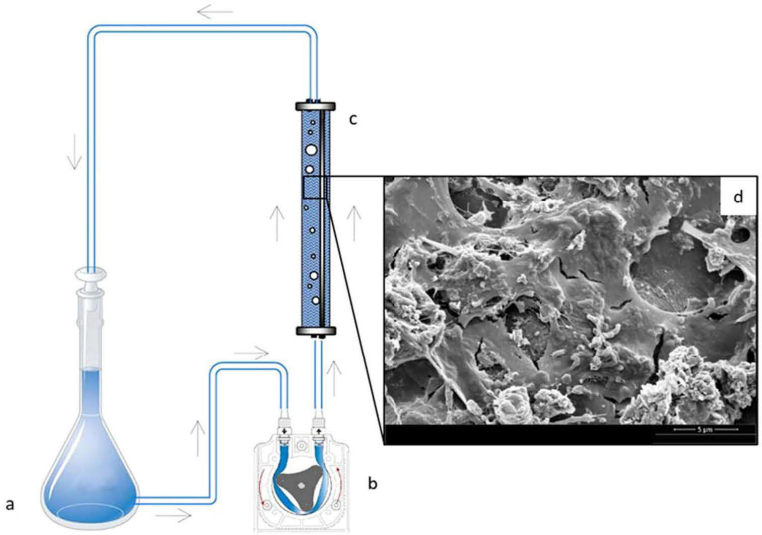


Fig. 2 Schematic of the model biofiltration system for monitoring pathogen survival. Arrows refer to water flow direction. **a** Liquid media bottle containing a simulated fresh water (1 L); **b** Peristaltic pump (35 mL min^{-1}); **c** Biofilter column containing the ceramic filter media on which a biofilm was established and **d** a scanning electron micrograph of the environmental biofilm established on the filter media (scale bar 5 µm).

Further to this, the removal of nitrate (denitrification) occurs under anoxic conditions through the following reaction:

$$NO_3^- \rightarrow N_2 \quad (2)$$

A metabolic interaction that precedes nitrification can occur when nitrite-oxidizing bacteria supply ammonia to ammonia-oxidizers. These interactions rely on the close proximity of cells that exchange metabolites, to enable efficient exchange by diffusion. The reduction of nitrate within this study indicates possible localised anoxic conditions within the biofilter even though the system was vented, therefore it is likely that oxygen diffusion gradients exist within the biofilm attached to the filter media[21]. Such localised diffusion gradients of oxygen within biofilms have been previously described, whereby aerobic microbial metabolism can protect anaerobic organisms deeper within the biofilm[29,55]. Therefore, this could have facilitated the survival of microorganisms that are able to convert nitrate into nitrogen and result in the reductions observed (Table 3). The effect of nutrients including carbon has been shown to impact the growth and survival of planktonic bacteria in water[56,57]. However, there was no significant reduction in carbon within this study (Table 3; $p > 0.05$), and hence the reduction of viable indicator species observed is more likely to have occurred through the direct action of the biofilm on the test species. There was no significant difference in TOC between the biofilter and empty filter column control. However, there was a significant difference in TIC after 24 hours of circulation. It is possible that the TOC increased from cell death which was then mineralised by the biofilters to result in the observed increase in TIC. Moreover, denitrification that was observed in this study has also been shown to be involved with the precipitation of TIC[44]. Therefore, this could be linked with the increase in DIC found in the biofilter systems (Table 3; $p < 0.05$).

Biofilters in a recirculation configuration can be used to manage water quality by reducing waterborne pathogens in stored water facilities. Such management of pathogens will reduce the risk of illness associated with contaminated water to the end users. Current approaches, such as slow sand filtration, have demonstrated effective reductions in viable pathogens such as *E. coli*. However, regrowth of *E. coli* within water storage tanks post slow sand filtration have been identified[58,59]. The use of recirculating biofiltration systems would offer practical solutions to the management of harvested rainwater, stored ground and surface waters. The use of multiple biofilters configured in series, could significantly scale-up the volumes of water that can be treated. Another practical application of this work is the use of recirculation biofiltration systems, in series, to reduce the biological burden of final effluent discharges. Potentially improving the water quality of designated inland bathing waters or catchments used for drinking water supply.

Overall, this study demonstrates that the process of biofiltration creates an unfavourable aquatic environment for the survival of the planktonic test pathogens and environmental indicator species investigated. However, the specific mechanisms that resulted in a reduction of the viability of waterborne bacterial pathogens are not yet known and further research is necessary. We clearly demonstrate that environmental biofilms significantly reduce the viability of waterborne pathogenic bacteria; however, the following points are critical to further elucidate and optimise this process:

1. Further research into the mechanism of action by which the biofilter is reducing the number of viable waterborne pathogens.
2. Characterisation of the longevity of pathogen removal, including study of potential VBNC bacteria within the system.
3. Investigation of biofilter microbial diversity through functional metagenomics.
4. The upscale of laboratory biofilters to test their performance within a community scale system.
5. Investigation of process parameters, such as contact time, and their impact on pathogen reduction.

The competition between the biofilm established on the filter media and the test pathogens, is a key factor for the reduction observed (Fig. 1 and Table 2). Species groups that are involved in the killing mechanisms within the biofilm could be investigated through the use of functional metagenomics, searching for the potential production of compounds that could inhibit the waterborne bacterial pathogens such as bacteriocins, antibiotics and toxins.

In relation to the removal of organic matter, the function and stability of biofilters has been previously studied through investigating the effect of dosing matured biofilters with nutrients, including ammonium (NH_4^+) and phosphate (PO_4^{3-})[22]. These studies concluded that the addition of PO_4^{3-} plays an important role in enhancing the removal of biodegradable dissolved organic carbon. Moreover, it has been shown that in drinking water systems, PO_4^{3-} is the critical limiting factor for limiting or promoting microbial growth[60,61]. Therefore, the effect of nutrient concentrations on the performance of biofilters in relation to the reduction of waterborne pathogens warrants further investigation. Nutrient deprivation within the biofilter could compromise one of the main mechanisms of pathogen control. Further work is required to investigate this, including the use of upscaled biofilter systems. This would serve to maintain the control of nutrients within the treated water, ultimately enhancing the control of potential pathogens.

The concept of controlling pathogens using biofilters without the aid of chemical or UV disinfection is a challenging one. Even so, this study demonstrates, under laboratory conditions, that biofilters can significantly reduce the levels of *E. coli*, enterococci (including *E. faecalis*) and *P. aeruginosa* within a fresh water source. Currently, for water to be biologically safe, the level of *E. coli* and enterococci (indicator species) must be 0 CFU 100 mL^{-1}. This study has demonstrated that biofiltration was able to reduce the viability of test pathogens and bacterial indicator species to very low levels, however this would not be classified as potable water. Nonetheless, the reduction of the indicator species observed in this study may be significant to reduce the risk of illness. The global effort into research and development on new approaches of drinking water treatment for developing communities and the remediation of emerging contaminants is crucial to progress towards achieving the 2030 United Nations (UN) Sustainable Development Goals (SDGs)[62]. This approach has the potential to significantly impact on the delivery of the sustainable development goal number 6: clean water and sanitation for all. Moreover, this would significantly reduce the load on other treatment methods such as UV water clarifiers that consume low levels of energy to reduce the remainder of the indicator species to potable water standards. Therefore, biofilters could be used as the main source of water treatment for low technology solutions for improving access to water free from biological contamination.

METHODS

Setup and the maturation of test biofiltration system

The laboratory test biofilters comprised a 500 mL PVC column (63 mm internal diameter, 160 mm length) containing 250 mL of ceramic filter media. The ceramic filter media had a particle size and density of 20 ± 1 mm and 1.16 g mL^{-1} respectively, and was sterilised by autoclaving before use. The resulting bed porosity was determined using methods outlined by McKie et al.[24] and calculated as 2.99. The filter columns containing filter media were connected to circulation tanks (25 L) and were allowed to mature over a period of 4 weeks to establish and achieve mature biofilters. Prior to the maturation process, the circulation tanks were filled with mains tap water and flocculated of residual chlorine via aeration for 24 h (termed recirculation water). Water taken from environmentally-derived surface water held in an urban drainage pond (N 51°29′56″, W 2°32′39″) (250 mL) was then added to inoculate the tanks with a source of environmental microorganisms to provide a seed community for the formation of biofilm on the filter media. At the start of the maturation process, a flow rate of 35 mL min^{-1} through the filter column was maintained via a multi-channel peristaltic pump (Watson-Marlow 505 S; Falmouth, UK). This ensured that the entire volume of water in the tank was recirculated through the filter column for every 12 h of operation. To further assist the maturation of the biofilm on the filter media, the recirculation water was supplemented with low levels of nutrients on a weekly basis equating to humic acids (50 mg week^{-1}) and NH_4CL (5 mg week^{-1}), over the four-week maturation period. The 4-week maturation period was based on experimental demonstration of the stabilisation of biofilm function, quantified by efficient removal of a fixed ammonium load over a 24 h period (in the form of 0.2 mg L^{-1} ammonium chloride). In addition, for biofilm maturation and preparation, an increase in the number of viable heterotrophic microorganisms present on the filter media after 4 weeks of circulation was also confirmed experimentally. Humic acids were extracted from the soil riparian zone surrounding the aforementioned environmental water source using a method outlined by Serra and Schnitzer.[63]

Bacterial strains and culture maintenance

The following bacterial strains were chosen because of their relevance as biological indicators of water quality and their potential to cause disease: *Escherichia coli* NCTC 10418, *Enterococcus faecalis* NCTC 12679 and *Pseudomonas aeruginosa* NCIMB 8295. Bacterial strains were isolated and maintained on cryopreservation beads (Microbank, Pro Lab Diagnostics, Canada) at −80 °C, and recovered by plating on nutrient agar (NA; Oxoid™, UK) as required.

Preparation of test pathogen monocultures

Overnight batch cultures of bacterial strains were prepared in 10 mL of nutrient broth (CM0067; Oxoid™, UK) for *E. coli* and *P. aeruginosa* and brain heart infusion broth (CM1135; Oxoid™, UK) for *E. feacalis* and incubated for 16–18 h at 37 °C in an orbital shaker (150 rpm) incubator. Cells were then centrifuged (5 min at 10,000 × *g*), washed twice and resuspended in 3 L of sterile SFW[64].

Bacterial challenge of test biofiltration system

Prior to testing, matured biofilters were disconnected from the circulation tanks, drained and re-connected to a double ported flask (3 × 1 L) containing the test pathogen challenge monoculture (Fig. 2). The test pathogens were circulated through the biofilter at a flow rate of 35 mL min^{-1}. To quantify the reduction of the test pathogens within the SFW, viable counts of *E. coli, E. faecalis and P. aeruginosa* were recorded over a 24 h duration when individually cycled through the biofilter and control systems. The biofilters were operated in circulation mode to monitor the efficiency of the biofilters to reduce a fixed microbiological load of the test pathogen monoculture and enable the direct comparison between experimental configurations and pathogen species. The SFW was sampled regularly over a 24-hour period; a 10 mL aliquot was taken from the SFW and enumerated using membrane filtration and plating onto selective and differential agar (*E. faecalis*; Slanetz and Bartley, Oxoid™, Basingstoke, UK. *E. coli;* Membrane Lactose Glucuronide Agar, Oxoid™, Basingstoke, UK. *P. aeruginosa*; Pseudomonas C-N Selective Agar, Oxoid™, Basingstoke, UK). A control filter was employed containing sterile ceramic filter media that was pre-soaked with SFW and drained prior to use. An additional control, consisting of an empty 500 mL plastic filter column was used to account for any pathogen reduction resulting from the experimental apparatus. For each of the experimental configurations (empty filter column; column containing sterile filter media; biofilter) three separate experiments were undertaken in parallel. This process was then repeated on three independent occasions (9 filters in total). Within each independent experimental run, all the microbiological sampling (and associated viable counting) was determined in triplicate.

The presence and viability of pathogens on the filter media

To determine if there was any accumulation or retention of the test pathogens on the biofilter media or sterile filter media following the 24 h circulation (Fig. 1), viable counts of *E. coli, E. faecalis* and *P. aeruginosa* were taken from the filter media within the filter columns (Fig. 2c). The filter media was removed from the column and suspended in 200 mL of phosphate buffered saline (PBS; Oxoid™, Basingstoke, UK), sonicated for 30 s and vortexed for 30 s (repeated 3 times). Aliquots of the suspension were enumerated using membrane filtration and plated onto selective and

differential agar (see section Bacterial challenge of test biofiltration system) to determine the number of test pathogens recovered per filter column.

Environmentally-derived surface water challenge of mature test biofiltration system

To further investigate the control of potential waterborne pathogens under conditions more representative to that found in the environment, environmentally-derived surface water was taken and used (section Setup and the maturation of test biofiltration system) to circulate through the biofilters for 24 h. Following the same preparation procedure outlined in section Bacterial challenge of test biofiltration system, the biofilters were connected to a double ported flask containing water taken from an environmental source known to be contain environmental *E. coli* and enterococci. Standard biological and physiochemical water quality indicators included in this study: *E. coli*, enterococci, heterotrophic plate counts (HPCs), ammonium, nitrite, nitrate were taken of the environmental water before and after 24 h circulation. For each of the experimental configurations (empty filter column and biofilters), six separate experiments were operated in parallel. Within each independent experimental run, all the microbiological sampling (and associated viable counting) and the physiochemical parameters were determined in triplicate.

An empty filter column was employed as a control to account for any change in the environmental water that was independent of the biofilter after 24 h of circulation.

Scanning electron microscopy

Established biofilms grown on the filter media were visualised using scanning electron microscopy (SEM). The biofilter media was fixed in 4% glutaraldehyde for 1 h, followed by three 1 h washes in 0.1 M phosphate buffer. Following fixation, the biofilter media was dehydrated by a series of 5 min washes in 30–100% ethanol and then a series of hexamethyldisilane washes with 100% ethanol. Each individual sample was mounted onto a stainless-steel stub, gold splutter coated and inserted into a stub holder on the cooler stage. The SEM (FEI Quanta FEG 650) used the Everhart Thornley detector for all imaging. The biofilm sample was analysed with an acceleration voltage of 5.00 kV and spot size 3.0.

Ion chromatography

Ion Chromatography (Metro HM, 850 Professional IC Anion) was used to quantify the level of anions and cations of relevant nutrients with regards to water quality and microbial growth; phosphate (PO_4^{3-}), nitrite (NO_2^-), nitrate (NO_3^-) and ammonium (NH_4^+). Samples were taken from the SFW and were filtered through a 0.2 µm filter immediately after collection to prevent any further microbial processing. Aqueous samples were then loaded onto an auto sampler in open top tubes and auto-injected. For anion analysis, a sodium carbonate (3.2 mM L^{-1}) and sodium bicarbonate (1.0 mM L^{-1}) mobile phase was used throughout. Background conductivity of the mobile phase was suppressed by a cation exchanger and regenerated using a dilute sulphuric acid (150 mM L^{-1}) and oxalic acid (100 mM L^{-1}) solution. For cations a nitric acid (0.7 mM L^{-1}) and dipicolonic acid (1.7 mM L^{-1}) mobile phase was used. IC certified standards (Fisher scientific) were used to generate standard curves from which the water samples were interpolated.

Total Carbon analysis

Total Carbon analysis (EnviroTOC, Elementar), was used to quantify the level total inorganic carbon (TIC) and total organic carbon (TOC). Samples were taken from the environmentally-derived surface water and were filtered through a 0.45 µm filter immediately after collection. Aqueous samples were then loaded onto an auto sampler in open top tubes and auto-injected.

Statistical analysis

Statistical analysis of all data was conducted using GraphPad Prism version 9.2.0 for Windows (GraphPad Software, San Diego, CA, USA). T-tests and two-way analysis of variance (ANOVA) with Tukey's post-hoc test were used where appropriate, with $p < 0.05$ regarded as significant.

DATA AVAILABILITY

All of the data generated and/or analysed during this study is available from the corresponding author upon reasonable request.

Received: 24 November 2021; Accepted: 10 June 2022;
Published online: 12 July 2022

REFERENCES

1. Holland, R. A. et al. Global impacts of energy demand on the freshwater resources of nations. *Proc. Natl Acad. Sci. U.S.A.* **112**, E6707–E6716 (2015).
2. Gleick, P. H. & Palaniappan, M. Peak water limits to freshwater withdrawal and use. *Proc. Natl Acad. Sci. U.S.A.* **107**, 11155–11162 (2010).
3. Oki, T. & Kanae, S. Global hydrological cycles and world water resources. *Science.* **313**, 1068–1072 (2006).
4. Postel, S. L., Daily, G. C. & Ehrlich, P. R. Human Appropriation of Renewable Fresh Water. *Science.* **271**, 785–788 (1996).
5. United Nations Children's Fund (UNICEF) & World Health Organization (WHO). Progress on household drinking water, sanitation and hygiene 2000-2017. Special focus on inequalities. https://www.unicef.org/media/55276/file/Progress on drinking water, sanitation and hygiene 2019.pdf (2019).
6. Prüss-Ustün, A. et al. Burden of disease from inadequate water, sanitation and hygiene for selected adverse health outcomes: An updated analysis with a focus on low- and middle-income countries. *Int. J. Hyg. Environ. Health* **222**, 765 (2019).
7. Caprioli, A., Morabito, S., Bruégre, H. & Oswald, E. Enterohaemorrhagic Escherichia coli: emerging issues on virulence and modes of transmission. *Vet. Res.* **36**, 289–311 (2005).
8. Vital, M., Fuchslin, H. P., Hammes, F. & Egli, T. Growth of Vibrio cholerae O1 Ogawa Eltor in freshwater. *Microbiology* **153**, 1993–2001 (2007).
9. Agudelo Higuita, N. I. & Huycke, M. M. Enterococcal Disease, Epidemiology, and Implications for Treatment. in Enterococci: From Commensals to Leading Causes of Drug Resistant Infection 47–72 (Massachusetts Eye and Ear Infirmary, 2014).
10. Paton, J. C. & Paton, A. W. Pathogenesis and diagnosis of Shiga toxin-producing Escherichia coli infections. *Clin. Microbiol. Rev.* **11**, 450–479 (1998).
11. Bellamy, W. D., Silverman, G. P., Hendricks, D. W. & Logsdon, G. S. Removing Giardia cysts with slow sand filtration. *J. Am. Water Works Assoc.* **77**, 52–60 (1985).
12. Fogel, D., Isaac-Renton, J., Guasparini, R., Moorehead, W. & Removing, O. J. giardia and cryptosporidium by slow sand filtration. *JAWWA, Res. Technol.* **3**, 77–84 (1993).
13. Hijnen, W. A. M., Schijven, J. F., Bonné, P., Visser, A. & Medema, G. J. Elimination of viruses, bacteria and protozoan oocysts by slow sand filtration. *Water Sci. Technol.* **50**, 147–154 (2004).
14. Campos, L. C., Su, M. F. J., Graham, N. J. D. & Smith, S. R. Biomass development in slow sand filters. *Water Res.* **36**, 4543–4551 (2002).
15. Basu, O. D., Dhawan, S. & Black, K. Applications of biofiltration in drinking water treatment - a review. *J. Chem. Technol. Biotechnol.* **91**, 585–595 (2016).
16. Terry, L. G. & Summers, R. S. Biodegradable organic matter and rapid-rate biofilter performance: A review. *Water Res.* **128**, 234–245 (2018).
17. Loh, Z. Z. et al. Shifting from conventional to organic filter media in wastewater biofiltration treatment: a review. *Appl. Sci. 2021, Vol. 11, Page 8650* **11**, 8650 (2021).
18. Bennett, A. Drinking water: Pathogen removal from water – technologies and techniques. *Filtr. Sep.* **45**, 14–16 (2008).
19. Di Cristo, C., Esposito, G. & Leopardi, A. Modelling trihalomethanes formation in water supply systems. *Environ. Technol.* **34**, 61–70 (2013).
20. Pooi, C. K. & Ng, H. Y. Review of low-cost point-of-use water treatment systems for developing communities. *npj Clean Water 2018 1* **1**, 11 (2018).
21. Flemming, H.-C. et al. Biofilms: an emergent form of bacterial life. *Nat. Rev. Microbiol.* **14**, 563–575 (2016).
22. Fu, J. et al. Pilot investigation of two-stage biofiltration for removal of natural organic matter in drinking water treatment. *Chemosphere* **166**, 311–322 (2017).
23. Chen, F. et al. Kinetics of natural organic matter (NOM) removal during drinking water biofiltration using different NOM characterization approaches. *Water Res.* **104**, 361–370 (2016).
24. McKie, M. J., Ziv-El, M. C., Taylor-Edmonds, L., Andrews, R. C. & Kirisits, M. J. Biofilter scaling procedures for organics removal: A potential alternative to piloting. *Water Res.* **151**, 87–97 (2019).
25. de Vries, J. Soil filtration of wastewater effluent and the mechanism of pore clogging. *J. Water Pollut. Control Fed.* **44**, 565–573 (1972).
26. Métivier, R., Bourven, I., Labanowski, J. & Guibaud, G. Interaction of erythromycin ethylsuccinate and acetaminophen with protein fraction of extracellular polymeric substances (EPS) from various bacterial aggregates. *Environ. Sci. Pollut. Res.* **20**, 7275–7285 (2013).

27. Writer, J. H., Barber, L. B., Ryan, J. N. & Bradley, P. M. Biodegradation and attenuation of steroidal hormones and alkylphenols by stream biofilms and sediments. *Environ. Sci. Technol.* **45**, 4370–4376 (2011).
28. Flemming, H.-C. Biofilms. in Encyclopedia of Life Sciences (John Wiley & Sons, Ltd, 2008). https://doi.org/10.1002/9780470015902.a0000342.pub2.
29. Kragh, K. N. et al. Role of multicellular aggregates in biofilm formation. *MBio* **7**, e00237 (2016).
30. Grumbein, S., Opitz, M. & Lieleg, O. Selected metal ions protect Bacillus subtilis biofilms from erosion †. *Metallomics* **6**, 1441 (2014).
31. Fu, J. et al. Removal of pharmaceuticals and personal care products by two-stage biofiltration for drinking water treatment. *Sci. Total Environ.* **664**, 240–248 (2019).
32. Nemani, V. A., McKie, M. J., Taylor-Edmonds, L. & Andrews, R. C. Impact of biofilter operation on microbial community structure and performance. *J. Water Process Eng.* **24**, 35–41 (2018).
33. Beutel, M. W. & Larson, L. Pathogen removal from urban pond outflow using rock biofilters. *Ecol. Eng.* **78**, 72–78 (2014).
34. Wendt, C. et al. Microbial removals by a novel biofilter water treatment system. *Am. J. Trop. Med. Hyg.* **92**, 765–772 (2015).
35. Granger, H. C., Stoddart, A. K. & Gagnon, G. A. Direct biofiltration for manganese removal from surface water. *J. Environ. Eng.* **140**, 04014006 (2014).
36. Srivastava, N. K. & Majumder, C. B. Novel biofiltration methods for the treatment of heavy metals from industrial wastewater. *J. Hazard. Mater.* **151**, 1–8 (2008).
37. Fu, J. et al. Removal of disinfection byproduct (DBP) precursors in water by two-stage biofiltration treatment. *Water Res.* **123**, 224–235 (2017).
38. McKie, M. J., Andrews, S. A. & Andrews, R. C. Conventional drinking water treatment and direct biofiltration for the removal of pharmaceuticals and artificial sweeteners: A pilot-scale approach. *Sci. Total Environ.* **544**, 10–17 (2016).
39. Crognale, S. et al. Biological As(III) oxidation in biofilters by using native groundwater microorganisms. *Sci. Total Environ.* **651**, 93–102 (2019).
40. Klayman, B. J., Volden, P. A., Stewart, P. S. & Camper, A. K. Escherichia coli O157:H7 requires colonizing partner to adhere and persist in a capillary flow cell. *Environ. Sci. Technol.* **43**, 2105–2111 (2009).
41. Bauman, W. J., Nocker, A., Jones, W. L. & Camper, A. K. Retention of a model pathogen in a porous media biofilm. *Biofouling* **25**, 229–240 (2009).
42. Nocker, A., Burr, M. & Camper, A. Pathogens in water and biofilms. In Microbiology of waterborne diseases: microbiological aspects and risks: Second Edition 3–32 (Academic Press, 2013). https://doi.org/10.1016/B978-0-12-415846-7.00001-9.
43. Li, J., McLellan, S. & Ogawa, S. Accumulation and fate of green fluorescent labeled Escherichia coli in laboratory-scale drinking water biofilters. *Water Res.* **40**, 3023–3028 (2006).
44. Rendueles, O. & Ghigo, J.-M. Mechanisms of competition in biofilm communities. *Microbiol. Spectr.* **3**, 1–14 (2015).
45. Hibbing, M. E., Fuqua, C., Parsek, M. R. & Peterson, S. B. Bacterial competition: Surviving and thriving in the microbial jungle. *Nat. Rev. Microbiol.* **8**, 15–25 (2010).
46. Aoki, S. K. et al. A widespread family of polymorphic contact-dependent toxin delivery systems in bacteria. *Nature* **468**, 439–442 (2010).
47. MacIntyre, D. L., Miyata, S. T., Kitaoka, M. & Pukatzki, S. The Vibrio cholerae type VI secretion system displays antimicrobial properties. *Proc. Natl Acad. Sci. U.S.A.* **107**, 19520–19524 (2010).
48. Ławniczak, Ł., Marecik, R. & Chrzanowski, Ł. Contributions of biosurfactants to natural or induced bioremediation. *Appl. Microbiol. Biotechnol.* **97**, 2327 (2013).
49. Cornforth, D. M. & Foster, K. R. Competition sensing: the social side of bacterial stress responses. *Nat. Rev. Microbiol. 2013 114* **11**, 285–293 (2013).
50. Legnani, P., Leoni, E., Rapuano, S., Turin, D. & Valenti, C. Survival and growth of Pseudomonas aeruginosa in natural mineral water: a 5-year study. *Int. J. Food Microbiol.* **53**, 153–158 (1999).
51. Moll, D. M., Summers, R. S., Fonseca, A. C. & Matheis, W. Impact of temperature on drinking water biofilter performance and microbial community structure. *Environ. Sci. Technol.* **33**, 2377–2382 (1999).
52. Hozalski, R. M., Bouwer, E. J. & Goel, S. Removal of natural organic matter (NOM) from drinking water supplies by ozone-biofiltration. *Water Sci. Technol.* **40**, 157–163 (1999).
53. Schmidt, K. D., Tümmler, B. & Römling, U. Comparative genome mapping of Pseudomonas aeruginosa PAO with P. aeruginosa C, which belongs to a major clone in cystic fibrosis patients and aquatic habitats. *J. Bacteriol.* **178**, 85 (1996).
54. Nigaud, Y. et al. Biofilm-induced modifications in the proteome of Pseudomonas aeruginosa planktonic cells. *Biochim. Biophys. Acta - Proteins Proteom.* **1804**, 957–966 (2010).
55. Von Ohle, C. et al. Real-time microsensor measurement of local metabolic activities in ex vivo dental biofilms exposed to sucrose and treated with chlorhexidine. *Appl. Environ. Microbiol.* **76**, 2326 (2010).
56. Nescerecka, A., Juhna, T. & Hammes, F. Identifying the underlying causes of biological instability in a full-scale drinking water supply system. *Water Res.* **135**, 11–21 (2018).
57. Prest, E. I., Hammes, F., Kötzsch, S., Van Loosdrecht, M. C. M. & Vrouwenvelder, J. S. A systematic approach for the assessment of bacterial growth-controlling factors linked to biological stability of drinking water in distribution systems. *Water Sci. Technol. Water Supply* **16**, 865–880 (2016).
58. Liang, K., Sobsey, M. & Stauber, C. E. Improving Household Drinking Water Quality: Use of Biosand Filter in Cambodia. https://scholarworks.gsu.edu/iph_facpub (2010).
59. Fabiszewski De Aceituno, A. M., Stauber, C. E., Walters, A. R., Meza Sanchez, R. E. & Sobsey, M. D. A randomized controlled trial of the plastic-housing biosand filter and its impact on diarrheal disease in Copan, Honduras. *Am. J. Trop. Med. Hyg.* **86**, 913–921 (2012).
60. Miettinen, I. T., Vartiainen, T. & Martikainen, P. J. Phosphorus and bacterial growth in drinking water. *Appl. Environ. Microbiol.* **63**, 3242–3245 (1997).
61. Keinänen, M. M. et al. The microbial community structure of drinking water biofilms can be affected by phosphorus availability. *Appl. Environ. Microbiol.* **68**, 434–439 (2002).
62. United Nations (UN). Transforming Our World: The 2030 Agenda for Sustainable Development. in A New Era in Global Health 529–567, https://doi.org/10.1891/9780826190123.ap02 (2018).
63. Serra, M. O. D. E. & Schnitzer, M. Extraction of humic acid by alkali and chelating resin. *Can. J. Soil Sci.* **52**, 365–374 (1972).
64. Smith, E. J., Davison, W. & Hamilton-Taylor, J. Methods for preparing synthetic freshwaters. *Water Res.* **36**, 1286–1296 (2002).
65. Sobsey, M. D. Managing Water in the Home: Accelerated Health Gains from Improved Water Supply Water, Sanitation and Health Department of Protection of the Human Environment World Health Organization Geneva. https://apps.who.int/iris/bitstream/handle/10665/67319/WHO_SDE_WSH_02.07.pdf?sequence=1&isAllowed=y (2002).
66. Carratalà, A. et al. Solar disinfection of viruses in polyethylene terephthalate bottles. *Appl. Environ. Microbiol.* **82**, 279–288 (2016).
67. Attisani, M. Can solar technology generate clean water for developing nations? *Renew. Energy Focus* **17**, 138–139 (2016).
68. Chaidez, C. et al. Point-of-use Unit Based on Gravity Ultrafiltration Removes Waterborne Gastrointestinal Pathogens from Untreated Water Sources in Rural Communities. *Wilderness Environ. Med.* **27**, 379–385 (2016).
69. Clayton, G. E., Thorn, R. M. S. & Reynolds, D. M. Development of a novel off-grid drinking water production system integrating electrochemically activated solutions and ultrafiltration membranes. *J. Water Process Eng.* **30**, 100480 (2017).
70. Baig, S. A., Mahmood, Q., Nawab, B., Shafqat, M. N. & Pervez, A. Improvement of drinking water quality by using plant biomass through household biosand filter - A decentralized approach. *Ecol. Eng.* **37**, 1842–1848 (2011).

ACKNOWLEDGEMENTS

Andrew Cox for providing technical advice. This research was funded by UWE, Bristol, and Origin Aqua Technologies as part of a partnership Ph.D. and partially supported by the Natural Environment Research Council, UK [NE/R003106/1].

AUTHOR CONTRIBUTIONS

J.A.C.S.: experimental method development, experimental work, data collection, and analysis, manuscript preparation and writing, review and editing. R.M.S.: funding acquisition, experimental conceptualisation and development, data analysis, project supervision and manuscript review and editing. G.M.R.: project supervision, data analysis and manuscript review and editing. D.T.: project supervision, data analysis, and manuscript review and editing. J.E.L.: experimental conceptualisation, manuscript review and editing. D.M.R.: funding acquisition, experimental conceptualisation and development, data analysis, project supervision and manuscript review and editing.

COMPETING INTERESTS

The authors declare no competing interests.

ADDITIONAL INFORMATION

Correspondence and requests for materials should be addressed to Darren M. Reynolds.

Reprints and permission information is available at http://www.nature.com/reprints

Publisher's note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Open Access This article is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons license, and indicate if changes were made. The images or other third party material in this article are included in the article's Creative Commons license, unless indicated otherwise in a credit line to the material. If material is not included in the article's Creative Commons license and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this license, visit http://creativecommons.org/licenses/by/4.0/.

© The Author(s) 2022

Appendix II Supplementary material

S1: Table of DNA yield from biofilm samples.

Sample Number	ID	DNA Concentration (ng μL^{-1})	Sample Volume (µL)	Sample Number	ID	DNA Concentration (ng μL^{-1})	Sample Volume (µL)
1	PD1	81.6	48	29	US1B-W3	60	20
2	PD2	57.6	48	30	US2B-W3	4.3	20
3	PD3	12	48	31	US3B-W3	3.65	20
4	LS1-W1	5.94	20	32	LS1-W4	24	20
5	LS2-W1	4.87	20	33	LS2-W4	15.9	20
6	LS3-W1	5.69	20	34	LS3-W4	13.8	20
7	US1T-W1	2.01	20	35	US1T-W4	4.83	20
8	US2T-W1	2.2	20	36	US2T-W4	21.9	20
9	US3T-W1	3.11	20	37	US3T-W4	10.3	20
10	US1B-W1	3.26	20	38	US1B-W4	3.92	20
11	US2B-W1	2.5	20	39	US2B-W4	43.9	20
12	US3B-W1	2.34	20	40	US3B-W4	59.5	20
13	LS1-W2	40.9	20	41	LS1-M3	14.5	45
14	LS2-W2	24.2	20	42	LS2-M3	13.4	45
15	LS3-W2	64.8	20	43	LS3-M3	2.4	45
16	US1T-W2	29.7	20	44	US1T-M3	8.32	45
17	US2T-W2	54.8	20	45	US2T-M3	4.44	45
18	US3T-W2	16	20	46	US3T-M3	4.64	45
19	US1B-W2	9.15	20	47	US1B-M3	19.9	45
20	US2B-W2	72.1	20	48	US2B-M3	3.1	45
21	US3B-W2	28	20	49	US3B-M3	8.8	45
22	PD4	18.5	40	50	Site 4	4.65	45
23	LS1-W3	40.9	20	51	Site 3	328	45
24	LS2-W3	10.2	20	52	Site 5	26	45
25	LS3-W3	18.6	20	53	Site 1	137	45
26	US1T-W3	41.7	20	54	Site 2	296	45
27	US2T-W3	4.55	20	55	Site 6	78	45
28	US3T-W3	7.67	20				

S2: DADA2 parametric error model: Points are the observed error rates for each consensus quality score. The black line shows the estimated error rates after convergence of the machine-learning algorithm. The red line shows the error rates expected under the nominal definition of the Q-score. Here the estimated error rates (black line) are a good fit to the observed rates (points).