
The University Of Sheffield.

# The Effects of Climate Change on Microbial Ecology and Water Quality of Drinking Water Distribution Systems

Carolina Calero Preciado

Thesis submitted for the degree of

Doctor of Philosophy

The University of Sheffield

Faculty of Engineering

Department of Civil and Structural Engineering

October 2020

# Abstract

Microorganisms inhabiting drinking water distribution systems (DWDS), particularly those forming biofilms attached to pipe surfaces, play a key role in determining the quality and safety of the final tap water supplied to consumers. Climate change is producing alterations in environmental conditions that can affect these systems and therefore its diverse microbiome, thus it may reduce the water quality and/or availability and accessibility. This may compromise the distribution of safe drinking water, resulting in detrimental consequences for public sanitation and human society.

In this research the effect of different processes to which DWDS are susceptible as a result of climate change has been investigated. A unique full-scale DWDS facility at the University of Sheffield, which allow for the study of both biofilm and planktonic communities, was used to simulate realistic and controlled conditions. Advanced molecular techniques combined with the characterisation of key water physico-chemical parameters were applied to obtain a complete overview of what happened in the systems under different experimental conditions. The first part of this work evaluated the impacts of increasing temperature as a consequence of global warming. For this, biofilm was grown at different temperatures in the test loop facility. The study of biofilm mobilisation events showed that under high temperatures there was an increased risk of water discoloration and metals release into the bulk water. In addition, temperature was demonstrated to be a key factor shaping the microbiome of DWDS. The second part of the research studied the effect of temperature increase on biofilm control strategies: flushing of pipes followed by hyperchlorination, only flushing and without mechanical or chemical treatment. This study demonstrated that the increase of water discolouration at higher temperatures when the biofilm is mobilised depended on the biofilm control strategy applied. Furthermore, the management strategy applied was observed to be critical in determining biofilm community structure and composition, while planktonic communities were less affected by temperature increase and/or management strategy. Finally, experiments were carried out to understand how different intermittent water supply (IWS) times (6 hours, 48 hours and 6 days) impact on the microbiome of DWDS. This study evidenced that different IWS times affect in different way several physico-chemical parameters, including the discolouration response during the supply restarting. Similarity, microbiological analyses showed how biofilm experience structural and/or compositional changes during different IWS events and that planktonic communities were affected when supply was restarted.

This research has improved the understanding of how different processes in relation to climate change can modify DWDS conditions and affect drinking water quality and safety. Results have provided new and valuable understanding that can be useful to adapt and mitigate the risks associated to climate change in DWDS and to protect the quality and safety of drinking water. In addition, it has highlighted the necessity of including biofilm monitoring and microbial culture-independent methods to generate more accurate microbial information and get an extensive view of what happens in the pipeline environment.

# Table of contents

# Acknowledgments

The research reported here was supported by the UK Engineering and Physical Sciences Research Council (EPSRC), EPSRC-LWEC Challenge Fellowship EP/N02950X/1. The laboratory work was performed at the NERC Biomolecular Analysis Facility at the University of Sheffield and supported by the Natural Environment Research Council, UK.

I would like to express my gratitude to my supervisors Dr. Isabel Douterelo, Prof. Joby Boxall and Dr. Víctor Soria Carrasco. Thank you for giving me the opportunity and for all your help, patience, support and guidance throughout this time. It had been a great privilege to learn from all of you about disciplines as different as microbiology, engineering and bioinformatics. Especially thanks to Isabel for having been more than just an ordinary supervisor. You have been the best transmitting your passion for science.

I am also extremely grateful to the people who have helped me during this time. Infinite thanks to Dr. Stewart Husband for always being available to answer my questions about turbidity stuff, for helping me in the RIG lab and for all the field trips in which I have learned so much about flushing trials and real drinking water distribution systems. Soledad Martínez, Gonzalo del Olmo, Esther Rosales and Berte Mekonen, it has been a pleasure learning together and having had a great time in and out of the lab.

Finally, I would like to thank my family and friends for all the support from the beginning to the end of this “adventure book”. Thank you for always being there, even when I was thousands of miles away. Thanks to my parents, my sister, and my brother, for your unconditional support in everything, for believing in me, and giving me encouragement when I needed it. Thank you, Mario, for always being with me, you are my perfect travel partner.

Gracias,

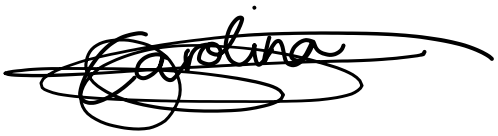
Carolina

# Declaration of Authorship

I, Carolina Calero Preciado, hereby declare that I am the sole author of this thesis titled “The Effects of Climate Change on Microbial Ecology and Water Quality of Drinking Water Distribution Systems” and that the contents of this research are result of my own work. I confirm that:

- The dissertation is original and has not been submitted for any other degree, diploma or other qualifications to this university or any other institution.
- This work was done wholly while in candidature for a research degree at this University.
- Where I have consulted the published work of others, this is always clearly attributed.
- Where I have quoted from the work of others, the source is always given. With the exception of such quotations, this thesis is entirely my own work.
- I have acknowledged all main sources of help.
- Where the thesis is based on work done by myself jointly with others, I have made clear exactly what was done by others and what I have contributed myself.

October 2020,



Carolina Calero Preciado

# Abbreviations and nomenclature

| | |
|---|---|
| % | Percentage |
| °C | Degrees Celsius |
| 2D | 2 dimensions |
| 3D | 3 dimensions |
| A | Adenine, nucleobase |
| ANOSIM | Analysis of similarities |
| AOB | Ammonia oxidising bacteria |
| AOC | Assimilable organic carbon |
| Bq | Becquerel |
| bs | Base pairs |
| BSA | Bovine serum albumin |
| C | Cytosine, nucleobase |
| cm | Centimetre |
| $C_T$ | Cycle threshold |
| CTAB | Hexadecyltmethyl ammonium bromide |
| DBP | Disinfection by product |
| DMA | District management area |
| DNA | Deoxyribonucleic acid |
| dNTP | Deoxyribonucleoside triphosphate |
| DO | Dissolved oxygen |
| DOC | Dissolved organic carbon |
| DWDS | Drinking water distribution system |
| e.g. | Exempli gratia, for example |
| EDTA | Ethylenediaminetetraacetic acid |
| Em | Emission |
| EPA | Environmental Protection Agency |
| EPS | Extracellular polymeric substances |
| *et al.* | And others |
| EU | European Union |
| Ex | Excitation |
| FCM | Flow cytometry |
| Fe | Iron |
| G | Guanine, nucleobase |
| Global R | Statistic from ANOSIM test (0 means no difference, and 1 completely different) |
| h | Hours |
| HDPE | High density polyethylene |
| HPC | High Performance Computing |
| i.e. | *Id est*, namely |
| ICC | Intact cell counts |
| ICP-MS | Inductively Coupled Plasma Mass Spectrometry |
| IPCC | Intergovernmental panel on climate change |
| ITS | Internal transcribed spacer |
| IWS | Intermittent water supply |
| KRI | Kroto Research Institute |

| | |
|---|---|
| L | Litre |
| LVF | Low varied flow profile |
| m | Metre |
| M | Molar |
| $m^2$ | Square metre |
| $m^3$ | Cubic metre |
| MAG | Magnification |
| MDPE | Medium density polyethylene |
| mg | Milligrams |
| MIC | Microbiologically influenced corrosion |
| Min | Minutes |
| mL | Mililitres |
| mm | Millimetres |
| $mm^2$ | Square millimetre |
| Mn | Manganese |
| MSCBS | The Ministry of Health of the Government of Spain |
| mW | milliwatt |
| N | Newton |
| n | Number of samples/replicates |
| NGS | Next Generation Sequencing |
| nm | Nanometres |
| nMDS | Non-metric multi-dimensional scaling |
| NOM | Natural Organic Matter |
| nrDNA | Nuclear ribosomal DNA |
| ns | Nanoseconds |
| nt | Nucleotide |
| NTM | Nontuberculous mycobacteria |
| NTU | Nephelometric Turbidity Unit |
| OP | Opportunistic pathogen |
| OTU | Operational Taxonomic Unit |
| p-value | Statistic showing significance |
| PBS | Phosphate buffer solution |
| PCR | Polymerase chain reaction |
| PI | Propidium iodide |
| POC | Particulate organic carbon |
| PODDS | Discolouration in Distribution Systems model |
| ppb | Parts per billion |
| PVC | Polyvinyl chloride |
| PWG | Pennine Water Group |
| Q | Flow |
| q-PCR | Quantitative polymerase chain reaction |
| QIIME2 | Quantitative Insights Into Microbial Ecology 2 program |
| $R^2$ | Co-efficient of determination of a linear regression |
| RNA | Ribonucleic acid |
| RPM | Revolutions per minute |
| rRNA | Ribosomal ribonucleic acid |
| s | Seconds |

| | |
|---|---|
| SDS | Sodium dodecyl sulfate |
| SEM | Scanning electron microscopy |
| SRB | sulphate-reducing bacteria |
| SUVA | Specific ultraviolet absorbance |
| T | Thymine |
| TC | Total Carbon |
| TCC | Total cell counts |
| THMs | Trihalomethanes |
| TOC | Total organic carbon |
| UK | United Kingdom |
| US | United States |
| USEPA | United states Environmental Protection Agency |
| UV | Ultraviolet light |
| UVA | Ultraviolet absorbance |
| v | Velocity |
| V | Volt |
| W | Statistic parameter from Mann-Whitney *U test* |
| WHO | World Health Organization |
| $X^2$ | Chi square, statistic parameter from Kruskal–Wallis test |
| Δ | Delta, indicates change in the value of a variable |
| µg | Micrograms |
| µL | Microlitres |
| µm | Micrometres |
| µS | Micro siemens |
| τ | Shear stress |
| χ2 | Chi-square, statistical output from Kruskal Wallis test |

# List of figures

## Supplementary figures within the appendices

# List of tables



**Supplementary tables within the appendices**

# Chapter 1

# Introduction

## 1.1 Introduction

Access to safe drinking water is vitally important, as it facilitates adequate sanitation and improves hygiene, thus reducing the spread of waterborne diseases. Climate change consequences are producing a widespread impact across Earth, including engineered environments such as drinking water distributions systems (DWDS). Climate change can affect the water sources providing potable water but also the way DWDS work by changing the environmental conditions within the pipes. For example, climate change is producing global warming by increasing the average temperature of the Earth and thus DWDS temperatures. Moreover, it can modify the hydrological cycles of water leading to water stress conditions and affect the water demand and hydraulic patters of water supply. All of these changes can modify the existing physico-chemical processes taking place in DWDS, but also the microbiome of these systems, which plays an essential role in determining the water quality and safety. Therefore, the understanding of how different aspects of climate change will influence DWDS and its microbiome is important in order to inform and plan effective strategies that will allow these distribution networks to ensure continued delivery of high-quality wholesome water.

## 1.2 Research aim and objectives

Despite the importance of the availability and supply of drinking water for human life, currently there are knowledge gaps regarding the effects of climate change on the DWDS microbiome and water quality. Therefore, research is needed to understand the behavior of microbial communities in such systems under different climate change scenarios to guarantee that safe water is delivered. This will help to protect public health and to effectively manage DWDS.

The overall aim of this research was to investigate how different processes associated to climate change may affect the microbial ecology and water quality in DWDS. To achieve this aim, this research integrated advanced molecular techniques and water engineering to study the three following specific aims:

1. To determine the effect of temperature increase on the microbial ecology and water quality of chlorinated DWDS. To achieve this aim, the objectives established were to understand how temperature affects (i) water quality physico-chemical parameters; (ii) biofilm mobilisation and water discolouration risk; (iii) the structure and composition of the overall microbiome of DWDS; and (iv) the occurrence of specific opportunistic pathogens of DWDS.

2. To evaluate the impact of temperature on biofilm control strategies in chlorinated DWDS. To achieve this, the objectives established were to understand the combined effect of temperature and biofilm management strategies on (i) water physico-chemical parameters; (ii) biofilm mobilisation and water discolouration risk; and (iii) the structure and composition of biofilm and planktonic communities.

3. To understand the impact of different intermittent water supply times in the microbiological ecology and water quality of chlorinated DWDS. To achieve this, the objectives established were to explore the effects of different times of intermittent water supply on (i) water physico-chemical characteristics, (ii) biofilm mobilisation and water discolouration risk and (iii) the structure and composition of microbial biofilm and planktonic communities.

## 1.3 Dissertation overview

To contribute in a novel way to the scientific research of DWDS, this thesis includes 7 chapters in which a review of the literature, experiments, methods, analyses, data, results and conclusions are shown. The thesis follows a 'publication format thesis' style, incorporating a collection of three manuscripts that are suitable for publication in a peer-reviewed journal. The dissertation is organized as follow:

⇒ ***Chapter 1*** corresponds to this introduction.

⇒ ***Chapter 2*** shows an overview of the literature and general aspects and on water distribution systems and climate change.

⇒ ***Chapter 3*** describes in detail the methods chosen and used to carry out this research.

⇒ ***Chapter 4*** presents the first manuscript derived from this research that is proposed for publication: "*The impacts of increasing temperature on water quality and microbial ecology of drinking water distribution systems*".

⇒ ***Chapter 5*** presents the second manuscript derived from this research that is proposed for publication: "*Effect of temperature on biofilm control strategies in chlorinated drinking water distribution systems*".

⇒ ***Chapter 6*** presents the third manuscript derived from this research that is proposed for publication: "*How intermittent water supply time impacts on the microbiome of drinking water distribution systems*?".

⇒ ***Chapter 7*** includes a final discussion, summaries the main conclusions from all this research and presents future research suggestion

⇒ ***Appendix A*** includes supplementary information for chapter 4.

⇒ ***Appendix B*** includes supplementary information for chapter 5.

⇒ ***Appendix C*** includes supplementary information for chapter 6.

⇒ ***Appendix D*** includes a statement with the contributions and co-authors

⇒ ***Appendix E*** includes scientific publications derived from this research.

# Chapter 2

# Background and Literature Review

## 2.1 Drinking water and its importance for public health

Water covers nearly 70 % of the surface of Planet Earth, but only a 2.5 % of it is freshwater, the rest is saline, and ocean based (Sigee, 2005). The greatest part of freshwater is trapped in glaciers and snowfields, and only a 1 % of the total freshwater is easily accessible. This makes that the available freshwater includes approximately an 0.01 % of the total planet's water, a very small fraction for the 7.8 billion people inhabiting the Earth (Sigee, 2005; Matta, 2010). Despite its scarcity, freshwater is fundamental resource and it brings a wide variety of services and benefits to human society, including its potabilization and consumption or its use for agriculture or industry (Selborne, 2000).

Drinking water is considered a basic human right and essential for public health since its inaccessibility or contamination are related to a poor sanitation and hygiene, which normally enhance the transmission of waterborne diseases (Liu *et al.*, 2012; WHO, 2017). For example, diarrhea affects 4 billion and kills 2.2 million people in the world per year, mostly children in developing countries who consume unsafe water (Levy, 2015; WHO, 2017); cholera, that is spread by ingesting water that has been contaminated by the bacterium *Vibrio cholerae*, affect between 1.3 and 4 million people and kill 21.000 143.000 people per year (Jutla *et al.*, 2013; WHO, 2017); or fluorosis, that is produced by the ingestion of drinking water contaminated with high levels of fluoride, affects millions of people around the world (Mandinic *et al.*, 2010). In addition, drinking water is key for protecting human health during all infectious disease outbreaks. For example, access to drinking water for a frequent handwashing has been essential for the control of the pandemic produced by the coronavirus disease in 2019/2020 (COVID-19) (WHO, 2020). Therefore, the supply of safe drinking water and the good sanitation have been demonstrated to be fundamental to avoid and control this and a wide range of human health concerns.

## 2.2 Drinking water supply

The distribution of drinking water is carried out through diverse engineering infrastructures that consist of an interconnected series of hydraulic components. They include reservoirs, storage tanks, pipes of different materials, pumps, valves, hydrants, water quality meters, fittings, and many other more hydraulic components (National Research Council, 2006).

Surface water and groundwater are both the most common sources for collecting water for its distribution (Ritter *et al.*, 2002). From its collection, the water is transported to a centralized treatment plant, where it is purified prior to its distribution to consumers (Figure 1, Figure 2). In order to remove all contaminants and reduce the turbidity of the raw water and make it safe for human consumption, a succession of treatment processes is applied. The most common processes include coagulation, flocculation, sedimentation, filtration and disinfection (Figure 2) (Gerba, 2009). Coagulation-flocculation process implies the addition of coagulants or precipitating agents, for example alum or ferric sulfate. These chemicals form precipitates, and dissolved and suspended solids absorb on their surface, promoting the formation of large particles. These particles are then and removed by sedimentation, a physical process that consist in the gravitation deposition of the particles that are denser that water. After sedimentation, the partially treated water is filtered to remove microorganisms and other impurities. The most common method for water filtration is slow sand filtration, in which the water by gravity goes through different layers. The sand filter is normally composed by a succession of layers with different pore sizes, and also include a biologically active layer called *schmutzdecke.* Finally, the filtered water is generally disinfected to remove pathogenic as well as other microorganism in water by adding chemical disinfectant. The most common disinfectant are chlorine and chloramines, although others such as ozone, chlorine dioxide, and ultraviolet (UV) light may be also used as an alternative or together with chemical disinfectants (Gerba, 2009; Gray, 2014; Purwoto, Antoro and Sembodo, 2019). Once the water is treated, it is distributed to consumers through complex pipe networks, normally divided into district management areas (DMAs). Pipes can be made of different materials such as cast iron, asbestos cement, or tarred steel, although in the last decades the new pipes are made of plastic materials such as polyvinyl chloride (PVC) or medium and high density polyethylene (MDPE/HDPE) (Figure 1, Figure 2) (Niquette, Servais and Savoir, 2000; Rezaei, Ryan and Stoianov, 2015).

Water often travel through many km of these pipes before it reaches the consumer taps and depending on the consumer demands and hydraulic regimens, the water age in the system can change. That is, if there is a low demand, the flow of the water will decrease, thus increasing the residence time of the water in the system (Twort, Ratnayaka and Brandt, 2000). Others environmental parameters can also affect DWDS and thus the distributed drinking water. In addition, occasional break of pipe or leaky pipes can allow potentially the intrusion of harmful compounds into the system (Collins *et al.*, 2010). All this makes that the quality of the water leaving the treatment plant may deteriorate and include contaminants, that can potentially compromise water safety. Therefore, a full knowledge of what happens and the conditions inside the DWDS, it is important to control potential deterioration of water quality and thus ensure the constant distribution of safe water for human health. Once the water has been used by the users, the effluent is channeled through a wastewater network to the wastewater treatment plants. There, a series of physical, chemical and biological processes are carried out to eliminate contaminants before being discharged or reused (Figure 1).

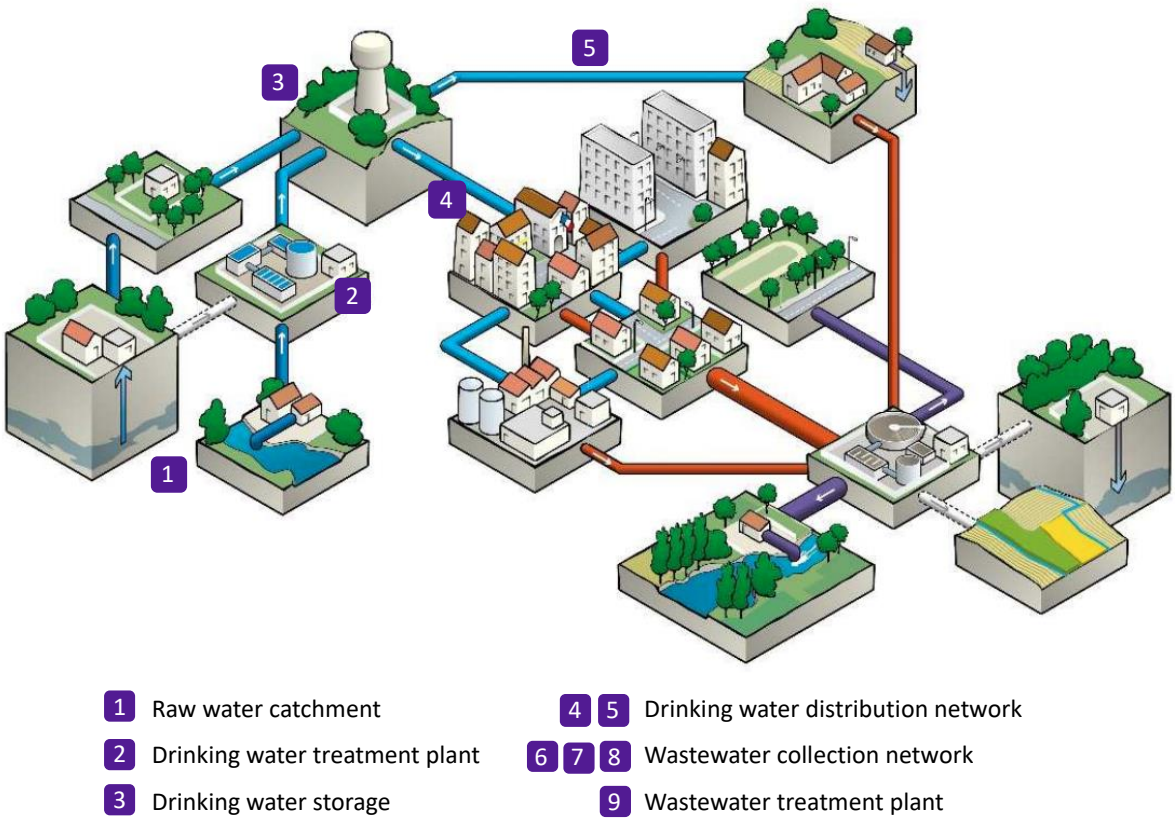



***Figure* 1**. Schema of a real DWDS. Adapted from MSCBS (2018).

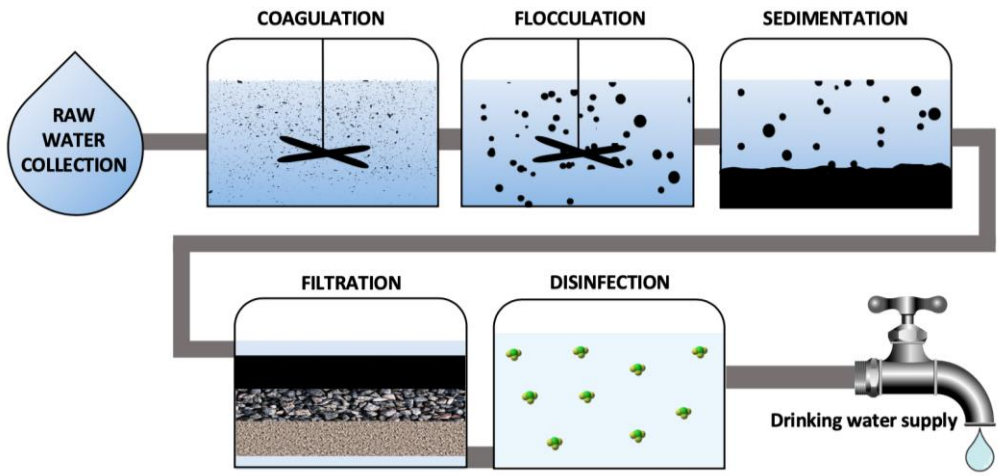



***Figure 2***. Schema of the most common steps during the water potabilization treatment process.

## 2.3 Drinking water contaminants

Despite drinking water treatment plants use many treatment methods to remove water contaminants and supply drinkable water to protect public health, no water treatment system is considered 100 % effective removing all contaminant particles from drinking water (Spellman, 2013; Cotruvo, Craun and Hearne, 2019). Contaminants and other external compounds can get into drinking water from natural sources, by human activities such as industrial and agricultural processes, or simply by intrusion during the water supply (Fawell and Nieuwenhuijsen, 2003). These drinking water harmful compounds can include (1) chemical, (2) radiological and (3) biological contaminants.

(1) There is a wide range of chemical substances that can alter the water safety, including both organic and inorganic compounds. Inorganic chemicals with a significative effect on water quality are naturally present in drinking water, for example fluoride, arsenic, nitrate, iron or manganese. However, human activities and/or water distribution through the systems can encourage the concentration of these compounds and introduce others such as mercury, lead or copper (Fawell and Nieuwenhuijsen, 2003; Sharma and Bhattacharya, 2017). Fluoride is present in many waters, and especially groundwaters that are usually exploited present higher fluoride concentrations. Medium-level ingestion of fluoride is known to cause dental problems, but long-term exposure can produce skeletal fluorosis or even can aggravate neuropathological lesions such as Alzheimer's disease and other forms of dementia (Fawell and Nieuwenhuijsen, 2003; Sharma and Bhattacharya,

2017; Cao *et al.*, 2019). Arsenic contamination in drinking water is responsible of important disease, especially in countries such as India or Bangladesh. The exposure to arsenic for long periods produces leads to arsenic poisoning or arsenicosis, that include a large range of adverse effects including stomach pain, nausea and vomiting, diarrhea, partial paralysis, hyperkeratosis or peripheral vascular disease. In addition, arsenic is the only contaminant whose exposure through drinking water has been demonstrated to be the cause of skin, lung or bladder cancer (Fawell and Nieuwenhuijsen, 2003; WHO, 2017). Nitrate can reach surface and groundwater sources as a consequence of its intrusion due to the use of fertilizers in agriculture. The reduction of nitrate produce nitrite, a toxic compound that can cause serious illness at higher levels. Shortness of breath, methemoglobinemia or blue-baby syndrome are some of the health problems associated with high levels of these compounds in drinking water (WHO, 2011). Iron and manganese can occur at high concentrations in some source waters, result of the use of iron coagulants or the corrosion of the pipes during the water distribution. Although these metals do not have a direct impact on human health, iron and manganese can affect the flavor and colour of the water and high levels of these contaminants can result in discolored water. This is the main cause of the loss of aesthetic acceptability, being one of the problems that produces the most complaints from consumers in the UK (Fawell and Nieuwenhuijsen, 2003; Husband and Boxall, 2011). Mercury is a powerful environmental contaminant that is mainly introduced into the drinking water by agricultural runoff and by leaking from factories and landfills. Human exposure to mercury, even at low concentrations, can cause serious health problems including the disruption of the endocrine system, neurological problems and impairment of brain functions, disorders in intrauterine development and early life or abortion (Counter and Buchanan, 2004; WHO, 2017). Lead water contamination is a common problem in older water systems since it was used widely to build DWDS due its good properties. However, it is a dangerous heavy metal that produce toxic effects on several organisms, and in humans it has been demonstrated that leads to neurological, renal, cardiovascular and reproductive problems, can produce a wide range of developmental difficulties in children (Schock, Hyland and Welch, 2008; EPA, 2016). Copper is also naturally present in water, but its concentration in DWDS often increase during the water distribution since it has been historically used to build elements of the systems because of its versatility. At lower doses copper poisoning include headache, nausea, vomiting or diarrhea, but a high concentrations copper can induce the DNA damage and enhance genotoxicity problems (WHO, 2004a).

Regarding organic contaminants in water, the most of them come from anthropogenic sources such as pesticides, herbicides, domestic and industrial wastes, etc. (Mandal *et al.*, 2016). Organochlorine pesticides (OCPs), polycyclic aromatic hydrocarbons (PAHs), acidic herbicides (compounds which include derivatives of phenol) or carbamate are common persistent organic pollutants in drinking water. In addition to promoting the biological growth in DWDS, it has been widely demonstrated that, due their hydrophobic characteristics, all these aromatic compounds can react with chemical disinfectants in water to form disinfection by products (DBPs). The main groups of DBPs formed in drinking water are trihalomethanes (THMs), haloacetic acids (HAAs) and haloacetonitriles (HANs) (Sadiq and Rodriguez, 2004; Prasad, 2020). The most common THMs include chloroform ($CHCl_3$), bromodichloromethane ($CHCl_2Br$), dibromochloromethane ($CHClBr_2$) and bromoform ($CHBr_3$), which have been related to human health problems such as arrhythmia or damage to the kidneys and livers. However, long-term exposure of these THMs can result in adverse effects on the central nervous system and it has been consistently associated with bladder cancer (WHO, 2005). HAAs include nine substances, although the five most commonly found in drinking water are monochloroacetic acid ($ClCH_2COOH$), dichloroacetic acid ($Cl_2CHCOOH$), trichloroacetic acid ($Cl_3CCOOH$), monobromoacetic acid ($BrCH_2COOH$) and dibromoacetic acid ($Br_2CHCOOH$). These compounds have irritating and corrosive effects on the eyes and skin, and at higher doses it has been demonstrated that can produce metabolic disorders in humans and therefore induce cancer and reproductive disorders (National Research Council, 2000). The most common HANs generated in drinking water during disinfection are bromoacetonitrile ($BrCH_2CN$), chloroacetonitrile ($ClCH_2CN$), dibromoacetonitrile ($C_2HBr_2N$), dichloroacetonitrile ($C_2HCl_2N$), trichloroacetonitrile ($CCl_3CN$), and bromochloroacetonitrile ($C_2HBrClN$) (Prasad, 2020). These nitrogenous DBPs have also toxicity effect on human health, causing kidney, liver, cardiovascular, intestinal or nervous system problems (WHO, 2004b; Muellner *et al.*, 2007).

(2) Radioactive materials occur naturally in all environments and hence into drinking water, although human activities have been demonstrated to increase their concentrations (Dinh Chau *et al.*, 2011). The risks associated with radionucleotides in DWDS are lower compared to other chemical substances, since their concentration and the radiation dose produced are lower than that received by other natural sources (WHO, 2017). However, radioactive isotopes of uranium and radium have been observed to produce toxic effects for human health through drinking water, such

as kidney disease, depression of the immune system, birth defects and bone or lung cancer (Fawell and Nieuwenhuijsen, 2003; Weir, 2004).

(3) Biological contaminants refer to presence of living microorganisms, which have been identified as the main cause of waterborne diseases, including cholera, typhoid, gastroenteritis or infectious hepatitis (Bain *et al.*, 2014; WHO, 2017). Microorganisms in drinking water can have various origins, including its entry into the treated water, by intrusion or contamination due to the pipe breakage and repair, or they can even grow in tanks, deposit, reservoirs or in the system during the water distribution (Liu *et al.*, 2018). The majority of the of microbial cells in DWDS are found forming biofilms attached to the inner pipe surfaces, although planktonic cells are also able to survive in bulk water (Flemming, Percival and Walker, 2002). Both, prokaryotes and eukaryotes microbial cells have been identified taking part of drinking water related environments worldwide (Bruno *et al.*, 2018).

Regarding prokaryotes, Bacteria have been the most widely studied microorganisms, probably due to the ease of cultivation of some groups under laboratory conditions, and they are the only microorganisms whose presence is used worldwide to establish the water quality (Fish *et al.*, 2015). A wide range of pathogenic bacteria have been identified contaminating drinking water, for example fecal coliforms, which the most common member is *Escherichia coli*. Most strains of this enterobacteria has been demonstrated to cause important illness such as diarrhea, urinary tract infections, meningitis or pneumonia (Ashbolt, 2015; WHO, 2017). Another enterobacteria that have been observed as a contaminant in drinking water are *Shigella* spp*.*, causing also serious intestinal diseases, bloody diarrhea, abdominal cramps and fever, and *Salmonella* spp. that can produce intestinal illness including gastroenteritis, bacteremia or septicemia (Cabral, 2010; Levantesi *et al.*, 2012). Proteobacteria phylum, although is also very common in drinking water, contains different groups that are important drinking water contaminants. For example, *Vibrio cholerae,* responsible for cholera which continues producing significant outbreaks in developing countries, or *Legionella pneumophila*, that is considered the major waterborne pathogen responsible of Pontiac fever and severe pneumonia (WHO, 2017). Non-tuberculous mycobacteria, belonging to Actinobacteria pylum, can be also potentially transmitted by drinking water and cause a range of diseases including respiratory or gastrointestinal problems that mainly affect immunocompromised patients (Vaerewijck *et al.*, 2005). Another group of bacteria that occasionally can cause contamination in drinking water are cyanobacteria. Although they are not infectious for humans, *Cyanobacteria* are

able to produce important cyanotoxins, for example microcystins, that may pose a risk for human health, such as liver damage, neurotoxicity or even tumor promotion (WHO, 2015; He *et al.*, 2016).

Several studies have also detected different Archaea groups being part of drinking water related environments, where can play important roles for example participating in important processes such as the nitrification (van der Wielen, Voost and van der Kooij, 2009; Sun *et al.*, 2013). However, archaeal communities have been observed to be less diverse in drinking water environments that bacterial communities (Lymperopoulou, Kormas and Karagouni, 2009; Fish *et al.*, 2015) and to date no indications have been found that the presence of any group of these microorganisms in drinking water supposes or is implicated in human diseases.

Organisms belonging to the Eukaryota domain can also be found in drinking water environments. The most identified eukaryotes in these systems are Fungi, which have been accepted as water contaminants in recent years (Hageskal, Lima and Skaar, 2009). A wide diversity of fungi has been isolated from drinking water, including parasitic or toxic species for human health. Among the most common are *Aspergillus fumigatus* or members of the genera *Penicillium*, *Cladosporium* or *Trichoderma. A. fumigatus* is well known to produce severe pulmonary infections, especially in immunocompromised patients (Novak Babič *et al.*, 2017; Garcia-Rubio and Alcazar-Fuoli, 2018). *Penicillium* produce a wide range of symptoms including pneumonia, endocarditis, urinary tract infections or endophtalmitis, while *Cladosporium* species are also related to respiratory disease. *Trichoderma* can produce human local infections, but several species such as *T. harzianum* have been associated to fatal disseminated disease (Guarro *et al.*, 1999; Hageskal, Lima and Skaar, 2009).

Other important Protista parasites such as *Giardia spp*. and *Cryptosporidium spp.* have been also identified contaminating drinking water. *Giardia spp*. is a human gastrointestinal parasite of humans which causes the disease known as giardiasis (Haque, 2007). *Cryptosporidium* oocysts are resistant to chlorine concentrations typically used in water treat and thus is an important waterborne pathogen of global concern. Especially the species *C. hominis* and *C. parvum* are known to produce severe respiratory and gastrointestinal illness (Leitch and He, 2012; Pignata *et al.*, 2019). Other protists like Amoebas are also found in DWDS, including pathogenic genera like *Acanthamoeba* or *Naegleria*. *Acanthamoeba* disease include serious keratitis, meningitis and in some cases pulmonary infections. In addition, this amoeba is an important host of pathogenic bacteria such as *Legionella pneumophillia*, and thus play an important role in its persistence in drinking water (Hoffmann and

Michel, 2001; Gomes *et al.*, 2018). In the same way, species of *Naegleria* such as *Naegleria fowleri* are important parasites detected in drinking water supplies, especially in those systems that exceed 25 °C. It causes meningoencephalitis, which produces extensive brain damage and is fatal in many cases (Cogo *et al.*, 2004; Wingender and Flemming, 2011).

Some organisms belonging to the Animalia kingdom have also been observed as sources of contaminants in DWDS, for example free-living nematodes. These organisms have not been seen as a direct threat to human health, and the presence of nematodes in drinking water is mainly an aesthetic problem that reduce the water acceptability to the consumer. However, despite nematodes are harmless by themselves, they may pose a risk to human health because some pathogens like *Mycobacterium* or *Salmonella* can colonise its gut can colonize its gut, thus transform nematodes into a source of harmful contaminants of drinking water (Buse *et al.*, 2013; Kos *et al.*, 2020).

In addition to the presence of living organisms, it has been observed that viruses can also generate biological contamination in drinking water. Several virus groups have been identified in DWDS including enteroviruses, adenovirus, hepatitis A and E viruses, astrovirus or rotavirus (Sinclair, Jones and Gerba, 2009). Viral pathogens related to waterborne transmission mainly produce gastrointestinal infections generating symptoms that include diarrhea abdominal cramping, vomiting and fever. However, viral infections of these viruses can also cause more severe disease, for example hepatitis produced by hepatitis A and E viruses, myocarditis or encephalitis by enteroviruses or pneumonia by adenoviruses (Gall *et al.*, 2015; WHO, 2017).

However, it is important to consider that not all microorganisms present in DWDS are harmful and some of them have not been observed to affect water quality or safety. Moreover, some bacteria and fungi observed in DWDW environments are capable of being part of biodegradation processes of pollutants and xenobiotic contaminants (Kent, Garcia and Martiny, 2018; Guha Roy, 2019; Mahmood, Al-Haideri and Hassan, 2019; Tsagkari and Sloan, 2019) and they could even be used for a probiotic approach to avoid quality problems and improve distributed water (Wang *et al.*, 2013).

## 2.4 Contaminants regulation

Despite the new technologies and methods for water purification, some contaminants, such as those mentioned above, may persist in the water that is distributed to consumers (Spellman, 2013;

Cotruvo, Craun and Hearne, 2019). Therefore, it has been necessary the establishment of legislation by health and government authorities, dictating the maximum permitted concentration of contaminants in drinking water that does not compromise the health of consumers. The World Health Organisation (WHO), the European Union (EU) Drinking Water Directive and the United States (US) Environmental Protection Agency (EPA) have implemented a minimum quality standard for water for human consumption. Table 1 shows a summary of the maximum values of the most relevant parameters and contaminants permitted by these authorities (EU, 1998; WHO, 2017; USEPA, 2018).

**Table 1.** Contaminants regulation and guidelines.

| **Parameter/Contaminant** | **WHO Guidelines** (WHO, 2017) | **EU Commission** (EU, 1998) | **USEPA Standards** (USEPA, 2018) |
|---|---|---|---|
| pH | 6.5 – 8.5 | - | 6.5 – 8.5 |
| Colour | - | - | 15 colour units |
| Conductivity | 250 µS/cm | 250 µS/cm | - |
| Turbidity | 5 NTU | 4 NTU | 5 NTU |
| Arsenic | 0.01 mg/L | 0.01 mg/L | 0.01 mg/L |
| Chlorine | 5 mg/L | - | 4 mg/L |
| Copper | 2 mg/L | 2 mg/L | 1.3 mg/L |
| Fluoride | 1.5 mg/L | 1.5 mg/L | 4 mg/L |
| Iron | 0.3 mg/L | 0.2 mg/L | 0.3 mg/L |
| Lead | 0.01 mg/L | 0.01 mg/L | 0.015 mg/L |
| Manganese | 0.5 mg/L | 0.05 mg/L | 0.05 mg/L |
| Mercury | 0.006 mg/L | 0.001 mg/L | 0.002 mg/L |
| Nitrate | 50 mg/L | 50 mg/L | 10 mg/L |
| Nitrite | 3 mg/L | 0.50 mg/L | 1 mg/L |
| Radium-226 | 1 Bq/L | - | 0.185 Bq/L |
| Radium-228 | 0.1 Bq/L | - | 0.185 Bq/L |
| Bromodichloromethane | 0.06 mg/L | - | 0 mg/L |
| Chloroform | 0.3 mg/L | - | 0.07 mg/L |
| Dichloroacetic acid | 0.05 mg/L | - | 0 mg/L |
| Dichloroacetonitrile | 0.02 mg/L | - | - |
| Monochloroacetic acid | 0.02 mg/L | - | 0.07 mg/L |
| Trichloroacetic acid | 0.2 mg/L | - | 0.02 mg/L |
| *Escherichia coli* | - | 0 in 250 ml | Less than 5.0 % samples total coliform-positive in a month |
| *Enterococci* | - | 0 in 250 ml | |
| *Pseudomonas aeruginosa* | - | 0 in 250 ml | |
| *Clostridium perfringens* | - | 0 in 100 ml | |
| Coliform bacteria | - | 0 in 100 ml | |
| Colony count 22 °C | - | 100/ml | |

## 2.5 Microbiology of drinking water distribution systems

As it has been reported, DWDS are not sterile environments and despite the oligotrophic conditions and the presence of disinfectant residual used in some countries, a wide variety of microorganisms inhabit DWDS, including bacteria, archaea, eukaryotes and viruses (Bruno *et al.*, 2018). Two different ways of life have been observed in these systems, planktonic cells and biofilm communities, which present very different structures (Douterelo *et al.*, 2016; Chen *et al.*, 2018).

Bulk water of DWDS contains a total cells concentration ranging from $10^3$ to $10^5$ cells/mL, less than 2 % of the total biomass of DWDS (Proctor and Hammes, 2015). Despite this low percentage, planktonic communities are the major contributor to microorganisms found in the tap water and they are normally used to establish the microbiological quality of the supplied drinking water (WHO, 2017; Chen *et al.*, 2020). Several environmental factors such as hydraulic regimens (Douterelo, Sharpe and Boxall, 2013), the type and amount of disinfectant (Wang *et al.*, 2014), different contaminant concentrations such as nitrite and nitrate (Nagymáté, Homonnay and Márialigeti, 2016) can shape planktonic communities of DWDS. However, it has been demonstrated that the continuous use of disinfectant residuals is able to control and reduce the concentration of planktonic microorganisms in these systems (Fish and Boxall, 2018).

Biofilm communities are the primary source of microorganisms in DWDS. Between $10^4$ and $10^7$ cells/$cm^2$ are attached to internal pipe surfaces, representing more than 98 % of the total biomass in these systems (Flemming, Percival and Walker, 2002; Batté *et al.*, 2003; Proctor and Hammes, 2015). Biofilm is defined as an assemblage of microorganisms attached to a surface and/or to each other (Figure 3) and enclosed in an self-produced matrix of hydrated extracellular polymer substances (EPS), and it is characterized by an structural, functional and metabolic heterogeneity and capable of use quorum sensing for intercellular communications (Flemming and Wingender, 2010; Farkas, Ciataras and Brandus, 2012).

The structure and composition of the EPS matrix depends on environmental conditions, nutrient availability and the microorganisms forming the biofilm, but in general it is mainly composed of water, exopolysaccharides, extracellular proteins, extracellular DNA and lipids (Flemming and Wingender, 2010). EPS matrix is the most extensive part of the biofilm, and it is responsible for adhesion and fixation of microbial cells to the pipe surface and between them. This allows for the

development and cohesion of the microbial consortium in different environments such as DWDS (Flemming, Percival and Walker, 2002). In addition to the structure and mechanical stability, biofilm EPS matrix provide a wide range of advantages for the microorganisms, including protection against chemicals, desiccation or oxidizing. EPS are also a rich source of nutrients and exoenzymes, that enhancing metabolic capacities helping the acquisition of these nutrients. Several exoenzymes can also help for complete degradation of complex compounds potentially toxic or bactericidal, for example antibiotics (Flemming, 2016; Santos *et al.*, 2018; Sivadon *et al.*, 2019). In addition, EPS matrix constitutes a reservoir of genes for horizontal gene transfer, which can help the ecological adaptation of microorganisms in situations of change or stress (Flemming and Wingender, 2010; Santos *et al.*, 2018).

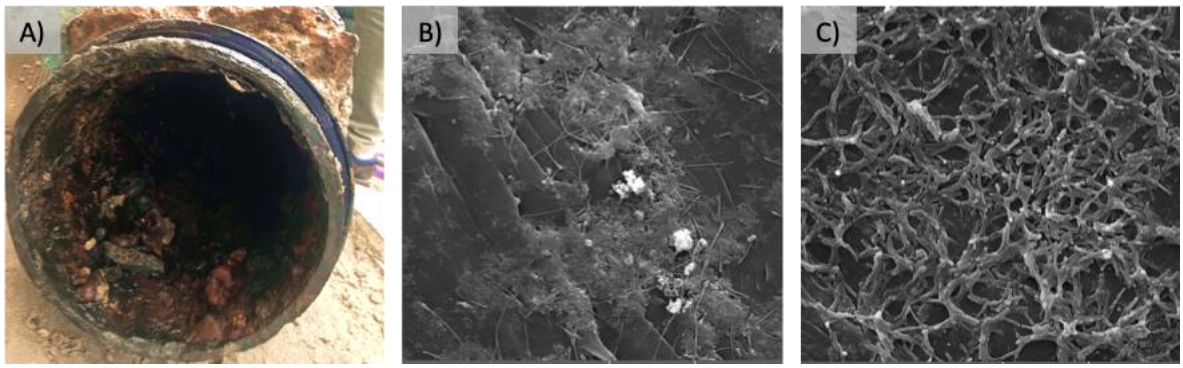



***Figure 3***. A) Image of biofilm accumulation inside a pipe from a real DWDS. B) and C) Scanning Electron Microscopy micrographs showing biofilm development attached to the pipe walls (MAG=5.00 kx).

### 2.5.1 Biofilm development

Biofilm formation is a dynamic process that includes a succession of physical, chemical and biological phenomena. They comprise an initial adhesion or attachment, the microcolony formation, the biofilm maturation to form three-dimensional structures, and the final equilibrium stage. Additionally, a detachment and biofilms dispersion process may also occur (O'Toole, Kaplan and Kolter, 2000; Santos *et al.*, 2018):

⇒ **Initial attachment.** Biofilm formation begins when any planktonic microorganism sense attractive environmental conditions from the pipe surface, such as hydrophobicity, surface charge, or the presence of organic and inorganic molecules (Davey and O'toole, 2000). This conditioned surface induces extracellular signals that attract to planktonic microorganisms to adhere to the pipe.

This first adhesion of these initial colonisers is via Van-der Waals physical forces, that are weak and reversible (Cowle *et al.*, 2014; Carniello *et al.*, 2018).

⇒ **Microcolony formation.** The initial colonisers activate the metabolic pathways for the biofilm formation, starting the cell division and the EPS secretions, thus leading to stronger bonds and irreversible adhesion to the pipe surface (López, Vlamakis and Kolter, 2010; Cowle *et al.*, 2014). These irreversible adhesions induce change in gene and protein expression in the microorganisms that produce a greater cellular division to colonise the surface (Melo and Bott, 1997; Chagnot *et al.*, 2013). This results in a more compact cell matrix embedded in EPS, which also play an important role for trapping and concentrating nutrients from the water and attracting secondary colonisers (O'Toole, Kaplan and Kolter, 2000; Chagnot *et al.*, 2013; Cowle *et al.*, 2014).

⇒ **Biofilm maturation.** Further colonisation and growth is induced by the EPS secretion and cell division, enhancing the biofilm development and leading to a thicker and denser structure (Chagnot *et al.*, 2013; Cowle *et al.*, 2014). At this point microorganisms start the adaptation to life in a biofilm and they create a favorable environment and developing certain abilities: increase the genic exchange, improve their metabolic and biodegradative capabilities, increase the resistance to UV light, etc. (O'Toole, Kaplan and Kolter, 2000; Givskov and Kjelleberg, 2007). Biofilm continues to develop until it adapts to the adverse conditions of water flow and the consequent boundary shear stress, thus reaching an equilibrium state and acquiring three-dimensional structure (Cowle *et al.*, 2014).

⇒ **Biofilm detachment/dispersion.** Mature biofilm may respond to changing environmental conditions and it can experience a contrary process in which the detachment of cells from the colony occurs (Melo and Bott, 1997; Stoodley *et al.*, 2002). The detachment can occur by erosion and sloughing due to increases in the boundary shear stress. However, biofilm detachment can also be a biological dispersal process to colonise new areas and thus spread through the entire pipeline system (Cowle *et al.*, 2014; Petrova and Sauer, 2016).

### 2.5.2 Biofilm related problems

Although biofilms are an intrinsic part of DWDS and they appear distributed throughout the system, it is increasingly known that their presence is associated with several problems in DWDS, which can deteriorate the quality and safety of drinking water (Simões and Simões, 2013).

⇒ **Aesthetic deterioration – Discolouration**

Biofilm accumulation at the pipe wall and its mobilisation has been demonstrated to be the main cause degradation of the aesthetic water quality in DWDS (Ginige, Wylie and Plumb, 2011; Husband and Boxall, 2011a). Biofilm can increase turbidity levels of drinking water by its own composition: microbial cells, EPS and associated organic material, metabolisms products, etc. (Douterelo, Sharpe and Boxall, 2014; Husband *et al.*, 2016; Fish, Osborn and Boxall, 2017). In addition to this, biofilm is known to facilitate the adsorption and entrapment of materials and particles from the bulk water, for example Fe and Mn, compounds that have been demonstrated to be one of the main causes of water discolouration in DWDS (Ginige, Wylie and Plumb, 2011; Husband and Boxall, 2011a; Husband *et al.*, 2016).

Under normal water supply conditions, a low-level background of particulate material is present in the bulk water since biofilms are adapted and in equilibrium with the hydrological forces, i.e. a balance between the biofilm cohesive retaining forces and the mobilising shear stress exists which keep them adhered (Verberk *et al.*, 2006; Vreeburg and Boxall, 2007). However, it has been demonstrated that increases in applied hydraulic cause shear stress excess the cohesive shear strength of accumulated material on pipe walls, i.e. the balance of established forces is broken leading to the biofilm mobilisation from the pipe wall into the water column (Vreeburg and Boxall, 2007). The mobilisation and presence of biofilm increase turbidity levels in drinking water, compromising the aesthetic quality of the water that reaches consumers. In fact, discolouration is one of the key problems causing consumer dissatisfaction in the UK, and currently water utilities make a great effort to improve operational strategies to manage and avoid discolouration in DWDS (Vreeburg and Boxall, 2007; Husband and Boxall, 2011; Paul *et al.*, 2012; Husband and Boxall, 2016). Currently, conceptual models have been developed and can be used to predict the discolouration response of distribution networks to changes in hydraulic condition, for example the Prediction of Discolouration in Distribution Systems model (PODDS) developed by the Pennine Water Group at the University of Sheffield (Husband and Boxall, 2010).

⇒ **Organoleptic – Taste and odour problems**

In addition to discolouration problems, other water organoleptic characteristics such as taste and odour may be affected by the presence of biofilm. It has been observed that the metabolic activity of the microorganisms forming the biofilm produce compounds that deteriorate the taste and odour

of the water (Prest *et al.*, 2016; Zhou *et al.*, 2017). Some bacteria and even several algae can produce these metabolites, but fungi have been observed to be the major source causing organoleptic problems (Zhou *et al.*, 2017). Important microbial metabolites that have been found to produce taste and odour in drinking water include geosmin, 2-methylisoborneol, isobutyl-3-methyoxypyrazin, 2-isopropyl-3-methoxypyrazine and β-cyclocitral (Lin, 1977; Zhou *et al.*, 2017). All of them mainly cause earthy and musty flavours, the major cause of consumer complaints. Although they have been identified, the mechanism of generation of most of these compounds in drinking water is still unknown and it cannot be controlled, posing a challenge to the water utilities (Zhou *et al.*, 2017; Zhang *et al.*, 2018).

⇒ **Pathogens reservoir**

The favourable micro-environment within the biofilms allows for the persistence of microorganisms that that by themselves would not survive in DWDS. They can accidentally enter the system and attach to pre-existing biofilm, where the advantages of this way of life (protection against chemical disinfectants, higher amount of nutrients, etc.) will allow these pathogens to survive and even proliferate in an environment that *a priori* is not favourable (Batté *et al.*, 2003; Simões, 2013). Biofilm of DWDS host a wide range of bacterial and fungal pathogens that are responsible for several waterborne diseases, such as *E. coli*, *Campylobacter* spp., or *Aspergillus fumigatus,* opportunistic pathogens (OPs) such as *Legionella pneumophila, Pseudomonas aeruginosa,* or *Mycobacterium avium* and other nontuberculous mycobacteria (NTM) (Wingender and Flemming, 2011; Wang *et al.*, 2012). Viruses, for example adenoviruses or enteroviruses, or even parasitic protozoa such as *Acanthamoeba spp.* or *Cryptosporidium parvum* (Percival, Walker and Hunter, 2000; Skraber *et al.*, 2005). The occurrence of these microorganisms in the biofilm of the DWDS is especially important if the biofilms detachment occurs and they end up reaching the tap. Particularly, it is critical in hospital water systems where immunocompromised patients are exposed and can develop nosocomial infections due to infection by these pathogens (Szewzyk *et al.*, 2000; Wingender and Flemming, 2011; Chaves Simões and Simões, 2013).

⇒ **Corrosion and pipe deterioration**

Microbial growth and biofilm development have been observed to contribute and to accelerate the corrosion on pipe surfaces, especially in iron and steel pipes. This process is known as biocorrosion or microbiologically influenced corrosion (MIC) (Teng, Guan and Zhu, 2008; Liduino *et al.*, 2019).

Biocorrosion include a wide range of electrochemical and biogeochemical processes including interaction between microorganisms and/or with the surfaces. The best known and the most severe biocorrosion process is that produced by sulphate-reducing bacteria (SRB) (Delaunois, Tosar and Vitry, 2014; Liduino *et al.*, 2019). Derived compounds from the metabolic activity of these bacteria include high concentrations of sulfide, which is highly reactive and corrosive. Acid-producing bacteria and iron-oxidizing bacteria are also important biocorrosive bacteria found in the biofilm of DWDS, which release organic acids and oxidizing dissolved ferrous iron, respectively (Liduino *et al.*, 2019).. Biocorrosion not only produce the pipe deterioration, it also can affect the water quality for example inducing the water discolouration by releasing the iron oxyhydroxides, and also by reactions with DBPs, nitrates or natural organic matter. In addition to this, material released into the bulk water during the corrosion process can re-precipitate forming tubercles that can obstruct the pipes (Teng, Guan and Zhu, 2008; Zhu *et al.*, 2014; Liduino *et al.*, 2019).

⇒ **Biofilm control and management**

Water companies invest time and money to control the presence and problems related to microorganisms and especially biofilm in DWDS (Isabel Douterelo *et al.*, 2016). Chemical methods have been the most widespread strategy used to control the microbial growth in DWDS. Free chlorine ($HOCl/OCl^-$) and chloramine (mainly monochloramine, $NH_2Cl$) are used as disinfection residual due to its rapid effectiveness, the broad range of activation and low cost (Fish and Boxall, 2018; Michael B Waak *et al.*, 2019). These methods have been shown to be effective for planktonic communities, and several studies have shown that these microbial communities of different DWDS from different locations remain stable over the time and show biological stability when these disinfectants are present in the system in constant doses and concentrations (Pinto, Heller and Bastos, 2012; Schwering *et al.*, 2013; Prest *et al.*, 2016). However, the addition of these chemicals produces potential problems related to human health such the as the formation of DBPs, the need for constant addition due to their degradation in the network and the associated economic impact and the environmental contamination (Garcia de Carellan *et al.*, 2012). Because of this, other countries mainly in Europe has implemented alternatives that do not have these drawbacks, for example removal of assimilable organic carbon (AOC) to limit the microbial growth (Van der Kooij *et al.*, 1999). However, the efficiency of all of these strategies in controlling biofilm development and growth is limited, and it is currently known that the biofilm presence in DWDS is inevitable (Schwering *et al.*, 2013; Waak *et al.*, 2019).

In order to minimise the risk associated with the presence of biofilms, water companies have also implemented different mechanical techniques including flushing, air scouring and pigging (Vreeburg, 2007; Vreeburg and Boxall, 2007). Flushing is the most used method and consists of increasing the water flow to produce an increase in the water velocity that will lead to further shear stress on the biofilms attached to the pipe surfaces (Friedman *et al.*, 2003; Vreeburg, 2007). Planned flushing trials are carried out by closing selected valves and opening hydrants to increase flow and rise the hydraulic forces within pipes (Douterelo, Husband and Boxall, 2014). Sometimes, flushing works can be applied in conjunction with high concentrations of disinfectant for more effective system cleaning (Chaves Simões and Simões, 2013; van Bel *et al.*, 2019). However, flushing programs present several drawbacks, including that they have been found not to be completely effective, and sometimes they only can partially remove biofilm from pipes (Douterelo, Sharpe and Boxall, 2013; Fish, 2013). In addition, for a flushing event a very large volume of drinking water is wasted and sometimes it can be difficult to apply in pipes with large diameters such as trunks mains because it is impossible to reach the necessary water velocity (Quarini *et al.*, 2010). Air scouring is a version of flushing but injecting filtered and compressed air onto the pipes (Kitney, Woulfe and Codd, 2001). As a consequence, "slug flows" are formed, which are driven along the pipes by the compressed air at high velocity removing biofilm, fouling and material sedimentation (Kitney, Woulfe and Codd, 2001; Pourcel, Smith and Duchesne, 2017). It has the advantage that can be used in pipes of large diameter and with low pressure. However, it also implies the use of large volumes of water in order to remove the air from the system, can produce potential pipe damage, and it requires additional equipment such as a compressor, air cooler and filters (Kitney, Woulfe and Codd, 2001; Rubulis *et al.*, 2008; Garcia de Carellan *et al.*, 2012). Pigging is another of the most used techniques and consist of the introduction inside the pipes of a solid device or material (the pig) that travels through the system (Tiratsoo, 1992; Garcia de Carellan *et al.*, 2012). The pig can be ice, a foam sponge or even smart devices that can also inspect and monitor the pipe conditions. The advantages of this method include the low volume of water required and that it can be applied in any pipe regardless of its length and/or diameter. However, it is more expensive than the other techniques because it needs specific installation and equipment for its insertion, such as launcher and catcher. In addition, it is necessary the pig design and its disinfection prior to use, and it is possible that the pig get lost inside the system, break or stick to the pipe, causing a pipe blockage or damage (Rubulis *et al.*, 2008; Garcia de Carellan *et al.*, 2012)

## 2.6 Drinking water and climate change

The Intergovernmental Panel on Climate Change (IPCC) has concluded that there are detectable effects and scientific evidences that human activities are affecting the global climate (IPCC, 2014b). Extreme temperature rise, weather events and severe natural hazards, sea-levels rise, or various forms of environmental degradation are some of the consequences that can be observed today. Climate change is therefore affecting the hydrological cycle and with it the availability of fresh water and with it the accessibility, the quality and the distribution of safe drinking water worldwide (McMichael, Woodruff and Hales, 2006; Bates *et al.*, 2008; WHO, 2017).

### 2.6.1 Temperature increase

The most obvious consequence of climate change is the increase of temperature. The IPPC estimates that during the 20$^{th}$ Century, the temperature in the Earth surfaces has increased 0.6 °C, and it has been suggested that in the next 100 years the temperature will rise by 1.4 to 5.8 °C, or even up to 11 °C in the worst case scenario with very high anthropogenic greenhouse emissions (Figure 4) (Bates *et al.*, 2008). In addition to the constant increase that occurs globally, the frequency and severity of extreme weather events are increasing, producing greater seasonal changes and extreme heatwaves that cause short periods with high temperature peaks. This happens especially in regions with desert and continental-like climates such a Mediterranean countries (Mesquita *et al.*, 2013; WHO, 2017), but as a consequence of climate change, these changes could be greater, longer, and they could happen in other climatic zones.

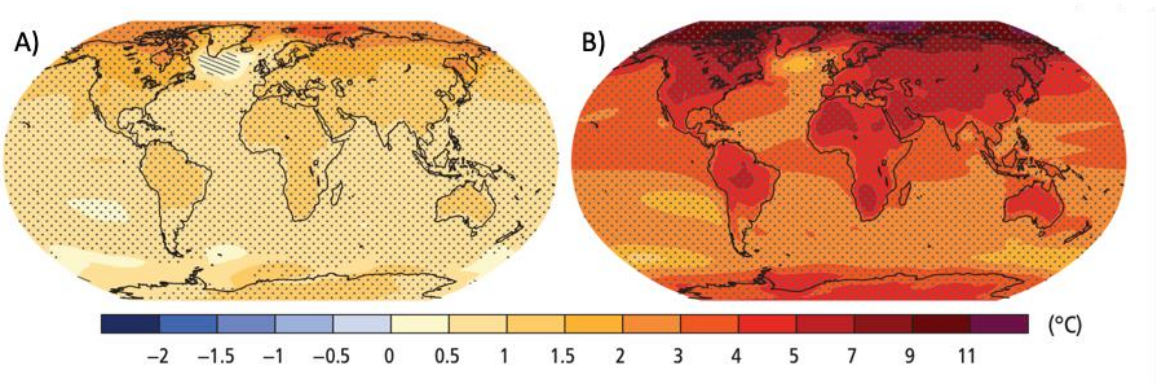



***Figure 4***. Projected change in average surface temperature (1986−2005 to 2081−2100) based on two different scenarios: A) low greenhouse emissions scenario and B) very high greenhouse emission scenario. Adapted from IPCC (2014).

The increase in ambient temperatures due to global warming will affect DWDS temperatures, since it has previously been demonstrated that both are positively related (Blokker and Pieterse-Quirijns, 2013). Thus, temperature increase can modify processes occurring in DWDS, affecting the physico-chemical parameters, biological processes, or endothermic reactions such as solubilisation, degradation, evaporation or dissolution, and therefore affect the integrity of DWDS and the supply of safe drinking water worldwide. For example, it has been demonstrated that the amount of disinfectant residual, which is used to limit growth of microorganisms, decrease at higher water temperatures and can affect the water quality allowing their growth (Hua *et al.*, 1999; Li *et al.*, 2018). Other parameters such as dissolved oxygen (DO) also decrease at higher water temperatures, which can compromise the water quality since DO depletion encourages the microbial reduction of nitrate to nitrite and sulphate to sulphide (Fisher, Kastl and Sathasivan, 2012; WHO, 2017). Conversely, other chemical reactions and microbial growth are generally accelerated when the temperature increase (Delpla *et al.*, 2009; Ashofteh, Rajaee and Golfam, 2017; Oreskes, 2018). In addition to increasing microbial growth rate, temperature could also shape the ecology of microbial communities in DWDS, producing changes in its structure and composition. For example, in marine aquatic environments microorganisms tended to form biofilms to a greater extent at higher temperatures (Kent, Garcia and Martiny, 2018), which in DWDS could lead to an increased risk of discolouration and an increase in all other biofilm-related problems. Moreover, in different freshwater ecosystems it has been demonstrated that temperature can favor the proliferation of microorganisms and even of specific species, including pathogens, opportunistic pathogens and viruses, which can compromise the safety of the water supplied (van der Wielen and van der Kooij, 2013; Pandey *et al.*, 2014). These changes in the DWDS microbiome can affect function and operation of currently DWDS as well as management practices to maintain these systems and optimum water quality. This will force water companies to select and/or modify the effective cleaning strategy to maintaining the biological safety of the water that reaches consumers' taps (e.g. add more disinfectant or apply more frequent flushing regimes to clean the pipes). Therefore, understand the consequences of global warming on the distribution of drinking water and its impact on water quality and safety is essential to protect public health.

### 2.6.2 Drinking water availability – Intermittent water supply

Currently, the availability of safe drinking water is an important problem in many parts of the world. The scarcity of water sources, the difficulty of access, deficiencies of DWDS infrastructures, the increase in water demand due to population growth, changing economic activity, land-use change and urbanization, and/or the high energy and economic cost necessary to distribute water are the main causes of drinking water being a limiting resource (Lee and Schwab, 2005; Simukonda, Farmani and Butler, 2018). Climate change is expected to aggravate these processes since it is currently impacting the hydrological cycle. Consequently, the frequently and severity of extreme weather events are increasing, including heavy rainfalls, floods, changes in water runoff or groundwater flows, or droughts (Bates *et al.*, 2008; Delpla *et al.*, 2009; Ashofteh, Rajaee and Golfam, 2017). This will lead to an increase in water stress worldwide, which according to European Environment Agency (www.eea.europa.eu) "occurs when the demand for water exceeds the available amount during a certain period of time or when poor water quality restricts its use" (Arnell and Delaney, 2006; Ashofteh, Rajaee and Golfam, 2017).

Intermittent Water Supplies (IWS) is a common practice adopted in some parts of the world to try to reduce the per capita drinking water demand and solve the water stress (Vairavamoorthy, Gorantiwar and Mohan, 2007). During IWS, DWDS do not supply water for different periods of time and/or the supply times are shortened. This is especially recurring in low-income countries where resources and infrastructures are limited and IWS is the only way to access water for millions of people (Kumpel and Nelson, 2016). However, the application of IWS is increasing in different parts of the world including more economically developed countries, because the consequences of climate change are aggravating the scarcity of water resources (Table 2). Currently, the WHO estimates that one-third of the world population is under IWS. The duration of IWS may range from few hours eventually or daily, to full days without supply as it happens in countries like India, Kenya or Bangladesh are currently operating severe intermittency water services (Table 2, Figure 5) (WHO and UNICEF, 2000; Kumpel and Nelson, 2016; WHO, 2017).

***Table 2***. Number of countries reporting IWS, population with IWS and the average and range of supply duration. Adapted from Kumpel and Nelson (2016).

| | **Countries** | **Population with IWS** | **Supply duration** | |
|---|---|---|---|---|
| | IWS/total | Millions | Average hours | Range |
| East Asia and Pacific | 9/32 | 15.0 | 16.7 | 1 – 23 |
| Europe and Central Asia | 17/41 | 25.4 | 13.0 | 0.2 – 23.7 |
| Latin America, North America and Caribbean | 8/21 | 28.4 | 16.0 | 2 – 24 |
| Middle East and Northern Africa | 1/2 | 4.6 | 3.0 | 3 – 3 |
| South Asia | 5/6 | 116.6 | 7.2 | 0.3 – 23 |
| Sub-Saharan Africa | 19/40 | 118.8 | 12.8 | 1 – 23.5 |
| Total | 59/142 | 308.9 | 12.5 | 0.2 – 24 |

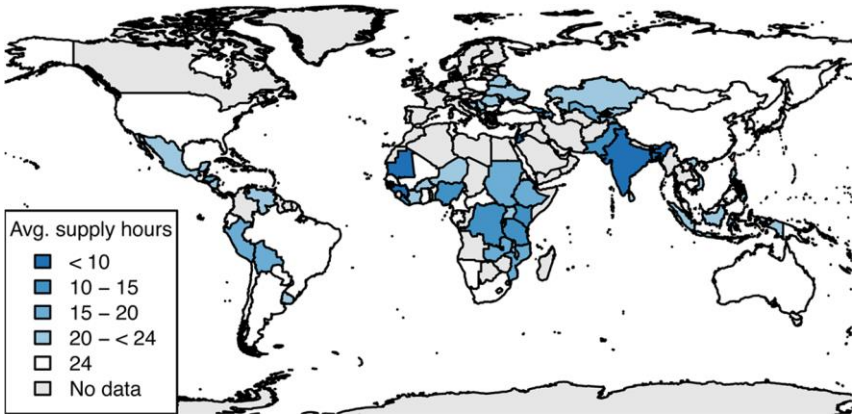



***Figure 5***. Average hours of water supply in the world. Taken from Kumpel and Nelson (2016).

Although IWS is a prevalent action around the world in conditions of scarcity or unavailability of water, this practice has been shown to have a large range of negative consequences (Christodoulou and Agathokleous, 2012). Firstly, IWS is related to damage to the infrastructure of the distribution networks, that can pose a challenge for the water utilities. It has been observed that the infrastructures deteriorate faster than in a system that operates under a continuous supply: there is a greater wear of the valves of the system; locating leaks or breaks in the pipes becomes more difficult; normally large deposits or storage tanks, and their consequent maintenance, are necessary; or an increase in disinfectant levels is necessary if the retention time in the systems increases (Charalambous, 2012; Christodoulou and Agathokleous, 2012; Ilaya Ayza, 2016).

Moreover, IWS has critical consequences and implications on the quality of the water that reaches consumers and therefore for public health, that are mainly associated to microbial presence of these systems (Kumpel and Nelson, 2016; Bivins *et al.*, 2017). When the water supply is turned off and an IWS event happens, different processes that can modify microbial communities and affect water quality and safety may occur:

(1) Loss of pressure. When supply stops the pressure of the system decays resulting in negative pressures in the pipes which can generate the intrusion of surrounding environmental contaminants from groundwater or sewage via leaking or junction points between pipes (Lee and Schwab, 2005; Vairavamoorthy, Gorantiwar and Mohan, 2007).

(2) Complete or partial emptying of pipes. Water inside the pipes increases its residence times before the system is completely emptied. Longer residence times in DWDS produce the decay of disinfectant that is used to limit microbial growth, thus favouring their proliferation. Longer residence times due to IWS could also generate more easily removable biofilms, since it has been observed that biofilms developed under low shear stress conditions have a less cohesive structure and detach more easily from the surface when shear stress changes (Manuel, Nunes and Melo, 2007; Douterelo, Sharpe and Boxall, 2013). In addition, if the pipes are completely drained, biofilms can experience dry periods that can affect its structure, as it happens to biofilms of other freshwater systems (Timoner *et al.*, 2014). Therefore, IWS events in DWDS can potentially lead to increase the growth rate of biofilm, change the structure of these communities and/or make them more easily detachable.

(3) System refilling and water supply restarting. During the process of supply restarting the system is refilled producing increases in water flows that can mobilise biofilm from the pipe walls into the bulk water (Kumpel and Nelson, 2016). Thus, this flushing effect in IWS when the water supply is restarted may have great potential water quality implications, for example increase the water discolouration and include microbial biomass and particles such a metals or inorganics into the final tap water.

Although there is evidence that the use of IWS has negative implications for water quality and its occurrence is increasing worldwide due to climate change, limited studies exist about this practice. Extensive research is necessary to determine what the risks of the IWS are, especially those related to microbiology of DWDS.

# Chapter 3

# Methodology

## 3.1 Experimental test loop facility

To develop this research and achieve aim and objectives, a full scale experimental DWDS located in The Civil and Structural Engineering Department of The University of Sheffield (Sheffield, UK) was used. This facility allows overcoming the limitations related to studying real DWDS. Studies in real systems are scarce because the difficulty of accessing and manipulating samples in real operational systems. In addition, most of previous microbial studies about DWDS were performed under idealised conditions using small scale bench reactors, staggered pipelines and biological inoculations, which do not reproduce the conditions of real DWDS and do not allow an accurate knowledge of the factors that affect the formation of biofilms or their composition (Deines *et al.*, 2010). Therefore, this facility overcomes the major barriers for DWDS studies, since it allows for representing the conditions within real pipe networks and enable the effects of abiotic factors to be explored in a controlled environment (Deines *et al.*, 2010; Gomes, Simões and Simões, 2014).

The facility is comprised of three loops of 9.5 x 21.4 m long coils of High-Density Polyethylene (HDPE) pipe (PE100 SDR17), with approximately a total length of 200 m (Figure 6). The internal diameter of the pipeline is 79.3 mm, except a pipe section of approximately 9 meter in each loop with a 50 mm of internal diameter, into which flow meters are fitted (pale blue pipe seen in Figure 6A). The system is usually run as three individual loops that are isolated using a series of manual valves. However, it is possible to open these valves and run it as a single large loop (Figure 6C). The system is fed with drinking water from the local DWDS, which has an upland peat runoff surface water source and is treated with chlorine. Drinking water is distributed and enters the building housing the experimental facility from a cast iron trunk main. There is no local DMA in place. Local drinking water feds individual enclosed reservoir tanks of an approximate capacity of 0.486 $m^3$, which are connected to each loop. From each tank, water can be re-circulated in each loop at different flow rates and pressure using individual speed pumps and several controlled valves installed throughout the

system (Figure 6B). In order to maintain baseline water quality parameters and preserve nutrient supply and disinfection residual, among other water quality parameters, the system retention time of the water is 24 hours, controlled with a trickle drain and feed-based system into the tanks. Schematic diagram of the DWDS experimental facility and images with details of the test loop facility are shown in Figure 6 and Figure 7.


***Figure 6.*** A) Full scale experimental DWDS facility; B) Idividual tanks and pumps of each loop; c) Manual valves and turbidity meters installed in the facility; D) Chorine meters and and turbidity meters installed in the facility.

The facility is within a temperature-controlled room and each tank has also its own immersion heater. This allows controlling and maintaining the temperature of the water entering the system and within it, as well as the water temperature in the facility during the experiments. Each loop of the experimental facility also includes other several monitoring equipment installed *in situ:* ATi A15/76 turbidity meters (ATi, Delph, UK), ATi Q45H-79 chlorine meters (ATi, Manchester, UK), pressure transducers (Gems™ Sensors and Controls, US) and flow meters (Flownetix, UK) (Figure 6A,C,D; Figure 7). All of these monitors provide and record continuous measurements thought the duration of the experiments of flow, pressure, turbidity, chlorine and temperature.

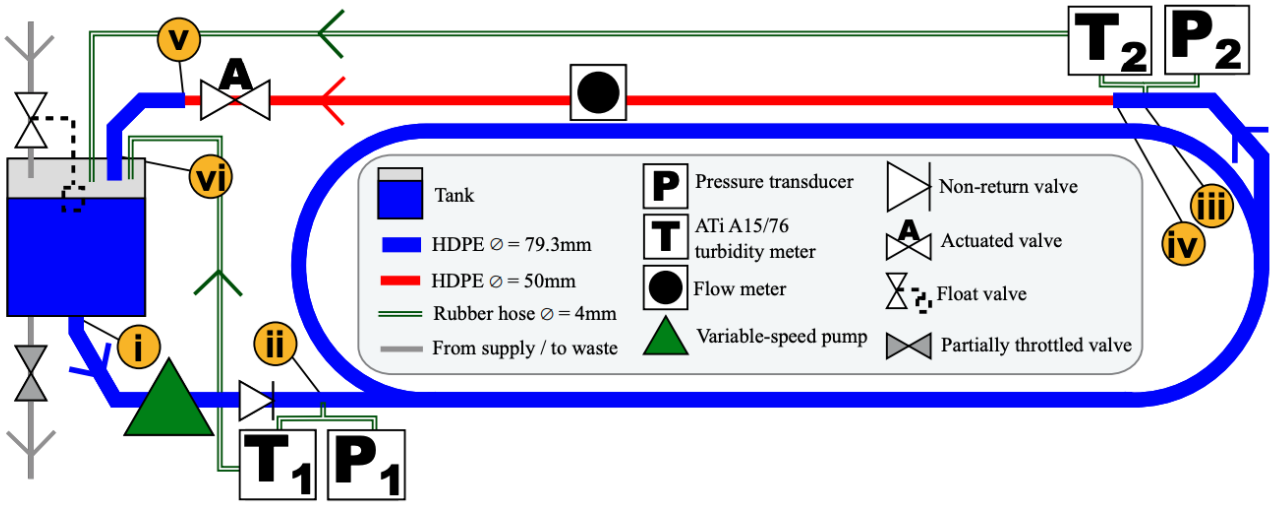



***Figure 7***. Schematic diagram of each pipe loop. Taken from Furnass *et al.* (2014) .

LabVIEW (version 8.2, National Instrument Corporation, UK) installed in a central computer is used to control pump and valve settings, enabling the simulation of real hydraulic demand patterns during the tests. For this research, a low varied flow (LVF) was applied for all experiments allow for biofilm development in the pipe walls (growth phase). LVF ranges from 0.2 to 0.5 L/s and is based on daily patterns observed in DWDS in the UK (Figure 8, Table 3) (Husband, Boxall and Saul, 2008).

In addition to simulating the hydraulic demands patterns of real systems, the facility allows to perform flushing trials in the test loop facility to investigate the mobilisation of material from the pipe wall into the bulk water. This mobilisation phase is based on the cohesive layer theory of PODDS model, which suggests that "layers of particulate material with a defined profile of shear strength properties accumulate continuously on pipe walls and are conditioned by hydraulic shear stress forces" (Husband and Boxall, 2010). Therefore, the mobilisation phases used in this research was

designed to use a series of 4 gradual steps of increasing shear stresses by flushing with an incrementally increasing flow rate (Figure 9). Flow increases were based on previous work in the same facility and their conversion rates shown in Table 3 (Husband, Boxall and Saul, 2008; Sharpe, 2012; Sharpe, Biggs and Boxall, 2017). For this, the flow rate measurements provided by online flow meters installed in the facility were converted to boundary shear stress by a calibrated standard curve (Figure 10). Each step is run 3 turnovers, i.e. the time that the total volume of the water needs for recirculate in the loop three times in order to provide enough time for mix and detect all material mobilised into the water (Sharpe *et al.*, 2010; R. Sharpe, 2012).

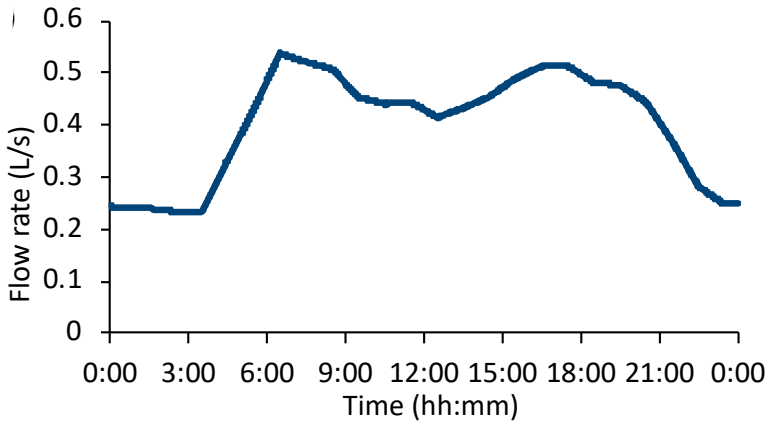



***Figure 8***. Low varied flow profile used during the growth phases which simulates a real hydraulic regime based on daily patterns observed in real DWDS in the UK (adapted from Husband, Boxall and Saul, 2008).

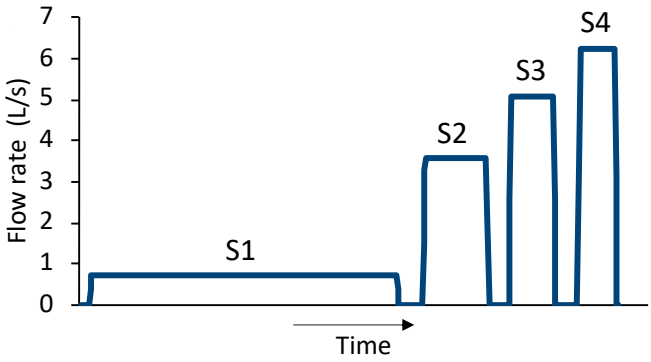



***Figure 9.*** Flushing gradual steps (S) applied in this research for a duration of 3 turnovers.

***Table 3.*** Flow rate conversions of the LVF applied during the growth phases in the experiments, and in each stage of the flushing steps.

| | Flow (Q) - (L/s) | Velocity (v) - (m/s) | Shear stress (τ) - ($N/m^2$) |
|---|---|---|---|
| Growth phases | 0.2 - 0.5 | 0.04 – 0.10 | 0.1 – 0.3 |
| Step 1 Flushing | 0.74 | 0.15 | 0.4 |
| Step 2 Flushing | 3.58 | 0.72 | 2.3 |
| Step 3 Flushing | 5.10 | 1.03 | 3.4 |
| Step 4 Flushing | 6.24 | 1.27 | 4.3 |

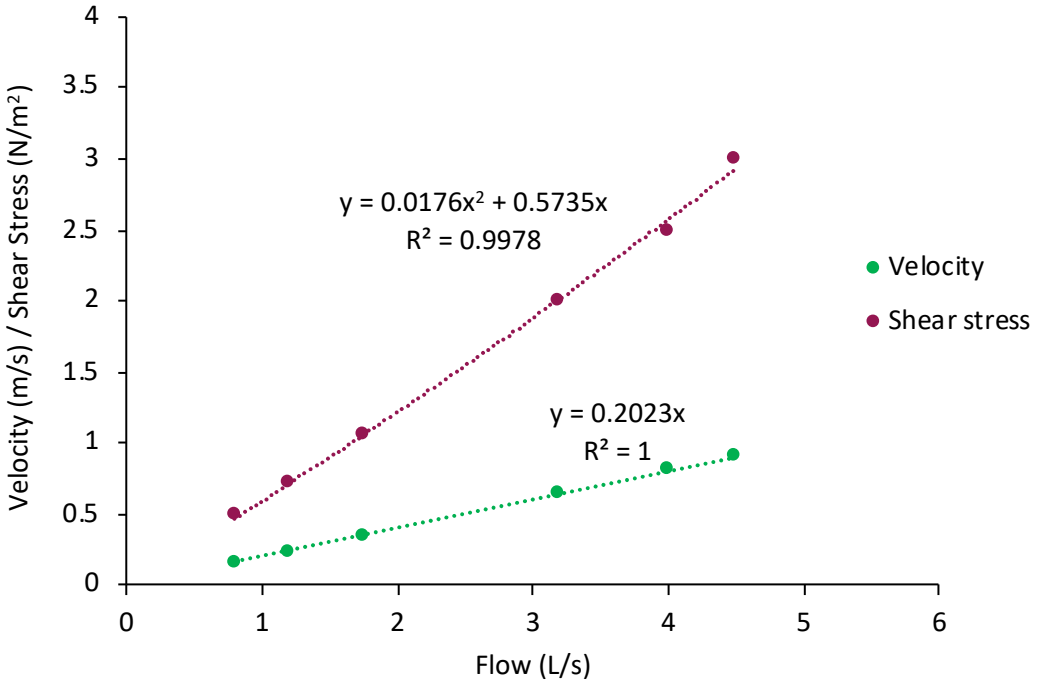



***Figure 10.*** Standard curves used for velocity and boundary shear stress calculations. Based on data published by Sharpe, Biggs and Boxall (2017).

Before all experiments, the entire system is filled and disinfected adding 20 mg/L of RODOLITE H (RODOL Ltd, Liverpool, UK), which is a solution of sodium hypochlorite with less than 16 % free available chlorine. The water with the RODOLITE H is recirculated at maximum flow rate (4.2 L/s) to flushing the pipes and left standing for 24 h with the disinfectant to clean the whole systems. Afterwards, the facility was filled and flushed again at 4.2 L/s with fresh tap water until the levels of free chlorine were similar to the fresh water entering the system (≈ 0.20 mg/L).

### 3.1.1 Coupons and removable pipe sections

In addition to simulating real conditions of DWDS and controlling all the experimental conditions, the other great advantage of the facility used in this research is the possibility of taking biofilm samples *in situ*. For this purpose, the facility has two different types of equipment:

⇒ **Coupons**

Each loop includes two pipe coils where it is possible to insert 2 x 27 removable HDPE Pennine Water Group (PWG) coupons (54 coupons per loop in total). Coupon consists of a portion of pipe of a total surface area of 314 $mm^2$ which fit with the internal surface and accurately following the pipe curvature, thus experiencing the same environment as the rest of the pipeline with a minimal impact on the system hydraulic as it is described by Deines et al. (2010). Coupons are fixed into the pipes with holders and clips as is showed in Figure 11, following a patter that enabled the investigation of biofilms around the entire pipe (top, medium and bottom positions) (Figure 12). The use of coupons has the advantage that the system does not need to be drained for the sampling, and the water continues in the loops. This makes the impact of sampling on the biofilm minimal, allowing for sampling campaigns at any time throughout the experiment. Coupons taken as samples are replaced with sterile coupons (see section 3.3 for sterilisation protocol).

***Figure 11.*** Coupons inserted and fixed into the pipes with holders and clips.

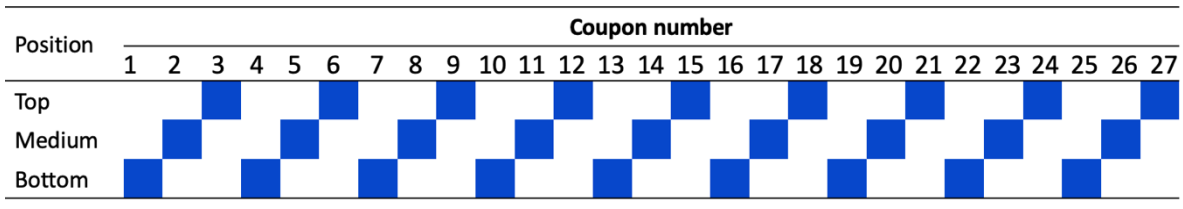



***Figure 12.*** Coupon location around the pipeline. Numbers indicate the coupon placement along the length of the pipeline and they are used for sample identification. Adapted from Test Loop Facility Manual (2014).

Each coupons is comprised of two parts making possible simultaneous visual and molecular analysis of biofilms developed on the pipe walls: (1) the outer coupon used to collect biofilm biomass for DNA-based analysis; and (2) an insert that can be removed from the coupon body for microscopy analysis (Deines et al., 2010). Details of the coupon's dimensions are shown in Figure 13.

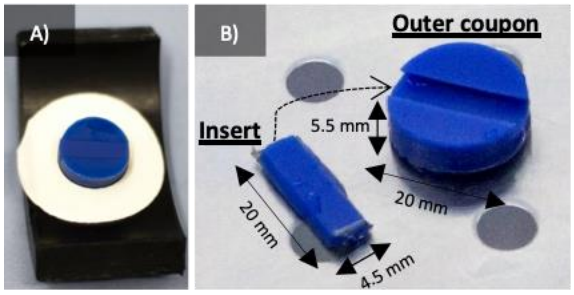



***Figure 13***. A) Coupon fixed into a mounting piece (black piece) with a circular rubber gasket in between (white piece) to ensure a watertight fit into the pipelines. B) Details and dimensions of the coupons: outer coupon surface area = 244 $mm^2$, and insert surface area = 90 $mm^2$.

⇒ **Removable pipe sections**

Instead of pipe coils with coupons, in each loop it is also possible to install 6 removable HDPE pipe sections of 0.5 m long (total surface area of 0.25 $m^2$) (Figure 14). These removable sections are designed with the aim of obtaining a larger biofilm surface. However, before sampling a section of pipe the system needs to be drained, and therefore sampling campaigns can only be carried out at the end of the experiments. In addition, it is necessary to control the drainage flow to avoid biofilm disturbance and removal from the pipe walls. A constant flow of 0.06 L/s, flow below the minimum of the LVF profile has been shown to be optimal for draining the loops.

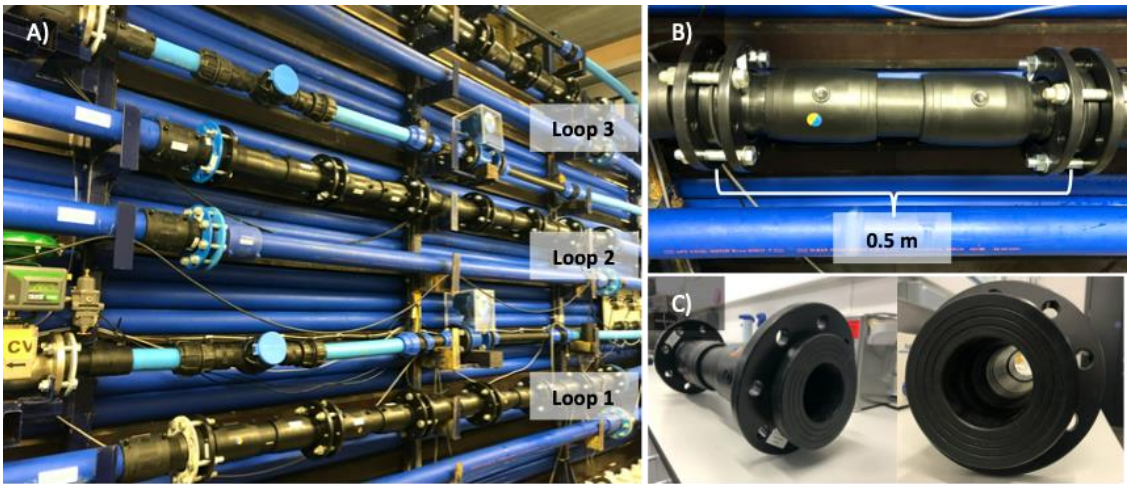



***Figure 14***. A) Pipe section inserted into the 3 loops; B) Detail of the section fixing system; C) Detail of the inside of a section.

## 3.2 Microbial communities sampling

### ⇒ Biofilm samples

For biofilm communities analysis using coupons, 3 biological replicates of 3 PWG coupons each (top, medium and bottom) were taken in each sampling point to obtain a representative sample of the entire pipe, as well as an optimal concentration of DNA for Next Generation Sequencing (NGS) if molecular analysis was carried out. Biofilm was removed from coupons following a standarised protocol and was suspended in 30 mL of phosphate-buffered saline (PBS) (Gibco®, Thermo Fisher Scientific, UK) solution using petri dishes and sterile nylon brushes (Sharma, Bhosle and Wagh, 1990; Deines *et al.*, 2010). For each coupon, 30 brushed were performed in each direction, rinsing the nylon brush in the PBS after every 10 brushes (Figure 15). Biofilm suspension was filtered through a 0.22 µm nitrocellulose membrane filters (Millipore Corp., USA) (Deines et al., 2010) and using a Microstat membrane filtration unit (Sartorius, UK). Filters were preserved in the dark and at - 20 °C prior to DNA extractions.

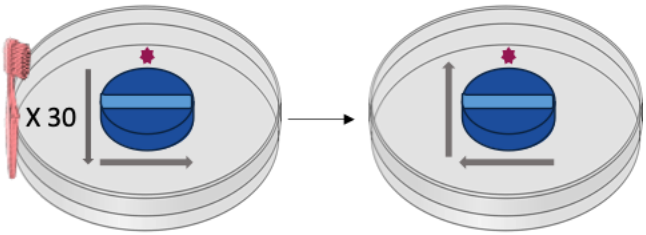



***Figure 15***. Standardised brushing for coupons: brush the coupon 30 times in each direction indicated by the arrows while holding the coupon with the sterile forceps at the point marked by the red dot. After every 10 brushes rinse the nylon brush in the PBS.

To obtain biofilm samples using the removable pipe sections, 500 ml of PBS (Gibco®, Thermo Fisher Scientific, UK) and a sterile nylon brush were used (Sharma, Bhosle and Wagh, 1990). For each end of the section, 50 ml of PBS were introduced four times (one for each quarter of the lateral surface) and standardized brushing was performed to remove the attached biofilm. Standarised brushing consist on brush the quarter of the pipe section 30 times in each direction: right, left in and out (Figure 16). After every 10 brushes, the brush was rinsed in the remaining 50 ml of PBS. Then, the two 250 ml biofilm suspensions from each end were pooled and filtered through a 0.22 µm nitrocellulose membrane filters (Millipore Corp., USA) and preserved in the dark at - 20 °C for subsequent DNA extraction.

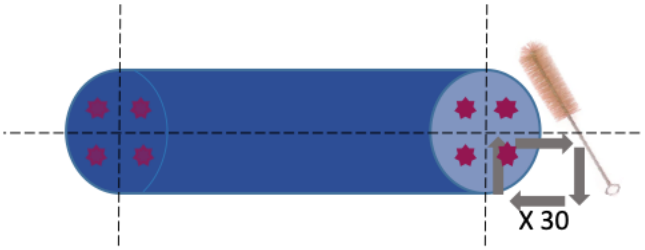



***Figure 16***. Standardised brushing for pipe sectoins: brush each quarter of each end of the section for 30 times in each direction indicated by the arrows (right, left, tin and out). Repeat in each quarter marked by the red dot. After every 10 brushes rinse the nylon brush in the PBS.

⇒ **Water samples**

To study planktonic communities, triplicates of water samples from each loop were taken in each sampling campaign. Samples were taken in polyethylene bottles of 10 L previously disinfected with virkon disinfectant powder (Sigma Aldrich, UK) for 24 hours and then rinsed with sterilised water. For small volume water (1 – 10 L) samples were filtered through 0.22 µm nitrocellulose membrane filters (Millipore Corp., USA) using a Microstat membrane filtration unit (Sartorius, UK). For larger volumes of water (> 10 L) cells in the bulk water samples were concentrated using a Tangential Flow Filtration (TFF) system following the manufacturer's guidelines (PALL Life Science, New York, USA) (Schwartz and Seeley, 2002) and then filtered through 0.22 µm nitrocellulose membrane filters (Millipore, Corp). Filters were preserved in the dark at - 20 °C for subsequent DNA extraction.

## 3.3 Material sterilisation

Before use, PWG coupons and nylon brushes were sterilized according to the following protocol: coupons and/or nylon brushes were placed in a sonicating water bath for 45 minutes with a 2% (w/v) sodium dodecyl sulfate (SDS) solution. Then, materials were sonicated for 15 minutes in distilled deionised water and they were allowed to air dry in a laminar flow cabinet. To finish, coupons and nylon brushes were autoclaved (Backhus *et al.*, 1997; Buss, Brantley and Liermann, 2003).

## 3.4 Water physicochemical analysis

During the experiments key water physicochemical parameter were analysed to obtain information of the inner pipe environment, depending on the requirements of the experiment. Turbidity and chlorine were continuously measured and recorder online at 1-minute intervals by the equipment

installed in the test loop facility, an ATi A15/76 turbidity monitor (ATi, Delph, UK) and an ATi Q45H-79 chlorine monitor (ATi, Manchester, UK) respectively. Additionally, triplicates of discrete water samples (n = 3) were collected at the time of each sampling event to analyse other physico-chemical parameters by handheld equipment. These include, pH and water temperature measured by using a Hanna portable meter HI 991003 (Hanna Instruments, Leighton Buzzard, UK) and free and total chlorine analysed with a Palintest CS100 chlorosense (Palintest, UK). Measurements were performed following the manufactures' protocols.

Other important parameters for water quality were measured, including iron (Fe) and manganese (Mn) concentration. The concentration of these metals were tested together with turbidity as indicators of water discolouration, since it has been observed that the fine particles of Fe/Mn are the main compounds that can cause discolouration in DWDS (Seth *et al.*, 2004; Husband, Boxall and Saul, 2008). For this research Fe and Mn concentrations were determined by the chemistry laboratories by The Kroto Research Institute (KRI) (The University of Sheffield, UK), by means of Inductively Coupled Plasma Mass Spectrometry (ICP-MS) analysis. Water samples were collected in 20 mL vials containing 5 M of nitric acid and then ions were monitored on a Perkin Elmer Elan DRC II (PerkinElmer, Inc., USA) (Sloetjes and Wittenburg, 2008). The system is equipped with nickel sampler and skimmer cones, a quartz torch and injector, a Meinhard concentric glass nebulizer, and a cyclonic spray chamber. A Cetac ASX-150 autosampler was used for samples introduction (Thermo Fisher Scientific, England), with 4 lines peristaltic pump set at 20 rpm (one line was used for the addition of the internal standard, $^{103}$Rh). The operating conditions are shown in Table 4. Data acquisition was via Elan NT software (tla.mpi.nl/tools/tla-tools/elan) (Sloetjes and Wittenburg, 2008).

***Table 4.*** Optimized operating Conditions for the ICP-MS.

| **Operation** | **Condition** |
|---|---|
| RF- power | 1150 watts |
| Plasma Gas Flow | 15 L/min |
| Nebulizer Gas Flow | 0.85 L/min |
| Auxillary Gas Flow | 1.20 L/min |
| Lens voltage | 8.25 V |
| Analog Stage Voltage | - 1850.00 V |
| Pulse Stage Voltage | 1500.00 V |
| Vacuum | $1x10^{-7}$ Torr |
| Acquisition Dead Time | 55 ns |

| | |
|---|---|
| Current Dead Time | 55 ns |
| Standard sweep and calibration range (element/mass): | |
| Fe 56.9 | 10 - 500 µg/L (ppb) |
| Mn 54.9 | 1 - 50 µg/L (ppb) |

Several analytical methods were also used in the experiments of this research to characterise Natural Organic Matter (NOM). NOM has been related to taste and odour problems and plays an important role as a precursor of DBPs, harmful compounds for human health that favour the development of diseases such as cancer, and thus strongly impacting the water quality (Matilainen *et al.*, 2011; Ibrahim and Aziz, 2014; WHO, 2017; Tsagkari and Sloan, 2019). Total organic carbon (TOC) and dissolved organic carbon (DOC) were used to quantify the amount of NOM, while the ultraviolet absorbance at 254 nm wavelength ($UVA_{254}$), the specific ultraviolet absorbance (SUVA) and the fluorescent properties of NOM were analysed to determine the nature and reactivity of the NOM content.

TOC and DOC were analysed by the analytical chemistry laboratories by KRI (Sheffield, UK). Water samples for both parameters were stored in 20 ml glass vials, and samples for DOC analysis were previously filtered thought 0.45 µm sterile filters to remove the particulate organic carbon (POC) (Karanfil, Erdogan and Schlautman, 2003). Then, samples were analysed using a Shimadzu TOC-$V_{CPH}/_{CPN}$ Analyzer (Shimadzu, Kyoto, Japan) following the manufacturer's protocol: samples were heated at 680 °C in an oxygen-rich environment inside combustion tube with platinum catalyst, and the total carbon (TC) of the sample is burned to form carbon dioxide. The carbon dioxide generated by this catalytic oxidation was detected using an infrared gas analyzer (NDIR). Then, after acidifying the sample (pH ranged 2-3), sparge gas was bubbled through the sample to eliminate the inorganic carbon (IC) component. The total carbon remaining in the sample was measured to determine the amount of carbon in the sample.

The $UVA_{254}$ of the water samples was quantified using a spectrophotometer (DR5000, Hach, USA). $UVA_{254}$ together with DOC was used to calculate SUVA, which is the average absorptive capacity of DOC molecules of a water samples, and it is used as a measure of DOC aromaticity (Weishaar *et al.*, 2003). For each water sample SUVA was calculated as the $UVA_{254}$ in a water sample normalized for DOC concentration:

$$SUVA_{\left(\frac{L}{mg-m}\right)} = \frac{UVA_{254\ (cm^{-1})}\ x\ 100}{DOC_{\ (MG/L)}}$$

Fluorescent properties of NOM were obtained via 3D-excitation-emission matrix (EEM), using a spectrofluorophotometer (RF-5301PC, Shimadzu, Japan) in Civil and Environmental Engineering Department of Sejong University (Seoul, South Korea). The EEM were measured every 10 nm over an excitation (ex) wavelengths from 220 to 400 nm, with an emission (em) range from 280 to 600 nm at 1 nm intervals. The fluorescence profiles monitored were as shown in (Figure 17): T1 peak and T2 peaks for protein-like material (ex 220-240 nm and em 330-360 nm, ex 270-280 nm,and em 330-360 nm, respectively), and A and C peaks for humic-like components (ex 230-260 nm, and em 400-450 nm, ex 300-340 nm, and em 400-450 nm) (Park *et al.*, 2016) (Figure 17). EEM contour plots were generated by utilising MATLAB 7.8 software (Mathworks, Natick, MA, USA).

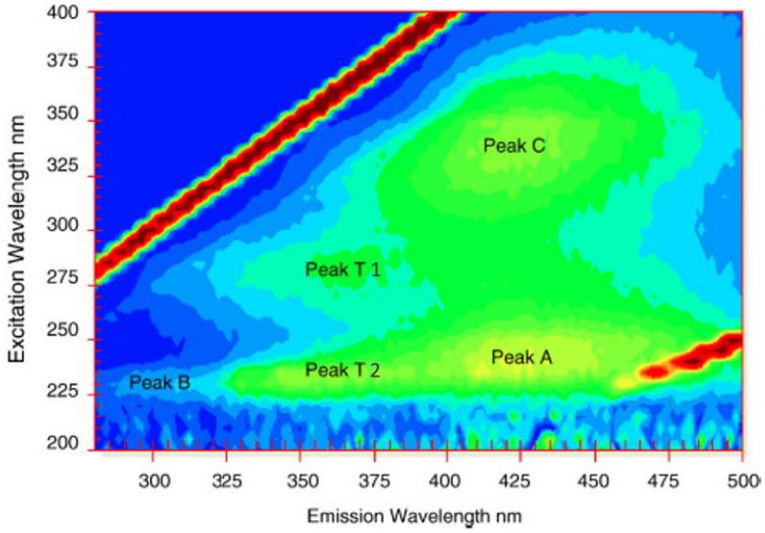



***Figure 17.*** Example of specific regions to determine organic matter characteristics. Regions T1 and T2 (protein-like substances), and regions A and C (humic-like substances) are shown. Extracted from Okache, Haggett and Ajmal (2015).

## 3.5 DNA extraction and purification protocol

DNA from all the filters where water and biofilm samples were concentrated, was extracted following the protocol based on hexadecylmethylammonium bromide (CTAB) and proteinase K chemical lysis, followed by DNA purification using phenol/isoamyl alcohol method (Zhou, Bruns and Tiedje, 1996; Neufeld *et al.*, 2007). This method provided an optimal DNA concentration and quality for both biofilm and water samples:

1) Filters were place into 15 mL sterile Falcon tubes.
2) 740 µL of SET lysis buffer and 90 ml of lysozyme 10 mg/mL (Sigma Aldrich Co.,UK) were added. SET lysis buffer consists of 40 mM EDTA (Ambion, Warrington, UK), 50mM Tris-HCl pH 9 (Sigma Aldrich Co., UK.) and 0.75 M sucrose.
3) Tubes were incubated at 37 °C for 30 minutes with rotation in a Hybaid hybridisation oven (Thermo Scientific, UK).
4) 90 mL of 10 % sodium dodecyl sulphate (SDS) (Sigma Aldrich Co., UK.) and 25 mL of proteinase K 20 mg/mL (Applied Biosystems, Life Technologies Ltd., UK) were added.
5) Tubes were incubated at 55 °C for 2 hours with rotation.
6) Lysates (i.e. supernatant) were withdrawn and transferred into 2 mL sterile Eppendorf tubes.
7) 137 µL 5M NaCl 1 % and 115 µL Hexadecyltmethyl ammonium bromide (CTAB) / NaCl solution (Sigma Aldrich Co., UK) were added to the
8) Tubes were incubated at 65 °C for 30 minutes with rotation.
9) In a fume hood, 838 mL µL of chloroform:isoamyl alcohol (24:1) (Sigma-Aldrich, UK) were added and the mix was centrifugate at maximum RPM for 5 minutes. The supernatant was extracted into a clear 2 mL tube and repeat the process.
10) DNA was precipitated adding 815 µL of 100 % isopropanol (Sigma-Aldrich, UK) and leave standing at -20 °C overnight.
11) Centrifugate the samples at maximum RPM for 30 minutes.
12) In a fume hood, the supernant was removed keeping the pellet (DNA).
13) DNA was washed twice with 1 mL of 70 % ethanol. To decant the ethanol, centrifugate at maximum RPM 10 minutes.
14) DNA was air dried and resuspended in 50 µL DEP-treated sterile water (Thermo Scientific, UK).

The concentration of purified DNA from each extraction was assessed fluorometrically by the HS dsDNA Assay kit and a Qubit 4 Fluorometer (Invitrogen by Thermo Fisher Scientific, Wilmington, USA) following the manufacturer's protocol.

## 3.6 Next Generation Sequencing

Traditionally most of the microbial research about DWDS have been based on studying microorganisms using culture-based methods, such as heterotrophic plate counts or fecal indicators (e.g., *Escherichia coli* or coliforms) (Douterelo *et al.*, 2014). In addition, culturing methods are the most used methods by water utilities as diagnostic tool since they have a relatively low cost, they are easy to perform and they are the only microbiological parameters that the legislation forces to control (Liu, Verberk and Van Dijk, 2013; Isabel Douterelo *et al.*, 2016). However, cultured-based techniques are only able to identify those microorganisms that can be grown under laboratory conditions in a certain type of nutrient media or environmental conditions. It is estimated that culture-based methods can only represent less than 1 % of the total microbial diversity in any given environmental sample (Riesenfeld, Schloss and Handelsman, 2004; Mutlu and Güven, 2015). In the last two decades, cultivation-independent molecular techniques have been developed that are aiding to overcome the limitations of culturing. The biggest change has come from advances in DNA-based molecular typing techniques such as sequencing, i.e. the process of determining the order of deoxyribonucleotides bases (dNTPs: adenine (A), thymine (T), guanine (G) and cytosine (C)) within a molecule of DNA, which determines the genetic code (DNA barcoding) (Yee and Tapani, 2017). Therefore, metagenomic sequencing allows the study of the entire genetic material contained in an environmental sample, including the uncultured microbial component (Sanschagrin and Yergeau, 2014; Garza and Dutilh, 2015).

The main question in microbial ecology is “who is there?” and DNA sequencing has been demonstrated to be an excellent tool to answer this. For this, molecular markers have been widely used in microbial ecology for phylogenetic studies (Sanschagrin and Yergeau, 2014). Molecular markers have regions highly conserved among phylogenetic groups and other hypervariable regions that can be used for species diversity identification (Chakravorty *et al.*, 2007; Liu *et al.*, 2012; Badotti *et al.*, 2017). For this research, the molecular marker-gene selected were 16S ribosomal RNA (rRNA) gen and the Internal Transcribed Spacer (ITS) nuclear ribosomal DNA (nrDNA) regions 1 and 2, for bacteria and fungi respectively. These were selected because bacteria are the most abundant group of microorganisms in these systems, and fungi have been observed to be key in a large number of processes that can affect water quality and safety (Zhou *et al.*, 2017; Douterelo, Fish and Boxall, 2018).

Traditionally DNA sequencing was performed using methods such as cloning, or Sanger sequencing of PCR amplicons based on capillary electrophoresis (Anderson and Schrijver, 2010). However, molecular advances in recent years have allowed the development of new technologies such as the next-generation sequencing (NGS). NGS is faster, provides greater sequencing depth and allows a much higher sequencing volume, therefore massive parallel studies can be carried out. In addition, it is much more economically effective (Sanschagrin and Yergeau, 2014). Illumina platform, the most used NGS platform worldwide (Schirmer *et al.*, 2015; Y. Lu *et al.*, 2016) was used in this research for samples sequencing. The samples in this study were sequenced using Illumina MiSeq® pair-end technology (www.illumina.com/systems/sequencing-platforms/miseq). This massively parallel sequencing platform allows high throughput sequencing and provides up to read 250 million reads of up to lengths up to 2 × 300 base pairs (bs) (Caporaso *et al.*, 2012; Ravi, Walton and Khosroheidari, 2018).

After the isolation of genomic DNA, Illumina technology workflow includes the NGS library preparation and the sequencing process. Library preparation consist of generate a collection of DNA fragments to which specialised adapters for Illumina to both fragment ends are ligated (Figure 18a). The adapters include the sequencing primer binding sites and the sites that allow DNA fragments to attach to the Illumina flow cell channels (solid support). If several sample libraries are pooled together and sequenced simultaneously during a single run on Illumina instruments (known as multiplex sequencing), individual barcode or index sequences are ligated to each DNA fragment for its identification. After adapter ligation, DNA fragments with adapters are amplified by the PCR technique and desired DNA fragments are separated by agarose gel electrophoresis and recovered by gel-purification (Schirmer *et al.*, 2015; Clark, Pazdernik and McGehee, 2019). Detailed protocols for all these processes and adapters information are available from Illumina's web site (www.illumina.com).

Libraries are loaded onto an Illumina flow cell that contain billions of nanowells (Figure 18b). Flow cells contains oligonucleotides bound to the surface that are complementary to the adapters previously ligated to the DNA template, and therefore DNA templates are attached to the flow cell via base pairing (Illumina, 2015). Then, the solid-phase bridge amplification starts. PCR steps are repeated several rounds to amplify the signal and resulting in a cluster of copies. After this, sequencing reagents are added and sequencing of the cluster stars (Figure 18c). Sequencing uses sequence-by-synthesis technology, i.e., single bases are detected as they are incorporated into

growing DNA strands. For this, fluorescently labelled reversible terminator-bound dNTPs are used for the polymerization, and since each nucleotide has an elongation terminator, only one base is incorporated per sequencing cycle (Schirmer *et al.*, 2015). When the nucleotide is incorporated, fluorophores are excited by laser sources (red laser for A and C bases and a green laser for G and T bases) and an optical sensor examine the flow cell to capture the signals produced by the new added bases. This optical signal is used to identify the base sequence. This is cyclically repeated until the desired sequence length is obtained. In the case of the paired-end sequence, after the forward strand sequence is obtained, another sequence primer starts the reverse strand sequencing of each fragment. Finally the optical digital image with the sequencing data is exported to an output text file (Illumina, 2015; Schirmer *et al.*, 2015; Y. Lu *et al.*, 2016; Clark, Pazdernik and McGehee, 2019).

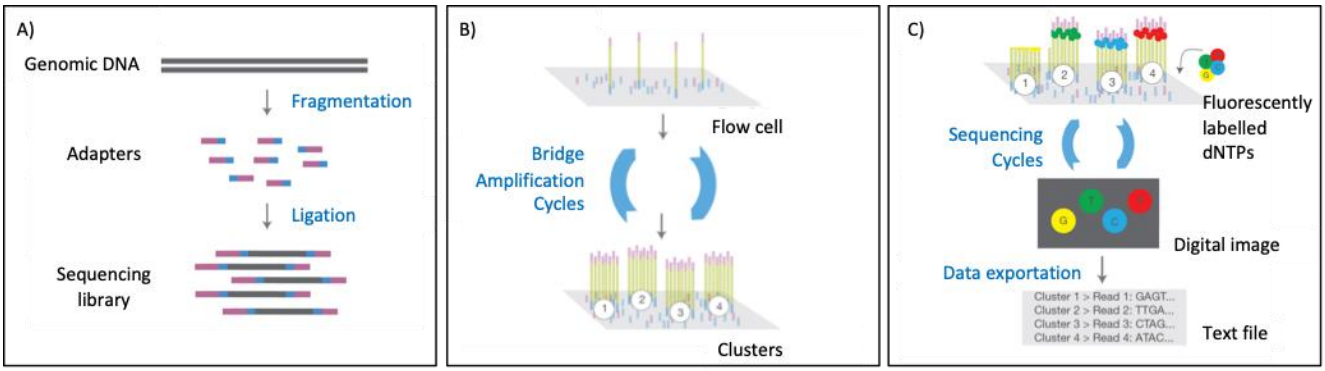



***Figure 18***. Outline of the Illumina sequencing process. A) Library preparation: genomic DNA sample is fragmentated specialised adapter are ligated to both fragments ends. B) Cluster amplification: library is loaded into an Illumina flow cell and fragments are amplificated by bridge amplification to form clonal clusters. C) Sequencing: sequencing reagents are added, and optical emissions are recoded to identify the base sequence of each cluster. Adapted from Illumina manual (2015).

### 3.6.1 Samples sequencing

For this research, genomic DNA isolated from all environmental samples was sent to Mr DNA Laboratory (www.mrdnalab.com, Shallowater, TX, USA) for NGS. DNA was sequences on the Illumina MiSeq platform following the manufacturer's protocols for pair-end sequencing. The bacterial 16S rRNA gene was amplified using the primers 28F (GAGTTTGATCNTGGCTCAG) and 519R (GTNTTACNGCGGCKGCTG) spanning the V1 to V3 hypervariable regions. For fungal communities, the ITS1-2 region was selected for amplification using the primers ITS1FBt1 (CTTGGTCATTTAGAGGAAGTAA)/ ITS2R (GCTGCGTTCTTCATCGATGC). These primers with barcodes inserted on the forward primer were used in a 30 PCR cycles using the HotStarTaq Plus Master Mix

Kit (Qiagen, USA). PCR conditions were: 94°C for 3 minutes, followed by 30 cycles of 94°C for 30 seconds, 53°C for 40 seconds and 72°C for 1 minute, after which a final elongation step at 72°C for 5 minutes was performed. PCR products were checked after the amplification in 2% agarose gel to determine the success of amplification and the relative intensity of bands. Several samples were pooled in equal proportions based on their molecular weight and DNA concentrations. Pooled samples were purified using calibrated Ampure XP beads (Beckman Coulter, Inc.) and the pooled and purified PCR product was used to prepare Illumina DNA library.

## 3.7 Bioinformatics

Illumina output text file with the raw sequences reads obtained during the sequencing process were analysed using a range of bioinformatic tools in order to study the structure and composition of microbial communities across the different experiments. A wide range of bioinformatics tools and platforms exist to analyse the sequencing outputs, and there is no standard method available that ensures maximum accuracy and efficiency in analyses (Nguyen *et al.*, 2016; Niu *et al.*, 2017). For the bioinformatics analysis in this research, a clustering-first or alignment-based approach was selected since it has been demonstrated to be the only reliable approach for studying microbial communities that have not been fully studied and described, such as the microbiome of DWDS (Goodrich *et al.*, 2014; Siegwald *et al.*, 2017). In addition, it is the most represented tool for targeted metagenomics analyses (Siegwald *et al.*, 2017). In the clustering-first approach sequencing reads are *de-novo* gathered into Operational Taxonomic Units (OTUs) based on their similarities. OTUs are therefore cluster of similar sequence variants that can be considered as a taxonomic representative member of the community. In microbial ecology, it has been established that sequences with 97 % similarity (or a maximum distance of 3 %) correspond to the same OTU (Blaxter *et al.*, 2005; Westcott and Schloss, 2015). This cutoff threshold value has been observed to be optimal for estimate and discriminate known species in data sets for both 16S rRNA and ITS1-2 (Blaalid *et al.*, 2013; Wang, Jordan and Mayer, 2015).

The Quantitative Insights Into Microbial Ecology 2 program (QIIME2, version 2019.7, qiime2.org) (Bolyen *et al.*, 2019) was selected in this research for bioinformatic analysis. QIIME 2 is defined as "a powerful, extensible, and decentralized microbiome analysis package with a focus on data and analysis transparency", and it is a very complete tool widely used in studies of microbial ecology since it is relatively easy to use and is more user-friendly than other similar programs. It is based on

a plugin architecture, which means that a large number of plugins are available for a wide range of functions. This allows the performance of different analyses within the same tool, as well as different analysis routes depending on the data set and the research objectives. In addition, QIIME2 allows the export of the data set at any time of the analysis for import into other programs if desired, for example statistical packages such as R. Another advantage of this tool is the possibility of analysing large data with a with a memory-efficient pipeline (Bolyen *et al.*, 2019; Estaki *et al.*, 2020).

### 3.7.1 Microbial community analysis workflow

Bioinformatic analysis was carried out using the High Performance Computing (HPC) cluster of The University of Sheffield (Sheffield, UK), due to the high computational requirements. Prior to import the data to QIIME2 for microbial analysis, raw data from Illumina was preprocessed. The quality control of the raw data was carried out using the FastQC software version 0.11.8 (bioinformatics.babraham.ac.uk) (Andrew, 2010) to analyse the number, the quality and the length of the initial reads. Then, sequencing errors, i.e. sequences that are not really part of the samples, for example those that may be present in the reagents or cross-contamination between samples during the sequencing process (Davis *et al.*, 2018) were removed using the BBDuk software version 37.95 (jgi.doe.gov/data-and-tools/bbtools/bb-tools-user-guide/bbduk-guide/). The same software was applied filter and trim sequences with a minimum length of 100 bp and/or average quality phred score below 20 (Cock *et al.*, 2009). Phred score is a measure logarithmically linked to the error probability in the identification of a nucleotide during sequencing, and a phred score of 20 represents a base call accuracy of 99 % (Ewing *et al.*, 1998; Cock *et al.*, 2009; Andrew, 2010). Then, sequencing reads were demultiplexed and depleted of barcodes by applying the sabre software (github.com/najoshi/sabre) (Joshi, 2011) (Figure 19a).

Afterwards, sequencing data was imported into the QIIME2 (Bolyen *et al.*, 2019) using the vsearch plug-in implemented (github.com/qiime2/q2-vsearch) (Rognes *et al.*, 2016) (Figure 19b). In QIIME2, pair-end sequences from Illumina were joined and dereplicated (i.e., to find the set of unique sequences) (Edgar, 2013) (Figure 20a). Then, chimeric sequences, which are sequences formed for two or more biological distinct parental sequences during the DNA amplification by PCR (Figure 20b) (Gonzalez, Zimmermann and Saiz-Jimenez, 2005; Edgar, 2014), were identified and filtered using the vsearch plug-in for this process in QIIME2. Finally, sequences were *de-novo* clustering by 97% similarity to obtain the OTUs using the vsearch plug-in for these process in QIIME2 (Figure 20c). The

taxonomic assignment of the final OTUs was carried out using the classify-consensus-vsearch method (Rognes *et al.*, 2016) of the feature-classifier plug-in in QIIME2 (github.com/qiime2/q2-feature-classifier) (Bokulich *et al.*, 2018). 16S sequences were compared against SILVA SSU r132 database (arb-silva.de) (Quast *et al.*, 2013) and ITS2 sequences against UNITE 8.0 (unite.ut.ee) (Kõljalg *et al.*, 2013).

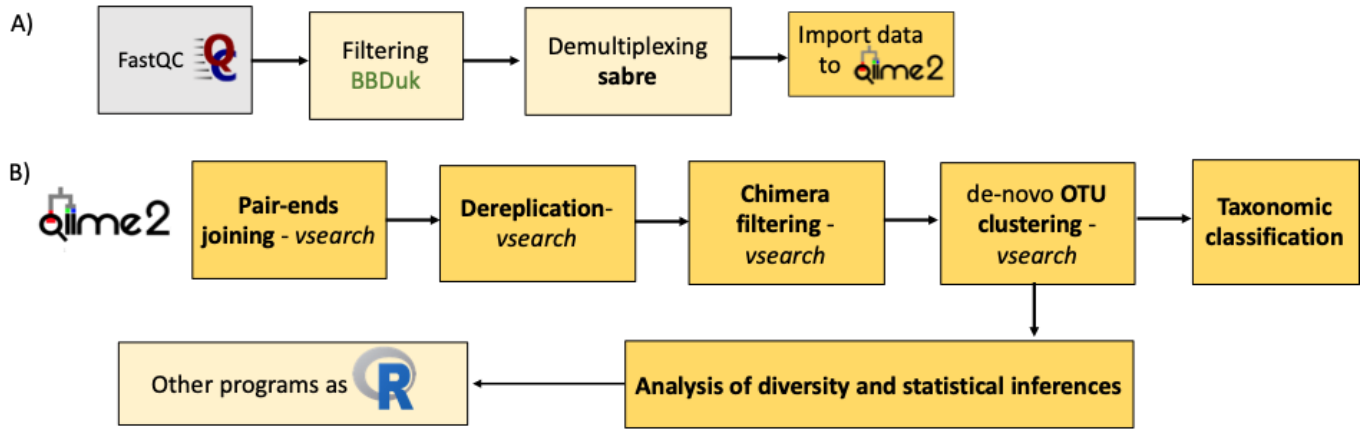



***Figure 19***. Schema of the bioinformatic analysis workflow. A) Data preprocessing performed before importing to QIIME 2. B) Analyses carried out in QIIME2 for the characterization of the microbial communities.

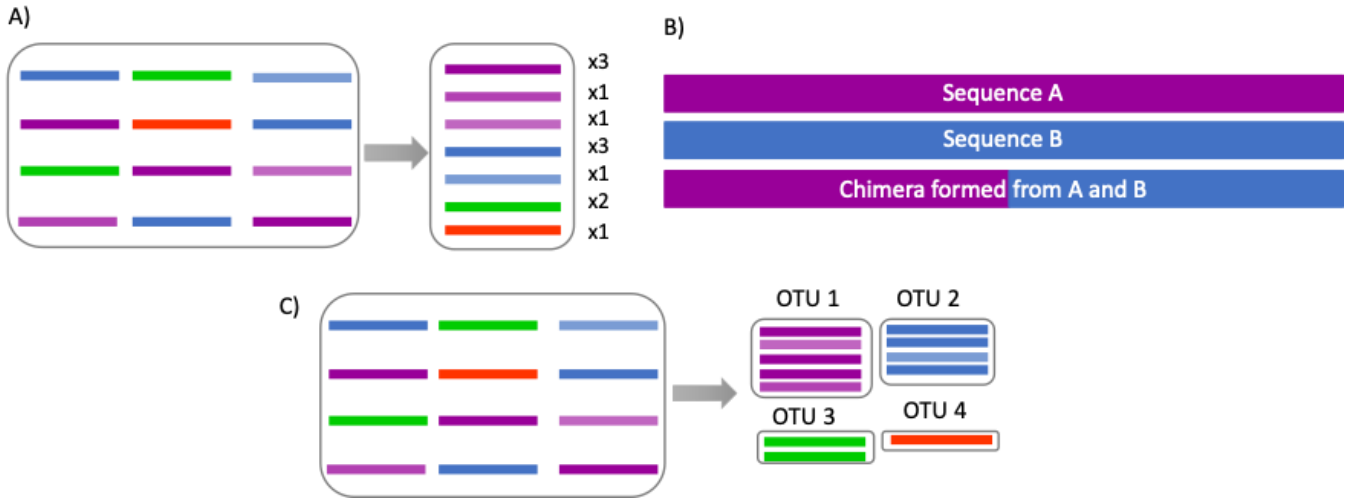



***Figure 20***. A) Scheme of the sequences dereplication process. B) Scheme of a chimera sequence. C) Scheme of the OTU clustering process.

To obtain quantitative measurements of the structure of bacterial and fungal communities, rarefied tables of relative abundance of 97 % OTUs were used for alpha and beta diversity calculations (Morris *et al.*, 2014). OTU tables were rarefied (i.e., standarised to the same the sampling depth)

since diversity metrics are sensitive to different sampling depths across different samples (Haynes, 2008). However, if the rarefaction value is higher that the read count for any sample, this sample is dropped from the analysis (Smith and Peay, 2014). Therefore, the rarefaction value selected was as high as possible to reattain all possible sequences per sample, and thus cover all diversity, while excluding as few samples as possible.

Alpha-diversity, which measures the internal diversity of each sample, was calculated as a measurement of Chao 1 index (richness estimator), Simpson index (dominance estimator) and Shannon index (diversity estimator which consider both richness and dominance) (Morris *et al.*, 2014) using the q2-diversity plug-in in QIIME2 (github.com/qiime2/q2-diversity). Alpha diversity indices are calculated as following:

⇒ $Chao1 = S_{obs} + \frac{F_1^2}{2F_2}$ , *where* $S_{obs}$ *is the number of OTUs in the sample, F1 is the number of singletons and F2 is the number of doubletons.*

⇒ $Simpson\ (D) = \frac{\sum n\ (n-1)}{N(N-1)}$ , *where n = the total number OTUs of a particular group and N is the total number of organisms of all groups.*

⇒ $Shannon\ (H') = -\sum_{i=1}^{s} p_i \ln p_i$, *where s is the number of OTUs and* $p_i$ *is the proportion of the community represented by* $OTU_i$.

For beta-diversity, which estimates the degree of differentiation between samples, the rarefied OTU table was quare-root transformed and then Bray-Curtis method was applied to construct the community similarity matrices using vegan package version 2.5-6 in R (github.com/vegandevs/vegan) (Oksanen *et al.*, 2019). Bray-Curtis resemblance matrices among temperatures were visualized by non-metric multidimensional scaling (nMDS) made with ggplot2 package version 3.2.1 in R (github.com/tidyverse/ggplot2) (Wickham and Chan, 2016).

## 3.8 Statistical analysis

All biological and physico-chemical parameters were measured in triplicate, and the mean and standard deviation were calculated to obtain a representative value. In the same way, the relative abundance of each OTU and the values of alpha diversity indices, were calculated as the mean of all replicates analysed for one sample. The normality of the data sets was tested before performing

significance tests by Shapiro-Wilkstest. Statistical differences between treatments in the experiments for all physico-chemical and biological parameters were tested via the non-parametric Kruskal–Wallis test by ranks. Then, if significant differences were observed, the non-parametric Mann-Whitney *U test* was used to compare samples pairwise. All statistical tests were carried out using R software version 3.6.1 (r-project.org) (R Core Team, 2014), and differences were considered statistically significant when p-value was ≤0.05.

For beta diversity, analysis of similarities (ANOSIM) was applied to Bray-Curtis distance matrices obtained from rarefied OTU tables to detect significant differences in biofilm and water microbial communities between the different treatments in each experiment. To establish the impact of the different treatments on the microbial communities, global-R statistic was calculated. global-R values ranges from 0 to 1, where 1 indicates that communities are totally different (Anderson and Walsh, 2013). Differences were considered statistically significant when p-value was ≤0.05, and all statistical tests were carried out using R software version 3.6.1 (r-project.org) (R Core Team, 2014).

## 3.9 Quantitative polymerase chain reaction (q-PCR)

The quantitative polymerase chain reaction (q-PCR), also known as real-time PCR, is another culture-independent technique most used in microbial ecology to quantify the abundance and expression of both taxonomic and functional genes (Smith and Osborn, 2009; Douterelo *et al.*, 2014). It has been demonstrated to be a robust, reproducible and sensitive tool to quantify, detect variation of specific genes or associate their abundance to different abiotic or biotic parameters, biological activities or environmental conditions (Smith and Osborn, 2009; Nolan, Huggett and Sanchez, 2013). The main disadvantage of the q-PCR is that it is aimed at detecting specific target gene of interest, and therefore PCR assays can only be used for targeting of known genes. However, its high specificity makes it possible to detect and discriminate from the domain level down to the quantification of individual species or phylotype, which with the current sequencing depth for example, cannot be achieved with good accuracy. Therefore, the combination of q-PCR with other techniques such as sequencing has allowed the study and an excellent characterisation of the majority of the environmental microbiome (Smith and Osborn, 2009; Gaebler *et al.*, 2019).

q-PCR combines the basic principles of the end-point PCR with fluorescent detection technologies that allows to monitor and record the accumulation of amplicons during each cycle of the

amplification (in real-time) and thus enabling the quantitative determination of the initial template gene numbers (Smith and Osborn, 2009; Biosystems, 2015). For the amplification, the steps performed are the same as during the traditional PCR: (1) Denaturing: the double-stranded target DNA is heated to separate to separate the double strand into two single strands; (2) Annealing: the temperature is lowered to enable the oligonucleotide primers targeting specific sequences to attach the template; (3) Extension/Elongation: temperature is increased to enable the Taq DNA polymerase enzyme start adding nucleotides and synthesise the complementary strand from each annealed primer (Joshi and Deshpande, 2010; Watson, 2012). This is repeated during multiple cycles and results in an exponential increase in amplicon copies. The key to the q-PCR is that this increase in the number of amplicon copies is detected by a fluorescent reporter that indicates amplicon accumulation during every cycle (Ginzinger, 2002; Smith and Osborn, 2009; Kralik and Ricchi, 2017; Magalhães *et al.*, 2019). Today, the reporters most commonly employed for q-PCR assays include SYBR green and TaqMan probe reagents (Ginzinger, 2002; Smith and Osborn, 2009):

⇒ SYBR Green reagents uses SYBR® Green I dye that binds to all double-stranded DNA via intercalation between adjacent base pairs to detect PCR products. When SYBR green bound to DNA, a fluorescent signal is emitted following light excitation (Figure 21). Therefore, as PCR product accumulate during PCR cycles, the fluorescence signal increases (Smith and Osborn, 2009; Biosystems, 2010; Kralik and Ricchi, 2017).

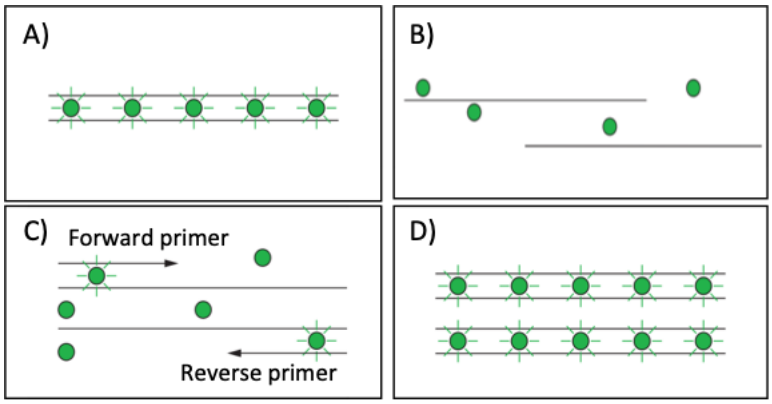



***Figure 21***. Schema of the principle of q-PCR process using SYBR Green reagents. A) The SYBR Green dye fluoresces when bound to double-stranded DNA. B) During the denaturation step the separation of DNA into single strains produce the SYBR Green releasing and thus the fluorescence is reduced. C) During the DNA extension the double strand is synthesised and SYBR Green binds to the PCR products. D) when the synthesis is complete, exponential amplification produces a significant increase in the emitted fluorescence. Adapted from Biosystems manual (2010).

The main advantage using SYBR green dyes is its low cost since it is not necessary to incorporate a fluorescent reporter system or fluorescently labeled probes for a specific sequence (Biosystems, 2015). However, since SYBR green binds to all double-stranded DNA the use of highly specific primers is needed to avoid non-target products as well as the primer-dimer formation that can overestimate the fluorescent signal. To avoid these false positive signals and check for nonspecific product formation, it is necessary to perform a post-PCR dissociation (melting) curve analysis and confirm that the fluorescence signal is generated only from target templates (Smith and Osborn, 2009). To obtain the melting curve, the double-stranded DNA is heated over a temperature gradient. As the temperature is raised, the double strand begins to denature resulting in a corresponding decline in fluorescence due to SYBR green dissociation from the double-stranded product (Giglio, Monis and Saint, 2003; Smith and Osborn, 2009). Melting temperature (Tm), i.e. the temperature at which one-half of the double-stranded template is disnatured, is then used to confirm that that the targeted product is present as well as to detect the presence of other non-target products. Visually, the change in fluorescence/change in temperature (-ΔF/ΔT) is plotted against temperature gradient to obtain a clear view of the melting dynamics (Giglio, Monis and Saint, 2003; Hochstrat, Wintgens and Corvini, 2015). In this way, a melting curve with a single peak indicates a good specificity of the q-PCR while several peaks show the presence of non-target products (Figure 22) (Smith and Osborn, 2009; Biosystems, 2010; Kralik and Ricchi, 2017).

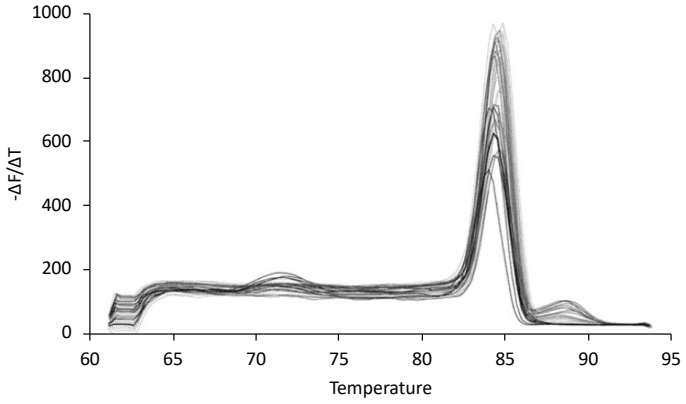



***Figure 22***. Example of a melting curve showing a single peak which indicates a good specificity of the q-PCR reaction. Adapted from (Hochstrat, Wintgens and Corvini, 2015).

⇒ TaqMan reagents consists of a fluorescently labelled probe that hybridizes to an additional conserved region that lies within the target amplicon sequence to detect a specific PCR product as it accumulates during the PCR (Smith and Osborn, 2009). The oligonucleotide TaqMan probe

is labelled with reporter dye bound to the 5' end, while the 3' end is tagged with a quencher molecule (Nagy *et al.*, 2017). If the probe is intact, the proximity between the of the quencher reduces the fluorescence emitted by the reporter. However, if the targeted DNA template is present, primers and probe bind to it during the annealing step (Smith and Osborn, 2009; Biosystems, 2010). Then, during the extension step, the fluorophore from the TaqMan probe is cleaved by the 5' nuclease activity of Taq DNA polymerase. This cleavage separates the reporter dye from the quencher, increasing the reporter dye signal since the fluorophore is no longer in close proximity to the quencher. In addition, cleavage removes the probe from the target template, and thus allows for the primer extension to the end and complete the amplification (i.e. the probe inclusion does not interfere with the PCR process (Biosystems, 2010). Therefore, the template amplification is measured by the increase and accumulation of the fluorophore during the extension stage of each PCR cycles. The use of a hybridization probe adds a high specificity a to this method and ensures that the fluorescent signal generated during q-PCR is derived only from amplification of the target sequence. This makes the q-PCR assays much more robust but also more expensive than when SYBR Green dye is used (Smith and Osborn, 2009; Biosystems, 2010; Kralik and Ricchi, 2017) (Figure 23).

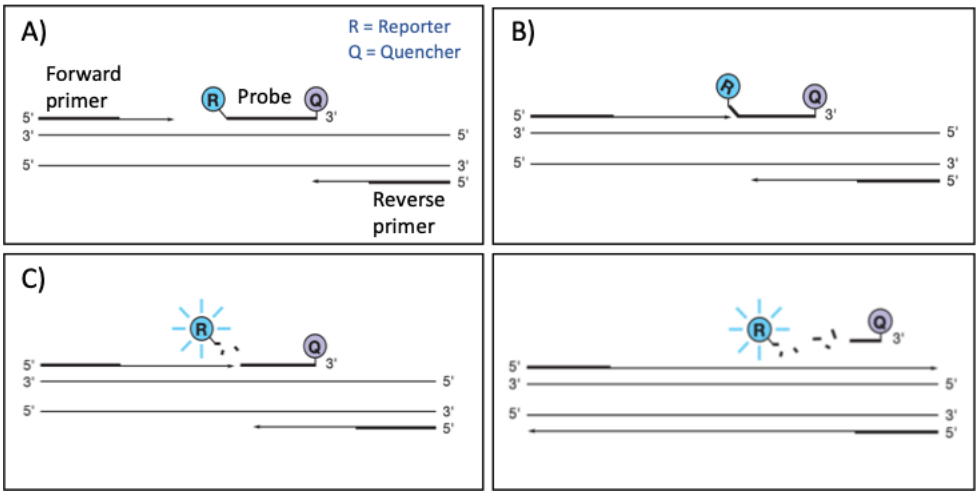



***Figure 23**.* Schema of the principle of q-PCR process using TaqMan reagents. A) Reporter and quencher are attached to the 5' and 3' TaqMan probe. B) Reporter dye and quencher proximity reduce the fluorescence signal. B) C) During the extension step, Taq DNA polymerase cleaves the reporter dye from the probe. D) Reporter dye emits fluorescence since it is no longer in close proximity to the quencher. Adapted from Biosystems manual (2010).

The quantification of the number of copies of the target template DNA is calculated using the cycle threshold ($C_T$) method. $C_T$ is the number of PCR cycles needed for the fluorescence signal of the amplified DNA to cross the fluorescence threshold, which is significantly above the background fluorescence (Kubista *et al.*, 2006; Smith and Osborn, 2009; Nolan, Huggett and Sanchez, 2013). When the $C_T$ value is reached, the exponential accumulation of PCR product is measured (Smith and Osborn, 2009). Therefore, the initial concentration of the target is determined by the $C_T$ value (the higher the initial concentrations of the target DNA, the earlier the $C_T$ value will be reached). Results from this calculation can be produce relative or absolute quantifications (Smith and Osborn, 2009; Biosystems, 2015).

Relative quantifications determine the variation of the target template with respect to a steady-state level. In absolute quantifications the number of target template is determined performing a standard curve (linear regression), which is normally based on the amplification of a serial dilutions of which the initial number of the target DNA template is known. Quantification of the target template is then determined comparing the values of the Ct value against the standard curve (Klein, 2002; Pfaffl, 2004; Smith and Osborn, 2009).

### 3.9.1 Quantitative PCR workflow

In this research, q-PCR was used to quantify changes in the number of gene copies of 6 opportunistic pathogens (OPs) reported previously in DWDS studies (van der Wielen and van der Kooij, 2013; Lu *et al.*, 2015; Qin *et al.*, 2017; Liu *et al.*, 2019). At genus level, the 16S rRNA gene of *Mycobacterium* spp. was targeted because many of their members of this NTM are responsible for causing a large number of diseases (Liu *et al.*, 2016). The 18S rRNA gene of *Acanthamoeba* spp. was selected since this genus of free-living amoeba act as an important host of other pathogens in drinking water (Dobrowsky *et al.*, 2016). At species level, the 16S rRNA gene of *Mycobacterium avium* complex, regA gene of *Pseudomonas aeruginosa*, mip gene of *Legionella pneumophilia*, and chiA gene of *Stenotrophomonas maltophilia* were quantified for having a global importance for human health and because their recognition as major agents of concern in drinking waters are currently increasing (Ashbolt, 2015; Benedict *et al.*, 2017). See Table 5 for details of the primer and probes used.

***Table 5***. Sequences of the primers and probes used for q-PCR (Adapted from van der Wielen and van der Kooij, 2013).

| Target microorganism | Primer and probe sequences |
|---|---|
| *Mycobacterium spp.* | F: 5'-GGGCGATACGGGCAGAC-3' |
| | R:5'-GAAACCGACACCTAGTACC-3' |
| | Probe: 5'-[FAM]CAGGGGAGACTGGAATTCCTGGTGTA[QSY]-3' |
| *M. avium* complex | F: 5'-GCCTCTTCGGAGGTACTCG-3' |
| | R: 5'-ACCAGAAGACATGCGTCTTG-3' |
| | Probe: 5'-[FAM]CAATCTGCCCTGCACTTCGGGATAAG[QSY]-3' |
| *P. aeruginosa* | F: 5'-ATCGAGTACCTGAACCGGC-3' |
| | R: 5'-TGGTGCAGTTCCTCATTGTC-3' |
| | Probe: 5'-[FAM]CCAGATGCTTTGCCTCAAC[QSY]-3' |
| *L. pneumophilia* | F: 5'-CCGATGCCACATCATTAGC-3' |
| | R: 5'-CCAATTGAGCGCCACTCATAG-3' |
| | Probe: 5'-[FAM]TGCCTTTAGCCATTGCTTCCG[QSY]-3' |
| *Acanthamoeba spp.* | F: 5'-CCCAGATCGTTTACCGTGAA-3' |
| | R: 5'-TAAATATTAATGCCCCCAACTATCC-3' |
| | Probe: 5'-[FAM]CTGCCACCGAATACATTAGCATGG[QSY]-3' |
| *S. maltophilia* | F: 5'-TACCACCCGTACCTGGACTT-3' |
| | R: 5'-ATCGCATCGTTGCTGTTGTA-3' |

Absolute quantifications were based on comparison of the value of sample cycle threshold ($C_T$; i.e the number of PCR cycles needed for the fluorescence signal of the amplified DNA to cross the fluorescence threshold, which is significantly above the background fluorescence) with the $C_T$ value of a calibration curve, based on known copy numbers of the target microorganisms (Kubista *et al.*, 2006; Biosystems, 2015). Calibration curves were generated by amplifying the specific fragments (vector plasmids) by conventional PCR. The amplified fragments were purified using a 2% agarose gel QIAquick Gel Extraction Kit (QIAGEN, UK), and serial dilutions (1:10) were prepared to obtain the standards. The number of copy gene copies in each standard was determined by quantifying que DNA concentration via Qubit® dsDNA HS Assay Kit (Life Technologies) and calculating gene copies using the following equation:

$$Gene\ number = \frac{Avogadro\ constant_{\ (copies/mol)}\ x\ DNA\ concentration\ of\ standard_{\ (g/\mu l)}}{MW_{\ (g/mol)}}$$

Where Avogadro's constant is 6.023 x $10^{23}$ and MW is the molecular weight of the target gene: length in base pair x 660 (average molecular weight of one base pair) (McKew and Smith, 2017).

Reaction mixtures for TaqMan assays contained 12.5 µl of iQ™ supermix (2x) (Bio-Rad Laboratories Ltd., Watford, UK), 0.2 µM of each primer and probe (Table 2), 0.4 mg/mL bovine serum albumin (BSA) and 1 µl of DNA template in a total volume of 25 µl. For the SYBR-Green reactions, each 25 µl mixture contained 12.5 µl of iQTM SYBR® Supermix (2x) (Bio-Rad Laboratories Ltd., Watford, UK), 0.2 µM of each primer, 0.4 mg/ml BSA and 1 µl DNA template.

For each qPCR run, a seven-point diluted plasmid standard curve, a negative control (DNA replaced with nuclease-free water) and the samples were run in triplicates. A melt curve was produced for the SYBR-Green qPCR run to verify the specificity of the primers. The amplification, detection and were performed in a QuantStudio™ 12K Flex Real-Time PCR System (Thermo Fisher Scientific Inc., UK). The thermal cycling processes applied are shown in Table 6.

***Table 6***. PCR amplification programs for the different target microorganisms (modified from van der Wielen and van der Kooij, 2013).

| **Target microorganism** | **PCR programme** |
|---|---|
| *Mycobacterium spp.* | 5 min 95 °C; 43 cycles: 20 sec 95°C, 1 min 60 °C |
| *M. avium* | 2 min 95 °C; 43 cycles: 20 sec 95 °C, 1 min 60 °C |
| *P. aeruginosa* | 2 min 95 °C; 43 cycles: 20 sec 95 °C, 1 min 60 °C |
| *L. pneumophilia* | 5 min 95 °C; 43 cycles: 20 sec 95 °C, 48 sec 60 °C |
| *Acanthamoeba spp.* | 30 sec 954 °C; 43 cycles: 20 sec 95 °C, 30 sec 62 °C, 40 sec 72 °C; 2 min 72 °C |
| *S. maltophilia* | 5 min 95 °C; 43 cycles: 30 sec 95 °C, 30 sec, 58 °C, 1 min 72 °C; 10 min 72 °C |

## 3.10 Flow cytometry

The flow cytometry (FCM) method consist on optical detection and quantification of individual suspended particles based on how they fluoresce when passing through a light source, normally a laser beam. These particles can be auto fluorescent or be made fluorescent by staining them with fluorescence dyes. They can include inorganic and organic compounds or microbials cells of bacteria, viruses or even protozoa (S Van Nevel *et al.*, 2017; McKinnon, 2018). This rapid technique generates reproducible and accurate measurements at a low economic cost for drinking water monitoring, and this has made FCM an accepted and widely used approach today for detection and enumeration of drinking water microbial populations (Van Nevel, De Roy and Boon, 2013; Safford and Bischel, 2019; Vembadi, Menachery and Qasaimeh, 2019). In addition, FCM can combine a different fluorescent dye enabling the analysis of different aspects such as presence, viability or activity of

the biomass (Ziglio *et al.*, 2002), thus providing very useful information that can be an indicator of problems occurring during the water distribution or the efficacy of water treatment.

For this research, FCM was used for absolute cell counting or total cell counting (TCC) and intact cell counting (ICC) in bulk water samples by using the combination of SYBR Green I and propidium iodide staining. SYBR Green I is an intercalating dye that enters and stains all bacterial cells with nucleic acids, independently of membrane integrity, and therefore can be used to obtain the total cell number in a sample (Gatza, Hammes and Prest, 2013; Habtewold, Duchateau and Christophides, 2016). SYBR Green I can be used in combination to propidium iodide (PI) is only able to penetrate and stain bacterial cells with damaged or compromised cell membranes. This offers the possibility of discriminating between bacterial cells with intact membranes and damaged membranes (Gatza, Hammes and Prest, 2013; S Van Nevel *et al.*, 2017) (Figure 24).

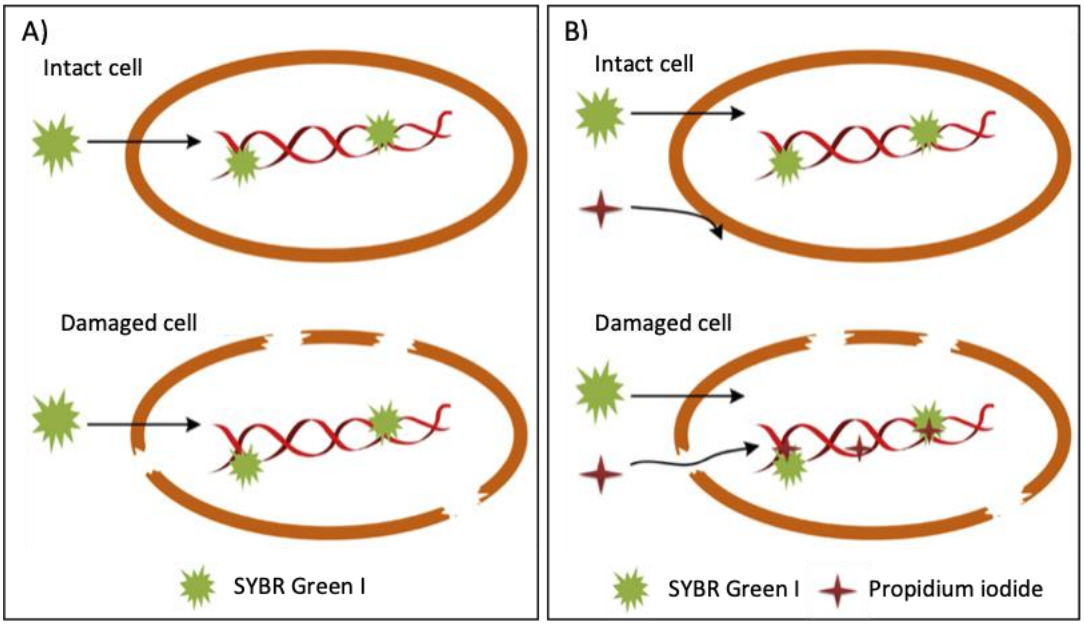



***Figure 24***. Schema of the principle of TCC and ICC measurements using FCM. A) For TCC, SYBR Green I enter and stain with nuclear double-stranded DNA of all cells independently of the membrane integrity. B) For ICC, damaged cells stain with both dyes (SYBR Green I and PI) while intact cells only stain with SYBR Green I and exclude PI, enabling the differentiation between intact and damage cells. Adapted from Van Nevel *et al.* (2017).

### 3.10.1 Flow cytometry workflow

Firstly, water samples were dechlorinated adding 1 % (w/v) sodium ascorbate solution to avoid the disinfectant affect the staining (Safford and Bischel, 2019). For TCC, SYBR® Green I (10000x stock, Invitrogen, UK) was used for staining nuclear double-stranded DNA, and diluted 1:100 with dimethyl sulfoxide (DMSO, Fisher Scientific, Fair Lawn, NJ), to make a working stock solution of a concentration of 100x. 5 µl of SYBR® Green I working stock solution were added to 500 µl of sample aliquot. For ICC, 1 part of propidium iodide (PI) (1.5 mM, Life Technologies Ltd., Paisley, UK), which is not is not permeant to live cells membrane (i.e. used to exclude damage cells), was added to 5 parts of SYBR® Green I working stock solution. 6 µL of this dye mix were then added to 500 µL of sample given a final concentration of 1x SYBR® Green I and 3 µM PI). Samples with the dyes mixes were vortexed and incubated at 36 °C for 10 minutes in the dark (Gillespie *et al.*, 2014; Prest *et al.*, 2014). After the incubation period, 50 µL of each sample was analysed using a BD Accuri™ C6 Cytometer (Becton Dickinson (BD) U.K. Ltd., Oxford, UK) equipped with a 50 mW laser emitting at a fixed wavelength of 488 nm.

The resulting flow cytometer data was processed and analysed using the BD Accuri™ C6 software (BD Biosciences, UK) following the protocol described by Prest *et al.* (2013). In brief, green fluorescence was collected at FL1 channel = 533 nm, and red fluorescence at FL3 channel >670 nm. The following instrument settings were applied to all samples: limit volume of 50 µl, fluidics speed of 35 µL/min (medium), and threshold value of 800 on FL1 channel. Fixed gates were determined as explained in Gatza, Hammes and Prest (2013) and they were used to distinct stained microbial cells from instrumental and water sample background. The counts of this gates after staining with SYBR® Green I or SYBR® Green I/PI were used to estimate the number of TCC and ICC per mL.

## 3.11 Scanning Electron Microscopy

Scanning electron microscopy (SEM) was used to obtain a visual characterization of the biofilms attached to the inner pipe surface. This technique only allows for a qualitative measurement of the biofilm physical structure providing visual data such as its heterogeneity, architecture or surface coverage. SEM analysis for this research were carried out at The Electron Microscopy Facility, at the Faculty of Science (FoS) of the University of Sheffield (UK).

In each sampling point in which samples were collected for SEM characterisation, PWG coupons inserts were removed and cells were initially fixed in 5 % formaldehyde solution (Fisher Scientific, UK) for 24 hours and preserved in PBS at 4 °C prior to visualizations. Afterwards, samples were prepared for SEM. They were fixed secondary in 1 % aqueous osmium tetroxide for 1 hour at room temperature and then washed twice with PBS for 10 minutes. Biofilms were then dehydrated through a graded series of ethanol washes at room temperature for 15 minutes: 75 %, 95 %, 100 % twice and 100 % ethanol dried over anhydrous copper sulphate. Samples were placed in 50/50 (v/v) mixture of 100 % ethanol/100% hexamethyldisilazane for 30 minutes followed by 30 minutes in 100 % hexamethyldisilazane and air-dried overnight. For visualization, samples were coated with approximately 25 nm of gold in an Edwards Gold Sputter Coater S150B (Edwards, UK). Micrographs of biofilms were obtained with a TESCAN Vega 3 LMU SEM (Girton, Cambridge, UK) at an accelerating voltage of 15 kV (Fischer *et al.*, 2012).

# Chapter 4

# The impacts of increasing temperature on water quality and microbial ecology of drinking water distribution system*

## 4.1 Abstract

Microbial communities play an important role within drinking water distribution systems, particularly those that are forming biofilms in the pipe walls which can alter the water quality if they are mobilised into the bulk water. Global climate change is brining changes in environmental conditions such as the increase of temperature that could affect these communities and thus the quality of the final tap water supplied to consumers. A full-scale DWDS facility, which allowing for the study of both biofilm and planktonic communities, was used to simulate temperature increasing produced by climate change. Molecular techniques combined with water physico-chemical characterisation were then applied to understand the effect of temperature increase on microbial ecology, water quality, and occurrence of several opportunistic pathogens inhabiting these systems. Results showed that different water quality parameters were affected by temperature, including turbidity response and metals concentration when biofilms were mobilised. Temperature also leaded to a change in the structure and/or composition of the bacterial and fungal communities in both habitats, and to higher relative abundance of *Mycobacterium* spp. and *M. avium* complex within biofilms. This research has significantly improved the understanding about the effects of increasing temperature on the microbiome of DWDS and provides an important knowledge regarding its consequences on drinking water quality.

**Keywords**: climate change, biofilms, discolouration, bacteria, fungi, opportunistic pathogens.

* This chapter has been adapted and submitted to *Water Research* journal as Calero-Preciado, C.; Boxall, J.; Soria-Carrasco, V.; Martínez, S.; Douterelo, I.: Temperature increase triggers changes in the microbial dynamics of drinking water distribution systems. See Appendix D for more information on contributions and co-authors.

## 4.2 Introduction

Drinking water after leaving the treatment plants, travels thousands of km through distribution systems until reaches consumers taps. Drinking water distribution systems (DWDS) are not pure and sterile environments (Liu, Verberk and Van Dijk, 2013; Husband *et al.*, 2016) and it is well known that microorganisms constitute an active part of these systems, including bacteria, archaea, eukaryotes and viruses (Pinto *et al.*, 2014). Microorganisms can inhabit DWDS in a planktonic way of life, but most of them prefer to live attached to internal pipe surfaces forming biofilms (Batté *et al.*, 2003; Douterelo, Husband and Boxall, 2014). These consortia of microorganisms embedded in a gelatinous matrix of extracellular polymeric substances (EPS), provide them several advantages such a protection against chemicals, exchange of genetic material or enhancing metabolic capacities helping the acquisition of nutrients (Flemming, 2002). This makes that more than 95% of the microbial biomass in DWDS can be found as biofilms (Liu *et al.*, 2016). Nevertheless, if the flow shear stress at the pipe wall exceeds the normal value, biofilms attached at the pipe wall can be mobilised into the bulk water (Husband and Boxall, 2011a). The mobilisation and presence of biofilms in bulk water have been associated with several problems in DWDS, which can alter the quality and safety of drinking water; including problems of discolouration, taste or odour, increase of metals and contaminants concentrations in water, release and proliferation of potential pathogens, or obstruction and the deterioration of pipes (Szewzyk *et al.*, 2000; Douterelo, Husband and Boxall, 2014; Nescerecka *et al.*, 2014; Husband *et al.*, 2016). It has been reported that microorganisms in DWDS, and especially those that form biofilms, are affected by and depend on several factors, such a pipe material, hydraulic conditions, the amount and type of disinfectant, concentration of organic and inorganic compounds including nutrients or contaminants (Batté *et al.*, 2003; Douterelo, Sharpe and Boxall, 2013; Mi *et al.*, 2015; Ren *et al.*, 2015). Another important factor is temperature, however research about the impacts of this variable on microbial ecology and water quality of DWDS is limited, and its implications for these systems have not been well recognized yet. Water temperature is an important parameter of water quality and its increase can modify processes occurring within DWDS (Delpla *et al.*, 2009; Bondank, Chester and Ruddell, 2018). For example, the amount and efficacy of disinfectant residual, which is used to limit growth of microorganisms, decreases at higher water temperatures, which could lead to microbial proliferation (Hua *et al.*, 1999; Li *et al.*, 2018). Other parameters such as dissolved oxygen (DO) in water, also decreases at higher water temperatures (Fisher, Kastl and Sathasivan, 2012; WHO, 2017). In addition, microbial

growth is generally accelerated when the temperature increases (Hallam *et al.*, 2001; Delpla *et al.*, 2009) and recent studies in marine aquatic environments showed that microorganisms tend to form and develop biofilms to a greater extent at higher temperatures (Kent, Garcia and Martiny, 2018).

Therefore, under higher temperatures, microbial growth rates of microorganisms in DWDS can change, producing more-developed biofilms and leading to problems like a greater risk of discolouration. Moreover, temperature changes can also modify the structure and composition of microbial communities in DWDS. Previously, it has been shown that microbial communities in these systems depends on the seasonal fluctuations, that are correlated with temperature (Liu *et al.*, 2016; Revetta *et al.*, 2016). Temperature has also been associated with a greater occurrence and proliferation of opportunistic pathogens (OPs) in biofilms (WHO, 2017). Several studies have demonstrated the incidence of OPs such a non-tuberculous mycobacteria (NTM) or *Legionella* spp. in DWDS, and its ability to proliferate in biofilm in these systems. In different freshwater ecosystems such a rivers, lakes, reservoirs or groundwater it has been observed that the growth of some of these pathogens depends on the temperature (Pandey *et al.*, 2014). In unchlorinated DWDS, higher temperature in the summer could have contributed to the growth of some OPs such a mycobacteria or *L. pneumophila* (van der Wielen and van der Kooij, 2013). Therefore, similar behaviour in response to temperature is expected in chlorinated DWDS. Thus, temperature can be an important determinant of microbial ecology of the DWDS and consequently of the water quality.

The Intergovernmental Panel on Climate Change (IPCC) has concluded that during the 20$^{th}$ Century, the temperature in the Earth surfaces has increased 0.6 °C, and there are detectable effects that in the next 100 years the average of global air temperature will rise by 1.4 to 5.8 °C (Bates *et al.*, 2008). This increase in ambient temperatures will affect water temperatures in DWDS, since it has previously been demonstrated that both are positively related (Sakaue et al., 2000; Blokker and Pieterse-Quirijns, 2013). Currently, the frequency and severity of extreme weather events are increasing, producing greater seasonal changes that cause short periods with high temperature peaks, especially in regions with desert and continental-like climates such a Mediterranean countries (Mesquita *et al.*, 2013; WHO, 2017). As a consequence of climate change, these changes could be greater, longer, and they could happen in other climatic zones, affecting the integrity of DWDS and the supply of safe drinking water worldwide.

New research is needed to understand how temperature rise due to climate change will influence the microbiome of DWDS. This will allow these systems to ensure continued delivery of high-quality wholesome water and protect public health under climate change. The aim of this research was to determine the effect of temperature increase on the microbial ecology and water quality of DWDS. To achieve this aim, the objectives established were: i) water physico-chemical characterisation; ii) the study of the structure and composition of microbial biofilm and planktonic communities and iii) the quantification of the occurrence of specific opportunistic pathogens in DWDS under different temperatures.

## 4.3 Materials and methods

### 4.3.1 Experimental DWDS facility and experiment conditions

Previous research in real DWDS showed that the average water temperature in UK is 8 °C in autumn-winter months and 16 °C in spring-summer months (Husband, Boxall and Saul, 2008). The World Health Organization (WHO) recommend that the temperature of drinking water should not exceed 25 °C to limit the growth of microorganisms and since different pathogens can proliferate at above this temperature (WHO, 2017). According to this, the temperatures selected to achieve the objectives of this research were 16 °C and 24 °C (an increase of another 8 °C).

The research was carried out using a full scale experimental DWDS at the University of Sheffield. This facility allows for simulating conditions in real DWDS and consists of three loops of 9.5 x 21.4 m long coils of High Density Polyethylene (HDPE) pipe (PE100 SDR17), with an internal diameter of 79.3 mm (Figure 25a). Each loop is connected to its own enclosed reservoir tank which is fed with water from local DWDS (Figure 25b). From each tank, water can be recirculated in each loop at different flow rates and pressure, using individual speed pumps (Figure 25b) and valves that are controlled by a central computer to enable simulation of real hydraulic demand patterns. The facility is within a temperature-controlled room and each tank has also its own immersion heater (Figure 25c). This allows controlling and maintaining the temperature of the water entering the system and within it, as well as the water temperature in the facility during the experiments.

In order to maintain baseline water quality parameters, the system retention time of the water was 24 hours, controlled with a trickle drain and feed-based system. Removable Pennine Water Group (PWG) coupons were inserted into the pipes to enable analysis of biofilms *in situ* (Figure 25d, e).

Each coupon has an outer part for DNA-based analysis and an insert for microscopy (Figure 25e), making possible simultaneous visual and molecular analysis of biofilms developed on the pipe walls (Deines *et al.*, 2010).

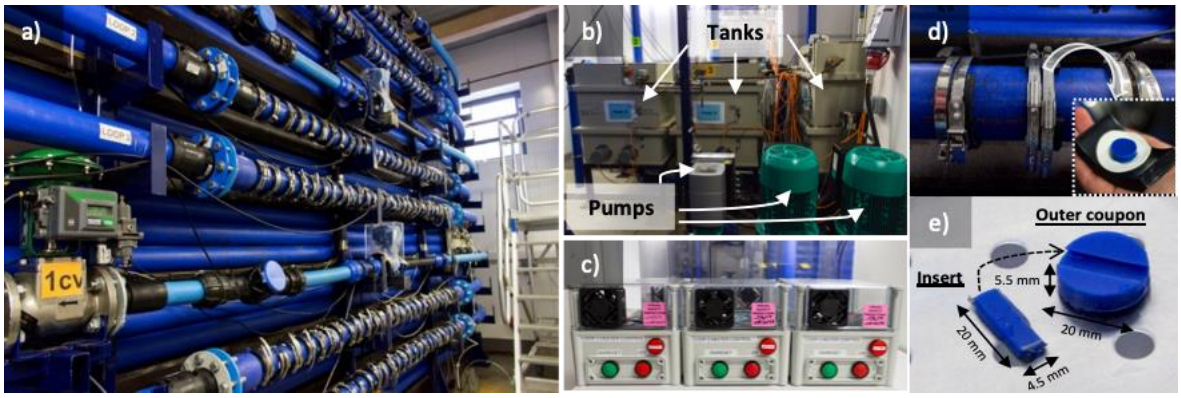



***Figure 25***. a) Full scale experimental DWDS facility; b) Idividual tanks and pumps of each loop; c) Controls of heater immersed in tanks; d) Coupons inserted and fixed into the pipes with holders and clips; e) details and dimensions of the coupons: insert for microscopy and outer for DNA analysis.

Before the experiment started, and in orther to clean the whole system, the facility was disinfected with 20mg/L of RODOLITE H (RODOL Ltd, Liverpool, UK), a sodium hypochlorite based solution with less than 16 % free chlorine. The pipes were flushed at a maximum flow rate (4.2 L/s) and left standing for 24 h with the disinfectant. Then, the facility was flushed again at the maximum flow rate with fresh tap water until the levels of free chlorine were within the limit recommended by WHO (no greater than 5 mg/L) (WHO, 2017) and showed close values to the tap water entering the system (≈ 0.2 mg/L). After disinfecting the system, sterile PWG coupons were inserted along the pipes.

The facility was run at the two temperatures selected (16 °C vs 24°C) for 30 days each to allow for biofilm development in the pipe walls (Figure 26). For these growth phases, a low varied flow (LVF), ranging from 0.2 to 0.5 L/s, was applied based on daily patterns observed in DWDS in the UK (Husband, Boxall and Saul, 2008) (Table 7). After growth phases, the system was flushed using the same protocol for both tests in order to remove and quantify loosely adhered material at the pipe walls at both temperatures (Figure 26). The flushing protocol consisted in 4 gradual steps by increasing the shear stress (τ) in the pipes: step 1=0.4 N/m$^2$, step 2=2.3 N/m$^2$, step 3=3.4 N/m$^2$, step 4=4.3 N/m$^2$ (Table 7). Each step was performed for a duration of 3 water turnovers (i.e. the time

that the total volume of the water needs for recirculate in the loop three times) to mix and detect all material mobilised into the water (Sharpe *et al.*, 2010; R. Sharpe, 2012).


***Figure 26***. Scheme of the experiment over time. Two independent tests were run at different temperatures for 30 days, first at 16 °C and then at 24 °C. Before each test, the system was disinfected with RODOLITE H. After each test, monitored managed flushing trials were applied to study the mobilization characteristics and composition of material attached to pipe surfaces.

***Table 7.*** Test loop facility phases flow rate conversions.

| | Flow (L/s) | Velocity (m/s) | Shear stress (N/m$^2$) |
|---|---|---|---|
| Growth phase | 0.2 - 0.5 | 0.04 – 0.10 | 0.1 – 0.3 |
| Step 1 Mobilisation phase | 0.74 | 0.15 | 0.4 |
| Step 2 Mobilisation phase | 3.58 | 0.72 | 2.3 |
| Step 3 Mobilisation phase | 5.10 | 1.03 | 3.4 |
| Step 4 Mobilisation phase | 6.24 | 1.27 | 4.3 |

### 4.3.2 Microbial communities sampling

To study planktonic and biofilm communities, water samples from each loop and PWG coupons were obtained on day 0 and every 10 days until day 30 during the growth phase and after the mobilisation phase at both temperatures. In each sampling event, 3 replicates of 2 L of bulk water were taken and filtered through 0.22 µm nitrocellulose membrane filters (Millipore Corp., USA). For the biofilm samples, 9 coupons were taken in each sampling point to make 3 replicates of 3 coupons in order to get enough DNA for Next Generation Sequencing (NGS). Biofilms were removed from coupons and suspended in 30 mL of phosphate-buffered saline (PBS) (Gibco®, Thermo Fisher Scientific, UK) solution using petri dishes and sterile nylon brushes, creating a biofilm suspension that was filtered through a 0.22 µm nitrocellulose membrane filters (Millipore Corp., USA) (Deines *et al.*, 2010). Filters of water and biofilm samples were preserved in the dark and at -20 °C prior to DNA extractions.

### 4.3.3 Water quality physico-chemical analysis

Turbidity and chlorine concentration were measured online every minute during the experiments by an ATi A15/76 turbidity monitor (ATi, Delph, UK) and an ATi Q45H-79 chlorine monitor (ATi, Manchester, UK) respectively, installed in the experimental facility. At the time of each sampling, triplicates of pH and water temperature were measured in using a Hanna portable meter HI 991003 (Hanna Instruments, Leighton Buzzard, UK) and free and total chlorine were tested with a Palintest CS100 chlorosense (Palintest, UK). In addition, at each sampling event three replicates of water samples were taken to analyse total organic carbon (TOC) to test the organics in water, and the concentration of iron (Fe) and manganese (Mn), the main compounds that can cause discolouration in DWDS (Boxall & Husband, 2007). Samples for TOC were stored in 20 ml glass vials and then analysed using a Shimadzu TOC-$V_{CPH}/_{CPN}$ Analyzer (Shimadzu, Kyoto, Japan) in Kroto Research Institute (KRI) (Sheffield, UK) following the manufacturer's protocol. The concentration of Fe and Mn was determined by Inductively Coupled Plasma Mass Spectrometry (ICP-MS) in KRI (Sheffield, UK). Water samples were collected in 20 mL vials containing 5 M of nitric acid, then ions were monitored on a Perkin Elmer Elan DRC II (PerkinElmer, Inc., USA) and data acquisition was via Elan NT software (tla.mpi.nl/tools/tla-tools/elan) (Sloetjes and Wittenburg, 2008).

### 4.3.4 Microscopy analysis

Biofilms were visually characterised by Scanning Electron Microscopy (SEM). Triplicates of coupons inserts were removed on day 0 (control) and day 30, fixed in 5% formaldehyde solution (Fisher Scientific, UK) for 24 hours and preserved in PBS at 4°C. Afterwards, the samples were fixed secondary in 1 % aqueous osmium tetroxide for 1 hour at room temperature and then washed twice with PBS for 10 minutes. Biofilms were then dehydrated through a graded series of ethanol washes at room temperature for 15 minutes: 75 %, 95 %, 100 % twice and 100 % ethanol dried over anhydrous copper sulphate. Samples were placed in 50/50 (v/v) mixture of 100 % ethanol/100% hexamethyldisilazane for 30 minutes followed by 30 minutes in 100 % hexamethyldisilazane and air-dried overnight. For visualization, samples were coated with approximately 25 nm of gold in an Edwards Gold Sputter Coater S150B (Edwards, UK). Micrographs of biofilms were obtained with a TESCAN Vega 3 LMU SEM (Girton, Cambridge, UK) at an accelerating voltage of 15 kV.

### 4.3.5 DNA extraction and sequencing

DNA from all the filters of water and biofilm samples was extracted following the protocol based on hexadecylmethylammonium bromide (CTAB) and proteinase K chemical lysis, followed by DNA purification using phenol/isoamyl alcohol method (Zhou, Bruns and Tiedje, 1996; Neufeld *et al.*, 2007). DNA concentration from each extraction was quantified fluorometrically using the HS dsDNA Assay kit with a Qubit 4 Fluorometer (Invitrogen by Thermo Fisher Scientific, Wilmington, USA).

Extracted DNA was sequenced by Mr DNA Laboratory (www.mrdnalab.com, Shallowater, TX, USA) on the Illumina MiSeq platform following the manufacturer's guidelines for pair-end sequencing. The bacterial 16S rRNA gene was amplified using the primers 28F (GAGTTTGATCNTGGCTCAG) and 519R (GTNTTACNGCGGCKGCTG) spanning the V1 to V3 hypervariable regions. For fungal analysis, primers ITS1FBt1 (CTTGGTCATTTAGAGGAAGTAA)/ ITS2R (GCTGCGTTCTTCATCGATGC) targeting the ITS1-2 regions were selected for amplification. These primers with a barcode inserted on the forward primer were used in a 30 PCR cycles using the HotStarTaq Plus Master Mix Kit (Qiagen, USA) and following these conditions: 94°C for 3 minutes, followed by 30 cycles of 94°C for 30 seconds, 53°C for 40 seconds and 72°C for 1 minute, after which a final elongation step at 72°C for 5 minutes was performed. PCR products were checked after the amplification in 2% agarose gel to determine the success of amplification and the relative intensity of bands. Several samples were pooled together in equal proportions based on their molecular weight and DNA concentrations. Pooled samples were purified using calibrated Ampure XP beads (Beckman Coulter, Inc.) and the pooled and purified PCR product was used to prepare Illumina DNA library.

### 4.3.6 Bioinformatics and community analysis

The quality control of the raw data from Illumina was carried out using the FastQC software version 0.11.8 (bioinformatics.babraham.ac.uk) (Andrew, 2010) in order to analyse the number, the quality and the length of the initial reads. BBDuk software version 37.95 (jgi.doe.gov/data-and-tools/bbtools/bb-tools-user-guide/bbduk-guide/) was used to remove sequencing errors (i.e. sequences that are not really part of the samples, for example those that may be present in the reagents or cross-contamination between samples during the sequencing process) (Davis *et al.*, 2018) and filter and trim sequences with an average quality phred score below 20 and/or a minimum length of 100 bp (Cock *et al.*, 2009). Afterwards, sequencing reads were demultiplexed and depleted

of barcodes by applying the sabre software (github.com/najoshi/sabre) (Joshi, 2011) and imported into the Quantitative Insights Into Microbial Ecology 2 program (QIIME2, version 2019.7, qiime2.org) (Bolyen *et al.*, 2019). Then, using the vsearch plug-in implemented in QIIME2 (github.com/qiime2/q2-vsearch) (Rognes *et al.*, 2016), pair-end sequences were joined and dereplicated. Chimeric sequences were identified and filtered and *de-novo* clustering by 97% similarity to obtain the Operational Taxonomic Units (OTUs) using the vsearch plug-ins for these processes in QIIME2. The taxonomic assignment of the final OTUs was carried out using the classify-consensus-vsearch method (Rognes *et al.*, 2016) of the feature-classifier plug-in in QIIME2 (github.com/qiime2/q2-feature-classifier) (Bokulich *et al.*, 2018). 16S sequences were compared against SILVA SSU r132 database (arb-silva.de) (Quast *et al.*, 2013) and ITS2 sequences against UNITE 8.0 (unite.ut.ee) (Kõljalg *et al.*, 2013).

To obtain quantitative measurements of the structure of bacterial and fungal communities at different temperatures, rarefied tables of relative abundance of 97% OTUs were used to calculate alpha and beta diversity (Morris *et al.*, 2014). Alpha-diversity, which measures the internal diversity of each sampel, was calculated as a measurement of Chao 1 index (richness estimator), Simpson index (dominance estimator) and Shannon index (diversity estimator which consider both richness and dominance) (Morris *et al.*, 2014) using the q2-diversity plug-in in QIIME2 (github.com/qiime2/q2-diversity). For beta-diversity, which estimates the degree of differentiation between samples, the rarefied OTU table was quare-root transformed and then Bray-Curtis method was applied to construct the community similarity matrices using vegan package version 2.5-6 in R (github.com/vegandevs/vegan) (Oksanen *et al.*, 2019). Bray-Curtis resemblance matrices among temperatures were visualized by non-metric multidimensional scaling (nMDS) made with ggplot2 package version 3.2.1 in R (github.com/tidyverse/ggplot2) (Wickham and Chan, 2016).

### 4.3.7 q-PCRs

q-PCR was performed to monitor changes in the gene copy number of 6 OPs previously reported in DWDS (van der Wielen and van der Kooij, 2013; Qin *et al.*, 2017; Liu *et al.*, 2019). The genetic markers used to study the OPs were as follows: the 16S rRNA gene of *Mycobacterium* spp and *Mycobacterium avium* complex respectively, the 18S rRNA gene of *Acanthamoeba* spp, regA gene of *Pseudomonas aeruginosa*, mip gene of *Legionella pneumophilia*, and chiA gene of *Stenotrophomonas maltophilia* The amplification, detection and quantification were performed in

a QuantStudio™ 12K Flex Real-Time PCR System (Thermo Fisher Scientific Inc., UK), following the protocol described by van der Wielen and van der Kooij (2013).

### 4.3.8 Statistical analysis

For each physico-chemical parameter measured in water samples, bacterial and fungal alpha diversity indices and qPCR results, the mean and standard deviation of the replicates were calculated. Prior to significance tests, to determine the normality of all data sets the Shapiro-Wilks test was applied to all data. The statistical differences between temperatures of all physico-chemical and biological parameters were tested via the non-parametric test Mann-Whitney *U test*. For beta diversity, analysis of similarities (ANOSIM) was applied to Bray-Curtis distance matrices in order to detect statistically significant differences in biofilm and water microbial communities between temperatures (p-value). To calculate the strength of impact of temperature on them, global-R statistic was calculated, which has a value from 0 to 1, indicating 1 that communities are totally different between temperatures and 0 that are identical (Anderson and Walsh, 2013). Differences were considered statistically significant when *p-value* was < 0.05, and all statistical tests were carried out using R software version 3.6.1 (r-project.org) (R Core Team, 2014).

## 4.4 Results

### 4.4.1 Water physico-chemical analysis

As shown in Table 8, water temperature was stable over the duration of each experiment, keeping values close to 16 °C and 24 °C for both tests. pH values ranged between 6.61 and 7.59 during the 30 days of biofilm growth phase. Regardless disinfectant residual, a high concentration of total chlorine (average at 16 °C = 0.93 mg/L, average at 24 °C = 0.80 mg/L) and free (average at 16 °C = 0.89 mg/L, average at 24 °C = 0.68 mg/L) was observed at both temperatures on day 0, associated with the process of disinfection of the system at the beginning of the experiment. During the growing phase and after mobilisation phase, average chlorine concentrations showed higher values at 16 °C (total chlorine average = 0.19 mg/L, n = 12; free chlorine average = 0.13 mg/L, n = 12) than at 24 °C (total chlorine average = 0.15 mg/L, n = 12; free chlorine average = 0.09 mg/L, n = 12). However, statistical test showed no significant differences for total and free chlorine (Mann-Whitney *U tests*, p-value >0.05). TOC, iron and manganese concentrations were similar during the

growth phases at both temperatures, ranging from 1.13-1.35 mg/L, 42.4-59.3 µg/L and 0.33-0.52 µg/L respectively, and no significant differences were found (Mann-Whitney *U tests*, p-value >0.05). Fe and Mn concentration increased considerably after the mobilisation phases at both temperatures, showing significantly higher values at 24 °C (Fe average = 81.2 µg/L, n = 3, Mn average = 1.2 µg/L, n = 3) than at 16 °C (Fe average = 53.7 µg/L, n = 3; Mn average = 0.69 µg/L, n = 3) (Mann-Whitney *U tests*, p-value ≤0.05) (Table 8). See appendix A.4 for supplementary information on the results of the statistical analysis.

***Table 8***. Water physico-chemical parameters measured on sampling days during the growth phase and after mobilisation phase (AM). All values represent an average of three water replicates analysis ± standard deviation.

| | | | **16 °C** | | |
|---|---|---|---|---|---|
| **Sampling day** | **0** | **10** | **20** | **30** | **AM** |
| **Temperature (°C)** | 15.7 ± 0.06 | 15.5 ± 0.07 | 15.9 ± 0.09 | 15.8 ± 0.15 | 15.6 ± 0.03 |
| **pH** | 7.59 ± 0.08 | 7.09 ± 0.11 | 6.78 ± 0.29 | 6.61 ± 0.16 | 6.53 ± 0.08 |
| **Total Cl (mg/L)** | 0.93 ± 0.06 | 0.19 ± 0.01 | 0.19 ± 0.01 | 0.22 ± 0.04 | 0.18 ± 0.02 |
| **Free Cl (mg/L)** | 0.89 ± 0.05 | 0.08 ± 0.01 | 0.11 ± 0.00 | 0.14 ± 0.02 | 0.18 ± 0.02 |
| **Fe (µg/L)** | 42.4 ± 0.65 | 41.3 ± 1.09 | 46.3 ± 0.92 | 44.6 ± 0.68 | 53.7 ± 2.09 |
| **Mn (µg/L)** | 0.33 ± 0.07 | 0.39 ± 0.02 | 0.52 ± 0.02 | 0.45 ± 0.00 | 0.69 ± 0.06 |
| **TOC (mg/L)** | 1.21 ± 0.00 | 1.21 ± 0.05 | 1.23 ± 0.05 | 1.13 ± 0.03 | 1.49 ± 0.04 |
| | | | **24 °C** | | |
| **Sampling day** | **0** | **10** | **20** | **30** | **AM** |
| **Temperature (°C)** | 23.1± 0.41 | 24.0 ± 0.03 | 23.8 ± 0.07 | 23.9 ± 0.06 | 23.4 ± 0.03 |
| **pH** | 6.79 ± 0.19 | 7.47 ± 0.21 | 6.92 ± 0.09 | 6.80 ± 0.16 | 6.93 ± 0.13 |
| **Total Cl (mg/L)** | 0.80 ± 0.01 | 0.13 ± 0.03 | 0.13 ± 0.01 | 0.13 ± 0.05 | 0.21 ± 0.01 |
| **Free Cl (mg/L)** | 0.68 ± 0.03 | 0.08 ± 0.04 | 0.08 ± 0.01 | 0.10 ± 0.02 | 0.11 ± 0.03 |
| **Fe (µg/L)** | 49.0 ± 0.63 | 49.9 ± 0.54 | 59.3 ± 0.83 | 45.1 ± 1.07 | 81.2 ± 1.34 |
| **Mn (µg/L)** | 0.39 ± 0.01 | 0.47 ± 0.01 | 0.42 ± 0.02 | 0.39 ± 0.02 | 1.20 ± 0.04 |
| **TOC (mg/L)** | 1.35 ± 0.05 | 1.44 ± 0.06 | 1.34 ± 0.06 | 1.31 ± 0.02 | 1.50 ± 0.02 |

#### 4.4.1.1 Turbidity

Online turbidity measurements during the growth phase, showed similar values at both temperatures, average of 0.043 ± 0.01 NTU at 16 °C and 0.042 ± 0.01 NTU at 24 °C. During the mobilisation phase when the shear stress (τ) increased, in the first two stages (τ = 0.4 N/m$^2$ and τ = 2.3 N/m$^2$) no statistical differences were found (Mann-Whitney *U test*, p-value > 0.05) between temperatures in the turbidity response of the last turnover of each stage, when the water with the mobilised material was mixed. However, in the last two stages (τ = 3.4 N/m$^2$ and τ = 4.3 N/m$^2$) statistical differences (Mann-Whitney *U tests*, p-value ≤0.05) in the turbidity response were observed at both temperatures. The response at both temperatures was linear between imposed shear stress and turbidity, $R^2$ = 0.9955 at 16 °C and $R^2$ = 0.9996 at 24 °C (Figure 27).

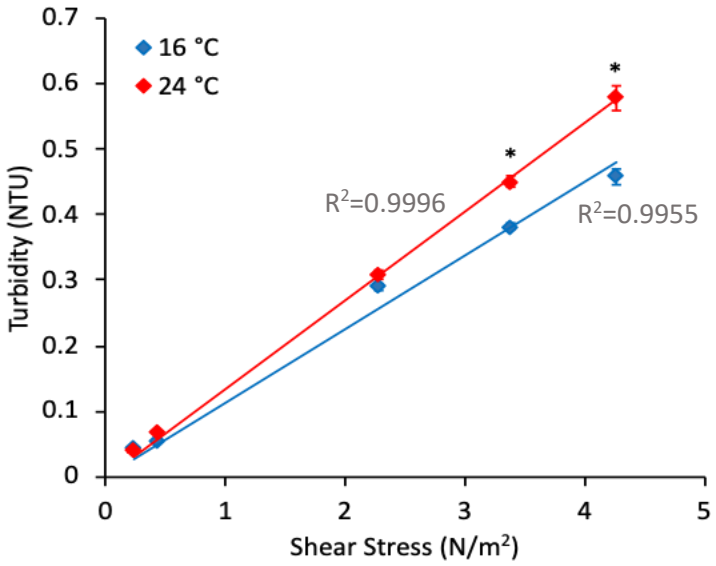



***Figure 27***. Average turbidity response during the last 24 hours of the growth phase (representative of the growth phase) and during the last turnover of each stage of the flushes (i.e. when the water with the mobilised material was mixed) at different temperatures. All values represent an average ± standard deviation. * p-value ≤0.05 from Mann–Whitney *U test*.

### 4.4.2 Scanning Electron Microscope

Figure 28 shows SEM micrographs of modified PWG pipe coupons on day 0 (control) and day 30 at 16 °C and 24 °C. Sterilized coupons at the beginning of the experiment showed no cells attached to the surface of the PWG coupon at 16 °C and 24 °C respectively (Figure 28a, b). Differences in the coupons surface coverage can be observed on day 30 at different temperatures (Figure 28c, d, e and

f). At 16 °C small patches of biofilm were developed on the surface of the coupon. However, at 24 °C a greater number of cells and more biofilm accumulation was observed on the surface of the coupon.

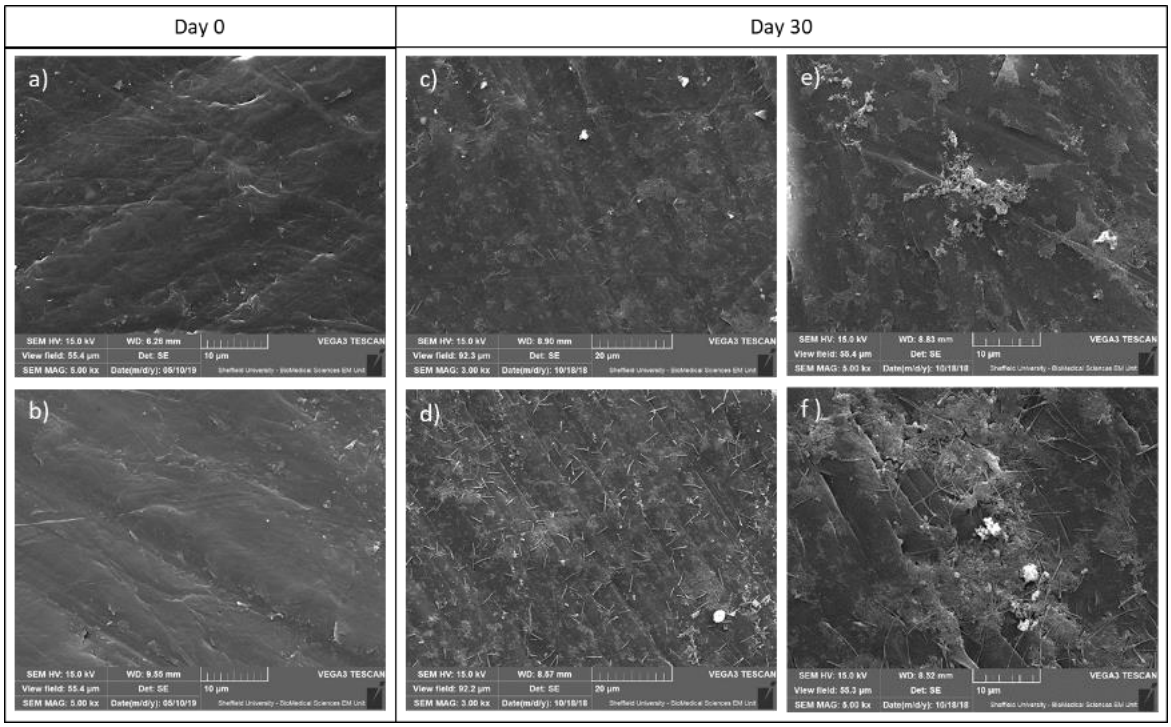



***Figure 28**.* SEM micrographs of biofilm developed in modified PWG coupons: (a) Control at 16 °C (day 0) (MAG = 5.00 kx); (b) Control at 24 °C (day 0) (MAG = 5.00 kx); (c) Day 30 at 16 °C (MAG = 3.00 kx); (d) Day 30 at 24 °C (MAG = 3.00 kx); (e) Day 30 at 16 °C (MAG = 5.00 kx); and (f) Day 30 at 24 °C (MAG = 5.00 kx). MAG=magnification.

### 4.4.3 Microbial community structure (bacteria and fungi)

#### 4.4.3.1 Alpha-diversity (diversity within samples)

Figure 29 shows the results for the diversity indices, Chao 1, Simpson and Shannon at genus level for bacteria and fungi. In general, fungal communities were more affected by temperature than bacteria during the growth phase. Alpha-diversity of the biofilm bacterial communities did not show significant changes between temperatures, during the growing phase (Mann-Whitney *U tests*, p-values >0.05). However, after the mobilisation phase, Chao 1 and Shannon indices showed lower significant values at higher temperature (Mann-Whitney *U tests*, p-values ≤0.05). Conversely, biofilm fungal communities showed significant differences for all the calculated indices over time (Mann-

Whitney *U tests*, p-values ≤0.05) with the exception of day 10 were no differences were observed for Chao 1 (Mann-Whitney *U test*, p-value >0.05). Regarding water communities (bacteria and fungi), diversity indices during the growth phase were not affected by the temperature increase (Mann-Whitney *U tests*, p-value >0.05). After flushing at 24 °C the Shannon index of bacterial communities decreased (Mann-Whitney *U tests*, p-value ≤0.05), and Chao 1 of fungal communities increased (Mann-Whitney *U tests*, p-value ≤0.05). See appendix A.4 for supplementary information on the results of the statistical analysis.

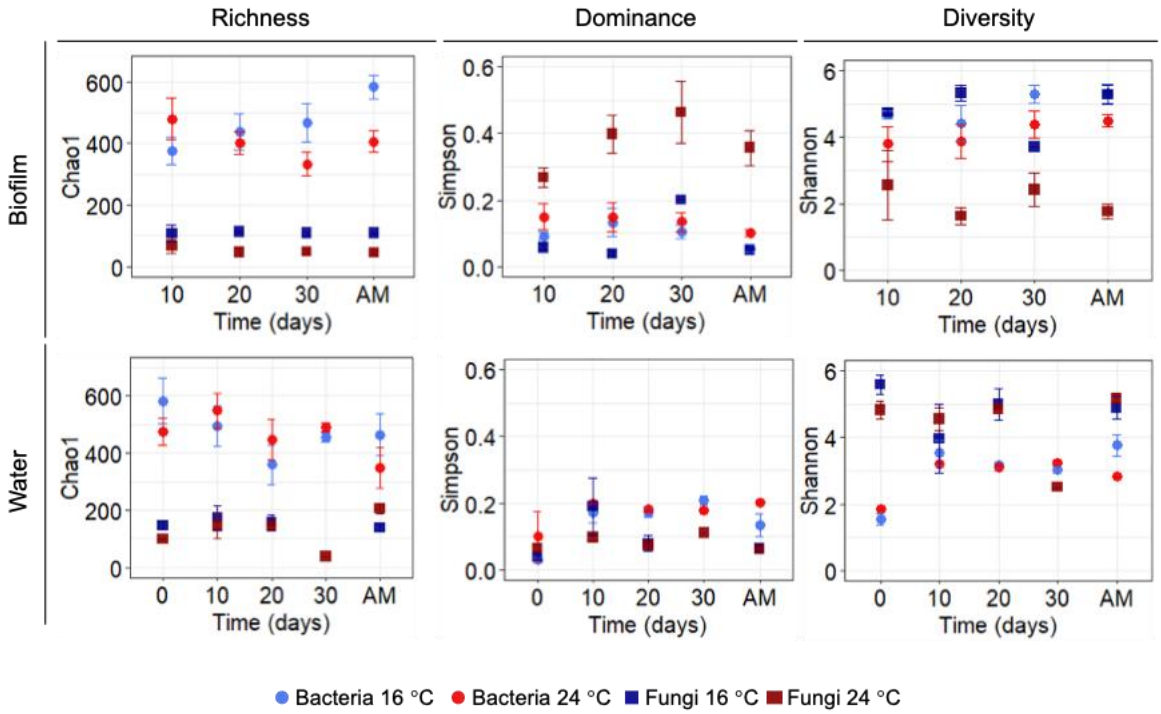



***Figure 29***. Chao 1 (richness), Simpson (dominance) and Shannon (diversity) indices for OTUs at 97 % cut off for bacteria and fungi in biofilm and water samples calculated for each sampling day during the growth phase and after mobilisation phase (AM). All values represent an average of three water replicates ± standard deviation.

### 4.4.3.2 Beta-diversity (diversity among/between samples)

Figure 30 shows nMDS plots with the resemblance between microbial communities at genus level at different temperatures. For biofilm, nMDS showed an evident clustering at different temperatures for both, bacteria and fungi. ANOSIM confirmed that temperature increase significantly affected for bacterial community structure (global-R = 0.66, p-value ≤0.05) and fungal communities (global-R = 0.45, p-value ≤0.05). For water samples, nMDS showed a clear separation for bacteria, but not for fungi. ANOSIM analysis for water samples showed that only bacterial

structure was significantly modified by the increase of temperature (global-R = 0.5, p-value ≤0.05), while fungal communities showed no significant differences between temperatures (global-R = 0.03, p-value >0.05).

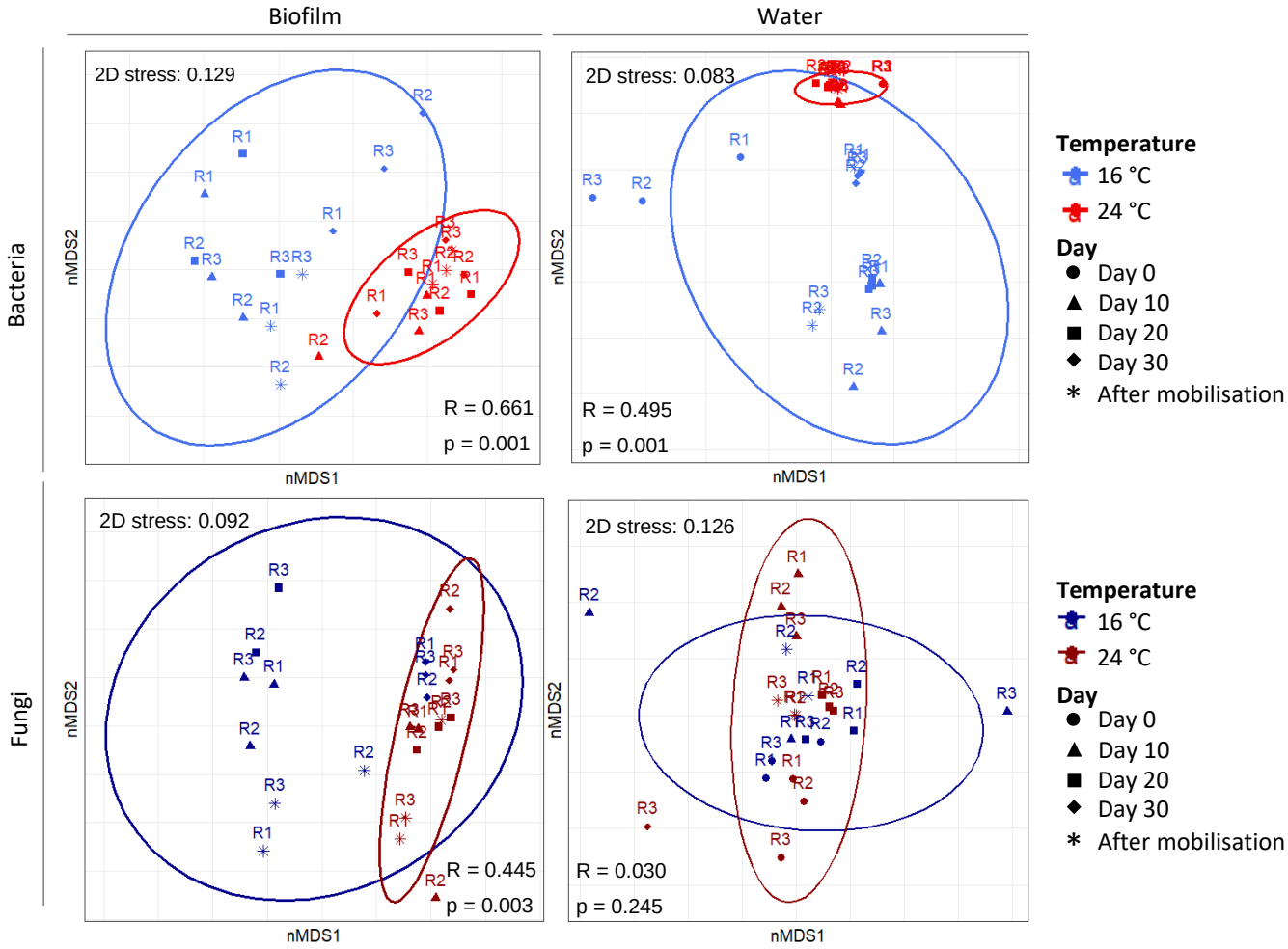



***Figure 30***. Two-dimensional plot of the non-multidimensional scaling (nMDS) analysis based on Bray–Curtis similarities of the relative abundance of bacteria and fungi in water and biofilm samples. The 3 replicates (R1, R2 and R3) per sampling day are represented.

### 4.4.4 Taxonomic profiles of microbial communities (bacteria and fungi)

Focusing on the most abundant taxa (average >1% relative abundance, n = 3), differences in the taxonomical composition of bacteria and fungi were detected in both biofilm and water samples at different temperatures (Figure 31, Figure 32).

At 16 °C, the dominant groups of bacteria (Figure 31) on the first stages of biofilm development (day 10 and 20) were a not defined (ND) genera belonging to the family of Burkholderiaceae

(averages 28.98 ± 6.30 % and 32.75 ± 22.81 % on day 10 and 20, respectively), followed by *Pseudomonas* (14.91 ± 8.90 % and 26.72 ± 11.13 %) and *Methylotenera* (9.98 ± 4.91 % and 8.89 ± 6.09 %). On day 30, *Pseudomonas* (18.49 ± 6.58 %) became the most abundant genera, followed by *Mucilaginibacter* (14.52 ± 7.74 %), *Delftia* (12.40 ± 0.08 %) and *Phreatobacter* (10.70 ± 7.60 %). After the mobilisation phase, biofilm bacterial communities were dominated by *Nevskia* (20.55 ± 6.92 %), *Sphingomonas* (14.70 ± 8.60 %) and *Phreatobacter* (14.18 ± 8.32 %). At 24 °C, biofilm bacterial communities showed a different taxonomic profile throughout the growth phase when compared with the 16 °C communities. The most representative genera at each sampled point was *Pseudomonas* (ranged from 61.20 % to 45.27 %). On day 10, *Pseudomonas* was followed by *Nevskia* (12.93 ± 11.22 %) and *Phreatobacter* (9.63 ± 5.83 %), and on day 20 and 30, *Phreatobacter* (6.88 ± 3.17 % and 11.31 ± 6.45 % respectively) and *Sphingomonas* (5.41 ± 0.61 % and 6.59 ± 2.23 %) were the following most abundant genera. After the mobilisation phase, the bacterial community showed a similar profile when compared with the growth phase, being *Pseudomonas* (56.51 ± 6.24 %) the most abundant genus, followed by *Sphingobium* (8.60 ± 7.85 %) and *Sphingomonas* (6.58 ± 3.59 %).

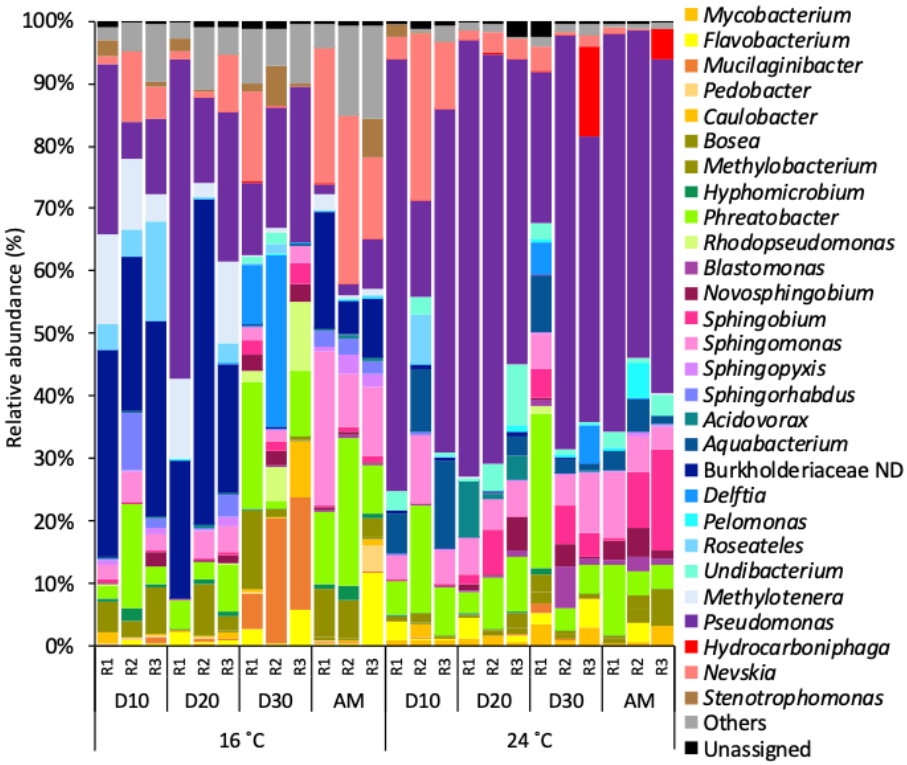



***Figure 31***. Relative abundance of bacterial genera (>1 % of the total sequences) at 16 °C and 24 °C in biofilm samples every 10 days (D) throughout the growth phase and after the mobilization phase (AM). The 3 replicates (R1, R2 and R3) per sampling point are represented. Remaining genera were combined in category "Others". Category "Unassigned" correspond to unidentified OTUs and "ND" means not defined at that level.

At 16 °C, for biofilm fungal communities (Figure 32), on days 10 and 20 *Aspergillus* (21.50 ± 13.03 % and 12.36 ± 8.6 %) and *Cladosporium* (21.44 ± 8.65 % and 16.55 ± 2.82 %) followed by *incertae sedis* of Helotiales (6.97 ± 4.24 %) and *Cystobasidium* (6.83 ± 9.65 %), respectively, were the most abundant taxa. On day 30, the relative abundance of *Fusarium* increased to become the most abundant genus (52.74 %) followed by the order Helotiales *incertae sedis* (9.07 %). After the mobilisation phase, Helotiales *incertae sedis* (18.81 ± 9.12 %), *Fusarium* (9.84 ± 15.84 %) and *Rhodotorula* (8.54 ± 3.57 %) dominated the community. At 24 °C, *Fusarium* (ranged from 86.97 % to 64.46%) was the most abundant taxa over the biofilm growth phase, followed by *Trichoderma* (26.05 ± 25.85 %) and Helotiales *incertae sedis* (10.47 ± 8.07 %) on day 10, and by Helotiales *incertae sedis* (11.03 ± 13.47 %) and *Cladosporium* (0.35 ± 0.30 %) on day 20. On day 30, the following genera, *Penicullium* (0.1 ± 0.00 %) and *Cladosporium* (0.05 ± 0.07 %), showed a very low relative abundance. After the mobilisation phase, Helotiales *incertae sedis* (49.80 ± 40.67 %) and *Fusarium* (48.32 ± 40.65 %) continued being the most abundant taxa.

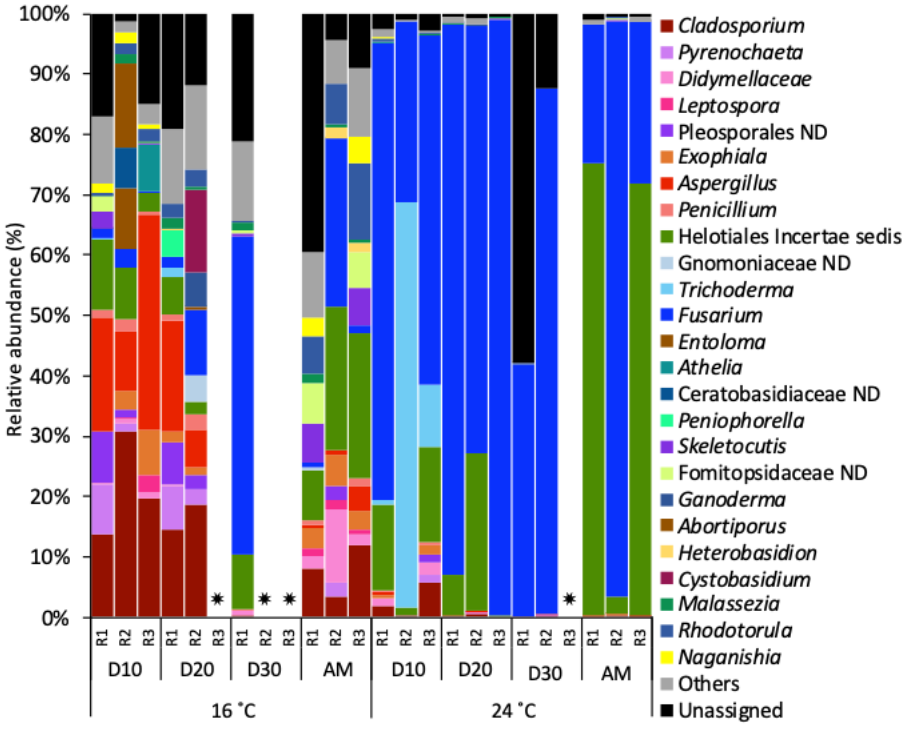



***Figure 32**.* Relative abundance of fungal genera (>1 % of the total sequences) at 16 °C and 24 °C in biofilm samples every 10 days (D) throughout the growth phase and after the mobilization phase (AM). The 3 replicates (R1, R2 and R3) per sampling point are represented. Remaining genera were combined in category "Others". Category "Unassigned" correspond to unidentified OTUs and "ND" means not defined at that level. ✶ Samples that did not amplify during the sequencing process.

Regarding water samples (Figure 33), at 16 °C bacterial communities were dominated by an uncultured bacterium belonging to the Obscuribacterales order (30.18 ± 3.9 %), followed by *Phreatobacter* (11.21 ± 4.7 %) and *Methylobacterium* (8.15 ± 1.46 %) on day 0. On days 10, 20 and 30 Burkholderiaceae (51.56 ± 8.38 %, 47.70 ± 4.52 %, 18.37 ± 2.58 %,) and *Phreatobacter* (22.28 ± 8.43 %, 30.17 ± 2.73 %, 60.90 ± 4.18 %) were the most abundant taxa. They were followed by *Sphingomonas* (5.45 ± 7.82 %) on day 10, *Methylotenera* (9.90 ± 5.63 %) on day 20 and *Sphingorhabdus* genus (6.32 ± 1.20 %) on day 30. After the mobilisation event, *Phreatobacter* (32.39 ± 15.76 %) continued to be the most abundant genus, followed by *Pseudomonas* (30.09 ± 11.25 %) and Burkholderiaceae NA (11.15 ± 1.64 %). At 24 °C on day 0 water communities were dominated by *Phreatobacter* (92.24 ± 0.98 %), followed by other genera with a very low relative abundance such as uncultured Obscuribacterales (1.50 ± 0.35 %) and *Hyphomicrobium* (1.15 ± 0.33 %). Then, over time on day 10, 20, 30 and after the mobilisation phase, water communities showed the same taxonomic profile, being *Phreatobacter* (ranging from 60.33 % to 45.30 %) the most abundant genus, followed by *Sphingomonas* (39.96 % - 17.15 %) and *Nevskia* (15.73 % - 9.08 %).

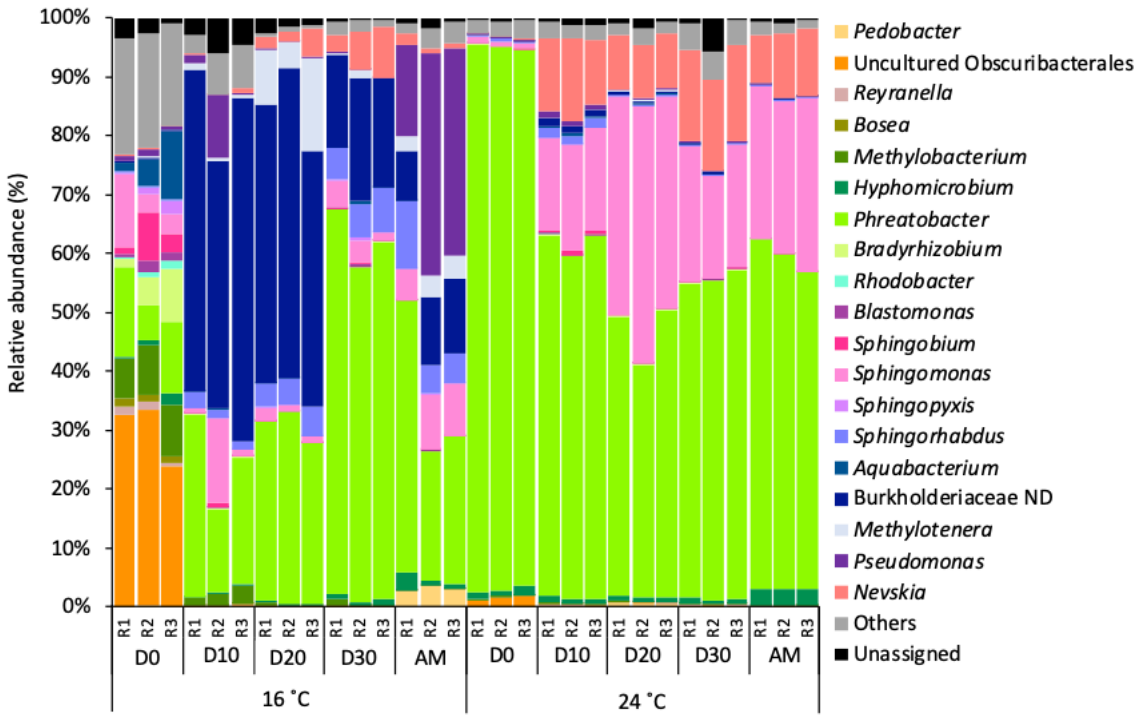



***Figure 33***. Relative abundance of bacterial genera (>1 % of the total sequences) at 16 °C and 24 °C in bulk water samples every 10 days (D) throughout the growth phase and after the mobilization phase (AM). The 3 replicates (R1, R2 and R3) per sampling point are represented. Remaining genera were combined in category "Others". Category "Unassigned" correspond to unidentified OTUs and "ND" means not defined at that level.

Regarding water fungal communities (Figure 34), a high percentage of unassigned sequences (ranged from 8.4% to 97.11%) was observed in all the samples, particularly at 24 °C. At 16 °C on day 0, planktonic fungal community was dominated by *Cladosporium* (18.65 ± 3.63 %) and an unidentified taxa belonging to the family Fomitopsidaceae (12.72 ± 5.62 %). On day 10 Helotiales *incertae sedis* (33.23 ± 44.76 %), another unidentified Ascomycota (21.93 ± 30.01%) and *Cladosporium* (9.87 ± 11.43 %) were the most abundant taxa. On day 20 Helotiales *incertae sedis* (33.49 ± 12.10 %) was followed by *Cladosporium* (10.16 ± 7.31 %) and *Fusarium* (5.58 ± 2.16 %), and on day 30 any water replicate amplified for fungi. After flushing, the community was dominated by Helotiales *incertae sedis* (17.29 ± 10.22 %) and *Fusarium* (6.13 ± 3.74 %). At 24 °C on day 0 the most abundant taxa were Family Debaryomycetaceae ND (27.81 ± 6.21 %) and Fomitopsidaceae ND (12.30 ± 4.14 %). On day 10 and 20 Helotiales *incertae sedis* (11.69 ± 4.99 % and 32.50 ± 1.96 %) became the most abundant taxa, followed by *Fusarium* (5.55 ± 2.21 % and 14.22 ± 1.36 %) and *Cladosporium* (4.79 ± 1.01 % and 4.49 ± 0.65 %). On day 30, the most abundant taxa identified were *Penicillium* (2.00 %) and *Fusarium* (0.40 %). After flushing, *Fusarium* (27.95 ± 3.47 %), Helotiales *incertae sedis* (15.42 ± 1.77 %) and *Cladosporium* (9.01 ± 3.16 %) were the most abundant taxa.

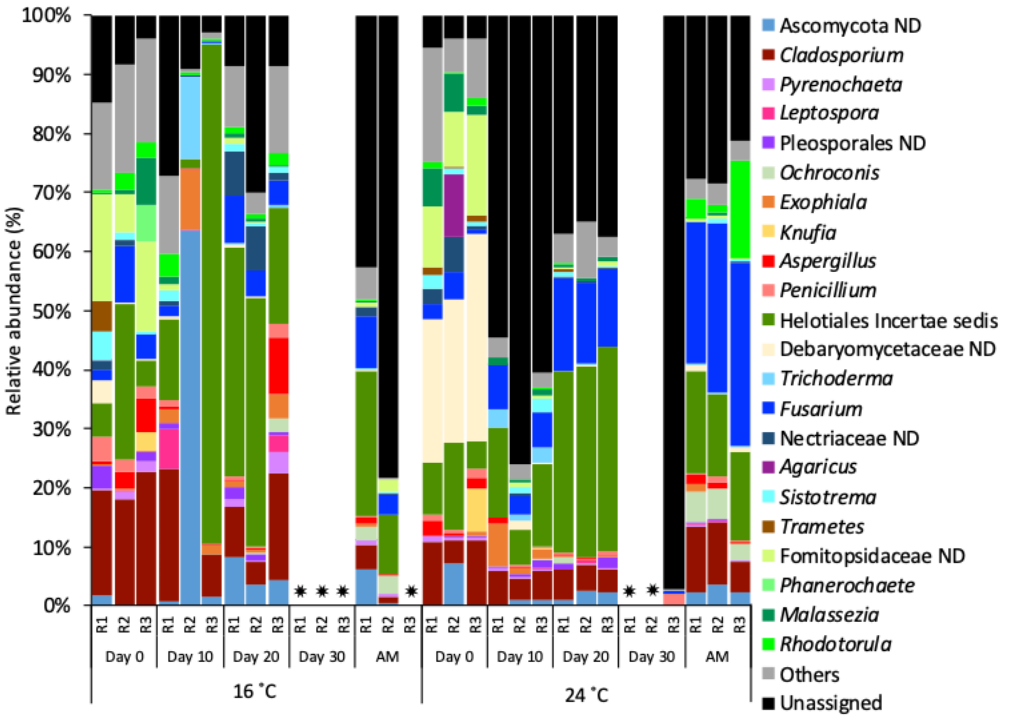



***Figure 34***. Relative abundance of fungal genera (>1 % of the total sequences) at 16 °C and 24 °C in bulk water samples every 10 days (D) throughout the growth phase and after the mobilization phase (AM). The 3 replicates (R1, R2 and R3) per sampling point are represented. Remaining genera were combined in category "Others". Category "Unassigned" correspond to unidentified OTUs and "ND" means not defined at that level.

### 4.4.5 Opportunistic pathogens occurrence analysed by q-PCR

The OPs gene copies number at 16°C and 24°C are shown in Table 1. The majority of OP selected showed no statistically significant difference between the different temperatures for water or biofilm samples. The results did show statistical differences between temperatures for *Mycobacterium* spp. on biofilm samples from day 30 (p-value≤0.05). However, in the rest of the biofilm samples and in water samples this microorganism did not show statistical differences. The gene copy number for *M. avium* complex showed statistical changes between temperatures; at 16°C the gene copies were below the limit of quantification, but they showed an increase at 24°C in all the samples analysed (biofilm and water). *P. aeruginosa*, *Acanthomoeba* spp. and *S. maltophilia* were detected and quantified in all samples of biofilm and water over time (except *Acanthomaeba* spp. after the mobilisation phase in both water and biofilm). *L. pneumophillia* was below quantification limit in every sampling point. Overall, the OPs gene copy number in biofilm samples tended to increase at 24°C (with 14 out of 19 relevant cases, but with only 5 statistically significant), yet not a clear pattern was observed with biofilm age. See appendix A.4 for supplementary information on the results of the statistical analysis.

***Table 9***. Occurrence of *Mycobacterium* spp., *M. avium* complex, *P. aeruginosa*, *L. pneumophillia*, *Acanthamoeba* spp. and S*. malthophilia* in biofilm and water samples at 16 °C and 24 °C. All values represent an average of three replicates ± standard deviation. "B.Q.L" correspond to number of genes below quantification limit.

| | | *Mycobacterium spp.* | | *M. avium* complex | | *P. aeruginosa* | |
|---|---|---|---|---|---|---|---|
| | | 16 °C | 24 °C | 16 °C | 24 °C | 16 °C | 24 °C |
| Biofilm (copies /cm$^2$) | Day 10 | 4.99E+02 ± 2.48E+02 | 9.02E+02 ± 1.26E+02 | B.Q.L | 1.94E+02 ± 2.91E+01 * | 2.32E+03 ± 3.33E+02 | 1.38E+03 ± 5.85E+00 |
| | Day 20 | 1.60E+03 ± 8.19E+02 | 7.77E+03 ± 5.5E+03 | B.Q.L | 3.12E+02 ± 9.21E+00 * | 4.95E+02 ± 3.21E+02 | 7.06E+02 ± 1.29E+01 |
| | Day 30 | 1.90E+03 ± 1.23E+03 | 1.14E+04 ± 2.82E+03 * | B.Q.L | 2.17E+02 ± 8.22E+01 * | 7.53E+02 ± 2.33E+02 | 1.41E+03 ± 1.52E+02 |
| | AM | 1.21E+03 ± 1.02E+03 | 4.37E+03 ± 2.70E+03 | B.Q.L. | 3.70E+02 ± 5.24E+01 * | 1.25E+03 ± 1.28E+02 | 146E+03 ±9.14E+01 |
| Water (copies /L) | Day 0 | 7.59E+03 ± 4.12E+03 | 2.42E+03 ± 3.97E+02 | B.Q.L. | 1.14E+02 ± 7.00E+01 * | 1.95E02 ± 1.02E+02 | 7.18E+02 ± 2.27E+02 |
| | Day 10 | 2.05E+04 ± 7.31E+02 | 1.73E+04 ± 2.59E+03 | B.Q.L. | 2.94E+02 ± 4.77E+01 * | 3.23E+02 ± 4.45E+01 | 1.50E+03 ± 4.89E+02 |
| | Day 20 | 1.02E+04 ± 3.20E+03 | 9.55E+03 ± 3.76E+03 | B.Q.L. | 2.20E+02 ± 5.47E+01 * | 3.21E+02 ± 1.92E+02 | 2.45E+03 ± 7.79E+02 |
| | Day 30 | 1.68E+04 ± 1.31E+03 | 1.42E+04 ± 2.34E+02 | B.Q.L. | 3.54E+02 ± 8.23E+01 * | 3.18E+03 ± 1.23E+03 | 2.81E+03 ± 3.44E+02 |
| | AM | 2.09E+04 ± 7.23E+03 | 3.85E+04 ± 3.40E+03 | B.Q.L. | 2.43E+02 ± 1.02E+02 * | 4.73E+03 ± 1.84E+03 | 3.10E+03 ± 1.15E+03 |
| | | *L. pneumophillia* | | *Acanthomaeba spp.* | | *S. maltophilia* | |
| | | 16 °C | 24 °C | 16 °C | 24 °C | 16 °C | 24 °C |
| Biofilm (copies /mm$^2$) | Day 10 | B.Q.L | B.Q.L | 1.74E+04 ± 5.71E+03 | 1.85E+04 ± 5.23E+03 | 2.17E+03 ± 1.20E+03 | 1.5E+03 ± 1.16E+03 |
| | Day 20 | B.Q.L | B.Q.L | 1.21E+04 ± 3.64E+03 | 2.14E+04 ± 8.03E+03 | 1.81E+03 ± 3.04E+02 | 1.95E+03 ± 1.91E+02 |
| | Day 30 | B.Q.L | B.Q.L | 7.89E+03 ± 1.58E+03 | 2.18E+04 ± 8.18E+03 | 3.09E+03 ± 1.35E+03 | 1.88E+03 ± 7.61E+02 |
| | AM | B.Q.L | B.Q.L | B.Q.L. | B.Q.D. | 2.84E+03 ± 7.84E+01 | 9.60E+02 ± 7.30E+02 |
| Water (copies /L) | Day 0 | B.Q.L. | B.Q.L. | 1.86E+04 ± 2.09E+03 | 1.18E+04 ± 6.46E+03 | 1.38E+03 ± 1.09E+03 | 1.91E+03 ± 1.39E+03 |
| | Day 10 | B.Q.L | B.Q.L | 1.73E+04 ± 7.85E+03 | 2.04E+04 ± 4.77E+03 | 7.32E+03 ± 2.24E+03 | 4.54E+03 ± 2.14E+03 |
| | Day 20 | B.Q.L | B.Q.L | 2.06E+04 ± 2.17E+03 | 3.30E+04 ± 1.32E+04 | 4.44E+03 ± 2.37E+03 | 6.96E+03 ± 2.46E+03 |
| | Day 30 | B.Q.L | B.Q.L | 1.50E+04 ± 2.31E+03 | 1.66E+04 ± 4.00E+03 | 4.28E+03 ± 1.86E+03 | 3.29E+03 ± 1.28E+03 |
| | AM | B.Q.L | B.Q.L | 2.55E+04 ± 4.10E+03 | 4.73E+04 ± 1.43E+04 | 8.64E+02 ± 0.00E+00 | 0.00E+00 ± 0.00E+00 |

## 4.5 Discussion

### 4.5.1 Effect of temperature rise on water physico-chemical parameters and water discolouration

Most water physico-chemical water characteristics during the growth phase were stable over time at both temperatures. TOC, which has not been assigned a limiting value for drinking water standards (WHO, 2017), showed values fluctuating in a similar way as has been reported in other studies in DWDS (Douterelo, Husband and Boxall, 2014; Li *et al.*, 2018). pH values were near to neutral, and within the standard range proposed by WHO for drinking water (6.5 to 8.5) (WHO, 2017). High free and total chlorine values on day 0 were associated with the disinfection process of the facility with high concentrations of RODOLITE that was carried out at the beginning of the experiment. These concentrations, as well as those of the following sampling points were within the limits recommended by WHO (2017) for drinking water (no greater than 5 mg/L). Previous research in DWDS reported that temperature was one the factor that most influenced chlorine decay concentrations in these systems (Powell *et al.*, 2000; Fisher, Kastl and Sathasivan, 2012; Karadirek *et al.*, 2016). The level of disinfectant plays an important role in DWDS by limiting the microbial growth and preventing the proliferation of pathogenic microorganisms (Donnermair and Blatchley, 2003). It has been observed previously that higher chlorine concentrations reduce the bacterial concentrations. For example Bertelli *et al.* (2018) observed this effect in the bulk water, and (Fish and Boxall, 2018) found this in biofilms using the same full scale experimental facility that was used in this study. In agreement with the results, although there was not significant differences in the chlorine concentration between the two temperatures, the slight reduction of disinfectant observed at higher temperatures is a reflexion of the 24h retention time of the water in the system, where new fresh drinking water was constantly introduced in the system. However, in real DWDS in which water travels through the network, the concentration of chlorine may decrease with water age, suggesting that in a scenario of increasing temperature it will be necessary to augment the doses of disinfectant to limit microbial growth. Higher concentrations of chlorine can produce problems such as the potential formation of DBP, which have been shown to have adverse effects on human health (WHO, 2017; Tsagkari and Sloan, 2019). Thus, the increase of temperature can pose an economic and technical challenge for water utilities to maintain concentrations throughout the entire distribution network without compromising the health of consumers.

When the process of water discolouration was evaluated, results showed similar concentrations of Fe and Mn during the growth phase at 16 °C and 24 °C. This was expected since the water source during the experiments was the same, the local DWDS, and water in the test loop facility was renovated every 24 hours. However, in response to the mobilisation phase the concentration of these metals in the discrete water samples increased significantly at both temperatures, and especially at 24 °C. These results were consistent with those from online turbidity monitoring. During the growth phase, there was no evidence that the temperature affected the turbidity in the system, but when the pipes were flushed, an increase in the turbidity response at 16 °C and 24 °C was clearly observed. This turbidity response showed statistical differences between temperatures in the last two steps of the flushing when the shear stress reached the highest values. Turbidity response, Fe and Mn concentrations were higher at the test run at 24 °C that at 16 °C, indicating greater material accumulation and attachment at higher temperatures in a chlorinated DWDS, as it was observed by Blokker and Schaap (2015) in unchlorinated DWDS. In addition, these results reinforce previous research which found a greater number of complaints related to discolouration at warmer temperatures (Horsley *et al*., 1998; Cook, Husband and Boxall, 2016). A higher accumulation of material could be indicative of greater biofilm formation on pipes. This was in agreement with SEM micrographs obtained from PWG coupons, which showed a greater accumulation of biofilm on the surface of the coupon at 24 °C. Previous research, reported a link between microbial growth and temperatures; Dukan *et al.*, (1996) noted an increased in bacterial number and biological activity with the increase of temperature using dynamic modelling of distribution networks; Hallam *et al.* (2001) concluded that biofilm potential growth in real chlorinated DWDS was enhanced at higher temperatures and its activity decreased with lower temperatures; and Kent, García and Martiny (2018) observed that warmer temperatures promote biofilm formation at the air-liquid interface in marine ecosystems. Thus, the increase in growth and microbial activity in DWDS biofilms due temperature rise may lead to a greater amount of EPS production, which might facilitate the adsorption and entrainment of material from the bulk water (Douterelo *et al.*, 2019). This would explain the greater concentration of metals and the greater turbidity that was observed in this study at 24°C. Our results confirm that higher temperatures promote biofilm growth and development on HDPE pipe walls and that if the material attached to the pipe walls is mobilised the risk of water discoloration is greater when the temperature increases.

### 4.5.2 Influence of temperature upon water and biofilm microbial community structure and taxonomical profiles

Microbial community structure characterisation by diversity indices showed that in biofilms differences were only observed for bacterial richness after the mobilisation phases, which was significantly higher at 16 °C. This suggest that at this temperature a lower number of OTUs at 97 % were removed with flushing. However, dominance and diversity followed the same patterns at both temperatures, with no differences between them during the growth phase or after mobilisation. This was in contrast with other studies that showed a reduction in bacterial diversity after flushes (Douterelo *et al.*, 2016). Biofilm fungal communities were statistically different between temperatures, showing at 24 °C lower richness and higher dominance over time during the growth phase, which resulted in lower diversity of these microorganisms at high temperatures. Ortiz-Vera *et al.* (2018) founded that fungal dominance and diversity in rivers were affected by temperature, and our results confirmed that in DWDS biofilms these microorganisms follow a similar pattern. The higher dominance and lower diversity due temperature rise could have important implications for water quality in DWDS. It has been demonstrated that fungi are related to several bioremediation processes thanks to the variety of extracellular and intracellular enzymes that they are able to produce (Jones, Lester and Voulvoulis, 2005; Olicón-Hernández, González-López and Aranda, 2017). This includes the degradation of pollutants, xenobiotics or pharmaceutical compounds such as antibiotics, that can remain in the water even after purification treatments (Jones, Lester and Voulvoulis, 2005; Guha Roy, 2019; Mahmood, Al-Haideri and Hassan, 2019). Therefore, the great reduction in fungal diversity due temperature rise could lead to the loss of these potential functions that could reduce water quality. In addition, this information suggests that, within biofilms, the structure of fungal communities were more affected by temperature than bacteria, which show that these microorganisms are an important part of the biological component of DWDS and it is necessary to carry out more research in the DWDS. Regarding water, no significant differences in diversity indices for bacteria and fungi microbial communities for water samples, showing the same structure at both temperatures in terms of richness, dominance and diversity. Thus, temperature increase mainly affected biofilms diversity when compared with water, especially shifting the fungal communities. This results also reinforces how important are biofilms in DWDS, although these communities are not usually monitored by water companies.

Despite the limited differences in alpha diversity, nMDS ordinations based on the relative abundance of microbial groups at genus level showed a clear clustering of biofilm samples at 16 °C and 24 °C for both bacteria and fungi. This indicated that the communities of both types of microorganisms in biofilms were statistically different at different temperatures over time, although less markedly in fungi than in bacteria. These differences in the microbial communities due to temperature were in agreement with previous studies. Stanish *et al.* (2016) reported that water temperature was an important factor for abundance and microbial community composition in tap water samples, and Ji *et al.* (2017) showed that temperature changed the characteristic of bacterial communities building plumbing systems. In the same way results from this study confirm that temperature is a driving factor changing the biofilm microbial community structure also in chlorinated DWDS, and the importance of controlling these communities. Regarding planktonic communities, temperature only had significant effect on bacterial composition since fungi did not show differences at 16 °C and 24 °C. In addition, the effect of temperature on bacterial communities in water was less marked than in biofilm. These results suggest that water communities, which are more affected and controlled by disinfection processes, are less likely to be modified by other factors such as temperature. However, in biofilms, in which a more favourable environment is generated, other abiotic factors such as temperature can modify these microbial communities. This information demonstrates again the importance of biofilms in water DWDS systems and the need to monitor and control them to avoid changes that may compromise water safety.

Changes in the structure of the microbial communities were visible also when the composition of the communities was analysed. Differences in microbial community composition between temperatures were observed when comparing taxonomic profiles of bacteria and fungi. Regarding biofilm bacterial communities, common genera found at similar relative abundance in samples at both temperatures included *Nevskia*, *Phreatobacter*, *Roseateles* on the first stage of biofilm development (days 10 and 20) or *Delftia* in more developed stages. All of them have not yet been related to adverse effects on water quality, and are usually reported as components of biofilm communities in DWDS at colder temperatures (Douterelo *et al.*, 2016; Douterelo, Fish and Boxall, 2018; van Assche *et al.*, 2019), and our results confirmed that these taxa also are part of the community core at warmer temperatures. Other taxa such a Burkholderiaceae ND, *Methylotenera* or *Methylobacterium,* observed before in DWDS (Inkinen *et al.*, 2016; Bruno *et al.*, 2018), decreased their relative abundance at 24 °C, indicating that these bacteria were in disadvantage by the

temperature increase and they were shifted from the community. Burkholderiaceae is known by the presence of ecologically extremely diverse organisms, and several phytopathogens and primary pathogens for animals and humans belong to this family (Coenye, 2014). In addition, it has been reported as one of the most common contaminant for distilled and sterile water (Ferranti *et al.,* 2014), and therefore the reduction of the relative abundance of Burkholderiaceae ND at higher temperatures that has been observed here, could be potentially beneficial for water quality. Contrary, the reduction of the relative abundance of *Methylotenera* and *Methylobacterium* genera due temperature rise does not suggest positive effects for water quality. Tsagkari and Sloan (2019) demonstrated that *Methylobacterium*, even at low concentrations, is able to decrease the concentration of trihalomethanes in drinking water. Trihalomethanes are DBP formed mainly when chlorine is used to disinfect drinking water and they are considered carcinogenic for humans (WHO, 2017; Tsagkari and Sloan, 2019). On the other hand, members belonging to *Methylotenera* have been attributed to play an important role in methanol-linked denitrification in lake sediment (Kalyuhznaya *et al.*, 2009). Thus, they are able to decrease compounds such a nitrite in water, that is quite often formed as a DBP in DWDS and that it is potentially harmful when ingested by humans (Rantanen *et al.*, 2018). Thus, these results demonstrate that the reduction in the relative abundance of these genera due the increase of temperature can affect different degradation processes of compounds that are harmful to humans, and therefore reduce the water quality and compromise the health of consumers. This results also shows that, since it is impossible to avoid colonization by microorganisms in DWDS, more research is needed to promote the development of beneficial microorganisms such as *Methylotenera* or *Methylobacterium*, that might lead to safer drinking water. The relative abundance of other genera included *Sphingobium* or *Undibacterium,* increased their relative abundance in biofilms at higher temperatures. They were previously reported with variable relative abundances in other studies in these systems (Zhang *et al.*, 2012; Douterelo, Sharpe and Boxall, 2013), and results confirmed that they were favored in DWDS biofilms by the rise in temperature. Regarding *Undibacterium*, some species have been isolated from water environments, including drinking water (Kämpfer *et al.*, 2007) or purified water (Eder *et al.*, 2011), but it has not been reported to produce adverse effects on water quality or human health. However, Vaz-Moreira, Nunes and Manpaia (2011) observed that the predominance of *Sphingobium* in tap water samples and in water from dental chairs were related to the higher prevalence of antibiotic resistance genes, which threatening the ability to treat common infectious diseases (Ventola, 2015; WHO, 2017). Therefore, the increase in the relative abundance of this genus that has been observed

at higher temperatures could be associated with the increase and spread of antibiotic resistance genes, thus reducing the safety of distributed water. Other important genera that increased their abundance in biofilms at 24 °C were *Sphingomonas* and *Pseudomonas,* indicating that warmer temperatures favored their growth. Their presence was expected at both temperatures, since they have been observed to be ubiquitous bacteria that appears in DWDS independently of the pipe material, source water or hydraulic conditions (Martiny *et al.*, 2005; Zhang *et al.*, 2012; Douterelo *et al.*, 2016). However, the increase in *Sphingomonas* and *Pseudomonas* due to temperature rise could have negative consequences in terms of human health risks, since several opportunistic pathogens belong to these genera (Zhang *et al.*, 2012; Liu *et al.*, 2016), and they could endanger consumers with compromised immune or pulmonary systems. In addition, it is known that *Sphingomona*s and *Pseudomonas,* play an important role in DWDS since they are considered as pioneers organisms involved in the initial stages of biofilm development. These two microorganisms are capable of secreting a high amount of EPS that enhance the growth of biofilms (Johnsen *et al.*, 2000; Simões, Simões and Vieira, 2007; Irie *et al.*, 2012; Navarro-Noya *et al.*, 2013). Therefore, the increase of these genera due temperature rise could lead to an increase in EPS production and this is in agreement with the hypothesis described above about the greater biofilm formation and the consequent greater turbidity response observed at higher temperatures. These results again confirm that the increase in temperature in these systems can compromise water quality. Moreover, since EPS can act as protection for the cells (Flemming, 2002), the potential largest production at higher temperatures would make biofilm communities more resistant against changes in the environmental pipe conditions. This would explain that the microbial compositions during the growth phase and after mobilization were more similar to each other at 24 °C than at 16 °C, for both biofilm and water samples, which means that the flush had a lesser effect on changing community composition when the temperature was higher. These results have important implications in terms of DWDS cleaning strategies for water utilities. Since at higher temperatures the composition of bacterial communities was not affected by flushing, this mechanical strategy decreases its effectiveness in changing and removing certain genera of microorganisms. For example, the relative abundance of *Pseudomonas* in biofilm decreased at 16 °C after the mobilization phase but not at 24 °C, so it continued to be the most abundant genus which can facilitate the biofilm regrowth after flushing.

Regarding fungi in biofilms, genera like *Aspergillus* or *Cladosporium* were relatively abundant at 16 °C on day 10 and 20. Although it has been shown that these fungi are part of DWDS microbiota, *Aspergillus* is known to cause aspergillosis, which includes different human diseases, from allergies to invasive diseases, which can mainly affect people with a compromised immune system (Richardson and Rautemaa-Richardson, 2019). *Cladosporium* species are mainly pathogens of plants, but they can produce some allergies in humans after long-term exposure (Ogórek *et al.*, 2012). Thus, the reduction in the relative abundance of these genera that has been observed in this study could be a positive consequence of the temperature increase in this system for water quality. However, these genera were replaced in the microbial community by *Fusarium,* that became the most abundant genera on day 30 at 16 °C. *Fusarium* has important implications for DWDS due to the pathogenic species belonging to this genus. Specifically, it has been reported a large range of problems caused by the presence of these microorganisms in drinking water related to nosocomial infections that mainly affect immunocompromised patients (Anaissie *et al.*, 2001). Therefore, although *Fusarium* displaces other fungal species that can cause problems, their proliferation can also affect water quality, especially if it is mobilised into the bulk water and reaches consumer taps. Moreover, from this study it was notable the dominance of *Fusarium* at 24 °C from the earliest stages of biofilm development, reducing the complexity of the fungal community during the 30 days of the growth phase. Previous research demonstrated that the optimal temperature for the growth of some species of these filamentous fungi ranged from 24.7 °C to 27.5 °C (Nazari *et al.*, 2018), which would explain its faster proliferation during the 24 °C test in thus study. Thus, the human health risk due its pathogenic species commented above is bigger at higher temperatures. In addition, it is also known the ability of *Fusarium* to produce EPS that enhance biofilm formation (Anaissie *et al.*, 2001; Sav *et al.*, 2018). As it happened with the bacterial species *Pseudomonas* and *Sphingomonas,* the high relative abundance of *Fusarium* also could explain the greater biofilm formation and the consequent greater turbidity response observed at higher temperatures.

The composition of planktonic communities, as alpha and beta diversity also indicated, was more similar at the different temperatures. For bacteria, *Phreatobacter* was one of the genera that dominated the community at both temperatures, although it increased the relative abundance at 24 °C. *Phreatobacter* has not been reported by many studies because it was described few years ago by Toth *et al.* (2014). This genus was isolated from ultrapure water of a water purification system and it was observed in a previous study to be more abundant in waters with low chlorine

concentrations (Toth *et al.*, 2014; Ma *et al.*, 2019; Perrin *et al.*, 2019). Due to this, although it could be a clean water bioindicator, the implications of this genus for water quality have not yet been observed, so more research is needed around this to understand its role in DWDS. However, results from this research confirms that the increase of temperature and its consequence decrease in chlorine can enhance the relative abundance of *Phreatobacter* in water communities of chlorinated DWDS. The main genera that dominated the community together with *Phreatobacter* were *Sphingomonas* and *Nevskia.* They tended to increase its relative abundance in bulk water samples when the temperature was higher, displacing other taxa such a Burkholderiacea NA as it happened in biofilm communities. Although these genera are widely distributed and can be commonly found in water with variable relative abundance, it has been observed that some *Sphingomonas* species can produce sporadic infections (Anaissie *et al.*, 2001; Steinberg and Burd, 2015). The high proliferation observed here due to the increase in temperature then also could affect water quality. Therefore, more research is needed to study at the species level and verify that water quality can be affected by these genera. On the other hand, as it was possible to observe with biofilm communities, *Pseudomonas* was detached from the pipe walls at 16 °C decreasing its relative abundance in biofilms and increasing in the bulk water after the mobilisation phase. However, this did not happen at 24 °C and this reinforce the hypothesis raised above that the mechanical cleaning strategies that are commonly used can be compromised by the increase in temperature, losing effectiveness to remove certain species that can favour the rapid regrowth of biofilms.

Planktonic fungal community taxonomic structure was not significantly affected by temperature, as indicated by alpha and beta diversity. The principal taxa, such a Helotiales incertae sedis, *Cladosporium* or *Fusarium*, that were also found in other DWDS (Richardson and Rautemaa-Richardson, 2019), appeared in water samples thorough growth phase with a similar relative abundance at both temperatures. This means that with the increase of temperature the planktonic fungal composition was not affected, and temperature is not a determinant factor for these microorganisms. This again reinforces the importance of biofilms in DWDS and shows that sampling only the tap water communities it is not possible to understand the behavior of the microbiome of DWDS. However, after the mobilisation phase, it was possible to observe an increase of *Fusarium* at 24 °C, possibly consequence of its high relative abundance in biofilms at this temperature and its detachment due flushing events. The mobilisation of *Fusarium* sp. in the water at higher

temperatures could have important consequences, due to the pathogenic species of this genus (Anaissie *et al.*, 2001) which can reach the tap of consumers.

### 4.5.3 Occurrence and variation with temperature of opportunistic pathogens in water and biofilm samples

Temperature increase due to climate change is expected to encourage the growth of OPs in water sources (van der Kooij and van der Wielen, 2014; Bédard *et al.*, 2015; Ji *et al.*, 2017). Results from this study showed no significant differences between temperatures in the abundance of several OPs such as *P. aeruginosa*, *S. maltophilia* and *Acanthamoeba spp*. Nevertheless, these three microorganisms tested positive in practically all biofilm and water samples when were analysed with qPCR. Similar values for these particular OPs have been also reported by van der Wielen and van der Kooij (2013) in unchlorinated systems at temperatures ranging from 8.7 to 14.8 °C, and this results confirms that they are able to survive in chlorinated system at 16 °C and 24 °C. Despite the absence of differences between temperatures, these results reinforce that this important OPs can survive under different conditions in DWDS and drinking water can act as a channel to transmit them. *P. aeruginosa* and *S. maltophilia* are bacteria commonly present in drinking water and biofilms DWDS (van der Wielen and van der Kooij, 2013; van der Wielen and Lut, 2016), although they are a common virulent respiratory pathogen that produce diseases such as pneumonia or cystic fibrosis (Morello *et al.*, 2011; Brooke, 2012). It was notable that their occurrence in bulk water did not increase after removing the material attached to pipes at any temperature, suggesting that flushing at both 16 °C and 24 °C did not remove these microorganisms from the biofilm. Contrary, *Acanthamoeba spp.* increased its occurrence in water and reduced it in biofilms after the mobilisation phase at both temperatures, but there were no significant differences between 16 °C and 24 °C. Thus, results show that *Acanthamoeba* spp. are likely to be mobilised when the flow changes as in events of flushing, and its mobilization could have negative consequences for human health. This free-living amoeba is a well know host of other pathogenic microorganisms but some species of this genus also have been associated to opportunistic and non-opportunistic infections in humans and other animal, although normally they mainly affect immunocompromised patients (Hoffmann and Michel, 2001; Guimaraes *et al.*, 2016). In addition, *Acanthamoeba* spp. are a well know hosts of *L. pneumophillia* (Dobrowsky *et al.*, 2016; Guimaraes *et al.*, 2016) which was also quantified in this study. *L. pneumophilllia* is an important human pathogen normally inhabits

drinking-water-related systems at temperatures between 25 °C and 45 °C, but it has been reported that can also survive at lower temperatures in DWDS, especially in biofilms on different pipe materials (Markku J Lehtola *et al.*, 2007; Gião *et al.*, 2009; Moritz, Flemming and Wingender, 2010). Although it is common in DWDS and that its guest *Acanthamoeba* spp. was detected, this OP was below q-PCR detection limit in in all samples at both temperatures, which is a positive aspect for the microbiological quality of this system.

Regarding NTM q-PCR results showed that *Mycobacterium* spp. was present in all biofilm and water samples analyzed in this study, with a number of genes within the range previously reported in other studies in unchlorinated systems (van der Wielen and van der Kooij, 2013; Michael B. Waak *et al.*, 2019). Although this genus is widespread in the environment, the presence of *Mycobacterium* spp. in drinking water have been linked to nosocomial infections (Liu *et al.*, 2016), so its presence at both temperatures observed in this study, is a negative implication in terms of water safety of chlorinated DWDS. Differences for *Mycobacterium* spp. gene copy number were only significant between both temperatures in biofilm samples on day 30. This indicates that temperature rise can promote pathogen hosting in biofilms after the initial development of attached microbial communities. In addition, it was also possible to observe the increase of this genus in bulk water after the mobilisation phase at both temperatures, suggesting that it can be mobilised into the bulk water if the flow shear stress at the pipe wall changes compromising the water safety.

For *M. avium* complex, there were not any quantification at 16 °C in biofilm and water samples, but this pathogen increased its occurrence and was detected at 24 °C in all sampling events. It has been reported that temperature had impact in the relative abundance of *M*. *avium* complex in tap water samples from a pilot-scale rigs that simulated a hot water plumbing system at temperatures between 39 °C and 51 °C (Dai *et al.,* 2018). Results from this study reinforce this previous research, and show that *M. avium* complex is also present in biofilms of DWDS and that less drastic changes in temperature (i.e. from 16 °C to 24 °C, temperatures that DWDS can reach) can also lead to an increase in the relative abundance of this OP.

Therefore, temperature is a determinant factor for the occurrence NTM in water and biofilms of DWDS, and that higher temperatures favoured its presence in the two habitats. Moreover, since the occurrence of *Mycobacterium spp*. could not be explained only with *M. avium* complex, this results also suggest that other uncertain species of mycobacteria are present in the samples analyzed in our study. Other pathogenic species belong to this genus, such a *M. gordonae,* have been also

detected in DWDS (Michael B. Waak *et al.*, 2019), and thus they could be part of the microbiome of these systems, which would have implications for water safety and human health. Thus, more studies are needed to identify all of this OPs and understand what factors enhance their development to try to avoid their presence in DWDS and safeguard the health of consumers.

## 4.6 Conclusion

From this research it can be concluded that:

- Higher temperatures increased biofilm development on HDPE pipes in a chlorinated DWDS, leading to a higher discolouration (quantified as turbidity, iron and manganese) due to simulated flushing.
- Temperature was a key factor for the structure and composition of the microbiome of the DWDS. Temperature affected the structure of bacterial and fungal communities in water, but changes were more marked in biofilms than in planktonic communities.
- In biofilms, the relative abundance of *Pseudomonas* and *Fusarium* was favoured by temperature increase. These two genera have an enhanced capability to promote biofilm development and compromising further water quality.
- The study of OPs showed that temperature rise increased the detection of *Mycobacterium* spp. in biofilms and favour the presence of *M. avium* complex in water and biofilm samples.

This study provides new insights on the consequences of climate change on the distribution of drinking water and its impact on water quality. It has been shown that the higher temperatures and extreme weather conditions projected will affect DWDS and further deteriorate the way water is delivered through these complex systems. More research in this direction is needed in order to get a good understanding about how these communities are able to adapt to higher temperatures leaded by earth's warming due to climate change in drinking-water-related environments. This knowledge can help to design of effective management strategies aimed at minimizing the water quality risks associated with climate change and microbial presence. Moreover, all of this remarks the importance of the understanding DWDS microbiome (water and biofilm) in order to prevent effectively problems related to human health and ensure the distribution of safe quality water.

# Chapter 5

# The effects of temperature on biofilm control strategies in chlorinated drinking water distribution systems*

## 5.1 Abstract

The development of microbial biofilms communities in drinking water distribution systems (DWDS) can be affected by different environmental factors, being temperature one of the key parameters influencing its characteristics. This study investigated the effect of different management strategies used to control biofilm development in DWDS when the temperature increase in the system. Using an experimental real scale chlorinated DWDS, three different management strategies were compared: flushing of pipes followed by hyperchlorination, only flushing and without mechanical or chemical treatment. Temperature rise from 16 °C to 24 °C promoted water discolouration events, although these were dependent on the control strategy applied. Results from DNA sequencing showed that when the temperature was increased, the management strategy applied was key in determining biofilm community structure, while planktonic communities were less affected by temperature changes and/or management strategy. Flushing, combined or not with hyperchlorination, was effective at reducing the abundance of microorganisms that can compromise water quality and safety such as the bacteria *Flavobacterium, Sphingobium* or *Methyloversatilis,* and the fungi *Fusarium* and *Cladosporium*. This study provides new knowledgeon the microbial ecology of DWDS, which can be useful to adapt/improve management strategies in real DWDS under a climate change scenario.

**Keywords:** climate change, flushing, hyperchlorination, discolouration, bacteria, fungi, metagenomics.

* This chapter will be adapted for a journal publication in *Environmental Science and Technology* journal as Calero-Preciado, C..; Soria-Carrasco, V.; Boxall, J.; Douterelo, I.: The effects of temperature on biofilm control strategies in chlorinated drinking water distribution systems. See Appendix D for more information on contributions and co-authors.

## 5.2 Introduction

Drinking Water Distribution Systems (DWDS) are large and complex engineering networks designed for the distribution and supply of drinking water. Before and during its distribution through DWDS, drinking water is treated by different processes in order to remove contaminants and microbes to make it safe for consumption. However, it has been widely observed that microorganisms are able to survive this disinfection processes and inhabit DWDS (Szewzyk *et al.*, 2000; Chaves Simões and Simões, 2013). Most of the microorganisms living in DWDS are attached to the pipe surfaces forming biofilms, which provide them protection and other physiological advantages over planktonic cells (Berry, Xi and Raskin, 2006; Henne *et al.*, 2012). Different environmental factors can affect biofilm development in DWDS, including source water characteristics, operating hydraulic regimes, different pipe materials and temperature (Douterelo, Sharpe and Boxall, 2013; Gomez-Alvarez *et al.*, 2015; Ren *et al.*, 2015; Calero Preciado *et al.*, 2019). Temperature has been found to be a key factor on which water quality of different environments depends (Whitehead *et al.*, 2009). For example, in in drinking-water related systems it has been observed that disinfectant concentration and water dissolved oxygen decrease with increasing temperature (Monteiro *et al.*, 2017; WHO, 2017; Li *et al.*, 2018). In a microbiological point of view, it has been demonstrated that higher temperatures, within physiological limits, stimulate the microbial growth and activity and enhance the biofilm development (Hallam *et al.*, 2001; Delpla *et al.*, 2009; Kent, Garcia and Martiny, 2018).

Currently, global climate change is expected to increase the ambient temperature, thus affecting drinking water temperature in the networks since they are positively related (Bates *et al.*, 2008; Blokker and Pieterse-Quirijns, 2013). This increase in temperature can lead to changes in microbial communities in DWDS and promote biofilm development. This further potential for biofilm development can affect the quality and safety of the water supplied to consumers. This is because although natural biofilm communities are part of DWDS, they are also the cause of a large number of problems contributing to the deterioration of water quality, especially if they are mobilised into the bulk water when the shear stress on the pipe wall changes (Husband and Boxall, 2011). The presence of biofilms and/or their metabolic activities can produce discolouration, taste or odour problems, it can promote the corrosion or the obstruction of the system or/and can compromise the water safety by acting as reservoir for pathogens (Batté *et al.*, 2003; Wingender and Flemming, 2011; Chaves Simões and Simões, 2013).

In order to minimise the risk associated with the presence of biofilms, water companies have implemented different operational and management strategies (Vreeburg, 2007; Garcia de Carellan *et al.*, 2012). Mechanical methods are the most widely used to control biofilms in DWDS, including flushing, water/air scouring and pigging (Vreeburg, 2007; Vreeburg and Boxall, 2007). Water flushing is the most common and longest applied method for cleaning the pipes in UK and consists of increasing the water flow to produce an increase in the velocity in the pipe that will lead to further shear stress on the biofilms attached to the pipe surfaces (Boxall, Skipworth and Saul, 2001; Friedman *et al.*, 2003; Vreeburg, 2007). In real operational networks planned flushing regimes are carried out by closing selected valves and opening hydrants to increase flow and rise the hydraulic forces within pipes (Douterelo, Husband and Boxall, 2014). Sometimes, flushing works can be applied in conjunction with high concentrations of disinfectant for more effective system cleaning, especially after pipe break and repair (Chaves Simões and Simões, 2013; van Bel *et al.*, 2019). Nevertheless, these cleaning methods have been found not to be completely effective, and sometimes they only can partially remove biofilm deposits from pipes (Douterelo, Sharpe and Boxall, 2013; Fish, 2013). In addition, it has been observed that microorganisms remaining attached to the pipe wall play a fundamental role and contribute to the biofilm regrowth (Douterelo, Husband and Boxall, 2014), thus selecting a good effective cleaning strategy can be vital to maintaining the biological safety of the water that reaches consumers' taps. This may be especially important in a climate change scenario since temperature could affect the microbial communities in different ways depending on the cleaning strategy.

This study aims to evaluate how the increase in temperature affects the microbial ecology, thus the water quality of DWDS under different management strategies. The management strategies tested included (1) cleaning based on the combination of mechanical and chemical techniques (i.e. flushing events combined with hyperchlorination); (2) only mechanical treatment (i.e. flushing); and (3) without applying any mechanical or chemical treatment (i.e. microbial community transition between temperatures). The objectives of this study were aimed at understanding the combined effect of temperature with management strategies on biofilm characteristics and its implication for water quality. This new understanding will provide valuable information to adapt and/or improve biofilm management strategies under a temperature increase scenario, thus helping to ensure the supply of safe drinking water to consumers.

## 5.3 Materials and methods

### 5.3.1 Experimental DWDS facility

The study was carried out in a full scale experimental DWDS facility located at the University of Sheffield. The facility has three independent loops made of High-Density Polyethylene (HDPE) pipes (PE100 SDR17) with an internal diameter of 79.3 mm and a length of 9.5 x 21.4 m coils (Figure 35A). Local drinking water is supplied using individual enclosed reservoir tanks, from where the water is recirculated in the system using individual speed pumps and several valves installed throughout the system (Figure 35B). LabVIEW (version 8.2, National Instrument Corporation, UK) installed in a central computer is used to control pump and valve settings, enabling the simulation of real hydraulic demand patterns during the tests. The facility has different monitoring equipment that provide online measurements of parameters including flow, pressure, turbidity, chlorine, or temperature. The facility is within a temperature-controlled room, and the temperature of the water entering the system and during the test is controlled by immersion heaters installed in each tank. In addition, the water has a retention time of 24 hours in the system, controlled with a trickle drain and feed-based system to maintain baseline water quality parameters.

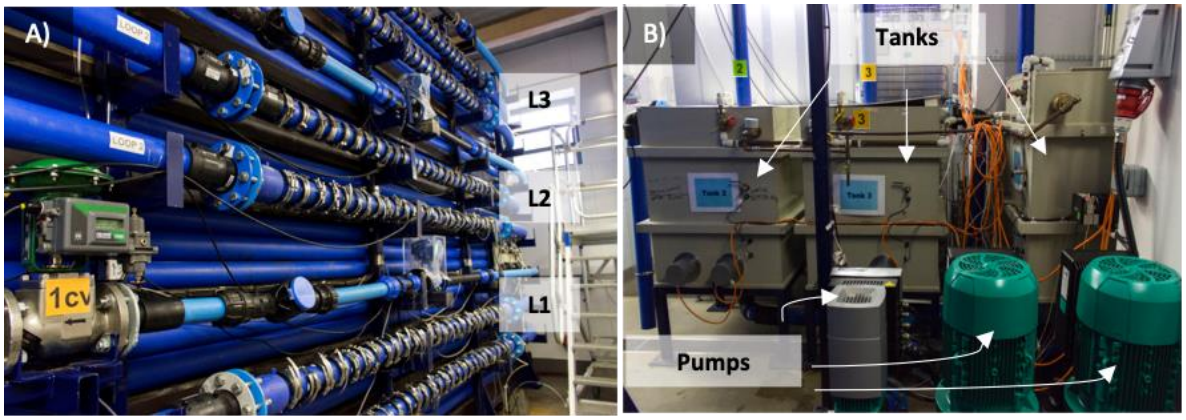



***Figure 35***. A) Full scale test facility composed of 3 loops (L1, L2 and L3); B) Idividual tanks and pumps.

Each loop has two coils where it is possible to insert 2 x 27 removable HDPE Pennine Water Group (PWG) coupons to facilitate the analysis of biofilms *in* situ (Figure 36A, B). Each coupon consists of a portion of pipe which fit with the internal surface and accurately following the pipe curvature, thus experiencing the same environment as the rest of the pipeline with a minimal impact on the system hydraulic. Coupons are comprised of two parts: (i) the outer coupon used to collect biofilm

biomass for DNA-based analysis; and (ii) and an insert that can be removed from the coupon body for microscopy analysis (Figure 36C) (Deines *et al.*, 2010).

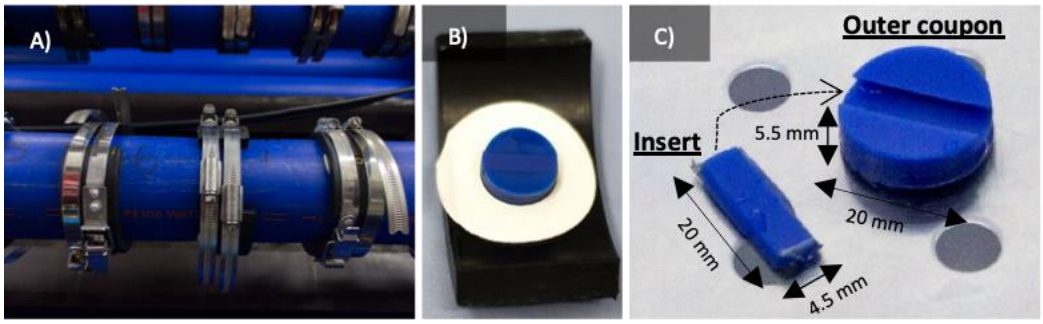



***Figure 36***. A) Coupons inserted and fixed into the pipes with holders and clips; B) coupon fixed in a coupon mounting; C) details and dimensions of the coupons: insert for microscopy and outer for DNA analysis.

### 5.3.2 Operational conditions

To study the effect of temperature under different management strategies, 2 different temperatures were selected to run experiments, 16 °C and 24 °C. 16 °C is the average UK water temperature during spring-summer months (Husband, Boxall and Saul, 2008), and 24 °C was selected based on the World Health Organization (WHO) recommendation that the drinking water temperature should not be greater than 25°C to limit the microbial growth and pathogen proliferation in drinking water systems (WHO, 2017).

Prior to the start of the experiments the facility was cleaned and disinfected adding to each loop 20 mg/L of RODOLITE H (sodium hypochlorite based solution with less than 16 % free chlorine) (RODOL Ltd, Liverpool, UK). Then, the system was flushed at a maximum flow rate (4.2 L/s) and left standing for 24 h with the disinfectant. After this time the facility was flushed again at 4.2 L/s with fresh tap water until the levels of free chlorine showed similar values to the local tap water entering the system (≈ 0.2 mg/L). After disinfecting the system, sterile PWG coupons were inserted along the pipes.

The 3 loops were run during 30 days at 16 °C to allow for biofilm development in the pipe walls. After this growth phase, each loop was subjected to different management strategy (Figure 37A). In loop 1 mechanical cleaning and chemical disinfection were applied. Pipes were flushed by increasing the flow in 4 gradual steps for a duration of 3 turnovers (Figure 37B, Table 10) and then a hyperchlorination of the system was performed adding 20mg/L of RODOLITE H. In loop 2, only a

mechanical cleaning procedure was performed, flushing the pipes by applying the same 4 gradual steps as explained for loop 1. In loop 3, no mechanical or chemical maintenance treatment was applied to study the natural transition of biofilm communities when temperature increases. After the 3 different treatments, new sterile PWG coupons were inserted along the pipes in the 3 loops and temperature was increased to run a second biofilm growth phase of 30 days at 24 °C. At the end of this growth phase, the 3 loops were flushed by applying the same 4 gradual steps by increasing the shear stress (τ) in the pipes: step 1=0.4 N/m$^2$, step 2=2.3 N/m$^2$, step 3=3.4 N/m$^2$, step 4=4.3 N/m$^2$. Each step was performed for a duration of 3 water turnovers (i.e. the time that the total volume of the water needs for recirculate in the loop three times) in order to remove and quantify loosely adhered material at the pipe walls (Sharpe et al., 2010) (Table 10). For biofilm growth phases at both temperatures, a low varied flow (LVF), ranging from 0.2 to 0.5 L/s, was applied which simulate a real hydraulic regime based on daily patterns observed in real DWDS in the UK (Husband, Boxall and Saul, 2008) (Table 10).

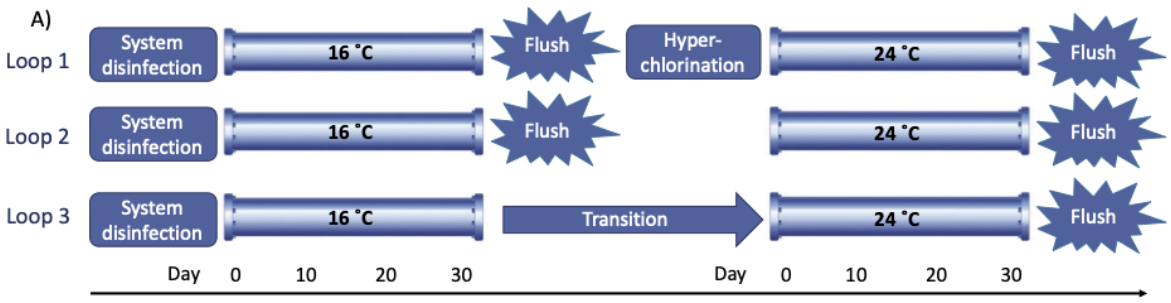



***Figure 37.*** A) Scheme of the test performed in the 3 loops over time.

***Table 10.*** Flow rate conversions applied during the growth phases and in each flushing step.

| | Flow (Q) - (L/s) | Velocity (v) - (m/s) | Shear stress (τ) - (N/m$^2$) |
|---|---|---|---|
| Growth phase | 0.2 - 0.5 | 0.04 – 0.10 | 0.1 – 0.3 |
| Step 1 Flushing | 0.74 | 0.15 | 0.4 |
| Step 2 Flushing | 3.58 | 0.72 | 2.3 |
| Step 3 Flushing | 5.10 | 1.03 | 3.4 |
| Step 4 Flushing | 6.24 | 1.27 | 4.3 |

### 5.3.3 Microbial communities sampling

To study planktonic and biofilm microbial communities, bulk water samples and PWG coupons were collected on days 0, 10, 20 and 30 throughout the growth phases and post-flushing events performed in each loop at both temperatures. For biofilm samples, 3 biological replicates of 3 PWG coupons each were taken in each sampling point to obtain an optimal concentration of DNA for Next Generation Sequencing (NGS). Since at 16 °C the 3 loops were running under the same conditions, one biological replicate was obtained from each loop. At 24 °C after the different management strategies the 3 replicates were obtained from each corresponding loop. Biofilm attached to the coupons was removed by standardized brushing in 30 mL of phosphate-buffered saline solution (PBS) (Gibco®, Thermo Fisher Scientific, UK), creating a biofilm suspension that was then filtered through a 0.22 µm nitrocellulose membrane filters (Millipore Corp., USA) (Deines *et al.*, 2010). For water samples, 3 replicates of 2 L of bulk water were taken at each sampling point and then filtered through 0.22 µm nitrocellulose membrane filters (Millipore Corp., USA). Filters of water and biofilm samples were preserved at -20 °C in the dark until the molecular analysis was carried out.

### 5.3.4 Water quality physico-chemical analysis

During the experiment, turbidity and chlorine online measurements were recorder at 1-minute intervals by the equipment installed in the system, an ATi A15/76 turbidity monitor (ATi, Delph, UK) and an ATi Q45H-79 chlorine monitor (ATi, Manchester, UK) respectively. Additionally, triplicates of discrete water samples were collected at the time of each sampling point to analyse several physico-chemical parameters. pH and water temperature were tested using a Hanna portable meter HI 991003 (Hanna Instruments, Leighton Buzzard, UK) and free and total chlorine were analysed with a Palintest CS100 chlorosense (Palintest, UK). Total organic carbon (TOC), and the concentration of iron (Fe) and manganese (Mn) (as water discolouration indicators), were also measured in triplicate at each sampling point by The Kroto Research Institute (KRI) (Sheffield, UK). Briefly, TOC samples were taken in 20 ml glass vials and then analysed using a Shimadzu TOC-$V_{CPH}$/$_{CPN}$ Analyzer (Shimadzu, Kyoto, Japan) following the protocol of the instruction manual. Inductively Coupled Plasma Mass Spectrometry (ICP-MS) was used to determine Fe and Mn concentrations in water. Samples were collected in 20 mL vials containing 5 M of nitric acid, then ions were monitored on a Perkin Elmer Elan DRC II (PerkinElmer, Inc., USA) and data acquisition was via Elan NT software (tla.mpi.nl/tools/tla-tools/elan) (Sloetjes and Wittenburg, 2008).

### 5.3.5 Microscopy analysis

For biofilm visualization, the inserts of the PWG coupons were analysed using Scanning Electron Microscopy (SEM). SEM analysis was performed electron microscopy facility, at the Faculty of Science (FoS) of the University of Sheffield (UK). Triplicates of PWG coupons inserts were removed on day 0 (control) and day 30 from each loop at both temperatures. Inserts were fixed for 24 hours in 5% formaldehyde solution (Fisher Scientific, UK) and then preserved in PBS at 4 °C. For visualization, inserts were secondary fixed in 1 % aqueous osmium tetroxide for 1 hour at room temperature and then washed twice with PBS for 10 minutes. Then, biofilms were dehydrated through a graded series of ethanol washes for 15 minutes: 75 %, 95 %, 100 % twice and 100 % ethanol dried over anhydrous copper sulphate. Samples were placed in 50/50 (v/v) mixture of 100 % ethanol/100% hexamethyldisilazane for 30 minutes followed by 30 minutes in 100 % hexamethyldisilazane and air-dried overnight. Samples were mounted onto SEM Pin stub specimen mounts and coated with approximately 25 nm of gold in an Edwards Gold Sputter Coater S150B (Edwards, UK). Micrographs of biofilms were obtained with a TESCAN Vega 3 LMU SEM (Girton, Cambridge, UK) at an accelerating voltage of 15 kV.

### 5.3.6 DNA extraction and sequencing

DNA from all the filters where water and biofilm samples were concentrated, was extracted following the protocol based on hexadecylmethylammonium bromide (CTAB) and proteinase K chemical lysis, followed by DNA purification using phenol/isoamyl alcohol method ((Zhou, Bruns and Tiedje, 1996; Neufeld *et al.*, 2007). In brief, filters were place into 15 mL sterile Falcon tubes and 740 mL of SET lysis buffer (40 mM EDTA, 50mM Tris-HCl, pH 9, 0.75 M sucrose) and 90 ml of lysozyme (10 mg/mL) were added. Tubes were incubated at 37 °C for 30 minutes with rotation in a Hybaid hybridisation oven (Thermo Scientific, UK). Then, 90 mL of 10 % sodium dodecyl sulphate (SDS) and 25 mL of proteinase K (20 mg/mL) were added and the tubes were again incubated at at 55 °C for 2 hours with rotation. Afterwards, lysates (i.e. supernatant) were withdrawn and transferred into 2 mL sterile Eppendorf tubes. 137 µL 5M NaCl and 115 µL CTAB/NaCl solution were added to the tubes and they were incubated at 65 °C for 30 minutes with rotation. 838 mL µL of chloroform:isoamyl alcohol (24:1) (Sigma-Aldrich, UK) were added twice to extract the supernatant. DNA was precipitated adding 815 µL of 100 % isopropanol (Sigma-Aldrich, UK) and finally washed twice with 1 mL of 70 % ethanol, air dried and resuspended in 50 µL DEP-treated sterile water (Thermo

Scientific, UK). The concentration of purified DNA from each extraction was assessed fluorometrically by the HS dsDNA Assay kit and a Qubit 4 Fluorometer (Invitrogen by Thermo Fisher Scientific, Wilmington, USA).

The sequencing of the purified DNA was performed by Mr DNA Laboratory (www.mrdnalab.com, Shallowater, TX, USA) on the Illumina MiSeq platform following the manufacturer's guidelines for pair-end sequencing. The 16S rRNA gene of bacteria was amplified using the primers 28F (GAGTTTGATCNTGGCTCAG) and 519R (GTNTTACNGCGGCKGCTG) spanning the V1 to V3 hypervariable regions. For fungal analysis, the ITS1-2 region was targeted for the amplification using the primers ITS1FBt1 (CTTGGTCATTTAGAGGAAGTAA)/ ITS2R (GCTGCGTTCTTCATCGATGC). Briefly, 30 PCR cycles were performed using the HotStarTaq Plus Master Mix Kit (Qiagen, USA) and the following protocol: 94 °C for 3 minutes, followed by 30 cycles of 94 °C for 30 seconds, 53 °C for 40 seconds, 72 °C for 1 minute and a final elongation step at 72°C for 5 minutes. PCR products were checked after the amplification in 2 % agarose gel to determine the success of amplification and the relative intensity of bands. Samples were pooled together in equal proportions based on their molecular weight and DNA concentrations. Pooled samples were purified using calibrated Ampure XP beads (Beckman Coulter, Inc.) and the pooled and purified PCR product was used to prepare Illumina DNA library.

### 5.3.7 Bioinformatics and microbial community analysis

Firstly, raw sequences obtained from Illumina were subjected to quality control to evaluate the number, the quality and the length of the initial reads using the FastQC software version 0.11.8 (bioinformatics.babraham.ac.uk) (Andrew, 2010). Sequences were then filtered and trimmed, removing those with an average quality phred score below 20 and/or a minimum length of 100 bp by applying BBDuk software version 37.95 (bbtools/bb-tools-user-guide/bbduk-guide/) (Cock *et al.*, 2009). BBDuk was also used to remove sequencing errors (Davis *et al.*, 2018). Subsequently, sequences were demultiplexed and depleted of sequencing barcodes using sabre software (github.com/najoshi/sabre) (Joshi, 2011) and imported into the Quantitative Insights Into Microbial Ecology 2 program (QIIME2, version 2019.7, qiime2.org) (Bolyen *et al.*, 2019). Then, within QIIME2 the implemented vsearch plug-in (github.com/qiime2/q2-vsearch) (Rognes *et al.*, 2016) was used to join pair-end sequences, dereplicate them, identify and filter chimeric sequences and to make *de-novo* clustering by 97% similarity to obtain the Operational Taxonomic Units (OTUs). OTUs

taxonomic assignment was performed using the classify-consensus-vsearch method (Rognes *et al.*, 2016) of the feature-classifier plug-in in QIIME2 (github.com/qiime2/q2-feature-classifier) (Bokulich *et al.*, 2018). 16S sequences were compared against SILVA SSU r132 database (arb-silva.de) (Quast *et al.*, 2013) and ITS2 sequences against UNITE 8.0 (unite.ut.ee) (Kõljalg *et al.*, 2013).

Biodiversity of bacterial and fungal communities was estimated via alpha and beta diversity using rarefied tables of relative abundance of OTUs at 97% sequence similarity cut off (Morris *et al.*, 2014). Alpha diversity (diversity within samples) was estimated by Chao1 (richness measurement), Simpson (dominance measurement) and Shannon (diversity measurement that consider both richness and dominance) indices, that were calculated applying the q2-diversity plug-in in QIIME2 (github.com/qiime2/q2-diversity). For beta diversity (degree of community differentiation between samples) rarefied OTU tables were square-root transformed and similarity matrices were constructed applying the Bray-Curtis method was using vegan package version 2.5-6 in R (github.com/vegandevs/vegan) (Oksanen *et al.*, 2019). Non-metric multidimensional scaling (nMDS) diagrams were built for the visualization of resemblance matrices with ggplot2 package version 3.2.1 in R (github.com/tidyverse/ggplot2) (Wickham and Chan, 2016).

### 5.3.8 Statistical analysis

Triplicates measurements (n = 3) were used for the mean and standard deviation calculation of all physico-chemical parameters and alpha diversity values. For 16 °C the average and standard deviation of the mean values of the 3 loops (n = 9) was calculated since the 3 loops were running under the same conditions during the growth phase.

Before the significance test, Shapiro-Wilks tests were used to test the normality of the data sets. Then, the non-parametric Kruskal–Wallis test by ranks was performed in order to detect significant differences between temperatures and different management strategies. Then, if significant, differences between samples were compared pairwise by performing the non-parametric Mann-Whitney *U test*. The analysis of similarities (ANOSIM) was calculating using the Bray-Curtis distance matrices to test beta diversity significant differences. The global-R statistics was calculated to detect the impact of the temperature increasing under different management strategies. global-R values range between 0 and 1, indicating 1 a total differentiation of the samples (Anderson and Walsh,

2013). Differences were considered statistically significant when p-value was ≤0.05, and all statistical tests were carried out using R software version 3.6.1 (r-project.org) (R Core Team, 2014).

## 5.4 Results

### 5.4.1 Water physico-chemical analysis

Results of all different water quality parameters measured through the experiment are shown in Table 11. Water temperature showed stable average values close to the 16 °C and 24 °C selected for the experiments. pH was near neutral showing values fluctuating from 6.67 to 7.47. Water disinfectant concentrations during the test showed higher average values of total and free chlorine on day 0 at 16 °C (total chlorine = 0.91 mg/L, free chlorine = 0.89 mg/L) and in loop 1 at 24 °C (total chlorine = 0.80 mg/L, free chlorine = 0.68 mg/L), consequence of the disinfection of the system carried out before the experiment, and of the hyperchlorination carried out in this loop. Then, the concentration of disinfectant was slightly higher in the test at 16 °C (total chlorine ranging 0.17 - 0.21 mg/L , and free chlorine 0.17 - 0.09 mg/L) than in the tests at 24 °C (total chlorine ranging 0.11 - 0.21 mg/L, and free chlorine 0.03 - 0.11 mg/L). When the values were statistically compared, no significant differences were observed (Kruskal-Wallis test, p-value >0.05). In the same way, TOC values did not show high fluctuations during the experiments, ranging from 1.16 to 1.50 mg/L, and no significant changes were found (Kruskal-Wallis test, p-value >0.05).

Regarding the concentration of Fe and Mn, similar values were observed during the growth phases, ranging Fe from 41.3 to 59.3 µg/L and Mn from 0.39 to 0.58 µg/L in all tests, and no significant differences were found (Kruskal-Wallis test, p-value >0.05). However, post-flushing events the concentration of these metals increased, showing higher average values after the tests at 24 °C than at 16 °C (Table 11). Statistical test showed significant differences in the concentration of both metals (Kruskal-Wallis test, p-value >0.05) and pairwise comparisons confirmed significant higher values (Mann-Whitney *U tests*, p-value ≤0.05) at 24 °C after all management strategies (i.e. loop 1: after flushing-hyperchlorination; loop 2: after flushing; loop 3: transition with no treatment) than at 16 °C. No significant differences (Mann-Whitney *U tests*, p-value >0.05) were observed when Fe and Mn concentrations were compared between each management strategy at 24 °C. See appendix B.4 for supplementary information on the results of the statistical analysis.

***Table 11.*** Water physico-chemical parameters measured on all sampling points. 16 °C values are the average ± standard deviation of the 3 replicates of the 3 loops during the growth phase (n = 9), and of the loops 1 and 2 post-flushing (n = 6, loop 3 was not flushed). The rest of values represent the average of 3 water replicates analysis ± standard deviation.

| | Sampling day | Temperature (°C) | pH | Total Cl (mg/L) | Free Cl (mg/L) | TOC (mg/L) | Fe (µg/L) | Mn (µg/L) |
|---|---|---|---|---|---|---|---|---|
| 16 °C | **0** | 16.0 ± 0.05 | 7.29 ± 0.08 | 0.91 ± 0.06 | 0.89 ± 0.05 | 1.16 ± 0.03 | 44.6 ± 0.64 | 0.39 ± 0.03 |
| | **10** | 15.7 ± 0.07 | 7.11 ± 0.09 | 0.17 ± 0.03 | 0.09 ± 0.01 | 1.20 ± 0.04 | 41.3 ± 0.22 | 0.48 ± 0.01 |
| | **20** | 16.0 ± 0.06 | 6.98 ± 0.23 | 0.19 ± 0.01 | 0.13 ± 0.00 | 1.23 ± 0.03 | 46.3 ± 0.92 | 0.43 ± 0.04 |
| | **30** | 15.8 ± 0.10 | 6.71 ± 0.19 | 0.21 ± 0.03 | 0.14 ± 0.02 | 1.15 ± 0.05 | 44.6 ± 0.97 | 0.45 ± 0.00 |
| | **Post-flushing** | 15.7 ± 0.05 | 6.67 ± 0.08 | 0.19 ± 0.02 | 0.17 ± 0.01 | 1.51 ± 0.06 | 52.3 ± 0.41 | 0.69 ± 0.06 |
| 24 °C Flushing + Chlorine | **0** | 23.1 ± 0.41 | 6.79 ± 0.19 | 0.80 ± 0.01 | 0.68 ± 0.03 | 1.35 ± 0.05 | 49.0 ± 0.63 | 0.39 ± 0.01 |
| | **10** | 24.0 ± 0.03 | 7.47 ± 0.21 | 0.13 ± 0.03 | 0.08 ± 0.04 | 1.44 ± 0.06 | 49.9 ± 0.54 | 0.47 ± 0.01 |
| | **20** | 23.8 ± 0.07 | 6.92 ± 0.09 | 0.13 ± 0.01 | 0.08 ± 0.01 | 1.34 ± 0.06 | 59.3 ± 0.83 | 0.42 ± 0.02 |
| | **30** | 23.9 ± 0.06 | 6.80 ± 0.16 | 0.13 ± 0.05 | 0.10 ± 0.02 | 1.31 ± 0.02 | 45.1 ± 1.07 | 0.39 ± 0.02 |
| | **Post-flushing** | 23.4 ± 0.03 | 6.93 ± 0.13 | 0.21 ± 0.01 | 0.11 ± 0.03 | 1.50 ± 0.02 | 81.2 ± 1.34 | 1.20 ± 0.04 |
| 24 °C Flushing | **0** | 23.1 ± 0.07 | 6.98 ± 0.16 | 0.12 ± 0.01 | 0.08 ± 0.01 | 1.37 ± 0.04 | 48.5 ± 0.32 | 0.43 ± 0.00 |
| | **10** | 24.0 ± 0.06 | 7.00 ± 0.02 | 0.11 ± 0.00 | 0.07 ± 0.00 | 1.32 ± 0.03 | 51.6 ± 0.85 | 0.47 ± 0.01 |
| | **20** | 23.7 ± 0.18 | 6.82 ± 0.11 | 0.15 ± 0.01 | 0.06 ± 0.01 | 1.40 ± 0.05 | 52.8 ± 1.03 | 0.42 ± 0.01 |
| | **30** | 24.2 ± 0.10 | 6.65 ± 0.01 | 0.12 ± 0.04 | 0.10 ± 0.01 | 1.23 ± 0.06 | 48.6 ± 0.37 | 0.39 ± 0.04 |
| | **Post-flushing** | 23.5 ± 0.03 | 6.91 ± 0.11 | 0.16 ± 0.00 | 0.10 ± 0.02 | 1.34 ± 0.06 | 85.49 ± 1.69 | 1.08 ± 0.07 |
| 24 °C Transition | **0** | 23.8 ± 0.18 | 6.8 ± 0.04 | 0.13 ± 0.01 | 0.07 ± 0.03 | 1.37 ± 0.06 | 47.8 ± 0.37 | 0.39 ± 0.01 |
| | **10** | 24.3 ± 0.07 | 7.05 ± 0.02 | 0.12 ± 0.02 | 0.03 ± 0.01 | 1.34 ± 0.03 | 52.7 ± 0.24 | 0.48 ± 0.03 |
| | **20** | 23.4 ± 0.07 | 7.25 ± 0.03 | 0.15 ± 0.00 | 0.05 ± 0.02 | 1.32 ± 0.01 | 45.5 ± 1.38 | 0.58 ± 0.12 |
| | **30** | 23.9 ± 0.28 | 6.99 ± 0.12 | 0.12 ± 0.01 | 0.05 ± 0.00 | 1.35 ± 0.03 | 48.7 ± 1.61 | 0.55 ± 0.02 |
| | **Post-flushing** | 22.9 ± 0.09 | 6.96 ± 0.04 | 0.15 ± 0.01 | 0.08 ± 0.00 | 1.34 ± 0.05 | 87.8 ± 1.96 | 1.24 ± 0.20 |

#### 5.4.1.1 Turbidity

Results of turbidity online measurements showed similar average values ranging from 0.041 to 0.043 ± 0.03 NTU during the last 24 hours of the growth phases in all tests (representative value of the entire growth phase, where shear stress (τ) = 0.1 – 0.3 N/m$^2$). However, during the flushing events when τ increased by 4 steps, different turbidity responses were observed (Figure 38). In stage 1 (τ = 0.4 N/m$^2$) the average turbidity response of the last turnover (i.e. when the water with the mobilised material was mixed) at 16 °C (in loop 1 and loop 2, which acted as replicates since both ran under the same conditions), at 24 °C after flushing-hyperchlorination, and at 24 °C after only flushing showed similar values. At 24 °C after transition, a slightly higher value was observed, but no significant differences were found (Kruskal-Wallis test, p-value >0.05). Turbidity responses in stages 2 (τ = 2.3 N/m$^2$) followed a similar trend, but in this case turbidity at 24 °C after transition showed a much higher average value compared to the other responses at 16 °C and 24 °C. Statistical analysis showed that the observed differences were significant (Kruskal-Wallis test, p-value ≤0.05; Mann-Whitney *U tests*, p-values ≤0.05). In stage 3 (τ = 3.4 N/m$^2$) and stage 4 (τ = 4.3 N/m$^2$) the highest turbidity response was recorded at 24 °C after transition, followed by 24 °C after only flushing and flushing-hyperchlorination. The lowest turbidity response was observed after 16 °C. Significant differences between turbidity responses were observed in this last stage (Kruskal-Wallis test, p-value ≤0.05). When the data set was compared pairwise, statistical analysis confirmed differences in turbidity responses between 16 °C and 24 °C after all the different management strategies (Mann-Whitney *U tests*, p-values ≤0.05). In addition, higher significant values were observed at 24 °C after transition than at 24 °C after flushing-hyperchlorination or only flushing (Mann-Whitney *U tests*, p-values ≤0.05). No statistical differences were found for turbidity response in stage 4 between 24 °C after flushing-hyperchlorination and only flushing (Mann-Whitney *U tests*, p-values >0.05). See appendix B.4 for supplementary information on the results of the statistical analysis.

The response at 16 °C and at 24 °C after the different biofilm management strategies was linear between imposed shear stress and turbidity, $R^2$ = 0.9955 at 16 °C (Loop 1), $R^2$ = 0.9647 at 16 °C (Loop 2), $R^2$ = 0.9996 at 24 °C after flushing-hyperchlorination, $R^2$ = 0.9961 at 24 °C after only flushing, and $R^2$ = 0.9299 at 24 °C after transition.

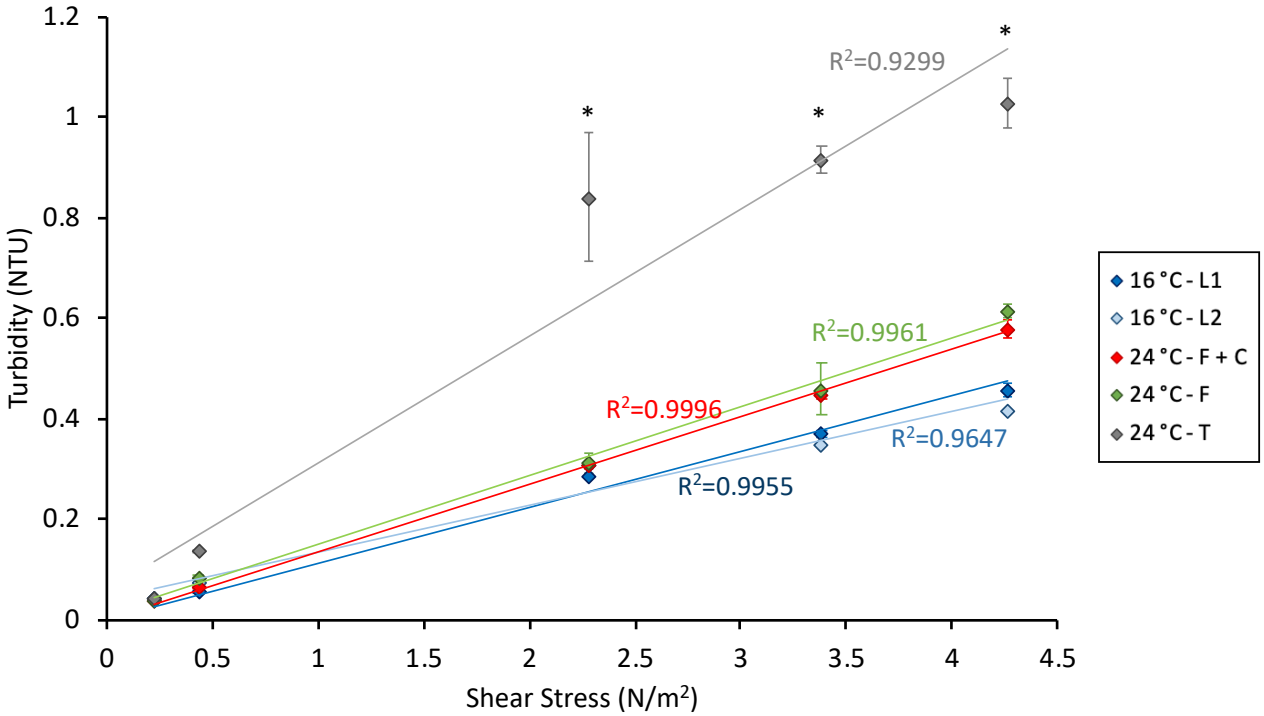



***Figure 38.*** Turbidity response during the last 24 hours of the growth phase and during the last turnover (i.e. when the water with the mobilised material was mixed) of the 4 stages of the flushing events at 16 °C (loop 1 (L1) and loop 2 (L2)) and at 24 °C after different management strategies: flushing + hyperchlorination (F + C), only flushing (F) and transition (T). All values represent an average ± standard deviation. * p-value ≤0.05 from Mann–Whitney *U test*.

### 5.4.2 SEM micrographs

SEM micrographs of PWG coupons on day 0 (control) and day 30 at 16 °C and 24 °C after the different managements strategies are shown in Figure 39. As it was expected, PWG coupons taken on day 0 from all tests did not show cells attached to the surface (Figure 39A, C, E, G). However, PWG coupons collected at the end of the biofilm growth phase presented different surface coverage. At 16 °C small areas of the coupon were covered by biofilm (Figure 39B), while at 24 °C after all management strategies were applied, bigger patches of biofilm accumulation were observed (Figure 39D, F, H). In addition, some visual differences could be observed in the biofilm structures at 24 °C. For example, biofilm developed at 24 °C after flushing-hyperchlorination visually presented a more regular and smoother structure (Figure 39D) when compared with after only flushing biofilm, which showed a more irregular structure (Figure 39F). Similarly, biofilms after the transition only showed a more developed, thicker and rougher structure (Figure 39H).

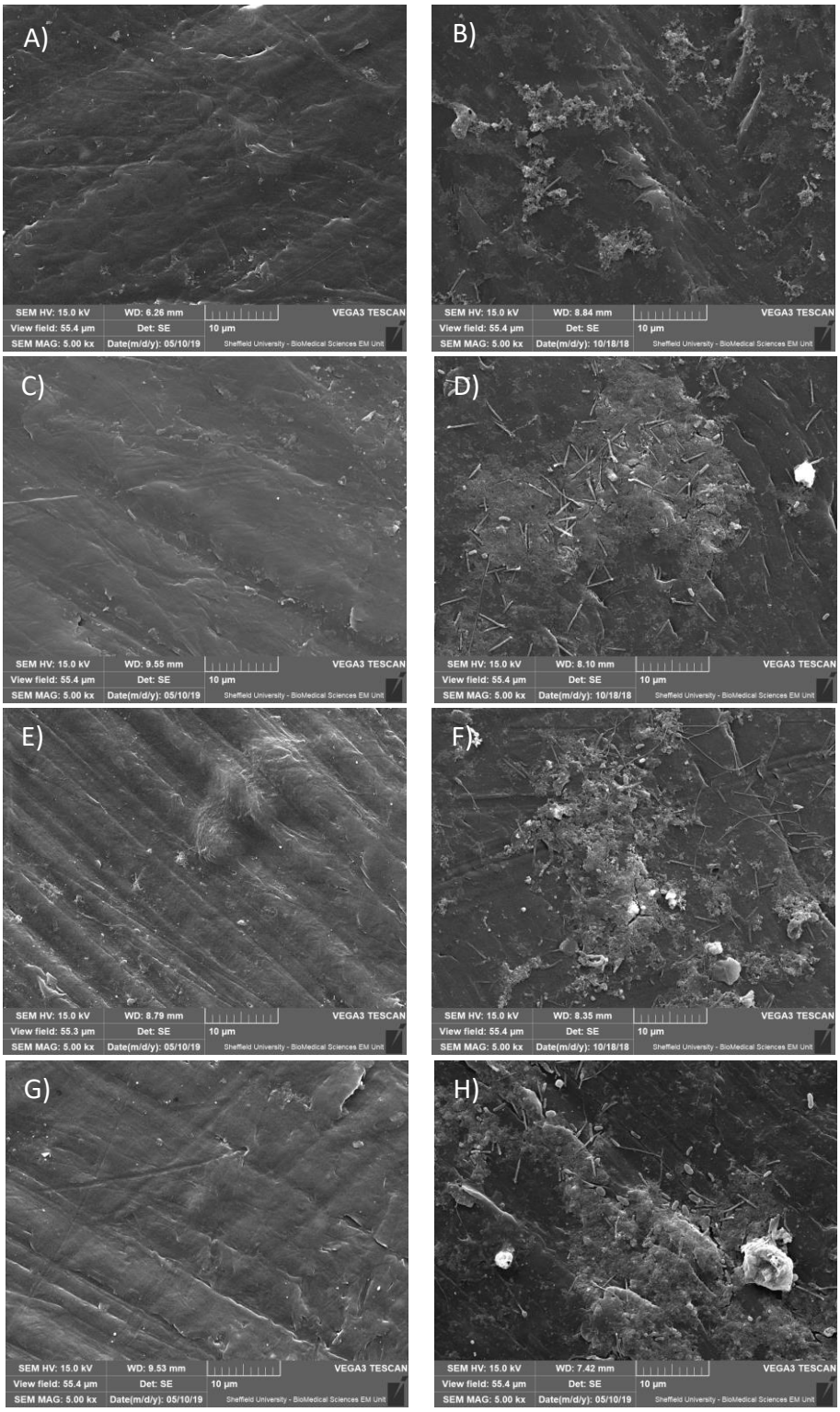



***Figure 39.*** SEM micrographs of developed biofilm in modified PWG coupons at 16 °C on day 0 (A) and on day 30 (B); at 24 °C after flushing-hyperchlorination on day 0 (C) and on day 30 (D); at 24 °C after only flushing on day 0 (E) and on day 30 (F); and at 24 °C after transition on day 0 (G) and on day 30 (H). MAG=5.00 kx in all micrographs.

### 5.4.3 Microbial community structure (bacteria and fungi)

#### 5.4.3.1 Alpha diversity (diversity within samples)

Results from alpha diversity analysis at genus level (Chao 1, Simpson and Shannon indices) for bacterial and fungal biofilm communities are shown in Figure 40. Overall, on days 10 and 20 during the growth phases biofilm bacterial communities did not show significant changes in alpha diversity indices (Kruskal-Wallis test, p-value >0.05), except on day 10 when Chao1 at 24 °C after transition showed a significant higher value compared to 16 °C (Mann-Whitney *U tests*, p-value ≤0.05). On day 30 and considering post-flushing events, significant differences in all indices were observed (Kruskal-Wallis test, p-value ≤0.05) in the samples. On day 30, Chao and Shannon indices were significantly higher at 24 °C after only flushing than at 16 °C, while Simpson was lower (Mann-Whitney *U tests*, p-values ≤0.05). Samples from post-flushing events showed lower significant values of Chao1 and Shannon at 16 °C than at 24 °C after flushing-hyperchlorination (Mann-Whitney *U tests*, p-values ≤0.05). See appendix B.4 for supplementary information on the results of the statistical analysis.

The diversity of biofilm fungal communities was more affected than bacteria, and significant differences between treatments were observed in all indices at each sampling point (Kruskal-Wallis test, p-value ≤0.05) (Figure 40). Samples at 16 °C showed higher Chao1, lower Simpson and higher Shannon compared to all samples at 24 °C after all management strategies (Mann-Whitney *U tests*, p-values ≤0.05), except Chao1 on day 10 at 24 °C after flushing-hyperchlorination (Mann-Whitney *U tests*, p-value >0.05). No significant differences were observed between samples at 24 °C after the different management strategies (Mann-Whitney *U tests*, p-values >0.05). See appendix B.4 for supplementary information on the results of the statistical analysis.

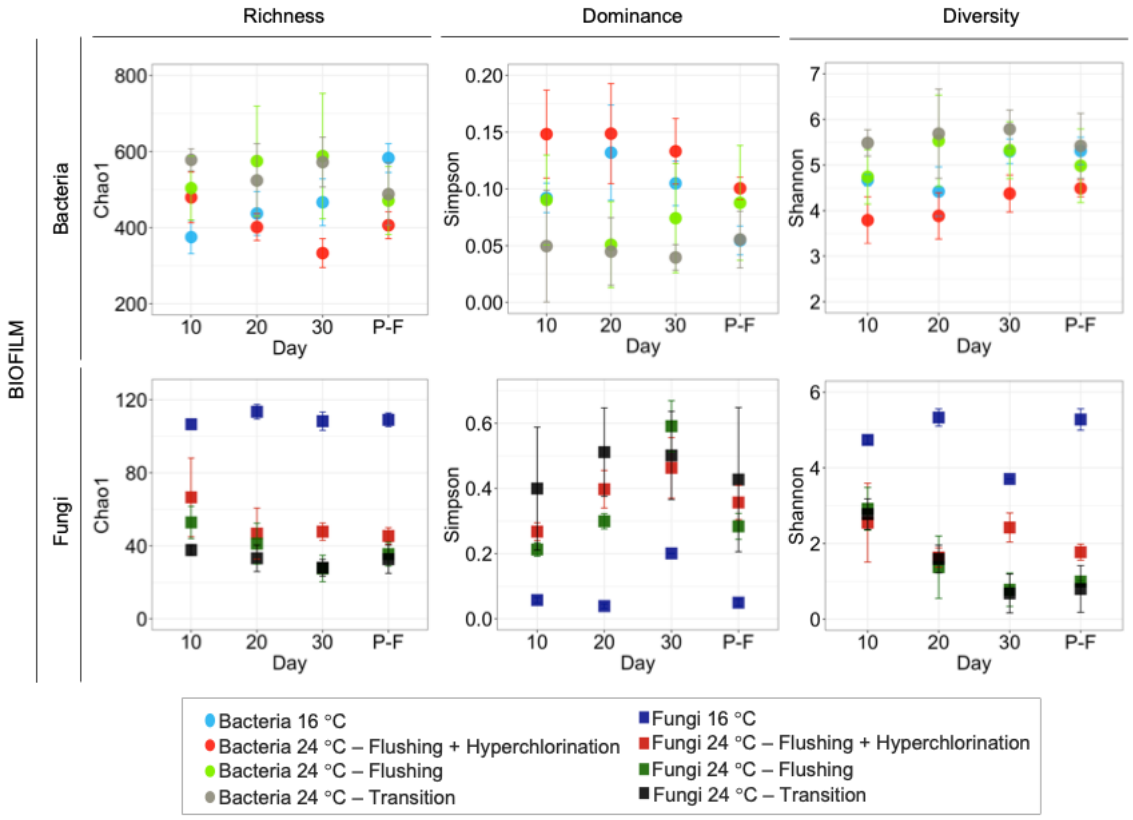



***Figure 40.*** Chao 1 (richness), Simpson (dominance) and Shannon (diversity) indices for OTUs at 97 % cut off for bacteria and fungi in biofilm samples calculated for each sampling day during the growth phases and post-flushing events (P-F). All values represent an average (n=3) and errors bars are ± standard deviation.

Regarding planktonic communities (Figure 41), no significant differences were observed in any index during the growth phases at 16 °C and 24 °C after different management strategies for bacteria and fungi (Kruskal-Wallis tests, p-value >0.05). However, in post-flushing event samples significant differences were found in the Chao1 index values for fungi, and in Shannon for bacteria and fungi (Kruskal-Wallis test, p-value ≤0.05). When samples were compared pairwise, Chao1 in fungal communities was significantly lower at 16 °C than at 24 °C after all management strategies (Mann-Whitney *U tests*, p-values ≤0.05). Shannon index was significantly lower in bacterial communities at 24 °C after flushing-hyperchlorination than at 16 °C. In fungal communities Shannon was statistically lower at 24 °C after transition than at 16 °C or at 24 °C after the other two strategies (Mann-Whitney *U tests*, p-values ≤0.05). See appendix B.4 for supplementary information on the results of the statistical analysis.

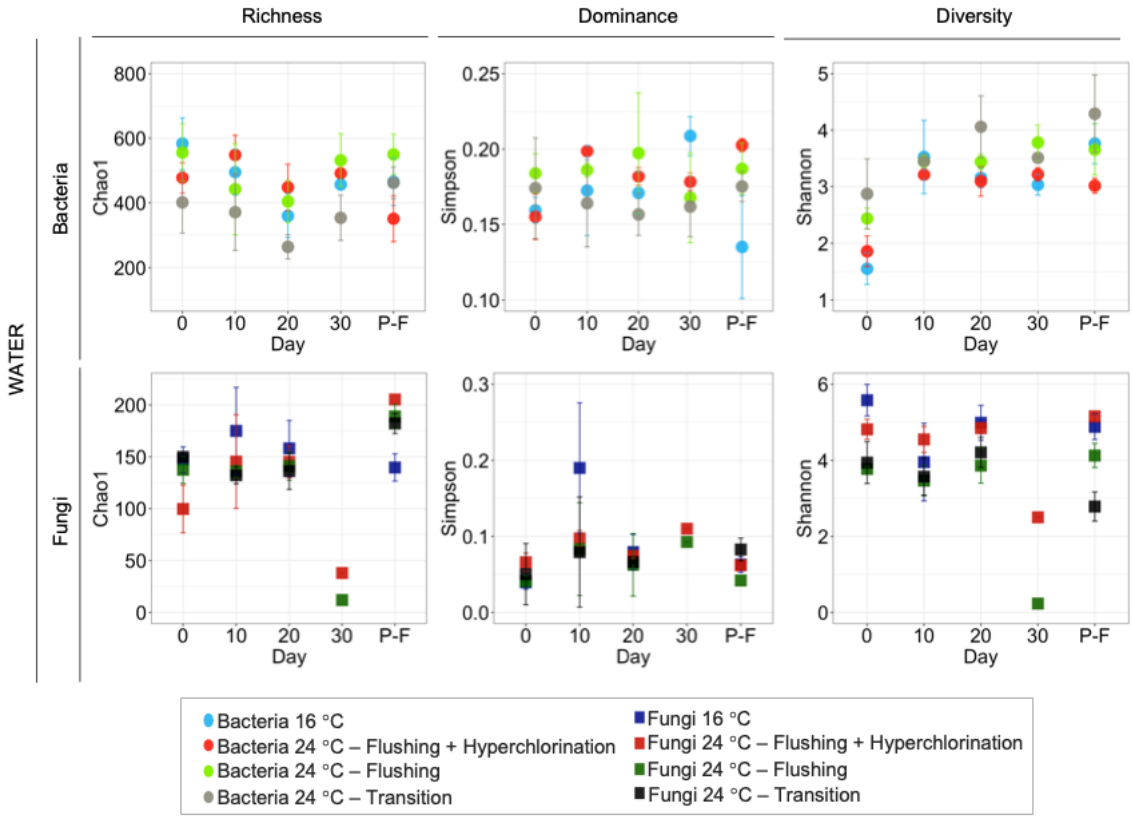



***Figure 41.*** Chao 1 (richness), Simpson (dominance) and Shannon (diversity) indices for OTUs at 97 % similarity cut off for bacteria and fungi in water samples calculated for each sampling day during the growth phases and post-flushing events (P-F). All values represent an average (n=3) ± standard deviation, except for fungi on day 30 where just 1 replicate amplified at 24 °C after flushing-hyperchlorination and at 24 °C after flushing. Neither replicate at 16 °C and at 24 °C transition amplified; thus, statistics could not be performed.

### 5.4.3.2 Beta diversity (diversity among/between samples)

nMDS diagrams with the resemblance matrices at 97 % similarity cut-off for bacteria and fungi communities at 16 °C and 24 °C after different management strategies are shown in Figure 42. nMDS for biofilm bacterial communities showed a clear separation of samples in relation to temperature and management strategy. This separation was confirmed statistically by ANOSIM analysis, which showed significant differences between temperatures and management strategies, as shown in Table 12. nMDS of biofilm fungal communities showed a clear clustering of samples when the factor analysed was the temperature. However, samples did not cluster according to different management treatments. This was confirmed by the ANOSIM test results, that concluded that there were significant differences in the structure of the communities at 16 °C compared to those at 24 °C, but not between the communities at 24 °C after the different management strategies (Table 12).

Conversely, bacterial and fungal planktonic communities did not clearly cluster in relation to different temperatures and/or management strategies in nMDS plots (Figure 42). ANOSIM analysis showed that only bacterial community structure was significantly affected by the increase of temperature after different management strategies (Table 12). Fungal communities showed no significant differences between temperatures and/or different management strategies (Table 12).

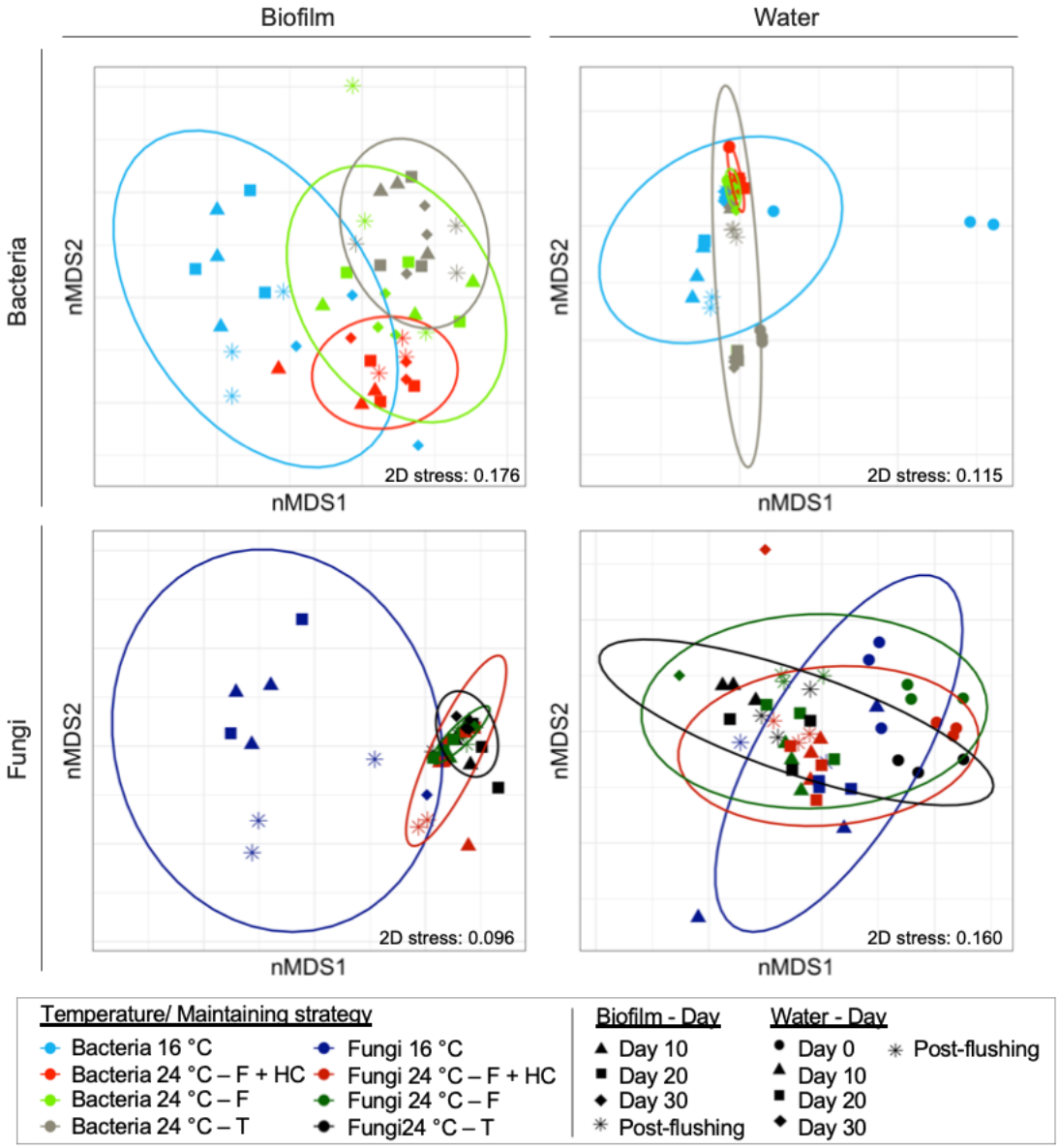



***Figure 42.*** Non-multidimensional scaling (nMDS) analysis based on Bray–Curtis similarity matrixes calculated with the relative abundance of bacteria and fungi at 97 % cut off in biofilm and water samples at 16 °C and 24 °C after the different management strategies: flushing-hyperchlorination (F + HC), only flushing (F) and transition (T). Colours represent the different types of treatment applied and symbols the different sampling points. The 3 replicates per sampling point are represented.

***Table 12.*** ANOSIM statistics calculated using the Bray-Curtis distance matrixes to test beta-diversity significant differences. Differences were considered statistically significant when p-value was ≤0.05. Global-R statistic determine the level of differentiation between treatments, indicating 0 no differentiation and 1 a total differentiation of the samples. * p-value ≤0.05.

| BIOFILM | Bacteria | | Fungi | |
|---|---|---|---|---|
| | p-value | global-R | p-value | global-R |
| 16 °C vs 24 °C Flushing-Hyperchlorination | 0.001* | 0.661 | 0.001* | 0.723 |
| 16 °C vs 24 °C Flushing | 0.001* | 0.571 | 0.001* | 0.691 |
| 16 °C - 24 °C Transition | 0.001* | 0.774 | 0.001* | 0.667 |
| 24 °C Flushing-Hyperchlorination vs 24 °C Flushing | 0.001* | 0.280 | 0.293 | 0.024 |
| 24 °C Flushing-Hyperchlorination vs 24 °C Transition | 0.001* | 0.797 | 0.429 | 0.004 |
| 24 °C Flushing vs 24 °C Transition | 0.001* | 0.194 | 0.521 | 0.015 |
| WATER | Bacteria | | Fungi | |
| | p-value | global-R | p-value | global-R |
| 16 °C vs 24 °C Flushing-Hyperchlorination | 0.001* | 0.492 | 0.093 | 0.062 |
| 16 °C vs 24°C Flushing | 0.001* | 0.407 | 0.427 | 0.008 |
| 16 °C - 24 °C Transition | 0.002* | 0.310 | 0.086 | 0.136 |
| 24 °C Flushing-Hyperchlorination vs 24 °C Flushing | 0.061 | 0.115 | 0.450 | 0.005 |
| 24 °C Flushing-Hyperchlorination vs 24 °C Transition | 0.083 | 0.209 | 0.187 | 0.059 |
| 24 °C Flushing vs 24 °C Transition | 0.101 | 0.118 | 0.790 | 0.037 |

## 5.4.4 Taxonomical analysis (bacteria and fungi)

### 5.4.4.1 Taxonomical analysis of biofilm communities

The results for biofilm bacterial and fungal community composition at genus level are shown in Figure 43 and Figure 44, respectively. Differences in taxonomic profiles were observed between different temperatures and types of management strategies applied.

Biofilm bacterial communities at 16 °C were dominated by a not defined genus belonging to the family Burkholderiaceae (ranging from 0.1 to 59.11%), followed by other genus such as *Pseudomonas* (1.51 - 35.97 %), *Methylonera* (0.24 – 22.71 %) or *Mucilaginibacter* (5.31-19.27 %) during the growth phase or *Sphingomonas* (8.57 – 24.91 %) in samples post-flushing event. Biofilm community samples at 24 °C after flushing-hyperchlorination were dominated by *Pseudomonas* (12.11 – 73.54 %) and other genera such as *Phreatobacter* (3.24 – 16.76 %) and *Sphingomonas* (1.98 – 10.57 %) presented high relative abundance. At 24 °C after only flushing *Pseudomonas* continued to be the most abundant genus (21.28 – 54.18 %), although with lower relative abundance. *Phreatobacter* (0.50 – 13.66 %) and *Sphingomonas* (0.69 – 9.93 %) were also present with similar relative abundances, and other genera such as *Aquabacterium* (0.44 – 15.82 %) and *Flavobacterium*

(0.25 – 17.82 %) increased its relative abundances at 24 °C after only flushing. At 24 °C after transition the relative abundance of *Pseudomonas* (12.53 – 44.97 %) decreased compared to communities at 24 °C after the other treatments, although it continued dominating the biofilm community. *Sphingobium* (2.26 – 16.84 %) and *Flavobacterium* (3.24 – 16.07 %) showed higher relative abundances at 24 °C after transition than after other management strategies. Other genera that considerably increased its relative abundances when the temperature was increased and after transition were *Methyloversatilis* (0.11 – 26.82 %) and *Pelomonas* (0.77 – 10.14 %) (Figure 43).

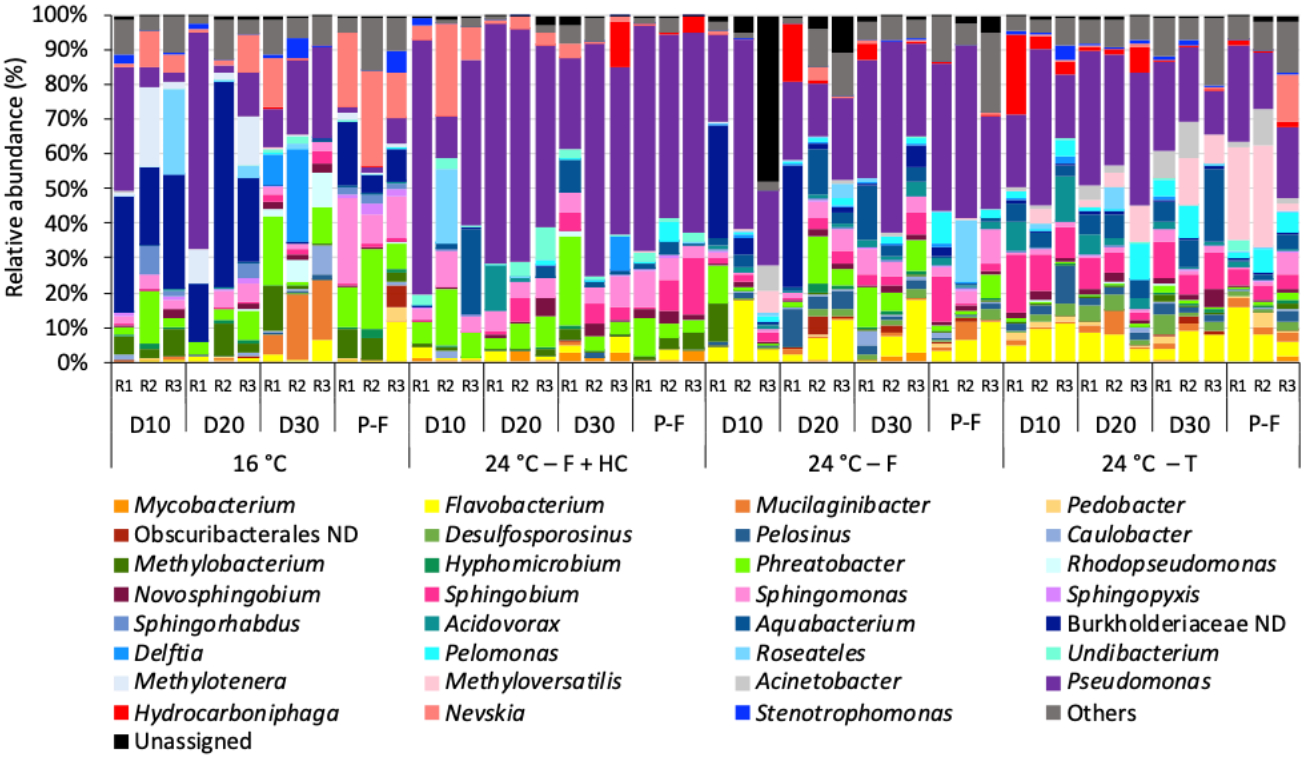



***Figure 43.*** Relative abundance of bacteria at genus level (>1 % of the total sequences) in biofilms from days (D) 10, 20, 30 and post-flushing (P-F) events at 16 °C and 24 °C after each management strategy: flushing - hyperchlorination (F+HC), only flushing (F) and transition (T). The 3 replicates (R1, R2 and R3) per sampling point are represented. Remaining genera were combined in category "Others". Category "Unassigned" correspond to unidentified OTUs and "ND" means not defined at that level.

Focusing on the most abundant fungi taxa in biofilm communities (Figure 44) differences were also observed. At 16 °C during the growth phase on days 10 and 20 *Cladosporium* (13.45 – 30.80 %) together with *Aspergillus* (0.20 – 19.26 %) were the dominant genera. However, on day 30 fungal communities at 16 °C were dominated by *Fusarium* (52.48 %) and *incertae sedis* of Helotiales (9.04 %). In post-flushing event samples, *incertae sedis* of Helotiales (10.96 – 26.45 %) together with *Fusarium* (0.50 – 27.50 %) and *Rhodotorula* (6.67 – 12.48 %) dominated the community structure.

Biofilm fungal communities at 24 °C after all management strategies was dominated mainly by *Fusarium* (51.34 – 97.48 %). At 24 °C after flushing-hyperchlorination treatment, other genera such as *Trichoderma* (1.2 – 66.68 %) on day 10 or *incertae sedis* of Helotiales (2.15 – 74.41 %) in post-flushing events samples presented a high relative abundance. At 24 °C after only flushing *Fusarium* appeared together with *incertae sedis* of Helotiales (4.34 – 36.34 %) on days 10 and 20. At 24 °C after transition, during the growth phase a not defined taxa belonging to the family Nectriaceae (1.23 – 31.33 %) increased its relative abundance, and in post-flushing event samples *incertae sedis* of Helotiales (10.33 – 26.92 %) presented also a high relative abundance.

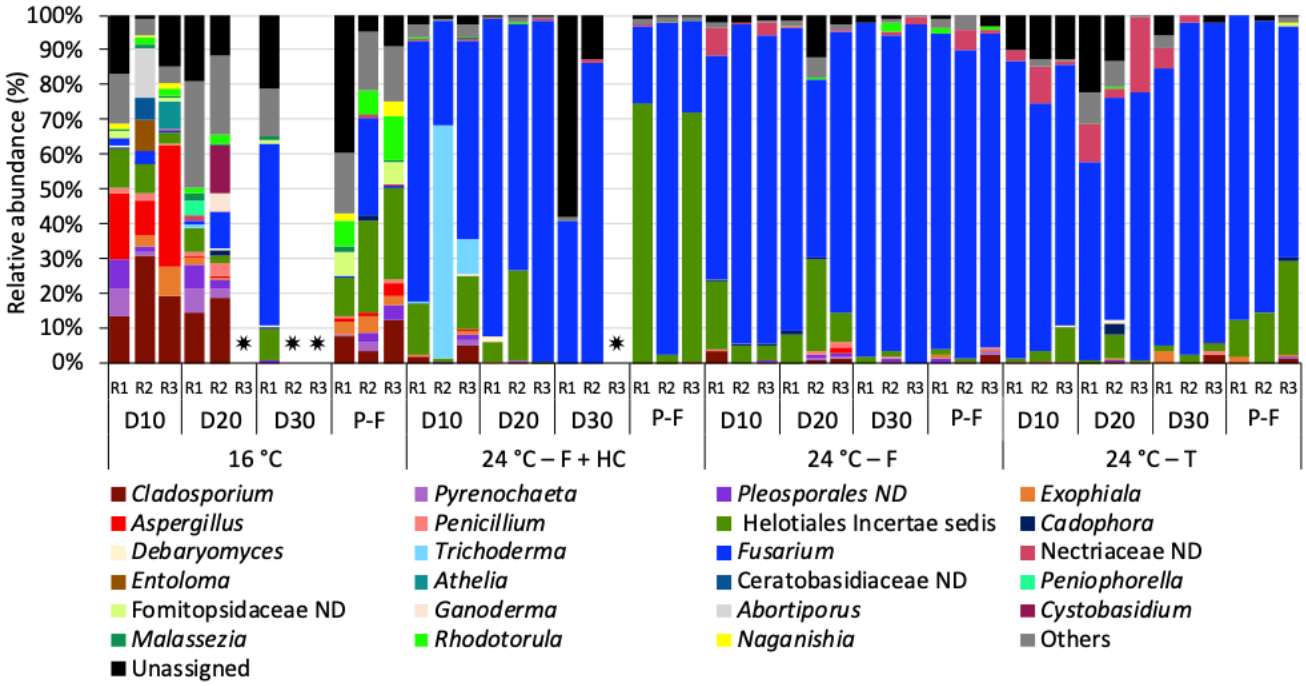



***Figure 44.*** Relative abundance of fungi at genus level (>1 % of the total sequences) in biofilms from days (D) 10, 20, 30 and post-flushing events (P-F) at 16 °C and 24 °C after each management strategy: flushing and hyperchlorination (F+HC), only flushing (F) and transition (T). The 3 replicates (R1, R2 and R3) per sampling point are represented. Remaining genera were combined in category "Others". Category "Unassigned" correspond to unidentified OTUs and "ND" means not defined at that level. ✷ Samples that did not amplify during the sequencing process.

### 5.4.4.2 Taxonomical analysis of planktonic communities

The taxonomical profiles of bacterial and fungal planktonic communities showed differences in composition in relation to temperature as shown in Figure 45 and Figure 46. At 16 °C on day 0, a not defined Obscuribacterales (28.64 – 34.48 %) was the most abundant taxa, followed by

*Phreatobacter* (2.50 – 25.94 %) and *Methylobacterium* (1.80 – 15.27 %). However, on day 10 and successive days, Burkholderiaceae ND (15.67 – 59.02 %) and *Phreatobacter* (13.56 – 66.02 %) were the dominant members of the community. In the post-flushing event samples, *Phreatobacter* (22.37 – 62.24 %) together with *Pseudomonas* (0.12 – 37.24 %), Burkholderiaceae ND (9.27 – 13.47 %) and *Sphingomonas* (5.66 – 9.66 %) dominated the community structure. Planktonic bacterial communities presented very similar profiles at 24 °C after all management strategies. The most abundant taxa over time during the growth phases and post-flushing events were *Phreatobacter* (38.65 – 92.84 %), *Sphingomonas* (6.05 – 37.29 %) and *Nevskia* (1.62 – 16.53 %) (Figure 45).

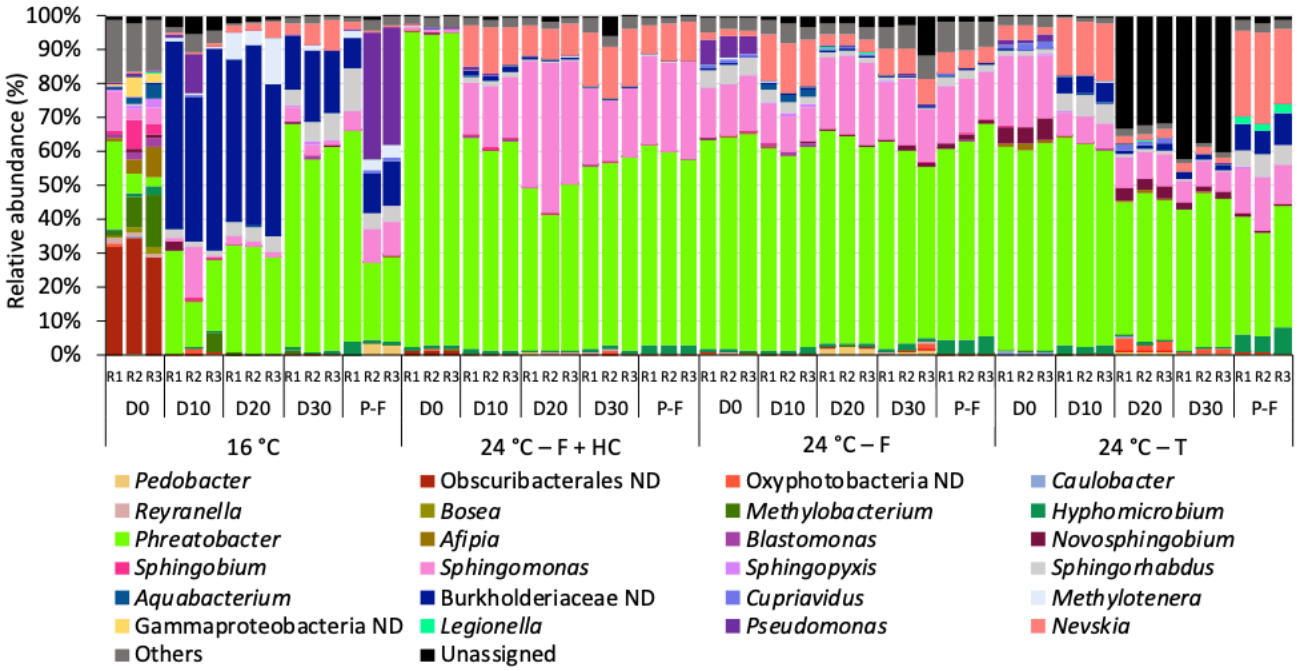



***Figure 45.*** Relative abundance of bacteria at genus level (>1 % of the total sequences) in water samples from days (D) 0, 10, 20, 30 and post-flushing (P-F) events at 16 °C and 24 °C after each management strategy: flushing and hyperchlorination (F+HC), only flushing (F) and transition (T). The 3 replicates (R1, R2 and R3) per sampling point are represented. Remaining genera were combined in category "Others". Category "Unassigned" correspond to unidentified OTUs and "ND" means not defined at that level.

For fungi, a high percentage of unassigned sequences (3.58 – 97.11 %) was observed in all the samples. Despite that all the profiles were similar, some changes were observed in the relative abundance over time with temperature and with the management strategy applied. At 16 °C Helotiales *incertae sedis* (1.24 – 82.84 %) and *Cladosporium* (2.59 – 22.74 %) were the predominant taxa over time. On day 0 samples, they appeared with an unidentified taxa belonging to the family Fomitopsidaceae (6.68 – 21.21 %), and on day 10 samples together with *Cadophora* (1.98 – 60.26

%). On day 20 and post-flushing event samples, *Fusarium* (3.74 – 7.77 %) and *Pyrenochaeta* (1.53 – 7.33 %) presented high relative abundances. On day 30 any water replicate amplified for fungi.

At 24 °C after all management strategies, Helotiales *incertae sedis* (1.24 – 82.84 %) and *Cladosporium* (2.59 – 27.97 %) continued to be the most abundant taxa during the growth phase in all the samples analysed. These two taxa, appeared in all samples together with *Fusarium* (1.71 – 29.87 %), which presented higher relative abundance compared to 16 °C samples. On day 0 samples, after all management strategies, *Debaryomyces* (3.76 – 35.17 %) and a not defined Fomitopsidaceae (2.38 – 20.50 %) also presented a high relative abundance. On day 10 after flushing-hyperchlorination and after only flushing, *Pyrenochaeta* (5.09 – 14.06 %) was relatively abundant. On day 20 after only flushing and after transition, it was notable the increase of not defined Nectriaceae (6.41 – 13.26 %). In post-flushing events samples, Helotiales *incertae sedis* (5.46 – 26.31 %)*, Cladosporium* (5.83 – 32.99 %)*, and Fusarium* (20.61 – 37. 36 %) -dominated the structure of fungal communities, together with *Rhodotorula* (1.67 – 16.51 %) that increased its relative abundance (Figure 46).

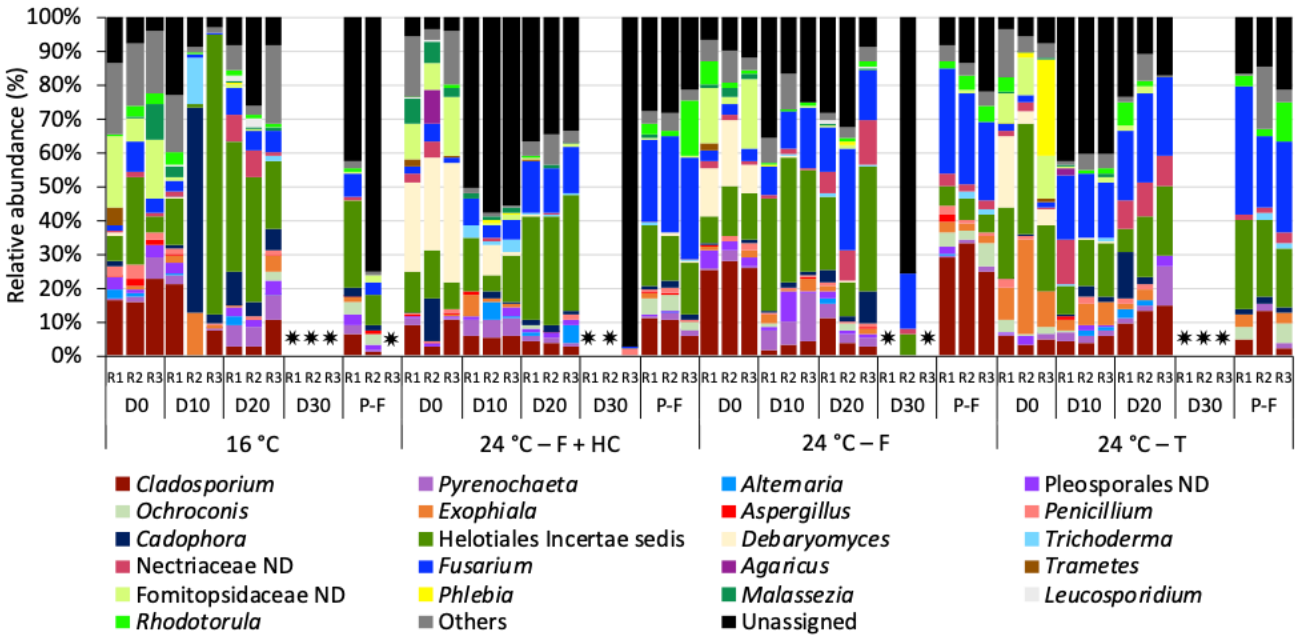



***Figure 46***. Relative abundance of fungi at genus level (>1 % of the total sequences) in water samples from days (D) 0, 10, 20, 30 and post-flushing (P-F) events at 16 °C and 24 °C after each management strategy: flushing and hyperchlorination (F+HC), only flushing (F) and transition (T). The 3 replicates (R1, R2 and R3) per sampling point are represented. Remaining genera were combined in category "Others". Category "Unassigned" correspond to unidentified OTUs and "ND" means not defined at that level. ✷ Samples that did not amplify during the sequencing process.

## 5.5 Discussion

### 5.5.1 Effect of different management strategies on physico-chemical analysis and water discolouration

When the temperature was increased, the different management strategies did not significantly affect physico-chemical parameters of the water, including pH, TOC or chlorine concentrations. However, turbidity and Fe and Mn concentrations, as the most direct measurements of water discolouration and key cause of consumer dissatisfaction (Husband and Boxall, 2011) showed changes. During the biofilm growth phase temperature did not affect turbidity levels in the bulk water, and Fe and Mn concentrations showed similar values at 16 °C and at 24 °C after all management strategies. Nevertheless, when the system was subjected to controlled and monitored flushes, a clear increase in turbidity and metal concentration at both temperatures was observed. This increase in water discolouration was expected since it has been shown that changes in hydraulic conditions, for example a rise in flow due operational flushing can produce the material mobilisation and (Husband and Boxall, 2011) and enhance Fe and Mn concentrations in chlorinated DWDS (Douterelo, Sharpe and Boxall, 2013).

When discolouration response post-flushing events was compared between temperatures, lower metal concentrations and turbidity were observed at 16 °C than at 24 °C after all the management strategies. These results suggested that there was a greater amount of material adhered to the pipe walls at higher temperatures. When biofilms were visually characterized, SEM micrographs were in accordance with this, showing a higher biofilm surface coverage at 24 °C and suggesting that temperature rise promote the biofilm development. Previous studies in DWDS have shown the potential influence of temperature on biofilm. Pintar and Slawson (2003) and Liu *et al.* (2016) observed that warmer temperatures due to seasonality were more optimal for microbial growth rate in DWDS and promote the biofilm formation. Similar results were reported by Hallam *et al.* (2001), that concluded that higher temperatures in DWDS lead to increase biofilm growth and activity. Moreover, higher biofilm activity has been associated with increased Fe and Mn deposition (Ginige, Wylie and Plumb, 2011). Therefore, in agreement with this previous research, it can be suggested that the increase in water discolouration observed in this study can be associated to enhance biofilm growth and the accumulation of metals under higher temperatures.

Moreover, when the different management strategies applied were compared between them, no differences were observed for Fe and Mn concentrations, suggesting that if biofilm detachment occurs none of these management strategies would reduce the risk of increasing metal concentration in the bulk water. This was in contrast with online turbidity measurements that showed a statistically higher turbidity response at 24 °C after transition than after flushing-hyperchlorination or only flushing. These higher turbidity values after the biofilm transition can be precisely attributed to absence of treatment between temperatures. Biofilm at 24 °C after transition had a total growth period of 2 months, one at 16 °C and the other at 24 °C, allowing for a greater biofilm development and accumulation of material, that was later removed with flushing leading to higher turbidity levels. This was also observed in SEM micrographs, which showed a more developed biofilms with a rough structure after the transition strategy. Therefore, from these results it can be suggested that a management/maintenance strategy of DWDS is required for biofilm control in a temperature rise scenario.

In this research, the application of flushing-hyperchlorination and only flushing has been observed to be effective, reducing the discolouration risk when hydraulic conditions change. This was in agreement with previous studies in DWDS, that showed that flushing was an effective mechanism managed to control biofilm accumulation and thus to minimize discoloration risk (Vreeburg *et al.*, 2008; Cook and Boxall, 2011). When these two strategies were compared, turbidity response did not show significant differences between them. It has been widely determined that microorganisms forming biofilms are more resistant and tolerate higher concentrations of disinfectant than planktonic communities (Bridier *et al.*, 2011). Specifically, in DWDS previous research found that chlorine was not effective to avoid biofilm formation in the pipe walls, and even high concentrations can favor its development (Srinivasan *et al.*, 2008). In the same way, results from this study suggested that the hyperchlorination after a flushing event did not affect the biofilm re-growth capacity and/or material accumulation in terms of quantity when the temperature increases, and thus does not decrease the discolouration risk.

Taking into account results reported here, it can be concluded that temperature increase lead to a greater biofilm development and higher metal accumulation, which encourage the risk of water discoloration. In addition, results seem to indicate that the temperature transition without applying a biofilm control strategy favor the risk of biofilm mobilisation, while the application of mechanical control strategies decrease the discolouration risk when the temperature increases.

### 5.5.2 Influence of different management strategies upon planktonic and biofilm microbial communities

Besides to further development of biofilms, a clear effect of temperature increase and management strategies was observed for the structure of bacterial communities in biofilm when microbial communities were characterised using molecular methods. Several biofilm-related studies have previously observed that different factors can shape the structure of bacterial communities. For example, Fish and Boxall (2018) demonstrated that chlorine concentration had a statistically significant impact on these communities and Douterelo, Sharpe and Boxall (2014) showed that bacterial community structure changed after flushing events. Results from this study have demonstrated that temperature is also a determining factor for biofilm bacterial communities in chlorinated DWDS, and that this change depended on the management strategy applied for biofilm control. Douterelo, Husband and Boxall (2014) demonstrated that microorganisms remaining attached to the pipe wall after a flushing event play a key role in biofilm regrowth. Thus, changes produced in bacterial biofilm community during the cleaning processes applied would have driven the differences observed in these communities at higher temperatures.

These changes in the structure were reflected in the taxonomical composition. The most notable change was the high relative abundance of *Pseudomonas* observed at 24 °C, especially after flushing-hyperchlorination and after only flushing, suggesting that these invasive biofilm control methods produced favoured its presence in biofilms. *Pseudomonas* spp. are able to synthesize large quantities of EPS contributing to biofilm formation and growth (Irie *et al.*, 2012; Liu *et al.*, 2020). Thus, its high relative abundance and the potential greatest production of EPS could have contributed to the more developed biofilms and the highest discolouration risk at 24 °C. In addition, the high relative abundance of *Pseudomonas* in biofilms at warmer temperatures also can have other detrimental consequences related to water safety. *Pseudomonas* is an ubiquitous genus that include species considered opportunistic pathogens such as *P. aeruginosa*, which has been previously observed in DWDS and can cause severe infections in healthy or in immunocompromised people (Jurgens, Sattar and Mah, 2008; Vaz-Moreira, Nunes and Manaia, 2012; J. Lu *et al.*, 2016). None of the management strategies studied here has been effective to avoid its increase in relative abundance with a temperature rise, and therefore further research in this way is needed the increase of the relative abundance of *Pseudomonas* can have several negative implications for water quality. Other genera including *Flavobacterium, Sphingobium* or *Methyloversatilis* were also

observed to increase their relative abundance when the temperature increased. The increase of *Flavobacterium* can suppose a risk por water quality because this genus include opportunistic pathogen species that have been related to human infections (Geldreich, 1996; Waśkiewicz and Irzykowska, 2014). *Flavobacterium* especially increased at 24 °C after only flushing and after transition, and lower relative abundance was observed after flushing-hyperchlorination. Bremer, Monk and Butler (2002) found using a drinking water bioreactor that *Flavobacterium* spp. decreased in number in biofilm samples when it was exposed to different free chlorine concentrations and concluded that this genus has a low resistance to disinfectant compared with other genera. Considering this, from this study it could be suggested that the high concentrations of chlorine during the hyperchlorination affected *Flavobacterium*, thus preventing its proliferation when the temperature increases. *Sphingobium* or *Methyloversatilis* followed a similar trend and increasing their relative abundance at higher temperatures. This genera have been observed that play an important role as antibiotic resistance genes (ARGs) reservoirs in DWDS from a wide range of regions (Vaz-Moreira, Nunes and Manaia, 2011; Narciso-da-Rocha, Vaz-Moreira and Manaia, 2014; Ma *et al.*, 2017). The increase of this bacterial genera in DWDS biofilms due temperature rise may cause public health concern, particularly if any cleaning strategy is applied since the highest relative abundance was observed at 24 °C after transition. Therefore, it could be concluded that the application of invasive management strategies used here (flushing or flushing together with hyperchlorination) prevent the increase of relative abundance of bacterial potentially pathogenic genera in biofilm communities when the temperature increases.

The structure of fungal communities in biofilms was also affected by the temperature increase, but no significant changes were observed between management strategies. Fungi are morphologically more complex and robust than bacteria, making them more resistant to certain changes in the environment and presenting a greater range of adaptations (Begon and Fitter, 1995; Denham, Wambaugh and Brown, 2019). Other studies in DWDS have shown that fungi in biofilm are not affected by different chlorine regimes, and that several species are resistant to different disinfection methods such as UV, chlorine or ozone (Hageskal *et al.*, 2012; Fish and Boxall, 2018). However, taking into account results from this study it could be suggested that temperature is one of the abiotic factors that most affects the structure of biofilm fungal communities in chlorinated DWDSs, while they show a high tolerance to hydraulic changes produced by flushing or to hyperchlorination.

Therefore, in a scenario of temperature increase the structure of fungal communities will change in the same way regardless of the management strategy applied in the system.

Overall, biofilm fungal communities at higher temperatures showed less richness and diversity and taxonomical analysis confirmed that *Fusarium* was the dominant genus in biofilm fungal communities when temperature increased after all management strategies. This is of concern for water quality since several *Fusarium* species are important plant pathogens and some of them also produce mycotoxins that cause severe human infections such as fusariosis, affecting mainly immunocompromised patients (Sautour *et al.*, 2012; Abdel-Azeem *et al.*, 2019). Moreover, the results reported here shown that *Fusarium* was dominant regardless of the applied management strategy, suggesting that none of these methods is effective modifying the taxonomical composition of fungi biofilms of DWDS when temperature increases. Further research will help to determine which *Fusarium* species were present, evaluate the real risk when the temperature increases, as well as understand the adaptability of this genus in DWDS and to know if any other biofilm control strategy is effective limiting its proliferation.

Planktonic communities, both bacteria and fungi, did not show significant changes in relation to the different management strategies applied. These strategies were aimed at controlling biofilm communities. Thus, it can be suggested that planktonic communities can be momentarily affected when material is mobilised from the pipe walls by flushing events or by the action of the high concentration of disinfectant during the hyperchlorination. Once the application of the management strategy is complete, the water run through the system and thus planktonic communities were expected to stabilize. Therefore, it is logical that these strategies had a minimal effect on planktonic communities compared to biofilms. Temperature increase also had no effect on the structure of fungal communities, as it occurred in biofilms communities, showing more resistance to environmental changes. However, temperature had a significant effect on planktonic bacterial communities, although less marked than in biofilm communities. This lower effect of temperature on water communities is probably because planktonic cells are less protected than biofilms against disinfectant action, and thus they effectively controlled in chlorinated DWDS, providing them more biological stability (Schwering *et al.*, 2013; Prest *et al.*, 2016). In agreement with these results, other studies have also showed changes in planktonic bacterial communities in DWDS related to temperature. McCoy and VanBriesen (2014) and Douterelo *et al.* (2017) observed a seasonal effect on planktonic communities in real DWDSs, in UK and USA respectively. In

agreement with this, results reported here have demonstrated that under controlled conditions, the increase of temperature have effect on planktonic bacterial communities regardless of the management strategies to which the system is subjected.

When the taxonomical composition was analysed, the most notable change with the temperature rise was the increase in the relative abundance of *Phreatobacter* and *Sphingomonas* bacteria, and *Fusarium* and *Cladosporium* fungi. *Phreatobacter* is a relatively new genus isolated from a ultrapure water of a Hungarian power plant (Toth *et al.*, 2014) and the information and research on this genre is limited. It has been observed that it is one of the most abundant bacterial genus in drinking water samples from different countries such as Paris, Belgium, Paris or Japan (Perrin et al., 2019; Van Assche et al., 2019; Rahmatika et al., 2020). Chlorine concentration or residual biodegradable organic matter in drinking water has been observed to influence in the relative abundance of *Phreatobacter* in other DWDSs (Stanish et al., 2016; Rahmatika et al., 2020). From this study, it can be suggested that temperature is another abiotic factor that determine the relative abundance of this novel genus in chlorinated DWDS, being the warmer temperatures the ones that most favour its presence. Regarding *Sphingomonas* sp., it has been observed widely distributed in DWDS although its presence has been related to water quality concerns since several species produce human disease, including nosocomial infection in immunocompromised individuals (Steinberg and Burd, 2015). Thus, the high relative abundance of this genus into the bulk water enhanced by higher temperature is not desirable, since it could lead to water quality problems. The increase in *Fusarium* genus, as previously reported, can also compromise the water quality. In the same way, *Cladosporium* spp. that has been observed in DWDS environments are also related to skin and pulmonary infections in humans (Tamsikar, Naidu and Singh, 2006; Novak Babič *et al.*, 2017). These demonstrated that although fungi are more resistant to environmental changes (Denham, Wambaugh and Brown, 2019), certain taxonomical groups can be affected by abiotic parameters such as temperature. In addition, despite the absence of differences between temperatures and/or management strategies, a large number of fungi that are able to produce mycotoxins including *Aspergillus*, *Alternaria*, *Penicillium*, *Fusarium*, *Cladosporium*, *Malassezia* or *Trichoderma* have been found in this study, in both biofilm and water samples. These results show that more studies are needed on these organisms in DWDS to a better knowledge about their possible implication in the water quality.

Therefore, it could be concluded that different management strategies are capable of directing the modification of biofilm bacterial communities when temperature increases, but not biofilm fungal communities. Planktonic communities were less affected by temperature, while the different management strategies for biofilm control have no effect on them. These results also reinforce the importance of biofilm communities in DWDS and demonstrate that the controls using tap water samples that normally are carried out for water companies to establish the quality and biological safety are not complete. Planktonic communities do not give a real overview about the microbiome of these systems and therefore, regulatory requirements for water quality should include biofilm monitoring.

## 5.6 Conclusion

This research has shown that:

- Temperature rise in DWDS promoted further biofilm development and thus higher water discolouration risk if hydraulic conditions changes.
- The absence of mechanical and/or chemical treatment for biofilms control when temperature increases (i.e., transition strategy) was related to greater water discolouration levels. Therefore, a biofilm management strategy is recommended under a temperature increase scenario to control biofilm communities and minimize water quality problems related to these communities.
- The application of flushing-hyperchlorination or only flushing led to a similar turbidity levels post-flushing events when temperature increased, suggesting that hyperchlorination did not reduce the discolouration risk.
- Temperature is a determining factor for the structure of biofilm communities in chlorinated DWDS. For bacteria the level of differentiation depended on the management strategy applied in the system when temperature increased, while fungi were not affected by the different treatments.
- Several bacterial and fungal genera potentially pathogenic or detrimental to the quality of DWDS increased its relative abundance in with the temperature rise, including *Pseudomonas*, *Flavobacterium, Sphingobium, Sphingomonas, Methyloversatilis, Fusarium* and *Cladosporium*. Flushing-hyperchlorination and only flushing treatment were found to be effective avoiding the further increase of these microorganisms.

# Chapter 6

# How intermittent water supply time impacts on the microbiome of drinking water distribution systems?*

## 6.1 Abstract

Intermittent water supply (IWS) is a prevalent practice in drinking water distribution systems (DWDS) that is being encouraged due to the effects of climate change. The application of IWS produces changes in the hydraulic conditions of the system that can affect microbial communities inhabiting the systems, particularly biofilms that can be mobilised and thus impact the water quality. In this research, a full-scale DWDS facility was used to simulate IWS events of different times (6 hours, 48 hours, and 6 days) and to establish their effect on microbial ecology and water quality when the water supply is restarted. Water was physico-chemically characterized, and Illumina sequencing was used to obtain a detailed analysis of biofilm and planktonic communities (bacteria and fungi) after the different IWS times. Results showed that IWS affect several physico-chemical parameters. It has been demonstrated that IWS promote the material mobilisation into the bulk water and that different IWS times produce different discolouration response. Microbiological analyses showed how biofilm experience structural and/or compositional changes during different IWS events and that planktonic communities are affected when supply is restarted. This research brings an important advance in knowledge about the effects of different IWS times and the risks associated with its application in chlorinated DWDS.

**Keywords:** microbial ecology, discolouration, climate change, biofilms**.**

* This chapter will be adapted for a journal publication in *Environmental Science & Technology journal* as Calero Preciado, C..; Husband, S.; Maeng, S.; Boxall, J.; Soria-Carrasco, V.; Douterelo, I.: How intermittent water supply time impacts on the microbiome of drinking water distribution systems?. See Appendix D for more information on contributions and co-authors.

## 6.2 Introduction

Contrary to Continuous Water Supply (CWS) which ensure the water delivery 24 hours a day, in Intermittent Water Supply (IWS), Drinking Water Distribution Systems (DWDSs) do not transport water for different periods of time. Although it has been demonstrated that the establishment of a DWDS operating continuously is critical for public health, CWS systems can become IWS for several reasons, and currently one-third of the world population is under IWS (WHO and UNICEF, 2000; WHO, 2017). The main cause for IWS is water stress, which according to European Environment Agency (EEA) "occurs when the demand for water exceeds the available amount during a certain period of time or when poor water quality restricts its use" (EEA, 2008). In developing countries in arid and tropical areas, water stress is caused by the scarce water sources and its difficult access, together with the deficiencies of DWDS infrastructures and/or the high energy and economic cost necessary to distribute water (Lee and Schwab, 2005; Simukonda, Farmani and Butler, 2018). This scenario is being encouraged by climate change since it is affecting the hydrological cycle (e.g. heavy rainfall, floods and droughts), thus lower water availability and promoting IWS (Vairavamoorthy, Gorantiwar and Pathirana, 2008; Delpla *et al.*, 2009; IPCC, 2014a; Bivins *et al.*, 2017). The consequences of climate change are also having effects on more developed countries. For example, this is currently happening Mediterranean regions in Europe, where climate change is intensifying drought episodes in the hottest months, making water a really scarce recurse (DG Environment - European Commission, 2007; Berbel and Esteban, 2019). In addition, DWDS of both developed and middle-incoming countries can experience IWS due to network maintenance works (e.g., pipe repair or replacement). In 2018/19 an average of 172 IWS events over 3 hours or longer occurred in UK per 1000 km of mains due to pipe bursts and repairs (Ofwat, 2019). Therefore, IWS is a common practice which is increasing in different parts of the world and its duration may range from few hours eventually or daily, to full days without supply as it happens in countries like India, Kenya or Bangladesh are currently operating severe intermittency water services (WHO, 2017).

Limited studies have demonstrated that the IWS application have important implications and consequences on water quality of DWDS, that are mainly associated to microbial presence of these systems (Kumpel and Kara L Nelson, 2016; Bivins *et al.*, 2017). DWDS are diverse microbial environments where the dominant form of life can be found as biofilms adhered at the pipe walls (Douterelo *et al.*, 2014; Liu *et al.*, 2014). Microorganisms forming biofilms are embedded in a

complex matrix of extracellular polymeric substances (EPS) that provides different advantages such as mechanical protection or better nutrients acquisition (Flemming and Wingender, 2010). This allows microorganisms survive and inhabit the DWDS despite of the oligotrophic conditions within the pipes, the action of the constant water flows or the disinfectants used in some countries. However, it has been widely reported that biofilms in DWDS are able to deteriorate the water quality, for example causing problems of discolouration if they are mobilized from the pipe walls when the shear stress increases, or they can encourage the occurrence of opportunistic pathogens (Nescerecka *et al.*, 2014; Husband *et al.*, 2016). Different factors can affect biofilms in DWDS, including the concentration of disinfectant, pipe material, concentration of different nutrients, temperature or hydraulic conditions (Douterelo, Sharpe and Boxall, 2013; Mi *et al.*, 2015; Calero Preciado *et al.*, 2019), and thus IWS conditions are susceptible to influence biofilms.

When the water supply is turned off and an IWS event happens, different processes that can modify biofilms and affect water quality in DWDS may occur, these include: (1) the loss of pressure, (2) complete or partial emptying of pipes, and (3) system refilling and water supply restarting. Firstly, the pressure of the system decays resulting in negative pressures in the pipes which can generate the intrusion of surrounding environmental contaminants via leaking pipes (Lee and Schwab, 2005; Vairavamoorthy, Gorantiwar and Mohan, 2007). Moreover, during an IWS event, the water inside the pipes increases its residence times before the system is completely emptied. Longer residence times in DWDS produce the decay of disinfectant that is used to limit microbial growth, thus favouring their proliferation (Hua *et al.*, 1999; Blokker, Vreeburg and Speight, 2014). For example, Lehtola *et al.* (2007) and Mesquita *et al.* (2013) demonstrated that water stagnation resulted in an increase on total cells and heterotrophic plate counts (HPC) in water and biofilms in DWDS. Longer residence times due to IWS could also generate more easily removable biofilms, since it has been observed that biofilms developed under low shear stress conditions have a less cohesive structure and detach more easily from the surface when shear stress changes (Manuel, Nunes and Melo, 2007; Douterelo, Sharpe and Boxall, 2013). In addition, if the pipes are completely drained, biofilms can experience dry periods that can affect its structure. This happened in Mediterranean intermittent freshwater streams where dry periods led to a changes in biofilm microbial community (Timoner *et al.*, 2014), and similar behaviour may occur in DWDS. Therefore, IWS events in DWDS can potentially lead to increase the growth rate of biofilm, change the structure of these communities and/or make them more easily detachable. Furthermore, during the process of supply

restarting the system is refilled producing increases in water flows that can mobilise this biofilm from the pipe walls and incorporate it into the bulk water (Kumpel and Kara L Nelson, 2016). Thus, this flushing effect in IWS when the water supply is restarted may have great potential water quality implications, for example increase the water discolouration and include microbial biomass and particles such a metals or inorganics into the final tap water.

Although IWS is a common application worldwide and there are evidences that can deteriorate the water quality and increase the microbial risk in DWDS, limited research exists about its consequences and the effects of different times of duration of this practice. In this study, advanced microbial methods were used to understand the effect of IWS on the characteristics and behaviour of microbial communities, particularly biofilms, and its implications for water quality and safety. Better understanding of IWS impact on the microbial ecology of DWDS will aid to minimize the risk associated to this practice to ensure the delivery safe water and protect public health. The aim of this research was to understand the impact of different IWS times in the microbiological ecology and water quality of chlorinated DWDS. This is a critical unknown in ensuring safe drinking water relevant under IWS conditions. The particular objectives of this study are hence to explore the effects of different times of IWS on: i) water physico-chemical characteristics; ii) biofilm mobilisation and discolouration risk; iii) structure and composition of microbial biofilm and planktonic communities.

## 6.3 Materials and methods

### 6.3.1 Experimental DWDS facility

A full-scale experimental DWDS at the University of Sheffield was used to perform experiments under different IWS times (Figure 47). The facility simulates the environmental conditions of real DWDS and include 3 independent loops of 9.5 x 21.4 m long coils of High-Density Polyethylene (HDPE) pipe (PE100 SDR17), with an internal diameter of 79.3 mm (Figure 47a). Each loop is connected to its own reservoir tank which are fed with local DWDS and is connected to individual speed pumps. Water is recirculated in system from these tanks at different flow rates and pressures via controlling the pumps and different control valves that are distributed in the loops. LabVIEW software (version 8.2, National Instrument Corporation, UK) is used to control the system and to enable simulation of real hydraulic demand patterns. The water retention time in the facility is 24

hours to maintain baseline water quality parameters, controlled with a trickle drain and feed-based system. To control the water temperature during the experiments, each tank has its own immersion heater to manipulate the water temperature entering the system. In addition, the facility is set within a temperature-controlled room equipped with various monitoring equipment. To enable the *in-situ* sampling of biofilms developed in the pipe walls, each loop has 6 removable HDPE pipe sections of 0.5 m long (Figure 47a, b, c).

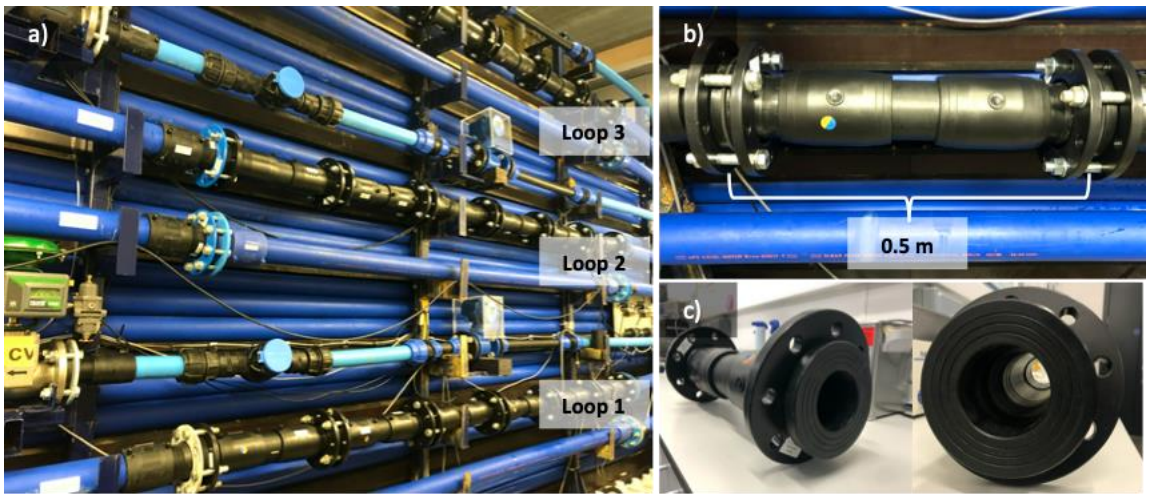



***Figure 47.*** a) Full scale experimental DWDS facility composed by 3 individual loops; b) Sections used for biofilm sampling inserted and fixed into the system; c) detail of the inside of a section.

Before the experiments, each loop was filled and disinfected adding 20 mg/L of RODOLITE H (RODOL Ltd, UK), which is a solution of sodium hypochlorite with less than 16% free available chlorine. The water with the RODOLITE H was recirculated at maximum flow rate (4.2 L/s) to flush the pipes to remove material attached to them and left standing for 24 h with the disinfectant to clean the whole system. Afterwards, the facility was filled and flushed again at 4.2 L/s with fresh tap water until the levels of free chlorine were similar to the average of the supplied local drinking water (≈ 0.20 mg/L).

### 6.3.2 Experimental design and conditions

To study the effect of IWS, the 3 loops were run under the same conditions to allow for biofilm development and then subjected to different IWS times. First, the 3 loops of the facility were operated for 60 days under the same conditions to allow for biofilm development on the pipe walls. The temperature selected was 20 °C and a Low Varied Flow (LVF) regime (ranging from 0.2 to 0.54 L/s) was applied, based on daily patterns observed in DWDS in the UK (Husband, Boxall and Saul, 2008). After the 60 days of biofilm growth phase, the system was stopped and the pipes were

drained at a constant flow of 0.06 L/s, flow below the minimum of the LVF profile to avoid remove material from the pipes walls. In Loop 1 the supply was stopped, and the pipes were drained for 6 hours, in loop 2 for 48 hours and in loop 3 for 144 hours (6 days) (Figure 48). These times were selected to simulate different periods of IWS, since the duration of IWS can range from few hours occasionally for repair works as in the UK, several hours every day or full days depending on the country or the purpose of carrying it out (Coelho *et al.*, 2003; Kumpel and Nelson, 2016).

After the different IWS times each loop was re-filled without using pumping at a constant flow of 0.2 L/s, the minimum flow of the LVF profile to avoid removing material attached to pipes. Once the pipes were full of water, the supply was re-started following scheduled stages combining different values of pressure and flow (Figure 48, Figure 49). First, the system was run with the peak values of pressure and flow of LVF (stage 0). After this, the pressure was increased (stage 1) and then a higher flow was applied (stages 2 and 3). Finally, an extra stage was performed to test the impact of very high flow after different times of IWS (stage 4) (Figure 49). Each stage was performed until flow and pressure values were stabilised and the water run for 3 turnovers in the system to provide enough time for the water to be mixed and turbidity to stabilise (Sharpe *et al.*, 2010). See appendix C.1 for supplementary information on experimental DWDS facility settings for each stage in the 3 loops.

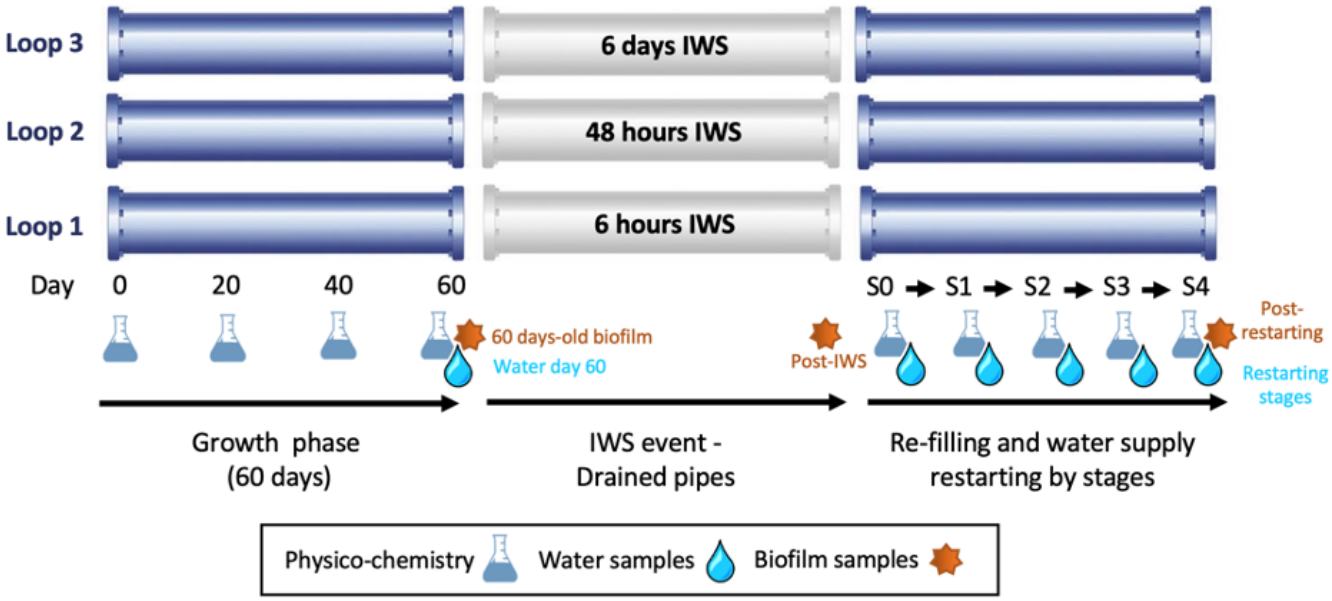



***Figure 48***. Schema of the experiment over time. The 3 loops were running under the same conditions for a growth phase of 60 days. Subsequently, each loop experienced a different IWS during which the supply was stopped, and the pipes drained. Finally, after each IWS, the loops were re-filled, and the supply was restarted by programmed steps (S). Sampling points for water physico-chemical analysis and water and biofilm samples for molecular analysis are indicated.

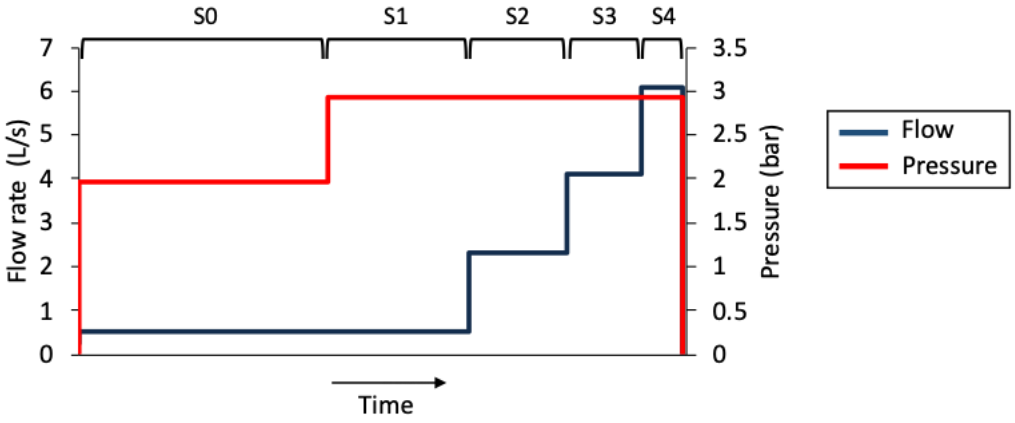



***Figure 49***. Theorical flow and pressure applied in each stage of the water supply restarting. Stage 0 (S0): Q = 0.54 L/s, p = 1.96 bar; Stage 1 (S1): Q = 0.54 L/s, p = 2.94 bar; Stage 2 (S2): Q = 2.3 L/s, p = 2.94 bar; Stage 3 (S3): Q = 4.1 L/s, p = 2.94 bar; Stage 4 (S4): Q = 6.1 L/s, p = 2.94 bar. See appendix C.2 for supplementary information on flow rate conversions of the water supply restarting for each stage in the 3 loops.

### 6.3.3 Water quality physico-chemical and biological analysis

A range of water quality parameters were analysed at the beginning of the experiment, every 20 days during the growth phase and in each stage of the water supply restarting after the different IWS times (Figure 48). These analyses were performed using discrete water samples in triplicates (n=3). pH and water temperature were tested in using a Hanna portable meter HI 991003 (Hanna Instruments, Leighton Buzzard, UK) and free and total chlorine concentrations were analysed with a Palintest CS100 chlorosense (Palintest, UK). Measurements were performed following the manufactures' protocols.

Several analytical methods were utilised to characterise Natural Organic Matter (NOM), which is related to taste and odour problems and plays an important role as a precursor of disinfection by-products (DBPs). DBPs are considered harmful compounds for public health since they can favour the development of diseases such as cancer, and thus strongly impacting the water quality (Matilainen *et al.*, 2011; Ibrahim and Aziz, 2014; WHO, 2017; Tsagkari and Sloan, 2019). Total organic carbon (TOC) and dissolved organic carbon (DOC) were used to quantify the amount of NOM, while the ultraviolet absorbance at 254 nm wavelength ($UVA_{254}$), the specific ultraviolet absorbance (SUVA) and the fluorescent properties of NOM were analysed to determine the nature and reactivity of the NOM content. TOC and DOC were analysed by the analytical chemistry laboratories at KRI (Sheffield, UK). Water samples for both parameters were stored in 20 ml glass vials, and samples for DOC analysis were previously filtered thought 0.45 µm sterile filters to remove the particulate

organic carbon (POC) (Karanfil, Erdogan and Schlautman, 2003). Then, samples were analysed using a Shimadzu TOC-$V_{CPH}/_{CPN}$ Analyzer (Shimadzu, Kyoto, Japan) manufacturer's the protocol. The ultraviolet absorbance at 254 nm ($UVA_{254}$) of the water samples was quantified using a spectrophotometer (DR5000, Hach, USA). $UVA_{254}$ together with DOC was used to calculate SUVA, which is the average absorptive capacity of DOC molecules of a water samples, and it is used as a measure of DOC aromaticity (Weishaar *et al.*, 2003). For each water sample SUVA was calculated as the $UVA_{254}$ in a water sample normalized for DOC concentration.

Fluorescent properties of NOM were obtained via 3D-excitation-emission matrix (EEM), using a spectrofluorophotometer (RF-5301PC, Shimadzu, Japan) in Civil and Environmental Engineering Department of Sejong University (Seoul, South Korea). The EEM were measured every 10 nm over an excitation (ex) wavelengths from 220 to 400 nm, with an emission (em) range from 280 to 600 nm at 1 nm intervals. The fluorescence profiles monitored in this study were: T1 peak and T2 peaks for protein-like material (ex 220-240 nm and em 330-360 nm, ex 270-280 nm,and em 330-360 nm, respectively), and A and C peaks for humic-like components (ex 230-260 nm, and em 400-450 nm, ex 300-340 nm, and em 400-450 nm) (Park *et al.*, 2016) (EEM contour plots were generated by utilising MATLAB 7.8 software (Mathworks, Natick, MA, USA).

Turbidity, as the most direct measure of key aesthetic parameter and key cause of consumer dissatisfaction (Husband and Boxall, 2011b), was measured continuously online throughout the experiment by an ATi A15/76 turbidity monitor (ATi, Delph, UK) installed in the experimental facility. The concentration of iron (Fe) and manganese (Mn) was tested together with turbidity as indicators of water discolouration (Seth *et al.*, 2004). Fe and Mn concentrations were determined by the chemistry laboratories at The Kroto Research Institute (KRI) (The University of Sheffield, UK), by means of Inductively Coupled Plasma Mass Spectrometry (ICP-MS) analysis. Water samples were collected in 20 mL vials containing 5 M of nitric acid and then ions were monitored on a Perkin Elmer Elan DRC II (PerkinElmer, Inc., USA) (Sloetjes and Wittenburg, 2008).

Flow cytometry (FCM) was used to quantify the amount and viability of microbial cells in suspension in the water during each experimental phase. In brief, water samples were dechlorinated adding 1 % (w/v) sodium ascorbate solution to avoid the disinfectant affect the staining (Safford and Bischel, 2019). For total cell counts (TCC), SYBR® Green I (10000x stock, Invitrogen, UK) was used for staining nuclear double-stranded DNA, and diluted 1:100 with dimethyl sulfoxide (DMSO, Fisher Scientific,

Fair Lawn, NJ), to make a working stock solution of a concentration of 100x. 5 µl of SYBR® Green I working stock solution were added to 500 µl of sample aliquot. For intact cell counts (ICC), 1 part of propidium iodide (PI) (1.5 mM, Life Technologies Ltd., Paisley, UK), which is not is not permeant to live cells membrane (i.e. used to exclude damage cells), was added to 5 parts of SYBR® Green I working stock solution. 6 µL of this dye mix were then added to 500 µL of sample given a final concentration of 1x SYBR® Green I and 3 µM PI). Samples with the dyes mixes were vortexed and incubated at 36 °C for 10 minutes in the dark (Gillespie *et al.*, 2014; Prest *et al.*, 2014). After the incubation period, 50 µL of each sample was analysed using a BD Accuri™ C6 Cytometer (Becton Dickinson (BD) U.K. Ltd., Oxford, UK) equipped with a 50 mW laser emitting at a fixed wavelength of 488 nm. The resulting flow cytometer data was processed and analysed using the BD Accuri™ C6 software (BD Biosciences, UK) following the protocol described by Prest *et al.* (2013).

### 6.3.4 Microbial communities sampling

To study the impact of the different IWS times on planktonic communities, 3 biological replicates of 10 L of bulk water were taken on day 60 (growth phase background, i.e. planktonic communities before applying the IWS) and during each stage of the water supply restarting after the different IWS times, as explained in Figure 48. For day 60, 1 replicate was obtained from each loop, since all the 3 loops were running under the same conditions during the growing phase. At each stage after the different IWS, water samples were collected from each loop when flow and pressure were stabilised and after 3 turnovers, the time that the total volume of the water needs for recirculate in the loop 3 times to homogenise (Sharpe *et al.*, 2010). For microbial analysis, cells in the water samples were concentrated using a Tangential Flow Filtration (TFF) system (PALL Life Science, New York, USA) (Schwartz and Seeley, 2002) and then filtered through 0.22 µm nitrocellulose membrane filters (Millipore, Corp). Filters were preserved at -20 °C prior DNA extractions were performed.

To study the critical but often over looked biofilm communities, 2 pipe sections (Figure 47b, c) were removed from each loop (0.25 $m^2$ of biofilm total area) on day 60 (to analyse biofilm development on the pipes before applying the IWS), post-IWS times (i.e. before supply restarting) and post-restarting (i.e. at the end of the water supply restarting) (Figure 48). Biofilm was removed from pipe sections and suspended in 500 ml of phosphate-buffered saline (PBS) (Gibco®, Thermo Fisher Scientific, UK) using a sterile nylon brush and a standardised brushing protocol, applyed to all samples. From each section, 2 x 250 mL biofilm suspensions were obtained, then pooled and filtered

through a 0.22 µm nitrocellulose membrane filters (Millipore Corp., USA) and preserve at -20 °C until DNA analysis.

### 6.3.5 DNA extraction and sequencing

DNA extraction of biofilm and water samples was carried out using a chemical lysis method with hexadecylmethylammonium bromide (CTAB) and proteinase K chemical, followed by DNA purification with phenol/isoamyl alcohol (Zhou, Bruns and Tiedje, 1996; Neufeld *et al.*, 2007). After the extractions, a Qubit 4 Fluorometer (Invitrogen by Thermo Fisher Scientific, Wilmington, USA) with a High Sensitivity dsDNA Assay kit was used for the quantification of DNA concentration from each sample.

Extracted DNA was sent to Mr DNA Laboratory (www.mrdnalab.com, Shallowater, TX, USA) for Next Generation Sequencing (NGS). DNA was sequences on the Illumina MiSeq platform following the manufacturer's protocols for pair-end sequencing. The bacterial 16S rRNA gene was amplified using the primers 28F (GAGTTTGATCNTGGCTCAG) and 519R (GTNTTACNGCGGCKGCTG) spanning the V1 to V3 hypervariable regions. For fungal communities, the ITS1-2 region was selected for amplification using the primers ITS1FBt1 (CTTGGTCATTTAGAGGAAGTAA)/ ITS2R (GCTGCGTTCTTCATCGATGC). These primers with barcodes inserted on the forward primer were used in a 30 PCR cycles using the HotStarTaq Plus Master Mix Kit (Qiagen, USA). PCR conditions were: 94°C for 3 minutes, followed by 30 cycles of 94°C for 30 seconds, 53°C for 40 seconds and 72°C for 1 minute, after which a final elongation step at 72°C for 5 minutes was performed. PCR products were checked after the amplification in 2% agarose gel to determine the success of amplification and the relative intensity of bands. Several samples were pooled in equal proportions based on their molecular weight and DNA concentrations. Pooled samples were purified using calibrated Ampure XP beads (Beckman Coulter, Inc.) and the pooled and purified PCR product was used to prepare Illumina DNA library.

### 6.3.6 Bioinformatics and community analysis

A range of bioinformatic tools were used in order to analyse the genetic material recovered from each environmental sample to study the community of microorganisms present after the different IWS times. The quality, number and length of raw sequences obtained with Illumina were checked using the FastQC software version 0.11.8 (bioinformatics.babraham.ac.uk) (Andrew, 2010). After

this, sequences were filter and trim with an average quality phred score below 20 and/or a minimum length of 100 bp and sequencing errors present in the samples were removed (Cock *et al.*, 2009; Davis *et al.*, 2018), applying BBDuk software version 37.95 (jgi.doe.gov/data-and-tools/bbtools/bb-tools-user-guide/bbduk-guide/). Then, sequencing reads were demultiplexed and barcodes were removed using sabre software (github.com/najoshi/sabre) (Joshi, 2011). Afterwards, the reads were imported into the Quantitative Insights Into Microbial Ecology 2 program (QIIME2, version 2019.7, qiime2.org) (Bolyen *et al.*, 2019), and using the implemented vsearch plug-in (github.com/qiime2/q2-vsearch) (Rognes *et al.*, 2016), pair-end sequences were joined and dereplicated. Chimeric sequences were identified and filtered and *de-novo* clustering by 97% similarity to obtain the Operational Taxonomic Units (OTUs) using the vsearch plug-ins for these processes in QIIME2. Finally, final OTUs were taxonomic assigned using the classify-consensus-vsearch method (Rognes *et al.*, 2016) of the feature-classifier plug-in in QIIME2 (github.com/qiime2/q2-feature-classifier) (Bokulich *et al.*, 2018). 16S reads were aligned against SILVA SSU r132 database (arb-silva.de) (Quast *et al.*, 2013) and ITS1-2 sequences against UNITE 8.0 (unite.ut.ee) (Kõljalg *et al.*, 2013).

For the estimation of quantitative measurements of the structure of bacterial and fungal communities after different IWS times, first the 97 % OTUs tables with the relative abundance were rarified and then used for alpha and beta diversity calculations (Morris *et al.*, 2014). Alpha diversity (within-sample diversity) was determined using Chao 1 index (richness estimator), Simpson index (dominance estimator) and Shannon index (diversity estimator, i.e. consider both richness and dominance) (Morris *et al.*, 2014) using the q2-diversity plug-in in QIIME2 (github.com/qiime2/q2-diversity). For beta diversity (the extent of change in communities composition), the rarefied 97 % cut off OTU table was transformed by square-root and then the community similarity matrices were constructed applying Bray-Curtis method using vegan package version 2.5-6 in R (github.com/vegandevs/vegan) (Oksanen *et al.*, 2019). For the visualization of Bray-Curtis resemblance matrices, non-metric multidimensional scaling (nMDS) were constructed with ggplot2 package version 3.2.1 in R (github.com/tidyverse/ggplot2) (Wickham and Chan, 2016).

### 6.3.7 Statistical analysis

All biological and physico-chemical parameters were measured in triplicate, and the mean and standard deviation were calculated. In the same way, the relative abundance of each OTU and the

values of alpha diversity indices, were calculated as the mean of all replicates analysed for one sample. The normality of the data sets was tested before performing significance tests by Shapiro-Wilks test. Statistical differences between IWS times of all physico-chemical and biological parameters were tested via the non-parametric Kruskal–Wallis test by ranks. Then, if significant differences were observed, the non-parametric Mann-Whitney *U test* was used to compare samples pairwise.

For beta diversity, analysis of similarities (ANOSIM) was applied to Bray-Curtis distance matrices to detect significant differences in biofilm and water microbial communities between IWS times. To establish the impact of different IWS times on them the global-R statistics was calculated, which has values ranging from 0 to 1, where 1 indicates that communities are totally different) (Anderson and Walsh, 2013). Differences were considered statistically significant when p-value was ≤0.05, and all statistical tests were carried out using R software version 3.6.1 (r-project.org) (R Core Team, 2014).

## 6.4 Results

### 6.4.1 Water physico-chemical and biological analysis

Results for water quality parameters during the growth phases and after different IWS times are shown in Table 13. In general, base physico-chemical analysis showed similarity between the 3 loops during the 60 days of growth phase, and differences were observed in several parameters after the different IWS times. Temperature was stable over time during the growth phase, average of 20.3 °C (n = 12) in loop 1, 20.2 °C in loop 2 (n = 12) and 20.3 °C in loop 3 (n = 12). However, after IWS was applied and the supply was restarted water temperature decreased slightly in the three loops, average of 17.7 °C (n = 15) in loop 1, 17.8 °C in loop 2 (n = 15) and 17.3 °C (n = 15) in loop 3. pH values showed limited changes during the test, ranging from 6.8 to 7.5 in the 3 loops. Water disinfectant concentrations were similar over time, total chlorine ranged between 0.35 and 0.54 mg/L and free chlorine between 0.29 and 0.50 mg/L.

Regarding NOM water quantification, during the growth phase TOC and DOC showed values ranging from 1.23 to 1.87 mg/L and 1.03 to 1.5 mg/L respectively (Table 13). After restarting the water supply, TOC and DOC showed values similar to those in the growth phase. Statistical analysis confirmed that TOC and DOC values did not change significantly when compared to day 60 (p-values >0.05). In addition, no significant differences were observed in any stage between the 3 IWS times

after the water supply restarting (p-values >0.05). Water SUVA showed averages of 1.47 L/mg-m in loop 1 (n = 12), 1.37 L/mg-m in loop 2 (n = 12) and 1.48 L/mg-m in loop 3 (n = 12) during the growth phase (Table 13, Figure 50). After the water supply restarting, the highest values for SUVA were observed after 6 hours of IWS, followed by 48 hours and 6 days. Statistical tests confirmed that significant differences were only detected after 6 hours of IWS when compared to day 60 (p-value ≤0.05). When water SUVA values after the 3 IWS times were compared together, significant differences were observed in stages 1, 2 and 4 (p-values ≤0.05), but not in stages 0 and 3 (p-values >0.05). Pairwise comparisons showed higher significant SUVA values in stage 1 after 6 hours of IWS, than after 48 hours and 6 days (p-values ≤0.05). In stages 2 and 4, significant differences were observed between all IWS times (p-values ≤0.05), showed after 6 hours the highest values followed by 48 hours and 6 days. See appendix C.6 for supplementary information on the results of the statistical analysis.

***Table 13.*** Water physico-chemical parameters measured every 20 days during the growth phase and in each stage of water supply restarting after different IWS periods. All values represent an average of three water replicates analysis ± standard deviation.

| IWS time | Sampling day | Temperature (°C) | pH | Total Cl (mg/L) | Free Cl (mg/L) | Fe (µg/L) | Mn (µg/L) | TOC (mg/L) | DOC (mg/L) | $UV_{254}$ ($cm^{-1}$) | $SUVA_{254}$ (L/mg-m) |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | Day 0 | 20.40 ± 1.01 | 6.88 ± 0.06 | 0.41 ± 0.06 | 0.38 ± 0.11 | 32.60 ± 2.31 | 0.55 ± 0.12 | 1.80 ± 0.05 | 1.47 ± 0.07 | 0.022 ± 0.001 | 1.48 ± 0.05 |
| | Day 20 | 21.13 ± 0.06 | 6.88 ± 0.02 | 0.38 ± 0.00 | 0.29 ± 0.02 | 34.19 ± 4.20 | 0.77 ± 0.08 | 1.64 ± 0.31 | 1.31 ± 0.21 | 0.017 ± 0.001 | 1.25 ± 0.17 |
| | Day 40 | 20.03 ± 0.06 | 6.87 ± 0.11 | 0.39 ± 0.03 | 0.35 ± 0.01 | 30.70 ± 4.73 | 0.58 ± 0.06 | 1.52 ± 0.24 | 1.31 ± 0.09 | 0.025 ± 0.005 | 1.88 ± 0.23 |
| | Day 60 | 19.53 ± 0.05 | 6.80 ± 0.02 | 0.38 ± 0.02 | 0.35 ± 0.01 | 37.94 ± 2.06 | 0.74 ± 0.08 | 1.23 ± 0.17 | 1.03 ± 0.05 | 0.013 ± 0.000 | 1.26 ± 0.04 |
| 6 hours | Stage 0 | 15.70 ± 0.21 | 6.87 ± 0.23 | 0.51 ± 0.01 | 0.48 ± 0.04 | 118.75 ± 8.47 | 1.27 ±0.24 | 1.31 ± 0.10 | 1.28 ± 0.10 | 0.016 ± 0.000 | 1.25 ± 0.09 |
| | Stage 1 | 17.90 ± 0.15 | 6.92 ± 0.05 | 0.53 ± 0.01 | 0.42 ± 0.09 | 125.70 ± 9.68 | 1.22 ± 0.15 | 1.34 ± 0.19 | 1.14 ± 0.14 | 0.026 ± 0.000 | 2.28 ± 0.20 |
| | Stage 2 | 18.03 ± 0.06 | 7.15 ± 0.08 | 0.47 ± 0.01 | 0.37 ± 0.06 | 143.55 ± 9.78 | 1.93 ± 0.16 | 1.32 ± 0.31 | 1.18 ± 0.06 | 0.035 ± 0.016 | 3.00 ± 0.27 |
| | Stage 3 | 18.57 ± 0.06 | 7.34 ± 0.06 | 0.45 ± 0.03 | 0.43 ± 0.01 | 146.53 ± 2.93 | 1.40 ± 0.12 | 1.45 ± 0.37 | 1.27 ± 0.20 | 0.026 ± 0.000 | 2.05 ± 0.30 |
| | Stage 4 | 18.40 ± 0 0.1 | 7.41 ± 0.02 | 0.40 ± 0.19 | 0.35 ± 0.06 | 143.90 ± 88.46 | 1.35 ± 0.14 | 1.49 ± 0.64 | 1.39 ± 0.11 | 0.033 ± 0.001 | 2.35 ± 0.18 |
| | Day 0 | 19.77 ± 0.25 | 7.00 ± 0.18 | 0.52 ± 0.03 | 0.50 ± 0.03 | 31.51 ± 4.21 | 0.77 ± 0.08 | 1.71 ± 0.31 | 1.34 ± 0.22 | 0.022 ± 0.000 | 1.65 ± 0.16 |
| | Day 20 | 21.27 ± 0.06 | 7.03 ± 0.06 | 0.43 ± 0.01 | 0.30 ± 0.02 | 31.20 ± 7.28 | 0.67 ± 0.06 | 1.62 ± 0.13 | 1.46 ± 0.09 | 0.017 ± 0.009 | 1.16 ± 0.06 |
| | Day 40 | 20.20 ± 0.00 | 6.94 ± 0.07 | 0.36 ± 0.02 | 0.30 ± 0.01 | 39.84 ± 7.16 | 0.49 ± 0.22 | 1.74 ± 0.14 | 1.40 ± 0.15 | 0.020 ± 0.000 | 1.43 ± 0.15 |
| | Day 60 | 19.50 ± 0.00 | 6.90 ± 0.13 | 0.35 ± 0.05 | 0.29 ± 0.04 | 39.90 ± 4.91 | 0.64 ± 0.17 | 1.48 ± 0.20 | 1.18 ± 0.18 | 0.015 ± 0.005 | 1.24 ± 0.15 |
| 48 hours | Stage 0 | 15.50 ± 0.61 | 7.22 ± 0.20 | 0.53 ± 0.04 | 0.46 ± 0.10 | 98.06 ± 26.31 | 0.71 ± 0.08 | 1.47 ± 0.25 | 1.17 ± 0.08 | 0.014 ± 0.001 | 1.16 ± 0.12 |
| | Stage 1 | 17.80 ± 0.17 | 7.12 ± 0.21 | 0.51 ± 0.06 | 0.45 ± 0.03 | 129.40 ± 23.10 | 1.30 ± 0.17 | 1.39 ± 0.03 | 1.29 ± 0.08 | 0.023 ± 0.001 | 1.81 ± 0.08 |
| | Stage 2 | 18.23 ± 0.15 | 7.19 ± 0.07 | 0.49 ± 0.12 | 0.37 ± 0.05 | 140.40 ± 12.31 | 1.90 ± 0.53 | 1.27 ± 0.14 | 1.21 ± 0.02 | 0.024 ± 0.000 | 1.99 ± 0.03 |
| | Stage 3 | 18.57 ± 0.15 | 7.20 ± 0.01 | 0.47 ± 0.02 | 0.33 ± 0.03 | 165.60 ± 22.94 | 2.07 ± 0.16 | 1.34 ± 0.16 | 1.32 ± 0.20 | 0.025 ± 0.001 | 1.87 ± 0.19 |
| | Stage 4 | 18.93 ± 0.06 | 6.35 ± 1.65 | 0.46 ± 0.02 | 0.30 ± 0.03 | 166.00 ± 26.53 | 2.06 ± 0.26 | 1.42 ± 0.07 | 1.36 ± 0.07 | 0.026 ± 0.000 | 1.91 ± 0.09 |
| | Day 0 | 19.87 ± 0.15 | 7.04 ± 0.07 | 0.54 ± 0.03 | 0.50 ± 0.01 | 28.19 ± 1.12 | 0.74 ± 0.09 | 1.79 ± 0.14 | 1.38 ± 0.18 | 0.022 ± 0.001 | 1.57 ± 0.16 |
| | Day 20 | 21.57 ± 0.06 | 7.04 ± 0.06 | 0.39 ± 0.01 | 0.33 ± 0.02 | 39.50 ± 2.18 | 0.82 ± 0.23 | 1.87 ± 0.23 | 1.43 ± 0.21 | 0.021 ± 0.006 | 1.45 ± 0.22 |
| | Day 40 | 20.43 ± 0.06 | 6.92 ± 0.08 | 0.31 ± 0.03 | 0.29 ± 0.00 | 37.37 ± 5.67 | 0.60 ± 0.14 | 1.70 ± 0.09 | 1.50 ± 0.09 | 0.027 ± 0.001 | 1.82 ± 0.06 |
| | Day 60 | 19.50 ± 000 | 6.95 ± 0.08 | 0.35 ± 0.03 | 0.29 ± 0.03 | 35.78 ± 4.65 | 0.80 ± 0.16 | 1.34 ± 0.19 | 1.14 ± 0.16 | 0.012 ± 0.004 | 1.09 ± 0.17 |
| 6 days | Stage 0 | 15.40 ± 0.30 | 7.55 ± 0.18 | 0.50 ± 0.00 | 0.44 ± 0.06 | 112.70 ± 7.40 | 2.39 ± 0.17 | 1.43 ± 0.17 | 1.40 ± 0.03 | 0.014 ± 0.000 | 1.00 ± 0.02 |
| | Stage 1 | 17.20 ± 0.26 | 7.41 ± 0.08 | 0.40 ± 0.05 | 0.31 ± 0.04 | 141.51 ± 24.00 | 2.49 ± 0.84 | 1.53 ± 0.11 | 1.38 ± 0.06 | 0.021 ± 0.001 | 1.55 ± 0.05 |
| | Stage 2 | 17.57 ± 0.12 | 7.24 ± 0.07 | 0.39 ± 0.01 | 0.30 ± 0.02 | 191.90 ± 27.35 | 2.55 ± 0.19 | 1.64 ± 0.13 | 1.36 ± 0.07 | 0.022 ± 0.000 | 1.62 ± 0.06 |
| | Stage 3 | 17.87 ± 0.15 | 7.40 ± 0.11 | 0.39 ± 0.01 | 0.32 ± 0.03 | 181.90 ± 50.36 | 3.13 ± 0.75 | 1.57 ± 0.09 | 1.47 ± 0.05 | 0.023 ± 0.000 | 1.57 ± 0.06 |
| | Stage 4 | 17.80 ± 0.17 | 7.43 ± 0.07 | 0.36 ± 0.02 | 0.30 ± 0.05 | 193.20 ± 51.76 | 2.97 ± 0.55 | 1.50 ± 0.08 | 1.47 ± 0.04 | 0.024 ± 0.000 | 1.63 ± 0.05 |

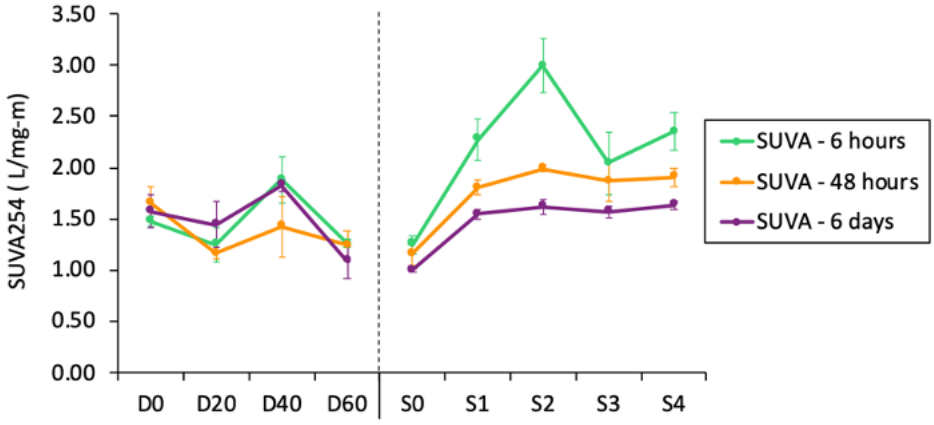



***Figure 50***. SUVA values every 20 days (D) during the growth phase and in each stage (S) after restarting the water supply after 6 hours, 48 hours and 6 days of IWS. All values represent an average of three water replicates analysis ± standard deviation.

EEM showed that protein-like components (T1 and T2) were predominant in all samples compared to humic-like components (A and C), which were not detected in any sample (Figure 52). T1 peaks (in relative fluorescence units) were relatively higher than T2 peaks on days 0 and 60 during the growth phase and after all the IWS periods applied (Figure 51). Overall, no important changes were observed between different IWS, result showed that after 48 h IWS T1 (peak average = 1875.25 ± 632.29, n = 4) and T2 (peak average = 992.50 ± 531.40, n = 4) were higher than after 6 h (T1 = 1575.25 ± 181.59 and T2 = 889.00 ± 199.27, n = 4) and 6 days (T1 = 1459.75 ± 335.47 and T2 = 923.50 ± 454.76, n = 4). These data only provide descriptive information since only 1 replicate could be analysed at each sampling point, and no results from stage 0 sample were obtained. Thus, it was not possible to calculate the standard deviation or perform statistical analysis to detect differences between the 3 times of IWS.

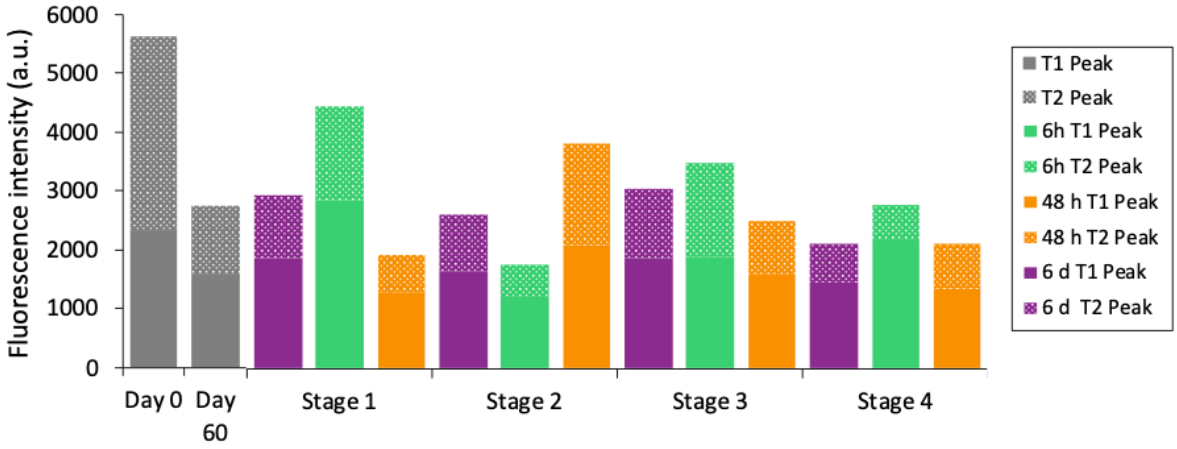



***Figure 51***. Fluorescence intensities at peak regions after different IWS times. Protein-like regions T1 and T2.

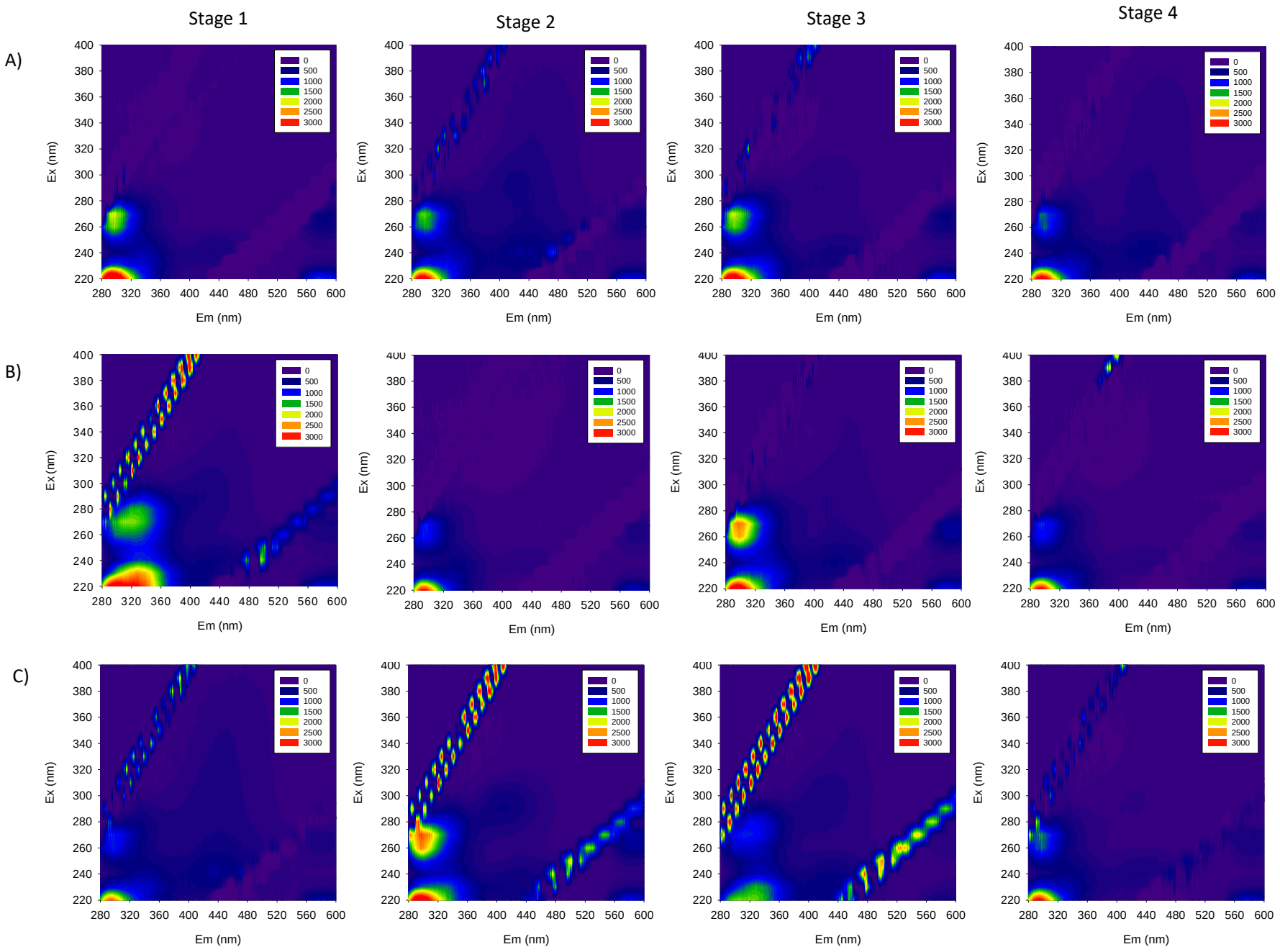



***Figure 52**.* Excitation-emission matrix spectroscopy contour plot of each stage after (A) 6 hours, (B) 48 hours and (C) 6 days of IWS.

#### 6.4.1.1 Water discolouration

Fe and Mn, concentration showed similar values for the 3 loops through the biofilm growth phase, ranging from 28.19 - 39.90 µg/L and 0.49 - 0.82µg/L respectively (Table 13, Figure 53), with no statistical difference between the 3 loops in any sampling point. However, when the water supply was restarted after the different IWS times, it was possible to observe an increase in the concentration of these metals. Overall, the highest Fe concentrations were observed after 6 days of IWS, followed by 48 hours and 6 hours, and statistical test reported significant differences between IWS times for stages 2, 3 and 4 (p-values ≤0.05). When samples were compared pairwise, significant differences were found between 6 hours and 6 days on these stages (p-values ≤0.05). Mn concentrations were statistically higher after 6 days than after 6 and 48 hours in all stages (p-values ≤0.05). Mn levels after 6 hours and 48 hours showed no significant differences in stages 0, 1 and 2 (p-values >0.05) but in stages 3 and 4 the concentration of this metal was statistically higher after 48 h than after 6h (p-values <0.05). See appendix C.6 for supplementary information on the results of the statistical analysis.

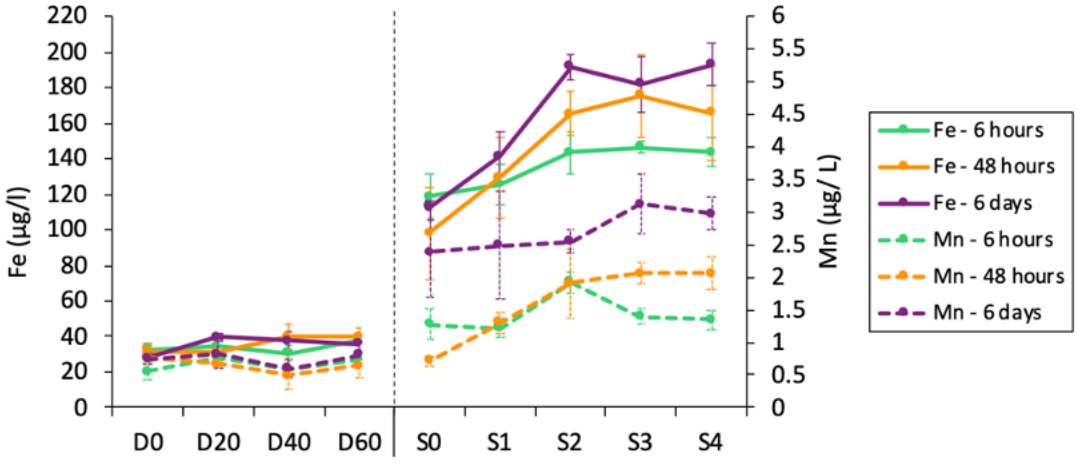



***Figure 53.*** Iron (Fe) and manganese (Mn) water concentrations every 20 days (D) during the growth phase and in each stage (S) during the water supply restarting after 6 hours, 48 hours and 6 days of IWS. All values represent an average of 3 water replicates analysis ± standard deviation.

Online turbidity measurements showed similar values in the 3 loops through the biofilm growth phase, average ranging from 0.049 to 0.051 NTU in the 3 loops. However, an increase in turbidity response when the water supply was restarted was observed after all the 3 IWS times (Figure 54). Peaks in turbidity response were detected immediately after restarting the water supply, which are related to the loss of material from the pipe walls when hydraulic conditions change. These turbidity

peaks are repeated cyclically due the water recirculation into the system, until the water is completely mixed, and turbidity values stabilise. This is typically observed during flushing events and it was expected based on PODDS modelling (Sharpe *et al.*, 2010; Husband *et al.*, 2016).

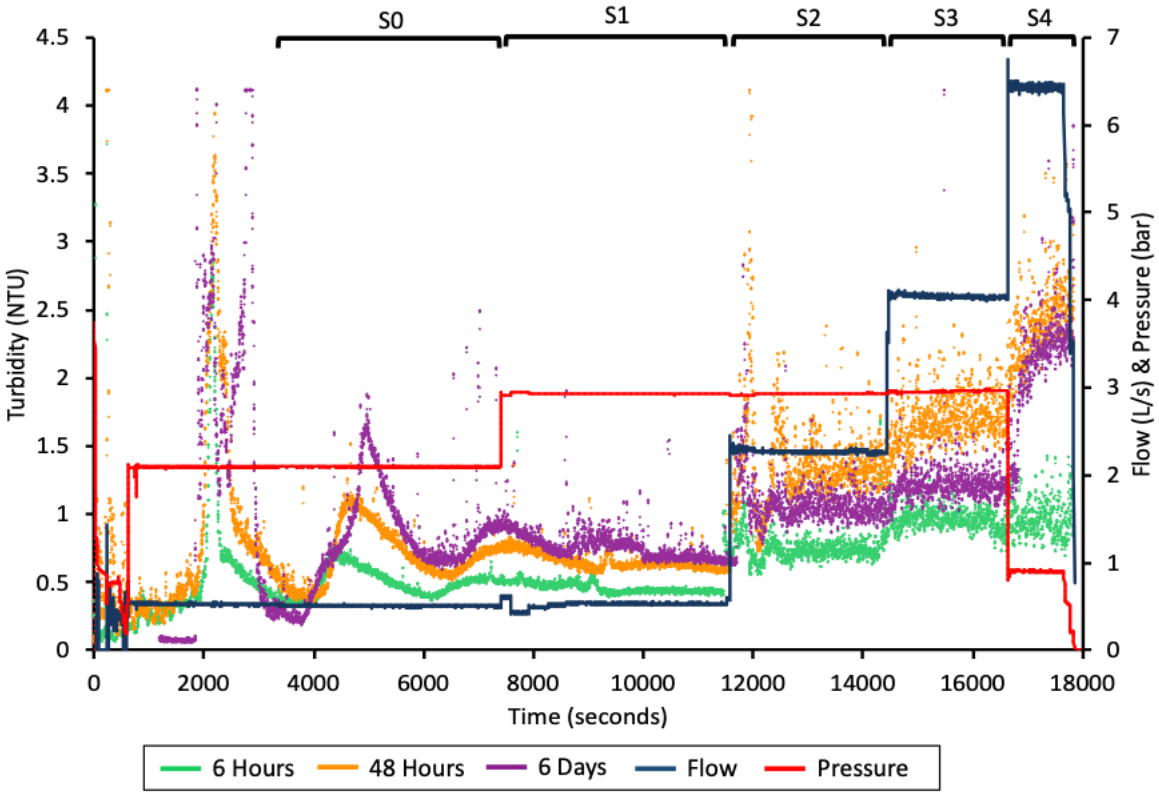



***Figure 54***. Turbidity response, flow rate and pressure profile during the water supply restarting by stages after different IWS times. S0 = Stage 0, S1 = Stage 1, S2 = Stage 2; S3 = Stage 3; S4 = Stage 4.

Turbidity levels of the last water turnover of each stage (i.e., when water with possible mobilised material was mixed (Figure 55) reported similar values for stages 0 and 1, while in stages 2, 3 and 4 showed higher turbidity levels after all IWS periods. In both stages 0 and 1, turbidity values were statistically lower after 6 hours than after 48 hours and 6 days of IWS (p-values ≤0.05). No statistical differences were found between 48 hours and 6 days (p-value >0.05). In stages 2 and 3, statistical differences between the 3 times were observed (p-values ≤0.05), presenting after 6 hours the lowest values, followed by 6 days and then by 48 hours, which presented the highest values. In stage 4, turbidity levels after 6 hours of IWS were statistically lower (p-values ≤0.05), than after 48 hours and 6 days. However, turbidity response did not show statistical differences between 48 h and 6 days in this stage (p-value >0.05) (Figure 54 and Figure 55). See appendix C.6 for supplementary information on the results of the statistical analysis.

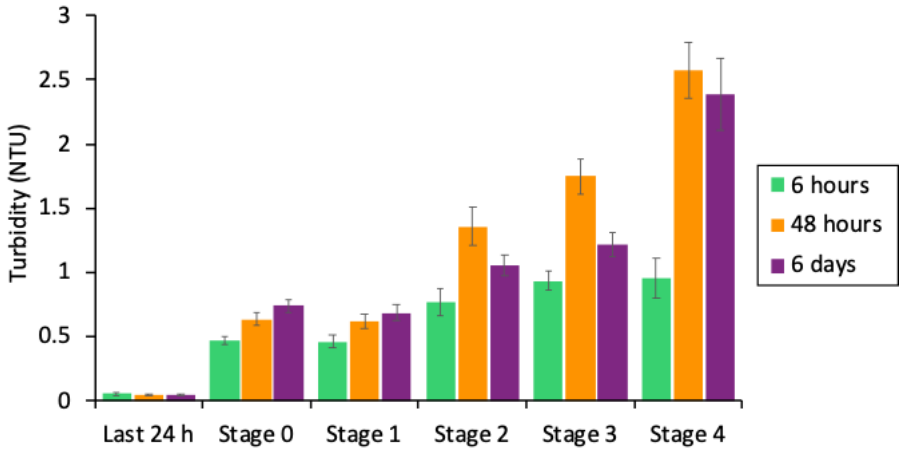



***Figure 55**. Average turbidity response during the last 24 hours of the growth phase (representative of the growth phase) and the last turnover of each stage of water supply restarting after different times of IWS.*

### 6.4.1.2 Flow cytometer counts

Results from FCM with TCC and ICC of planktonic cells during the growth phase and at each stage of the water supply restarting after different IWS times are shown in Figure 56. Overall, higher TCC were observed in all stages after the 3 IWS periods than during the growth phase, except in stage 0. When the different IWS times were compared between them, higher significant values of TTC and ICC in stages 1, 2, 3 and 4 were obtained after 6 days of IWS than after 6 hours and 48 hours (p-values ≤0.05). No significant changes were observed between 6 hours and 48 hours for TTC and ICC (p-value >0.05). See appendix C.3 for supplementary information on the results of the statistical analysis.

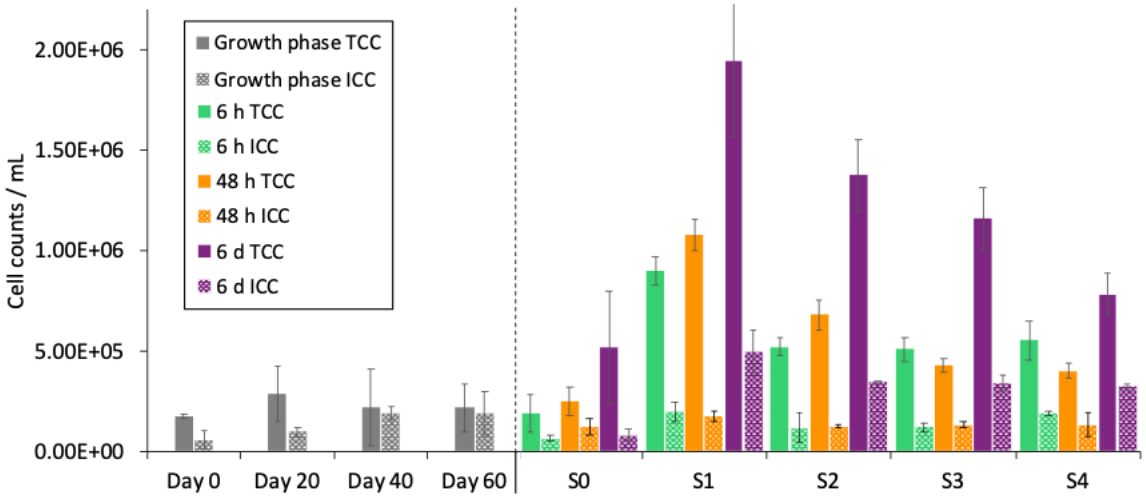



***Figure 56**. Total cell counts (TCC) and intact cell counts (ICC) of planktonic cells in the bulk water during the growth phase and in each stage (S) after different IWS periods. All values represent an average of three water replicates ± standard deviation.*

### 6.4.2 Microbial community structure (bacteria and fungi)

#### 6.4.2.1 Alpha diversity (diversity within samples)

Chao 1, Simpson, and Shannon indices were used to estimate the richness, dominance and diversity, respectively. Figure 57 shows the results for these diversity indices at genus level for bacteria and fungi in all biofilm and water samples. No significant differences were observed in any index for bacteria biofilm communities in samples post-IWS and post-restarting the water supply between the different IWS times (p-values >0.05). For fungi biofilm communities no significant differences were found between the 3 IWS times in samples post-IWS (p-value >0.05). Post-restarting the water supply, Chao 1 for fungi in biofilm did not show significant differences between IWS times, but Simpson was significant higher after 48 hours followed by 6 hours and 6 days (p-values ≤0.05). Consequently, Shannon index presented significant lower values after 48 hours than after 6 hours or 6 days (p-values ≤0.05). See appendix C.6 for supplementary information on the results of the statistical analysis.

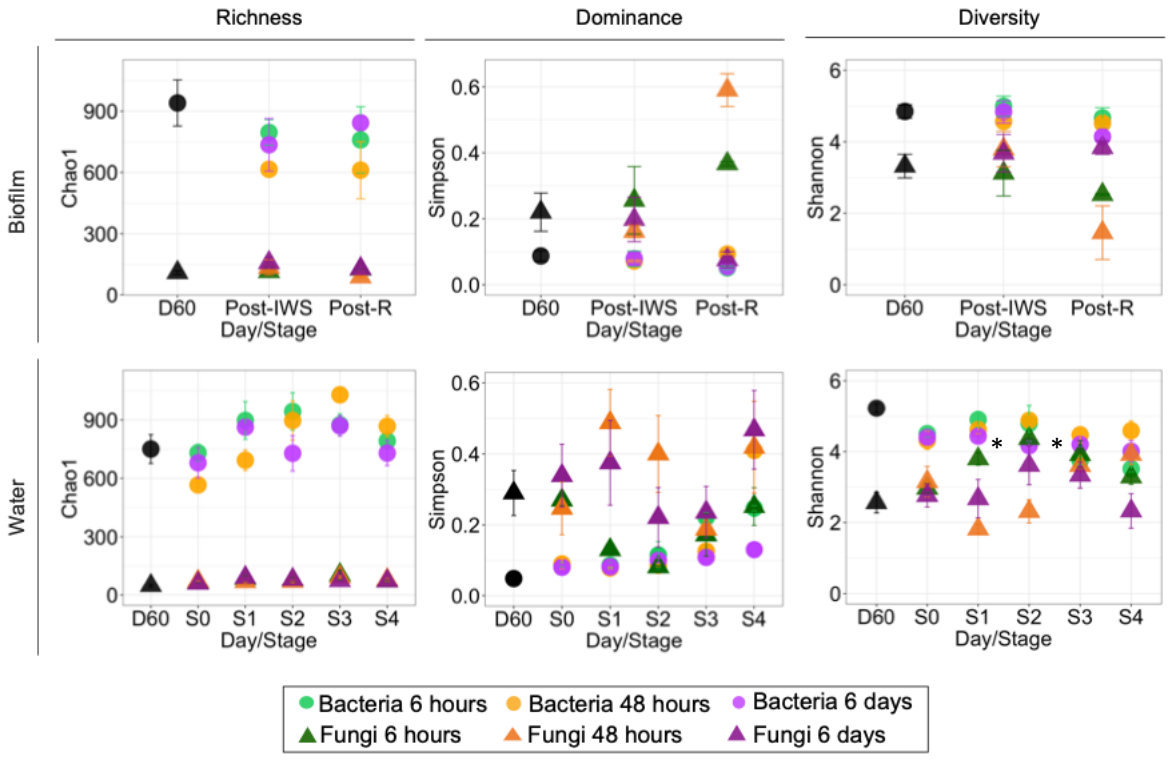



***Figure 57***. Chao 1 (richness), Simpson (dominance) and Shannon (diversity) indices for OTUs at 97 % cut off for bacteria and fungi in biofilm and water samples. Biofilm samples are from day 60 (D60), post-IWS times (i.e., before the supply restarting) and post-restarting the water supply (Post-R) after different IWS times. Water samples are from day 60 (D60) and from each stage (S) of water supply restarting after different IWS times. All values represent an average (n=3) ± standard deviation.

Planktonic bacterial communities were not affected by IWS events, and no significant differences were observed in any index between water samples from the 3 IWS times at all the different stages of the water supply restarting (p-values >0.05). Planktonic fungal communities did not show significant differences for Chao 1 at any stage of water supply restarting between the different IWS times (p-value >0.05). Simpson and Shannon indices did not show significant differences in stages 0, 3 and 4 (p-value >0.05) during the water supply restarting, but significant changes were observed for these indices in stages 1 and 2. Overall, fungal community after 6 hours presented lower significant values for Simpson, and con consequently higher significant Shannon values (p-values ≤0.05) than fungal communities after 48 hours or 6 days. See appendix C.6 for supplementary information on the results of the statistical analysis.

#### 6.4.2.2 Beta diversity (diversity between samples)

nMDS plots with the resemblance of bacteria and fungi communities at genus level in biofilm samples and water samples after the different IWS times are shown in Figure 58. For biofilm samples, no clear separation of the bacterial and fungal communities was observed. The ANOSIM analysis (Table 14) confirmed that no significant differences were found for bacteria between 60-day old biofilms and after 6 hours of IWS. However, the bacterial community structure was significantly affected after 48 hours and after 6 days of IWS. In addition, bacterial community structure showed significant differences between the different IWS times (Table 14). For fungi in biofilm, ANOSIM showed significant differences between 60-day old samples and after 48 hours and 6 days of IWS. No significant differences were found between 60-day old biofilm and after 6 hours. When the structure of biofilm fungal communities was compared between the different IWS times, ANOSIM did not show significant differences between them (Table 14).

Regarding planktonic communities, it was possible to observe an evident clustering at genus level for bacterial communities for the 3 IWS times. The ANOSIM analysis confirmed that water samples from day 60 had a significant different bacterial community structure when compared with samples after all IWS times. Significant differences were also found when compared bacterial communities after the 3 IWS times between them (Table 14). For planktonic fungal communities, no clear separation was observed in the nMDS. ANOSIM confirmed the absence of significant differences between fungal communities of day 60 and after all IWS times. Between the different IWS times, no differences were observed between plektonic fungal communities after 48 hours compared to 6

hours 6 days. However, significant changes were observed for planktonic fungi between in water samples after 6 hours and 6 days of IWS (Table 14).

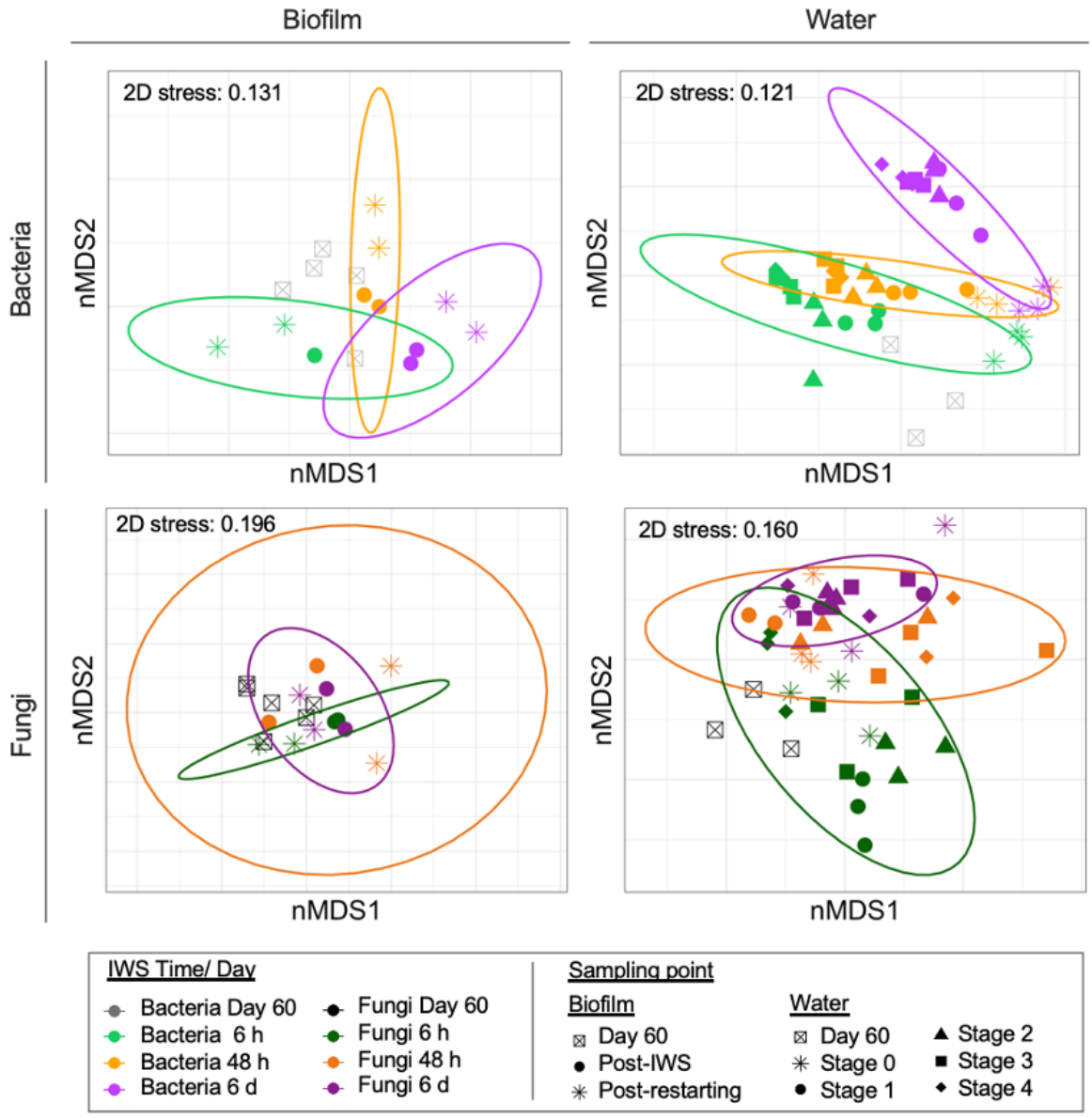



***Figure 58***. Two-dimensional diagrams of the non-multidimensional scaling (nMDS) analysis based on Bray–Curtis similarities of the relative abundance of bacteria and fungi at 97 % cut off in bulk water and biofilm samples from the different sampling points: day 60, post-IWS events and after restarting the water supply for biofilm samples; day 60 and each stage of water supply restarting for water samples. All replicates per sampling point are represented. Symbols are based the day/stage of sampling and different colours represent the different IWS times applied in this study.

***Table 14.*** ANOSIM statistics calculated using the Bray-Curtis distance matrixes to test beta-diversity significant differences. Differences were considered statistically significant when p-value was ≤0.05. Global-R statistic determine the level of differentiation between treatments, indicating 0 no differentiation and 1 a total differentiation of the samples.

| BIOFILM | Bacteria | | Fungi | |
|---|---|---|---|---|
| | p-value | global-R | p-value | global-R |
| Day 60 vs 6 hours IWS | 0.074 | 0.294 | 0.106 | 0.283 |
| Day 60 vs 48 hours IWS | 0.008* | 0.373 | 0.037* | 0.357 |
| Day 60 vs 6 days IWS | 0.003* | 0.865 | 0.072* | 0.262 |
| 6 hours IWS vs 48 hours IWS | 0.0025* | 0.729 | 0.584 | 0.071 |
| 6 hours IWS vs 6 days IWS | 0.0028* | 0.875 | 0.149 | 0.250 |
| 6 days IWS vs 48 hours IWS | 0.0029* | 0.552 | 0.447 | 0.026 |
| WATER | Bacteria | | Fungi | |
| | p-value | global-R | p-value | global-R |
| Day 60 vs 6 hours IWS | 0.001* | 0.558 | 0.400 | 0.400 |
| Day 60 vs 48 hours IWS | 0.004* | 0.707 | 0.196 | 0.201 |
| Day 60 vs 6 days IWS | 0.005* | 0.659 | 0.078 | 0.360 |
| 6 hours IWS vs 48 hours IWS | 0.007* | 0.164 | 0.069 | 0.140 |
| 6 hours IWS vs 6 days IWS | 0.001* | 0.569 | 0.001* | 0.259 |
| 6 days IWS vs 48 hours IWS | 0.001* | 0.439 | 0.083 | 0.066 |

### 6.4.3 Microbial community composition (bacterial and fungi)

#### 6.4.3.1 Taxonomical analysis of biofilm communities

Differences in bacterial and fungal composition at genus level were observed in biofilm communities between 60-days old biofilms, biofilm samples post-IWS and biofilm samples post-restarting the water supply after the different IWS times (Figure 59, Figure 60).

**Figure 59** shows the taxonomical analysis of bacteria in all biofilm samples. Overall, 60-days old biofilm samples in the 3 loops were dominated by *Phreatobacter* (average of 29.2%) and a not defined taxa belonging to the order Obscuribacterales (17.5 %). After 6 hours of IWS (both post-IWS and post-restarting the water supply biofilm samples showed similar bacterial profiles to those of day 60: *Phreatobacter* (24.1 %), *Flavobacterium* (18.2 %) and an identify taxa within the order Obscuribacterales (8.7 %) presented high relative abundances. *Pseudomonas* presented a high relative abundance only in samples post-IWS (14.64 %), while post-restarting its relative abundance decreased (1.70 %). After 48 hours of IWS biofilm bacterial community composition changed when compared to day 60 or after 6 hours of IWS communities. In samples post-IWS, *Aquabacterium* (30.1 %) dominated the community together with *Phreatobacter* (20.4 %), *Pseudomonas* (17.5 %) and a

not defined genus belonging to the class Obscuribacterales (14.6 %). The analysis of biofilm samples obtained post-restarting the water supply after 48 hours of IWS showed that a not defined Obscuribacterales (37.2 %) became the most abundant taxa, followed by *Aquabacterium* (15.5 %), *Phreatobacter* (7.3 %) and *Pseudomonas* (4.5 %). After 6 days of IWS, samples post-IWS showed that *Aquabacterium* (35.7 %) was the most abundant genus together with *Pseudomonas* (11.0 %), *Phreatobacter* (10.7 %) and *Mycobacterium* (9.4 %). In samples post-restarting the water supply after 6 days of IWS *Sphingomonas* (26.8 %) was the most abundant genus, together with *Mycobacterium* (16.9 %) and *Pseudomonas* (15.5 %).

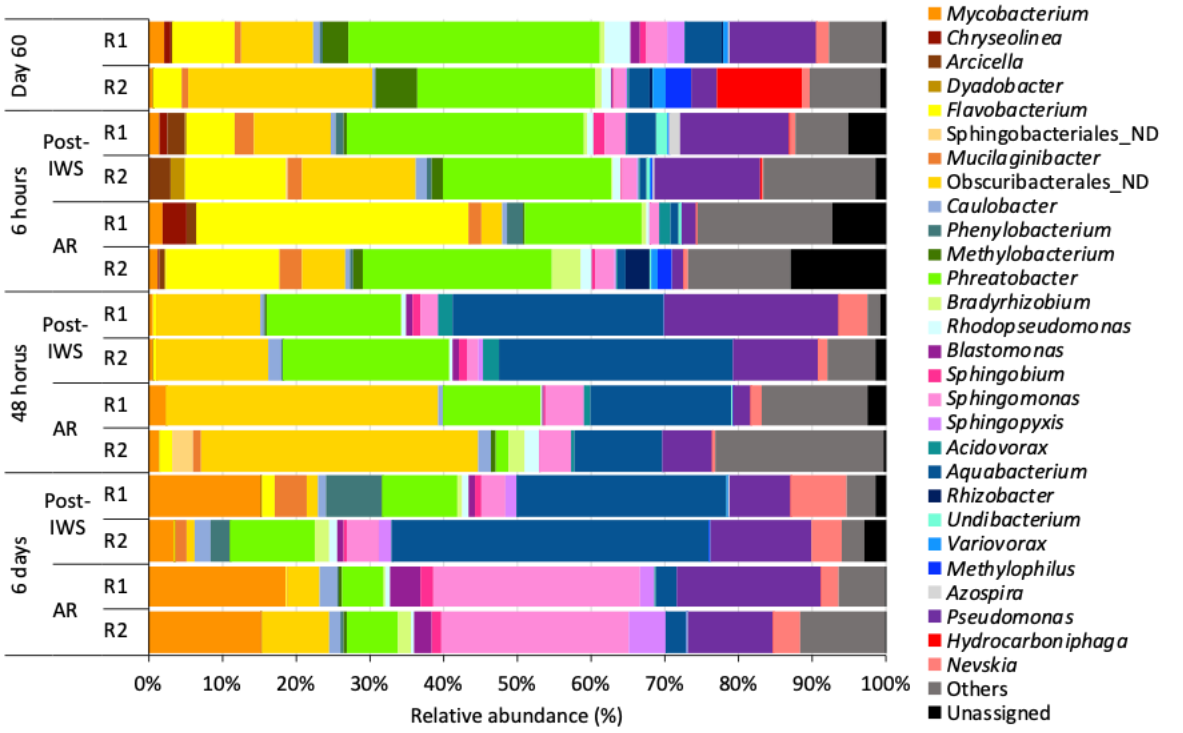



***Figure 59.*** Relative abundance of bacteria at genus level (>1 % of the total sequences) of biofilm samples of day 60, post-IWS and after restarting the water supply (AR) after 6 hours, 48 hours and 6 days of IWS. The 2 biological replicates (R1 and R2) are represented. For day 60, R1 and R2 are the average of relative abundances from samples from the 3 loops (n = 3). Remaining genera were combined in category "Others". Category "Unassigned" corresponds to unidentified OTUs and "ND" indicates not defined at that level.

Figure 60 shows the taxonomical analysis of fungi in biofilm samples. In 60-days old biofilms the most abundant genera were *Cladosporium* (average of 19.7 %) and *Cadophora* (22.2 %), followed by *Ochroconis* (8.5 %), *Exophiala* (7.9 %) and *Penicillium* (7.4 %). After 6 hours of IWS, in samples post-IWS a not defined taxa belonging to the family Nectriaceae was the dominant taxa (47.6 %), followed by other taxonomic groups with lower relative abundance such as *Cadophora* (9.30 %).

Post-restarting the water supply after 6 hours the relative abundance of *Cadophora* (66.46 %) increased becoming predominant in the community, followed by *Ochroconis* (10.46 %). After 48 hours IWS and post-IWS, the community was dominated by *Cadophora* (18.9 %), *Cladosporium* (18.8 %), a not defined genus belonging to the order Pleosporales (17.4 %) and *Exophiala* (16.3 %). Post-restarting the water supply after 48 hours of IWS, fungal community was mainly dominated by *Ochroconis* (42.4 %) and *Penicillium* (40.5 %). After 6 days of IWS, in biofilms post-IWS the most abundant genera were again *Ochroconis* (24.7 %) and *Cladosporium* (13.8 %). Post-restarting the water supply after 6 days *Cladosporium* (20.4 %) became the most abundant genera, and other such as *Ochroconis* (8.3 %) or *Debaryomyces* (8.0 %) presented high relative abundances.

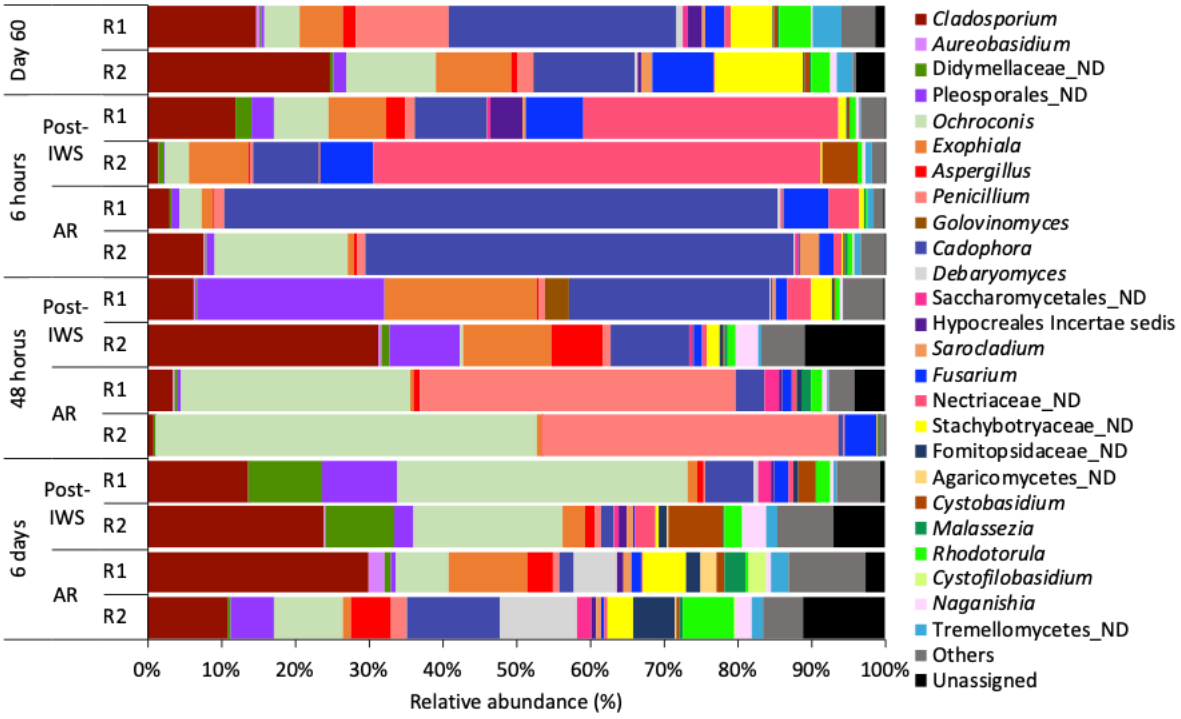



***Figure 60**.* Relative abundance of fungi at genus level (>1 % of the total sequences) of biofilm samples of day 60, post-IWS and after restarting the water supply (AR) after 6 hour, 48 hours and 6 days of IWS. The 2 biological replicates (R1 and R2) are represented. For day 60, R1 and R2 are the average of relative abundances from each sample from each loop (n = 3). Remaining genera were combined in category "Others". Category "Unassigned" corresponds to unidentified OTUs and "ND" indicates not defined at that level.

### 6.4.3.2 Taxonomical analysis of planktonic communities

Differences in bacterial and fungal composition at genus level were observed in planktonic communities when the water supply was restarted after different IWS times (Figure 61, Figure 62). Taxonomical analysis of bacteria in water samples (Figure 61) showed that on day 60 of the growth phase the 3 loops presented similar planktonic communities, and several genera such a *Reyranella*,

*Phreatobacter*, *Nevskia*, *Sphingomonas*, *Methylobacterium* or *Cupriavidus,* were present with average relative abundances between 12.5 % and 6.9 %. After 6 hours and 48 hours of IWS, bacterial planktonic communities followed a similar pattern of change through the stages of the water supply restarting. In stage 0, *Sphingomonas* (average 36.6 – 40.8 %) and *Methylobacterium* (24.6 – 26.1 %) were the most abundant genera. However, their relative abundance in samples from stage 1 to 4 decreased, whilst *Phreatobacter* became dominant (45.5 – 70.2 %). It was notable the increase of the relative abundance of *Mycobacterium* (6.3 –11.6 %) and the taxa belonging to the order Obscuribacterales (6.3 – 11.3 %) in stages 2, 3 and 4 after 48 hours of IWS. After 6 days of IWS in stage 0, as it occurred in the other two IWS times, *Sphingomonas* (44. 6 %) and *Methylobacterium* (23.1 %) were the most abundant bacterial genera in planktonic communities. From stages 1 to 4 *Sphingomonas* (34.1 – 47.6 %) continued to be the most abundant genus, and it was followed by *Nevskia* (6.3 – 25.1 %) and *Phreatobacter* (13.3 – 21.8 %).

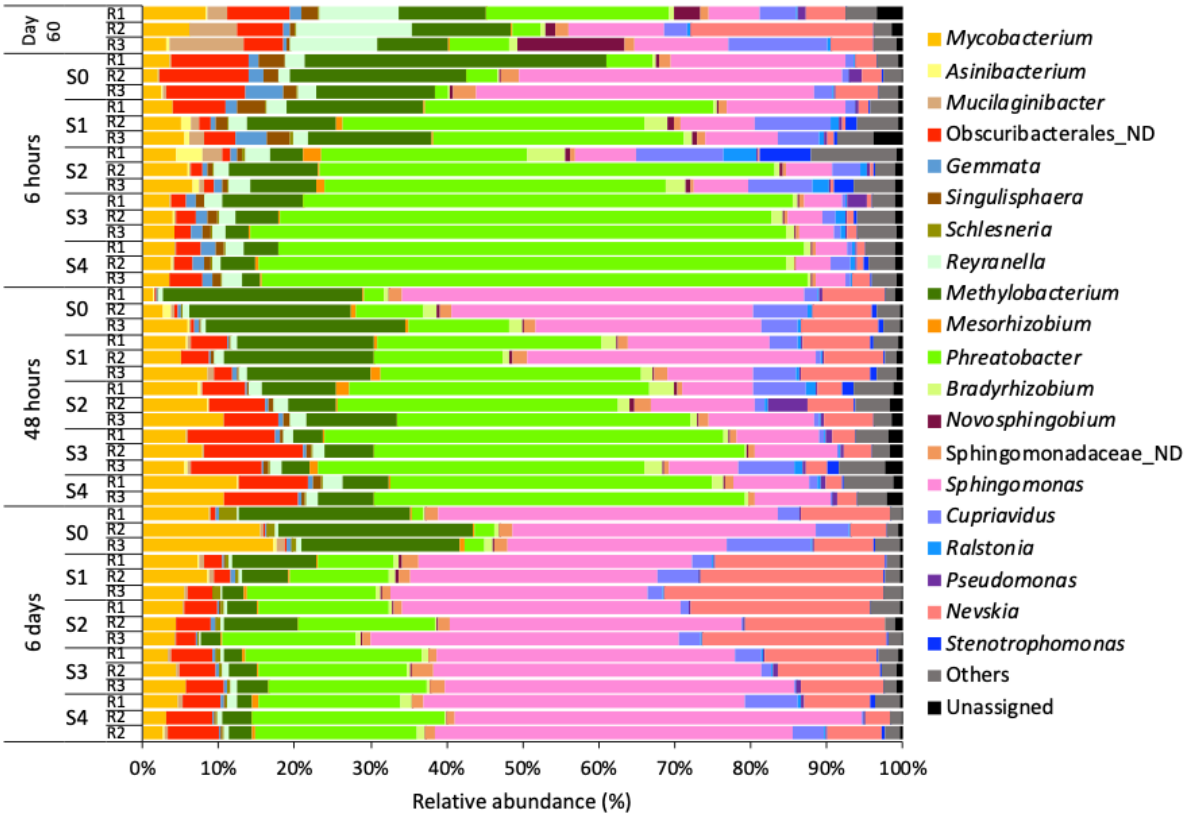



***Figure 61.*** Relative abundance of bacteria at genus level (>1 % of the total sequences) of water samples from day 60 and each stage of water supply restarting after 6 hours, 48 hours and 6 days of IWS. The 3 biological replicates (R1, R2 and R3) per sampling point are represented. For day 60, each replicate was taken from each loop since they ran under the same conditions during the 60 days. Remaining genera were combined in category "Others". Category "Unassigned" corresponds to unidentified OTUs and "ND" indicates not defined at that level.

Fungal planktonic composition (Figure 62) did not show significant changes during the water supply restarting after the different IWS times. On day 60, the most abundant planktonic fungi in the 3 loops were *Cadophora* (average of 54.8 %), a not defined taxa belonging to Sordariomycetes class (19.28 %) and *Exophiala* (16.98 %). After the 3 IWS, the taxonomical profile of planktonic fungi was similar: *Cadophora* (average 42.5 %) dominated the community with high relative abundance, followed by *Exophiala* (17.4 %) and *Cladosporium* (11.4 %), which also presented a high relative abundance.

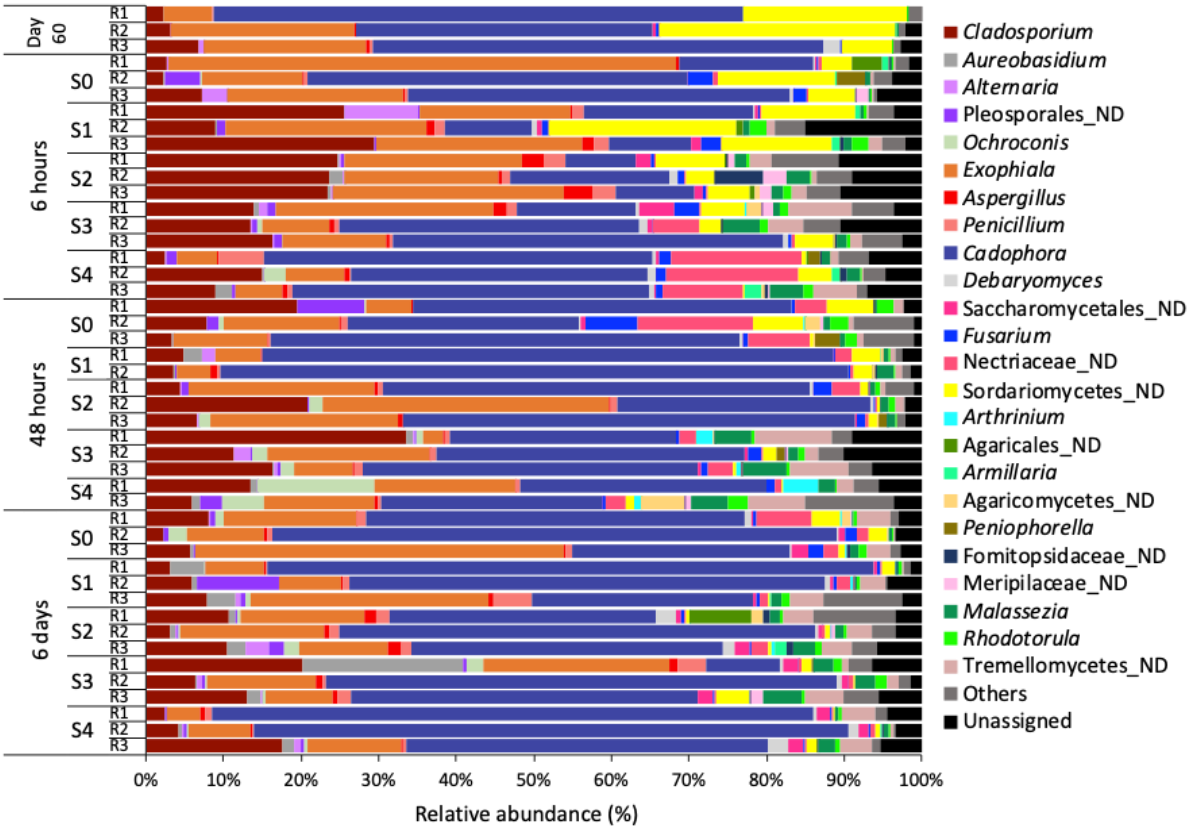



***Figure 62***. Relative abundance of fungi at genus level (>1 % of the total sequences) of water samples from day 60 and each stage of water supply restarting after 6 hours, 48 hours and 6 days of IWS. The 3 biological replicates (R1, R2 and R3) per sampling point are represented. For day 60, each replicate was taken from each loop since they ran under the same conditions during the 60 days. Remaining genera were combined in category "Others". Category "Unassigned" corresponds to unidentified OTUs and "ND" indicates not defined at that level.

## 6.5 Discussion

### 6.5.1 Effect of different IWS times on water quality

This study has characterised the water NOM after different IWS times by using measurements of TOC and DOC, SUVA and EEM. These parameters are used as a water quality indicator since they can provide measurements of the concentration and type of the NOM present in the water, which can react with different disinfectant used in drinking water and lead to the formation of the harmful DBPs (Matilainen *et al.*, 2011). Specifically, the hydrophobic humic fraction of NOM (i.e. aromatic compounds that contains a significant amounts of aromatic rings or unsaturated carbon bonds) is more reactive with oxidants like chlorine and thus it can promote the potential formation of DBPs (Reckhow, Singer and Malcolm, 1990; Weishaar *et al.*, 2003). The concentration of NOM was determined by TOC and DOC, and results throughout the experiment showed typical concentrations reported previously for drinking water (Douterelo, Husband and Boxall, 2014; Li *et al.*, 2018), and no significant differences were observed in any stage after restarting the water supply after the 3 different IWS times. This suggests that there is no a DBPs risk associated with the increase of NOM concentration in this system after different IWS times. Then, the nature and reactivity of the NOM after the different IWS times was tested using SUVA and EEM. Descriptive analysis of EEM indicated that protein-like components were predominant in all samples after the 3 IWS times, while humic-like components were not detected. This higher proportion of non-humic substances is a positive aspect in terms of water quality since these hydrophilic substances are less reactive and do not tend to form DBPs (Weishaar *et al.*, 2003). SUVA was also used in this study as a measurement of the content of the aromaticity of organic compounds. It has been observed that SUVA <2 L/mg-m indicates high fraction of hydrophilic non-humic matter, while SUVA values between 2 - 4 L/mg-m are indicative of the presence of hydrophobic humic fraction and aromatic compounds (Parsons *et al.*, 2004; EPA, 2012). Almost all SUVA values obtained at all the sample points during the growth phases and after 48 hours and 6 days of IWS were under 2 L/mg-m, similar values to those reported in other studies in chlorinated DWDS (Goslan *et al.*, 2009; Park *et al.*, 2016). This was in agreement with EEM results, indicating a higher proportion of non-humic substances in the bulk water. However, samples after 6 hours of IWS showed higher SUVA values, up to 3.00 ± 0.27 L/mg-m, which were statistically significant from stage 1 of the water supply restarting. These highest SUVA values observed after 6 hours of IWS indicated a higher proportion of aromatic organic compounds, which

can potentially react with chlorine to form harmful DBPs. Thus, these results suggest a higher risk of DBPs formation after short IWS events.

This information is useful not only for DWDS operated under IWS regimes, also water companies performing under CWS should take into account this potential risk related to NOM since the interruption of supply for these short times is a common practice for maintenance works. Moreover, in chlorinated DWDS after these repair or maintenance works, in which short IWS events occur, high concentrations of chlorine are added in order to ensure the microbiological safety of the drinking water (Van Nevel *et al.*, 2017; van Bel *et al.*, 2019). Thus, the increase of proportion of hydrophobic humic fraction observed after short IWS times, together with the addition of high disinfectant concentrations can lead to a high DBPs formation potential, compromising the quality of the supplied water. This is the first time that SUVA and EEM analysis are carried out in IWS experiments, yet we can conclude that they can give a rapid determination of the proportion of humic and non-humic organic matter present in the water.

### 6.5.2 Effect of IWS on water discoloration and biofilm cell mobilisation

The effect of the different IWS times on water discolouration was evaluated using online turbidity measurements and the concentration of Fe and Mn of discrete water samples throughout the experiment. Results showed similar concentrations of Fe and Mn during the growth phase in the 3 loops and a significant increase in the concentrations after the 3 IWS times. These results were consistent with those from online turbidity monitoring (Figure 54 and Figure 55) showing an increase in the turbidity response when the water supply was restarted after the 3 IWS. Other studies in DWDS operating IWS have also observed an increase of the risk of water discolouration. Tokajian and Hashwa (2003) found high turbidity levels when the flow was restarted in a small DWDS in Lebanon supplied twice a week, and Cerrato *et al.* (2006), studying a DWDS in Honduras, observed higher concentrations of Fe and Mn during intermittent flow conditions than in continuous cycle in polyvinyl chloride (PVC) and iron pipes. Thus, result from this study were in agreement with this previous research but also provide new information about the risk of discolouration during IWS events.

Firstly, the increase in turbidity levels and concentration of Fe and Mn after the 3 IWS times was observed from stage 0. In this stage the system was run under the peak values of hydraulic conditions of the biofilm growth phases, i.e. conditions that during the growth phase did not disturb

the biofilm. However, these same conditions after applying the 3 IWS removed material from the pipe walls increasing the water discolouration. It has been observed that biofilms developed under the constant influence of a variable flow are more resistant than those formed in stagnant conditions, which detach more easily from pipe surfaces when shear stress changes (Manuel, Nunes and Melo, 2007). Taking into account this, it could be suggested that the potential biofilm developed during the IWS periods (without hydraulic influence) was more easily removable from the pipe walls. Thus, the entry of water into the system and/or the normal hydraulic conditions produced a flushing effect on the pipes after the IWS events, increasing the water discolouration. Moreover, Fe and Mn concentrations and turbidity values did not change when an increase in pressure was applied in stage 1 and similar values for these parameters were observed for stages 0 and 1 with no differences between the 3 IWS times. However, the increase in flow applied in stages 2, 3 and 4 resulted in additional mobilisation of material from the pipe walls in each stage. This increase in turbidity response as the flow increased was expected since it has been widely demonstrated that higher shear stresses generate the mobilization of the biofilm from the pipe walls (Husband *et al.*, 2016). Therefore, this study has demonstrated how the increase in pressure did not promote material mobilization, and only the increases in flow promoted the discolouration response during the water supply restarting after IWS events.

When the 3 IWS times were compared between them, turbidity response and the concentration of Fe and Mn after 48 hours and 6 days showed statistical higher values than after 6 hours. It has been observed that in absence of water flow biofilms are able to growth and develop in a greater extent, since they are not under the influence of the hydraulic dynamics of the system (Simões and Simões, 2013). Thus, results seem to indicate that longer times of IWS lead to a potential greater biofilm development attached the pipe walls during the interruption periods. This was supported by FCM results that showed higher values of TCC mobilised into the bulk water during the supply restarting after longer IWS. Previous studies have also observed changes in the number of microorganisms in samples from IWS events using culture-based methods. For example, Coelho *et al.* (2003) observed that HPC was higher when the flow was restarted after IWS events in different DWDS operating from 10-12 hours per 48 hours to twice per week in different locations; and Kumpel and Nelson (2013) reported a greater presence of total coliforms and *E. coli* in an IWS system than in an CWS system by using the most probable number method. Results from this research have reinforced these limited previous studies using FCM technique, which has been shown to be more sensitive,

reproducible and faster than others for routine drinking water monitoring (Van Nevel *et al.*, 2017). Regarding ICC, similar values were observed for all IWS times applied. This reduction in cell viability regardless of the IWS time can be explained by the combination of the mechanical action of flushing during the supply restarting together with the effect of the disinfectant in the water that fill the system (Nescerecka *et al.*, 2014).

According to the proposed hypothesis, a greater biofilm growth and higher discolouration response would be expected after 6 days than after 48 hours of IWS, but turbidity results did not show this trend until the last stage of the water supply restarting, when a very high flow was applied, and turbidity response after 6 days increased reaching values similar to 48 hours. This suggested that the material attached to the pipe walls after 6 days of IWS was more difficult to remove, and higher flows were needed to detach it and increase the discolouration response. This increased resistance of biofilms to detachment from the pipe wall after longer IWS periods could be explained by a drying process. It has been demonstrated that more than 90 % of the wet weight of biofilms is water, and thus the absence of water can change the biofilm compactness (Schmitt and Flemming, 1999). When the biofilm dries out, its internal structure changes and the forces established between the cells and the surface are strengthened. This makes the biofilm become more compact making more difficult for the water to intrude inside of the biofilm (Melo, 2005). Then, this would make biofilm more resistant to be detach from the pipe walls. IWS events have previously been related to biofilm drying processes, for example Cerrato *et al.* (2006) observed in a system operating under IWS that when plastic pipe surfaces were dried, the biofilm morphology was affected changing its texture and colour. Similarly, Gião and Keevil (2013) reported that the absence of water stresses the microbial cells and changes the morphology and structure of the DWDS biofilms from stainless steel and polytetrafluoroethylene surfaces.

Therefore, results from this research have suggested that longer IWS lead to a greater biofilm development during the drained period, and thus to higher discolouration risk when the water supply is restarted. However, these longer times also produce the biofilm drying out, making its structure change, and becomes more difficult to detach from the pipe wall. Consequently, higher shear stress is required to remove biofilm from the pipe walls. In terms of water quality, the high turbidity levels and the increase of Fe and Mn besides discolouration, can enhance taste or odour problems, promote the deterioration and obstruction of pipes (McNeill and Edwards, 2001). In addition, microbial biofilms can be hosts of opportunistic pathogens, and its mobilisation after IWS

events can encourage the occurrence of this microorganisms harmful to human health in the final tap water (Douterelo, Sharpe and Boxall, 2014; Nescerecka *et al.*, 2014; Husband *et al.*, 2016). Therefore, it can be concluded that the risk associated with material mobilization after an IWS event is greater after longer periods of interruption.

### 6.5.3 Effect of different IWS times on microbial community

When microbial communities were characterised by using genetic markers for bacteria and fungi, results showed how different IWS times did not significantly influenced bacterial richness and/or diversity of biofilm communities. However, differences in the structure between the 3 IWS times were reported by beta-diversity, showing that longer IWS times produce bigger changes in the structure of these communities. These results were expected since it has been widely demonstrated that biofilm bacterial communities are usually affected by changes in abiotic factors, including changes in hydraulic regimes (Douterelo, Sharpe and Boxall, 2013; Mi *et al.*, 2015; Ren *et al.*, 2015) like the ones that happen during IWS events. Structural changes in these communities were reflected in the taxonomical profiles after the different IWS times. After 6 hours of IWS the composition of biofilm bacterial community was the most similar to those of 60-day old biofilm, suggesting that the longer the IWS time, the greater changes occur in its taxonomical composition. However, some changes could also be observed after 6 hours of IWS, and in both post-IWS and post-restarting samples it was notable the increase in the relative abundance of *Flavobacterium* genus. *Flavobacterium* has been previously reported forming part of the microbiota of DWDS (Douterelo, Husband and Boxall, 2014). However, *Flavobacterium* sp. can promote the presence of *Legionella pneumophila* in biofilms, the agent of different types of pneumonia in humans (Navarro-Noya *et al.*, 2013). Therefore, the increase of *Flavobacterium* in biofilm observed after this short IWS time could be a potential risk for water safety since the biofilm could act as a potential reservoir of pathogens that can be then mobilised into the bulk water.

After 48 hours of IWS, a more differentiated composition could be observed in biofilm samples. In both post-IWS and post-restarting samples, the most important change was the increase of the relative abundance of *Aquabacterium* and the nor defined Obscuribacterales. These microorganisms have been observed in drinking water related environments but they have not been identified as disease-causing pathogens in humans (Kalmbach *et al.*, 2000; Keller *et al.*, 2010; Di Rienzi *et al.*, 2013; Isabel Douterelo *et al.*, 2016; Bruno *et al.*, 2018). Thus, their high relative

abundance observed after 48 hours of IWS a priori would not have a negative implication for water safety.

After 6 days of IWS biofilm bacterial communities showed a different composition, and several genera such as *Shphingomonas* and *Mycobacterium* presented high relative abundances in biofilms compared to the other IWS times. *Mycobacterium* appears widely distributed in DWDS with different characteristics, but this genus includes important opportunistic pathogens and its presence in drinking water have been related to nosocomial infections (Vaerewijck *et al.*, 2005; Hilborn *et al.*, 2006; Liu *et al.*, 2016; WHO, 2017). *Shphingomonas* is well recognized for its ability for EPS formation contributing to the biofilm formation in DWDS, and some species of this genus are also involved in the infection of immunocompromised patients in hospitals (Johnsen *et al.*, 2000; Zhang *et al.*, 2012; Steinberg and Burd, 2015). Thus, the high relative abundance of *Shphingomonas* in biofilms after 6 days of IWS could facilitate the biofilm re-growth when the water is supplied again, leading to more developed biofilms and the associated consequences for water quality. In addition, the increase of the relative abundance of these both genera in biofilm after longer IWS times could have important implications for water safety and human health if it is mobilised into the bulk water and reaches consumers taps.

In fact, *Mycobacterium* presented slightly higher relative abundance, and *Sphingomonas* increased its relative abundance in planktonic communities when the water supply was restarted after the longest IWS time. These results indicated that in addition to the load of these genera from the incoming water, a large proportion was removed from the biofilm when the water supply was restarted. Considering this, it could be suggested that longer IWS favor the risk associated to the presence of these potential pathogenic bacteria when the water supply is restarted. Similarly, *Phreatobacter*, that showed higher relative abundances in biofilm communities after 6 hours and 48 hours of IWS times, was the dominant genera in planktonic communities when the water supply was restarted after short IWS times. Limited information exits about this novel genus with only 3 species, but it has not been related to human health problems (Toth *et al.*, 2014) and thus it presence would not have implications for water safety. Therefore, these results seem to indicate that the different structures of bacterial planktonic community observed after the different IWS were related to the biofilm mobilisation when the water supply is restarted. This also reinforces the importance of studying biofilm communities in DWDS, since changes on these communities can modify the water safety.

Regarding fungi, results indicated that the structure of both biofilm and planktonic fungal communities was less affected by IWS than bacteria by IWS events. Douterelo, Fish and Boxall (2018) suggested that fungal communities of DWDS have more resilience to changes in the environment and they persist better than bacteria over time. Results obtained here are in accordance to this previous research and show that fungi have a higher resistance capacity to different IWS times in DWDS. Despite this, it was possible to observe several changes for fungi, for example the increase in dominance and diversity reduction in biofilm samples post-restarting the water supply after the shorter IWS times. This suggested that after these short IWS times, some fungi groups were more resistant to increases in flow during the water supply restarting and remained attached to the pipe walls becoming dominant. Taxonomical analysis confirmed that after 6 hours of IWS *Cadophora* was the genus that became dominant when the water supply was restarted. *Cadophora* has been observed previously in DWDS after flushing events of plastic pipes (Douterelo *et al.*, 2020), and although is widely recognized as a phytopathogenic fungus, it has not been related to human health problems (Travadon *et al.*, 2015). Therefore, the dominance of this fungal genus in the biofilm community after 6 hours of IWS should not be a potential risk for water safety. After 48 hours of IWS, *Ochroconis* and *Penicillium* were the dominant genera in biofilm favoured when water supply was restarted. Different species of *Ochroconis* have been detected forming exclusively part of the biofilm of different DWDS (Heinrichs *et al.*, 2013; Wang *et al.*, 2018), and *Penicillium* is one of the most isolated fungal specie from drinking water related environments in Europe (Lyratzopoulos *et al.*, 2002; Novak Babič *et al.*, 2017). However, these two fungal genera contains different pathogenic species that can produce systemic or local infections in humans (Lyratzopoulos *et al.*, 2002; Novak Babič *et al.*, 2017). Therefore, their dominance in the biofilm after 48 hours of IWS could be a potential risk to the water microbiological safety.

After 6 days of IWS, as diversity indices showed there was not a specific genus that mainly dominated the fungal community as it happened with the other IWS times. However, *Ochroconis* also presented high relative abundances after this longer IWS times together with *Cladosporium.* Some species of *Cladosporium* have been also shown to produce skin and lungs infections, affecting severely asthmatics and people with respiratory diseases (Assress *et al.*, 2019). Therefore, results of biofilm communities showed that, in general, longer IWS (48 hours and 6 days) led to increase the relative abundance of fungal genera that contains human pathogenic species such as *Ochroconis*

*Cladosporium* or *Penicillium.* More research is needed using precise molecular techniques and focusing on these microorganisms to assess the potential risk of these fungi for water safety.

The taxonomy composition of planktonic fungal communities showed limited changes between the 3 IWS times. Common genera such as *Cadophora*, *Exophiala, Cladosporium* that showed similar high relative abundances, have been observed as common inhabitants of DWDS (Gonçalves, Paterson and Lima, 2006; Isabel Douterelo *et al.*, 2016; Richardson and Rautemaa-Richardson, 2019). It could be observed small changes such as the increase in the relative abundance of *Cladosporium,* that increased after the 3 IWS times. This was possibly because it was also a common genus in biofilm communities, and as it happened with bacterial communities, it was removed from the pipe walls with the water supply restarting.

All these results provide for the first-time information about the effect of different IWS times on the structure and composition of microbial communities in a chlorinated DWDS of MDPE pipes. However, further investigation based on these results is necessary to keep moving forward and obtain a complete understanding of what happens in the pipes environment during IWS events and to understand how different IWS times affect systems with other characteristics, such as different pipe materials or unchlorinated systems. In addition, here it has been observed that different times of IWS affect the bacterial and fungal community that remains attached to the pipe walls, so it is necessary to see how these different biofilm communities would affect the biofilm regrowth in the system and the effect of different supply and intermittent cycles. This will help improve the management of this practice, which is increasingly common worldwide, and to minimise the associated risk to ensure the delivery safe water and protect public health.

## 6.6 Conclusion

From this study it can be concluded that:

- Shorter IWS (6 hours) are related to higher proportion of aromatic organic compounds in the water during the supply restarting, and thus with a greater potential formation of DBPs.
- When the water supply is restarted after an IWS event, the increase in pressure do not produce the material mobilisation from the pipe walls, while increases in flow promote the discolouration.

- Longer IWS lead to a greater biofilm development during the drained period, and thus to higher discolouration risk. However, after extremely long IWS times biofilm could also experience a drying process, making its structure change and becomes more difficult to remove from the pipe wall. Consequently, higher shear stress is required to remove biofilm from the pipe walls.
- IWS events produce changes in the structure and taxonomical profiles of biofilm and planktonic communities of DWDS:
    - The greatest structural and compositional changes are observed after longer times of IWS.
    - Bacterial communities are more affected than fungi, that show more resistance to IWS events.
    - Changes in planktonic communities observed after the different IWS are associated to the biofilm mobilisation when the water supply is restarted.
    - Different potentially pathogenic microbial species and/or detrimental to water quality were favoured and increased its relative abundance in biofilm samples after IWS, especially when the IWS time is longer. These include *Flavobacterium* after 6 hours of IWS, *Ochroconis* and *Penicillium* after 48 hours of IWS, and *Mycobacterium*, *Sphingomonas*, *Ochroconis* and *Cladosporium* after 6 days of IWS.

# Chapter 7

# Final discussion and conclusions

## 7.1 Overall discussion

The overall research aim of this thesis was to investigate how different processes associated to climate change may affect the microbial ecology and water quality in DWDS. Water engineering and advanced molecular techniques were used during the development of this research to achieve this aim. The experimental studies were carried out using a unique full-scale DWDS facility at the University of Sheffield in order to ensure controlled yet fully representative conditions of real DWDS. The microbial ecology of both planktonic and biofilm communities was studied under the different experimental condition applying advanced methodological techniques such as next generation sequencing and q-PCR. This, together with the characterisation of key water physico-chemical parameters, provided a novel knowledge and to get a better understanding about the effect of different climate change scenarios to which DWDS are susceptible.

Results from this research showed that the investigated processes associated to climate change are able to modify environmental conditions and therefore microbial communities of DWDS. It has been observed that under different climate change scenarios, the physicochemical factors of water quality can be affected, for example chlorine concentration, whose concentration decreased slightly when the temperature increased from 16 °C to 24 °C. However, the most important changes observed in this study were related to microbial communities and their mobilisation during flushing events at different temperatures, after different management strategies or after re-starting the water supply after different times of IWS.

Temperature has been observed to be a determinant abiotic factor that can affect and produce changes in the DWDS microbiome and affects the quality of drinking water over different management strategies or IWS times. The link between microbial growth and temperature has been previously suggested, observing changes between seasonal fluctuations (Hallam *et al.*, 2001; Delpla *et al.*, 2009), and in marine aquatic systems a greater development of biofilm was observed at warmer temperatures (Kent, García and Martiny, 2018). Accordingly, in this research it was

observed that higher temperatures promoted a greater biofilm development on HDPE pipe walls. As a consequence of this, higher turbidity response and Fe and Mn concentrations in the water were observed when the material attached to the pipe walls was mobilised. These results confirmed that the greater number of complaints about discolouration reported at warmer temperatures (Horsley *et al*., 1998; Cook, Husband and Boxall, 2016) are related to the microbial communities behaviour when the temperature increases. The study of the temperature rise together with different biofilm control strategies showed results in the same direction. Temperature was the key factor in the development of the biofilm and the turbidity response of the water regardless of the management strategy applied. However, this study also showed that if no biofilm management strategy is applied, the risk of water discoloration increases. This is in agreement with other authors who concluded that the application of management strategies such as the flushings are necessary tools for cleaning and controlling biofilms in DWDS to minimise the water discoloration risk (Vreeburg *et al.*, 2008; Cook and Boxall, 2011). Furthermore, this study has shown for the first time that the flushing strategy is also effective reducing the discolouration risk if the temperature of the system increases. However, the application of the chemical treatment of hyperchlorination together with flushing did not generate changes regarding the risk of discoloration. The use of chlorine as a disinfectant in drinking water is effective for the control of planktonic communities in the water column, but biofilms are more resistant and tolerate higher concentrations (Bridier *et al.*, 2011). According to this, results seem to indicate that in a temperature increase scenario, the hyperchlorination after a flushing event does not affect the biofilm re-growth capacity and/or material accumulation in terms of quantity, and thus do not decrease the discolouration risk. In the same way, the mobilisation of the biofilm adhered to the internal pipe walls was the main cause of the changes in the water quality after the supply restarting after different times of IWS. In agreement to previous observations (Tokajian and Hashwa, 2003; Cerrato *et al.*, 2006), IWS events have been observed as a key process increasing the biofilm detachment and therefore the amount of materials suspended in the water. Result showed that the material mobilisation after IWS was related to the increased flow and the consequent increase in shear stress (Husband *et al.*, 2016), while the increase in pressure had no effect on biofilm detachment. Moreover, as it happened with the temperature, the different interruption periods of the system were key in determining the behavior of the biofilm, and therefore the risk of discoloration. After 6 days and 48 hours of interruption, higher turbidity response and total cell counts in the water were found when the supply was re-started than after 6 hours. These results seem to indicate that longer times of IWS lead to a potential greater biofilm

development attached the pipe walls during the interruption periods. This greater growth could be associated with the absence hydraulic dynamics of the system (Simões and Simões, 2013). However, turbidity response when the supply was restarted after 48 hours showed higher values than after 6 days, until the last stage of the water supply restarting, when a very high flow was applied and turbidity response after 6 days increased reaching values similar to 48 hours. It was suggested that the long drainage period could lead to a drying process of the biofilm. It has been observed that when the biofilm dries, its structure becomes more compact (Melo, 2005) and thus could be more resistant to be detach from the pipe walls. Consequently, after long IWS times a higher flow (i.e., greater shear stress) would be necessary to detach the biofilm that was formed during the supply interruption period. Summarising, all these results obtained in this research agree that the mobilization of biofilm communities is closely related to the process of water discolouration (Husband *et al.*, 2016). In addition, it has been demonstrated that the different processes related to climate change that have been studied can have an impact on the biofilm and its mobilisation, thus affecting the risk of discoloration and therefore the supply of good quality water.

In addition to the influence of the different scenarios in the discolouration process, this research has provided new valuable information about the microbial ecology of these systems. The results have shown that the structure and composition of the microbial communities are affected by the different climate change processes. In general, it has been observed that the different experimentation scenarios mainly affect the biofilm communities, while planktonic communities are more resistant to changes in abiotic factors. This can be explained because the disinfection processes are directed and are more effective in the control of planktonic communities (Bridier *et al.*, 2011), and therefore are less likely to be modified by other factors. Temperature has been shown to be a driving parameter changing the biofilm microbial community structure and composition in chlorinated DWDS. Moreover, these changes due temperature increase have been shown to depend on the biofilm control strategy applied in the system. Similarly, biofilm communities showed different structures after different IWS times. Within biofilms, it was possible to observe differences in the behavior of bacterial and fungal communities. Changes in structure and composition were especially notable for bacteria, while fungi were fairly much constant irrespective of different conditions. Temperature increase produced significant changes in structure of biofilm bacterial. It was also possible to verify that depending on the management strategy applied in the system, this increase in temperature affects these communities differently. Similarity, IWS events affected

bacteria in biofilm communities, and result showed that longer IWS times produced bigger changes in the structure of bacteria of these communities. It has been widely demonstrated that biofilm bacterial communities are usually affected by changes in abiotic factors, including pipe material, concentration of disinfectant, nutrient availability or hydraulic regimes (Douterelo, Sharpe and Boxall, 2013; Mi *et al.*, 2015; Ren *et al.*, 2015). Accordingly, the results obtained from this research show that bacterial communities are also susceptible to being affected by changes in conditions produced by processes related to climate change. Nevertheless, fungal communities of the biofilm showed a different behaviour to the different processes studied. Temperature increase was key shaping the composition of fungal communities, greatly reducing the diversity of fungal species. However, fungi were fairly much constant to the changes produced by the different management strategies or the different times of IWS. Different studies have shown that fungi have a great capacity for adaptation and therefore are more resistant to changes in the environment and disinfection methods (Hageskal *et al.*, 2012; Fish and Boxall, 2018). This is possibly because fungi are morphologically more complex and robust than bacteria (Begon and Fitter, 1995; Denham, Wambaugh and Brown, 2019). Therefore, this research has shown that temperature is one of the most important factors that can impact the biofilm fungal communities in chlorinated DWDS. Regarding taxonomical profiles, the different experiments of this research showed species of bacteria and fungi that almost always appear as part of the microbiome of the system, independently of the climate change scenario. These included some bacteria like *Pseudomonas*, *Sphingomonas*, *Sphingobium*, *Nevskia*, or *Phreatobacter*, and fungi like *Aspergillus*, *Fusarium*, *Cladosporium*, or *Exophiala*. All these species of microorganisms have been previously observed as part of the microbiome of different DWDS (Douterelo *et al.*, 2016; Douterelo, Fish and Boxall, 2018; Van Assche *et al.*, 2019; Liu *et al.*, 2020). In addition, this and support observations from previous studies that suggested the presence of a core microbial community in DWDS regardless of the environmental conditions (Douterelo *et al.*, 2020; Del Olmo *et al.*, 2021). Furthermore, it has been observed how different processes of climate change can favor some microbial species. These microorganisms are able to adapt to changing conditions and become dominant in the community, displacing others that are decreasing in relative abundance. For example, temperature has been shown to favor an increase in the relative abundance of *Pseudomonas*, *Sphingomonas* or *Fusarium,* which are producers of EPS, thus promoting the greater development of the biofilm that has been observed at high temperature (Flemming, 2002; Irie *et al.*, 2012; Navarro-Noya *et al.*, 2013). Contrary, it has been observed that the application of flushing as a management strategy reduce

the abundance of microorganisms that can compromise water quality and safety such as the bacteria *Flavobacterium, Sphingobium* or *Methyloversatillis* (Narciso-da-Rocha, Vaz-Moreira and Manaia, 2014; Waśkiewicz and Irzykowska, 2014). Finally, IWS events also produced changes in the microbial community composition. It was observed that potentially pathogenic microbial species and/or detrimental to water quality were favoured and increased its relative abundance in biofilm samples after IWS, especially when the supply interruption was longer. These include *Flavobacterium* after 6 hours of IWS, *Ochroconis* and *Penicillium* after 48 hours of IWS, and *Mycobacterium*, *Sphingomonas*, *Ochroconis* and *Cladosporium* after 6 days of IWS.

In summary, this research has demonstrated that the different climate change processes studied can alter the microbial communities and water quality of chlorinated DWDS. This can pose an economic and technical challenge for water utilities to maintain the supply of good quality and safe drinking water. In addition, all these results reinforce the importance of including the study of microbial communities, and especially those of the biofilm, to characterise and address problems in DWDS.

## 7.2 Conclusions

The first specific aim was to determine the effect of temperature increase on the microbial ecology and water quality of chlorinated DWDS (Chapter 4). For this, microbial communities and water physico-chemical properties were characterised at two different temperatures, 16 °C and 24 °C. Results from this research showed that temperature is a key factor for bacterial and fungal communities, affecting their structure and/or composition in water but mainly in biofilms of these systems. Differences in alpha- and beta-diversity reflects that under different temperatures the ecological processes structuring the microbial communities in this chlorinated DWDS are different. In biofilm, bacterial genera such as *Pseudomonas* or *Sphingomonas* and fungi such as *Fusarium* are the most favoured by temperature, promoting the greatest development of biofilms and compromising the water safety. However, temperature has a minor effect for planktonic communities, which shows the importance of biofilms in DWDS. In addition, the study of the occurrence of OPs revealed that temperature rise leads to an increase of *Mycobacterium spp*. in biofilms and favour the presence of *M. avium* complex in water and biofilm of this system, and thus these microorganisms could compromise the security of the water that is supplied. The impacts were not only microbial, with physical mobilisation showing higher discolouration response and

metals release (Fe and Mn) due to the increased temperature. This research has provided an important knowledge about how temperature can shape the structure and composition of microbial communities in DWDS and its implication in water quality parameters.

The second specific objective of this thesis was to evaluate the impact of different biofilm control strategies on the microbiome and water quality when temperature increases in chlorinated DWDS (Chapter 5). The different management strategies compared were flushing of pipes followed by hyperchlorination, only flushing and temperature transition without mechanical or chemical treatment. Results from this research reinforced that temperature is a determining factor for DWDS microbiome and especially for biofilms. It has been shown how the different management strategies contribute to shaping bacterial communities when temperature increases. Flushing combined or not with hyperchlorination was effective at reducing the abundance of microorganisms that can compromise water quality and safety such as the bacteria *Flavobacterium, Sphingobium* or *Methyloversatilis.* However, fungal communities showed a different behavior. Although they were affected by the temperature rise, increasing the relative abundance of genera such us *Fusarium* and *Cladosporium,* no effects were observed between the different management strategies applied. In addition, this showed that the absence of mechanical and/or chemical treatment when the temperature increased was related to greater water turbidity response during the flushing events. Therefore, it has been demonstrated that the application of a biofilm management strategy is vital to reduce water discolouration events under a scenario of increased temperature. This has provided new knowledge and valuable information about the combined effect of temperature with biofilm control strategies that can be used to adapt and/or improve the management of DWDS to the current climate change, and thus helping to ensure the supply of safe drinking water to consumers.

Finally, the third objective studied was to understand the impact of different intermittent water supply times in the microbiological ecology and water quality of chlorinated DWDS (6 hours, 48 hours, and 6 days) (Chapter 6). The application of this practice is increasingly common due to the increase in water stress as a result of climate change. This study showed that several physico-chemical water quality parameters were affected by different IWS times. The shortest period of interruption was related with higher proportion of aromatic organic compounds in the bulk water. However, measurements of water turbidity and metal release reported higher water discolouration risk after longer IWS times when the water supply was re-started. In the same way, longer IWS times were associated to a higher concentration of total cells in the bulk water after restarting the supply.

These results indicated that longer IWS lead to a greater biofilm development in the pipe walls. However, the higher shear stress required to remove the biofilm from the pipe walls after longer IWS times suggested that biofilm could also experience a drying process after extremely long IWS times, making its structure change and becoming more difficult to remove from the pipe wall. Another important finding from this research was that raising pressure did not mobilise material after any period of intermittence, it was observed that mobilisation was always due to the change in flow/shear stress. Microbial analysis showed that the microbiome of these systems was affected by the different IWS times. Bacteria in of both biofilm and planktonic communities showed different structures after the water supply restarting depending on the IWS time. However, it has been shown that the structure of fungal communities was less affected by IWS events. In addition, results have demonstrated that IWS events are able to change the taxonomical composition of biofilm and planktonic communities of DWDS. In biofilms, the biggest compositional changes were observed after longer periods of IWS. Bacterial genera such as *Flavobacterium* after 6 hours of IWS, *Aquabacterium* after 48 hour or *Mycobacterium* after 6 days increased its relative abundance in biofilm samples. For fungi, it was observed that longer IWS leaded to increase the relative abundance of genera that contains human pathogenic species such as *Ochroconis Cladosporium* or *Penicillium*. Planktonic communities after restarting the water supply IWS also showed changes after the different times, *Phreatobacter* for example was the dominant genus after short times, while *Sphingomonas* dominated after 6 days of IWS. This study has made it possible to get new knowledge about the effects of IWS in DWDS. Importantly, and for the first time, this research has demonstrated that the duration of IWS events influences water quality and microbial safety, although these results can also be applied to any interruption to supply. This will help improve the management of this practice, which is increasingly common worldwide, and to minimise the associated risk to ensure the delivery safe water and protect public health.

In combination, this thesis has provided novel and valuable information and it has improved the understanding of how different processes in relation to climate change can modify DWDS conditions and affect drinking water quality and safety. The three investigations presented here highlighted the necessity of including biofilm monitoring and microbial culture-independent methods to generate more accurate microbial information and get an extensive view of what happens in the pipeline environment. Such research into the microbiome in DWDS is essential to understand and

hence to mitigate risks and adapt this fundamental transport infrastructure to prevent the effects of unavoidable climate change and therefore protect the quality and safety of drinking water.

## 7.3 Future research

Taking into account the outputs from this thesis, new research lines of research can be proposed with new experiments to in order to have a more complete and accurate knowledge on the effects of climate change on DWDS. Regarding temperature increase, on this research, two different temperatures were selected, 16 °C and 24 °C, based on the temperature of the real systems in the UK, and the predictions about the increase in the earth's temperature (Bates *et al.*, 2008; Husband, Boxall and Saul, 2008; WHO, 2017). This has been of great value in observing that this abiotic factor has a direct effect on microbial communities and water quality. However, several studies have demonstrated the transition of microbial taxa across temperature gradients in different environments (Brock *et al.*, 2003; Everroad *et al.*, 2012). Therefore, it would be of great interest to carry out a similar experiment but establishing a gradual change in temperature over time in the test loop facility, from colder temperatures typical of the winter-autumn months, to higher temperatures that are typical of other locations such as the Mediterranean area (Mesquita *et al.*, 2013; Del Olmo *et al.*, 2021). This would provide a comprehensive understanding of how the microbial communities of DWDS adapt to changes in environmental temperature. Likewise, since biofilms are in continuous development and it has been showed that matures biofilms in DWDS needed years to be established (Martiny *et al.*, 2003), increasing the biofilm growth phase to more than 30 days would provide a broader view on temporal dynamics and temperature. In regard to the temperature increase and biofilm control strategies, a similar study could be suggested focused on other biofilm management techniques to which real DWDS are also subjected. This includes mainly mechanical techniques, such as air scouring and pigging (Vreeburg, 2007; Vreeburg and Boxall, 2007). This knowledge would increase the information on the different techniques and will to design effective management strategies aimed at minimising the water quality risks associated with climate change and microbial presence.

Concerning IWS, the research carried out in this thesis has demonstrated for the first time that different IWS produce different effects on the microbiota and water quality of chlorinated DWDS. However, as there is limited information in the literature on this, further investigation based on these results is necessary to keep moving forward and obtain a complete understanding of what

happens in the pipes environment during IWS events. First of all, it would be important to know how these events and the different times affect systems with different characteristics. For example, it would be interesting in non-chlorinated or chloraminated systems since the results showed changes in measures of aromatic content, which could react with disinfectants leading to DBPs. Moreover, in this first experimental study about IWS, a single intermittent supply cycle has been studied. However, real systems where IWS is applied typically can experience continuous supply and interruption cycles. The extension of this study using several cycles of IWS will contribute with new information on the drying and rehydration processes of the biofilm during the cycles, its consequences on material detachment during continuous supply re-starts, etc. In addition, results from this research showed that different times of IWS affect the bacterial and fungal communities that remains attached to the pipe walls, so it is necessary to see how these different biofilm communities would affect the biofilm regrowth dynamics during the IWS cycles. Finally, one of the consequences of IWS events is the decrease in pressure in the pipes, which has been shown to be related to the intrusion of surrounding environmental contaminants via leaking pipes (Lee and Schwab, 2005; Vairavamoorthy *et al.*, 2007). Therefore, further investigation incorporating the intrusion of contaminants will help to understand another of the risks derived from the practice of IWS. For this, the use of new facilities is proposed, for example The National Distributed Water Infrastructure Facility at The Integrated Civil and Infrastructure Research Centre (ICAIR) (Sheffield, UK), which allows to explore the interaction of pipes in their buried environment (icair.ac.uk).

Finally, future studies should expand on the findings from this research for example applying other molecular techniques for the study and characterization of microbial communities, for example, the use of whole metagenome sequencing. The study of the entire microbial genome has the advantage of identifying genes not previously described, including those related to new organisms and functions (Hugenholtz, Goebel and Pace, 1998; Mutlu and Güven, 2015). With this molecular tool we therefore can start to provide answers not only to “who is there?” but also “what are they able to do?” and “who is doing what?” (Kunin *et al.*, 2008; Thomas, Gilbert and Meyer, 2014). This information would allow to know the functional genetic capabilities of the microbial communities and to establish patterns of microbial behaviour under the different processes related to climate change. This will contribute to the knowledge of these systems and the design of strategies to mitigate the effects related to microorganisms and climate change. that will improve knowledge of these systems.

# Reference list


Abdel-Azeem, A. M., Abdel-Azeem, M. A., Darwish, A. G., Nafady, N. A. and Ibrahim, N. A. (2019) 'Fusarium: Biodiversity, Ecological Significances, and Industrial Applications BT - Recent Advancement in White Biotechnology Through Fungi: Volume 1: Diversity and Enzymes Perspectives', in Yadav, A. N., Mishra, S., Singh, S., and Gupta, A. (eds). Cham: Springer International Publishing, pp. 201–261. doi: 10.1007/978-3-030-10480-1_6.

Anaissie, E. J., Kuchar, R. T., Rex, J. H., Francesconi, A., Kasai, M., Müller, F.-M. C., Mario, L.-C., Summerbell, R. C., Dignani, M. C., Chanock, S. J. and Walsh, T. J. (2001) 'Fusariosis Associated with Pathogenic Fusarium Species Colonization of a Hospital Water System: A New Paradigm for the Epidemiology of Opportunistic Mold Infections', *Clinical Infectious Diseases*, 33(11), pp. 1871–1878. doi: 10.1086/324501.

Anderson, M. J. and Walsh, D. C. I. (2013) 'PERMANOVA, ANOSIM, and the Mantel test in the face of heterogeneous dispersions: What null hypothesis are you testing?', *Ecological Monographs*. John Wiley & Sons, Ltd, 83(4), pp. 557–574. doi: 10.1890/12-2010.1.

Anderson, M. W. and Schrijver, I. (2010) 'Next generation DNA sequencing and the future of genomic medicine', *Genes*. MDPI, 1(1), pp. 38–69. doi: 10.3390/genes1010038.

Andrew, S. (2010) 'A quality control tool for high throughput sequence data', *Fast QC*, 532.

Arnell, N. W. and Delaney, E. K. (2006) 'Adapting to climate change: public water supply in England and Wales', *Climatic Change*. Springer, 78(2–4), pp. 227–255.

Ashbolt, N. J. (2015) 'Microbial Contamination of Drinking Water and Human Health from Community Water Systems', *Current environmental health reports*. Springer International Publishing, 2(1), pp. 95–106. doi: 10.1007/s40572-014-0037-5.

Ashofteh, P. S., Rajaee, T. and Golfam, P. (2017) 'Assessment of Water Resources Development Projects under Conditions of Climate Change Using Efficiency Indexes (EIs)', *Water Resources Management*. Water Resources Management, 31(12), pp. 3723–3744. doi: 10.1007/s11269-017-1701-y.

Van Assche, A., Crauwels, S., De Brabanter, J., Willems, K. A. and Lievens, B. (2019) 'Characterization of the bacterial community composition in water of drinking water production and distribution systems in Flanders, Belgium', *MicrobiologyOpen*. 2018/10/14. John Wiley and Sons Inc., 8(5), pp. e00726–e00726. doi: 10.1002/mbo3.726.

Assress, H. A., Selvarajan, R., Nyoni, H., Ntushelo, K., Mamba, B. B. and Msagati, T. A. M. (2019) 'Diversity, co-occurrence and implications of fungal communities in Wastewater treatment plants', *Scientific reports*. Nature Publishing Group, 9(1), pp. 1–15.

Backhus, D. A., Picardal, F. W., Johnson, S., Knowles, T., Collins, R., Radue, A. and Kim, S. (1997) 'Soil- and surfactant-enhanced reductive dechlorination of carbon tetrachloride in the presence of Shewanella putrefaciens 200', *Journal of contaminant hydrology*. Elsevier, 28(4), pp. 337–361.

Badotti, F., de Oliveira, F. S., Garcia, C. F., Vaz, A. B. M., Fonseca, P. L. C., Nahum, L. A., Oliveira, G. and Góes-Neto, A. (2017) 'Effectiveness of ITS and sub-regions as DNA barcode markers for the identification of Basidiomycota (Fungi)', *BMC Microbiology*, 17(1), p. 42. doi: 10.1186/s12866-

017-0958-x.

Bain, R., Cronk, R., Wright, J., Yang, H., Slaymaker, T. and Bartram, J. (2014) 'Fecal contamination of drinking-water in low- and middle-income countries: a systematic review and meta-analysis', *PLoS medicine*. Public Library of Science, 11(5), pp. e1001644–e1001644. doi: 10.1371/journal.pmed.1001644.

Bates, B. C., Kundzewicz, Z. W., Wu, S. and Palutikof, J. P. (2008) *Climate Change and Water:IPCC Technical Paper VI*, *Climate change and water*. doi: 10.1016/j.jmb.2010.08.039.

Batté, M., Appenzeller, B. M. R., Grandjean, D., Fass, S., Gauthier, V., Jorand, F., Mathieu, L., Boualam, M., Saby, S. and Block, J. C. (2003) 'Biofilms in drinking water distribution systems', *Reviews in Environmental Science and Biotechnology*, 2(2–4), pp. 147–168. doi: 10.1023/B:RESB.0000040456.71537.29.

Bédard, E., Fey, S., Charron, D., Lalancette, C., Cantin, P., Dolcé, P., Laferrière, C., Déziel, E. and Prévost, M. (2015) 'Temperature diagnostic to identify high risk areas and optimize Legionella pneumophila surveillance in hot water distribution systems', *Water Research*. Pergamon, 71, pp. 244–256. doi: 10.1016/J.WATRES.2015.01.006.

Begon, M. and Fitter, A. H. (1995) *Advances in ecological research*. Academic Press.

van Bel, N., Hornstra, L. M., van der Veen, A. and Medema, G. (2019) 'Efficacy of flushing and chlorination in removing microorganisms from a pilot drinking water distribution system', *Water (Switzerland)*, 11(5), pp. 1–18. doi: 10.3390/w11050903.

Benedict, K. M., Reses, H., Vigar, M., Roth, D. M., Roberts, V. A., Mattioli, M., Cooley, L. A., Hilborn, E. D., Wade, T. J., Fullerton, K. E., Yoder, J. S. and Hill, V. R. (2017) 'Surveillance for Waterborne Disease Outbreaks Associated with Drinking Water - United States, 2013-2014', *MMWR. Morbidity and mortality weekly report*. Centers for Disease Control and Prevention, 66(44), pp. 1216–1221. doi: 10.15585/mmwr.mm6644a3.

Berbel, J. and Esteban, E. (2019) 'Droughts as a catalyst for water policy change. Analysis of Spain, Australia (MDB), and California', *Global Environmental Change*. Pergamon, 58, p. 101969. doi: 10.1016/J.GLOENVCHA.2019.101969.

Berry, D., Xi, C. and Raskin, L. (2006) 'Microbial ecology of drinking water distribution systems', *Current Opinion in Biotechnology*, 17(3), pp. 297–302. doi: 10.1016/j.copbio.2006.05.007.

Bertelli, C., Courtois, S., Rosikiewicz, M., Piriou, P., Aeby, S., Robert, S., Loret, J. F. and Greub, G. (2018) 'Reduced chlorine in drinking water distribution systems impacts bacterial biodiversity in biofilms', *Frontiers in Microbiology*, 9(OCT), pp. 1–11. doi: 10.3389/fmicb.2018.02520.

Biosystems, A. (2010) 'StepOne and StepOnePlus real-time PCR systems reagent guide manual'. Applied Biosystems Foster City, CA.

Biosystems, A. (2015) 'Real-time PCR handbook', *Life Thecnologies: Carlsbad, CA, USA*.

Bivins, A. W., Sumner, T., Kumpel, E., Howard, G., Cumming, O., Ross, I., Nelson, K. and Brown, J. (2017) 'Estimating Infection Risks and the Global Burden of Diarrheal Disease Attributable to Intermittent Water Supply Using QMRA', *Environmental Science & Technology*. American Chemical Society, 51(13), pp. 7542–7551. doi: 10.1021/acs.est.7b01014.

Blaalid, R., Kumar, S., Nilsson, R. H., Abarenkov, K., Kirk, P. M. and Kauserud, H. (2013) 'ITS 1 versus ITS 2 as DNA metabarcodes for fungi', *Molecular ecology resources*. Wiley Online Library, 13(2),

pp. 218–224.

Blaxter, M., Mann, J., Chapman, T., Thomas, F., Whitton, C., Floyd, R. and Abebe, E. (2005) 'Defining operational taxonomic units using DNA barcode data', *Philosophical Transactions of the Royal Society B: Biological Sciences*. The Royal Society London, 360(1462), pp. 1935–1943.

Blokker, E. J. M. and Schaap, P. G. (2015) 'Temperature Influences Discolouration risk', *Procedia Engineering*. Elsevier, 119, pp. 280–289. doi: 10.1016/J.PROENG.2015.08.887.

Blokker, M., Vreeburg, J. and Speight, V. (2014) 'Residual chlorine in the extremities of the drinking water distribution system: the influence of stochastic water demands', *Procedia Engineering*. Elsevier, 70, pp. 172–180.

Bokulich, N. A., Kaehler, B. D., Rideout, J. R., Dillon, M., Bolyen, E., Knight, R., Huttley, G. A. and Gregory Caporaso, J. (2018) 'Optimizing taxonomic classification of marker-gene amplicon sequences with QIIME 2's q2-feature-classifier plugin', *Microbiome*, 6(1), p. 90. doi: 10.1186/s40168-018-0470-z.

Bolyen, E., Rideout, J. R., Dillon, M. R., Bokulich, N. A., Abnet, C. C., Al-Ghalith, G. A., Alexander, H., Alm, E. J., Arumugam, M., Asnicar, F., Bai, Y., Bisanz, J. E., Bittinger, K., Brejnrod, A., Brislawn, C. J., Brown, C. T., Callahan, B. J., Caraballo-Rodríguez, A. M., Chase, J., Cope, E. K., Da Silva, R., Diener, C., Dorrestein, P. C., Douglas, G. M., Durall, D. M., Duvallet, C., Edwardson, C. F., Ernst, M., Estaki, M., Fouquier, J., Gauglitz, J. M., Gibbons, S. M., Gibson, D. L., Gonzalez, A., Gorlick, K., Guo, J., Hillmann, B., Holmes, S., Holste, H., Huttenhower, C., Huttley, G. A., Janssen, S., Jarmusch, A. K., Jiang, L., Kaehler, B. D., Kang, K. Bin, Keefe, C. R., Keim, P., Kelley, S. T., Knights, D., Koester, I., Kosciolek, T., Kreps, J., Langille, M. G. I., Lee, J., Ley, R., Liu, Y.-X., Loftfield, E., Lozupone, C., Maher, M., Marotz, C., Martin, B. D., McDonald, D., McIver, L. J., Melnik, A. V, Metcalf, J. L., Morgan, S. C., Morton, J. T., Naimey, A. T., Navas-Molina, J. A., Nothias, L. F., Orchanian, S. B., Pearson, T., Peoples, S. L., Petras, D., Preuss, M. L., Pruesse, E., Rasmussen, L. B., Rivers, A., Robeson, M. S., Rosenthal, P., Segata, N., Shaffer, M., Shiffer, A., Sinha, R., Song, S. J., Spear, J. R., Swafford, A. D., Thompson, L. R., Torres, P. J., Trinh, P., Tripathi, A., Turnbaugh, P. J., Ul-Hasan, S., van der Hooft, J. J. J., Vargas, F., Vázquez-Baeza, Y., Vogtmann, E., von Hippel, M., Walters, W., Wan, Y., Wang, M., Warren, J., Weber, K. C., Williamson, C. H. D., Willis, A. D., Xu, Z. Z., Zaneveld, J. R., Zhang, Y., Zhu, Q., Knight, R. and Caporaso, J. G. (2019) 'Reproducible, interactive, scalable and extensible microbiome data science using QIIME 2', *Nature Biotechnology*, 37(8), pp. 852–857. doi: 10.1038/s41587-019-0209-9.

Bondank, E. N., Chester, M. V. and Ruddell, B. L. (2018) 'Water Distribution System Failure Risks with Increasing Temperatures', *Environmental Science and Technology*. American Chemical Society, 52(17), pp. 9605–9614. doi: 10.1021/acs.est.7b01591.

Boxall, J. B., Skipworth, P. J. and Saul, A. J. (2001) 'A novel approach to modelling sediment movement in distribution mains based on particle characteristics', *Water software systems*, 1, pp. 263–273.

Bremer, P. J., Monk, I. and Butler, R. (2002) 'Inactivation of Listeria monocytogenes/Flavobacterium spp. biofilms using chlorine: impact of substrate, pH, time and concentration', *Letters in applied microbiology*. Wiley Online Library, 35(4), pp. 321–325.

Bridier, A., Briandet, R., Thomas, V. and Dubois-Brissonnet, F. (2011) 'Resistance of bacterial biofilms to disinfectants: a review', *Biofouling*. Taylor & Francis, 27(9), pp. 1017–1032.

Brock, T. D., Madigan, M. T., Martinko, J. M. and Parker, J. (2003) *Brock biology of microorganisms*.

Upper Saddle River (NJ): Prentice-Hall, 2003.

Brooke, J. S. (2012) 'Stenotrophomonas maltophilia: an emerging global opportunistic pathogen', *Clinical microbiology reviews*. American Society for Microbiology, 25(1), pp. 2–41. doi: 10.1128/CMR.00019-11.

Bruno, A., Sandionigi, A., Bernasconi, M., Panio, A., Labra, M. and Casiraghi, M. (2018) 'Changes in the Drinking Water Microbiome: Effects of Water Treatments Along the Flow of Two Drinking Water Treatment Plants in a Urbanized Area, Milan (Italy)', *Frontiers in Microbiology*, 9(October), pp. 1–12. doi: 10.3389/fmicb.2018.02557.

Buse, H. Y., Lu, J., Struewing, I. T. and Ashbolt, N. J. (2013) 'Eukaryotic diversity in premise drinking water using 18S rDNA sequencing: implications for health risks', *Environmental Science and Pollution Research*, 20(9), pp. 6351–6366. doi: 10.1007/s11356-013-1646-5.

Buss, H. L., Brantley, S. L. and Liermann, L. J. (2003) 'Nondestructive methods for removal of bacteria from silicate surfaces', *Geomicrobiology Journal*. Taylor & Francis, 20(1), pp. 25–42.

Cabral, J. P. S. (2010) 'Water microbiology. Bacterial pathogens and water', *International journal of environmental research and public health*. 2010/10/15. Molecular Diversity Preservation International (MDPI), 7(10), pp. 3657–3703. doi: 10.3390/ijerph7103657.

Calero Preciado, C., Boxall, J., Soria-Carrasco, V. and Douterelo, I. (2019) 'Effect of temperature increase in bacterial and fungal communities of chlorinated drinking water distribution systems', *Access Microbiology*. Microbiology Society, 1(1A), p. 506. doi: 10.1099/acmi.ac2019.po0304.

Cao, K., Xiang, J., Dong, Y.-T., Xu, Y., Li, Y., Song, H., Zeng, X.-X., Ran, L.-Y., Hong, W. and Guan, Z.-Z. (2019) 'Exposure to fluoride aggravates the impairment in learning and memory and neuropathological lesions in mice carrying the APP/PS1 double-transgenic mutation', *Alzheimer's Research & Therapy*, 11(1), p. 35. doi: 10.1186/s13195-019-0490-3.

Caporaso, J. G., Lauber, C. L., Walters, W. A., Berg-Lyons, D., Huntley, J., Fierer, N., Owens, S. M., Betley, J., Fraser, L. and Bauer, M. (2012) 'Ultra-high-throughput microbial community analysis on the Illumina HiSeq and MiSeq platforms', *The ISME journal*. Nature Publishing Group, 6(8), pp. 1621–1624.

Carniello, V., Peterson, B. W., van der Mei, H. C. and Busscher, H. J. (2018) 'Physico-chemistry from initial bacterial adhesion to surface-programmed biofilm growth', *Advances in Colloid and Interface Science*, 261, pp. 1–14. doi: https://doi.org/10.1016/j.cis.2018.10.005.

Cerrato, J. M., Reyes, L. P., Alvarado, C. N. and Dietrich, A. M. (2006) 'Effect of PVC and iron materials on Mn(II) deposition in drinking water distribution systems', *Water Research*, 40(14), pp. 2720–2726. doi: https://doi.org/10.1016/j.watres.2006.04.035.

Chagnot, C., Zorgani, M. A., Astruc, T. and Desvaux, M. (2013) 'Proteinaceous determinants of surface colonization in bacteria: bacterial adhesion and biofilm formation from a protein secretion perspective', *Frontiers in microbiology*. Frontiers Media S.A., 4, p. 303. doi: 10.3389/fmicb.2013.00303.

Chakravorty, S., Helb, D., Burday, M., Connell, N. and Alland, D. (2007) 'A detailed analysis of 16S ribosomal RNA gene segments for the diagnosis of pathogenic bacteria', *Journal of microbiological methods*. 2007/02/22, 69(2), pp. 330–339. doi: 10.1016/j.mimet.2007.02.005.

Charalambous, B. (2012) 'The effects of intermittent supply on water distribution networks', *Water*

*Loss*.

Chaves Simões, L. and Simões, M. (2013) 'Biofilms in drinking water: problems and solutions', *RSC Advances*. The Royal Society of Chemistry, 3(8), pp. 2520–2533. doi: 10.1039/C2RA22243D.

Chen, J., Li, N., Xie, S. and Chen, C. (2018) 'Biofilm and planktonic bacterial communities in a drinking water distribution system supplied with untreated groundwater', *Archives of Microbiology*. Springer Berlin Heidelberg, 200(9), pp. 1323–1331. doi: 10.1007/s00203-018-1546-7.

Chen, L., Ling, F., Bakker, G., Liu, W.-T., Medema, G., van der Meer, W. and Liu, G. (2020) 'Assessing the transition effects in a drinking water distribution system caused by changing supply water quality: an indirect approach by characterizing suspended solids', *Water Research*, 168, p. 115159. doi: https://doi.org/10.1016/j.watres.2019.115159.

Christodoulou, S. and Agathokleous, A. (2012) 'A study on the effects of intermittent water supply on the vulnerability of urban water distribution networks', *Water Science and Technology: Water Supply*. IWA Publishing, 12(4), pp. 523–530.

Clark, D. P., Pazdernik, N. J. and McGehee, M. R. (2019) 'Chapter 8 - DNA Sequencing', in Clark, D. P., Pazdernik, N. J., and McGehee, M. R. B. T.-M. B. (Third E. (eds). Academic Cell, pp. 240–269. doi: https://doi.org/10.1016/B978-0-12-813288-3.00008-2.

Cock, P. J. A., Fields, C. J., Goto, N., Heuer, M. L. and Rice, P. M. (2009) 'The Sanger FASTQ file format for sequences with quality scores, and the Solexa/Illumina FASTQ variants', *Nucleic Acids Research*, 38(6), pp. 1767–1771. doi: 10.1093/nar/gkp1137.

Coelho, S. T., James, S., Sunna, N., Abu Jaish, A. and Chatila, J. (2003) 'Controlling water quality in intermittent supply systems', *Water Supply*, 3(1–2), pp. 119–125. doi: 10.2166/ws.2003.0094.

Coenye, T. (2014) 'The Family Burkholderiaceae', in Rosenberg, E., DeLong, E. F., Lory, S., Stackebrandt, E., and Thompson, F. (eds) *The Prokaryotes: Alphaproteobacteria and Betaproteobacteria*. Berlin, Heidelberg: Springer Berlin Heidelberg, pp. 759–776. doi: 10.1007/978-3-642-30197-1_239.

Cogo, P. E., Scagli, M., Gatti, S., Rossetti, F., Alaggio, R., Laverda, A. M., Zhou, L., Xiao, L. and Visvesvara, G. S. (2004) 'Fatal Naegleria fowleri meningoencephalitis, Italy', *Emerging infectious diseases*. Centers for Disease Control and Prevention, 10(10), pp. 1835–1837. doi: 10.3201/eid1010.040273.

Collins, R., Boxall, J., Besner, M.-C., Beck, S. and Karney, B. (2010) 'Intrusion modelling and the effect of ground water conditions', in *Water Distribution Systems Analysis 2010*, pp. 585–594.

Cook, D. M., Husband, P. S. and Boxall, J. B. (2016) 'Operational management of trunk main discolouration risk', *Urban Water Journal*. Taylor & Francis, 13(4), pp. 382–395. doi: 10.1080/1573062X.2014.993994.

Cook, M. D. and Boxall, J. B. (2011) 'Discoloration Material Accumulation in Water Distribution Systems', *Journal of Pipeline Systems Engineering and Practice*. American Society of Civil Engineers, 2(4), pp. 113–122. doi: 10.1061/(ASCE)PS.1949-1204.0000083.

Cotruvo, J., Craun, G. F. and Hearne, N. (2019) *Providing safe drinking water in small systems: Technology, operations, and economics*. Routledge.

Council, N. R. (2000) *Hormonally active agents in the environment*. National Academies Press.

Counter, S. A. and Buchanan, L. H. (2004) 'Mercury exposure in children: a review', *Toxicology and*

*applied pharmacology*. Elsevier, 198(2), pp. 209–230.

Cowle, M. W., Babatunde, A. O., Rauen, W. B., Bockelmann-Evans, B. N. and Barton, A. F. (2014) 'Biofilm development in water distribution and drainage systems: dynamics and implications for hydraulic efficiency', *Environmental Technology Reviews*. Taylor & Francis, 3(1), pp. 31–47.

Dai, D., Rhoads, W. J., Edwards, M. A. and Pruden, A. (2018) 'Shotgun Metagenomics Reveals Taxonomic and Functional Shifts in Hot water microbiome due to temperature setting and stagnation', *Frontiers in Microbiology*, 9(NOV), pp. 1–17. doi: 10.3389/fmicb.2018.02695.

Davey, M. E. and O'toole, G. A. (2000) 'Microbial biofilms: from ecology to molecular genetics', *Microbiology and molecular biology reviews : MMBR*. American Society for Microbiology, 64(4), pp. 847–867. doi: 10.1128/mmbr.64.4.847-867.2000.

Davis, N. M., Proctor, D. M., Holmes, S. P., Relman, D. A. and Callahan, B. J. (2018) 'Simple statistical identification and removal of contaminant sequences in marker-gene and metagenomics data', *Microbiome*, 6(1), p. 226. doi: 10.1186/s40168-018-0605-2.

Deines, P., Sekar, R., Husband, P. S., Boxall, J. B., Osborn, A. M. and Biggs, C. A. (2010) 'A new coupon design for simultaneous analysis of in situ microbial biofilm formation and community structure in drinking water distribution systems', *Applied Microbiology and Biotechnology*, 87(2), pp. 749–756. doi: 10.1007/s00253-010-2510-x.

Delaunois, F., Tosar, F. and Vitry, V. (2014) 'Corrosion behaviour and biocorrosion of galvanized steel water distribution systems', *Bioelectrochemistry*, 97, pp. 110–119. doi: https://doi.org/10.1016/j.bioelechem.2014.01.003.

Delpla, I., Jung, A. V., Baures, E., Clement, M. and Thomas, O. (2009) 'Impacts of climate change on surface water quality in relation to drinking water production', *Environment International*. Elsevier Ltd, 35(8), pp. 1225–1233. doi: 10.1016/j.envint.2009.07.001.

Denham, S. T., Wambaugh, M. A. and Brown, J. C. S. (2019) 'How Environmental Fungi Cause a Range of Clinical Outcomes in Susceptible Hosts', *Journal of Molecular Biology*, 431(16), pp. 2982–3009. doi: https://doi.org/10.1016/j.jmb.2019.05.003.

Dinh Chau, N., Dulinski, M., Jodlowski, P., Nowak, J., Rozanski, K., Sleziak, M. and Wachniew, P. (2011) 'Natural radioactivity in groundwater–a review', *Isotopes in Environmental and Health Studies*. Taylor & Francis, 47(4), pp. 415–437.

Dobrowsky, P. H., Khan, S., Cloete, T. E. and Khan, W. (2016) 'Molecular detection of Acanthamoeba spp., Naegleria fowleri and Vermamoeba (Hartmannella) vermiformis as vectors for Legionella spp. in untreated and solar pasteurized harvested rainwater', *Parasites & vectors*. BioMed Central, 9(1), p. 539. doi: 10.1186/s13071-016-1829-2.

Donnermair, M. M. and Blatchley, E. R. (2003) 'Disinfection efficacy of organic chloramines', *Water Research*, 37(7), pp. 1557–1570. doi: 10.1016/S0043-1354(02)00522-5.

Douterelo, I., Boxall, J. B., Deines, P., Sekar, R., Fish, K. E. and Biggs, C. A. (2014) 'Methodological approaches for studying the microbial ecology of drinking water distribution systems', *Water Research*. Elsevier Ltd, 65, pp. 134–156. doi: 10.1016/j.watres.2014.07.008.

Douterelo, I., Dutilh, B. E., Arkhipova, K., Calero, C. and Husband, S. (2020) 'Microbial diversity, ecological networks and functional traits associated to materials used in drinking water distribution systems', *Water Research*, 173, p. 115586. doi:

https://doi.org/10.1016/j.watres.2020.115586.

Douterelo, I., Fish, K. E. and Boxall, J. B. (2018) 'Succession of bacterial and fungal communities within biofilms of a chlorinated drinking water distribution system', *Water Research*. Elsevier Ltd, 141, pp. 74–85. doi: 10.1016/j.watres.2018.04.058.

Douterelo, I., Husband, S. and Boxall, J. B. (2014) 'The bacteriological composition of biomass recovered by flushing an operational drinking water distribution system', *Water Research*. Elsevier Ltd, 54, pp. 100–114. doi: 10.1016/j.watres.2014.01.049.

Douterelo, I, Husband, S., Loza, V. and Boxall, J. (2016) 'Dynamics of Biofilm Regrowth in Drinking Water Distribution Systems', *Applied and environmental microbiology*. American Society for Microbiology, 82(14), pp. 4155–4168. doi: 10.1128/AEM.00109-16.

Douterelo, Isabel, Jackson, M., Solomon, C. and Boxall, J. (2016) 'Microbial analysis of in situ biofilm formation in drinking water distribution systems: implications for monitoring and control of drinking water quality', *Applied Microbiology and Biotechnology*, 100(7), pp. 3301–3311. doi: 10.1007/s00253-015-7155-3.

Douterelo, I., Jackson, M., Solomon, C. and Boxall, J. (2017) 'Spatial and temporal analogies in microbial communities in natural drinking water biofilms', *Science of the Total Environment*. Elsevier B.V., 581–582, pp. 277–288. doi: 10.1016/j.scitotenv.2016.12.118.

Douterelo, I., Sharpe, R. and Boxall, J. (2014) 'Bacterial community dynamics during the early stages of biofilm formation in a chlorinated experimental drinking water distribution system: Implications for drinking water discolouration', *Journal of Applied Microbiology*, 117(1), pp. 286–301. doi: 10.1111/jam.12516.

Douterelo, I., Sharpe, R. L. and Boxall, J. B. (2013) 'Influence of hydraulic regimes on bacterial community structure and composition in an experimental drinking water distribution system', *Water Research*. Elsevier Ltd, 47(2), pp. 503–516. doi: 10.1016/j.watres.2012.09.053.

Douterelo, I., Sharpe, R. L., Husband, S., Fish, K. E. and Boxall, J. B. (2019) 'Understanding microbial ecology to improve management of drinking water distribution systems', *Wiley Interdisciplinary Reviews: Water*, 6(1), p. e01325. doi: 10.1002/wat2.1325.

Dukan, S., Levi, Y., Piriou, P., Guyon, F. and Villon, P. (1996) 'Dynamic modelling of bacterial growth in drinking water networks', *Water Research*. Pergamon, 30(9), pp. 1991–2002. doi: 10.1016/0043-1354(96)00021-8.

Eder, W., Wanner, G., Ludwig, W., Busse, H. J., Ziemke-Kägeler, F. and Lang, E. (2011) 'Description of Undibacterium oligocarboniphilum sp. nov., isolated from purified water, and Undibacterium pigrum strain CCUG 49012 as the type strain of Undibacterium parvum sp. nov., and emended descriptions of the genus Undibacterium and the species Undibacterium pigrum', *International Journal of Systematic and Evolutionary Microbiology*. Microbiology Society, 61(2), pp. 384–391. doi: 10.1099/ijs.0.018648-0.

Edgar, R. C. (2013) 'UPARSE: highly accurate OTU sequences from microbial amplicon reads', *Nature Methods*, 10(10), pp. 996–998. doi: 10.1038/nmeth.2604.

Edgar, R. C. (2014) 'Taxonomy benchmark tests, USEARCH manual v8. 1'.

EPA (2012) 'EPA drinking water guidance on disinfection by-products advice note no. 4. Version 2. Disinfection By-products in Drinking Water'.

EPA (2016) 'Basic Information about Lead in Drinking Water'.

Estaki, M., Jiang, L., Bokulich, N. A., McDonald, D., González, A., Kosciolek, T., Martino, C., Zhu, Q., Birmingham, A., Vázquez-Baeza, Y., Dillon, M. R., Bolyen, E., Caporaso, J. G. and Knight, R. (2020) 'QIIME 2 Enables Comprehensive End-to-End Analysis of Diverse Microbiome Data and Comparative Studies with Publicly Available Data', *Current Protocols in Bioinformatics*. John Wiley & Sons, Ltd, 70(1), p. e100. doi: 10.1002/cpbi.100.

EU (1998) 'Council Directive 98/83/EC of 3 November 1998 on the quality of water intended for human consumption', *Off. J. Eur. Communities L*, 330, pp. 32–54.

Everroad, R. C., Otaki, H., Matsuura, K. and Haruta, S. (2012) 'Diversification of bacterial community composition along a temperature gradient at a thermal spring', *Microbes and environments*. 2012/05/17. Japanese Society of Microbial Ecology/The Japanese Society of Soil Microbiology, 27(4), pp. 374–381. doi: 10.1264/jsme2.me11350.

Ewing, B., Hillier, L., Wendl, M. C. and Green, P. (1998) 'Base-calling of automated sequencer traces usingPhred. I. Accuracy assessment', *Genome research*. Cold Spring Harbor Lab, 8(3), pp. 175–185.

Farkas, A., Ciataras, D. and Brandus, B. (2012) 'Biofilms impact on drinking water quality', *Ecological Water Quality—Water Treatment and Reuse. Rijeka: InTech*, pp. 141–154.

Fawell, J. and Nieuwenhuijsen, M. J. (2003) 'Contaminants in drinking water: Environmental pollution and health', *British Medical Bulletin*, 68(1), pp. 199–208. doi: 10.1093/bmb/ldg027.

Ferranti, G., Marchesi, I., Favale, M., Borella, P. and Bargellini, A. (2014) 'Aetiology, source and prevention of waterborne healthcare-associated infections: a review', *Journal of Medical Microbiology*. Microbiology Society, 63(10), pp. 1247–1259. doi: 10.1099/jmm.0.075713-0.

Fischer, E. R., Hansen, B. T., Nair, V., Hoyt, F. H. and Dorward, D. W. (2012) 'Scanning electron microscopy', *Current protocols in microbiology*, Chapter 2, p. Unit2B.2-2B.2. doi: 10.1002/9780471729259.mc02b02s25.

Fish, K. (2013) 'The impact of hydraulic regime upon biofilms in drinking water distribution systems'. University of Sheffield.

Fish, K. E. and Boxall, J. B. (2018) 'Biofilm microbiome (re)growth dynamics in drinking water distribution systems are impacted by chlorine concentration', *Frontiers in Microbiology*, 9(OCT), pp. 1–21. doi: 10.3389/fmicb.2018.02519.

Fish, K. E., Collins, R., Green, N. H., Sharpe, R. L., Douterelo, I., Osborn, A. M. and Boxall, J. B. (2015) 'Characterisation of the physical composition and microbial community structure of biofilms within a model full-scale drinking water distribution system', *PLoS One*. Public Library of Science, 10(2), p. e0115824.

Fish, K., Osborn, A. M. and Boxall, J. B. (2017) 'Biofilm structures (EPS and bacterial communities) in drinking water distribution systems are conditioned by hydraulics and influence discolouration', *Science of the Total Environment*. The Authors, 593–594, pp. 571–580. doi: 10.1016/j.scitotenv.2017.03.176.

Fisher, I., Kastl, G. and Sathasivan, A. (2012) 'A suitable model of combined effects of temperature and initial condition on chlorine bulk decay in water distribution systems', *Water Research*. Elsevier Ltd, 46(10), pp. 3293–3303. doi: 10.1016/j.watres.2012.03.017.

Flemming, H.-C. (2002) 'Biofouling in water systems -- cases, causes and countermeasures', *Applied Microbiology and Biotechnology*, 59(6), pp. 629–640. doi: 10.1007/s00253-002-1066-9.

Flemming, H.-C. (2016) 'EPS-Then and Now', *Microorganisms*. MDPI, 4(4), p. 41. doi: 10.3390/microorganisms4040041.

Flemming, H.-C., Percival, S. L. and Walker, J. T. (2002) 'Contamination potential of biofilms in water distribution systems', *Water science and technology: water supply*. IWA Publishing, 2(1), pp. 271–280.

Flemming, H.-C. and Wingender, J. (2010) 'The biofilm matrix', *Nature Reviews Microbiology*, 8(9), pp. 623–633. doi: 10.1038/nrmicro2415.

Friedman, M., Martel, K., Hill, A., Holt, D., Smith, S., Ta, T., Sherwin, C., Hiltebrand, D., Pommerenk, P. and Hinedi, Z. (2003) *Establishing site-specific flushing velocities*. AWWA Research Foundation Denver.

Furnass, W., Douterelo, I., Collins, R., Mounce, S. and Boxall, J. (2014) 'Controlled, Realistic-scale, Experimental Study of How the Quantity and Erodibility of Discolouration Material Varies with Shear Strength', *Procedia Engineering*, 89, pp. 135–142. doi: https://doi.org/10.1016/j.proeng.2014.11.169.

Gaebler, C., Lorenzi, J. C. C., Oliveira, T. Y., Nogueira, L., Ramos, V., Lu, C.-L., Pai, J. A., Mendoza, P., Jankovic, M., Caskey, M. and Nussenzweig, M. C. (2019) 'Combination of quadruplex qPCR and next-generation sequencing for qualitative and quantitative analysis of the HIV-1 latent reservoir', *Journal of Experimental Medicine*, 216(10), pp. 2253–2264. doi: 10.1084/jem.20190896.

Gall, A. M., Mariñas, B. J., Lu, Y. and Shisler, J. L. (2015) 'Waterborne Viruses: A Barrier to Safe Drinking Water', *PLoS pathogens*. Public Library of Science, 11(6), pp. e1004867–e1004867. doi: 10.1371/journal.ppat.1004867.

Garcia-Rubio, R. and Alcazar-Fuoli, L. B. T.-R. M. in L. S. (2018) 'Diseases Caused by Aspergillus fumigatus', in. Elsevier. doi: https://doi.org/10.1016/B978-0-12-809633-8.12078-3.

Garcia de Carellan, I., Catton, P., Selcuk, C. and Gan, T.-H. (2012) 'Methods for detection and cleaning of fouling in pipelines', *Emerging Technologies in Non-Destructive Testing V*. CRC Press, p. 231.

Garza, D. R. and Dutilh, B. E. (2015) 'From cultured to uncultured genome sequences: metagenomics and modeling microbial ecosystems', *Cellular and molecular life sciences : CMLS*. 2015/08/09. Springer Basel, 72(22), pp. 4287–4308. doi: 10.1007/s00018-015-2004-1.

Gatza, E., Hammes, F. and Prest, E. (2013) 'Assessing water quality with the BD Accuri™ C6 flow cytometer', *White paper. BD Biosciences*.

Geldreich, E. E. (1996) *Microbial quality of water supply in distribution systems*. CRC Press.

Gerba, C. P. (2009) 'Chapter 25 - Drinking Water Treatment', in Maier, R. M., Pepper, I. L., and Gerba, C. P. B. T.-E. M. (Second E. (eds). San Diego: Academic Press, pp. 531–538. doi: https://doi.org/10.1016/B978-0-12-370519-8.00025-0.

Gião, M. S. and Keevil, C. W. (2013) 'Hydrodynamic shear stress to remove Listeria monocytogenes biofilms from stainless steel and polytetrafluoroethylene surfaces', *Journal of Applied Microbiology*. John Wiley & Sons, Ltd, 114(1), pp. 256–265. doi: 10.1111/jam.12032.

Gião, M. S., Wilks, S. A., Azevedo, N. F., Vieira, M. J. and Keevil, C. W. (2009) 'Comparison between

standard culture and peptide nucleic acid 16S rRNA hybridization quantification to study the influence of physico-chemical parameters on Legionella pneumophila survival in drinking water biofilms', *Biofouling*. Taylor & Francis, 25(4), pp. 335–343. doi: 10.1080/08927010902802232.

Giglio, S., Monis, P. T. and Saint, C. P. (2003) 'Demonstration of preferential binding of SYBR Green I to specific DNA fragments in real-time multiplex PCR', *Nucleic acids research*. Oxford University Press, 31(22), pp. e136–e136.

Gillespie, S., Lipphaus, P., Green, J., Parsons, S., Weir, P., Juskowiak, K., Jefferson, B., Jarvis, P. and Nocker, A. (2014) 'Assessing microbiological water quality in drinking water distribution systems with disinfectant residual using flow cytometry', *Water Research*. Pergamon, 65, pp. 224–234. doi: 10.1016/J.WATRES.2014.07.029.

Ginige, M. P., Wylie, J. and Plumb, J. (2011) 'Influence of biofilms on iron and manganese deposition in drinking water distribution systems', *Biofouling*. Taylor & Francis, 27(2), pp. 151–163. doi: 10.1080/08927014.2010.547576.

Ginzinger, D. G. (2002) 'Gene quantification using real-time quantitative PCR: An emerging technology hits the mainstream', *Experimental Hematology*, 30(6), pp. 503–512. doi: https://doi.org/10.1016/S0301-472X(02)00806-8.

Givskov, M. and Kjelleberg, S. (2007) *The biofilm mode of life: mechanisms and adaptations*. Horizon Bioscience.

Gomes, I. B., Simões, M. and Simões, L. C. (2014) 'An overview on the reactors to study drinking water biofilms', *Water Research*, 62, pp. 63–87. doi: https://doi.org/10.1016/j.watres.2014.05.039.

Gomes, T. S., Gjiknuri, J., Magnet, A., Vaccaro, L., Ollero, D., Izquierdo, F., Fenoy, S., Hurtado, C. and Del Águila, C. (2018) 'The Influence of Acanthamoeba-Legionella Interaction in the Virulence of Two Different Legionella Species', *Frontiers in microbiology*. Frontiers Media S.A., 9, p. 2962. doi: 10.3389/fmicb.2018.02962.

Gomez-Alvarez, V., Humrighouse, B. W., Revetta, R. P. and Santo Domingo, J. W. (2015) 'Bacterial composition in a metropolitan drinking water distribution system utilizing different source waters', *Journal of water and health*. IWA Publishing, 13(1), pp. 140–151.

Gonçalves, A. B., Paterson, R. R. M. and Lima, N. (2006) 'Survey and significance of filamentous fungi from tap water', *International Journal of Hygiene and Environmental Health*, 209(3), pp. 257–264. doi: https://doi.org/10.1016/j.ijheh.2005.12.001.

Gonzalez, J. M., Zimmermann, J. and Saiz-Jimenez, C. (2005) 'Evaluating putative chimeric sequences from PCR-amplified products', *Bioinformatics*. Oxford University Press, 21(3), pp. 333–337.

Goodrich, J. K., Di Rienzi, S. C., Poole, A. C., Koren, O., Walters, W. A., Caporaso, J. G., Knight, R. and Ley, R. E. (2014) 'Conducting a microbiome study', *Cell*. Elsevier, 158(2), pp. 250–262.

Goslan, E. H., Krasner, S. W., Bower, M., Rocks, S. A., Holmes, P., Levy, L. S. and Parsons, S. A. (2009) 'A comparison of disinfection by-products found in chlorinated and chloraminated drinking waters in Scotland', *Water Research*, 43(18), pp. 4698–4706. doi: https://doi.org/10.1016/j.watres.2009.07.029.

Gray, N. F. (2014) 'Filtration methods', in *Microbiology of Waterborne Diseases*. Elsevier, pp. 631–650.

Guarro, J., Antolín-Ayala, M. I., Gené, J., Gutiérrez-Calzada, J., Nieves-Díez, C. and Ortoneda, M. (1999) 'Fatal case of Trichoderma harzianum infection in a renal transplant recipient', *Journal of clinical microbiology*. American Society for Microbiology, 37(11), pp. 3751–3755.

Guha Roy, A. (2019) 'Antibiotics in water', *Nature Sustainability*, 2(5), p. 356. doi: 10.1038/s41893-019-0295-1.

Guimaraes, A. J., Gomes, K. X., Cortines, J. R., Peralta, J. M. and Peralta, R. H. S. (2016) 'Acanthamoeba spp. as a universal host for pathogenic microorganisms: One bridge from environment to host virulence', *Microbiological Research*. Urban & Fischer, 193, pp. 30–38. doi: 10.1016/J.MICRES.2016.08.001.

Habtewold, T., Duchateau, L. and Christophides, G. K. (2016) 'Flow cytometry analysis of the microbiota associated with the midguts of vector mosquitoes', *Parasites & Vectors*, 9(1), p. 167. doi: 10.1186/s13071-016-1438-0.

Hageskal, G., Lima, N. and Skaar, I. (2009) 'The study of fungi in drinking water', *Mycological research*. Elsevier, 113(2), pp. 165–172.

Hageskal, G., Tryland, I., Liltved, H. and Skaar, I. (2012) 'No simple solution to waterborne fungi: various responses to water disinfection methods', *Water Science and Technology: Water Supply*. IWA Publishing, 12(2), pp. 220–226.

Hallam, N. ., West, J. ., Forster, C. . and Simms, J. (2001) 'The potential for biofilm growth in water distribution systems', *Water Research*. Pergamon, 35(17), pp. 4063–4071. doi: 10.1016/S0043-1354(01)00248-2.

Haque, R. (2007) 'Human intestinal parasites', *Journal of health, population, and nutrition*. International Centre for Diarrhoeal Disease Research, Bangladesh, 25(4), pp. 387–391.

Haynes, M. (2008) 'Metagenomics', in Fath, B. B. T.-E. of E. (Second E. (ed.). Oxford: Elsevier, pp. 153–156. doi: https://doi.org/10.1016/B978-0-444-63768-0.00933-1.

He, X., Liu, Y.-L., Conklin, A., Westrick, J., Weavers, L. K., Dionysiou, D. D., Lenhart, J. J., Mouser, P. J., Szlag, D. and Walker, H. W. (2016) 'Toxic cyanobacteria and drinking water: Impacts, detection, and treatment', *Harmful algae*. Elsevier, 54, pp. 174–193.

Heinrichs, G., Hübner, I., Schmidt, C. K., de Hoog, G. S. and Haase, G. (2013) 'Analysis of Black Fungal Biofilms Occurring at Domestic Water Taps (II): Potential Routes of Entry', *Mycopathologia*, 175(5), pp. 399–412. doi: 10.1007/s11046-013-9619-2.

Henne, K., Kahlisch, L., Brettar, I. and Höfle, M. G. (2012) 'Analysis of structure and composition of bacterial core communities in mature drinking water biofilms and bulk water of a citywide network in Germany', *Appl. Environ. Microbiol.* Am Soc Microbiol, 78(10), pp. 3530–3538.

Hilborn, E. D., Covert, T. C., Yakrus, M. A., Harris, S. I., Donnelly, S. F., Rice, E. W., Toney, S., Bailey, S. A. and Stelma, G. N. (2006) 'Persistence of Nontuberculous Mycobacteria in a Drinking Water System after Addition of Filtration Treatment', *Applied and Environmental Microbiology*, 72(9), pp. 5864 LP – 5869. doi: 10.1128/AEM.00759-06.

Hochstrat, R., Wintgens, T. and Corvini, P. (2015) *Immobilised biocatalysts for bioremediation of groundwater and wastewater*. Iwa Publishing.

Hoffmann, R. and Michel, R. (2001) 'Distribution of free-living amoebae (FLA) during preparation and supply of drinking water', *International Journal of Hygiene and Environmental Health*. doi:

10.1078/S1438-4639(04)70031-0.

Hua, F., West, J. ., Barker, R. . and Forster, C. . (1999) 'Modelling of chlorine decay in municipal water supplies', *Water Research*. Pergamon, 33(12), pp. 2735–2746. doi: 10.1016/S0043-1354(98)00519-3.

Hugenholtz, P., Goebel, B. M. and Pace, N. R. (1998) 'Impact of culture-independent studies on the emerging phylogenetic view of bacterial diversity', *Journal of bacteriology*. Am Soc Microbiol, 180(18), pp. 4765–4774.

Husband, P. S. and Boxall, J. B. (2011a) 'Asset deterioration and discolouration in water distribution systems', *Water Research*. Pergamon, 45(1), pp. 113–124. doi: 10.1016/J.WATRES.2010.08.021.

Husband, P. S. and Boxall, J. B. (2011b) 'Asset deterioration and discolouration in water distribution systems', *Water Research*. Elsevier Ltd, 45(1), pp. 113–124. doi: 10.1016/j.watres.2010.08.021.

Husband, P. S., Boxall, J. B. and Saul, A. J. (2008) 'Laboratory studies investigating the processes leading to discolouration in water distribution networks', *Water Research*. Pergamon, 42(16), pp. 4309–4318. doi: 10.1016/J.WATRES.2008.07.026.

Husband, S. and Boxall, J. (2016) 'Understanding and managing discolouration risk in trunk mains', *Water Research*, 107, pp. 127–140. doi: https://doi.org/10.1016/j.watres.2016.10.049.

Husband, S. and Boxall, J. B. (2010) 'Field Studies of Discoloration in Water Distribution Systems: Model Verification and Practical Implications', *Journal of Environmental Engineering*. American Society of Civil Engineers, 136(1), pp. 86–94. doi: 10.1061/(ASCE)EE.1943-7870.0000115.

Husband, S., Fish, K. E., Douterelo, I. and Boxall, J. (2016) 'Linking discolouration modelling and biofilm behaviour within drinking water distribution systems', *Water Science and Technology: Water Supply*, 16(4), pp. 942–950. doi: 10.2166/ws.2016.045.

Ibrahim, N. and Aziz, H. A. (2014) 'Trends on natural organic matter in drinking water sources and its treatment', *International Journal of Scientific Research in Environmental Sciences*. International Journals of Scientific Research Publications (IJSRP), 2(3), p. 94.

Ilaya Ayza, A. E. (2016) 'Propuesta para la transición de un sistema con suministro de agua intermitente a suministro continuo'.

Illumina, I. (2015) 'An introduction to next-generation sequencing technology'.

Inkinen, J., Jayaprakash, B., Santo Domingo, J. W., Keinänen-Toivola, M. M., Ryu, H. and Pitkänen, T. (2016) 'Diversity of ribosomal 16S DNA- and RNA-based bacterial community in an office building drinking water system', *Journal of Applied Microbiology*. John Wiley & Sons, Ltd (10.1111), 120(6), pp. 1723–1738. doi: 10.1111/jam.13144.

IPCC (2014a) *Climate Change 2014: Impacts, Adaptation, and Vulnerability. Part A: Global and Sectoral Aspects. Contribution of Working Group II to the Fifth Assessment Report of the Intergovernmental Panel on Climate Change [Field, C.B., V.R. Barros, D.J. Dokken, K.J.* Cambridge, United Kingdom and New York, NY, USA: Cambridge University Press.

IPCC (2014b) *Climate Change 2014: Synthesis Report. Contribution of Working Groups I, II and III to the Fifth Assessment Report of the Intergovernmental Panel on Climate Change*, *Journal of Crystal Growth*. Edited by R. K. P. and L. A. M. (eds. ). [Core Writing Team. Geneva, Switzerland. doi: 10.1016/S0022-0248(00)00575-3.

Irie, Y., Borlee, B. R., O'Connor, J. R., Hill, P. J., Harwood, C. S., Wozniak, D. J. and Parsek, M. R. (2012)

'Self-produced exopolysaccharide is a signal that stimulates biofilm formation in Pseudomonas aeruginosa', *Proceedings of the National Academy of Sciences of the United States of America*. 2012/11/21. National Academy of Sciences, 109(50), pp. 20632–20636. doi: 10.1073/pnas.1217993109.

Ji, P., Rhoads, W. J., Edwards, M. A. and Pruden, A. (2017) 'Impact of water heater temperature setting and water use frequency on the building plumbing microbiome', *The ISME journal*. 2017/03/10. Nature Publishing Group, 11(6), pp. 1318–1330. doi: 10.1038/ismej.2017.14.

Johnsen, A. R., Hausner, M., Schnell, A. and Wuertz, S. (2000) 'Evaluation of fluorescently labeled lectins for noninvasive localization of extracellular polymeric substances in Sphingomonas biofilms', *Applied and environmental microbiology*. American Society for Microbiology, 66(8), pp. 3487–3491. doi: 10.1128/aem.66.8.3487-3491.2000.

Jones, O. A., Lester, J. N. and Voulvoulis, N. (2005) 'Pharmaceuticals: a threat to drinking water?', *Trends in Biotechnology*. Elsevier Current Trends, 23(4), pp. 163–167. doi: 10.1016/J.TIBTECH.2005.02.001.

Joshi, M. and Deshpande, J. D. (2010) 'Polymerase chain reaction: methods, principles and application', *International Journal of Biomedical Research*, 2(1), pp. 81–97.

Joshi, N. (2011) 'Sabre: a barcode demultiplexing and trimming tool for FastQ files', *GitHub: San Francisco, CA, USA*.

Jurgens, D. J., Sattar, S. A. and Mah, T. F. (2008) 'Chloraminated drinking water does not generate bacterial resistance to antibiotics in Pseudomonas aeruginosa biofilms', *Letters in Applied Microbiology*. John Wiley & Sons, Ltd, 46(5), pp. 562–567. doi: 10.1111/j.1472-765X.2008.02354.x.

Jutla, A., Whitcombe, E., Hasan, N., Haley, B., Akanda, A., Huq, A., Alam, M., Sack, R. B. and Colwell, R. (2013) 'Environmental factors influencing epidemic cholera', *The American journal of tropical medicine and hygiene*. ASTMH, 89(3), pp. 597–607.

Kalmbach, S., Manz, W., Bendinger, B. and Szewzyk, U. (2000) 'In situ probing reveals Aquabacterium commune as a widespread and highly abundant bacterial species in drinking water biofilms', *Water Research*, 34(2), pp. 575–581. doi: https://doi.org/10.1016/S0043-1354(99)00179-7.

Kalyuhznaya, M. G., Martens-Habbena, W., Wang, T., Hackett, M., Stolyar, S. M., Stahl, D. A., Lidstrom, M. E. and Chistoserdova, L. (2009) 'Methylophilaceae link methanol oxidation to denitrification in freshwater lake sediment as suggested by stable isotope probing and pure culture analysis', *Environmental Microbiology Reports*. John Wiley & Sons, Ltd, 1(5), pp. 385–392. doi: 10.1111/j.1758-2229.2009.00046.x.

Kämpfer, P., Rosselló-Mora, R., Hermansson, M., Persson, F., Huber, B., Falsen, E. and Busse, H. J. (2007) 'Undibacterium pigrum gen. nov., sp. nov., isolated from drinking water', *International Journal of Systematic and Evolutionary Microbiology*. Microbiology Society, 57(7), pp. 1510–1515. doi: 10.1099/ijs.0.64785-0.

Karadirek, I. E., Kara, S., Muhammetoglu, A., Muhammetoglu, H. and Soyupak, S. (2016) 'Management of chlorine dosing rates in urban water distribution networks using online continuous monitoring and modeling', *Urban Water Journal*. Taylor & Francis, 13(4), pp. 345–359. doi: 10.1080/1573062X.2014.992916.

Karanfil, T., Erdogan, I. and Schlautman, M. A. (2003) 'Selecting Filter Membranes for measuring

DOC and UV254', *Journal - AWWA*. John Wiley & Sons, Ltd, 95(3), pp. 86–100. doi: 10.1002/j.1551-8833.2003.tb10317.x.

Keller, P. M., Rampini, S. K., Büchler, A. C., Eich, G., Wanner, R. M., Speck, R. F., Böttger, E. C. and Bloemberg, G. V (2010) 'Recognition of potentially novel human disease-associated pathogens by implementation of systematic 16S rRNA gene sequencing in the diagnostic laboratory', *Journal of clinical microbiology*. 2010/07/14. American Society for Microbiology (ASM), 48(9), pp. 3397–3402. doi: 10.1128/JCM.01098-10.

Kent, A. G., Garcia, C. A. and Martiny, A. C. (2018) 'Increased biofilm formation due to high-temperature adaptation in marine Roseobacter', *Nature Microbiology*, 3(9), pp. 989–995. doi: 10.1038/s41564-018-0213-8.

Kitney, P., Woulfe, R. and Codd, S. (2001) 'AIR SCOURING OF WATER MAINS-AN ASSET MANAGEMENT APPROACH', in *64th Annual Water Industry Engineers and Operators Conference, Bendingo, Australia*.

Klein, D. (2002) 'Quantification using real-time PCR technology: applications and limitations', *Trends in molecular medicine*. Elsevier, 8(6), pp. 257–260.

Kõljalg, U., Nilsson, R. H., Abarenkov, K., Tedersoo, L., Taylor, A. F. S., Bahram, M., Bates, S. T., Bruns, T. D., Bengtsson-Palme, J., Callaghan, T. M., Douglas, B., Drenkhan, T., Eberhardt, U., Dueñas, M., Grebenc, T., Griffith, G. W., Hartmann, M., Kirk, P. M., Kohout, P., Larsson, E., Lindahl, B. D., Lücking, R., Martín, M. P., Matheny, P. B., Nguyen, N. H., Niskanen, T., Oja, J., Peay, K. G., Peintner, U., Peterson, M., Põldmaa, K., Saag, L., Saar, I., Schüßler, A., Scott, J. A., Senés, C., Smith, M. E., Suija, A., Taylor, D. L., Telleria, M. T., Weiss, M. and Larsson, K.-H. (2013) 'Towards a unified paradigm for sequence-based identification of fungi', *Molecular Ecology*. John Wiley & Sons, Ltd (10.1111), 22(21), pp. 5271–5277. doi: 10.1111/mec.12481.

Van der Kooij, D., Van Lieverloo, J. H. M., Schellart, J. A. and Hiemstra, P. (1999) 'Distributing drinking water without disinfectant: highest achievement or height of folly?', *Journal of Water Supply: Research and Technology—AQUA*. IWA Publishing, 48(1), pp. 31–37.

Kos, J., Brmež, M., Markić, M. and Sipos, L. (2020) 'The Mortality of Nematodes in Drinking Water in the Presence of Ozone, Chlorine Dioxide, and Chlorine', *Ozone: Science & Engineering*. Taylor & Francis, 42(2), pp. 120–127. doi: 10.1080/01919512.2019.1605877.

Kralik, P. and Ricchi, M. (2017) 'A Basic Guide to Real Time PCR in Microbial Diagnostics: Definitions, Parameters, and Everything', *Frontiers in microbiology*. Frontiers Media S.A., 8, p. 108. doi: 10.3389/fmicb.2017.00108.

Kubista, M., Andrade, J. M., Bengtsson, M., Forootan, A., Jonák, J., Lind, K., Sindelka, R., Sjöback, R., Sjögreen, B., Strömbom, L., Ståhlberg, A. and Zoric, N. (2006) 'The real-time polymerase chain reaction', *Molecular Aspects of Medicine*, 27(2), pp. 95–125. doi: https://doi.org/10.1016/j.mam.2005.12.007.

Kumpel, E. and Nelson, K. L. (2013) 'Comparing microbial water quality in an intermittent and continuous piped water supply', *Water Research*. Pergamon, 47(14), pp. 5176–5188. doi: 10.1016/J.WATRES.2013.05.058.

Kumpel, E. and Nelson, Kara L. (2016) 'Intermittent Water Supply: Prevalence, Practice, and Microbial Water Quality', *Environmental Science and Technology*, 50(2), pp. 542–553. doi: 10.1021/acs.est.5b03973.

Kumpel, E. and Nelson, Kara L (2016) 'Intermittent Water Supply: Prevalence, Practice, and Microbial Water Quality', *Environmental Science & Technology*. American Chemical Society, 50(2), pp. 542–553. doi: 10.1021/acs.est.5b03973.

Kunin, V., Copeland, A., Lapidus, A., Mavromatis, K. and Hugenholtz, P. (2008) 'A bioinformatician's guide to metagenomics', *Microbiology and molecular biology reviews*. Am Soc Microbiol, 72(4), pp. 557–578.

Lee, E. J. and Schwab, K. J. (2005) 'Deficiencies in drinking water distribution systems in developing countries', *Journal of Water and Health*, 3(2), pp. 109–127. doi: 10.2166/wh.2005.0012.

Lehtola, Markku J., Miettinen, I. T., Hirvonen, A., Vartiainen, T. and Martikainen, P. J. (2007) 'Estimates of microbial quality and concentration of copper in distributed drinking water are highly dependent on sampling strategy', *International Journal of Hygiene and Environmental Health*. doi: 10.1016/j.ijheh.2006.11.011.

Lehtola, Markku J, Torvinen, E., Kusnetsov, J., Pitkänen, T., Maunula, L., von Bonsdorff, C.-H., Martikainen, P. J., Wilks, S. A., Keevil, C. W. and Miettinen, I. T. (2007) 'Survival of Mycobacterium avium, Legionella pneumophila, Escherichia coli, and caliciviruses in drinking water-associated biofilms grown under high-shear turbulent flow', *Applied and environmental microbiology*. 2007/03/02. American Society for Microbiology, 73(9), pp. 2854–2859. doi: 10.1128/AEM.02916-06.

Leitch, G. J. and He, Q. (2012) 'Cryptosporidiosis-an overview', *Journal of biomedical research*. 2012/02/21. Editorial Department of Journal of Biomedical Research, 25(1), pp. 1–16. doi: 10.1016/S1674-8301(11)60001-8.

Levantesi, C., Bonadonna, L., Briancesco, R., Grohmann, E., Toze, S. and Tandoi, V. (2012) 'Salmonella in surface and drinking water: Occurrence and water-mediated transmission', *Food Research International*, 45(2), pp. 587–602. doi: https://doi.org/10.1016/j.foodres.2011.06.037.

Levy, K. (2015) 'Does Poor Water Quality Cause Diarrheal Disease?', *The American journal of tropical medicine and hygiene*. 2015/10/05. The American Society of Tropical Medicine and Hygiene, 93(5), pp. 899–900. doi: 10.4269/ajtmh.15-0689.

Li, W., Zhang, J., Wang, F., Qian, L., Zhou, Y., Qi, W. and Chen, J. (2018) 'Effect of disinfectant residual on the interaction between bacterial growth and assimilable organic carbon in a drinking water distribution system', *Chemosphere*. Elsevier Ltd, 202, pp. 586–597. doi: 10.1016/j.chemosphere.2018.03.056.

Liduino, V. S., Cravo-Laureau, C., Noel, C., Carbon, A., Duran, R., Lutterbach, M. T. and Camporese Sérvulo, E. F. (2019) 'Comparison of flow regimes on biocorrosion of steel pipe weldments: Community composition and diversity of biofilms', *International Biodeterioration & Biodegradation*, 143, p. 104717. doi: https://doi.org/10.1016/j.ibiod.2019.104717.

Lin, S. D. (1977) 'Tastes and odors in water supplies: a review', *Circular no. 127*. Illinois State Water Survey.

Liu, G., Verberk, J. Q. J. C. and Van Dijk, J. C. (2013) 'Bacteriology of drinking water distribution systems: An integral and multidimensional review', *Applied Microbiology and Biotechnology*, 97(21), pp. 9265–9276. doi: 10.1007/s00253-013-5217-y.

Liu, G., Zhang, Y., van der Mark, E., Magic-Knezev, A., Pinto, A., van den Bogert, B., Liu, W., van der Meer, W. and Medema, G. (2018) 'Assessing the origin of bacteria in tap water and distribution

system in an unchlorinated drinking water system by SourceTracker using microbial community fingerprints', *Water Research*, 138, pp. 86–96. doi: https://doi.org/10.1016/j.watres.2018.03.043.

Liu, L., Johnson, H. L., Cousens, S., Perin, J., Scott, S., Lawn, J. E., Rudan, I., Campbell, H., Cibulskis, R. and Li, M. (2012) 'Global, regional, and national causes of child mortality: an updated systematic analysis for 2010 with time trends since 2000', *The Lancet*. Elsevier, 379(9832), pp. 2151–2161.

Liu, L., Xing, X., Hu, C., Wang, H. and Lyu, L. (2019) 'Effect of sequential UV/free chlorine disinfection on opportunistic pathogens and microbial community structure in simulated drinking water distribution systems', *Chemosphere*. doi: 10.1016/j.chemosphere.2018.12.067.

Liu, R., Zhu, J., Yu, Z., Joshi, D. R., Zhang, H., Lin, W. and Yang, M. (2014) 'Molecular analysis of long-term biofilm formation on PVC and cast iron surfaces in drinking water distribution system', *Journal of Environmental Sciences (China)*. The Research Centre for Eco-Environmental Sciences, Chinese Academy of Sciences, 26(4), pp. 865–874. doi: 10.1016/S1001-0742(13)60481-7.

Liu, S., Gunawan, C., Barraud, N., Rice, S. A., Harry, E. J. and Amal, R. (2016) 'Understanding, monitoring, and controlling biofilm growth in drinking water distribution systems', *Environmental Science and Technology*, 50(17), pp. 8954–8976. doi: 10.1021/acs.est.6b00835.

Liu, W., Li, L., Khan, M. A. and Zhu, F. (2012) 'Popular molecular markers in bacteria', *Molecular Genetics, Microbiology and Virology*, 27(3), pp. 103–107. doi: 10.3103/S0891416812030056.

Liu, Y., Shan, R., Chen, G. and Liu, L. (2020) 'Linking flow velocity-regulated EPS production with early-stage biofilm formation in drinking water distribution systems', *Water Supply*. doi: 10.2166/ws.2020.039.

López, D., Vlamakis, H. and Kolter, R. (2010) 'Biofilms', *Cold Spring Harbor perspectives in biology*. 2010/06/02. Cold Spring Harbor Laboratory Press, 2(7), pp. a000398–a000398. doi: 10.1101/cshperspect.a000398.

Lu, J., Struewing, I., Vereen, E., Kirby, A. E., Levy, K., Moe, C. and Ashbolt, N. (2016) 'Molecular Detection of Legionella spp. and their associations with Mycobacterium spp., Pseudomonas aeruginosa and amoeba hosts in a drinking water distribution system', *Journal of Applied Microbiology*. John Wiley & Sons, Ltd, 120(2), pp. 509–521. doi: 10.1111/jam.12996.

Lu, J., Struewing, I., Yelton, S. and Ashbolt, N. (2015) 'Molecular survey of occurrence and quantity of Legionella spp., Mycobacterium spp., Pseudomonas aeruginosa and amoeba hosts in municipal drinking water storage tank sediments', *Journal of Applied Microbiology*, 119(1), pp. 278–288. doi: 10.1111/jam.12831.

Lu, Y., Shen, Y., Warren, W. and Walter, R. (2016) 'Next Generation Sequencing in Aquatic Models', *Next Generation Sequencing-Advances, Applications and Challenges*. IntechOpen, pp. 61–79.

Lymperopoulou, D. S., Kormas, K. A. and Karagouni, A. D. (2009) 'Variability of prokaryotic community structure in a drinking water reservoir (Marathonas, Greece)', *Microbes and environments*. Japanese Society of Microbial Ecology/Japanese Society of Soil Microbiology …, p. 1109300325.

Lyratzopoulos, G., Ellis, M., Nerringer, R. and Denning, D. W. (2002) 'Invasive Infection due to Penicillium Species other than P. marneffei', *Journal of Infection*. Elsevier, 45(3), pp. 184–195. doi: 10.1053/jinf.2002.1056.

Ma, L., Li, B., Jiang, X.-T., Wang, Y.-L., Xia, Y., Li, A.-D. and Zhang, T. (2017) 'Catalogue of antibiotic resistome and host-tracking in drinking water deciphered by a large scale survey', *Microbiome*, 5(1), p. 154. doi: 10.1186/s40168-017-0369-0.

Ma, X., Li, G., Yu, Y., Chen, R., Zhang, Y., Tao, H., Zhang, G. and Shi, B. (2019) 'Spatial variation of loose deposit characteristics in a 40 km long operational drinking water distribution system', *Environmental Science: Water Research & Technology*. The Royal Society of Chemistry, 5(10), pp. 1689–1698. doi: 10.1039/C9EW00391F.

Magalhães, A. P., França, Â., Pereira, M. O. and Cerca, N. (2019) 'RNA-based qPCR as a tool to quantify and to characterize dual-species biofilms', *Scientific Reports*, 9(1), p. 13639. doi: 10.1038/s41598-019-50094-3.

Mahmood, A. R., Al-Haideri, H. H. and Hassan, F. M. (2019) 'Detection of Antibiotics in Drinking Water Treatment Plants in Baghdad City, Iraq', *Advances in Public Health*. Edited by J. Khubchandani. Hindawi, 2019, p. 7851354. doi: 10.1155/2019/7851354.

Mandal, S., Kunhikrishnan, A., Bolan, N. S., Wijesekara, H. and Naidu, R. (2016) 'Application of biochar produced from biowaste materials for environmental protection and sustainable agriculture production', in *Environmental Materials and Waste*. Elsevier, pp. 73–89.

Mandinic, Z., Curcic, M., Antonijevic, B., Carevic, M., Mandic, J., Djukic-Cosic, D. and Lekic, C. P. (2010) 'Fluoride in drinking water and dental fluorosis', *Science of the total environment*. Elsevier, 408(17), pp. 3507–3512.

Manuel, C.M., Nunes, O. C. and Melo, L. F. (2007) 'Dynamics of drinking water biofilm in flow/non-flow conditions', *Water Research*. Pergamon, 41(3), pp. 551–562. doi: 10.1016/J.WATRES.2006.11.007.

Manuel, C. M., Nunes, O. C. and Melo, L. F. (2007) 'Dynamics of drinking water biofilm in flow/non-flow conditions', *Water Research*, 41(3), pp. 551–562. doi: 10.1016/j.watres.2006.11.007.

Martiny, A. C., Albrechtsen, H.-J., Arvin, E. and Molin, S. (2005) 'Identification of Bacteria in Biofilm and Bulk Water Samples from a Nonchlorinated Model Drinking Water Distribution System: Detection of a Large Nitrite-Oxidizing Population Associated with <em>Nitrospira</em> spp.', *Applied and Environmental Microbiology*, 71(12), pp. 8611 LP – 8617. doi: 10.1128/AEM.71.12.8611-8617.2005.

Martiny, A. C., Jørgensen, T. M., Albrechtsen, H.-J., Arvin, E. and Molin, S. (2003) 'Long-term succession of structure and diversity of a biofilm formed in a model drinking water distribution system', *Appl. Environ. Microbiol.* Am Soc Microbiol, 69(11), pp. 6899–6907.

Matilainen, A., Gjessing, E. T., Lahtinen, T., Hed, L., Bhatnagar, A. and Sillanpää, M. (2011) 'An overview of the methods used in the characterisation of natural organic matter (NOM) in relation to drinking water treatment', *Chemosphere*, 83(11), pp. 1431–1442. doi: https://doi.org/10.1016/j.chemosphere.2011.01.018.

Matta, G. (2010) 'Freshwater: Resources and Pollution Gagan Matta', *Environment Conservation Journal*, 11(3), pp. 161–169.

McCoy, S. T. and VanBriesen, J. M. (2014) 'Comparing spatial and temporal diversity of bacteria in a chlorinated drinking water distribution system', *Environmental Engineering Science*. Mary Ann Liebert, Inc. 140 Huguenot Street, 3rd Floor New Rochelle, NY 10801 USA, 31(1), pp. 32–41.

McKew, B. A. and Smith, C. J. (2017) 'Real-Time PCR Approaches for Analysis of Hydrocarbon-Degrading Bacterial Communities BT - Hydrocarbon and Lipid Microbiology Protocols: Microbial Quantitation, Community Profiling and Array Approaches', in McGenity, T. J., Timmis, K. N., and Nogales, B. (eds). Berlin, Heidelberg: Springer Berlin Heidelberg, pp. 45–64. doi: 10.1007/8623_2015_64.

McKinnon, K. M. (2018) 'Flow Cytometry: An Overview', *Current protocols in immunology*, 120, pp. 5.1.1-5.1.11. doi: 10.1002/cpim.40.

McMichael, A. J., Woodruff, R. E. and Hales, S. (2006) 'Climate change and human health: Present and future risks', *Lancet*, 367(9513), pp. 859–869. doi: 10.1016/S0140-6736(06)68079-3.

McNeill, L. S. and Edwards, M. (2001) 'Iron pipe corrosion in distribution systems', *Journal-American Water Works Association*. Wiley Online Library, 93(7), pp. 88–100.

Melo, L. F. (2005) 'Biofilm physical structure, internal diffusivity and tortuosity', *Water Science and Technology*, 52(7), pp. 77–84. doi: 10.2166/wst.2005.0184.

Melo, L. F. and Bott, T. R. (1997) 'Biofouling in water systems', *Experimental Thermal and Fluid Science*, 14(4), pp. 375–381. doi: https://doi.org/10.1016/S0894-1777(96)00139-2.

Mesquita, E., João, M. R., Menaia, J., Kardinaal, E., Eikebrokk, B. and Smeets, P. (2013) 'Adapted operation of drinking water systems to cope with climate change', p. 103.

Mi, Z., Dai, Y., Xie, S., Chen, C. and Zhang, X. (2015) 'Impact of disinfection on drinking water biofilm bacterial community', *Journal of Environmental Sciences*. Elsevier, 37, pp. 200–205. doi: 10.1016/J.JES.2015.04.008.

Mirjam Blokker, E. J. and Pieterse-Quirijns, E. J. (2013) 'Modeling temperature in the drinking water distribution system', *Journal - American Water Works Association*, 105(1), pp. 35–36. doi: 10.5942/jawwa.2013.105.0011.

Monteiro, L., Figueiredo, D., Covas, D. and Menaia, J. (2017) 'Integrating water temperature in chlorine decay modelling: a case study', *Urban Water Journal*. Taylor & Francis, 14(10), pp. 1097–1101. doi: 10.1080/1573062X.2017.1363249.

Morello, E., Saussereau, E., Maura, D., Huerre, M., Touqui, L. and Debarbieux, L. (2011) 'Pulmonary bacteriophage therapy on Pseudomonas aeruginosa cystic fibrosis strains: first steps towards treatment and prevention', *PloS one*. Public Library of Science, 6(2), pp. e16963–e16963. doi: 10.1371/journal.pone.0016963.

Moritz, M. M., Flemming, H.-C. and Wingender, J. (2010) 'Integration of Pseudomonas aeruginosa and Legionella pneumophila in drinking water biofilms grown on domestic plumbing materials', *International Journal of Hygiene and Environmental Health*. Urban & Fischer, 213(3), pp. 190–197. doi: 10.1016/J.IJHEH.2010.05.003.

Morris, E. K., Caruso, T., Buscot, F., Fischer, M., Hancock, C., Maier, T. S., Meiners, T., Müller, C., Obermaier, E., Prati, D., Socher, S. A., Sonnemann, I., Wäschke, N., Wubet, T., Wurst, S. and Rillig, M. C. (2014) 'Choosing and using diversity indices: insights for ecological applications from the German Biodiversity Exploratories', *Ecology and Evolution*. John Wiley & Sons, Ltd, 4(18), pp. 3514–3524. doi: 10.1002/ece3.1155.

MSCBS, M. de S. C. y B. S. de E. (2018) 'Informe Técnico de Calidad del agua de consumo humano en España 2018'.

Muellner, M. G., Wagner, E. D., McCalla, K., Richardson, S. D., Woo, Y.-T. and Plewa, M. J. (2007) 'Haloacetonitriles vs. regulated haloacetic acids: are nitrogen-containing DBPs more toxic?', *Environmental science & technology*. ACS Publications, 41(2), pp. 645–651.

Mutlu, M. B. and Güven, K. (2015) 'Bacterial diversity in Çamaltı saltern, Turkey', *Polish Journal of Microbiology*. -, 64(1), pp. 37–45.

Nagy, A., Vitásková, E., Černíková, L., Křivda, V., Jiřincová, H., Sedlák, K., Horníčková, J. and Havlíčková, M. (2017) 'Evaluation of TaqMan qPCR System Integrating Two Identically Labelled Hydrolysis Probes in Single Assay', *Scientific Reports*, 7(1), p. 41392. doi: 10.1038/srep41392.

Nagymáté, Z., Homonnay, Z. G. and Márialigeti, K. (2016) 'Investigation of Archaeal and Bacterial community structure of five different small drinking water networks with special regard to the nitrifying microorganisms', *Microbiological Research*. Elsevier, 188, pp. 80–89.

Narciso-da-Rocha, C., Vaz-Moreira, I. and Manaia, C. M. (2014) 'Genotypic diversity and antibiotic resistance in Sphingomonadaceae isolated from hospital tap water', *Science of The Total Environment*, 466–467, pp. 127–135. doi: https://doi.org/10.1016/j.scitotenv.2013.06.109.

National Research Council (2006) *Drinking Water Distribution Systems: Assessing and Reducing Risks*. Washington, DC: The National Academies Press. doi: 10.17226/11728.

Navarro-Noya, Y. E., Suárez-Arriaga, M. C., Rojas-Valdes, A., Montoya-Ciriaco, N. M., Gómez-Acata, S., Fernández-Luqueño, F. and Dendooven, L. (2013) 'Pyrosequencing Analysis of the Bacterial Community in Drinking Water Wells', *Microbial Ecology*, 66(1), pp. 19–29. doi: 10.1007/s00248-013-0222-3.

Nazari, L., Pattori, E., Manstretta, V., Terzi, V., Morcia, C., Somma, S., Moretti, A., Ritieni, A. and Rossi, V. (2018) 'Effect of temperature on growth, wheat head infection, and nivalenol production by Fusarium poae', *Food Microbiology*. Academic Press, 76, pp. 83–90. doi: 10.1016/J.FM.2018.04.015.

Nescerecka, A., Rubulis, J., Vital, M., Juhna, T. and Hammes, F. (2014) 'Biological instability in a chlorinated drinking water distribution network', *PLoS ONE*, 9(5), pp. 1–11. doi: 10.1371/journal.pone.0096354.

Neufeld, J. D., Vohra, J., Dumont, M. G., Lueders, T., Manefield, M., Friedrich, M. W. and Murrell, J. C. (2007) 'DNA stable-isotope probing', *Nature protocols*. Nature Publishing Group, 2(4), pp. 860–866.

Van Nevel, Sam, Buysschaert, B., De Roy, K., De Gusseme, B., Clement, L. and Boon, N. (2017) 'Flow cytometry for immediate follow-up of drinking water networks after maintenance', *Water Research*, 111, pp. 66–73. doi: https://doi.org/10.1016/j.watres.2016.12.040.

Van Nevel, S, Koetzsch, S., Proctor, C. R., Besmer, M. D., Prest, E. I., Vrouwenvelder, J. S., Knezev, A., Boon, N. and Hammes, F. (2017) 'Flow cytometric bacterial cell counts challenge conventional heterotrophic plate counts for routine microbiological drinking water monitoring', *Water Research*, 113, pp. 191–206. doi: https://doi.org/10.1016/j.watres.2017.01.065.

Van Nevel, S., De Roy, K. and Boon, N. (2013) 'Bacterial invasion potential in water is determined by nutrient availability and the indigenous community', *FEMS microbiology ecology*. Blackwell Publishing Ltd Oxford, UK, 85(3), pp. 593–603.

Nguyen, N.-P., Warnow, T., Pop, M. and White, B. (2016) 'A perspective on 16S rRNA operational

taxonomic unit clustering using sequence similarity', *npj Biofilms and Microbiomes*, 2(1), p. 16004. doi: 10.1038/npjbiofilms.2016.4.

Niquette, P., Servais, P. and Savoir, R. (2000) 'Impacts of pipe materials on densities of fixed bacterial biomass in a drinking water distribution system', *Water Research*, 34(6), pp. 1952–1956. doi: https://doi.org/10.1016/S0043-1354(99)00307-3.

Niu, S.-Y., Yang, J., McDermaid, A., Zhao, J., Kang, Y. and Ma, Q. (2017) 'Bioinformatics tools for quantitative and functional metagenome and metatranscriptome data analysis in microbes', *Briefings in Bioinformatics*, 19(6), pp. 1415–1429. doi: 10.1093/bib/bbx051.

Nolan, T., Huggett, J. F. and Sanchez, E. (2013) 'Good practice guide for the application of quantitative PCR (qPCR)', *Teddington: LGC*.

Novak Babič, M., Gunde-Cimerman, N., Vargha, M., Tischner, Z., Magyar, D., Veríssimo, C., Sabino, R., Viegas, C., Meyer, W. and Brandão, J. (2017) 'Fungal Contaminants in Drinking Water Regulation? A Tale of Ecology, Exposure, Purification and Clinical Relevance', *International Journal of Environmental Research and Public Health*. 2017/06/13. Edited by W. Ahmed and D. J. Beale. MDPI, 14(6), p. 636. doi: 10.3390/ijerph14060636.

O'Toole, G., Kaplan, H. B. and Kolter, R. (2000) 'Biofilm Formation as Microbial Development', *Annual Review of Microbiology*. Annual Reviews, 54(1), pp. 49–79. doi: 10.1146/annurev.micro.54.1.49.

Ogórek, R., Lejman, A., Pusz, W., Miłuch, A. and Miodyńska, P. (2012) 'Charakterystyka i taksonomia grzybów z rodzaju cladosporium', *Mikologia Lekarska*, 19(2), pp. 80–85.

Okache, J., Haggett, B. and Ajmal, T. (2015) 'UV LED Fluorescence Based Method For Detecting Organic Contaminants In Wa-ter: A Review', *Trans Mach Des*, 3(1), pp. 1–10.

Oksanen, J., Blanchet, F. G., Friendly, M., Kindt, R., Legendre, P., McGlinn, D., Minchin, P. R., O'Hara, R. B., Simpson, G. L. and Solymos, P. (2019) 'vegan: Community Ecology Package. R package version 2.5-6. 2019'.

Olicón-Hernández, D. R., González-López, J. and Aranda, E. (2017) 'Overview on the Biochemical Potential of Filamentous Fungi to Degrade Pharmaceutical Compounds', *Frontiers in microbiology*. Frontiers Media S.A., 8, p. 1792. doi: 10.3389/fmicb.2017.01792.

Del Olmo, G., Husband, S., Sánchez Briones, C., Soriano, A., Calero Preciado, C., Macian, J. and Douterelo, I. (2021) 'The microbial ecology of a Mediterranean chlorinated drinking water distribution systems in the city of Valencia (Spain)', *Science of The Total Environment*, 754, p. 142016. doi: https://doi.org/10.1016/j.scitotenv.2020.142016.

Oreskes, N. (2018) 'The Scientific Consensus on Climate Change: How Do We Know We're Not Wrong? BT - Climate Modelling: Philosophical and Conceptual Issues', in A. Lloyd, E. and Winsberg, E. (eds). Cham: Springer International Publishing, pp. 31–64. doi: 10.1007/978-3-319-65058-6_2.

Ortiz-Vera, M. P., Olchanheski, L. R., da Silva, E. G., de Lima, F. R., Martinez, L. R. del P. R., Sato, M. I. Z., Jaffé, R., Alves, R., Ichiwaki, S., Padilla, G. and Araújo, W. L. (2018) 'Influence of water quality on diversity and composition of fungal communities in a tropical river', *Scientific Reports*, 8(1), pp. 1–9. doi: 10.1038/s41598-018-33162-y.

Pandey, P. K., Kass, P. H., Soupir, M. L., Biswas, S. and Singh, V. P. (2014) 'Contamination of water

resources by pathogenic bacteria', *AMB Express*, 4(1), pp. 1–16. doi: 10.1186/s13568-014-0051-x.

Park, J. W., Kim, H. C., Meyer, A. S., Kim, S. and Maeng, S. K. (2016) 'Influences of NOM composition and bacteriological characteristics on biological stability in a full-scale drinking water treatment plant', *Chemosphere*. Elsevier Ltd, 160, pp. 189–198. doi: 10.1016/j.chemosphere.2016.06.079.

Parsons, S. A., Jefferson, B., Goslan, E. H., Jarvis, P. R. and Fearing, D. A. (2004) 'Natural organic matter–the relationship between character and treatability', *Water Science and Technology: Water Supply*. Iwa Publishing, 4(5–6), pp. 43–48.

Paul, E., Ochoa, J. C., Pechaud, Y., Liu, Y. and Liné, A. (2012) 'Effect of shear stress and growth conditions on detachment and physical properties of biofilms', *Water Research*, 46(17), pp. 5499–5508. doi: https://doi.org/10.1016/j.watres.2012.07.029.

Percival, S. L., Walker, J. T. and Hunter, P. R. (2000) *Microbiological aspects of biofilms and drinking water*. CRC press.

Perrin, Y., Bouchon, D., Delafont, V., Moulin, L. and Héchard, Y. (2019) 'Microbiome of drinking water: A full-scale spatio-temporal study to monitor water quality in the Paris distribution system', *Water Research*, 149, pp. 375–385. doi: 10.1016/j.watres.2018.11.013.

Petrova, O. E. and Sauer, K. (2016) 'Escaping the biofilm in more than one way: desorption, detachment or dispersion', *Current opinion in microbiology*. 2016/01/29, 30, pp. 67–78. doi: 10.1016/j.mib.2016.01.004.

Pfaffl, M. W. (2004) 'Quantification strategies in real-time PCR', *AZ of quantitative PCR*. La Jolla, 1, pp. 89–113.

Pignata, C., Bonetta, Silvia, Bonetta, Sara, Cacciò, S. M., Sannella, A. R., Gilli, G. and Carraro, E. (2019) 'Cryptosporidium Oocyst Contamination in Drinking Water: A Case Study in Italy', *International journal of environmental research and public health*. MDPI, 16(11), p. 2055. doi: 10.3390/ijerph16112055.

Pintar, K. D. M. and Slawson, R. M. (2003) 'Effect of temperature and disinfection strategies on ammonia-oxidizing bacteria in a bench-scale drinking water distribution system', *Water Research*, 37(8), pp. 1805–1817. doi: 10.1016/S0043-1354(02)00538-9.

Pinto, A., Schroeder, J., Lunn, M., Sloan, W. and Raskin, L. (2014) 'Spatial-Temporal Survey and Occupancy-Abundance Modeling To', *Mbio*, 5(3), pp. 1–13. doi: 10.1128/mBio.01135-14.Editor.

Pinto, V. G., Heller, L. and Bastos, R. K. X. (2012) 'Drinking water standards in South American countries: convergences and divergences', *Journal of water and health*. IWA Publishing, 10(2), pp. 295–310.

Pourcel, F., Smith, I. E. and Duchesne, S. (2017) 'Slug Flow Simulation, A Way to Improve Air Scouring of Water Mains?', *Procedia Engineering*, 186, pp. 601–608. doi: https://doi.org/10.1016/j.proeng.2017.03.276.

Powell, J. C., Hallam, N. B., West, J. R., Forster, C. F. and Simms, J. (2000) 'Factors which control bulk chlorine decay rates', *Water Research*. Pergamon, 34(1), pp. 117–126. doi: 10.1016/S0043-1354(99)00097-4.

Prasad, M. N. V. (2020) *Disinfection By-products in Drinking Water: Detection and Treatment*. Butterworth-Heinemann.

Prest, E. I., El-Chakhtoura, J., Hammes, F., Saikaly, P. E., van Loosdrecht, M. C. M. and Vrouwenvelder, J. S. (2014) 'Combining flow cytometry and 16S rRNA gene pyrosequencing: A promising approach for drinking water monitoring and characterization', *Water Research*. Pergamon, 63, pp. 179–189. doi: 10.1016/J.WATRES.2014.06.020.

Prest, E. I., Hammes, F., Kötzsch, S., van Loosdrecht, M. C. M. and Vrouwenvelder, J. S. (2013) 'Monitoring microbiological changes in drinking water systems using a fast and reproducible flow cytometric method', *Water Research*, 47(19), pp. 7131–7142. doi: https://doi.org/10.1016/j.watres.2013.07.051.

Prest, E. I., Hammes, F., van Loosdrecht, M. C. M. and Vrouwenvelder, J. S. (2016) 'Biological Stability of Drinking Water: Controlling Factors, Methods, and Challenges ', *Frontiers in Microbiology* , p. 45.

Proctor, C. R. and Hammes, F. (2015) 'Drinking water microbiology—from measurement to management', *Current Opinion in Biotechnology*, 33, pp. 87–94. doi: https://doi.org/10.1016/j.copbio.2014.12.014.

Purwoto, S., Antoro, R. and Sembodo, B. P. (2019) 'Pre Treatment of Raw Water Through the Coagulation Process, Filtration, Absorption, and Ion Exchange in Drinking Water', *Water and Energy International*. Central Board of Irrigation and Power, 62(4), pp. 61–65.

Qin, K., Struewing, I., Domingo, J. S., Lytle, D. and Lu, J. (2017) 'Opportunistic Pathogens and Microbial Communities and Their Associations with Sediment Physical Parameters in Drinking Water Storage Tank Sediments', *Pathogens (Basel, Switzerland)*. MDPI, 6(4), p. 54. doi: 10.3390/pathogens6040054.

Quarini, G., Ainslie, E., Herbert, M., Deans, T., Ash, D., Rhys, D., Haskins, N., Norton, G., Andrews, S. and Smith, M. (2010) 'Investigation and development of an innovative pigging technique for the water-supply industry', *Proceedings of the Institution of Mechanical Engineers, Part E: Journal of Process Mechanical Engineering*. IMECHE, 224(2), pp. 79–89. doi: 10.1243/09544089JPME312.

Quast, C., Pruesse, E., Yilmaz, P., Gerken, J., Schweer, T., Yarza, P., Peplies, J. and Glöckner, F. O. (2013) 'The SILVA ribosomal RNA gene database project: improved data processing and web-based tools', *Nucleic acids research*. 2012/11/28. Oxford University Press, 41(Database issue), pp. D590–D596. doi: 10.1093/nar/gks1219.

Rahmatika, I., Kasuga, I., Kurisu, F. and Furumai, H. (2020) 'Impacts of Organic Matter Migrating from Pipe Materials on Microbial Regrowth in Drinking Water', *Journal of Water and Environment Technology*. Japan Society on Water Environment, 18(1), pp. 45–53.

Rantanen, P.-L., Mellin, I., Keinänen-Toivola, M. M., Ahonen, M. and Vahala, R. (2018) 'The Seasonality of Nitrite Concentrations in a Chloraminated Drinking Water Distribution System', *International journal of environmental research and public health*. MDPI, 15(8), p. 1756. doi: 10.3390/ijerph15081756.

Ravi, R. K., Walton, K. and Khosroheidari, M. (2018) 'MiSeq: a next generation sequencing platform for genomic analysis', in *Disease Gene Identification*. Springer, pp. 223–232.

Reckhow, D. A., Singer, P. C. and Malcolm, R. L. (1990) 'Chlorination of humic materials: byproduct formation and chemical interpretations', *Environmental Science & Technology*. American Chemical Society, 24(11), pp. 1655–1664. doi: 10.1021/es00081a005.

Ren, H., Wang, W., Liu, Y., Liu, S., Lou, L., Cheng, D., He, X., Zhou, X., Qiu, S., Fu, L., Liu, J. and Hu, B.

(2015) 'Pyrosequencing analysis of bacterial communities in biofilms from different pipe materials in a city drinking water distribution system of East China', *Applied Microbiology and Biotechnology*, 99(24), pp. 10713–10724. doi: 10.1007/s00253-015-6885-6.

Revetta, R. P., Gomez-Alvarez, V., Gerke, T. L., Santo Domingo, J. W. and Ashbolt, N. J. (2016) 'Changes in bacterial composition of biofilm in a metropolitan drinking water distribution system', *Journal of Applied Microbiology*, 121(1), pp. 294–305. doi: 10.1111/jam.13150.

Rezaei, H., Ryan, B. and Stoianov, I. (2015) 'Pipe Failure Analysis and Impact of Dynamic Hydraulic Conditions in Water Supply Networks', *Procedia Engineering*, 119, pp. 253–262. doi: https://doi.org/10.1016/j.proeng.2015.08.883.

Richardson, M. and Rautemaa-Richardson, R. (2019) 'Exposure to Aspergillus in Home and Healthcare Facilities' Water Environments: Focus on Biofilms', *Microorganisms*. Multidisciplinary Digital Publishing Institute, 7(1), p. 7. doi: 10.3390/microorganisms7010007.

Di Rienzi, S. C., Sharon, I., Wrighton, K. C., Koren, O., Hug, L. A., Thomas, B. C., Goodrich, J. K., Bell, J. T., Spector, T. D., Banfield, J. F. and Ley, R. E. (2013) 'The human gut and groundwater harbor non-photosynthetic bacteria belonging to a new candidate phylum sibling to Cyanobacteria', *eLife*. Edited by R. Kolter. eLife Sciences Publications, Ltd, 2, p. e01102. doi: 10.7554/eLife.01102.

Riesenfeld, C. S., Schloss, P. D. and Handelsman, J. (2004) 'Metagenomics: genomic analysis of microbial communities', *Annu. Rev. Genet.* Annual Reviews, 38, pp. 525–552.

Ritter, L., Solomon, K., Sibley, P., Hall, K., Keen, P., Mattu, G. and Linton, B. (2002) 'SOURCES, PATHWAYS, AND RELATIVE RISKS OF CONTAMINANTS IN SURFACE WATER AND GROUNDWATER: A PERSPECTIVE PREPARED FOR THE WALKERTON INQUIRY', *Journal of Toxicology and Environmental Health, Part A*. Taylor & Francis, 65(1), pp. 1–142. doi: 10.1080/152873902753338572.

Rognes, T., Flouri, T., Nichols, B., Quince, C. and Mahé, F. (2016) 'VSEARCH: a versatile open source tool for metagenomics', *PeerJ*. PeerJ Inc., 4, pp. e2584–e2584. doi: 10.7717/peerj.2584.

Rubulis, J., Verberk, J., Vreeburg, J., Gruškevica, K. and Juhna, T. (2008) 'Chemical and microbial composition of loose deposits in drinking water distribution systems', in *Proceedings of the, 7th International Conference on Environmental Engineering, May*. Citeseer, pp. 22–23.

Sadiq, R. and Rodriguez, M. J. (2004) 'Disinfection by-products (DBPs) in drinking water and predictive models for their occurrence: a review', *Science of the total Environment*. Elsevier, 321(1–3), pp. 21–46.

Safford, H. R. and Bischel, H. N. (2019) 'Flow cytometry applications in water treatment, distribution, and reuse: A review', *Water research*. Elsevier, 151, pp. 110–133.

Sanschagrin, S. and Yergeau, E. (2014) 'Next-generation sequencing of 16S ribosomal RNA gene amplicons', *Journal of visualized experiments : JoVE*. MyJove Corporation, (90), p. 51709. doi: 10.3791/51709.

Santos, A. L. S. Dos, Galdino, A. C. M., Mello, T. P. de, Ramos, L. de S., Branquinha, M. H., Bolognese, A. M., Columbano Neto, J. and Roudbary, M. (2018) 'What are the advantages of living in a community? A microbial biofilm perspective!', *Memorias do Instituto Oswaldo Cruz*. Instituto Oswaldo Cruz, Ministério da Saúde, 113(9), pp. e180212–e180212. doi: 10.1590/0074-02760180212.

Sautour, M., Edel-Hermann, V., Steinberg, C., Sixt, N., Laurent, J., Dalle, F., Aho, S., Hartemann, P., L'Ollivier, C., Goyer, M. and Bonnin, A. (2012) 'Fusarium species recovered from the water distribution system of a French university hospital', *International Journal of Hygiene and Environmental Health*, 215(3), pp. 286–292. doi: https://doi.org/10.1016/j.ijheh.2011.11.003.

Sav, H., Rafati, H., Öz, Y., Dalyan-Cilo, B., Ener, B., Mohammadi, F., Ilkit, M., van Diepeningen, A. D. and Seyedmousavi, S. (2018) 'Biofilm Formation and Resistance to Fungicides in Clinically Relevant Members of the Fungal Genus Fusarium', *Journal of fungi (Basel, Switzerland)*. MDPI, 4(1), p. 16. doi: 10.3390/jof4010016.

Schirmer, M., Ijaz, U. Z., D'Amore, R., Hall, N., Sloan, W. T. and Quince, C. (2015) 'Insight into biases and sequencing errors for amplicon sequencing with the Illumina MiSeq platform', *Nucleic Acids Research*, 43(6), pp. e37–e37. doi: 10.1093/nar/gku1341.

Schmitt, J. and Flemming, H.-C. (1999) 'Water binding in biofilms', *Water Science and Technology*, 39(7), pp. 77–82. doi: https://doi.org/10.1016/S0273-1223(99)00153-5.

Schock, M. R., Hyland, R. N. and Welch, M. M. (2008) 'Occurrence of Contaminant Accumulation in Lead Pipe Scales from Domestic Drinking-Water Distribution Systems', *Environmental Science & Technology*. American Chemical Society, 42(12), pp. 4285–4291. doi: 10.1021/es702488v.

Schwartz, L. and Seeley, K. (2002) 'Introduction to tangential flow filtration for laboratory and process development applications', *Pall Scientific & Technical Report, PN*, 33213.

Schwering, M., Song, J., Louie, M., Turner, R. J. and Ceri, H. (2013) 'Multi-species biofilms defined from drinking water microorganisms provide increased protection against chlorine disinfection', *Biofouling*. Taylor & Francis, 29(8), pp. 917–928. doi: 10.1080/08927014.2013.816298.

Selborne, Lord (2000) *The ethics of freshwater use: a survey*. Unesco France.

Seth, A., Bachmann, R., Boxall, J., Saul, A. and Edyvean, R. (2004) 'Characterisation of materials causing discolouration in potable water systems', *Water Science and Technology*, 49(2), pp. 27–32. doi: 10.2166/wst.2004.0080.

Sharma, M. O., Bhosle, N. B. and Wagh, A. B. (1990) 'Methods for removal and estimation of microfouling biomass'. NISCAIR-CSIR, India.

Sharma, S. and Bhattacharya, A. (2017) 'Drinking water contamination and treatment techniques', *Applied Water Science*. Springer, 7(3), pp. 1043–1067.

Sharpe, R. (2012) 'Laboratory investigations into processes causing discoloured potable water'. University of Sheffield.

Sharpe, R. L. (2012) 'LABORATORY INVESTIGATIONS INTO PROCESSES CAUSING DISCOLOURED POTABLE WATER', (October).

Sharpe, R. L., Biggs, C. A. and Boxall, J. B. (2017) 'Hydraulic conditioning to manage potable water discolouration', *Proceedings of the Institution of Civil Engineers - Water Management*, 172(1), pp. 3–13. doi: 10.1680/jwama.16.00038.

Sharpe, R. L., Smith, C. J., Boxall, J. B. and Biggs, C. A. (2010) 'Pilot scale laboratory investigations into the impact of steady state conditioning flow on potable water discolouration', in *Water Distribution Systems Analysis 2010*, pp. 494–506.

Siegwald, L., Touzet, H., Lemoine, Y., Hot, D., Audebert, C. and Caboche, S. (2017) 'Assessment of common and emerging bioinformatics pipelines for targeted metagenomics', *PLoS One*. Public

Library of Science San Francisco, CA USA, 12(1), p. e0169563.

Sigee, D. (2005) *Freshwater microbiology: biodiversity and dynamic interactions of microorganisms in the aquatic environment*. John Wiley & Sons.

Simões, L. C. (2013) 'Biofilms in drinking water'. Nova Science Publishers.

Simões, L. C., Simões, M. and Vieira, M. J. (2007) 'Biofilm interactions between distinct bacterial genera isolated from drinking water', *Applied and Environmental Microbiology*, 73(19), pp. 6192–6200. doi: 10.1128/AEM.00837-07.

Simukonda, K., Farmani, R. and Butler, D. (2018) 'Intermittent water supply systems: causal factors, problems and solution options', *Urban Water Journal*. Taylor & Francis, 15(5), pp. 488–500. doi: 10.1080/1573062X.2018.1483522.

Sinclair, R. G., Jones, E. L. and Gerba, C. P. (2009) 'Viruses in recreational water-borne disease outbreaks: a review', *Journal of Applied Microbiology*. John Wiley & Sons, Ltd, 107(6), pp. 1769–1780. doi: 10.1111/j.1365-2672.2009.04367.x.

Sivadon, P., Barnier, C., Urios, L. and Grimaud, R. (2019) 'Biofilm formation as a microbial strategy to assimilate particulate substrates', *Environmental Microbiology Reports*. John Wiley & Sons, Ltd, 11(6), pp. 749–764. doi: 10.1111/1758-2229.12785.

Skraber, S., Schijven, J., Gantzer, C. and Husman, A. M. D. R. (2005) 'Pathogenic viruses in drinking-water biofilms: a public health risk?', *Biofilms*. Cambridge University Press, 2(2), p. 105.

Sloetjes, H. and Wittenburg, P. (2008) 'Annotation by category - ELAN and ISO DCR', pp. 816–820.

Smith, C. J. and Osborn, A. M. (2009) 'Advantages and limitations of quantitative PCR (Q-PCR)-based approaches in microbial ecology', *FEMS Microbiology Ecology*, 67(1), pp. 6–20. doi: 10.1111/j.1574-6941.2008.00629.x.

Smith, D. P. and Peay, K. G. (2014) 'Sequence depth, not PCR replication, improves ecological inference from next generation DNA sequencing', *PloS one*. Public Library of Science, 9(2), p. e90234.

Spellman, F. R. (2013) *Handbook of water and wastewater treatment plant operations*. CRC press.

Srinivasan, S., Harrington, G. W., Xagoraraki, I. and Goel, R. (2008) 'Factors affecting bulk to total bacteria ratio in drinking water distribution systems', *Water research*. Elsevier, 42(13), pp. 3393–3404.

Stanish, L. F., Hull, N. M., Robertson, C. E., Harris, J. K., Stevens, M. J., Spear, J. R. and Pace, N. R. (2016) 'Factors Influencing Bacterial Diversity and Community Composition in Municipal Drinking Waters in the Ohio River Basin, USA', *PloS one*, 11(6), p. e0157966. doi: 10.1371/journal.pone.0157966.

Steinberg, J. P. and Burd, E. M. (2015) '238 - Other Gram-Negative and Gram-Variable Bacilli', in Bennett, J. E., Dolin, R., and Blaser Douglas, and Bennett's Principles and Practice of Infectious Diseases (Eighth Edition), M. J. B. T.-M. (eds). Philadelphia: Content Repository Only!, pp. 2667-2683.e4. doi: https://doi.org/10.1016/B978-1-4557-4801-3.00238-1.

Stoodley, P., Cargo, R., Rupp, C. J., Wilson, S. and Klapper, I. (2002) 'Biofilm material properties as related to shear-induced deformation and detachment phenomena', *Journal of Industrial Microbiology and Biotechnology*, 29(6), pp. 361–367. doi: 10.1038/sj.jim.7000282.

Sun, W., Xia, C., Xu, M., Guo, J., Wang, A. and Sun, G. (2013) 'Distribution and abundance of archaeal and bacterial ammonia oxidizers in the sediments of the Dongjiang River, a drinking water supply for Hong Kong', *Microbes and environments*. 2013/11/19. Japanese Society of Microbial Ecology/The Japanese Society of Soil Microbiology, 28(4), pp. 457–465. doi: 10.1264/jsme2.me13066.

Szewzyk, U., Szewzyk, R., Manz, W. and Schleifer, K.-H. (2000) 'Microbiological Safety of Drinking Water', *Annual Review of Microbiology*. Annual Reviews, 54(1), pp. 81–127. doi: 10.1146/annurev.micro.54.1.81.

Tamsikar, J., Naidu, J. and Singh, S. M. (2006) 'Phaeohyphomycotic sebaceous cyst due to Cladosporium cladosporioides: case report and review of literature', *Journal de Mycologie Médicale*. Elsevier, 16(1), pp. 55–57.

Team, R. C. (2014) 'R: a language and environment for statistical computing. Version 3.1. 2 [computer program]. R Foundation for Statistical Computing, Vienna, Austria'.

Teng, F., Guan, Y. T. and Zhu, W. P. (2008) 'Effect of biofilm on cast iron pipe corrosion in drinking water distribution system: Corrosion scales characterization and microbial community structure investigation', *Corrosion Science*, 50(10), pp. 2816–2823. doi: https://doi.org/10.1016/j.corsci.2008.07.008.

Thomas, T., Gilbert, J. and Meyer, F. (2014) 'Metagenomics: A guide from sampling to data analysis', *The Role of Bioinformatics in Agriculture*. BioMed Central Ltd, 2(1), pp. 357–383. doi: 10.1201/b16568.

Timoner, X., Borrego, C. M., Acuña, V. and Sabater, S. (2014) 'The dynamics of biofilm bacterial communities is driven by flow wax and wane in a temporary stream', *Limnology and Oceanography*. John Wiley & Sons, Ltd, 59(6), pp. 2057–2067. doi: 10.4319/lo.2014.59.6.2057.

Tiratsoo, J. N. H. (1992) *Pipeline pigging technology*. Gulf Professional Publishing.

Tokajian, S. and Hashwa, F. (2003) 'Water quality problems associated with intermittent water supply', *Water Science and Technology*, 47(3), pp. 229–234. doi: 10.2166/wst.2003.0200.

Toth, E. M., Vengring, A., Homonnay, Z. G., Keki, Z., Sproer, C., Borsodi, A. K., Marialigeti, K. and Schumann, P. (2014) 'Phreatobacter oligotrophus gen. nov., sp. nov., an alphaproteobacterium isolated from ultrapure water of the water purification system of a power plant', *INTERNATIONAL JOURNAL OF SYSTEMATIC AND EVOLUTIONARY MICROBIOLOGY*. Microbiology Society, 64(Pt 3), pp. 839–845. doi: 10.1099/ijs.0.053843-0.

Travadon, R., Lawrence, D. P., Rooney-Latham, S., Gubler, W. D., Wilcox, W. F., Rolshausen, P. E. and Baumgartner, K. (2015) 'Cadophora species associated with wood-decay of grapevine in North America', *Fungal Biology*, 119(1), pp. 53–66. doi: https://doi.org/10.1016/j.funbio.2014.11.002.

Tsagkari, E. and Sloan, W. T. (2019) 'Impact of Methylobacterium in the drinking water microbiome on removal of trihalomethanes', *International Biodeterioration and Biodegradation*. Elsevier Ltd, 141(August 2018), pp. 10–16. doi: 10.1016/j.ibiod.2018.07.015.

Twort, A. C., Ratnayaka, D. D. and Brandt, M. J. (2000) *Water supply*. Elsevier.

USEPA (2018) '2018 Edition of the Drinking Water Standards and Health Advisories Tables', (March).

Vaerewijck, M. J. M., Huys, G., Palomino, J. C., Swings, J. and Portaels, F. (2005) 'Mycobacteria in drinking water distribution systems: ecology and significance for human health', *FEMS*

*Microbiology Reviews*, 29(5), pp. 911–934. doi: 10.1016/j.femsre.2005.02.001.

Vairavamoorthy, K., Gorantiwar, S. D. and Mohan, S. (2007) 'Intermittent Water Supply under Water Scarcity Situations', *Water International*. Routledge, 32(1), pp. 121–132. doi: 10.1080/02508060708691969.

Vairavamoorthy, K., Gorantiwar, S. D. and Pathirana, A. (2008) 'Managing urban water supplies in developing countries – Climate change and water scarcity scenarios', *Physics and Chemistry of the Earth, Parts A/B/C*. Pergamon, 33(5), pp. 330–339. doi: 10.1016/J.PCE.2008.02.008.

Vaz-Moreira, I., Nunes, O. C. and Manaia, C. M. (2011) 'Diversity and Antibiotic Resistance Patterns of Sphingomonadaceae Isolates from Drinking Water', *Applied and Environmental Microbiology*, 77(16), pp. 5697 LP – 5706. doi: 10.1128/AEM.00579-11.

Vaz-Moreira, I., Nunes, O. C. and Manaia, C. M. (2012) 'Diversity and antibiotic resistance in Pseudomonas spp. from drinking water', *Science of The Total Environment*, 426, pp. 366–374. doi: https://doi.org/10.1016/j.scitotenv.2012.03.046.

Vembadi, A., Menachery, A. and Qasaimeh, M. A. (2019) 'Cell Cytometry: Review and Perspective on Biotechnological Advances ', *Frontiers in Bioengineering and Biotechnology* , p. 147.

Ventola, C. L. (2015) 'The antibiotic resistance crisis: causes and threats.', *P & T journal*. MediMedia, USA, 40(4), pp. 277–283. doi: Article.

Verberk, J. Q. J. C., Hamilton, L. A., O'Halloran, K. J., van der Horst, W. and Vreeburg, J. (2006) 'Analysis of particle numbers, size and composition in drinking water transportation pipelines: results of online measurements', *Water Supply*, 6(4), pp. 35–43. doi: 10.2166/ws.2006.902.

Vreeburg, I. J. H. G. and Boxall, D. J. B. (2007) 'Discolouration in potable water distribution systems: A review', *Water Research*, 41(3), pp. 519–529. doi: https://doi.org/10.1016/j.watres.2006.09.028.

Vreeburg, J. (2007) 'Discolouration in drinking water systems: a particular approach'.

Vreeburg, J. H. G., Schaap, P. G., Bergmans, B. and Van Dijk, J. C. (2008) 'How Effective Is Flushing of Cast Iron Pipes?', *Water Distribution Systems Analysis 2008*. (Proceedings), pp. 1–12. doi: doi:10.1061/41024(340)48.

Waak, Michael B, Hozalski, R. M., Hallé, C. and LaPara, T. M. (2019) 'Comparison of the microbiomes of two drinking water distribution systems—with and without residual chloramine disinfection', *Microbiome*, 7(1), p. 87. doi: 10.1186/s40168-019-0707-5.

Waak, Michael B., Lapara, T. M., Hallé, C. and Hozalski, R. M. (2019) 'Nontuberculous Mycobacteria in Two Drinking Water Distribution Systems and the Role of Residual Disinfection', *Environmental Science and Technology*, 53(15), pp. 8563–8573. doi: 10.1021/acs.est.9b01945.

Wang, H., Edwards, M. A., Falkinham, J. O. and Pruden, A. (2013) 'Probiotic Approach to Pathogen Control in Premise Plumbing Systems? A Review', *Environmental Science & Technology*. American Chemical Society, 47(18), pp. 10117–10128. doi: 10.1021/es402455r.

Wang, H., Edwards, M., Falkinham, J. O. and Pruden, A. (2012) 'Molecular survey of the occurrence of Legionella spp., Mycobacterium spp., Pseudomonas aeruginosa, and amoeba hosts in two chloraminated drinking water distribution systems', *Applied and environmental microbiology*. Am Soc Microbiol, 78(17), pp. 6285–6294.

Wang, H., Proctor, C. R., Edwards, M. A., Pryor, M., Santo Domingo, J. W., Ryu, H., Camper, A. K.,

Olson, A. and Pruden, A. (2014) 'Microbial community response to chlorine conversion in a chloraminated drinking water distribution system', *Environmental science & technology*. ACS Publications, 48(18), pp. 10624–10633.

Wang, X., Cai, W., van den Ende, A. H. G. G., Zhang, J., Xie, T., Xi, L., Li, X., Sun, J. and de Hoog, S. (2018) 'Indoor wet cells as a habitat for melanized fungi, opportunistic pathogens on humans and other vertebrates', *Scientific Reports*, 8(1), p. 7685. doi: 10.1038/s41598-018-26071-7.

Wang, X., Jordan, I. K. and Mayer, L. W. (2015) 'Chapter 29 - A Phylogenetic Perspective on Molecular Epidemiology', in Tang, Y.-W., Sussman, M., Liu, D., Poxton, I., and Schwartzman, J. B. T.-M. M. M. (Second E. (eds). Boston: Academic Press, pp. 517–536. doi: https://doi.org/10.1016/B978-0-12-397169-2.00029-9.

Waśkiewicz, A. and Irzykowska, L. (2014) 'Flavobacterium spp. – Characteristics, Occurrence, and Toxicity', in Batt, C. A. and Tortorello, M. L. B. T.-E. of F. M. (Second E. (eds). Oxford: Academic Press, pp. 938–942. doi: https://doi.org/10.1016/B978-0-12-384730-0.00126-9.

Watson, J. D. (2012) *The polymerase chain reaction*. Springer Science & Business Media.

Weir, E. (2004) 'Uranium in drinking water, naturally', *CMAJ : Canadian Medical Association journal = journal de l'Association medicale canadienne*. Canadian Medical Association, 170(6), pp. 951–952. doi: 10.1503/cmaj.1040214.

Weishaar, J. L., Aiken, G. R., Bergamaschi, B. A., Fram, M. S., Fujii, R. and Mopper, K. (2003) 'Evaluation of Specific Ultraviolet Absorbance as an Indicator of the Chemical Composition and Reactivity of Dissolved Organic Carbon', *Environmental Science & Technology*. American Chemical Society, 37(20), pp. 4702–4708. doi: 10.1021/es030360x.

Westcott, S. L. and Schloss, P. D. (2015) 'De novo clustering methods outperform reference-based methods for assigning 16S rRNA gene sequences to operational taxonomic units', *PeerJ*. PeerJ Inc., 3, p. e1487.

Whitehead, P. G., Wilby, R. L., Battarbee, R. W., Kernan, M. and Wade, A. J. (2009) 'A review of the potential impacts of climate change on surface water quality', *Hydrological Sciences Journal*. Taylor & Francis, 54(1), pp. 101–123. doi: 10.1623/hysj.54.1.101.

WHO (2004a) 'Copper in Drinking-Water: Background Document for Development of WHO Guidelines for Drinking-Water Quality'. World Health Organization Geneva.

WHO (2004b) 'Halogenated Acetonitriles in Drinking-water (Background document for development of WHO Guidelines for Drinking-water Quality)'.

WHO (2005) 'Trihalomethanes in Drinking Water. Background document for development of WHO Guidelines for Drinking-water Quality.'

WHO (2011) 'Nitrite in drinking-water background document for development of WHO guidelines for drinking-water quality', *Geneva: World Health Organization (WHO/SDE/WSH/07.01/16/Rev/1)*.

WHO (2015) *Management of cyanobacteria in drinking-water supplies: Information for regulators and water suppliers*. World Health Organization.

WHO (2017) *WHO drinking water guideline*. doi: 10.1016/S1462-0758(00)00006-6.

WHO (2020) *Water, sanitation, hygiene, and waste management for the COVID-19 virus: interim guidance, 23 April 2020*. World Health Organization.

W. H. O. and UNICEF (2000) *Global water supply and sanitation assessment 2000 report*.

Wickham, H. and Chan, W. (2016) 'Rstudio (2016). Ggplot. R package version 2.21', *Online: https://github. com/tidyverse/ggplot2*.

van der Wielen, P. W. J. J. and van der Kooij, D. (2013) 'Nontuberculous mycobacteria, fungi, and opportunistic pathogens in unchlorinated drinking water in The Netherlands', *Applied and environmental microbiology*. 2012/11/16. American Society for Microbiology, 79(3), pp. 825–834. doi: 10.1128/AEM.02748-12.

van der Wielen, P. W. J. J. and Lut, M. C. (2016) 'Distribution of microbial activity and specific microorganisms across sediment size fractions and pipe wall biofilm in a drinking water distribution system', *Water Supply*, 16(4), pp. 896–904. doi: 10.2166/ws.2016.023.

van der Wielen, P. W. J. J., Voost, S. and van der Kooij, D. (2009) 'Ammonia-Oxidizing Bacteria and Archaea in Groundwater Treatment and Drinking Water Distribution Systems', *Applied and Environmental Microbiology*, 75(14), pp. 4687 LP – 4695. doi: 10.1128/AEM.00387-09.

Wingender, J. and Flemming, H.-C. (2011) 'Biofilms in drinking water and their role as reservoir for pathogens', *International Journal of Hygiene and Environmental Health*, 214(6), pp. 417–423. doi: https://doi.org/10.1016/j.ijheh.2011.05.009.

Yee, L. L. W. and Tapani, T. M. (2017) *Bioinformatics: A Practical Handbook Of Next Generation Sequencing And Its Applications*. # N/A.

Zhang, H., Ma, P., Shu, J., Yang, B. and Huang, J. (2018) 'Rapid detection of taste and odor compounds in water using the newly invented chemi-ionization technique coupled with time-of-flight mass spectrometry', *Analytica Chimica Acta*, 1035, pp. 119–128. doi: https://doi.org/10.1016/j.aca.2018.07.051.

Zhang, M., Liu, W., Nie, X., Li, C., Gu, J. and Zhang, C. (2012) 'Molecular analysis of bacterial communities in biofilms of a drinking water clearwell', *Microbes and Environments*, 27(4), pp. 443–448. doi: 10.1264/jsme2.ME12035.

Zhou, J., Bruns, M. A. and Tiedje, J. M. (1996) 'DNA recovery from soils of diverse composition', *Applied and Environmental Microbiology*, 62(2), pp. 316–322.

Zhou, X., Zhang, K., Zhang, T., Li, C. and Mao, X. (2017) 'An ignored and potential source of taste and odor (T&O) issues—biofilms in drinking water distribution system (DWDS)', *Applied microbiology and biotechnology*. Springer, 101(9), pp. 3537–3550.

Zhu, Y., Wang, H., Li, X., Hu, C., Yang, M. and Qu, J. (2014) 'Characterization of biofilm and corrosion of cast iron pipes in drinking water distribution system with UV/Cl2 disinfection', *Water Research*, 60, pp. 174–181. doi: https://doi.org/10.1016/j.watres.2014.04.035.

Ziglio, G., Andreottola, G., Barbesti, S., Boschetti, G., Bruni, L., Foladori, P. and Villa, R. (2002) 'Assessment of activated sludge viability with flow cytometry', *Water Research*. Elsevier, 36(2), pp. 460–468.

# Appendices

## Appendix A. Supplementary material for Chapter 4

This appendix includes supplementary tables and figures with the results used for the analysis discussed in chapter 4 of this manuscript, "*The impacts of increasing temperature on water quality and microbial ecology of drinking water distribution system*".

### A.1 DNA concentration

***Table S 1.*** DNA concentration from each biofilm sample at 16 °C and 24 °C. (-) No results, (AM) After mobilization phase.

| Sample Nº | Sample ID | Temperature (°C) | Day | Replicate | [DNA] ng/µL |
|---|---|---|---|---|---|
| 1 | B 16 °C D0 R1 | 16 °C | 0 | R1 | - |
| 2 | B 16 °C D0 R2 | 16 °C | 0 | R2 | - |
| 3 | B 16 °C D0 R3 | 16 °C | 0 | R3 | - |
| 4 | B 24 °C D0 R1 | 24 °C | 0 | R1 | - |
| 5 | B 24 °C D0 R1 | 24 °C | 0 | R2 | - |
| 6 | B 24 °C D0 R1 | 24 °C | 0 | R3 | - |
| 7 | B 16 °C D10 R1 | 16 °C | 10 | R1 | 1.90 |
| 8 | B 16 °C D10 R2 | 16 °C | 10 | R2 | 2.80 |
| 9 | B 16 °C D10 R3 | 16 °C | 10 | R3 | 2.30 |
| 10 | B 24 °C D10 R1 | 24 °C | 10 | R1 | 1.01 |
| 11 | B 24 °C D10 R2 | 24 °C | 10 | R2 | 2.87 |
| 12 | B 24 °C D10 R3 | 24 °C | 10 | R3 | 2.38 |
| 13 | B 16 °C D20 R1 | 16 °C | 20 | R1 | 1.29 |
| 14 | B 16 °C D20 R2 | 16 °C | 20 | R2 | 1.50 |
| 15 | B 16 °C D20 R3 | 16 °C | 20 | R3 | 1.99 |
| 16 | B 24 °C D20 R1 | 24 °C | 20 | R1 | 17.59 |
| 17 | B 24 °C D20 R2 | 24 °C | 20 | R2 | 16.57 |
| 18 | B 24 °C D20 R3 | 24 °C | 20 | R3 | 78.82 |
| 19 | B 16 °C D30 R1 | 16 °C | 30 | R1 | 7.35 |
| 20 | B 16 °C D30 R2 | 16 °C | 30 | R2 | 19.58 |
| 21 | B 16 °C D30 R3 | 16 °C | 30 | R3 | 51.98 |
| 22 | B 24 °C D30 R1 | 24 °C | 30 | R1 | 28.40 |
| 23 | B 24 °C D30 R2 | 24 °C | 30 | R2 | 26.34 |

| | | | | | |
|---|---|---|---|---|---|
| 24 | B 24 °C D30 R3 | 24 °C | 30 | R3 | 2.85 |
| 25 | B 16 °C AF R1 | 16 °C | AM | R1 | 2.36 |
| 26 | B 16 °C AF R2 | 16 °C | AM | R2 | 2.32 |
| 27 | B 16 °C AF R3 | 16 °C | AM | R3 | 5.12 |
| 28 | B 24 °C AF R1 | 24 °C | AM | R1 | 4.86 |
| 29 | B 24 °C AF R2 | 24 °C | AM | R2 | 3.30 |
| 30 | B 24 °C AF R3 | 24 °C | AM | R3 | 2.44 |

***Table S 2***. DNA concentration from each water sample at 16 °C and 24 °C. (AM) After mobilization phase.

| Sample Nº | Sample ID | Temperature (°C) | Day | Replicate | [DNA] ng/µL |
|---|---|---|---|---|---|
| 31 | W 16 °C D0 R1 | 16 °C | 0 | R1 | 51.93 |
| 32 | W 16 °C D0 R2 | 16 °C | 0 | R2 | 21.1 |
| 33 | W 16 °C D0 R3 | 16 °C | 0 | R3 | 20.27 |
| 34 | W 24 °C D0 R1 | 24 °C | 0 | R1 | 17.39 |
| 35 | W 24 °C D0 R1 | 24 °C | 0 | R2 | 24.11 |
| 36 | W 24 °C D0 R1 | 24 °C | 0 | R3 | 22.25 |
| 37 | W 16 °C D10 R1 | 16 °C | 10 | R1 | 55.65 |
| 38 | W 16 °C D10 R2 | 16 °C | 10 | R2 | 117.7 |
| 39 | W 16 °C D10 R3 | 16 °C | 10 | R3 | 110.3 |
| 40 | W 24 °C D10 R1 | 24 °C | 10 | R1 | 20.04 |
| 41 | W 24 °C D10 R2 | 24 °C | 10 | R2 | 24.01 |
| 42 | W 24 °C D10 R3 | 24 °C | 10 | R3 | 36.12 |
| 43 | W 16 °C D20 R1 | 16 °C | 20 | R1 | 60.37 |
| 44 | W 16 °C D20 R2 | 16 °C | 20 | R2 | 76.8 |
| 45 | W 16 °C D20 R3 | 16 °C | 20 | R3 | 81.62 |
| 46 | W 24 °C D20 R1 | 24 °C | 20 | R1 | 79.15 |
| 47 | W 24 °C D20 R2 | 24 °C | 20 | R2 | 36.57 |
| 48 | W 24 °C D20 R3 | 24 °C | 20 | R3 | 79.03 |
| 49 | W 16 °C D30 R1 | 16 °C | 30 | R1 | 27.99 |
| 50 | W 16 °C D30 R2 | 16 °C | 30 | R2 | 7.74 |
| 51 | W 16 °C D30 R3 | 16 °C | 30 | R3 | 22.52 |
| 52 | W 24 °C D30 R1 | 24 °C | 30 | R1 | 35.71 |
| 53 | W 24 °C D30 R2 | 24 °C | 30 | R2 | 31.09 |
| 54 | W 24 °C D30 R3 | 24 °C | 30 | R3 | 33.84 |
| 55 | W 16 °C AF R1 | 16 °C | AM | R1 | 124.7 |
| 56 | W 16 °C AF R2 | 16 °C | AM | R2 | 187.3 |
| 57 | W 16 °C AF R3 | 16 °C | AM | R3 | 176.7 |
| 58 | W 24 °C AF R1 | 24 °C | AM | R1 | 137.4 |

| | | | | | |
|---|---|---|---|---|---|
| 59 | W 24 °C AF R2 | 24 °C | AM | R2 | 191.3 |
| 60 | W 24 °C AF R3 | 24 °C | AM | R3 | 166.1 |

## A.2 Sequences counts

***Table S 3***. Sequence count during each step of bioinformatic analysis of the bacterial 16S rRNA gene in all biofilm and water samples at 16 °C and 24 °C. (-) Samples that did not amplify or were excluded during the analysis for not having enough readings.

| Sample ID | Raw reads | Filtered and imported to QIIME2 | After join pair-ends | After dereplication | After clustering (97%) | After chimera filtering | Rarefied | Total OTUs observed |
|---|---|---|---|---|---|---|---|---|
| B 16 °C D10 R1 | 90654 | 57811 | 25498 | 25498 | 25498 | 23937 | 9854 | 329 |
| B 16 °C D10 R2 | 86375 | 53532 | 26774 | 26774 | 26774 | 25527 | 9854 | 204 |
| B 16 °C D10 R3 | 58467 | 25624 | 10383 | 10383 | 10383 | 9854 | 9854 | 339 |
| B 24 °C D10 R1 | 119559 | 86716 | 40053 | 40053 | 40053 | 36681 | 9854 | 239 |
| B 24 °C D10 R2 | 100450 | 67607 | 32266 | 32266 | 32266 | 29676 | 9854 | 257 |
| B 24 °C D10 R3 | 143669 | 110826 | 48742 | 48742 | 48742 | 45702 | 9854 | 157 |
| B 16 °C D20 R1 | 63306 | 30641 | 12533 | 12533 | 12533 | 11540 | 9854 | 125 |
| B 16 °C D20 R2 | 100292 | 67627 | 25541 | 25541 | 25541 | 24157 | 9854 | 335 |
| B 16 °C D20 R3 | 102204 | 69539 | 29148 | 29148 | 29148 | 26317 | 9854 | 377 |
| B 24 °C D20 R1 | 92378 | 59713 | 21657 | 21657 | 21657 | 20910 | 9854 | 155 |
| B 24 °C D20 R2 | 148416 | 115751 | 47890 | 47890 | 47890 | 44270 | 9854 | 201 |
| B 24 °C D20 R3 | 123752 | 91087 | 39391 | 39391 | 39391 | 33719 | 9854 | 215 |
| B 16 °C D30 R1 | 98498 | 68448 | 24660 | 24660 | 24660 | 22167 | 9854 | 393 |
| B 16 °C D30 R2 | 103155 | 73105 | 21179 | 21179 | 21179 | 18987 | 9854 | 267 |
| B 16 °C D30 R3 | 83209 | 53159 | 21081 | 21081 | 21081 | 18163 | 9854 | 250 |
| B 24 °C D30 R1 | 63053 | 33003 | 12601 | 12601 | 12601 | 11873 | 9854 | 254 |
| B 24 °C D30 R2 | 106780 | 76730 | 26345 | 26345 | 26345 | 23871 | 9854 | 204 |
| B 24 °C D30 R3 | 74908 | 44858 | 13987 | 13987 | 13987 | 11991 | 9854 | 184 |
| B 16 °C AF R1 | 101126 | 68461 | 33367 | 33367 | 33367 | 31267 | 9854 | 324 |
| B 16 °C AF R2 | 63313 | 30648 | 15250 | 15250 | 15250 | 14367 | 9854 | 352 |
| B 16 °C AF R3 | 86112 | 53447 | 24243 | 24243 | 24243 | 21931 | 9854 | 364 |
| B 24 °C AF R1 | 102228 | 69563 | 28974 | 28974 | 28974 | 25765 | 9854 | 208 |
| B 24 °C AF R2 | 98473 | 65808 | 28939 | 28939 | 28939 | 25458 | 9854 | 213 |
| B 24 °C AF R3 | 82766 | 50101 | 22055 | 22055 | 22055 | 18899 | 9854 | 225 |
| W 16 °C D0 R1 | 103546 | 58130 | 30835 | 30835 | 30835 | 29017 | 9854 | 772 |
| W 16 °C D0 R2 | 108289 | 54253 | 26617 | 26617 | 26617 | 24741 | 9854 | 706 |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| W 16 °C D0 R3 | 110607 | 55843 | 25465 | 25465 | 25465 | 23332 | 9854 | 581 |
| W 24 °C D0 R1 | 92612 | 70321 | 45353 | 45353 | 45353 | 44453 | 9854 | 222 |
| W 24 °C D0 R1 | 97295 | 78187 | 49330 | 49330 | 49330 | 48210 | 9854 | 218 |
| W 24 °C D0 R1 | 113712 | 66268 | 42349 | 42349 | 42349 | 41441 | 9854 | 257 |
| W 16 °C D10 R1 | 90973 | 112292 | 51047 | 51047 | 51047 | 47697 | 9854 | 180 |
| W 16 °C D10 R2 | 87096 | 83323 | 39837 | 39837 | 39837 | 36247 | 9854 | 253 |
| W 16 °C D10 R3 | 88686 | 207910 | 65752 | 65752 | 65752 | 61507 | 9854 | 269 |
| W 24 °C D10 R1 | 103164 | 57199 | 37592 | 37592 | 37592 | 35435 | 9854 | 245 |
| W 24 °C D10 R2 | 111030 | 70383 | 46158 | 46158 | 46158 | 43468 | 9854 | 257 |
| W 24 °C D10 R3 | 99111 | 58433 | 38999 | 38999 | 38999 | 35778 | 9854 | 270 |
| W 16 °C D20 R1 | 145135 | 118806 | 54499 | 54499 | 54499 | 50772 | 9854 | 154 |
| W 16 °C D20 R2 | 116166 | 93391 | 42593 | 42593 | 42593 | 39880 | 9854 | 159 |
| W 16 °C D20 R3 | 240753 | 74727 | 34978 | 34978 | 34978 | 32727 | 9854 | 160 |
| W 24 °C D20 R1 | 90042 | 59852 | 38456 | 38456 | 38456 | 35625 | 9854 | 208 |
| W 24 °C D20 R2 | 103226 | 69793 | 48328 | 48328 | 48328 | 43770 | 9854 | 277 |
| W 24 °C D20 R3 | 91276 | 49698 | 33604 | 33604 | 33604 | 30819 | 9854 | 196 |
| W 16 °C D30 R1 | 148856 | 91164 | 46360 | 46360 | 46360 | 43558 | 9854 | 160 |
| W 16 °C D30 R2 | 123441 | 81700 | 37497 | 37497 | 37497 | 34945 | 9854 | 172 |
| W 16 °C D30 R3 | 104777 | 95054 | 43827 | 43827 | 43827 | 41531 | 9854 | 141 |
| W 24 °C D30 R1 | 89902 | 64826 | 37638 | 37638 | 37638 | 35232 | 9854 | 224 |
| W 24 °C D30 R2 | 99843 | 74058 | 43199 | 43199 | 43199 | 40506 | 9854 | 202 |
| W 24 °C D30 R3 | 79748 | 70407 | 41122 | 41122 | 41122 | 38537 | 9854 | 203 |
| W 16 °C AF R1 | 121214 | 70881 | 46666 | 46666 | 46666 | 42260 | 9854 | 146 |
| W 16 °C AF R2 | 111750 | 75624 | 40007 | 40007 | 40007 | 36397 | 9854 | 213 |
| W 16 °C AF R3 | 125104 | 77942 | 41583 | 41583 | 41583 | 37595 | 9854 | 211 |
| W 24 °C AF R1 | 94876 | 59947 | 41714 | 41714 | 41714 | 37138 | 9854 | 147 |
| W 24 °C AF R2 | 104108 | 64630 | 44141 | 44141 | 44141 | 39938 | 9854 | 139 |
| W 24 °C AF R3 | 100457 | 81047 | 54482 | 54482 | 54482 | 49931 | 9854 | 140 |

***Table S 4.*** Sequence count during each step of bioinformatic analysis of the fungal ITS1-2 region in all biofilm and water samples at 16 °C and 24 °C. (-) Samples that did not amplify or were excluded during the analysis for not having enough readings.

| Sample ID | Raw reads | Filtered and imported to QIIME2 | After join pair-ends | After dereplication | After clustering (97%) | After chimera filtering | Rarefied | Total OTUs observed |
|---|---|---|---|---|---|---|---|---|
| B 16 °C D10 R1 | 33476 | 20363 | 10160 | 10160 | 10160 | 10138 | 5702 | 110 |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| B 16 °C D10 R2 | 35243 | 22130 | 11015 | 11015 | 11015 | 11006 | 5702 | 92 |
| B 16 °C D10 R3 | 51327 | 38214 | 19107 | 19107 | 19107 | 19017 | 5702 | 99 |
| B 24 °C D10 R1 | 36289 | 18140 | 9140 | 9140 | 9140 | 9123 | 5702 | 104 |
| B 24 °C D10 R2 | 66985 | 64876 | 31472 | 31472 | 31472 | 31096 | 5702 | 64 |
| B 24 °C D10 R3 | 39603 | 26490 | 13204 | 13204 | 13204 | 12701 | 5702 | 102 |
| B 16 °C D20 R1 | 119118 | 99843 | 49921 | 49921 | 49921 | 49123 | 5702 | 136 |
| B 16 °C D20 R2 | 83599 | 64324 | 32110 | 32110 | 32110 | 32030 | 5702 | 141 |
| B 16 °C D20 R3 | - | - | - | - | - | - | - | - |
| B 24 °C D20 R1 | 82637 | 63362 | 31446 | 31446 | 31446 | 31276 | 5702 | 57 |
| B 24 °C D20 R2 | 99175 | 79900 | 39526 | 39526 | 39526 | 39226 | 5702 | 69 |
| B 24 °C D20 R3 | 74288 | 55013 | 27236 | 27236 | 27236 | 27123 | 5702 | 55 |
| B 16 °C D30 R1 | 72130 | 50525 | 1195 | 1195 | 1195 | 1011 | - | |
| B 16 °C D30 R2 | 48577 | 26972 | 482 | 482 | 482 | 417 | - | - |
| B 16 °C D30 R3 | 50784 | 29179 | 492 | 492 | 492 | 435 | - | - |
| B 24 °C D30 R1 | 155620 | 134015 | 6258 | 6257 | 6257 | 5969 | 5702 | 42 |
| B 24 °C D30 R2 | 224547 | 202942 | 4016 | 4016 | 4016 | 3609 | - | - |
| B 24 °C D30 R3 | 54948 | 33343 | 649 | 649 | 649 | 562 | - | - |
| B 16 °C AF R1 | 127405 | 108130 | 51574 | 51574 | 51574 | 51299 | 5702 | 155 |
| B 16 °C AF R2 | 71460 | 52185 | 25049 | 25049 | 25049 | 25183 | 5702 | 122 |
| B 16 °C AF R3 | 107740 | 88465 | 43141 | 43141 | 43141 | 43983 | 5702 | 164 |
| B 24 °C AF R1 | 217800 | 198525 | 93673 | 93673 | 93673 | 92710 | 5702 | 64 |
| B 24 °C AF R2 | 72890 | 53615 | 26802 | 26802 | 26802 | 26673 | 5702 | 72 |
| B 24 °C AF R3 | 184030 | 164755 | 80930 | 80930 | 80930 | 80187 | 5702 | 64 |
| W 16 °C D0 R1 | 121078 | 101803 | 47311 | 47311 | 47311 | 47170 | 5702 | 195 |
| W 16 °C D0 R2 | 88977 | 69702 | 33967 | 33967 | 33967 | 33741 | 5702 | 151 |
| W 16 °C D0 R3 | 51542 | 32267 | 15470 | 15470 | 15470 | 15431 | 5702 | 149 |
| W 24 °C D0 R1 | 105273 | 85998 | 41474 | 41474 | 41474 | 41310 | 5702 | 131 |
| W 24 °C D0 R1 | 141050 | 121775 | 58559 | 58559 | 58559 | 58205 | 5702 | 108 |
| W 24 °C D0 R1 | 119732 | 100457 | 49244 | 49244 | 49244 | 49111 | 5702 | 122 |
| W 16 °C D10 R1 | 92193 | 79080 | 38360 | 38360 | 38360 | 37941 | 5702 | 256 |
| W 16 °C D10 R2 | 250662 | 237549 | 117611 | 117611 | 117611 | 116806 | 5702 | 151 |
| W 16 °C D10 R3 | 157941 | 144828 | 70127 | 70127 | 70127 | 69873 | 5702 | 149 |
| W 24 °C D10 R1 | 63939 | 50826 | 24615 | 24615 | 24615 | 24562 | 5702 | 91 |
| W 24 °C D10 R2 | 138901 | 125788 | 59520 | 59520 | 59520 | 58706 | 5702 | 232 |
| W 24 °C D10 R3 | 116915 | 103802 | 49168 | 49168 | 49168 | 48694 | 5702 | 225 |
| W 16 °C D20 R1 | 69449 | 56336 | 27550 | 27550 | 27550 | 27054 | 5702 | 215 |
| W 16 °C D20 R2 | 66311 | 53198 | 25566 | 25566 | 25566 | 25247 | 5702 | 225 |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| W 16 °C D20 R3 | 52672 | 39559 | 17196 | 17196 | 17196 | 16779 | 5702 | 209 |
| W 24 °C D20 R1 | 70515 | 57402 | 27734 | 27734 | 27734 | 27409 | 5702 | 244 |
| W 24 °C D20 R2 | 69113 | 56000 | 26014 | 26014 | 26014 | 25852 | 5702 | 164 |
| W 24 °C D20 R3 | 90444 | 77331 | 36033 | 36033 | 36033 | 35403 | 5702 | 227 |
| W 16 °C D30 R1 | - | - | - | - | - | - | - | - |
| W 16 °C D30 R2 | - | - | - | - | - | - | - | - |
| W 16 °C D30 R3 | - | - | - | - | - | - | - | - |
| W 24 °C D30 R1 | - | - | - | - | - | - | - | - |
| W 24 °C D30 R2 | - | - | - | - | - | - | - | - |
| W 24 °C D30 R3 | 96667 | 75062 | 14351 | 14351 | 14351 | 13356 | 5702 | 55 |
| W 16 °C AF R1 | 81171 | 68058 | 32326 | 32326 | 32326 | 32079 | 5702 | 228 |
| W 16 °C AF R2 | 174353 | 161240 | 76121 | 76121 | 76121 | 75648 | 5702 | 179 |
| W 16 °C AF R3 | - | - | - | - | - | - | - | - |
| W 24 °C AF R1 | 51868 | 38755 | 18248 | 18248 | 18248 | 18080 | 5702 | 245 |
| W 24 °C AF R2 | 55335 | 42222 | 19283 | 19283 | 19283 | 19120 | 5702 | 227 |
| W 24 °C AF R3 | 39451 | 26338 | 12320 | 12320 | 12320 | 12183 | 5702 | 208 |

## A.3 Rarefaction curves

⇒ **Bacteria**

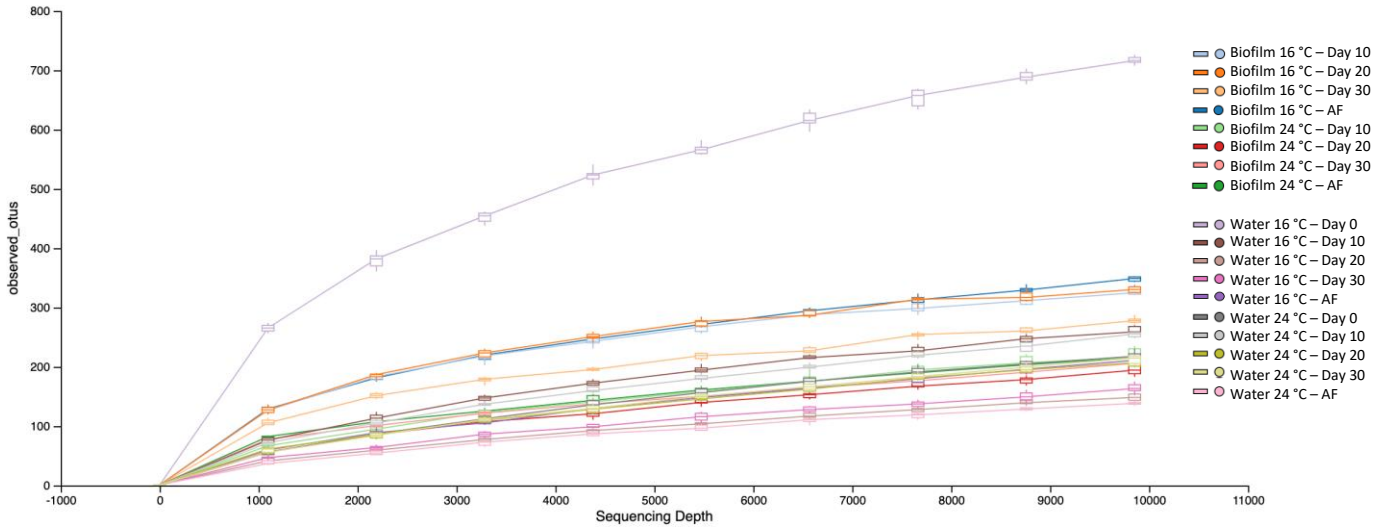



***Figure S 1.*** Rarefaction curves of observed OTUS for bacteria in all biofilm and water samples at 16 °C and 24 °C, applying a sequencing depth of 9854 sequences. Each curve represents an average of all biological replicates of each sample.

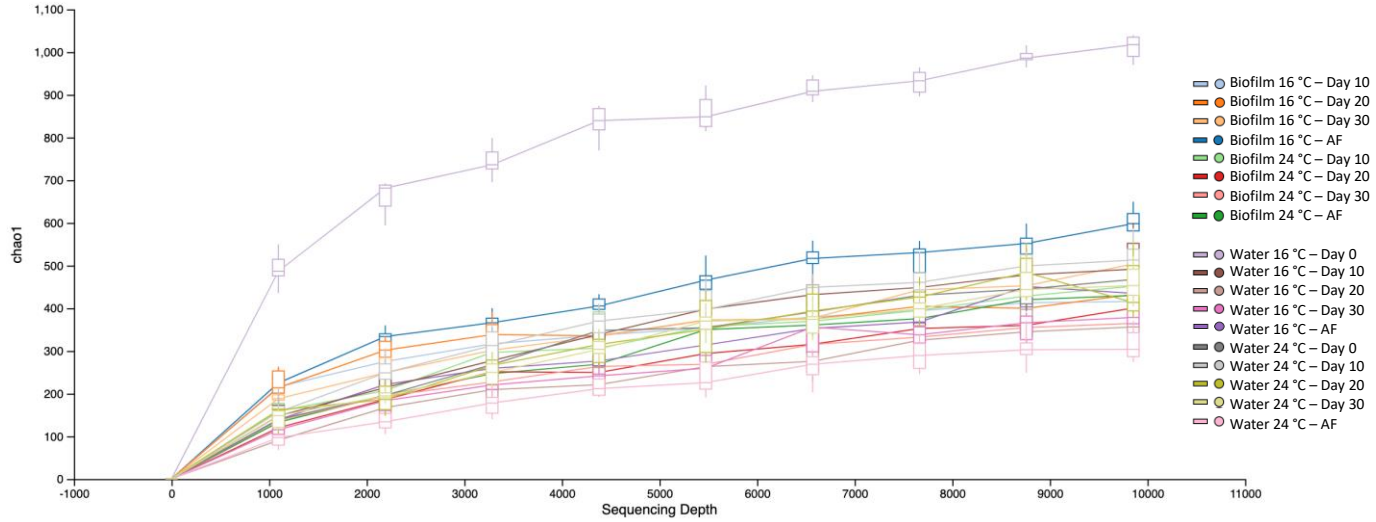



***Figure S 2***. Rarefaction curves of Chao1 index for bacteria in all biofilm and water samples at 16 °C and 24 °C, applying a sequencing depth of 9854 sequences. Each curve represents an average of all biological replicates of each sample.

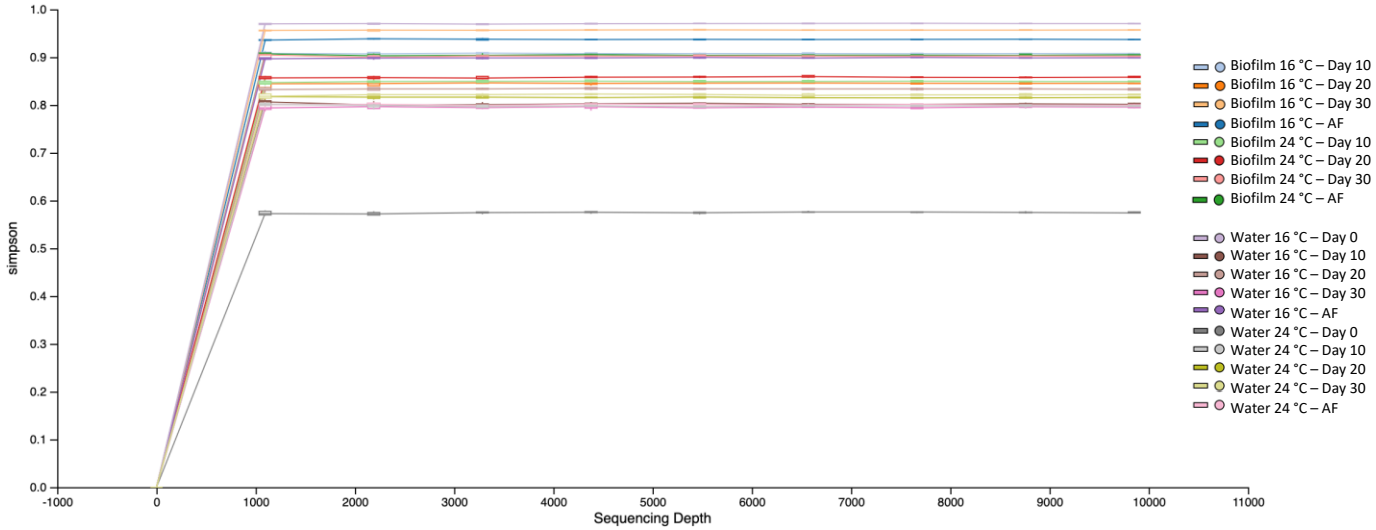



***Figure S 3.*** Rarefaction curves of Simpson index for bacteria in all biofilm and water samples at 16 °C and 24 °C, applying a sequencing depth of 9854 sequences. Each curve represents an average of all biological replicates of each sample.

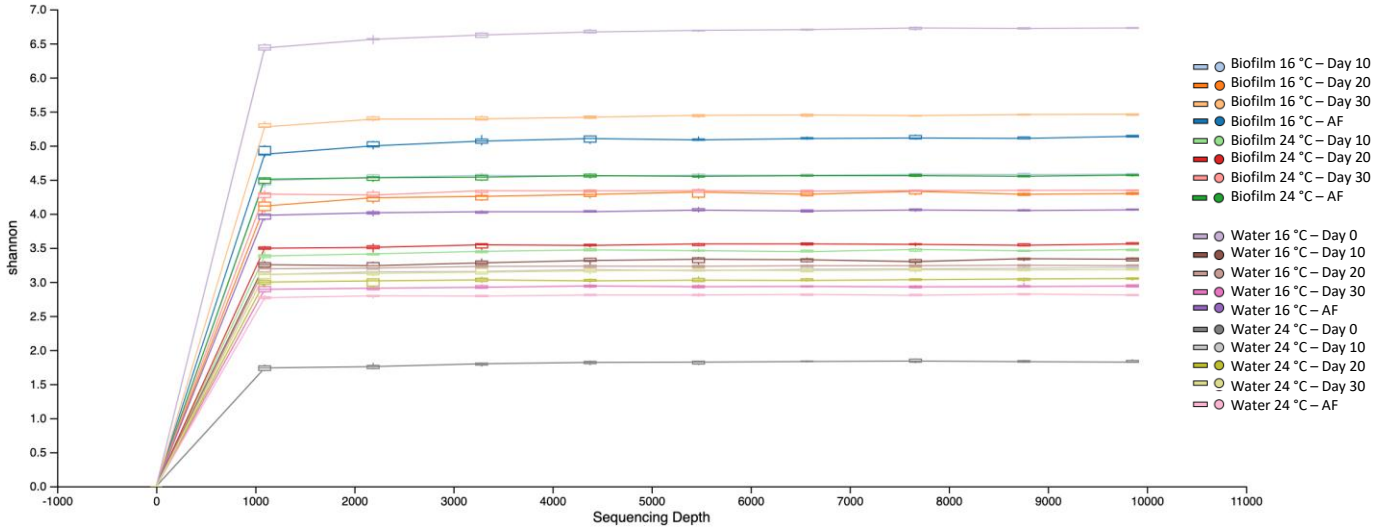



***Figure S 4.*** Rarefaction curves of Shannon index for bacteria in all biofilm and water samples at 16 °C and 24 °C, applying a sequencing depth of 9854 sequences. Each curve represents an average of all biological replicates of each sample.

⇒ **Fungi**

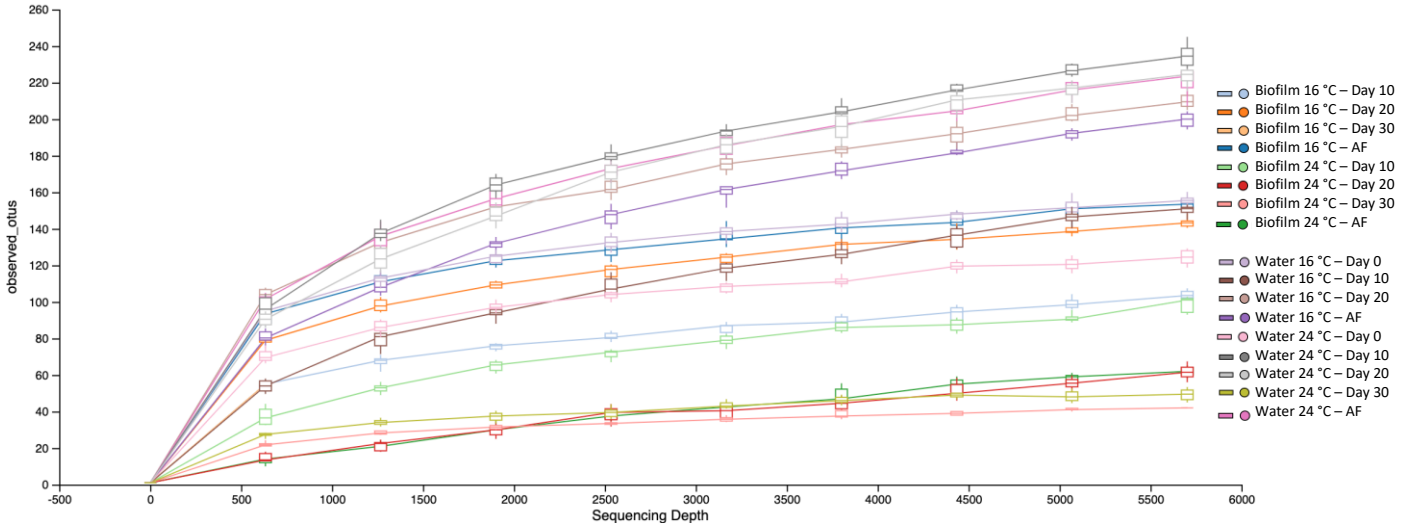



***Figure S 5***. Rarefaction curves of observed OTUS for fungi in all biofilm and water samples at 16 °C and 24 °C, applying a sequencing depth of 5702 sequences. Each curve represents an average of all biological replicates of each sample.

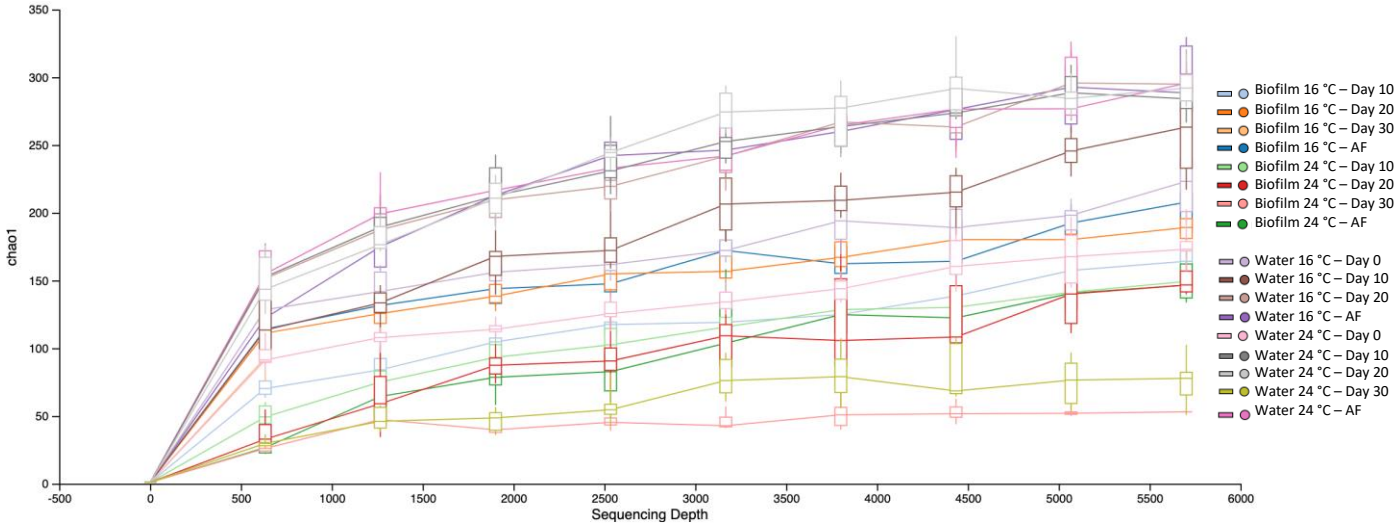



***Figure S 6.*** Rarefaction curves of Chao1 index for fungi in all biofilm and water samples at 16 °C and 24 °C, applying a sequencing depth of 5702 sequences. Each curve represents an average of all biological replicates of each sample.

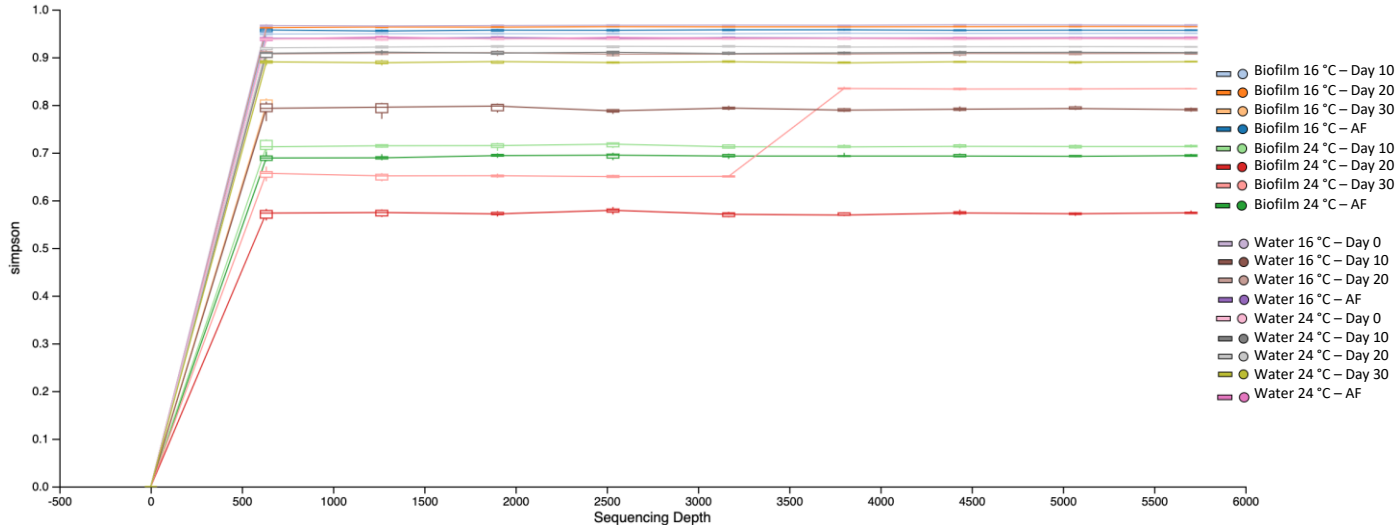



***Figure S 7.*** Rarefaction curves of Simpson index for fungi in all biofilm and water samples at 16 °C and 24 °C, applying a sequencing depth of 5702 sequences. Each curve represents an average of all biological replicates of each sample.

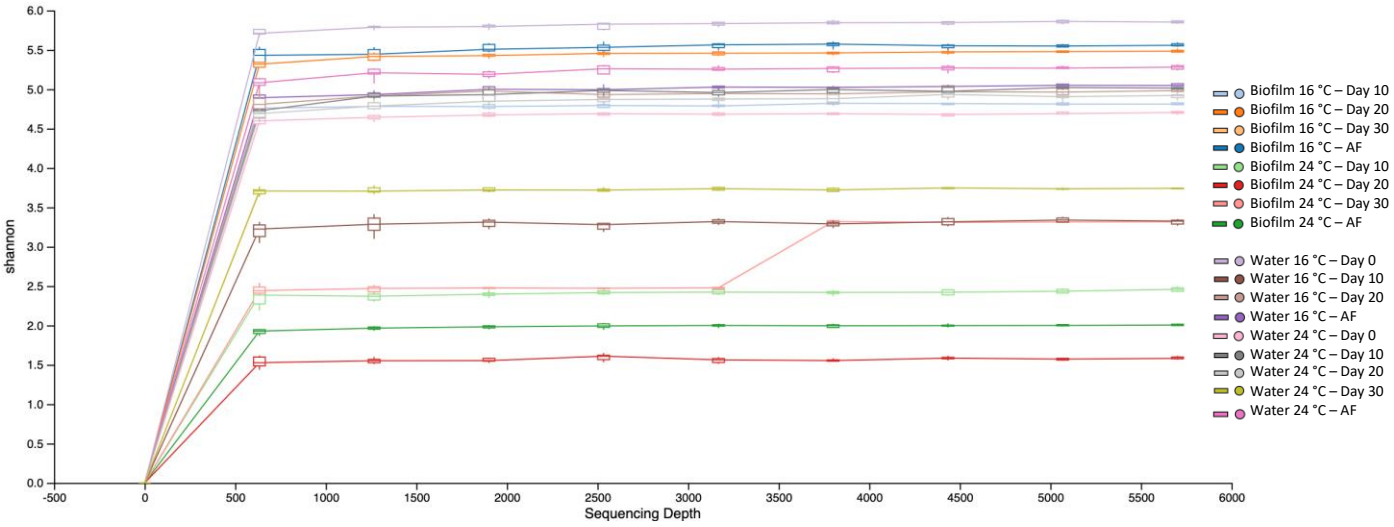



***Figure S 8.*** Rarefaction curves of Shannon index for fungi in all biofilm and water samples at 16 °C and 24 °C, applying a sequencing depth of 5702 sequences. Each curve represents an average of all biological replicates of each sample.

## A.4 Statistical analysis

***Table S 5***. Results from the Mann-Whitney *U* test to determine statistical differences in water-physico chemical parameters between temperatures. Differences were considered statistically significant when p-value was ≤0.05.

| **Mann-Whitney *U* test** | **Total chlorine** | **Free chlorine** | **Fe** | **Mn** | **TOC** |
|---|---|---|---|---|---|
| Day 0 | W = 7.5<br>p-value = 0.26 | W = 9<br>p-value = 0.1 | W = 0<br>p-value = 0.1 | W = 9<br>p-value = 0.01 | W = 0<br>p-value = 0.1 |
| Day 10 | W = 9<br>p-value = 0.07 | W = 6<br>p-value = 0.65 | W = 0<br>p-value = 0.1 | W = 0<br>p-value = 0.1 | W = 0<br>p-value = 0.1 |
| Day 20 | W = 9<br>p-value = 0.07 | W = 9<br>p-value = 0.65 | W = 0<br>p-value = 0.1 | W = 0<br>p-value = 0.1 | W = 1<br>p-value = 0.1 |
| Day 30 | W = 8<br>p-value = 0.18 | W = 9<br>p-value = 0.08 | W = 0<br>p-value = 0.1 | W = 9<br>p-value = 0.1 | W = 0<br>p-value = 0.1 |
| AF | W = 2<br>p-value = 0.34 | W = 9<br>p-value = 0.07 | W = 0<br>p-value = 0.05 | W = 0<br>p-value = 0.05 | W = 9<br>p-value = 0.1 |

***Table S 6***. Results from the Mann-Whitney *U* test to determine statistical differences between temperatures in turbidity response in each step of the mobilisation phase. Differences were considered statistically significant when p-value was ≤0.05.

| **Mann-Whitney *U* test** | **Turbidity** |
|---|---|
| Last 24 hours | W = 15254<br>p-value = 0.79 |
| Step 1 | W = 1135<br>p-value = 0.67 |
| Step 2 | W = 1201<br>p-value = 0.115 |
| Step 3 | W = 354<br>p-value = 0.0009469 |
| Step 4 | W = 276<br>p-value = 0.0005879 |

***Table S 7***. Results from the Mann-Whitney *U* test to determine statistical differences in bacterial alpha diversity indices between temperatures. Differences were considered statistically significant when p-value was ≤0.05.

| **Mann-Whitney *U* test** | | **Bacteria** | | |
|---|---|---|---|---|
| | | **Chao1** | **Simpson** | **Shannon** |
| Biofilm | Day 10 | W = 2<br>p-value = 0.4 | W = 2<br>p-value = 0.4 | W = 9<br>p-value = 0.01 |
| | Day 20 | W = 6<br>p-value = 0.35 | W = 5<br>p-value = 0.5 | W = 6<br>p-value = 0.35 |
| | Day 30 | W = 8<br>p-value = 0.1 | W = 3<br>p-value = 0.35 | W = 8<br>p-value = 0.1 |
| | AF | W = 9<br>p-value = 0.05 | W = 0<br>p-value = 0.06 | W = 9<br>p-value = 0.05 |
| Water | Day 0 | W = 7<br>p-value = 0.9 | W = 3<br>p-value = 0.8 | W = 6<br>p-value = 0.7 |
| | Day 10 | W = 3<br>p-value = 0.8 | W = 4<br>p-value = 0.5 | W = 6<br>p-value = 0.35 |
| | Day 20 | W = 2<br>p-value = 0.2 | W = 3<br>p-value = 0.35 | W = 5<br>p-value = 0.5 |
| | Day 30 | W = 2<br>p-value = 0.2 | W = 8<br>p-value = 0.1 | W = 2<br>p-value = 0.2 |
| | AF | W = 8<br>p-value = 0.1 | W = 2<br>p-value = 0.2 | W = 9<br>p-value = 0.05 |

***Table S 8.*** Results from the Mann-Whitney U test to determine statistical differences in fungal diversity indices between temperatures. Differences were considered statistically significant when p-value was ≤0.05

| **Mann-Whitney *U* test** | | **Fungi** | | |
|---|---|---|---|---|
| | | **Chao1** | **Simpson** | **Shannon** |
| Biofilm | Day 10 | W = 6<br>p-value = 0.35 | W = 0<br>p-value = 0.05 | W = 9<br>p-value = 0.05 |
| | Day 20 | W = 9<br>p-value = 0.05 | W = 0<br>p-value = 0.05 | W = 9<br>p-value = 0.05 |
| | Day 30 | W = 9<br>p-value = 0.05 | W = 0<br>p-value = 0.02 | W = 9<br>p-value = 0.35 |
| | AF | W = 9<br>p-value = 0.05 | W = 0<br>p-value = 0.05 | W = 9<br>p-value = 0.05 |
| Water | Day 0 | W = 7<br>p-value = 0.06 | W = 3<br>p-value = 0.95 | W = 0<br>p-value = 0.1 |
| | Day 10 | W = 3<br>p-value = 0.5 | W = 4<br>p-value = 0.35 | W = 6<br>p-value = 0.35 |
| | Day 20 | W = 2<br>p-value = 0.5 | W = 3<br>p-value = 0.35 | W = 5<br>p-value = 0.5 |
| | Day 30 | - | - | - |
| | AF | W = 0<br>p-value = 0.05 | W = 4<br>p-value = 0.5 | W = 2<br>p-value = 0.2 |

# Appendix B. Supplementary material for Chapter 5

This appendix includes supplementary tables and figures with the results used for the analysis discussed in chapter 5 of this manuscript, "The effects of temperature n biofilm control strategies in chlorinated drinking water systems".

## B.1 DNA concentration

***Table S 9*.** DNA concentration from each biofilm sample at 16 °C and 24 °C after different management strategies. (-) No results or treatment; F + HC: after flushing and hyperchlorination; F: after only flushing; T: after transition; P-F: post-flushing.

| Sample Nº | Sample ID | Temperature (°C) | Day | Treatment | Loop | Replicate | [DNA] ng/µL |
|---|---|---|---|---|---|---|---|
| 1 | B 16 °C D0 R1 | 16 °C | 0 | - | 1 | R1 | - |
| 2 | B 16 °C D0 R2 | 16 °C | 0 | - | 2 | R2 | - |
| 3 | B 16 °C D0 R3 | 16 °C | 0 | - | 3 | R3 | - |
| 4 | B 24 °C D0 L1 R1 | 24 °C | 0 | F + HC | 1 | R1 | - |
| 5 | B 24 °C D0 L1 R2 | 24 °C | 0 | F + HC | 1 | R2 | - |
| 6 | B 24 °C D0 L1 R3 | 24 °C | 0 | F + HC | 1 | R3 | - |
| 7 | B 24 °C D0 L2 R1 | 24 °C | 0 | F | 2 | R1 | - |
| 8 | B 24 °C D0 L2 R2 | 24 °C | 0 | F | 2 | R2 | - |
| 9 | B 24 °C D0 L2 R3 | 24 °C | 0 | F | 2 | R3 | - |
| 10 | B 24 °C D0 L3 R1 | 24 °C | 0 | T | 3 | R1 | - |
| 11 | B 24 °C D0 L3 R2 | 24 °C | 0 | T | 3 | R2 | - |
| 12 | B 24 °C D0 L3 R3 | 24 °C | 0 | T | 3 | R3 | - |
| 13 | B 16 °C D10 R1 | 16 °C | 10 | - | 1 | R1 | 1.90 |
| 14 | B 16 °C D10 R2 | 16 °C | 10 | - | 2 | R2 | 2.80 |
| 15 | B 16 °C D10 R3 | 16 °C | 10 | - | 3 | R3 | 2.30 |
| 16 | B 24 °C D10 L1 R1 | 24 °C | 10 | F + HC | 1 | R1 | 1.01 |
| 17 | B 24 °C D10 L1 R2 | 24 °C | 10 | F + HC | 1 | R2 | 2.87 |
| 18 | B 24 °C D10 L1 R3 | 24 °C | 10 | F + HC | 1 | R3 | 2.38 |
| 19 | B 24 °C D10 L2 R1 | 24 °C | 10 | F | 2 | R1 | 9.30 |
| 20 | B 24 °C D10 L2 R2 | 24 °C | 10 | F | 2 | R2 | 3.56 |
| 21 | B 24 °C D10 L2 R3 | 24 °C | 10 | F | 2 | R3 | 2.99 |
| 22 | B 24 °C D10 L3 R1 | 24 °C | 10 | T | 3 | R1 | 26.54 |
| 23 | B 24 °C D10 L3 R2 | 24 °C | 10 | T | 3 | R2 | 34.14 |
| 24 | B 24 °C D10 L3 R3 | 24 °C | 10 | T | 3 | R3 | 11.56 |
| 25 | B 16 °C D20 R1 | 16 °C | 20 | - | 1 | R1 | 1.29 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| 26 | B 16 °C D20 R2 | 16 °C | 20 | - | 2 | R2 | 1.50 |
| 27 | B 16 °C D20 R3 | 16 °C | 20 | - | 3 | R3 | 1.99 |
| 28 | B 24 °C D20 L1 R1 | 24 °C | 20 | F + HC | 1 | R1 | 17.59 |
| 29 | B 24 °C D20 L1 R2 | 24 °C | 20 | F + HC | 1 | R2 | 16.57 |
| 30 | B 24 °C D20 L1 R3 | 24 °C | 20 | F + HC | 1 | R3 | 78.82 |
| 31 | B 24 °C D20 L2 R1 | 24 °C | 20 | F | 2 | R1 | 29.26 |
| 32 | B 24 °C D20 L2 R2 | 24 °C | 20 | F | 2 | R2 | 3.47 |
| 33 | B 24 °C D20 L2 R3 | 24 °C | 20 | F | 2 | R3 | 8.23 |
| 34 | B 24 °C D20 L3 R1 | 24 °C | 20 | T | 3 | R1 | 41.33 |
| 35 | B 24 °C D20 L3 R2 | 24 °C | 20 | T | 3 | R2 | 30.93 |
| 36 | B 24 °C D20 L3 R3 | 24 °C | 20 | T | 3 | R3 | 36.74 |
| 37 | B 16 °C D30 R1 | 16 °C | 30 | - | 1 | R1 | 7.35 |
| 38 | B 16 °C D30 R2 | 16 °C | 30 | - | 2 | R2 | 19.58 |
| 39 | B 16 °C D30 R3 | 16 °C | 30 | - | 3 | R3 | 51.98 |
| 40 | B 24 °C D30 L1 R1 | 24 °C | 30 | F + HC | 1 | R1 | 28.40 |
| 41 | B 24 °C D30 L1 R2 | 24 °C | 30 | F + HC | 1 | R2 | 26.34 |
| 42 | B 24 °C D30 L1 R3 | 24 °C | 30 | F + HC | 1 | R3 | 2.85 |
| 43 | B 24 °C D30 L2 R1 | 24 °C | 30 | F | 2 | R1 | 2.84 |
| 44 | B 24 °C D30 L2 R2 | 24 °C | 30 | F | 2 | R2 | 3.17 |
| 45 | B 24 °C D30 L2 R3 | 24 °C | 30 | F | 2 | R3 | 1.94 |
| 46 | B 24 °C D30 L3 R1 | 24 °C | 30 | T | 3 | R1 | 4.03 |
| 47 | B 24 °C D30 L3 R2 | 24 °C | 30 | T | 3 | R2 | 20.45 |
| 48 | B 24 °C D30 L3 R3 | 24 °C | 30 | T | 3 | R3 | 8.66 |
| 49 | B 16 °C AF R1 | 16 °C | P-F | - | 1 | R1 | 2.36 |
| 50 | B 16 °C AF R2 | 16 °C | P-F | - | 2 | R2 | 2.32 |
| 51 | B 16 °C AF R3 | 16 °C | P-F | - | 3 | R3 | 5.12 |
| 52 | B 24 °C AF L1 R1 | 24 °C | P-F | F + HC | 1 | R1 | 4.86 |
| 53 | B 24 °C AF L1 R2 | 24 °C | P-F | F + HC | 1 | R2 | 3.30 |
| 54 | B 24 °C AF L1 R3 | 24 °C | P-F | F + HC | 1 | R3 | 2.44 |
| 55 | B 24 °C AF L2 R1 | 24 °C | P-F | F | 2 | R1 | 7.54 |
| 56 | B 24 °C AF L2 R2 | 24 °C | P-F | F | 2 | R2 | 15.82 |
| 57 | B 24 °C AF L2 R3 | 24 °C | P-F | F | 2 | R3 | 4.34 |
| 58 | B 24 °C AF L3 R1 | 24 °C | P-F | T | 3 | R1 | 4.27 |
| 59 | B 24 °C AF L3 R2 | 24 °C | P-F | T | 3 | R2 | 3.61 |
| 60 | B 24 °C AF L3 R3 | 24 °C | P-F | T | 3 | R3 | 1.70 |

***Table S 10***. DNA concentration from each water sample at 16 °C and 24 °C after different management strategies. (-) No results or treatment; F + HC: after flushing and hyperchlorination; F: after only flushing; T: after transition; P-F: post-flushing.

| Sample Nº | Sample ID | Temperature (°C) | Day | Treatment | Loop | Replicate | [DNA] ng/µL |
|---|---|---|---|---|---|---|---|
| 61 | W 16 °C D0 R1 | 16 °C | 0 | - | 1 | R1 | 51.93 |
| 62 | W 16 °C D0 R2 | 16 °C | 0 | - | 2 | R2 | 21.1 |
| 63 | W 16 °C D0 R3 | 16 °C | 0 | - | 3 | R3 | 20.27 |
| 64 | W 24 °C D0 L1 R1 | 24 °C | 0 | F + HC | 1 | R1 | 17.39 |
| 65 | W 24 °C D0 L1 R2 | 24 °C | 0 | F + HC | 1 | R2 | 24.11 |
| 66 | W 24 °C D0 L1 R3 | 24 °C | 0 | F + HC | 1 | R3 | 22.25 |
| 67 | W 24 °C D0 L2 R1 | 24 °C | 0 | F | 2 | R1 | 35.56 |
| 68 | W 24 °C D0 L2 R2 | 24 °C | 0 | F | 2 | R2 | 25.5 |
| 69 | W 24 °C D0 L2 R3 | 24 °C | 0 | F | 2 | R3 | 36.19 |
| 70 | W 24 °C D0 L3 R1 | 24 °C | 0 | T | 3 | R1 | 123.3 |
| 71 | W 24 °C D0 L3 R2 | 24 °C | 0 | T | 3 | R2 | 109.9 |
| 72 | W 24 °C D0 L3 R3 | 24 °C | 0 | T | 3 | R3 | 124.5 |
| 73 | W 16 °C D10 R1 | 16 °C | 10 | - | 1 | R1 | 55.65 |
| 74 | W 16 °C D10 R2 | 16 °C | 10 | - | 2 | R2 | 117.7 |
| 75 | W 16 °C D10 R3 | 16 °C | 10 | - | 3 | R3 | 110.3 |
| 76 | W 24 °C D10 L1 R1 | 24 °C | 10 | F + HC | 1 | R1 | 20.04 |
| 77 | W 24 °C D10 L1 R2 | 24 °C | 10 | F + HC | 1 | R2 | 24.01 |
| 78 | W 24 °C D10 L1 R3 | 24 °C | 10 | F + HC | 1 | R3 | 36.12 |
| 79 | W 24 °C D10 L2 R1 | 24 °C | 10 | F | 2 | R1 | 55.93 |
| 80 | W 24 °C D10 L2 R2 | 24 °C | 10 | F | 2 | R2 | 56.4 |
| 81 | W 24 °C D10 L2 R3 | 24 °C | 10 | F | 2 | R3 | 45.63 |
| 82 | W 24 °C D10 L3 R1 | 24 °C | 10 | T | 3 | R1 | 61.77 |
| 83 | W 24 °C D10 L3 R2 | 24 °C | 10 | T | 3 | R2 | 58.97 |
| 84 | W 24 °C D10 L3 R3 | 24 °C | 10 | T | 3 | R3 | 65.57 |
| 85 | W 16 °C D20 R1 | 16 °C | 20 | - | 1 | R1 | 60.37 |
| 86 | W 16 °C D20 R2 | 16 °C | 20 | - | 2 | R2 | 76.8 |
| 87 | W 16 °C D20 R3 | 16 °C | 20 | - | 3 | R3 | 81.62 |
| 88 | W 24 °C D20 L1 R1 | 24 °C | 20 | F + HC | 1 | R1 | 79.15 |
| 89 | W 24 °C D20 L1 R2 | 24 °C | 20 | F + HC | 1 | R2 | 36.57 |
| 90 | W 24 °C D20 L1 R3 | 24 °C | 20 | F + HC | 1 | R3 | 79.03 |
| 91 | W 24 °C D20 L2 R1 | 24 °C | 20 | F | 2 | R1 | 70.47 |
| 92 | W 24 °C D20 L2 R2 | 24 °C | 20 | F | 2 | R2 | 61.93 |
| 93 | W 24 °C D20 L2 R3 | 24 °C | 20 | F | 2 | R3 | 67 |
| 94 | W 24 °C D20 L3 R1 | 24 °C | 20 | T | 3 | R1 | 52.32 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| 95 | W 24 °C D20 L3 R2 | 24 °C | 20 | T | 3 | R2 | 94.65 |
| 96 | W 24 °C D20 L3 R3 | 24 °C | 20 | T | 3 | R3 | 82.03 |
| 97 | W 16 °C D30 R1 | 16 °C | 30 | - | 1 | R1 | 27.99 |
| 98 | W 16 °C D30 R2 | 16 °C | 30 | - | 2 | R2 | 7.74 |
| 99 | W 16 °C D30 R3 | 16 °C | 30 | - | 3 | R3 | 22.52 |
| 100 | W 24 °C D30 L1 R1 | 24 °C | 30 | F + HC | 1 | R1 | 35.71 |
| 101 | W 24 °C D30 L1 R2 | 24 °C | 30 | F + HC | 1 | R2 | 31.09 |
| 102 | W 24 °C D30 L1 R3 | 24 °C | 30 | F + HC | 1 | R3 | 33.84 |
| 103 | W 24 °C D30 L2 R1 | 24 °C | 30 | F | 2 | R1 | 18.53 |
| 104 | W 24 °C D30 L2 R2 | 24 °C | 30 | F | 2 | R2 | 24.32 |
| 105 | W 24 °C D30 L2 R3 | 24 °C | 30 | F | 2 | R3 | 20.24 |
| 106 | W 24 °C D30 L3 R1 | 24 °C | 30 | T | 3 | R1 | 57.68 |
| 107 | W 24 °C D30 L3 R2 | 24 °C | 30 | T | 3 | R2 | 44.61 |
| 108 | W 24 °C D30 L3 R3 | 24 °C | 30 | T | 3 | R3 | 58.75 |
| 109 | W 16 °C AF R1 | 16 °C | AF | - | 1 | R1 | 124.7 |
| 110 | W 16 °C AF R2 | 16 °C | AF | - | 2 | R2 | 187.3 |
| 111 | W 16 °C AF R3 | 16 °C | AF | - | 3 | R3 | 176.7 |
| 112 | W 24 °C AF L1 R1 | 24 °C | AF | F + HC | 1 | R1 | 137.4 |
| 113 | W 24 °C AF L1 R2 | 24 °C | AF | F + HC | 1 | R2 | 191.3 |
| 114 | W 24 °C AF L1 R3 | 24 °C | AF | F + HC | 1 | R3 | 166.1 |
| 115 | W 24 °C AF L2 R1 | 24 °C | AF | F | 2 | R1 | 204.9 |
| 116 | W 24 °C AF L2 R2 | 24 °C | AF | F | 2 | R2 | 298.1 |
| 117 | W 24 °C AF L2 R3 | 24 °C | AF | F | 2 | R3 | 231.5 |
| 118 | W 24 °C AF L3 R1 | 24 °C | AF | T | 3 | R1 | 196.5 |
| 119 | W 24 °C AF L3 R2 | 24 °C | AF | T | 3 | R2 | 260.4 |
| 120 | W 24 °C AF L3 R3 | 24 °C | AF | T | 3 | R3 | 216 |

## B.2 Sequences counts

***Table S 11.*** Sequence counts during each step of bioinformatic analysis of the bacterial 16S rRNA gene in all biofilm and water samples at 16 °C and 24 °C after different management strategies.

| Sample ID | Raw reads | Filtered and imported to QIIME2 | After join pair-ends | After dereplication | After clustering (97%) | After chimera filtering | Rarefied | Total OTUs observed |
|---|---|---|---|---|---|---|---|---|
| B 16 °C D10 R1 | 90654 | 57811 | 25498 | 25498 | 25498 | 23371 | 8131 | 307 |
| B 16 °C D10 R2 | 86375 | 53532 | 26774 | 26774 | 26774 | 24408 | 8131 | 191 |
| B 16 °C D10 R3 | 58467 | 25624 | 10383 | 10383 | 10383 | 9802 | 8131 | 305 |
| B 24 °C D10 L1 R1 | 119559 | 86716 | 40053 | 40053 | 40053 | 36170 | 8131 | 215 |
| B 24 °C D10 L1 R2 | 100450 | 67607 | 32266 | 32266 | 32266 | 28722 | 8131 | 248 |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| B 24 °C D10 L1 R3 | 143669 | 110826 | 48742 | 48742 | 48742 | 45693 | 8131 | 143 |
| B 24 °C D10 L2 R1 | 124924 | 92259 | 37141 | 37141 | 37141 | 33174 | 8131 | 314 |
| B 24 °C D10 L2 R2 | 100549 | 67884 | 27233 | 27233 | 27233 | 24201 | 8131 | 286 |
| B 24 °C D10 L2 R3 | 129204 | 96539 | 45850 | 45850 | 45850 | 42798 | 8131 | 214 |
| B 24 °C D10 L3 R1 | 90605 | 57940 | 22720 | 22720 | 22720 | 19944 | 8131 | 264 |
| B 24 °C D10 L3 R2 | 85950 | 53285 | 22002 | 22002 | 22002 | 18818 | 8131 | 307 |
| B 24 °C D10 L3 R3 | 99470 | 66805 | 23378 | 23378 | 23378 | 19785 | 8131 | 316 |
| B 16 °C D20 R1 | 63306 | 30641 | 12533 | 12533 | 12533 | 11310 | 8131 | 143 |
| B 16 °C D20 R2 | 100292 | 67627 | 25541 | 25541 | 25541 | 23960 | 8131 | 317 |
| B 16 °C D20 R3 | 102204 | 69539 | 29148 | 29148 | 29148 | 25206 | 8131 | 343 |
| B 24 °C D20 L1 R1 | 92378 | 59713 | 21657 | 21657 | 21657 | 20828 | 8131 | 110 |
| B 24 °C D20 L1 R2 | 148416 | 115751 | 47890 | 47890 | 47890 | 44287 | 8131 | 187 |
| B 24 °C D20 L1 R3 | 123752 | 91087 | 39391 | 39391 | 39391 | 34614 | 8131 | 217 |
| B 24 °C D20 L2 R1 | 127732 | 95067 | 36055 | 36055 | 36055 | 32081 | 8131 | 239 |
| B 24 °C D20 L2 R2 | 95963 | 63298 | 26382 | 26382 | 26382 | 23574 | 8131 | 419 |
| B 24 °C D20 L2 R3 | 86587 | 53922 | 20812 | 20812 | 20812 | 18541 | 8131 | 320 |
| B 24 °C D20 L3 R1 | 80386 | 47721 | 17108 | 17108 | 17108 | 14972 | 8131 | 306 |
| B 24 °C D20 L3 R2 | 101143 | 68478 | 24514 | 24514 | 24514 | 21168 | 8131 | 342 |
| B 24 °C D20 L3 R3 | 69182 | 36517 | 11923 | 11923 | 11923 | 10523 | 8131 | 284 |
| B 16 °C D30 R1 | 98498 | 68448 | 24660 | 24660 | 24660 | 22790 | 8131 | 371 |
| B 16 °C D30 R2 | 103155 | 73105 | 21179 | 21179 | 21179 | 19364 | 8131 | 265 |
| B 16 °C D30 R3 | 83209 | 53159 | 21081 | 21081 | 21081 | 18530 | 8131 | 250 |
| B 24 °C D30 L1 R1 | 63053 | 33003 | 12601 | 12601 | 12601 | 11970 | 8131 | 243 |
| B 24 °C D30 L1 R2 | 106780 | 76730 | 26345 | 26345 | 26345 | 23942 | 8131 | 194 |
| B 24 °C D30 L1 R3 | 74908 | 44858 | 13987 | 13987 | 13987 | 12423 | 8131 | 180 |
| B 24 °C D30 L2 R1 | 84378 | 54328 | 17275 | 17275 | 17275 | 15507 | 8131 | 275 |
| B 24 °C D30 L2 R2 | 60940 | 30890 | 9532 | 9532 | 9532 | 8674 | 8131 | 298 |
| B 24 °C D30 L2 R3 | 80726 | 50676 | 16150 | 16150 | 16150 | 14344 | 8131 | 398 |
| B 24 °C D30 L3 R1 | 70504 | 40454 | 11868 | 11868 | 11868 | 10170 | 8131 | 365 |
| B 24 °C D30 L3 R2 | 69593 | 39543 | 11904 | 11904 | 11904 | 9682 | 8131 | 307 |
| B 24 °C D30 L3 R3 | 62018 | 31968 | 9149 | 9149 | 9149 | 8131 | 8131 | 298 |
| B 16 °C AF R1 | 101126 | 68461 | 33367 | 33367 | 33367 | 31079 | 8131 | 297 |
| B 16 °C AF R2 | 63313 | 30648 | 15250 | 15250 | 15250 | 14338 | 8131 | 331 |
| B 16 °C AF R3 | 86112 | 53447 | 24243 | 24243 | 24243 | 21691 | 8131 | 322 |
| B 24 °C AF L1 R1 | 102228 | 69563 | 28974 | 28974 | 28974 | 25892 | 8131 | 188 |
| B 24 °C AF L1 R2 | 98473 | 65808 | 28939 | 28939 | 28939 | 25570 | 8131 | 211 |
| B 24 °C AF L1 R3 | 82766 | 50101 | 22055 | 22055 | 22055 | 19342 | 8131 | 207 |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| B 24 °C AF L2 R1 | 96640 | 63975 | 26805 | 26805 | 26805 | 24010 | 8131 | 327 |
| B 24 °C AF L2 R2 | 120743 | 88078 | 31388 | 31388 | 31388 | 29115 | 8131 | 219 |
| B 24 °C AF L2 R3 | 109367 | 76702 | 28270 | 28270 | 28270 | 25590 | 8131 | 341 |
| B 24 °C AF L3 R1 | 95399 | 62734 | 23482 | 23482 | 23482 | 20532 | 8131 | 250 |
| B 24 °C AF L3 R2 | 87682 | 55017 | 20378 | 20378 | 20378 | 17378 | 8131 | 296 |
| B 24 °C AF L3 R3 | 105268 | 72603 | 26946 | 26946 | 26946 | 22934 | 8131 | 373 |
| W 16 °C D0 R1 | 103546 | 70881 | 46666 | 46666 | 46666 | 42013 | 8131 | 737 |
| W 16 °C D0 R2 | 108289 | 75624 | 40007 | 40007 | 40007 | 36130 | 8131 | 708 |
| W 16 °C D0 R3 | 110607 | 77942 | 41583 | 41583 | 41583 | 37358 | 8131 | 568 |
| W 24 °C D0 L1 R1 | 92612 | 59947 | 41714 | 41714 | 41714 | 36998 | 8131 | 185 |
| W 24 °C D0 L1 R2 | 97295 | 64630 | 44141 | 44141 | 44141 | 39859 | 8131 | 203 |
| W 24 °C D0 L1 R3 | 113712 | 81047 | 54482 | 54482 | 54482 | 49800 | 8131 | 219 |
| W 24 °C D0 L2 R1 | 94594 | 61929 | 40985 | 40985 | 40985 | 36451 | 8131 | 247 |
| W 24 °C D0 L2 R2 | 94968 | 62303 | 41537 | 41537 | 41537 | 36007 | 8131 | 229 |
| W 24 °C D0 L2 R3 | 95757 | 63092 | 42372 | 42372 | 42372 | 37420 | 8131 | 227 |
| W 24 °C D0 L3 R1 | 98070 | 65405 | 39819 | 39819 | 39819 | 35052 | 8131 | 160 |
| W 24 °C D0 L3 R2 | 106337 | 73672 | 44189 | 44189 | 44189 | 39325 | 8131 | 175 |
| W 24 °C D0 L3 R3 | 89698 | 57033 | 34437 | 34437 | 34437 | 30619 | 8131 | 204 |
| W 16 °C D10 R1 | 90973 | 58130 | 30835 | 30835 | 30835 | 28998 | 8131 | 169 |
| W 16 °C D10 R2 | 87096 | 54253 | 26617 | 26617 | 26617 | 24211 | 8131 | 244 |
| W 16 °C D10 R3 | 88686 | 55843 | 25465 | 25465 | 25465 | 21380 | 8131 | 252 |
| W 24 °C D10 L1 R1 | 103164 | 70321 | 45353 | 45353 | 45353 | 44438 | 8131 | 222 |
| W 24 °C D10 L1 R2 | 111030 | 78187 | 49330 | 49330 | 49330 | 48216 | 8131 | 243 |
| W 24 °C D10 L1 R3 | 99111 | 66268 | 42349 | 42349 | 42349 | 41438 | 8131 | 277 |
| W 24 °C D10 L2 R1 | 110704 | 77861 | 47037 | 47037 | 47037 | 44143 | 8131 | 174 |
| W 24 °C D10 L2 R2 | 88890 | 56047 | 34146 | 34146 | 34146 | 32222 | 8131 | 202 |
| W 24 °C D10 L2 R3 | 75001 | 42158 | 25778 | 25778 | 25778 | 24445 | 8131 | 217 |
| W 24 °C D10 L3 R1 | 95868 | 63025 | 35803 | 35803 | 35803 | 32566 | 8131 | 139 |
| W 24 °C D10 L3 R2 | 106418 | 73575 | 41720 | 41720 | 41720 | 37462 | 8131 | 175 |
| W 24 °C D10 L3 R3 | 110580 | 77737 | 43984 | 43984 | 43984 | 39505 | 8131 | 208 |
| W 16 °C D20 R1 | 145135 | 112292 | 51047 | 51047 | 51047 | 47628 | 8131 | 145 |
| W 16 °C D20 R2 | 116166 | 83323 | 39837 | 39837 | 39837 | 36159 | 8131 | 145 |
| W 16 °C D20 R3 | 240753 | 207910 | 65752 | 65752 | 65752 | 61400 | 8131 | 134 |
| W 24 °C D20 L1 R1 | 90042 | 57199 | 37592 | 37592 | 37592 | 35309 | 8131 | 203 |
| W 24 °C D20 L1 R2 | 103226 | 70383 | 46158 | 46158 | 46158 | 43292 | 8131 | 238 |
| W 24 °C D20 L1 R3 | 91276 | 58433 | 38999 | 38999 | 38999 | 35709 | 8131 | 163 |
| W 24 °C D20 L2 R1 | 114441 | 81598 | 50714 | 50714 | 50714 | 45087 | 8131 | 203 |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| W 24 °C D20 L2 R2 | 131510 | 98667 | 59363 | 59363 | 59363 | 52245 | 8131 | 194 |
| W 24 °C D20 L2 R3 | 102214 | 69371 | 43629 | 43629 | 43629 | 38293 | 8131 | 215 |
| W 24 °C D20 L3 R1 | 78295 | 45452 | 28202 | 28202 | 28202 | 26932 | 8131 | 146 |
| W 24 °C D20 L3 R2 | 141037 | 108194 | 67274 | 67274 | 67274 | 62095 | 8131 | 144 |
| W 24 °C D20 L3 R3 | 122985 | 90142 | 55596 | 55596 | 55596 | 51139 | 8131 | 152 |
| W 16 °C D30 R1 | 148856 | 118806 | 54499 | 54499 | 54499 | 50216 | 8131 | 147 |
| W 16 °C D30 R2 | 123441 | 93391 | 42593 | 42593 | 42593 | 39600 | 8131 | 155 |
| W 16 °C D30 R3 | 104777 | 74727 | 34978 | 34978 | 34978 | 32019 | 8131 | 135 |
| W 24 °C D30 L1 R1 | 89902 | 59852 | 38456 | 38456 | 38456 | 35505 | 8131 | 195 |
| W 24 °C D30 L1 R2 | 99843 | 69793 | 48328 | 48328 | 48328 | 43479 | 8131 | 176 |
| W 24 °C D30 L1 R3 | 79748 | 49698 | 33604 | 33604 | 33604 | 30700 | 8131 | 193 |
| W 24 °C D30 L2 R1 | 100970 | 70920 | 48381 | 48381 | 48381 | 44509 | 8131 | 234 |
| W 24 °C D30 L2 R2 | 62436 | 32386 | 21738 | 21738 | 21738 | 20200 | 8131 | 240 |
| W 24 °C D30 L2 R3 | 80646 | 50596 | 34072 | 34072 | 34072 | 31400 | 8131 | 236 |
| W 24 °C D30 L3 R1 | 95746 | 65696 | 45165 | 45165 | 45165 | 42522 | 8131 | 100 |
| W 24 °C D30 L3 R2 | 103186 | 73136 | 50309 | 50309 | 50309 | 46714 | 8131 | 109 |
| W 24 °C D30 L3 R3 | 83032 | 52982 | 36539 | 36539 | 36539 | 34426 | 8131 | 120 |
| W 16 °C AF R1 | 121214 | 91164 | 46360 | 46360 | 46360 | 43527 | 8131 | 140 |
| W 16 °C AF R2 | 111750 | 81700 | 37497 | 37497 | 37497 | 34811 | 8131 | 190 |
| W 16 °C AF R3 | 125104 | 95054 | 43827 | 43827 | 43827 | 41546 | 8131 | 188 |
| W 24 °C AF L1 R1 | 94876 | 64826 | 37638 | 37638 | 37638 | 35179 | 8131 | 147 |
| W 24 °C AF L1 R2 | 104108 | 74058 | 43199 | 43199 | 43199 | 40471 | 8131 | 136 |
| W 24 °C AF L1 R3 | 100457 | 70407 | 41122 | 41122 | 41122 | 38459 | 8131 | 124 |
| W 24 °C AF L2 R1 | 92766 | 62716 | 37646 | 37646 | 37646 | 35381 | 8131 | 280 |
| W 24 °C AF L2 R2 | 97763 | 67713 | 39932 | 39932 | 39932 | 36972 | 8131 | 292 |
| W 24 °C AF L2 R3 | 93850 | 63800 | 38314 | 38314 | 38314 | 35504 | 8131 | 267 |
| W 24 °C AF L3 R1 | 90917 | 60867 | 39008 | 39008 | 39008 | 38085 | 8131 | 198 |
| W 24 °C AF L3 R2 | 114275 | 84225 | 54761 | 54761 | 54761 | 52899 | 8131 | 208 |
| W 24 °C AF L3 R3 | 126626 | 96576 | 63074 | 63074 | 63074 | 60683 | 8131 | 188 |

***Table S 12.*** Sequence counts during each step of bioinformatic analysis of the fungal ITS1-2 region in all biofilm and water samples at 16 °C and 24 °C after different management strategies. (-) Samples that did not amplify or were excluded during the analysis for not having enough readings.

| Sample ID | Raw reads | Filtered and imported to QIIME2 | After join pair-ends | After dereplication | After clustering (97%) | After chimera filtering | Rarefied | Total OTUs observed |
|---|---|---|---|---|---|---|---|---|
| B 16 °C D10 R1 | 33476 | 20363 | 10160 | 10160 | 10160 | 10138 | 1011 | 64 |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| B 16 °C D10 R2 | 35243 | 22130 | 11015 | 11015 | 11015 | 11006 | 1011 | 46 |
| B 16 °C D10 R3 | 51327 | 38214 | 19107 | 19107 | 19107 | 19017 | 1011 | 66 |
| B 24 °C D10 L1 R1 | 36289 | 18140 | 9140 | 9140 | 9140 | 9123 | 1011 | 47 |
| B 24 °C D10 L1 R2 | 66985 | 64876 | 31472 | 31472 | 31472 | 31096 | 1011 | 20 |
| B 24 °C D10 L1 R3 | 39603 | 26490 | 13204 | 13204 | 13204 | 12701 | 1011 | 48 |
| B 24 °C D10 L2 R1 | 99098 | 79823 | 38458 | 38458 | 38458 | 38024 | 1011 | 40 |
| B 24 °C D10 L2 R2 | 78109 | 58834 | 27968 | 27968 | 27968 | 27818 | 1011 | 19 |
| B 24 °C D10 L2 R3 | 117500 | 98225 | 47211 | 47211 | 47211 | 47760 | 1011 | 91 |
| B 24 °C D10 L3 R1 | 72100 | 52825 | 26072 | 26072 | 26072 | 26892 | 1011 | 20 |
| B 24 °C D10 L3 R2 | 75271 | 55996 | 25148 | 25148 | 25148 | 24157 | 1011 | 18 |
| B 24 °C D10 L3 R3 | 113864 | 94589 | 43040 | 43040 | 43040 | 42544 | 1011 | 24 |
| B 16 °C D20 R1 | 119118 | 99843 | 49921 | 49921 | 49921 | 49123 | 1011 | 92 |
| B 16 °C D20 R2 | 83599 | 64324 | 32110 | 32110 | 32110 | 32030 | 1011 | 90 |
| B 16 °C D20 R3 | - | - | - | - | - | - | - | - |
| B 24 °C D20 L1 R1 | 82637 | 63362 | 31446 | 31446 | 31446 | 31276 | 1011 | 23 |
| B 24 °C D20 L1 R2 | 99175 | 79900 | 39526 | 39526 | 39526 | 39226 | 1011 | 29 |
| B 24 °C D20 L1 R3 | 74288 | 55013 | 27236 | 27236 | 27236 | 27123 | 1011 | 13 |
| B 24 °C D20 L2 R1 | 100536 | 81261 | 40024 | 40024 | 40024 | 39754 | 1011 | 21 |
| B 24 °C D20 L2 R2 | 85192 | 65917 | 32671 | 32671 | 32671 | 32304 | 1011 | 62 |
| B 24 °C D20 L2 R3 | 111357 | 92082 | 45785 | 45785 | 45785 | 45259 | 1011 | 82 |
| B 24 °C D20 L3 R1 | 64244 | 44969 | 22248 | 22248 | 22248 | 21622 | 1011 | 23 |
| B 24 °C D20 L3 R2 | 127660 | 108385 | 54912 | 54912 | 54912 | 54434 | 1011 | 41 |
| B 24 °C D20 L3 R3 | 65347 | 46072 | 22389 | 22389 | 22389 | 22772 | 1011 | 28 |
| B 16 °C D30 R1 | 72130 | 50525 | 1195 | 1195 | 1195 | 1011 | 1011 | 73 |
| B 16 °C D30 R2 | 48577 | 26972 | 482 | 482 | 482 | 417 | - | - |
| B 16 °C D30 R3 | 50784 | 29179 | 492 | 492 | 492 | 435 | - | - |
| B 24 °C D30 L1 R1 | 155620 | 134015 | 6258 | 6257 | 6257 | 5969 | 1011 | 37 |
| B 24 °C D30 L1 R2 | 224547 | 202942 | 4016 | 4016 | 4016 | 3609 | 1011 | 29 |
| B 24 °C D30 L1 R3 | 54948 | 33343 | 649 | 649 | 649 | 562 | - | - |
| B 24 °C D30 L2 R1 | 181244 | 159639 | 3248 | 3248 | 3248 | 2995 | 1011 | 31 |
| B 24 °C D30 L2 R2 | 167160 | 145555 | 9662 | 9662 | 9662 | 9382 | 1011 | 24 |
| B 24 °C D30 L2 R3 | 130964 | 109359 | 2204 | 2204 | 2204 | 1901 | 1011 | 47 |
| B 24 °C D30 L3 R1 | 147988 | 126383 | 5695 | 5695 | 5695 | 5411 | 1011 | 42 |
| B 24 °C D30 L3 R2 | 150688 | 129083 | 10863 | 10863 | 10863 | 10595 | 1011 | 39 |
| B 24 °C D30 L3 R3 | 149519 | 127914 | 2890 | 2890 | 2890 | 2753 | 1011 | 40 |
| B 16 °C AF R1 | 127405 | 108130 | 51574 | 51574 | 51574 | 51299 | 1011 | 112 |
| B 16 °C AF R2 | 71460 | 52185 | 25049 | 25049 | 25049 | 25183 | 1011 | 81 |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| B 16 °C AF R3 | 107740 | 88465 | 43141 | 43141 | 43141 | 43983 | 1011 | 114 |
| B 24 °C AF L1 R1 | 217800 | 198525 | 93673 | 93673 | 93673 | 92710 | 1011 | 21 |
| B 24 °C AF L1 R2 | 72890 | 53615 | 26802 | 26802 | 26802 | 26673 | 1011 | 21 |
| B 24 °C AF L1 R3 | 184030 | 164755 | 80930 | 80930 | 80930 | 80187 | 1011 | 23 |
| B 24 °C AF L2 R1 | 396309 | 377034 | 187496 | 187496 | 187496 | 186916 | 1011 | 19 |
| B 24 °C AF L2 R2 | 74979 | 55704 | 27818 | 27818 | 27818 | 27300 | 1011 | 22 |
| B 24 °C AF L2 R3 | 89400 | 70125 | 33755 | 33755 | 33755 | 33587 | 1011 | 43 |
| B 24 °C AF L3 R1 | 72332 | 53057 | 25334 | 25334 | 25334 | 25191 | 1011 | 18 |
| B 24 °C AF L3 R2 | 93903 | 74628 | 36357 | 36357 | 36357 | 36110 | 1011 | 26 |
| B 24 °C AF L3 R3 | 86073 | 66798 | 32528 | 32528 | 32528 | 32238 | 1011 | 54 |
| W 16 °C D0 R1 | 121078 | 101803 | 47311 | 47311 | 47311 | 47170 | 1011 | 131 |
| W 16 °C D0 R2 | 88977 | 69702 | 33967 | 33967 | 33967 | 33741 | 1011 | 100 |
| W 16 °C D0 R3 | 51542 | 32267 | 15470 | 15470 | 15470 | 15431 | 1011 | 96 |
| W 24 °C D0 L1 R1 | 105273 | 85998 | 41474 | 41474 | 41474 | 41310 | 1011 | 96 |
| W 24 °C D0 L1 R2 | 141050 | 121775 | 58559 | 58559 | 58559 | 58205 | 1011 | 68 |
| W 24 °C D0 L1 R3 | 119732 | 100457 | 49244 | 49244 | 49244 | 49111 | 1011 | 78 |
| W 24 °C D0 L2 R1 | 235055 | 215780 | 102713 | 102713 | 102713 | 102052 | 1011 | 102 |
| W 24 °C D0 L2 R2 | 147900 | 128625 | 62423 | 62423 | 62423 | 62165 | 1011 | 115 |
| W 24 °C D0 L2 R3 | 148739 | 129464 | 73611 | 73611 | 73611 | 72076 | 1011 | 121 |
| W 24 °C D0 L3 R1 | 142316 | 123041 | 58323 | 58323 | 58323 | 58018 | 1011 | 102 |
| W 24 °C D0 L3 R2 | 183135 | 163860 | 79156 | 79156 | 79156 | 78588 | 1011 | 102 |
| W 24 °C D0 L3 R3 | 196381 | 177106 | 85187 | 85187 | 85187 | 84750 | 1011 | 87 |
| W 16 °C D10 R1 | 92193 | 79080 | 38360 | 38360 | 38360 | 37941 | 1011 | 170 |
| W 16 °C D10 R2 | 250662 | 237549 | 117611 | 117611 | 117611 | 116806 | 1011 | 72 |
| W 16 °C D10 R3 | 157941 | 144828 | 70127 | 70127 | 70127 | 69873 | 1011 | 57 |
| W 24 °C D10 L1 R1 | 63939 | 50826 | 24615 | 24615 | 24615 | 24562 | 1011 | 45 |
| W 24 °C D10 L1 R2 | 138901 | 125788 | 59520 | 59520 | 59520 | 58706 | 1011 | 111 |
| W 24 °C D10 L1 R3 | 116915 | 103802 | 49168 | 49168 | 49168 | 48694 | 1011 | 137 |
| W 24 °C D10 L2 R1 | 91892 | 78779 | 37555 | 37555 | 37555 | 37279 | 1011 | 86 |
| W 24 °C D10 L2 R2 | 103522 | 90409 | 42357 | 42357 | 42357 | 42039 | 1011 | 101 |
| W 24 °C D10 L2 R3 | 87941 | 74828 | 35231 | 35231 | 35231 | 34933 | 1011 | 86 |
| W 24 °C D10 L3 R1 | 72392 | 59279 | 28626 | 28626 | 28626 | 28313 | 1011 | 91 |
| W 24 °C D10 L3 R2 | 83486 | 70373 | 33503 | 33503 | 33503 | 33265 | 1011 | 83 |
| W 24 °C D10 L3 R3 | 94444 | 81331 | 38335 | 38335 | 38335 | 38083 | 1011 | 101 |
| W 16 °C D20 R1 | 69449 | 56336 | 27550 | 27550 | 27550 | 27054 | 1011 | 118 |
| W 16 °C D20 R2 | 66311 | 53198 | 25566 | 25566 | 25566 | 25247 | 1011 | 98 |
| W 16 °C D20 R3 | 52672 | 39559 | 17196 | 17196 | 17196 | 16779 | 1011 | 124 |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| W 24 °C D20 L1 R1 | 70515 | 57402 | 27734 | 27734 | 27734 | 27409 | 1011 | 117 |
| W 24 °C D20 L1 R2 | 69113 | 56000 | 26014 | 26014 | 26014 | 25852 | 1011 | 81 |
| W 24 °C D20 L1 R3 | 90444 | 77331 | 36033 | 36033 | 36033 | 35403 | 1011 | 112 |
| W 24 °C D20 L2 R1 | 69267 | 56154 | 26033 | 26033 | 26033 | 26638 | 1011 | 106 |
| W 24 °C D20 L2 R2 | 108579 | 95466 | 45785 | 45785 | 45785 | 44527 | 1011 | 125 |
| W 24 °C D20 L2 R3 | 89301 | 76188 | 36343 | 36343 | 36343 | 35497 | 1011 | 111 |
| W 24 °C D20 L3 R1 | 141292 | 128179 | 62335 | 62335 | 62335 | 62091 | 1011 | 68 |
| W 24 °C D20 L3 R2 | 156037 | 142924 | 69895 | 69895 | 69895 | 69526 | 1011 | 62 |
| W 24 °C D20 L3 R3 | 181004 | 167891 | 82212 | 82212 | 82212 | 81967 | 1011 | 48 |
| W 16 °C D30 R1 | - | - | - | - | - | - | - | - |
| W 16 °C D30 R2 | - | - | - | - | - | - | - | - |
| W 16 °C D30 R3 | - | - | - | - | - | - | - | - |
| W 24 °C D30 L1 R1 | - | - | - | - | - | - | - | - |
| W 24 °C D30 L1 R2 | - | - | - | - | - | - | - | - |
| W 24 °C D30 L1 R3 | 96667 | 75062 | 14351 | 14351 | 14351 | 13356 | 1011 | 38 |
| W 24 °C D30 L2 R1 | - | - | - | - | - | - | - | - |
| W 24 °C D30 L2 R2 | 79534 | 57929 | 6615 | 6615 | 6615 | 6263 | 1011 | 41 |
| W 24 °C D30 L2 R3 | - | - | - | - | - | - | - | - |
| W 24 °C D30 L3 R1 | - | - | - | - | - | - | - | - |
| W 24 °C D30 L3 R2 | - | - | - | - | - | - | - | - |
| W 24 °C D30 L3 R3 | - | - | - | - | - | - | - | - |
| W 16 °C AF R1 | 81171 | 68058 | 32326 | 32326 | 32326 | 32079 | 1011 | 116 |
| W 16 °C AF R2 | 174353 | 161240 | 76121 | 76121 | 76121 | 75648 | 1011 | 81 |
| W 16 °C AF R3 | - | - | - | - | - | - | - | - |
| W 24 °C AF L1 R1 | 51868 | 38755 | 18248 | 18248 | 18248 | 18080 | 1011 | 147 |
| W 24 °C AF L1 R2 | 55335 | 42222 | 19283 | 19283 | 19283 | 19120 | 1011 | 116 |
| W 24 °C AF L1 R3 | 39451 | 26338 | 12320 | 12320 | 12320 | 12183 | 1011 | 105 |
| W 24 °C AF L2 R1 | 78879 | 65766 | 30726 | 30726 | 30726 | 30551 | 1011 | 101 |
| W 24 °C AF L2 R2 | 83440 | 70327 | 33311 | 33311 | 33311 | 33043 | 1011 | 115 |
| W 24 °C AF L2 R3 | 104023 | 90910 | 38559 | 38559 | 38559 | 38211 | 1011 | 107 |
| W 24 °C AF L3 R1 | 145434 | 132321 | 63883 | 63883 | 63883 | 63637 | 1011 | 52 |
| W 24 °C AF L3 R2 | 165247 | 152134 | 73076 | 73076 | 73076 | 72783 | 1011 | 77 |
| W 24 °C AF L3 R3 | 148222 | 135109 | 65617 | 65617 | 65617 | 65327 | 1011 | 70 |

## B.3 Rarefaction curves

⇒ **Bacteria**

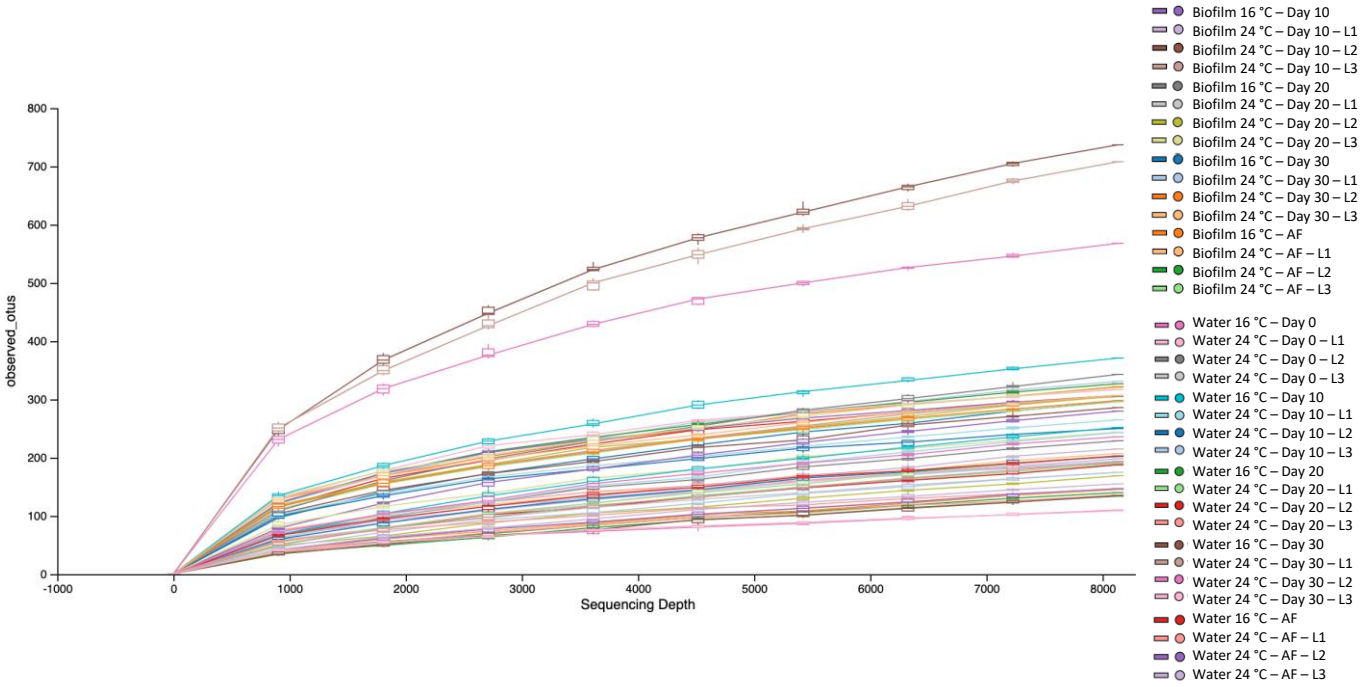



***Figure S 9.*** Rarefaction curves of observed OTUS for bacteria in all biofilm and water samples at 16 °C and 24 °C after different management strategies, applying a sequencing depth of 8131 sequences. Each curve represents an average of all biological replicates of each sample.

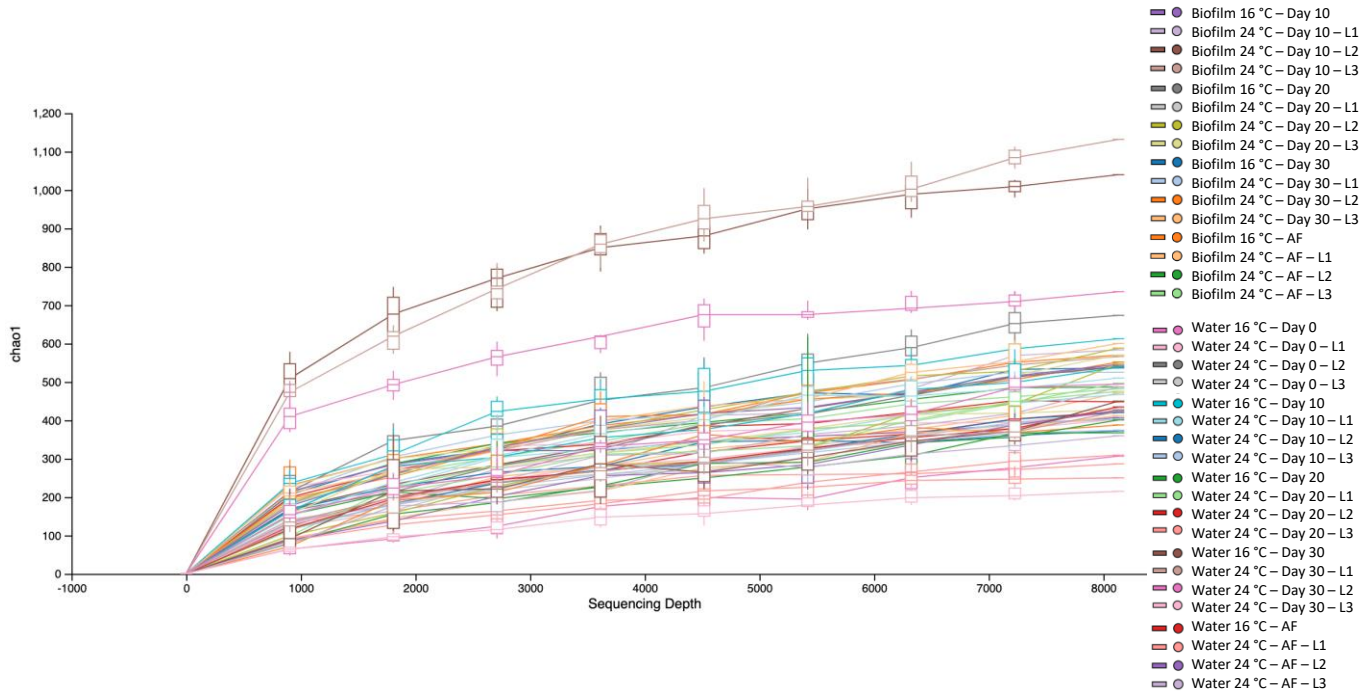



***Figure S 10***. Rarefaction curves of Chao1 index for bacteria in all biofilm and water samples at 16 °C and 24 °C after different management strategies, applying a sequencing depth of 8131 sequences. Each curve represents an average of all biological replicates of each sample.

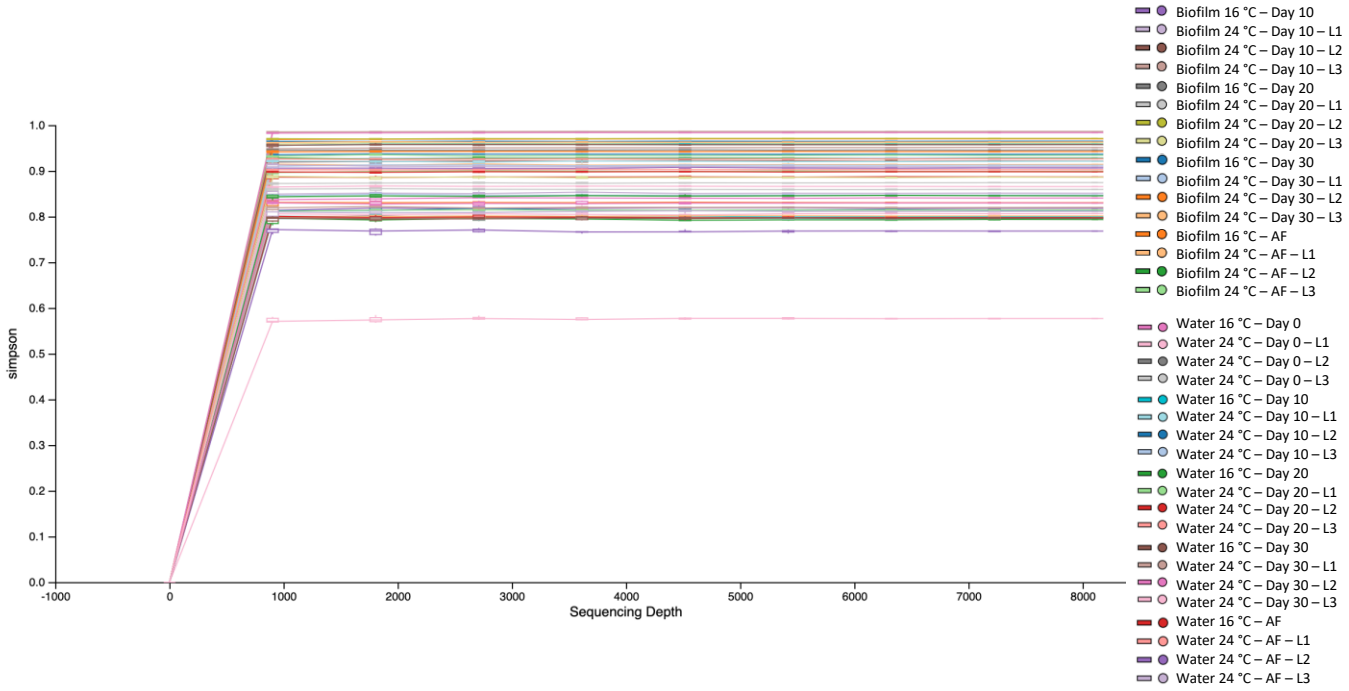



***Figure S 11*** Rarefaction curves of Simpson index for bacteria in all biofilm and water samples at 16 °C and 24 °C after different management strategies, applying a sequencing depth of 8131 sequences. Each curve represents an average of all biological replicates of each sample.

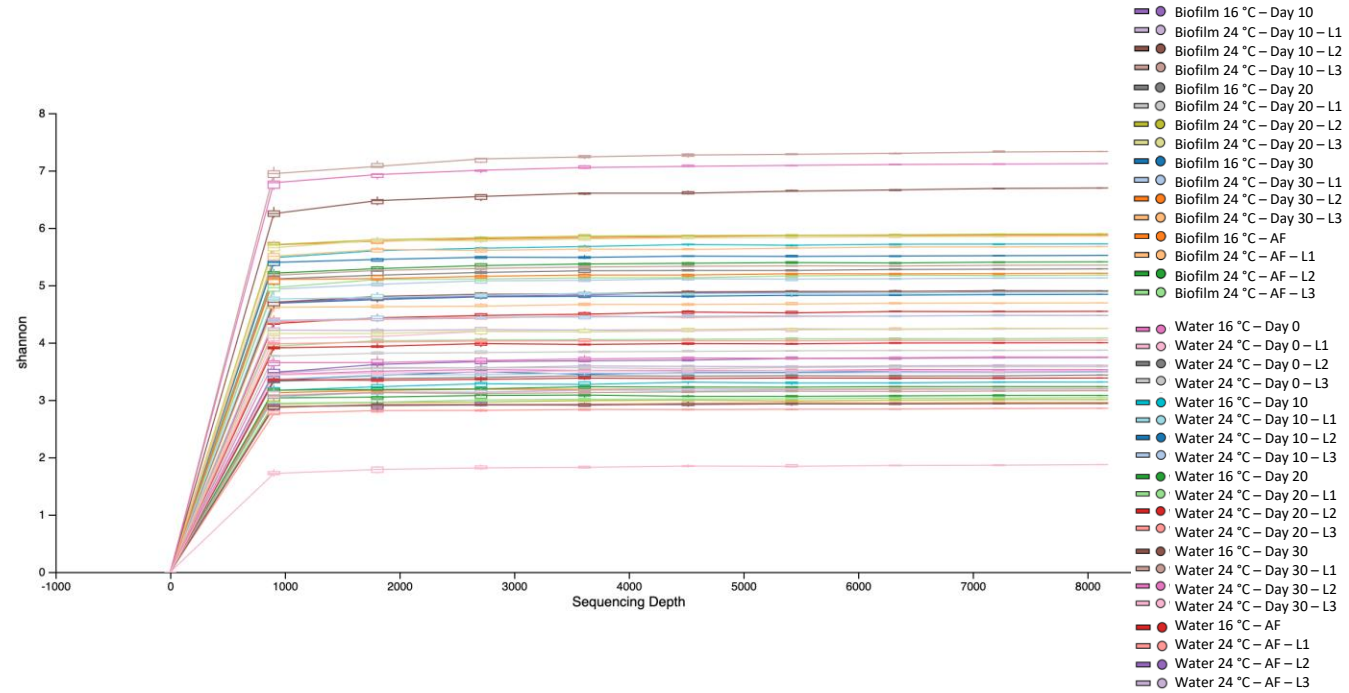



***Figure S 12.*** Rarefaction curves of Shannon index for bacteria in all biofilm and water samples at 16 °C and 24 °C after different management strategies, applying a sequencing depth of 8131 sequences. Each curve represents an average of all biological replicates of each sample.

⇒ **Fungi**

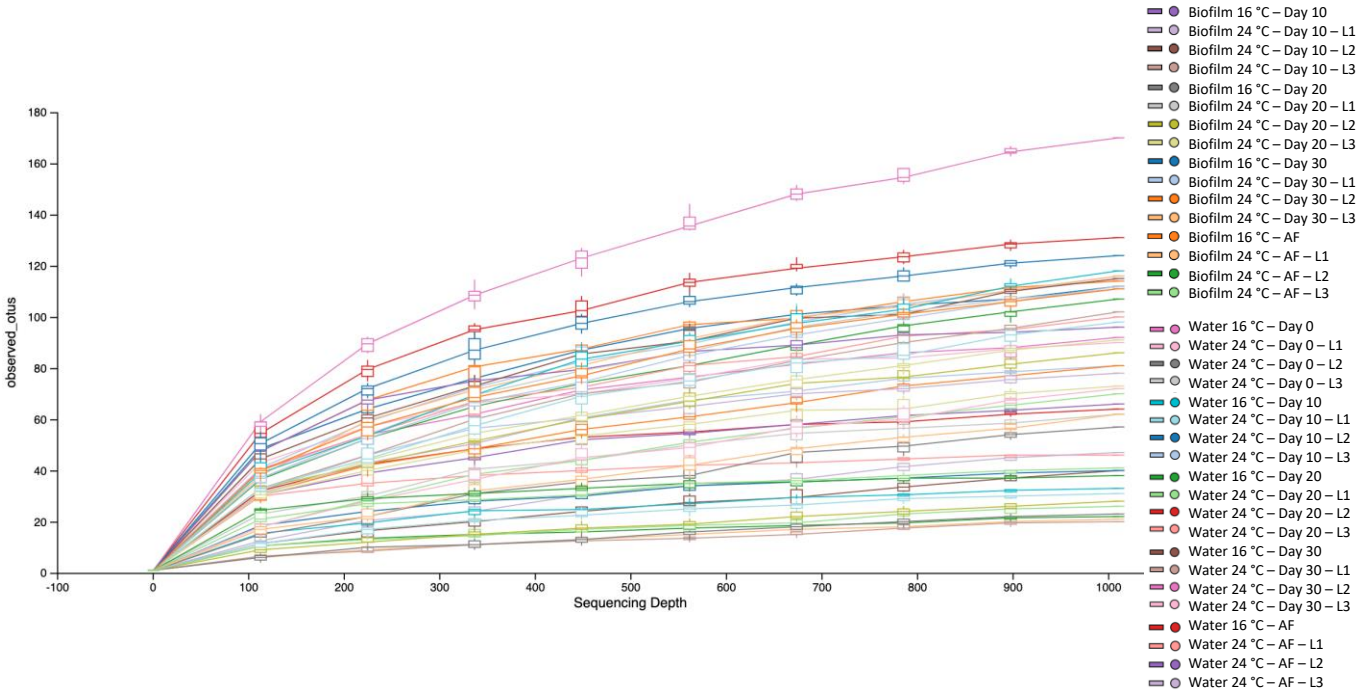



***Figure S 13***. Rarefaction curves of observed OTUS for fungi in all biofilm and water samples at 16 °C and 24 °C after different management strategies, applying a sequencing depth of 1011 sequences. Each curve represents an average of all biological replicates of each sample.

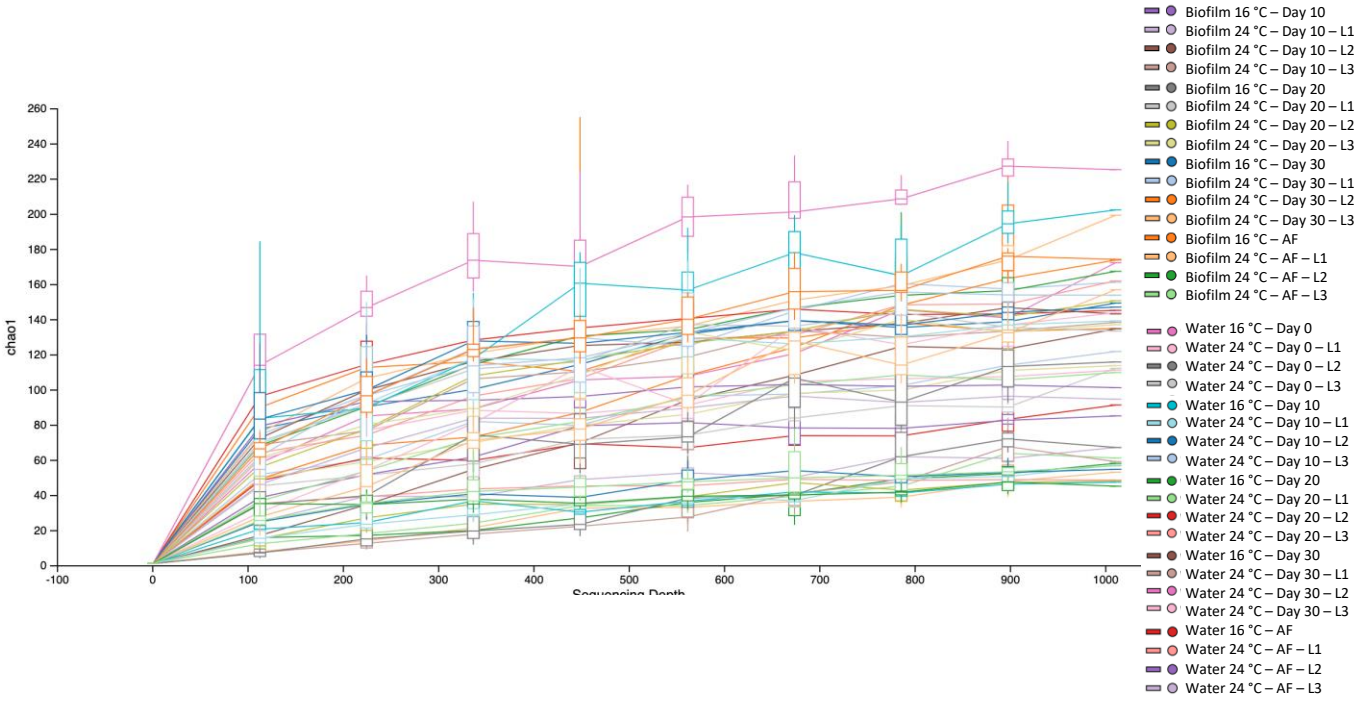



***Figure S 14***. Rarefaction curves of Chao1 index for fungi in all biofilm and water samples at 16 °C and 24 °C after different management strategies, applying a sequencing depth of 1011 sequences. Each curve represents an average of all biological replicates of each sample.

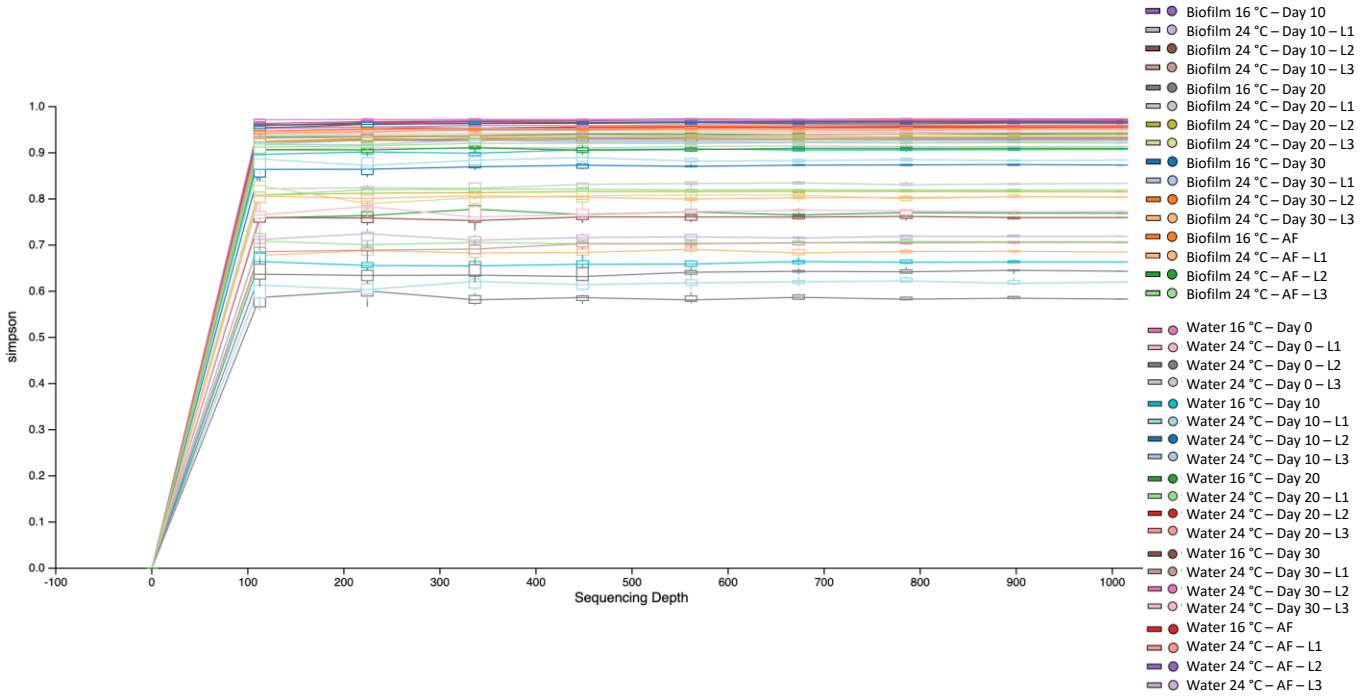



***Figure S 15.*** Rarefaction curves of Simpson index for fungi in all biofilm and water samples at 16 °C and 24 °C after different management strategies, applying a sequencing depth of 1011 sequences. Each curve represents an average of all biological replicates of each sample.

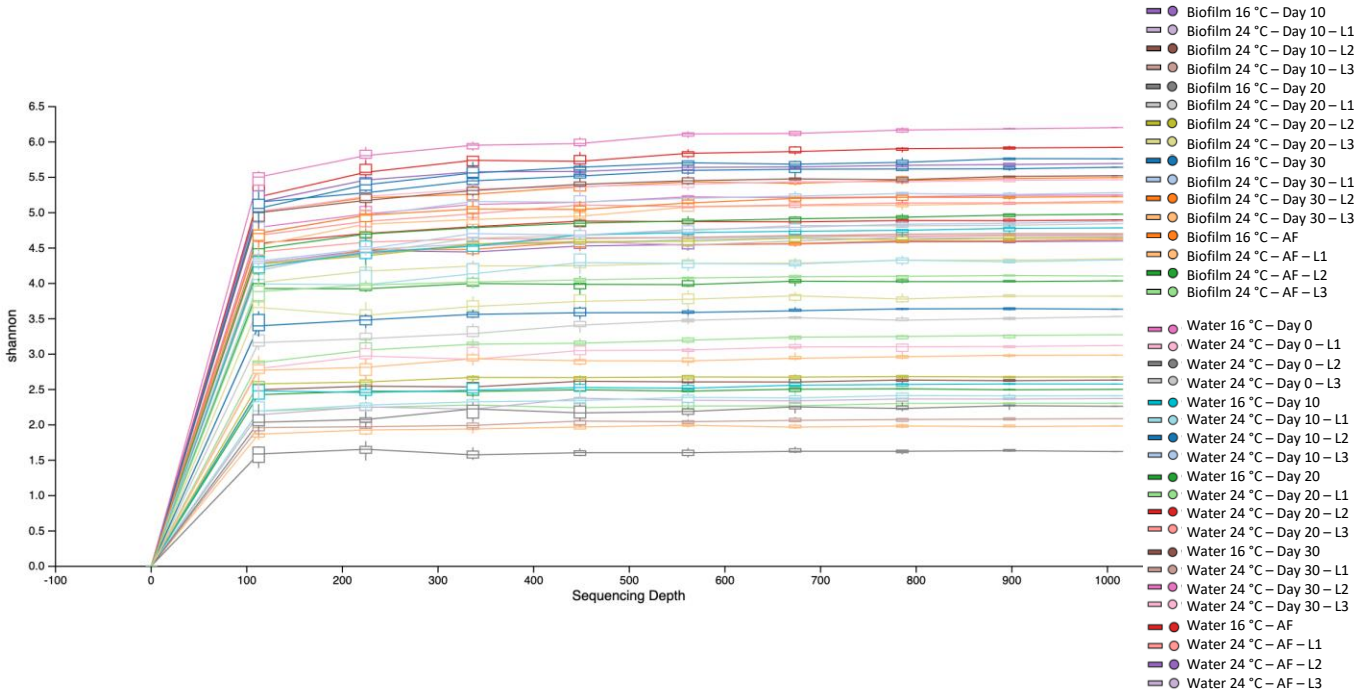



***Figure S 16***. Rarefaction curves of Shannon index for fungi in all biofilm and water samples at 16 °C and 24 °C after different management strategies, applying a sequencing depth of 1011 sequences. Each curve represents an average of all biological replicates of each sample.

## B.4 Statistical analysis

***Table S 13***. Results from the Kruskal-Wallis test to determine statistical differences in water-physico chemical parameters between temperatures and management strategies. Differences were considered statistically significant when p-value was ≤0.05.

| Kruskal-Wallis test | Total chlorine | Free chlorine | Fe | Mn | TOC |
|---|---|---|---|---|---|
| Day 0 | $X^2$ = 9.4<br>p-value = 0.22 | $X^2$ = 9.5<br>p-value = 0.23 | $X^2$ = 6.2<br>p-value = 0.09 | $X^2$ = 10.4<br>p-value = 0.15 | $X^2$ = 8.2<br>p-value = 0.06 |
| Day 10 | $X^2$ = 8.7<br>p-value = 0.17 | $X^2$ = 7.3<br>p-value = 0.06 | $X^2$ = 10.3<br>p-value = 0.07 | $X^2$ = 10.4<br>p-value = 0.06 | $X^2$ = 6.7<br>p-value = 0.08 |
| Day 20 | $X^2$ = 8.8<br>p-value = 0.03 | $X^2$ = 5.9<br>p-value = 0.11 | $X^2$ = 9.9<br>p-value = 0.10 | $X^2$ = 8.1<br>p-value = 0.06 | $X^2$ = 6.00<br>p-value = 0.11 |
| Day 30 | $X^2$ = 5.7<br>p-value = 0.12 | $X^2$ = 9.1<br>p-value = 0.27 | $X^2$ = 8.9<br>p-value = 0.30 | $X^2$ = 9.5<br>p-value = 0.06 | $X^2$ = 7.2<br>p-value = 0.06 |
| P-F | $X^2$ = 9.2<br>p-value = 0.09 | $X^2$ = 8.1<br>p-value = 0.06 | $X^2$ = 9.4<br>p-value = 0.02 | $X^2$ = 8.81<br>p-value = 0.03 | $X^2$ = 6.7<br>p-value = 0.08 |

***Table S 14***. Results from the Mann-Whitney U test for pairwise comparisons to determine statistical differences in Fe and Mn concentrations between temperatures and management strategies. Differences were considered statistically significant when p-value was ≤0.05.

| Mann-Whitney *U* test | Fe | Mn |
|---|---|---|
| 16 °C vs 24 °C Flushing-Hyperchlorination | W = 0<br>p-value = 0.05 | W = 0<br>p-value = 0.05 |
| 16 °C vs 24 °C Flushing | W = 0<br>p-value = 0.05 | W = 0<br>p-value = 0.05 |
| 16 °C - 24 °C Transition | W = 0<br>p-value = 0.05 | W = 0<br>p-value = 0.032 |
| 24 °C Flushing-Hyperchlorination vs 24 °C Flushing | W = 0<br>p-value = 0.1 | W = 4<br>p-value = 1 |
| 24 °C Flushing-Hyperchlorination vs 24 °C Transition | W = 0<br>p-value = 0.1 | W = 0<br>p-value = 0.06 |
| 24 °C Flushing vs 24 °C Transition | W = 5<br>p-value = 1 | W = 0<br>p-value = 0.06 |

***Table S 15***. Results from Kruskal-Wallis test to determine statistical differences in turbidity response between temperatures and management strategies. Differences were considered statistically significant when p-value was ≤0.05.

| **Kruskal-Wallis test** | **Turbidity** |
|---|---|
| Last 24 hours | $X^2$ = 245.8<br>p-value = 0.067 |
| Step 1 | $X^2$ = 187.6<br>p-value = 0.058 |
| Step 2 | $X^2$ = 117.3<br>p-value = <2e-16 |
| Step 3 | $X^2$ = 127.9<br>p-value = <2e-16 |
| Step 4 | $X^2$ = 87.8<br>p-value = <2e-16 |

***Table S 16***. Results from the Mann Whitney U test to determine statistical differences in turbidity response between temperatures and management strategies. Differences were considered statistically significant when p-value was ≤0.05.

| **Mann-Whitney *U* test** | **Step 2** | **Step 3** | **Step 4** |
|---|---|---|---|
| 16 °C vs 24 °C Flushing-Hyperchlorination | W = 1201.1<br>p-value = 0.12 | W = 404.0<br>p-value = 0.03 | W = 277.0<br>p-value = 0.003 |
| 16 °C vs 24 °C Flushing | W = 1705.5<br>p-value = 0.09 | W = 1447.0<br>p-value = 3.07e-13 | W = 179.0<br>p-value = 1.71e-6 |
| 16 °C - 24 °C Transition | W = 0<br>p-value = < 2.2e-16 | W = 0<br>p-value = 3.07e-14 | W = 0<br>p-value = 2.07e-12 |
| 24 °C Flushing-Hyperchlorination vs 24 °C Flushing | W = 1205.6<br>p-value = 0.09 | W = 1479.0<br>p-value = 0.7 | W = 3.71e+02<br>p-value = 0.06 |
| 24 °C Flushing-Hyperchlorination vs 24 °C Transition | W = 0<br>p-value = < 2.2e-16 | W = 0<br>p-value = 3.07e-14 | W = 17<br>p-value = 1.39e-11 |
| 24 °C Flushing vs 24 °C Transition | W = 100.0<br>p-value = < 2.2e-16 | W = 0<br>p-value = 2.98e-13 | W = 0<br>p-value = 3.01e-12 |

***Table S 17***. Results from Kruskal-Wallis test to determine statistical differences in bacterial alpha diversity indices between temperatures and management strategies. Differences were considered statistically significant when p-value was ≤0.05.

| **Kruskal-Wallis test** | | **Bacteria** | | |
|---|---|---|---|---|
| | | **Chao1** | **Simpson** | **Shannon** |
| Biofilm | Day 10 | $X^2$ = 5.6<br>p-value = 0.13 | $X^2$ = 7<br>p-value = 0.07 | $X^2$ = 8.7<br>p-value = 0.06 |
| | Day 20 | $X^2$ = 5.2<br>p-value = 0.15 | $X^2$ = 5.9<br>p-value = 0.12 | $X^2$ = 6.0<br>p-value = 0.11 |
| | Day 30 | $X^2$ = 7.0<br>p-value = 0.05 | $X^2$ = 6.4<br>p-value = 0.05 | $X^2$ = 5.4<br>p-value = 0.05 |
| | P-F | $X^2$ = 5.7<br>p-value = 0.02 | $X^2$ = 5.2<br>p-value = 0.05 | $X^2$ = 4.1<br>p-value = 0.02 |
| Water | Day 0 | $X^2$ = 4.8<br>p-value = 0.18 | $X^2$ = 9.4<br>p-value = 0.2 | $X^2$ = 10.4<br>p-value = 0.1 |
| | Day 10 | $X^2$ = 3.2<br>p-value = 0.36 | $X^2$ = 5.4<br>p-value = 0.14 | $X^2$ = 4.4<br>p-value = 0.2 |
| | Day 20 | $X^2$ = 5.5<br>p-value = 0.14 | $X^2$ = 7.82<br>p-value = 0.06 | $X^2$ = 9.3<br>p-value = 0.2 |
| | Day 30 | $X^2$ = 7.8<br>p-value = 0.06 | $X^2$ = 4.12<br>p-value = 0.08 | $X^2$ = 8.8<br>p-value = 0.7 |
| | P-F | $X^2$ = 4.9<br>p-value = 0.17 | $X^2$ = 7.5<br>p-value = 0.06 | $X^2$ = 9.5<br>p-value = 0.02 |

***Table S 18***. Results from Mann-Whitney U test for pairwise comparisons to determine statistical differences in alpha diversity indices of bacteria biofilm day 30 samples between temperatures and management strategies. Differences were considered statistically significant when p-value was ≤0.05.

| **Mann-Whitney *U* test<br>Bacteria - Biofilm Day 30** | **Chao1** | **Simpson** | **Shannon** |
|---|---|---|---|
| 16 °C vs 24 °C Flushing-Hyperchlorination | W = 2<br>p-value = 0.4 | W = 2<br>p-value = 0.2 | W = 9<br>p-value = 1 |
| 16 °C vs 24 °C Flushing | W = 1<br>p-value = 0.05 | W = 0<br>p-value = 0.05 | W = 4<br>p-value = 0.05 |
| 16 °C - 24 °C Transition | W = 0<br>p-value = 0.1 | W = 9<br>p-value = 0.1 | W = 0<br>p-value = 0.1 |
| 24 °C Flushing-Hyperchlorination vs 24 °C Flushing | W = 0<br>p-value = 0.2 | W = 7<br>p-value = 0.4 | W = 0<br>p-value = 0.1 |
| 24 °C Flushing-Hyperchlorination vs 24 °C Transition | W = 0<br>p-value = 0.1 | W = 9<br>p-value = 0.1 | W = 0<br>p-value = 0.1 |

| | | | |
|---|---|---|---|
| 24 °C Flushing vs 24 °C Transition | W = 5<br>p-value = 1 | W = 7<br>p-value = 0.4 | W = 2<br>p-value = 0.4 |

***Table S 19***. Results from Mann-Whitney U test for pairwise comparisons to determine statistical differences in alpha diversity indices of bacteria biofilm post-flushing samples between temperatures and management strategies. Differences were considered statistically significant when p-value was ≤0.05.

| **Mann-Whitney *U* test<br>Bacteria - Biofilm Post-flushing** | **Chao1** | **Simpson** | **Shannon** |
|---|---|---|---|
| 16 °C vs 24 °C Flushing-Hyperchlorination | W = 9<br>p-value = 0.05 | W = 0<br>p-value = 1 | W = 9<br>p-value = 0.05 |
| 16 °C vs 24 °C Flushing | W = 8<br>p-value = 0.2 | W = 2<br>p-value = 0.4 | W = 5<br>p-value = 1 |
| 16 °C - 24 °C Transition | W = 7<br>p-value = 0.4 | W = 4<br>p-value = 1 | W = 4<br>p-value = 1 |
| 24 °C Flushing-Hyperchlorination vs 24 °C Flushing | W = 2<br>p-value = 0.4 | W = 6<br>p-value = 0.7 | W = 3<br>p-value = 0.7 |
| 24 °C Flushing-Hyperchlorination vs 24 °C Transition | W = 1<br>p-value = 0.2 | W = 9<br>p-value = 0.1 | W = 0<br>p-value = 0.1 |
| 24 °C Flushing vs 24 °C Transition | W = 4<br>p-value = 1 | W = 6<br>p-value = 0.7 | W = 4<br>p-value = 1 |

***Table S 20***. Results from Mann-Whitney U test for pairwise comparisons to determine statistical differences in Shannon index of bacteria water post-flushing samples between temperatures and management strategies. Differences were considered statistically significant when p-value was ≤0.05.

| **Mann-Whitney *U* test<br>Bacteria- Water port-flushing** | **Shannon** |
|---|---|
| 16 °C vs 24 °C Flushing-Hyperchlorination | W = 9<br>p-value = 0.05 |
| 16 °C vs 24 °C Flushing | W = 6<br>p-value = 0.7 |
| 16 °C - 24 °C Transition | W = 0<br>p-value = 0.1 |
| 24 °C Flushing-Hyperchlorination vs 24 °C Flushing | W = 0<br>p-value = 0.1 |
| 24 °C Flushing-Hyperchlorination vs 24 °C Transition | W = 0<br>p-value = 0.1 |
| 24 °C Flushing vs 24 °C Transition | W = 0<br>p-value = 0.1 |

***Table S 21***. Results from Kruskal-Wallis test to determine statistical differences in fungal alpha diversity indices between temperatures and management strategies. Differences were considered statistically significant when p-value was ≤0.05.

| **Kruskal-Wallis test** | | **Fungi** | | |
|---|---|---|---|---|
| | | **chao1** | **simpson** | **shannon** |
| Biofilm | Day 10 | $X^2$ = 5.8<br>p-value = 0.12 | $X^2$ = 7.2<br>p-value = 0.05 | $X^2$ = 7.7<br>p-value = 0.05 |
| | Day 20 | $X^2$ = 3.4<br>p-value = 0.05 | $X^2$ = 5.75<br>p-value = 0.02 | $X^2$ = 6.28<br>p-value = 0.05 |
| | Day 30 | $X^2$ = 1.19<br>p-value = 0.04 | $X^2$ = 2.54<br>p-value = 0.05 | $X^2$ = 1.89<br>p-value = 0.05 |
| | P-F | $X^2$ = 6.5<br>p-value = 0.05 | $X^2$ = 6.9<br>p-value = 0.05 | $X^2$ = 7.77<br>p-value = 0.04 |
| Water | Day 0 | $X^2$ = 3.9<br>p-value = 0.26 | $X^2$ = 5.15<br>p-value = 0.16 | $X^2$ = 4.12<br>p-value = 0.24 |
| | Day 10 | $X^2$ = 0.43<br>p-value = 0.9 | $X^2$ = 3.8<br>p-value = 0.2 | $X^2$ = 3.2<br>p-value = 0.36 |
| | Day 20 | $X^2$ = 2.4<br>p-value = 0.48 | $X^2$ = 4.6<br>p-value = 0.20 | $X^2$ = 5.6<br>p-value = 0.13 |
| | Day 30 | - | - | - |
| | P-F | $X^2$ = 9.8<br>p-value = **0.05** | $X^2$ = 1.6<br>p-value = 0.4 | $X^2$ = 7.6<br>p-value = 0.04 |

***Table S 22***. Results from Mann-Whitney U test for pairwise comparisons to determine statistical differences in alpha diversity indices of fungi-biofilm day 10 samples between temperatures and management strategies. Differences were considered statistically significant when p-value was ≤0.05.

| **Mann-Whitney *U* test<br>Fungi - Biofilm Day 10** | **Chao1** | **Simpson** | **Shannon** |
|---|---|---|---|
| 16 °C vs 24 °C Flushing-Hyperchlorination | W = 6<br>p-value = 0.65 | W = 0<br>p-value = 0.1 | W = 9<br>p-value = 0.1 |
| 16 °C vs 24 °C Flushing | W = 1<br>p-value = 0.05 | W = 0<br>p-value = 0.05 | W = 9<br>p-value = 0.05 |
| 16 °C - 24 °C Transition | W = 9<br>p-value = 0.03 | W = 0<br>p-value = 0.05 | W = 9<br>p-value = 0.05 |
| 24 °C Flushing-Hyperchlorination vs 24 °C Flushing | W = 5<br>p-value = 1 | W = 2<br>p-value = 0.4 | W = 7<br>p-value = 0.4 |
| 24 °C Flushing-Hyperchlorination vs 24 °C Transition | W = 8<br>p-value = 0.18 | W = 2<br>p-value = 0.4 | W = 8<br>p-value = 0.2 |

| | | | |
|---|---|---|---|
| 24 °C Flushing vs 24 °C Transition | W = 9<br>p-value = 0.07 | W = 4<br>p-value = 1 | W = 5<br>p-value = 1 |

***Table S 23***. Results from Mann-Whitney U test for pairwise comparisons to determine statistical differences in alpha diversity indices of fungi-biofilm day 20 samples between temperatures and management strategies. Differences were considered statistically significant when p-value was ≤0.05.

| **Mann-Whitney *U* test<br>Fungi - Biofilm Day 20** | **Chao1** | **Simpson** | **Shannon** |
|---|---|---|---|
| 16 °C vs 24 °C Flushing-Hyperchlorination | W = 1<br>p-value = 0.05 | W = 5<br>p-value = 0.05 | W = 1<br>p-value = 0.05 |
| 16 °C vs 24 °C Flushing | W = 0<br>p-value = 0.05 | W = 0<br>p-value = 0.05 | W = 0<br>p-value = 0.05 |
| 16 °C - 24 °C Transition | W = 9<br>p-value = 0.03 | W = 4<br>p-value = 0.05 | W = 5<br>p-value = 0.05 |
| 24 °C Flushing-Hyperchlorination vs 24 °C Flushing | W = 2.5<br>p-value = 0.5 | W = 7<br>p-value = 0.4 | W = 1<br>p-value = 0.2 |
| 24 °C Flushing-Hyperchlorination vs 24 °C Transition | W = 5<br>p-value = 1 | W = 8<br>p-value = 0.2 | W = 1<br>p-value = 0.2 |
| 24 °C Flushing vs 24 °C Transition | W = 7<br>p-value = 0.4 | W = 6<br>p-value = 0.7 | W = 4<br>p-value = 1 |

***Table S 24***. Results from Mann-Whitney U test for pairwise comparisons to determine statistical differences in alpha diversity indices of fungi-biofilm day 30 samples between temperatures and management strategies. Differences were considered statistically significant when p-value was ≤0.05.

| **Mann-Whitney *U* test<br>Fungi - Biofilm Day 30** | **Chao1** | **Simpson** | **Shannon** |
|---|---|---|---|
| 16 °C vs 24 °C Flushing-Hyperchlorination | W = 0<br>p-value = 0.05 | W = 5<br>p-value = 0.05 | W = 1<br>p-value = 0.05 |
| 16 °C vs 24 °C Flushing | W = 1<br>p-value = 0.05 | W = 9<br>p-value = 0.05 | W = 1<br>p-value = 0.05 |
| 16 °C - 24 °C Transition | W = 0<br>p-value = 0.05 | W = 1<br>p-value = 0.05 | W = 1<br>p-value = 0.05 |
| 24 °C Flushing-Hyperchlorination vs 24 °C Flushing | W = 7<br>p-value = 0.4 | W = 6<br>p-value = 0.7 | W = 4<br>p-value = 1 |
| 24 °C Flushing-Hyperchlorination vs 24 °C Transition | W = 7<br>p-value = 0.4 | W = 6<br>p-value = 0.7 | W = 4<br>p-value = 1 |
| 24 °C Flushing vs 24 °C Transition | W = 4<br>p-value = 1 | W = 7<br>p-value = 0.4 | W = 2<br>p-value = 0.4 |

***Table S 25***. Results from Mann-Whitney U test for pairwise comparisons to determine statistical differences in alpha diversity indices of fungi-biofilm post-flushing samples between temperatures and management strategies. Differences were considered statistically significant when p-value was ≤0.05.

| **Mann-Whitney *U* test<br>Fungi - Biofilm Post-flushing** | **Chao1** | **Simpson** | **Shannon** |
|---|---|---|---|
| 16 °C vs 24 °C Flushing-Hyperchlorination | W = 9<br>p-value = 0.05 | W = 0<br>p-value = 0.05 | W = 9<br>p-value = 0.05 |
| 16 °C vs 24 °C Flushing | W = 9<br>p-value = 0.05 | W = 0<br>p-value = 0.05 | W = 9<br>p-value = 0.05 |
| 16 °C - 24 °C Transition | W = 9<br>p-value = 0.05 | W = 0<br>p-value = 0.05 | W = 9<br>p-value = 0.05 |
| 24 °C Flushing-Hyperchlorination vs 24 °C Flushing | W = 4<br>p-value = 1 | W = 3<br>p-value = 0.7 | W = 6<br>p-value = 0.7 |
| 24 °C Flushing-Hyperchlorination vs 24 °C Transition | W = 5<br>p-value = 1 | W = 4<br>p-value = 1 | W = 5<br>p-value = 1 |
| 24 °C Flushing vs 24 °C Transition | W = 6.5<br>p-value = 0.50 | W = 6<br>p-value = 0.7 | W = 3<br>p-value = 0.7 |

***Table S 26***. Results from Mann-Whitney U test for pairwise comparisons to determine statistical differences in alpha diversity indices of fungi-water post-flushing samples between temperatures and management strategies. Differences were considered statistically significant when p-value was ≤0.05.

| **Mann-Whitney *U* test<br>Fungi - Water Post-flushing** | **Chao1** | **Shannon** |
|---|---|---|
| 16 °C vs 24 °C Flushing-Hyperchlorination | W = 1<br>p-value = 0.05 | W = 2<br>p-value = 0.2 |
| 16 °C vs 24 °C Flushing | W =<br>p-va3lue = 0.05 | W = 3<br>p-value = 0.8 |
| 16 °C - 24 °C Transition | W = 1<br>p-value = 0.05 | W = 2<br>p-value = 0.2 |
| 24 °C Flushing-Hyperchlorination vs 24 °C Flushing | W = 6<br>p-value = 0.7 | W = 5<br>p-value = 0.05 |
| 24 °C Flushing-Hyperchlorination vs 24 °C Transition | W = 9<br>p-value = 0.1 | W = 7<br>p-value = 0.05 |
| 24 °C Flushing vs 24 °C Transition | W = 7<br>p-value = 0.4 | W = 7<br>p-value = 0.05 |

# Appendix C. Supplementary material for Chapter 6

This appendix includes supplementary tables and figures with the results used for the analysis discussed in chapter 6 of this manuscript, "*How intermittent water supply time impacts on the microbiome of drinking water distribution systems*?".

## C.1 Settings of the experimental DWDS facility for water supply restarting by stages

***Table S 27.*** Test loop facility conditions and setting for each loop and stage for the water supply restarting.

| Loop | Stage | Conditions | Settings |
|---|---|---|---|
| 1 (6 hours) | 0 | Q = 0.54 L/s<br>p = 20 m | Valve control signal = 5.60 mA<br>Pump control signal = 11.70 mA |
| | 1 | Q = 0.54 L/s<br>p = 30 m | Valve control signal = 5.16 mA<br>Pump control signal = 15.00 mA |
| | 2 | Q =2.3 L/s<br>p = 30 m | Valve control signal = 9.53 mA<br>Pump control signal = 16.00 mA |
| | 3 | Q = 4.1 L/s<br>p = 30 m | Valve control signal = 11.00 mA<br>Pump control signal = 18.00 mA |
| | 4 | Q = 6.1 L/s<br>p = 30 m | Valve control signal = 13.07 mA<br>Pump control signal = 20 mA |
| 2 (48 hours) | 0 | Q = 0.54 L/s<br>p = 20 m | Valve control signal = 5.80 mA<br>Pump control signal = 9.10 mA |
| | 1 | Q = 0.54 L/s<br>p = 30 m | Valve control signal = 5.45 mA<br>Pump control signal = 11.60 mA |
| | 2 | Q =2.3 L/s<br>p = 30 m | Valve control signal = 9.40 mA<br>Pump control signal = 12.80 mA |
| | 3 | Q = 4.1 L/s<br>p = 30 m | Valve control signal = 11.90 mA<br>Pump control signal = 14.90 mA |
| | 4 | Q = 6.1 L/s<br>p = 30 m | Valve control signal = 14.03 mA<br>Pump control signal = 16.80 mA |
| 3 (6 days) | 0 | Q = 0.54 L/s<br>p = 20 m | Valve control signal = 5.50 mA<br>Pump control signal = 9.20 mA |
| | 1 | Q = 0.54 L/s<br>p = 30 m | Valve control signal = 5.20 mA<br>Pump control signal = 11.00 mA |
| | 2 | Q =2.3 L/s<br>p = 30 m | Valve control signal = 9.18 mA<br>Pump control signal = 12.60 mA |
| | 3 | Q = 4.1 L/s<br>p = 30 m | Valve control signal = 11.86 mA<br>Pump control signal = 14.90 mA |
| | 4 | Q = 6.1 L/s<br>p = 30 m | Valve control signal = 14.00 mA<br>Pump control signal = 16.95 mA |

## C.2 Flow rate conversions for each stage of the water supply restarting

**Table S 28.** Flow rate conversions for the growth phase and each stage of the water supply restarting.

| | Flow (Q) - (L/s) | Velocity (v) - (m/s) | Shear stress (τ) - ($N/m^2$) |
|---|---|---|---|
| Growth phase | 0.20 - 0.54 | 0.04 – 0.10 | 0.1 – 0.3 |
| Stage 0 | 0.54 | 0.10 | 0.3 |
| Stage 1 | 0.54 | 0.10 | 0.3 |
| Stage 2 | 2.30 | 0.47 | 1.4 |
| Stage 3 | 4.10 | 0.83 | 2.3 |
| Stage 4 | 6.10 | 1.23 | 4.2 |

## C.3 DNA concentration

***Table S 29.*** DNA concentration from each biofilm sample after different IWS times.

| Sample Nº | Sample ID | IWS Time | Day/Stage | Loop | Replicate | [DNA]ng/µL |
|---|---|---|---|---|---|---|
| 1 | D60 B L1 R1 | - | Day 60 | 1 | R1 | 244.70 |
| 2 | D60 B L1 R2 | - | Day 60 | 1 | R2 | 147.50 |
| 3 | 6h P-IWS R1 | 6 hours | Post-IWS | 1 | R1 | 224.45 |
| 4 | 6h P-IWS R2 | 6 hours | Post-IWS | 1 | R2 | 327.20 |
| 5 | 6h AF R1 | 6 hours | After water supply restarting | 1 | R1 | 93.37 |
| 6 | 6h AF R2 | 6 hours | After water supply restarting | 1 | R2 | 96.85 |
| 7 | D60 L2 R1 | - | Day 60 | 2 | R1 | 244.30 |
| 8 | D60 L2 R2 | - | Day 60 | 2 | R2 | 110.90 |
| 9 | 48h P-IWS R1 | 48 hours | Post-IWS | 2 | R1 | 893.30 |
| 10 | 48h P-IWS R2 | 48 hours | Post-IWS | 2 | R2 | 245.00 |
| 11 | 48h AF R1 | 48 hours | After water supply restarting | 2 | R1 | 115.20 |
| 12 | 48h AF R2 | 48 hours | After water supply restarting | 2 | R2 | 153.40 |
| 13 | D60 L3 R1 | - | Day 60 | 3 | R1 | 95.83 |
| 14 | D60 L3 R2 | - | Day 60 | 3 | R2 | 335.40 |
| 15 | 6d P-IWS R1 | 6 days | Post-IWS | 3 | R1 | 242.80 |
| 16 | 6d P-IWS R2 | 6 days | Post-IWS | 3 | R2 | 112.30 |
| 17 | 6d AF R1 | 6 days | After water supply restarting | 3 | R1 | 75.19 |
| 18 | 6d AF R2 | 6 days | After water supply restarting | 3 | R2 | 163.50 |

***Table S 30***. DNA concentration from each water sample after different IWS times.

| Sample Nº | Sample ID | IWS Time | Day/Stage | Loop | Replicate | [DNA] ng/µL |
|---|---|---|---|---|---|---|
| 19 | D60 W L1 R1 | - | Day 60 | 1 | R1 | 31.85 |

| | | | | | | |
|---|---|---|---|---|---|---|
| 20 | D60 W L2 R2 | - | Day 60 | 2 | R2 | 46.3 |
| 21 | D60 W L3 R3 | - | Day 60 | 3 | R3 | 99.29 |
| 22 | 6h S0 R1 | 6 hours | Stage 0 | 1 | R1 | 39.56 |
| 23 | 6h S0 R2 | 6 hours | Stage 0 | 1 | R2 | 33.05 |
| 24 | 6h S0 R3 | 6 hours | Stage 0 | 1 | R3 | 91.70 |
| 25 | 6h S1 R1 | 6 hours | Stage 1 | 1 | R1 | 32.27 |
| 26 | 6h S1 R2 | 6 hours | Stage 1 | 1 | R2 | 30.44 |
| 27 | 6h S1 R3 | 6 hours | Stage 1 | 1 | R3 | 34.37 |
| 28 | 6h S2 R1 | 6 hours | Stage 2 | 1 | R1 | 45.91 |
| 29 | 6h S2 R2 | 6 hours | Stage 2 | 1 | R2 | 20.71 |
| 30 | 6h S2 R3 | 6 hours | Stage 2 | 1 | R3 | 32.39 |
| 31 | 6h S3 R1 | 6 hours | Stage 3 | 1 | R1 | 24.26 |
| 32 | 6h S3 R2 | 6 hours | Stage 3 | 1 | R2 | 21.11 |
| 33 | 6h S3 R3 | 6 hours | Stage 3 | 1 | R3 | 16.15 |
| 34 | 6h S4 R1 | 6 hours | Stage 4 | 1 | R1 | 16.93 |
| 35 | 6h S4 R2 | 6 hours | Stage 4 | 1 | R2 | 18.16 |
| 36 | 6h S4 R3 | 6 hours | Stage 4 | 1 | R3 | 15.54 |
| 37 | 48h S0 R1 | 48 hours | Stage 0 | 2 | R1 | 86.58 |
| 38 | 48h S0 R2 | 48 hours | Stage 0 | 2 | R2 | 46.84 |
| 39 | 48h S0 R3 | 48 hours | Stage 0 | 2 | R3 | 42.56 |
| 40 | 48h S1 R1 | 48 hours | Stage 1 | 2 | R1 | 33.54 |
| 41 | 48h S1 R2 | 48 hours | Stage 1 | 2 | R2 | 37.69 |
| 42 | 48h S1 R3 | 48 hours | Stage 1 | 2 | R3 | 25.34 |
| 43 | 48h S2 R1 | 48 hours | Stage 2 | 2 | R1 | 23.5 |
| 44 | 48h S2 R2 | 48 hours | Stage 2 | 2 | R2 | 27.56 |
| 45 | 48h S2 R3 | 48 hours | Stage 2 | 2 | R3 | 34.17 |
| 46 | 48h S3 R1 | 48 hours | Stage 3 | 2 | R1 | 7.33 |
| 47 | 48h S3 R2 | 48 hours | Stage 3 | 2 | R2 | 8.7 |
| 48 | 48h S3 R3 | 48 hours | Stage 3 | 2 | R3 | 11.37 |
| 49 | 48h S4 R1 | 48 hours | Stage 4 | 2 | R1 | 28.52 |
| 50 | 48h S4 R2 | 48 hours | Stage 4 | 2 | R2 | 10.59 |
| 51 | 48h S4 R3 | 48 hours | Stage 4 | 2 | R3 | 7.34 |
| 52 | 6d S0 R1 | 6 days | Stage 0 | 3 | R1 | 161.3 |
| 53 | 6d S0 R2 | 6 days | Stage 0 | 3 | R2 | 239.5 |
| 54 | 6d S0 R3 | 6 days | Stage 0 | 3 | R3 | 42.95 |
| 55 | 6d S1 R1 | 6 days | Stage 1 | 3 | R1 | 65.85 |
| 56 | 6d S1 R2 | 6 days | Stage 1 | 3 | R2 | 33.2 |
| 57 | 6d S1 R3 | 6 days | Stage 1 | 3 | R3 | 68.51 |
| 58 | 6d S2 R1 | 6 days | Stage 2 | 3 | R1 | 33.53 |

| | | | | | | |
|---|---|---|---|---|---|---|
| 59 | 6d S2 R2 | 6 days | Stage 2 | 3 | R2 | 52.72 |
| 60 | 6d S2 R3 | 6 days | Stage 2 | 3 | R3 | 40.94 |
| 61 | 6d S3 R1 | 6 days | Stage 3 | 3 | R1 | 42.52 |
| 62 | 6d S3 R2 | 6 days | Stage 3 | 3 | R2 | 39.99 |
| 63 | 6d S3 R3 | 6 days | Stage 3 | 3 | R3 | 37.65 |
| 64 | 6d S4 R1 | 6 days | Stage 4 | 3 | R1 | 36.06 |
| 65 | 6d S4 R2 | 6 days | Stage 4 | 3 | R2 | 37.33 |
| 66 | 6d S4 R3 | 6 days | Stage 4 | 3 | R3 | 57.39 |

## C.4 Sequences counts

***Table S 31***. Sequence count during each step of bioinformatic analysis of the bacterial 16S rRNA gene in all biofilm and water samples after different IWS times.

| Sample ID | Raw reads | Filtered and imported to QIIME2 | After join pair-ends | After dereplication | After clustering (97%) | After chimera filtering | Rarefied | Total OTUs observed |
|---|---|---|---|---|---|---|---|---|
| D60 B L1 R1 | 73208 | 60916 | 34730 | 34730 | 34730 | 27728 | 15846 | 362 |
| D60 B L1 R2 | 50617 | 38325 | 20194 | 20194 | 20194 | 15846 | 15846 | 421 |
| 6h P-IWS R1 | 67620 | 55328 | 36323 | 35956 | 35956 | 31456 | 15846 | 365 |
| 6h P-IWS R2 | 77204 | 64912 | 423402 | 41245 | 41245 | 37987 | 15846 | 341 |
| 6h AF R1 | 103352 | 91060 | 45514 | 45514 | 45514 | 39230 | 15846 | 302 |
| 6h AF R2 | 46215 | 33923 | 18756 | 18756 | 18756 | 16250 | 15846 | 534 |
| D60 L2 R1 | 54570 | 42278 | 22748 | 22748 | 22748 | 17246 | 15846 | 607 |
| D60 L2 R2 | 54123 | 41831 | 24621 | 24621 | 24621 | 17878 | 15846 | 554 |
| 48h P-IWS R1 | 72742 | 60450 | 28509 | 28509 | 28509 | 22347 | 15846 | 288 |
| 48h P-IWS R2 | 63429 | 51137 | 24793 | 24793 | 24793 | 20917 | 15846 | 282 |
| 48h AF R1 | 107526 | 95234 | 50106 | 50106 | 50106 | 46248 | 15846 | 286 |
| 48h AF R2 | 93327 | 81035 | 44843 | 44843 | 44843 | 40618 | 15846 | 274 |
| D60 L3 R1 | 77453 | 65161 | 39975 | 39975 | 39975 | 33579 | 15846 | 328 |
| D60 L3 R2 | 96905 | 84613 | 42917 | 42917 | 42917 | 30514 | 15846 | 399 |
| 6d P-IWS R1 | 69126 | 56834 | 27454 | 27454 | 27454 | 20901 | 15846 | 400 |
| 6d P-IWS R2 | 117382 | 105090 | 48062 | 48062 | 48062 | 39998 | 15846 | 304 |
| 6d AF R1 | 101674 | 89382 | 51712 | 51712 | 51712 | 44397 | 15846 | 396 |
| 6d AF R2 | 93938 | 81646 | 49937 | 49937 | 49937 | 41408 | 15846 | 394 |
| D60 W L1 R1 | 109505 | 86188 | 50163 | 50163 | 50163 | 44028 | 15846 | 441 |
| D60 W L2 R2 | 96705 | 73388 | 42435 | 42435 | 42435 | 37822 | 15846 | 322 |
| D60 W L3 R3 | 101356 | 78039 | 43112 | 43112 | 43112 | 37260 | 15846 | 356 |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| 6h S0 R1 | 96217 | 72900 | 46561 | 46561 | 46561 | 41767 | 15846 | 356 |
| 6h S0 R2 | 90267 | 66950 | 44320 | 44320 | 44320 | 39859 | 15846 | 314 |
| 6h S0 R3 | 100942 | 77625 | 49388 | 49388 | 49388 | 43756 | 15846 | 373 |
| 6h S1 R1 | 103950 | 80633 | 52741 | 52741 | 52741 | 46963 | 15846 | 393 |
| 6h S1 R2 | 110055 | 86738 | 47933 | 47933 | 47933 | 41379 | 15846 | 447 |
| 6h S1 R3 | 125226 | 101909 | 59736 | 59736 | 59736 | 52025 | 15846 | 535 |
| 6h S2 R1 | 102604 | 79287 | 38788 | 38788 | 38788 | 33691 | 15846 | 505 |
| 6h S2 R2 | 117347 | 94030 | 59485 | 59485 | 59485 | 53520 | 15846 | 414 |
| 6h S2 R3 | 112107 | 88790 | 49245 | 49245 | 49245 | 42643 | 15846 | 484 |
| 6h S3 R1 | 101420 | 78103 | 52229 | 52229 | 52229 | 47274 | 15846 | 382 |
| 6h S3 R2 | 92994 | 69677 | 44590 | 44590 | 44590 | 39987 | 15846 | 481 |
| 6h S3 R3 | 92525 | 69208 | 45552 | 45552 | 45552 | 42091 | 15846 | 470 |
| 6h S4 R1 | 84937 | 61620 | 38865 | 38865 | 38865 | 35708 | 15846 | 403 |
| 6h S4 R2 | 86126 | 62809 | 41158 | 41158 | 41158 | 37180 | 15846 | 453 |
| 6h S4 R3 | 78546 | 55229 | 36773 | 36773 | 36773 | 34206 | 15846 | 391 |
| 48h S0 R1 | 86565 | 63248 | 41837 | 41837 | 41837 | 37017 | 15846 | 241 |
| 48h S0 R2 | 63889 | 40572 | 23896 | 23896 | 23896 | 20789 | 15846 | 332 |
| 48h S0 R3 | 77847 | 54530 | 32882 | 32882 | 32882 | 28082 | 15846 | 337 |
| 48h S1 R1 | 81476 | 58159 | 35567 | 35567 | 35567 | 30784 | 15846 | 414 |
| 48h S1 R2 | 101173 | 77856 | 51371 | 51371 | 51371 | 45424 | 15846 | 315 |
| 48h S1 R3 | 79323 | 56006 | 33487 | 33487 | 33487 | 29859 | 15846 | 383 |
| 48h S2 R1 | 103147 | 79830 | 46604 | 46604 | 46604 | 40802 | 15846 | 472 |
| 48h S2 R2 | 90688 | 67371 | 41603 | 41603 | 41603 | 36109 | 15846 | 449 |
| 48h S2 R3 | 93104 | 69787 | 45055 | 45055 | 45055 | 39947 | 15846 | 413 |
| 48h S3 R1 | 93148 | 69831 | 45823 | 45823 | 45823 | 41069 | 15846 | 489 |
| 48h S3 R2 | 81903 | 58586 | 38244 | 38244 | 38244 | 34442 | 15846 | 411 |
| 48h S3 R3 | 121035 | 97718 | 56480 | 56480 | 56480 | 50290 | 15846 | 519 |
| 48h S4 R1 | 103579 | 80262 | 49064 | 49064 | 49064 | 44460 | 15846 | 523 |
| 48h S4 R2 | 31351 | 8034 | 4403 | 4403 | 4403 | 4100 | - | - |
| 48h S4 R3 | 87172 | 74880 | 49481 | 49481 | 49481 | 45361 | 15846 | 438 |
| 6d S0 R1 | 71421 | 59129 | 37348 | 37348 | 37348 | 33309 | 15846 | 262 |
| 6d S0 R2 | 93017 | 80725 | 50175 | 50175 | 50175 | 44869 | 15846 | 311 |
| 6d S0 R3 | 87087 | 74795 | 41694 | 41694 | 41694 | 37311 | 15846 | 377 |
| 6d S1 R1 | 78840 | 66548 | 41487 | 41487 | 41487 | 36505 | 15846 | 350 |
| 6d S1 R2 | 66056 | 53764 | 32448 | 32448 | 32448 | 29066 | 15846 | 383 |
| 6d S1 R3 | 83558 | 71266 | 44708 | 44708 | 44708 | 39463 | 15846 | 396 |
| 6d S2 R1 | 93237 | 80945 | 51394 | 51394 | 51394 | 45882 | 15846 | 389 |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| 6d S2 R2 | 85724 | 73432 | 49498 | 49498 | 49498 | 43515 | 15846 | 375 |
| 6d S2 R3 | 70307 | 58015 | 37281 | 37281 | 37281 | 34570 | 15846 | 296 |
| 6d S3 R1 | 99822 | 87530 | 56552 | 56552 | 56552 | 50553 | 15846 | 456 |
| 6d S3 R2 | 84623 | 72331 | 48661 | 48661 | 48661 | 44013 | 15846 | 408 |
| 6d S3 R3 | 101180 | 88888 | 60988 | 60988 | 60988 | 54721 | 15846 | 375 |
| 6d S4 R1 | 76471 | 64179 | 40255 | 40255 | 40255 | 35874 | 15846 | 432 |
| 6d S4 R2 | 75372 | 63080 | 44769 | 44769 | 44769 | 40976 | 15846 | 311 |
| 6d S4 R3 | 75754 | 63462 | 41444 | 41444 | 41444 | 37069 | 15846 | 368 |

***Table S 32***. Sequence count during each step of bioinformatic analysis of the fungal ITS1-2 region in all biofilm and water samples after different IWS times. (-) Samples that did not amplify or were excluded during the analysis for not having enough readings.

| Sample ID | Raw reads | Filtered and imported to QIIME2 | After join pair-ends | After dereplication | After clustering (97%) | After chimera filtering | Rarefied | Total OTUs observed |
|---|---|---|---|---|---|---|---|---|
| D60 B L1 R1 | 248296 | 229324 | 79433 | 79426 | 79426 | 77934 | 12029 | 280 |
| D60 B L1 R2 | 188897 | 169925 | 70823 | 70816 | 70816 | 70212 | 12029 | 191 |
| 6h P-IWS R1 | 297617 | 278645 | 90456 | 90387 | 90387 | 90380 | 12029 | 187 |
| 6h P-IWS R2 | 328844 | 309872 | 156872 | 156821 | 156821 | 156819 | 12029 | 232 |
| 6h AF R1 | 357937 | 338965 | 162815 | 162809 | 162809 | 162605 | 12029 | 171 |
| 6h AF R2 | 376954 | 357982 | 198676 | 198670 | 198670 | 197675 | 12029 | 266 |
| D60 L2 R1 | 73612 | 54640 | 26043 | 26039 | 26039 | 25394 | 12029 | 315 |
| D60 L2 R2 | 90646 | 71674 | 23924 | 23922 | 23922 | 23460 | 12029 | 382 |
| 48h P-IWS R1 | 120956 | 101984 | 49457 | 49455 | 49455 | 49236 | 12029 | 236 |
| 48h P-IWS R2 | 67203 | 48231 | 21564 | 21561 | 21561 | 21124 | 12029 | 481 |
| 48h AF R1 | 98880 | 79908 | 37074 | 37072 | 37072 | 36883 | 12029 | 260 |
| 48h AF R2 | 298204 | 279232 | 124901 | 124899 | 124899 | 124739 | 12029 | 175 |
| D60 L3 R1 | 78545 | 59573 | 27200 | 27199 | 27199 | 26677 | 1029 | 328 |
| D60 L3 R2 | 98953 | 79981 | 39006 | 39004 | 39004 | 38759 | 12029 | 286 |
| 6d P-IWS R1 | 151030 | 132058 | 47213 | 47205 | 47205 | 45808 | 12029 | 296 |
| 6d P-IWS R2 | 150671 | 131699 | 43111 | 43108 | 43108 | 41268 | 12029 | 282 |
| 6d AF R1 | 81062 | 62090 | 30664 | 30662 | 30662 | 30503 | 12029 | 350 |
| 6d AF R2 | 83217 | 64245 | 40806 | 40805 | 40805 | 40676 | 12029 | 294 |
| D60 W L1 R1 | 111618 | 98506 | 48664 | 48661 | 48661 | 48523 | 12029 | 119 |
| D60 W L2 R2 | 57127 | 44015 | 12144 | 12143 | 12143 | 12029 | 12029 | 164 |
| D60 W L3 R3 | 82832 | 69720 | 34355 | 34353 | 34353 | 34202 | 12029 | 137 |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| 6h S0 R1 | 131091 | 117979 | 57455 | 57453 | 57453 | 57147 | 12029 | 193 |
| 6h S0 R2 | 133907 | 120795 | 46593 | 46591 | 46591 | 46487 | 12029 | 227 |
| 6h S0 R3 | 135912 | 122800 | 60112 | 60108 | 60108 | 59796 | 12029 | 232 |
| 6h S1 R1 | 126594 | 113482 | 53660 | 53657 | 53657 | 53473 | 12029 | 259 |
| 6h S1 R2 | 102906 | 89794 | 44134 | 44131 | 44131 | 43954 | 12029 | 258 |
| 6h S1 R3 | 100072 | 86960 | 42169 | 42167 | 42167 | 41974 | 12029 | 236 |
| 6h S2 R1 | 111627 | 98515 | 40854 | 40851 | 40851 | 40644 | 12029 | 317 |
| 6h S2 R2 | 83535 | 70423 | 34914 | 34913 | 34913 | 34753 | 12029 | 230 |
| 6h S2 R3 | 69878 | 56766 | 28226 | 28226 | 28226 | 28030 | 12029 | 265 |
| 6h S3 R1 | 118774 | 105662 | 43481 | 43479 | 43479 | 43273 | 12029 | 302 |
| 6h S3 R2 | 77091 | 63979 | 27107 | 27105 | 27105 | 26972 | 12029 | 303 |
| 6h S3 R3 | 150750 | 137638 | 59816 | 59812 | 59812 | 59599 | 12029 | 276 |
| 6h S4 R1 | 150749 | 137637 | 44357 | 44354 | 44354 | 44215 | 12029 | 242 |
| 6h S4 R2 | 157254 | 144142 | 59578 | 59578 | 59578 | 59438 | 12029 | 257 |
| 6h S4 R3 | 166240 | 153128 | 47205 | 47204 | 47204 | 47062 | 12029 | 251 |
| 48h S0 R1 | 100864 | 87752 | 36366 | 36366 | 36366 | 36192 | 12029 | 224 |
| 48h S0 R2 | 138615 | 125503 | 44438 | 44435 | 44435 | 44245 | 12029 | 282 |
| 48h S0 R3 | 160463 | 147351 | 67753 | 67751 | 67751 | 67577 | 12029 | 234 |
| 48h S1 R1 | 154812 | 141700 | 30560 | 30558 | 30558 | 30393 | 12029 | 249 |
| 48h S1 R2 | 135057 | 121945 | 47101 | 47100 | 47100 | 47024 | 12029 | 236 |
| 48h S1 R3 | - | - | - | - | - | - | - | - |
| 48h S2 R1 | 142552 | 129440 | 41685 | 41685 | 41685 | 41432 | 12029 | 231 |
| 48h S2 R2 | 93304 | 80192 | 39688 | 39687 | 39687 | 39529 | 12029 | 200 |
| 48h S2 R3 | 131125 | 118013 | 71328 | 71326 | 71326 | 71211 | 12029 | 224 |
| 48h S3 R1 | 58783 | 45671 | 24556 | 24554 | 24554 | 24436 | 12029 | 365 |
| 48h S3 R2 | 74844 | 61732 | 30397 | 30397 | 30397 | 30327 | 12029 | 190 |
| 48h S3 R3 | 66435 | 53323 | 26744 | 26744 | 26744 | 26601 | 12029 | 303 |
| 48h S4 R1 | 67260 | 54148 | 26392 | 26392 | 26392 | 26269 | 12029 | 240 |
| 48h S4 R2 | - | - | - | - | - | - | - | - |
| 48h S4 R3 | 68134 | 49162 | 20353 | 20352 | 20352 | 20249 | 12029 | 279 |
| 6d S0 R1 | 80683 | 61711 | 25717 | 25716 | 25716 | 25430 | 12029 | 256 |
| 6d S0 R2 | 123355 | 104383 | 48892 | 48889 | 48889 | 48620 | 12029 | 214 |
| 6d S0 R3 | 112031 | 93059 | 45465 | 45464 | 45464 | 45003 | 12029 | 279 |
| 6d S1 R1 | 137440 | 118468 | 58026 | 58024 | 58024 | 57778 | 12029 | 201 |
| 6d S1 R2 | 107231 | 88259 | 43130 | 43127 | 43127 | 42876 | 12029 | 234 |
| 6d S1 R3 | 86543 | 67571 | 31036 | 31029 | 31029 | 30714 | 12029 | 325 |
| 6d S2 R1 | 113931 | 94959 | 41831 | 41830 | 41830 | 41642 | 12029 | 309 |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| 6d S2 R2 | 111442 | 92470 | 40202 | 40201 | 40201 | 40987 | 12029 | 244 |
| 6d S2 R3 | 104701 | 85729 | 31657 | 31656 | 31656 | 31518 | 12029 | 288 |
| 6d S3 R1 | 95969 | 76997 | 37463 | 37462 | 37462 | 37163 | 12029 | 264 |
| 6d S3 R2 | 89334 | 70362 | 34173 | 34171 | 34171 | 34005 | 12029 | 224 |
| 6d S3 R3 | 119582 | 100610 | 39234 | 39234 | 39234 | 38977 | 12029 | 245 |
| 6d S4 R1 | 96909 | 77937 | 33282 | 33281 | 33281 | 33211 | 12029 | 209 |
| 6d S4 R2 | 120529 | 101557 | 50600 | 50600 | 50600 | 50348 | 12029 | 231 |
| 6d S4 R3 | 85576 | 66604 | 33070 | 33068 | 33068 | 32889 | 12029 | 269 |

## C.5 Rarefaction curves

⇒ **Bacteria**

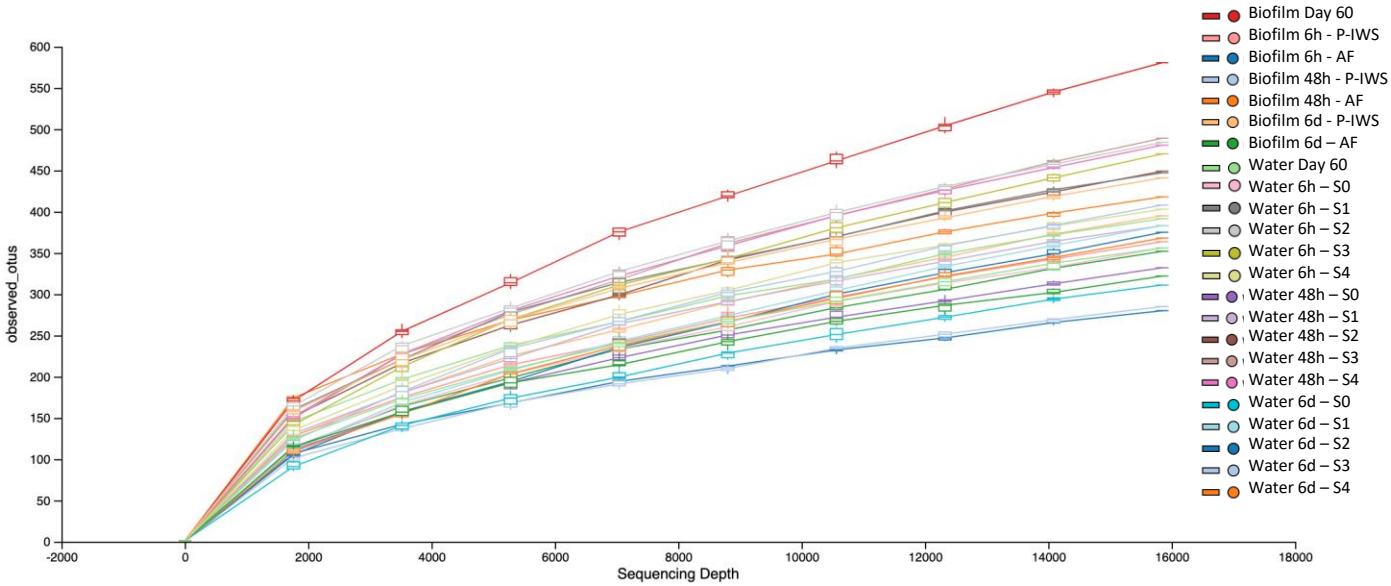



***Figure S 17.*** Rarefaction curves of observed OTUS for bacteria in all biofilm and water samples after different IWS times, applying a sequencing depth of 15846 sequences. Each curve represents an average of all biological replicates of each sample.

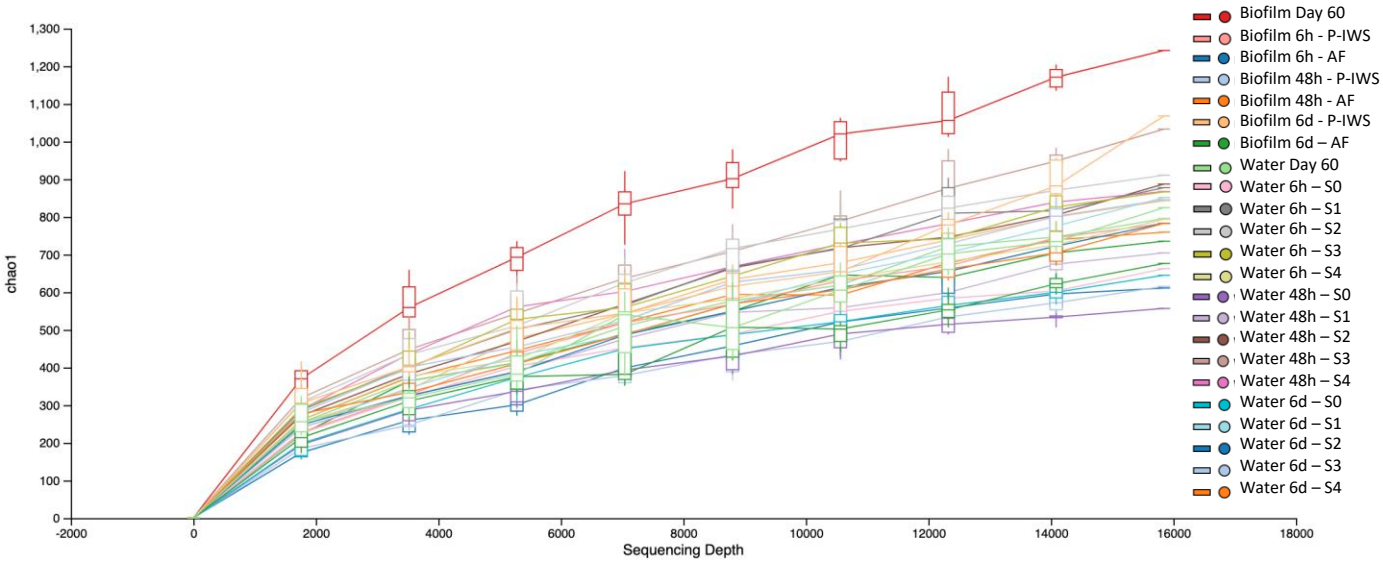



***Figure S 18***. Rarefaction curves of Chao1 index for bacteria in all biofilm and water samples after different IWS times, applying a sequencing depth of 15846 sequences. Each curve represents an average of all biological replicates of each sample.

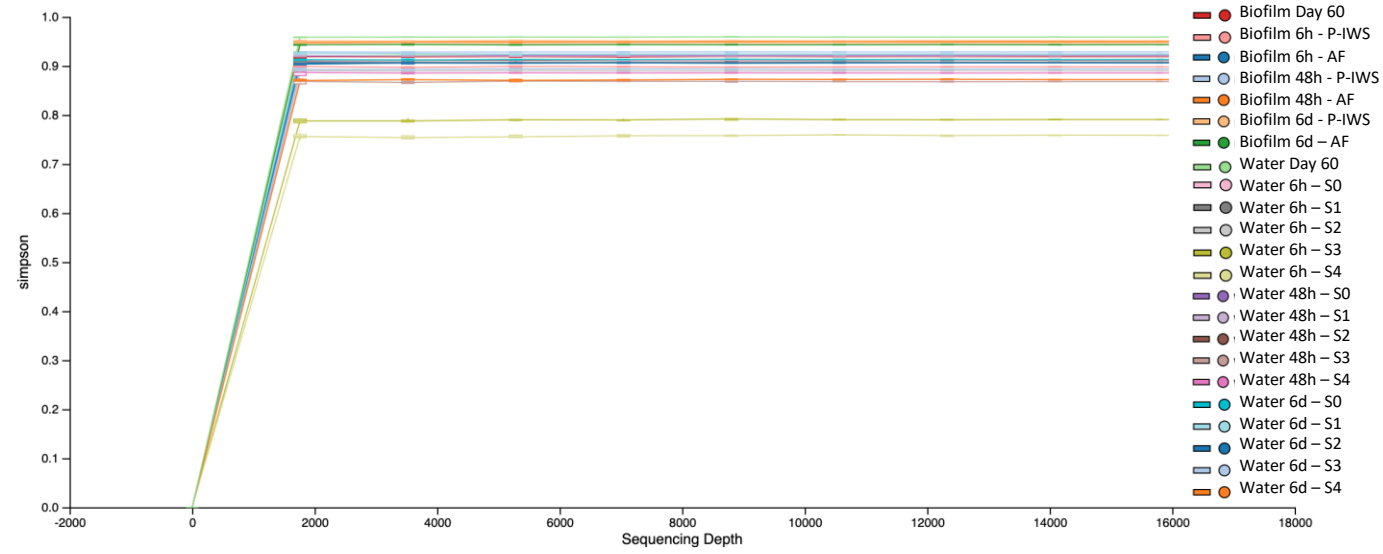



***Figure S 19***. Rarefaction curves of Simpson index for bacteria in all biofilm and water samples after different IWS times, applying a. sequencing depth of 15846 sequences. Each curve represents an average of all biological replicates of each sample.

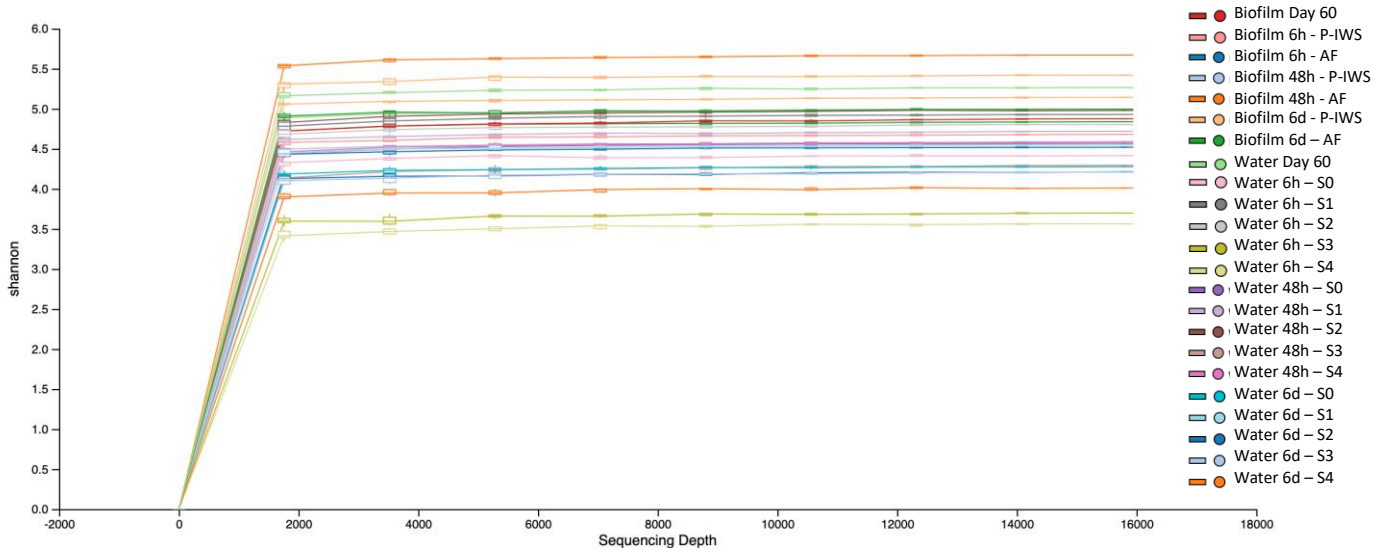



***Figure S 20***. Rarefaction curves of Shannon index for bacteria in all biofilm and water samples after different IWS times, applying a. sequencing depth of 15846 sequences. Each curve represents an average of all biological replicates of each sample.

⇒ **Fungi**

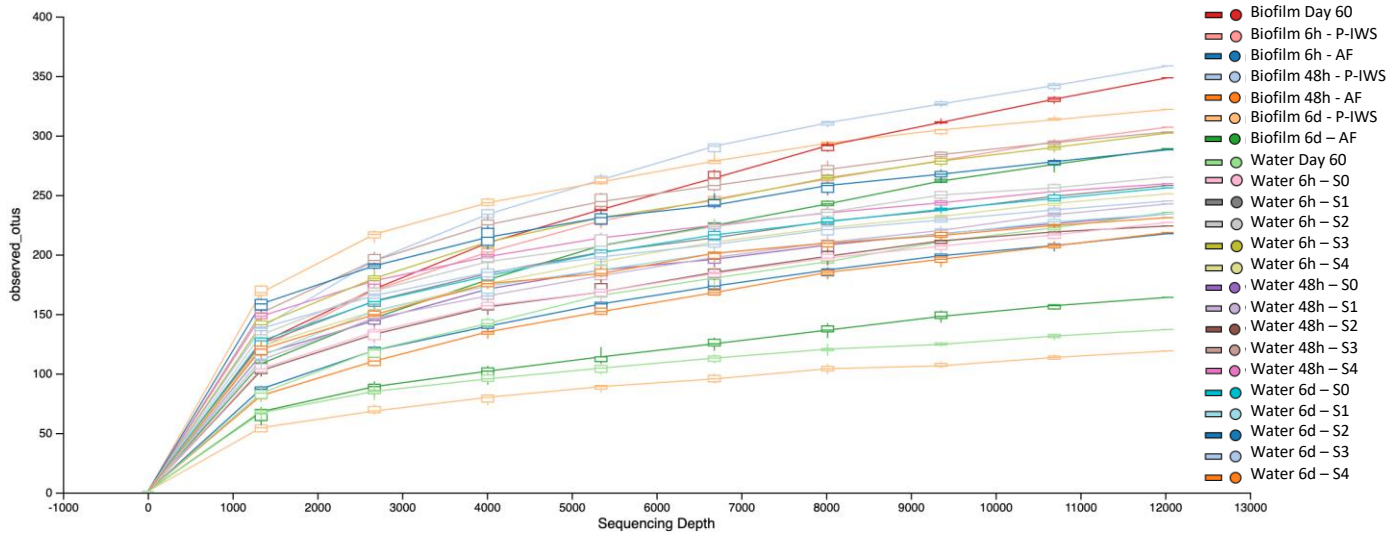



***Figure S 21.*** Rarefaction curves of observed OTUS for fungi in all biofilm and water samples after different IWS times, applying a sequencing depth of 12029 sequences. Each curve represents an average of all biological replicates of each sample.

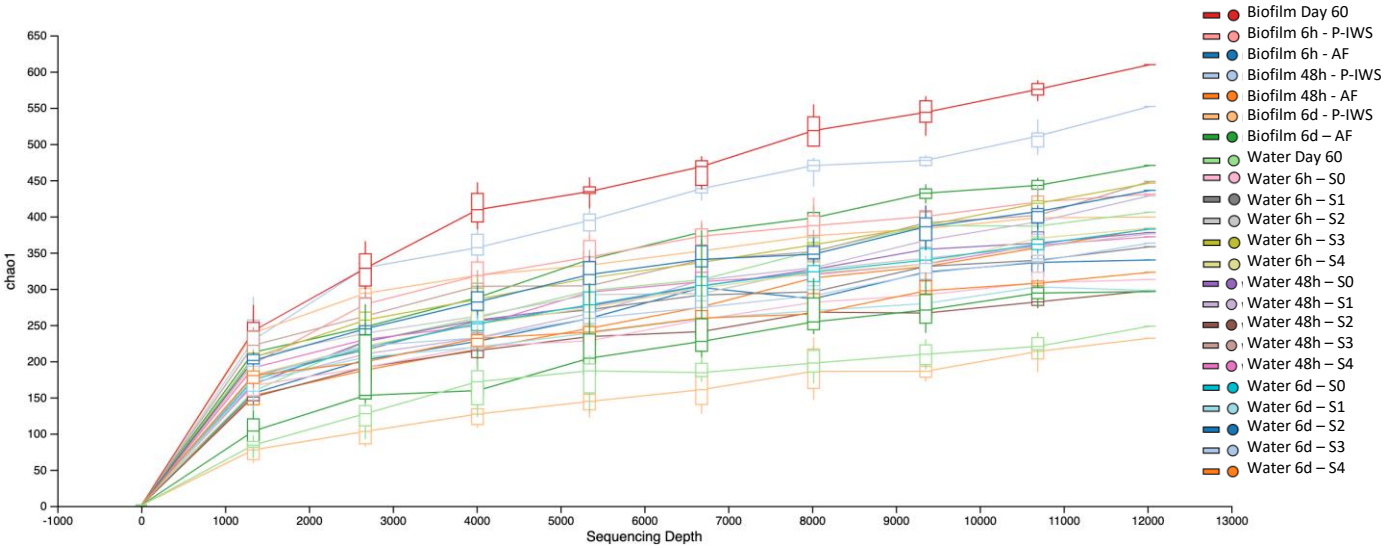



***Figure S 22.*** Rarefaction curves of Chao1 index for fungi in all biofilm and water samples after different IWS times, applying a sequencing depth of 12029 sequences. Each curve represents an average of all biological replicates of each sample.

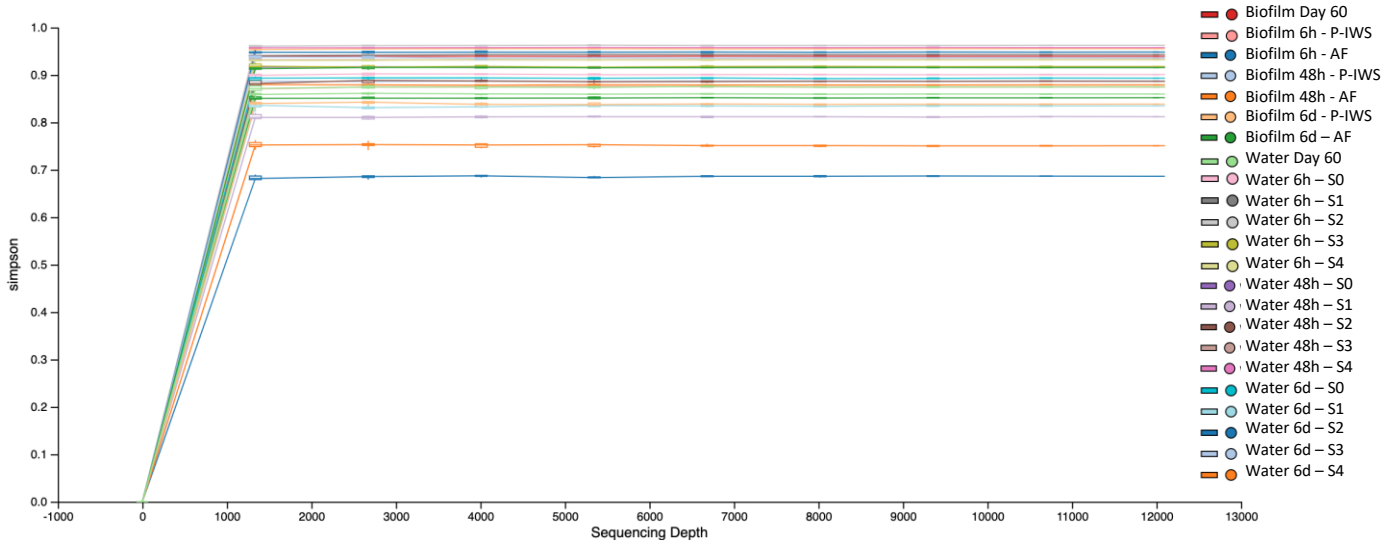



***Figure S 23.*** Rarefaction curves of Simpson index for fungi in all biofilm and water samples after different IWS times, applying a sequencing depth of 12029 sequences. Each curve represents an average of all biological replicates of each sample.

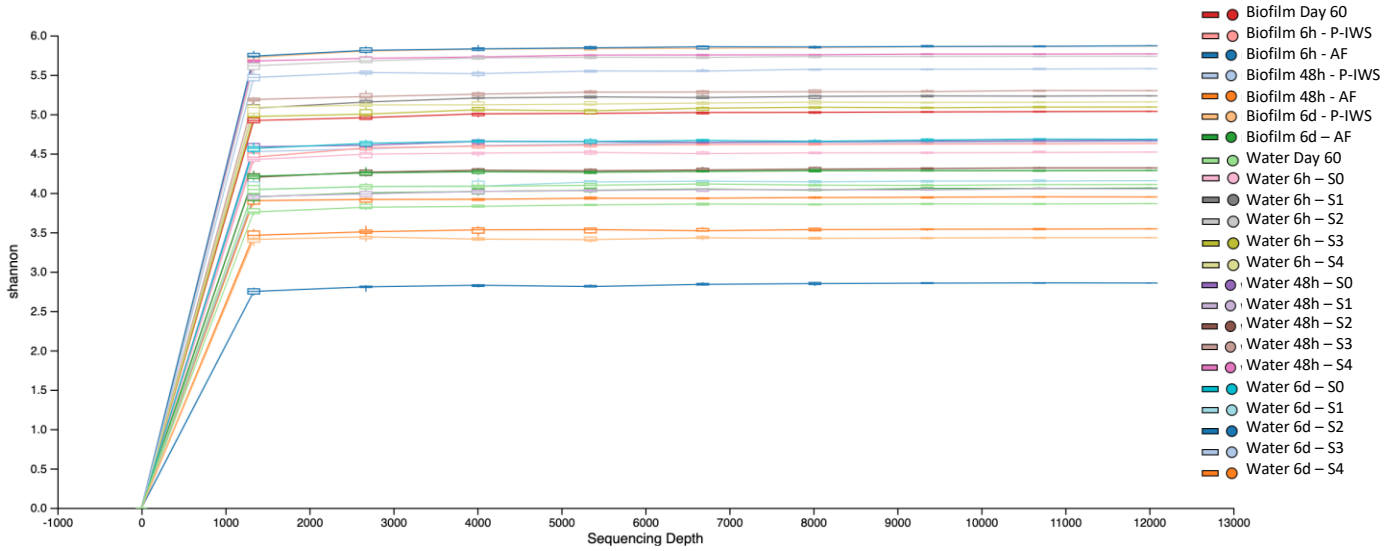



***Figure S 24***. Rarefaction curves of Shannon index for fungi in all biofilm and water samples after different IWS times, applying a sequencing depth of 12029 sequences. Each curve represents an average of all biological replicates of each sample.

## C.6 Statistical analysis

***Table S 33***. Results from the Kruskal-Wallis test to determine statistical differences in water-physico chemical parameters between IWS times. Differences were considered statistically significant when p-value was ≤0.05.

| Kruskal-Wallis test | Total chlorine | Free chlorine | Fe | Mn | TOC | DOC | $SUVA_{254}$ |
|---|---|---|---|---|---|---|---|
| Day 0 | $X^2$ = 5.5 p-value = 0.06 | $X^2$ = 6.0 p-value = 0.06 | $X^2$ = 6.5 p-value = 0.07 | $X^2$ = 6.3 p-value = 0.07 | $X^2$ = 5.5 p-value = 0.06 | $X^2$ = 6.3 p-value = 0.07 | $X^2$ = 1.14 p-value = 0.56 |
| Day 20 | $X^2$ = 6.5 p-value = 0.07 | $X^2$ = 6.1 p-value = 0.07 | $X^2$ = 6.3 p-value = 0.07 | $X^2$ = 5.9 p-value = 0.06 | $X^2$ = 6.3 p-value = 0.07 | $X^2$ = 6.7 p-value = 0.08 | $X^2$ = 3.5 p-value = 0.16 |
| Day 40 | $X^2$ = 5.6 p-value = 0.06 | $X^2$ = 6.3 p-value = 0.07 | $X^2$ = 6.1 p-value = 0.08 | $X^2$ = 6.5 p-value = 0.07 | $X^2$ = 4.5 p-value = 0.3 | $X^2$ = p-value = | $X^2$ = 5.8 p-value = 0.06 |
| Day 60 | $X^2$ = 5.8 p-value = 0.06 | $X^2$ = 6.5 p-value = 0.07 | $X^2$ = 5.9 p-value = 0.06 | $X^2$ = 6.3 p-value = 0.07 | $X^2$ = 3.2 p-value = 0.6 | $X^2$ = 6.5 p-value = 0.07 | $X^2$ = 1.2 p-value = 0.54 |
| Stage 0 | $X^2$ = 5.9 p-value = 0.06 | $X^2$ = 7.2 p-value = 008 | $X^2$ = 1.9 p-value = 0.39 | $X^2$ = 7.2 p-value = 0.03 | $X^2$ = 2.6 p-value = 0.87 | $X^2$ = 5.1 p-value = 0.07 | $X^2$ = 5.9 p-value = 0.6 |
| Stage 1 | $X^2$ = 5.5 p-value = 0.07 | $X^2$ = 6.3 p-value = 0.07 | $X^2$ = 1.1 p-value = 0.58 | $X^2$ = 5.9 p-value = 0.05 | $X^2$ = 4.2 p-value = 0.07 | $X^2$ = 1.8 p-value = 0.39 | $X^2$ = 5.9 p-value = 0.05 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| Stage 2 | $X^2$ = 4.3 p-value = 0.1 | $X^2$ = 4.5 p-value = 0.1 | $X^2$ = 7.2 p-value = 0.02 | $X^2$ = 6.8 p-value = 0.05 | $X^2$ = 5.4 p-value = 0.06 | $X^2$ = 5.4 p-value = 0.06 | $X^2$ = 7.2 p-value = 0.03 |
| Stage 3 | $X^2$ = 5.5 p-value = 0.06 | $X^2$ = 5.3 p-value = 0.08 | $X^2$ = 5.9 p-value = 0.05 | $X^2$ = 5.9 p-value = 0.05 | $X^2$ = 1.8 p-value = 0.39 | $X^2$ = 1.4 p-value = 0.49 | $X^2$ = 6.06 p-value = 0.07 |
| Stage 4 | $X^2$ = 5.5 p-value = 0.07 | $X^2$ = 5.1 p-value = 0.09 | $X^2$ = 7.2 p-value = 0.02 | $X^2$ = 7.2 p-value = 0.03 | $X^2$ = 1.8 p-value = 0.38 | $X^2$ = 5.06 p-value = 0.07 | $X^2$ = 7.2 p-value = 0.03 |

***Table S 34***. Results from the Mann-Whitney U test for pairwise comparisons to determine statistical differences in Fe concentration between IWS times. Differences were considered statistically significant when p-value was ≤0.05.

| **Mann-Whitney *U* test** | **Stage 2** | **Stage 3** | **Stage 4** |
|---|---|---|---|
| 6 hours vs 48 hours | W =3<br>p-value = 0.7 | W = 9<br>p-value = 0.1 | W = 7<br>p-value = 0.4 |
| 6 hours vs 6 days | W = 9<br>p-value = 0.05 | W = 9<br>p-value = 0.05 | W = 9<br>p-value = 0.05 |
| 48 hours vs 6 days | W = 0<br>p-value = 1 | W = 2<br>p-value = 0.4 | W = 2<br>p-value = 0.4 |

***Table S 35***. Results from the Mann-Whitney U test for pairwise comparisons to determine statistical differences in Mn concentration between IWS times. Differences were considered statistically significant when p-value was ≤0.05.

| **Mann-Whitney *U* test** | **Stage 0** | **Stage 1** | **Stage 2** | **Stage 3** | **Stage 4** |
|---|---|---|---|---|---|
| 6 hours vs 48 hours | W = 0<br>p-value = 1 | W = 7<br>p-value = 0.4 | W = 3<br>p-value = 0.7 | W = 9<br>p-value = 0.05 | W = 9<br>p-value = 0.05 |
| 6 hours vs 6 days | W = 9<br>p-value = 0.05 | W = 9<br>p-value = 0.05 | W = 9<br>p-value = 0.05 | W = 9<br>p-value = 0.05 | W = 9<br>p-value = 0.05 |
| 48 hours vs 6 days | W = 9<br>p-value = 0.05 | W = 9<br>p-value = 0.05 | W = 9<br>p-value = 0.05 | W = 0<br>p-value = 0.05 | W = 0<br>p-value = 0.05 |

***Table S 36***. Results from the Mann-Whitney U test for pairwise comparisons to determine statistical differences in $SUVA_{234}$ concentration between IWS times. Differences were considered statistically significant when p-value was ≤0.05.

| **Mann-Whitney *U* test** | **Stage 1** | **Stage 2** | **Stage 4** |
|---|---|---|---|
| 6 hours vs 48 hours | W = 0<br>p-value = 0.05 | W = 0<br>p-value = 0.05 | W = 0<br>p-value = 0.05 |
| 6 hours vs 6 days | W = 0<br>p-value = 0.05 | W = 0<br>p-value = 0.05 | W = 0<br>p-value = 0.05 |
| 48 hours vs 6 days | W = 9<br>p-value = 0.1 | W = 9<br>p-value = 0.05 | W = 0<br>p-value = 0.05 |

***Table S 37***. Results from Kruskal-Wallis test to determine statistical differences in turbidity response between IWS times. Differences were considered statistically significant when p-value was ≤0.05.

| **Mann-Whitney *U* test** | **Turbidity** |
|---|---|
| Last 24 hours | $X^2$ = 10.2<br>p-value = 0.1 |
| Stage 0 | $X^2$ = 26.7<br>p-value = 1.05e-6 |
| Stage 1 | $X^2$ = 32.1<br>p-value = 3.05e-8 |
| Stage 2 | $X^2$ = 37.31<br>p-value = 7.8e-11 |
| Stage 3 | $X^2$ = 35.7<br>p-value = 1.63e-11 |
| Stage 4 | $X^2$ = 36.8<br>p-value = 3.05e-11 |

***Table S 38***. Results from the Mann Whitney U test to determine statistical differences in turbidity response between IWS times. Differences were considered statistically significant when p-value was ≤0.05.

| **Mann-Whitney *U* test** | **Stage 0** | **Stage 1** | **Stage 2** | **Stage 3** | **Stage 4** |
|---|---|---|---|---|---|
| 6 hours vs 48 hours | W = 852670<br>p-value = 2.2e-16 | W = 965785<br>p-value = 2.2e-16 | W = 905356<br>p-value = 2.2e-16 | W = 912846<br>p-value = 2.2e-16 | W = 876989<br>p-value = 2.2e-16 |
| 6 hours vs 6 days | W = 896347<br>p-value = 1.3e-17 | W = 765201<br>p-value = 1.5e-11 | W = 889891<br>p-value = 2.2e-16 | W = 856136<br>p-value = 2.2e-16 | W = 960325<br>p-value = 2.2e-16 |
| 48 hours vs 6 days | W = 10365<br>p-value = 0.07 | W = 23657<br>p-value = 0.08 | W = 891751<br>p-value = 2.2e-16 | W = 789569<br>p-value = 2.2e-16 | W = 10265<br>p-value = 0.07 |

***Table S 39***. Results from Kruskal-Wallis test to determine statistical differences in total and intact cell counts between IWS times. Differences were considered statistically significant when p-value was ≤0.05.

| **Kruskal-Wallis test** | **Total Cell Counts** | **Intact Cell Counts** |
|---|---|---|
| Day 0 | $X^2$ = 8.5<br>p-value = 0.2 | $X^2$ = 6.3<br>p-value = 1 |
| Day 20 | $X^2$ = 9.8<br>p-value = 0.4 | $X^2$ = 9.6<br>p-value = 0.3 |
| Day 40 | $X^2$ = 9.3<br>p-value = 0.3 | $X^2$ = 10.1<br>p-value = 0.07 |

| | | |
|---|---|---|
| Day 60 | $X^2$ = 9.46<br>p-value = 0.2 | $X^2$ = 9.2<br>p-value = 0.3 |
| Stage 0 | $X^2$ = 6.23<br>p-value = 0.1 | $X^2$ = 8.6<br>p-value = 0.08 |
| Stage 1 | $X^2$ = 10.4<br>p-value = 0.02 | $X^2$ = 9.67<br>p-value = 0.02 |
| Stage 2 | $X^2$ = 10.5<br>p-value = 0.02 | $X^2$ = 8.9<br>p-value = 0.03 |
| Stage 3 | $X^2$ = 9.8<br>p-value = 0.02 | $X^2$ = 10.0<br>p-value = 0.02 |
| Stage 4 | $X^2$ = 9.9<br>p-value = 0.02 | $X^2$ = 9.7<br>p-value = 0.05 |

***Table S 40***. Results from the Mann Whitney U test to determine statistical differences in total and intact cell counts between IWS times. Differences were considered statistically significant when p-value was ≤0.05.

| **Mann-Whitney *U* test** | **Stage 1** | | **Stage 2** | | **Stage 3** | | **Stage 3** | |
|---|---|---|---|---|---|---|---|---|
| | **Total** | **Intact** | **Total** | **Intact** | **Total** | **Intact** | **Total** | **Intact** |
| 6 hours vs 48 hours | W = 9<br>p-value = 1 | W = 0<br>p-value = 0.05 | W = 9<br>p-value = 1 | W = 0<br>p-value = 0.05 | W = 1.5<br>p-value = 0.13 | W = 0<br>p-value = 0.03 | W = 1<br>p-value = 0.2 | W = 0<br>p-value = 0.05 |
| 6 hours vs 6 days | W = 9<br>p-value = 0.05 | W = 9<br>p-value = 0.05 | W = 0<br>p-value = 0.05 | W = 0<br>p-value = 0.05 | W = 9<br>p-value = 0.05 | W = 0<br>p-value = 0.03 | W = 9<br>p-value = 0.05 | W = 0<br>p-value = 0.05 |
| 48 hours vs 6 days | W = 0<br>p-value = 0.05 | W = 0<br>p-value = 0.05 | W = 0<br>p-value = 0.05 | W = 0<br>p-value = 0.05 | W = 0<br>p-value = 0.05 | W = 0<br>p-value = 0.05 | W = 0<br>p-value = 0.05 | W = 0<br>p-value = 0.05 |

***Table S 41***. Results from Kruskal-Wallis test to determine statistical differences in bacterial alpha diversity indices between IWS times. Differences were considered statistically significant when p-value was ≤0.05.

| **Kruskal-Wallis test** | | **Bacteria** | | |
|---|---|---|---|---|
| | | **Chao1** | **Simpson** | **Shannon** |
| Biofilm | Day 60 | $X^2$ = 1<br>p-value = 0.2 | $X^2$ = 0<br>p-value = 0.1 | $X^2$ = 9<br>p-value = 0.1 |
| | Post-IWS | $X^2$ = 0<br>p-value = 0.2 | X2 = 0<br>p-value = 0.1 | $X^2$ = 0<br>p-value = 0.06 |
| | After restarting | $X^2$ = 0<br>p-value = 0.35 | $X^2$ = 0<br>p-value = 0.5 | $X^2$ = 0<br>p-value = 0.35 |

| | | | | |
|---|---|---|---|---|
| Water | Day 60 | $X^2 = 1$<br>p-value = 0.1 | $X^2 = 0$<br>p-value = 0.1 | $X^2 = 0$<br>p-value = 0.1 |
| | Stage 0 | $X^2 = 0$<br>p-value = 0.9 | $X^2 = 1$<br>p-value = 0.8 | $X^2 = 1$<br>p-value = 0.7 |
| | Stage 1 | $X^2 = 1$<br>p-value = 0.8 | $X^2 = 0$<br>p-value = 0.5 | $X^2 = 1$<br>p-value = 0.35 |
| | Stage 2 | $X^2 = 0$<br>p-value = 0.2 | $X^2 = 1$<br>p-value = 0.35 | $X^2 = 0$<br>p-value = 0.5 |
| | Stage 3 | $X^2 = 0$<br>p-value = 0.2 | $X^2 = 1$<br>p-value = 0.2 | $X^2 = 0$<br>p-value = 0.2 |
| | Stage 4 | $X^2 = 1$<br>p-value = 0.1 | $X^2 = 0$<br>p-value = 0.1 | $X^2 = 1$<br>p-value = 0.1 |

***Table S 42***. Results from Kruskal-Wallis test to determine statistical differences in fungal alpha diversity indices between IWS times. Differences were considered statistically significant when p-value was ≤0.05.

| **Kruskal-Wallis test** | | **Fungi** | | |
|---|---|---|---|---|
| | | **Chao1** | **Simpson** | **Shannon** |
| Biofilm | Day 60 | $X^2 = 0$<br>p-value = 0.1 | $X^2 = 0$<br>p-value = 0.1 | $X^2 = 0$<br>p-value = 0.1 |
| | Post-IWS | $X^2 = 1$<br>p-value = 0.2 | $X^2 = 2$<br>p-value = 0.06 | $X^2 = 2$<br>p-value = 0.07 |
| | After restarting | $X^2 = 0$<br>p-value = 0.1 | $X^2 = 0$<br>p-value = 0.05 | $X^2 = 0$<br>p-value = 0.05 |
| Water | Day 60 | $X^2 = 0$<br>p-value = 0.06 | $X^2 = 0$<br>p-value = 1 | $X^2 = 1$<br>p-value = 0.1 |
| | Stage 0 | $X^2 = 0$<br>p-value = 0.06 | $X^2 = 0$<br>p-value = 0.95 | $X^2 = 0$<br>p-value = 0.5 |
| | Stage 1 | $X^2 = 1$<br>p-value = 0.5 | $X^2 = 0$<br>p-value = 0.05 | $X^2 = 0$<br>p-value = 0.05 |
| | Stage 2 | $X^2 = 1$<br>p-value = 0.5 | $X^2 = 0$<br>p-value = 0.05 | $X^2 = 0$<br>p-value = 0.05 |
| | Stage 3 | $X^2 = 0$<br>p-value = 0.1 | $X^2 = 1$<br>p-value = 0.1 | $X^2 = 0$<br>p-value = 0.05 |
| | Stage 4 | $X^2 = 0$<br>p-value = 0.1 | $X^2 = 0$<br>p-value = 1 | $X^2 = 1$<br>p-value = 0.1 |

***Table S 43***. Results from the Mann Whitney U test to determine statistical differences in Simpson and Shannon indices in fungi-biofilm after restarting samples between IWS times. Differences were considered statistically significant when p-value was ≤0.05.

| **Mann-Whitney *U* test** | **Fungi - Biofilm After restarting** | |
|---|---|---|
| | **Simpson** | **Shannon** |
| 6 hours vs 48 hours | W = 9<br>p-value = 0.03 | W = 0<br>p-value = 0.05 |
| 6 hours vs 6 days | W = 1<br>p-value = 0.05 | W = 0<br>p-value = 0.05 |
| 48 hours vs 6 days | W = 1<br>p-value = 0.05 | W = 1<br>p-value = 0.05 |

***Table S 44***. Results from the Mann Whitney U test to determine statistical differences in Simpson and Shannon indices in fungi-water stages 1 and 2 between IWS times. Differences were considered statistically significant when p-value was ≤0.05.

| **Mann-Whitney *U* test** | **Fungi - Water Stage 1** | | **Fungi - Water Stage 2** | |
|---|---|---|---|---|
| | **Simpson** | **Shannon** | **Simpson** | **Shannon** |
| 6 hours vs 48 hours | W = 0<br>p-value = 0.05 | W = 1<br>p-value = 0.05 | W = 9<br>p-value = 0.05 | W = 0<br>p-value = 0.03 |
| 6 hours vs 6 days | W = 0<br>p-value = 0.05 | W = 0<br>p-value = 0.05 | W = 0<br>p-value = 0.05 | W = 9<br>p-value = 0.05 |
| 48 hours vs 6 days | W = 9<br>p-value = 0.65 | W = 0<br>p-value = 0.1 | W = 7<br>p-value = 0.04 | W = 0<br>p-value = 0.1 |

# Appendix D. Contributions and co-authors

Summary of contributions and co-authors.

| | |
|---|---|
| Chapter 1 | My own work |
| Chapter 2 | My own work |
| Chapter 3 | My own work |
| Chapter 4 | My own work. An adapted version of this chapter was submitted in September 2020 to *Water Research* journal as Calero-Preciado, C.; Boxall, J.; Soria-Carrasco, V.; Martínez, S.; Douterelo, I.: Temperature increase triggers changes in the microbial dynamics of drinking water distribution systems. The contributions of the co-authors based on this chapter were the following: I carried out the experiment, analysed the results and wrote this chapter. Afterwards, from this chapter I drafted a first version for the journal publication. Dr. Isabel Douterelo was the principal investigator of the study, supervised all the analyses, the interpretation of results and the writing. Prof. Joby Boxall was involved in the design of the experiment, contributed to the interpretation of results and supervised the analyses and the writing. Dr. Víctor Soria-Carrasco was involved in the bioinformatic analysis. |
| Chapter 5 | My own work. This chapter will be adapted for a journal publication in *Environmental Science and Technology journal* as Calero-Preciado, C..; Soria-Carrasco, V.; Boxall, J.; Douterelo, I.: The effects of temperature on biofilm control strategies in chlorinated drinking water distribution systems. |
| Chapter 6 | My own work. This chapter will be adapted for a journal publication in *Environmental Science & Technology journal* as Calero Preciado, C..; Husband, S.; Maeng, S.; Boxall, J.; Soria-Carrasco, V.; Douterelo, I.: How intermittent water supply time impacts on the microbiome of drinking water distribution systems? Input from co-authors to adapt for publication is pending. |
| Chapter 7 | My own work |

# Appendix E. Scientific publications

Scientific publications derived from or related to the development of this thesis are detailed below.

## E.1 Journal publications

Del Olmo, G., Husband, S., Briones, C.S., Soriano, A., Calero, C., Macian, J. and Douterelo, I., 2020. The microbial ecology of a Mediterranean chlorinated drinking water distribution systems in the city of Valencia (Spain). *Science of The Total Environment*, p.142016.

Douterelo, I., Dutilh, B.E., Arkhipova, K., Calero, C. and Husband, S., 2020. Microbial diversity, ecological networks and functional traits associated to materials used in drinking water distribution systems. *Water Research*, *173*, p.115586.

Douterelo, I., Calero-Preciado, C., Soria-Carrasco, V. and Boxall, J.B., 2018. Whole metagenome sequencing of chlorinated drinking water distribution systems. *Environmental Science: Water Research & Technology*, *4*(12), pp.2080-2091.

## E.2 Conferences proceedings papers

Del, G., Rosales, E., Del, E., Jensen, H., Calero, C., Ahmad, A., Gaskin, P. and Douterelo, I., 2020. Influence of phosphate dosing on biofilm development on Drinking Water Distribution Systems infrastructure surfaces. *Access Microbiology*, *2*(7A), p.393.

Preciado CC, Boxall J, Soria-Carrasco V & Douterelo I (2019) Effect of temperature increase in bacterial and fungal communities of chlorinated drinking water distribution systems. Access Microbiology, 1(1A).

Douterelo, I., Calero, C. and Husband, S.P., 2018, July. Monitoring biofilm communities in operational drinking water distribution systems and the impact on water quality. In *WDSA/CCWI Joint Conference Proceedings* (Vol. 1).

## E.3 Conferences

Calero *et al.* (2019). Effect of temperature increase on microbial communities in chlorinated drinking water distribution systems. Poster and oral presentation at the 9th *International Young Water Professionals Conference.* Toronto, Canada.

Calero *et al.* (2019). Influence of intermittent water supply in microbial biofilm mobilization. Oral presentation at the *1st IWS Conference*. Kampala, Uganda.

Calero *et al.* (2019). Effect of temperature increase in bacterial and fungal communities of chlorinated drinking water distribution systems. Poster session at the *Microbial Society Conference*. Belfast, UK.

Calero *et al.* (2019). Microbial communities response to the increase in temperature in drinking water distribution systems. Poster session at the *8th Congress of European Microbiologists – FEMS 2019*. Glasgow, Scotland.

Calero *et al.* (2019). Drinking water distribution systems and climate change: microbiome and water quality. Poster and oral presentation at the *PGR Conference of University of Sheffield*. Sheffield, UK.

Martínez *et al.* (2019). Microbial characterization of secondary drinking water distribution systems in Uruguay". Oral presentation at the *IWA Biofilms 2019 Conference*: *Biofilms and their interaction with surfaces*. Santiago, Chile.

Douterelo *et al.* (2019) Biofilm adhesion properties at a nano and molecular scale for safe drinking water supply. Oral presentation at the *IWA Biofilms 2019 Conference*: *Biofilms and their interaction with surfaces*. Santiago, Chile.

Rosales *et al.* (2019). Influence of phosphate dosing to prevent plumbosolvency on biofilm formation and risk of mobilisation in an experimental chlorinated DWDS. Poster session at the *6th European Congress on Biofilms: EUROBIOFILMS*. Glasgow, Scotland.

Calero *et al.* (2018). Microbial traits in chlorinated Drinking Water Distribution Systems. Poster session at the *Microbial Society Conference*. Birmingham, UK.

Douterelo *et al.* (2018). Monitoring Biofilm Communities in Operational DWDS and the Impact on Water Quality. Oral presentation at the *WDSA/CCWI Conference*. Kingston, Canada.

Douterelo *et al.* (2018). Application and integration of biofilm ecology to inform management of DWDS. Oral presentation at the *Biofilms 8 International Conference*. Aarhus, Denmark.

Douterelo *et al.* (2017). Metagenomics put to work: use of microbial genes to understand DWDS. Oral presentation at the *Water Quality Technology Conference*. Portland, Oregon, United States.