Biofouling in open recirculating cooling systems

Characterization and control of biofilms and Legionella pneumophila

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Dissertation

for the purpose of obtaining the degree of doctor
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chair of the Board for Doctorates
to be defended publicly on
Monday 21 June 2021 at 10:00 o'clock

—

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CONTENTS

SUMMARY

Open recirculating cooling systems have been vital elements in industry since the early 20th century. Their purpose is to release excess heat from processes through water evaporation so that production can be carried out at optimal temperature. With the global development of industrial activities such as manufacturing, electricity and chemical production, the demand for cooling capacity keeps increasing. Biofouling is one of the main phenomena negatively affecting the performance of wet cooling systems. This phenomenon leads to: (i) loss of heat transfer efficiency, (ii) clogging, (iii) microbiologically influenced corrosion, and (iv) health risk associated to the development of pathogens. Controlling bacterial growth in open recirculating cooling systems is very challenging, and is generally performed via dosage of disinfectants such as sodium hypochlorite. Due to biofouling and the increasing concerns linked to chemical consumption and water discharge, attention has been given to investigate more sustainable approaches to cooling systems operation. Knowledge on the bacterial communities, disinfection impact and biofilm composition is however limited and a more in-depth characterization of biofouling is required to effectively establish new control strategies.

The objectives of this thesis were to contribute to the available knowledge on biofouling in cooling systems and to investigate sustainable and predictable alternative operations, providing results of direct relevance to practice. Attention was given to the identification of factors selecting the microbiome of cooling water subjected to conventional operation, characterization of cooling tower biofilms without disinfection, and assessment of the impact of temperature on biofilm composition. Then, alternative approaches to biofouling control were tested at pilot-scale. Phosphorus depletion was investigated as a solution for limiting biofilm growth and high pH was considered as a *Legionella pneumophila* control method. These studies were performed with the use of recent analytical methods such as next generation amplicon sequencing (NGS), quantitative polymerase chain reaction (qPCR), and flow-cytometry, which allowed collection of valuable data and a better characterization of biofouling.

In order to gain insight on bacterial growth in cooling systems, the first step was to explore the bacterial changes happening along an operating industrial process. The study described in **Chapter 2** was performed over a period of 5 months on a full-scale cooling system (25 MW cooling capacity) subjected to residual chlorine. The implementation of a mass balance based equation combining several analysing techniques showed that (i) the input feed water microbiome defined the cooling water bacterial community members, and (ii) the variations in net decay of bacterial members caused by disinfection was the main factor shaping the community structure. In addition to determine the impact of biocides on the selection of cooling water bacteria, the study revealed that the established mass balance is a useful tool to apply to characterization studies in general, as it can avoid misinterpretation of relative abundance data. One bacterial order, Obscuribacterales, was showing growth despite the

presence of residual chlorine in the system, suggesting a higher resistance to the disinfectant than other community members.

Substantial knowledge has been acquired in the last decades on biofilm population and extracellular polymeric substances (EPS). Still, many questions remain to be answered regarding composition and adaptation to external conditions and stresses. In cooling towers, a temperature gradient occurs at the surface of the heat-exchanger and in the bulk water. The bacteria involved in biofilm development are subjected to this heat gradient, which can affect their selection and the type of EPS they produce. A laboratory heat exchanger module was developed in **Chapter 3** for the assessment of temperature on biofilm composition. A strong shift in bacterial composition occurred with the increase in bulk water temperature from 20 °C to 27 °C. Biofilm grown at elevated temperature also displayed a protein to polysaccharide ratio in EPS significantly higher than in the EPS of the reference biofilm grown at 20 °C. These variations are of interest for future investigations, e.g. of cleaning strategies, and should be compared to full-scale heat-exchanger biofilms. Biofilm development also strongly depends on nutrients available for growth. The additional study of **Chapter 4** revealed the presence of sialic acids in EPS of biofilms collected from cooling tower pilots, under depleted nutrient condition and enriched nutrient condition. The presence of sialic acids, and specifically the conservation of production pathways under nutrient limitation, suggest (i) an important role of these compounds in the structural cohesion or protection of the biofilm layer, and (ii) their wide-spread occurrence in environmental biofilms.

With the aim of reducing chemical consumption during operation, alternatives to disinfection were investigated for the control of cooling system microbiology. Depletion of phosphorus, a vital element for growth of microorganisms, was proposed as a preventive method against biofilm formation (**Chapter 4**). The assumption was tested in pilot-scale cooling towers operated with reverse osmosis permeate supplemented in nutrients. P-limitation did not restrict biofilm growth and caused an even greater volume of organic matter accumulation per unit of active biomass in the biofilm. Based on the results, it was concluded that P removal alone will not be an adequate strategy for biofouling control in cooling systems. **Chapter 5** explored the effect of alkaline pH on *L. pneumophila* and other pathogenic genera. This second pilot-scale study revealed that *L. pneumophila* growth was effectively controlled at pH 9.6 with the use of demineralized water as feed water. Relative abundance in potentially pathogenic genera strongly declined compared to conventional operation with sodium hypochlorite dosage. Alkaline cooling system conditioning therefore represents an attractive alternative to disinfection for the limitation of health risks associated to *L. pneumophila*.

Based on these findings, recommendations for further investigations are provided in **Chapter 6**. Characterization of Obscuribacterales could shed light on survival of microorganisms to chlorination. Assessment of potential contamination of the cooling water by wind-carried particles, rain or mist in terms of airborne bacteria and nutrients would be useful in the selection of water treatment. Biofilm formation and the inhibition of *L. pneumophila* growth at high pH should be further explored and would highly contribute to current knowledge on this pathogenic species. Results and associated messages of this PhD thesis show that it is

possible to operate cooling systems without disinfection, with combination of adapted feed water treatments and alkaline conditions so no health threat linked to pathogenic bacteria is caused to the surrounding human population, and no significant biofouling occurs. In general, this thesis provides guidance to cooling experts, and any owner of an open recirculating cooling tower, in the selection of adapted feed water treatment and operational conditions to avoid biofouling.

SAMENVATTING

Open recirculerende koelsystemen zijn zeer belangrijke systemen in de industrie vanaf het begin van de 20ste eeuw. Hun doeleinde is om extra warmte van processen vrij te maken door verdamping van water zodat productie op optimale temperatuur kan uitgevoerd worden. Met de wereldwijde ontwikkeling van industriële activiteiten zoals elektriciteit en chemicaliën productie, blijft de behoefte aan koeling capaciteit stijgen. Biofouling is één van de belangrijkste verschijnselen die een negatieve invloed heeft op de werking van natte koelsystemen. Dit verschijnsel leidt tot: (i) het verlies van warmteoverdrachtsefficiëntie, (ii) verstopping, (iii) microbiële corrosie, en (iv) gezondheidsrisico in verband met de groei van ziekteverwekkers. De beperking van bacteriële groei in een open recirculerend koelsysteem is een uitdaging, en het is meestal uitgevoerd door het doseren van desinfectiemiddelen zoals natriumhypochloriet. Tegenwoordig is er een toenemende zorg over het verbruik en afvoer van chemicaliën, daarom wordt er meer aandacht besteed aan het onderzoeken van een duurzamere werking van koelsystemen. Kennis over de bacteriële gemeenschappen, impact van desinfectie en biofilmsamenstelling is echter beperkt en een meer diepgaande karakterisering van biofouling is vereist om effectieve nieuwe controlestrategieën vast te stellen.

De doelen van dit proefschrift waren om bij te dragen aan de actuele kennis over biofouling in koelsystemen, en duurzame en voorspelbare alternatieve werkingen te onderzoeken, met resultaten die direct relevant zijn voor de praktijk. Er is aandacht besteed aan de identificatie van factoren die het microbioom selecteren in koelwater dat aan een conventionele werking is onderworpen, karakterisering van biofilms van koeltorens zonder desinfectie en beoordeling van het effect van temperatuur op de samenstelling van biofilms. Vervolgens werden alternatieve benaderingen voor de beheersing van biofouling getest op pilotschaal. Fosfordepletie werd onderzocht als een oplossing voor het beperken van de groei van biofilms en een hoge pH werd beschouwd als methode om *Legionella pneumophila* te beheersen. Deze studies werden uitgevoerd met behulp van recente analysemethoden zoals next generation amplicon sequencing (NGS), quantitative polymerase chain reaction (qPCR), en flowcytometrie, waardoor waardevolle gegevens konden worden verzameld en biofouling beter kon worden gekarakteriseerd.

Om inzicht te krijgen in bacteriële groei in koelsystemen, was de eerste stap het verkennen van de bacteriële veranderingen die plaatsvinden tijdens een industrieel proces. De in **Hoofdstuk 2** beschreven studie werd uitgevoerd over een periode van 5 maanden op een industrieel koelsysteem (koelvermogen van 25 MW) dat werd blootgesteld aan residuaal chloor. De implementatie van een op massabalans gebaseerde vergelijking die verschillende analysetechnieken combineert, toonde aan dat (i) het microbioom van het invoer water de samenstelling van de bacteriële gemeenschap in het koelwater bepaalde, en (ii) de variaties in netto afbraak van bacteriële soorten veroorzaakt door desinfectie de belangrijkste factor

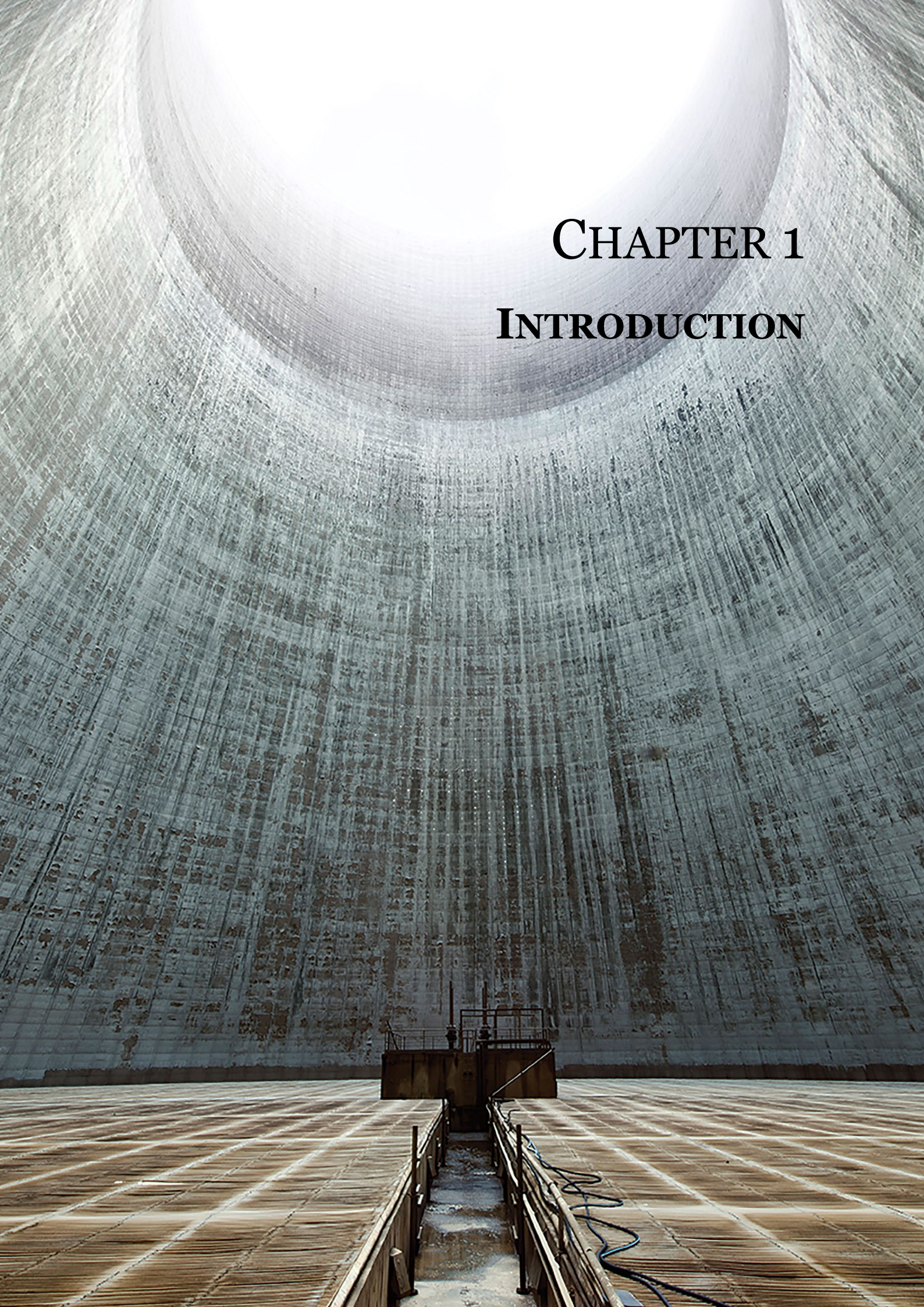
was die de gemeenschapsstructuur vormgaf. Naast het bepalen van de impact van biociden op de selectie van koelwaterbacteriën, toonde de studie aan dat de opgestelde massabalans een nuttig hulpmiddel is om toe te passen op karakteriseringsstudies in het algemeen, omdat de verkeerde interpretatie van relatieve overvloedsgegevens voorkomen kan worden. Een bacteriële orde, Obscuribacterales, vertoonde groei ondanks de aanwezigheid van residuaal chloor in het systeem, wat wijst op een hogere weerstand tegen het ontsmettingsmiddel dan andere leden van de gemeenschap.

In de afgelopen decennia is substantiële kennis opgedaan over biofilmpopulatie en extracellulaire polymere stoffen. Maar veel vragen met betrekking tot samenstelling, en aanpassing aan externe omstandigheden en spanningen zijn nog onbeantwoord. Bij koeltorens treedt een temperatuurgradiënt op aan het oppervlak van de warmtewisselaar en in het bulkwater. De bacteriën die betrokken zijn bij de ontwikkeling van de biofilms worden onderworpen aan deze hitte gradiënt, die hun selectie en het type geproduceerde EPS kan beïnvloeden. In **Hoofdstuk 3** was een laboratorium warmtewisselaarmodule ontwikkeld om het temperatuureffect op de biofilmsamenstelling te beoordelen. Bij de temperatuurstijging (20 ˚C tot 27 ˚C) trad een sterke verschuiving op in de bacteriesamenstelling. Biofilms die op verhoogde temperatuur waren gegroeid vertoonde ook een proteïne tot polysacharide verhouding in EPS die hoger was dan in de EPS van de 20 ˚C gegroeid referentiebiofilms. Deze variaties zijn van belang voor toekomstige onderzoeken, bijvoorbeeld voor reinigingsstrategieën, en moet worden vergeleken met biofilms uit warmtewisselaars op industriële schaal. De ontwikkeling van biofilms is ook sterk afhankelijk van de voedingsstoffen die beschikbaar zijn voor groei. De aanvullende studie van **Hoofdstuk 4** onthulde de aanwezigheid van siaalzuren in EPS van biofilms verzameld uit een pilotschaal koeltoren, onder verarmde nutriëntenconditie en verrijkte nutriëntenconditie. De aanwezigheid van siaalzuren, met name het behoud van de bijbehorende productieroutes onder nutriëntenbeperking, suggereert (i) een belangrijke rol van deze verbindingen in de structurele cohesie of bescherming van de biofilmlaag, en (ii) hun wijdverbreide voorkomen in natuurlijke biofilms.

Alternatieven voor desinfectie zijn onderzocht voor de beheersing van de microbiologie van het koelsystem. De uitputting van fosfor, een essentieel element voor de groei van microorganismen, is aangedragen als een preventieve methode tegen biofilmvorming (**Hoofdstuk 4**). De hypothese is getest in koeltorens op pilotschaal die worden bedreven met permeaat van omgekeerde osmose aangevuld met nutriënten. P-uitputting beperkte de groei van de biofilm niet en veroorzaakte een nog grotere ophoping van organische stof per eenheid actieve biomassa in de biofilm. Op basis van de resultaten werd geconcludeerd dat verwijdering van fosfor opzich zelf geen adequate strategie zal zijn voor het beheersen van biofouling in koelsystemen. **Hoofdstuk 5** onderzocht het effect van alkalische pH op *L. pneumophila* en andere pathogene genera. Deze pilotschaal studie onthulde dat de groei van *L. pneumophila* effectief werd beheerst bij pH 9.6 met het gebruik van gedemineraliseerd water als voedingswater. De relatieve aanwezigheid van potentieel pathogene genera nam sterk af in vergelijking met conventionele operatie met natriumhypochlorietdosering.

Alkalische conditionering van het koelsysteem vormt daarom een aantrekkelijk alternatief voor desinfectie om de gezondheidsrisico's van *L. pneumophila* te beperken.

Op basis van deze bevindingen worden aanbevelingen voor verder onderzoek gegeven in **Hoofdstuk 6**. Karakterisering van Obscuribacterales zou licht kunnen werpen op de overleving van micro-organismen bij chloor dosering. Beoordeling van mogelijke vervuiling van het koelwater door deeltjes in de lucht, regen of mist in termen van bacteriën en nutriënten zou nuttig zijn bij de keuze van een waterbehandeling strategie. Biofilmvorming en de beperking van de groei van *L. pneumophila* bij hoge pH moeten verder worden onderzocht en zouden in hoge mate bijdragen aan de huidige kennis over deze pathogene species. De resultaten en de bijbehorende boodschappen van dit proefschrift laten zien dat het mogelijk is om koelsystemen te bedienen zonder desinfectie, met een combinatie van aangepaste voedingswaterbehandelingen en alkalische omstandigheden, zodat er geen gezondheidsbedreiging door pathogene bacteriën wordt veroorzaakt voor de omringende menselijke bevolking, en geen significante biofouling. In het algemeen geeft dit proefschrift richtlijnen voor experts van koelsystemen, en ook voor eigenaren van recirculerende koeltorens, bij het selecteren van aangepaste invoerwater behandeling en operationele toestanden om biofouling te voorkomen.



CHAPTER 1

INTRODUCTION

1.1. CONTEXT

The research presented in this thesis was instigated by a partnership between TU Delft and Evides Industriewater early 2016, with the aim of gaining knowledge and operation of cooling systems. Evides is one of the leading companies in the Dutch water sector with activities ranging from drinking water and process water distribution to wastewater management. The branch Industriewater is responsible for the collection and treatment of various water sources such as fresh or brackish surface waters, groundwater or seawater, and their distribution to industries from various sectors. A large part of this supplied water is used by industrial customers as cooling water, necessary for the operation of their industrial sites. In this context, Evides Industriewater chose to diversify its business activities in the cooling water field, by creating a sustainable and economical balance between pre-treatment and conditioning, in order to offer additional services to current and potential customers. The research collaboration with TU Delft falls within this framework and plays an important role by bringing added value to the already long-established cooling technology, through the definition and filling of knowledge gaps as well as operational improvement.

1.2. WET COOLING SYSTEMS: AN ESSENTIAL TECHNOLOGY FACING CHALLENGES

1.2.1. General introduction to wet cooling systems

Industry accounts for about 40% of total freshwater abstraction in Europe, with existent disparities between countries. In the Netherlands, the total annual withdrawal of surface water and groundwater reached 8093 million m^3 in 2018, with 71% used as cooling water in the process industry and for the production of electricity (European Commission 2020) [1]. This incredibly high water requirement illustrates the importance of a continuous and sufficient supply of cooling water in an industrialised country's economy. In this context, wet cooling systems play an indispensable role by providing efficient cooling to industrial sites such as refinery, chemical, petrochemical and powerplants, allowing a good, economic operation of their production processes (Schulze et al. 2018).

Several types of wet cooling systems are being used (European Commission 2001). The once-through technology draws large volumes of water to feed the heat exchangers, and directly discharges it back to the water body at an elevated temperature. Open recirculating systems, also called evaporative systems, recirculate the water and involve direct contact with ambient air for cooling. Closed-loop technology recirculates water in a piping system, avoiding direct contact between cooling water and air. Some hybrid systems combine characteristics of both open and closed-loop cooling. A more recent type of wet cooling, adiabatic cooling systems, operates similarly as closed-loop with addition of a pre-cooling step of the ambient air. The choice of a cooling system is based on many parameters such as required cooling capacity,

[1] Water abstraction corresponds to the amount of water withdrawn from a water body for further use, such as cooling. This is not to be mistaken with water consumption, defined by the total amount of water withdrawn minus the total amount of water discharged. In the case of wet cooling, a large fraction of abstracted water is directly returned to the water body.

water availability, space, climate, or environmental requirements, and can be determined following available guidelines (European Commission 2001).

1.2.2. Open recirculating cooling system

Open recirculating cooling (Figure 1.1) is one of the most commonly used wet cooling technology for the release of heat from industrial fluids and processes in the world due to its high cooling efficiency. This technology takes advantage of the property of water to change state when exposed to an input of heat energy.

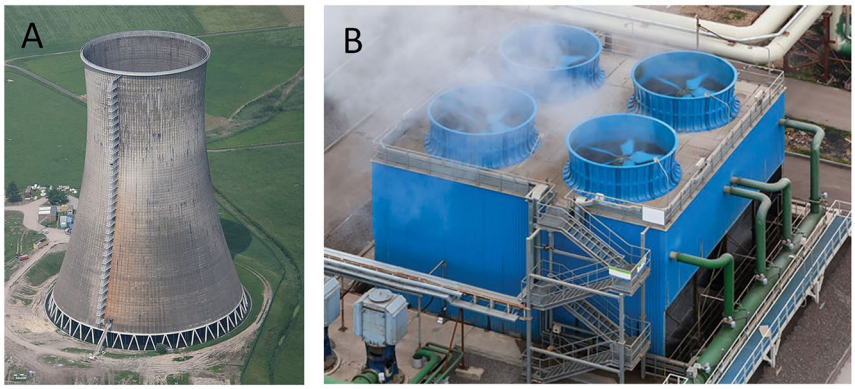


Figure 1.1. (A) 122-meter-tall natural draft cooling tower (Tim Reckmann) and (B) 10-meter-tall induced draft counterflow cooling tower (https://water.aecsi.us).

Contrarily to once-through cooling systems, built in great numbers before the 70s but causing thermal pollution in the immediate environment, open recirculating cooling systems consist in the recirculation of a water stream in a secondary circuit. Cooling water is pumped through heat exchangers where heat is absorbed from primary processes, and is sprayed from the top of a tower where part of the warm water evaporates in presence of an upward air flow. Packing fill materials located in the tower allows optimal contact between the air and the water, necessary for efficient cooling. To prevent droplets from exiting the system, drift eliminators are often installed above the spray nozzles. The cooled water is then collected in a basin at the bottom of the tower before to be pumped back to the heat exchangers. A simplified diagram of a cooling tower operation is shown in Figure 1.2. The excess energy is therefore released from the system through evaporation (Hill et al. 2013).

The most familiar and visible evaporative cooling towers are natural draft cooling towers which consist in up to 200m-high concrete chimneys (Figure 1.1.A). These large systems are easily spotted from afar and are mainly associated to power plants despite their frequent use in other industries such as petroleum refineries and chemical factories. Their hyperbolic structure allows the heated air to rise up, pulling cold air from the open base, which results in a continuous air flow used as water coolant. They are often applied for high rejected heat capacity, exceeding 200 MW.

A more recent type, induced draft cooling towers (Figure 1.1.B), makes use of a fan to induce an air flow through the system (Figure 1.2). This design consumes more energy but allows the construction of smaller structures, up to 20m-high, and a better control of the cooling

efficiency (Hill et al. 2013). Induced draft cooling towers are nowadays preferred in various industrial activities, for rejected heat capacity up to 100 MW, and are also very common in the urban environment, due to lower space requirement.

A wide range of materials are used in these systems. Heat exchanger tubes are often made of stainless steel or copper-nickel alloys, sometimes even titanium for its corrosion-resistant properties. The packing fill consists in thermoplastic polymers arranged in layers, while reinforced concrete is often chosen for the main structure, cooling basin and circulating water conduits (European Commission 2001). Flows and temperatures strongly vary depending on the localisation and process requirements.

In this thesis, field studies were performed exclusively on counterflow draft cooling towers used for industrial purposes. Other less common designs are described elsewhere (European Commission 2001, Hill et al. 2013).

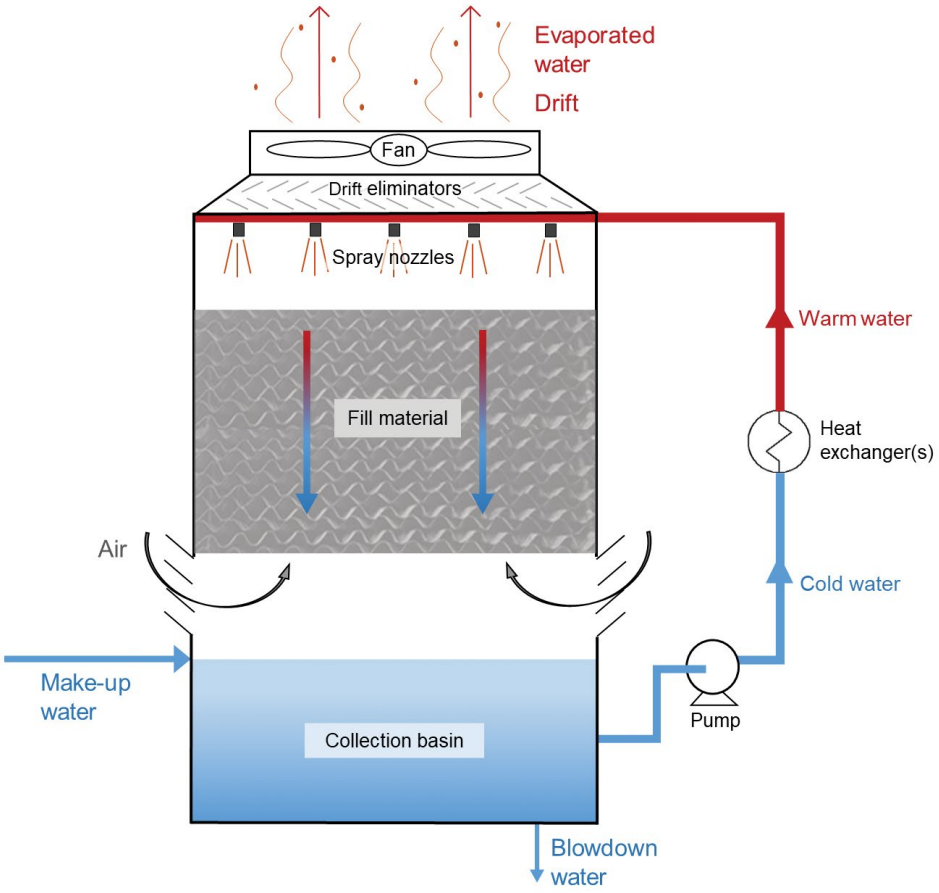


Figure 1.2. Schematic diagram of a counterflow induced draft cooling tower, a common type of design of open recirculating cooling systems.

1.2.3. Main challenges related to open recirculating cooling systems

The evaporation of water from cooling towers results in concentrating minerals and organic matter in the recirculating water. Some quality parameters are indicated in Table 1.1. Electrical conductivity is generally used as water quality indicator in full-scale systems and is maintained within a certain range during cooling tower operation, rarely exceeding 4500

µS/cm in freshwater-fed systems (Koeman-Stein et al. 2016). This key parameter is particularly important as a too high value will increase the risk of scale formation, eventually affecting the performance of the cooling system and resulting in higher operational costs (Walker et al. 2012). On the other hand, a low conductivity implies a greater water consumption. When the electrical conductivity threshold is reached, part of the concentrated water gets discharged as blowdown water and is replaced by fresh make-up water to conserve a constant water quality over time (Figure 1.2). Make-up water must comply to quality constraints regarding electrical conductivity, chloride, phosphates and total organics (Groot et al. 2015). The cycle of concentration (COC) is defined as the ratio of concentrations of dissolved solids in the blowdown water to that in the make-up water, but is often approximated by the ratio of electrical conductivities. COC vary between 3 and 5 in most surface water-fed cooling systems (Lv et al. 2018), and it can reach much higher values when high-quality make-up water is used. Cooling systems fed with seawater are operated with a COC lower than 2 (Al-Bloushi et al. 2018).

The concentration of minerals and organic matter in the water and their deposition on the surfaces cause several detrimental effects on the equipment and overall cooling system operation. Corrosion strongly affects metallic parts, including heat exchangers, and causes adverse effect such as water contamination by dissolved metals, obstruction, or even mechanical failures (Campbell 1980). This phenomenon strongly depends on the chemical composition of the water and the type of metals used. Some water parameters are well known to aggravate corrosion, such as high chloride content and extreme pHs (Campbell 1980). Another undesired consequence of an increased salt concentration is the formation of scale resulting from an increase in water hardness. Calcium and magnesium carbonates, sulphates or phosphates precipitate and form mineral films, promoted by factors such as high surface temperature or alkaline pH (Ali et al. 2020). Accumulation of inorganic scale on heat exchangers notably reduces heat transfer efficiency and can accelerate corrosion. Finally, the increasing concentration of nutrients and organics in the cooling water, resulting from the evaporation of water, favours multiplication of living organisms, eventually leading to biofilm formation. Biofilm is defined as the accumulation of microorganisms and extracellular materials on a surface. When biofilm formation or growth of planktonic microbial cells is considered unacceptable and threatens the operational reliability of a process or the health of surrounding human population, as it is the case in evaporative cooling systems, it is referred to as biofouling (Vrouwenvelder et al. 2007). Biofouling results in clogging of equipment parts, loss of heat transfer capacity, and can enhance corrosion when in contact with metallic parts of the system (Melo and Bott 1997). It can also lead to the release of pathogen via aerosol formation. A more detailed description of microbial growth factors and associated risks is provided in a later section. Other types of fouling such as particulate and organic fouling can also occur.

Corrosion, scaling and biofouling can take place individually, simultaneously and affect each other. They have disastrous consequences on system operation and connected processes. Extremely high operational cost – attributed to energy consumption increase, early renewal of process parts and increased interruption of operation – is unavoidable in absence of mitigation methods.

Table 1.1. Composition of 7 different cooling waters from previously published studies: CT1 - (Paranjape et al. 2020b), CT2 - (Pinel et al. 2020a), CT3 - (Farahani et al. 2016), CT4 - (Groot et al. 2015), CT5 - (Zhang et al. 2008), CT6 - (Wang et al. 2006), and CT7 - (Kim et al. 2001).

Parameter	Unit	CT1	CT2	CT3	CT4	CT5	CT6	CT7
Temp.	°C	9-29	15-25	-	20-30	5-21	4-40	-
pH	-	8.2-8.9	7.5-7.9	6.7-7.2	7.5-8.5	8.2-8.75	7.8-9.3	8.2
COD	mg/L	-	-	181	-	3-4.8	0-10	-
TOC	mg/L	6-41	34-56	-	40-60	-	-	-
EC	µS/cm	600-1400	3000-3500	2928	3500-4500	1300-2000	1650-2100	2150
Turbidity	NTU	1-58	1-13	73.6	-	5-23	-	-
TSS	mg/L	<100	-	-	<15	7-22	5-80	-
Alkalinity	mg_{CaCO3}/L	-	800-1300	1350	-	350-370	-	188
Chloride	mg/L	-	400-550	-	400–600	-	-	404
Phosphate	mg/L	<3	6-9	-	5–15	1.1	-	-

1.2.4. Conventional fouling mitigation strategy in industrial open recirculating cooling systems

To prevent these detrimental phenomena, chemicals are dosed to the cooling water and key water quality parameters are monitored. Conventional conditioning of cooling water systems includes corrosion inhibitors, antiscalants, biocides and dispersants (Wagner et al. 2018). Often used corrosion and scaling inhibitors in industry comprise polyphosphates, phosphonates, silicates and copolymers. To limit biofouling and pathogen growth, cooling systems make use of either oxidative or non-oxidative biocides. The first ones include compounds such as sodium hypochlorite, chlorine dioxide, monochloramine or ozone. Active chlorine, very strong oxidant, is the most frequently employed antimicrobial compound (Groshart and Balk 2003). Non-oxidative biocides such as 2,2-Dibromo-3-nitrilopropionamide (DBNPA) or quaternary ammonium compounds are less common. Biocides are applied at either continuous, shock or intermittent dosing. In addition to inhibitors, dosage of acid is applied to maintain the pH between 7 and 8 to allow appropriate disinfection. A more exhaustive list of commonly used chemicals for biological growth control is available elsewhere (Wagner et al. 2018).

Online monitoring parameters include pH, electrical conductivity, temperature and oxidation-reduction potential (ORP). The latter is used as an indicator of the efficiency of oxidative disinfection in cooling water and helps controlling the dosage of biocides. An ORP between 550 and 650 mV is considered optimal for efficient disinfection without inducing corrosion of the equipment. Monitoring and dosages are nowadays performed and adjusted by automated control systems based on feedback loops. Additional offline analyses of the water quality are frequently performed such as water hardness, as indication of scaling potential, or adenosine triphosphate, to approximate the growth of active microorganisms.

1.2.5. Need for more environmentally friendly and sustainable mitigation strategies

The efforts implemented to optimize cooling system performance while reducing the dosage of chemicals are motivated by significant downsides of the conventional conditioning. Optimal operation of cooling towers relies on a fragile balance of dosed chemicals which requires constant adjustment (Hill et al. 2013). Side-reactions are induced by the physicochemical properties of the recirculating water lowering the efficacy of chemicals and negatively affecting the system (Wagner et al. 2018). For instance, a high concentration of organic compounds in feed water reduces the disinfection power of biocides, which react with suspended matter to form unwanted halogenated by-products (Groshart and Balk 2003). An increase in biocide dosage can contribute to corrosion of heat exchangers. The use of phosphorus-based corrosion inhibitors contributes to an increase in nutrient content of the water, enhancing microbial growth (Sweity et al. 2013). As a consequence, an imbalance in conditioning triggered by e.g., a sudden change in make-up water quality or accumulation of rain water, can affect the equipment and bacterial stability of the cooling water. Eventually, these chemicals and their by-products are discharged in the cooling tower blowdown water, either to the environment or to a nearby treatment plant. Ecological impacts were anticipated by early cooling system researchers regarding the discharge of halogenated disinfection by-products and other compounds of environmental concern (Bott 1998). It is reasonable to assume that alternatives will need to be implemented in the near future to meet upcoming stricter regulations' requirements on blowdown water discharge and reduced freshwater availability. Treatment technologies are being developed to allow reuse of cooling tower blowdown water in industry with the aim of reducing freshwater footprint (Bisselink et al. 2016, Zhang et al. 2008). In this context, high concentrations of chemicals in the discharged water greatly hamper desalination efficiency (Wagner et al. 2018) and necessitate additional treatment steps.

1.3. MICROBIAL GROWTH IN OPEN RECIRCULATING COOLING SYSTEMS

1.3.1. Growth promoting factors and associated risks

The composition of make-up water plays a decisive role on the operational performance. Evaporative cooling systems are also strongly affected by seasonal variations, sunlight and air quality due to their open design. Wind and precipitation bring particles, pollens, insects and airborne microorganisms, contributing to cooling water quality deterioration. Exposure to sunlight promotes algal proliferation (Hauer 2010), sometimes visible in the packing fill of the towers (Figure 1.3.A). Next to external influences, cooling systems create an optimal environment for growth of microorganisms through the combination of favourable physical and chemical factors (Di Pippo et al. 2018). In addition to the increase in organic matter and nutrients caused by concentration of feed water compounds and inhibitors, cooling systems operate at temperatures suitable for most mesophilic organisms. Typical cooling water temperatures are within a range of 10 to 35 °C depending on external conditions, with a temperature difference of 5 to 10 °C. Acid dosage maintains the pH close to neutrality and significant volumes of air passing through the tower provides a continuous aeration. These

properties, and a retention time of several days in the system, would promote rapid proliferation of aerobic microorganisms without disinfection.

Among these microorganisms, a special attention is given to *Legionella pneumophila*, a bacterial pathogen thriving in cooling water systems (Figure 1.3.B). Growth of pathogens, especially *L. pneumophila*, is a public health concern as they can be carried by aerosols sprayed from a cooling tower and spread illnesses over several kilometres. Contamination occurs when aerosols get inhaled by the population in the vicinity of cooling towers (Paschke et al. 2019). Inhalation of *Legionella* can lead to the development of Legionnaires' disease, a severe form of pneumonia with symptoms such as muscle aches, cough, strong headache and tiredness, with a case fatality rate of 10-15% (Di Pippo et al. 2018). Pontiac fever, a milder non-pneumonia illness causing muscle ache and fever, is another disease associated with this pathogenic species. Biocide dosages are usually efficient in maintaining a low abundance of *L. pneumophila* in cooling waters. However, outbreaks of Legionnaires' disease associated to biologically unstable evaporative cooling systems still occur (Bartram et al. 2007) and are expected to increase with the rising number of cooling systems (Paschke et al. 2019). Although *L. pneumophila* is the most frequently reported cooling tower pathogen, it is noteworthy to mention that other waterborne pathogens can cause health-related issues when spread to the surrounding areas. It is e.g. the case for *Pseudomonas* and non-tuberculosis *Mycobacteria*, among which some species are responsible for pulmonary infections and bronchiectasis (Pagnier et al. 2009, Pereira et al. 2018, Rossolini and Mantengoli 2005, Torvinen et al. 2014).

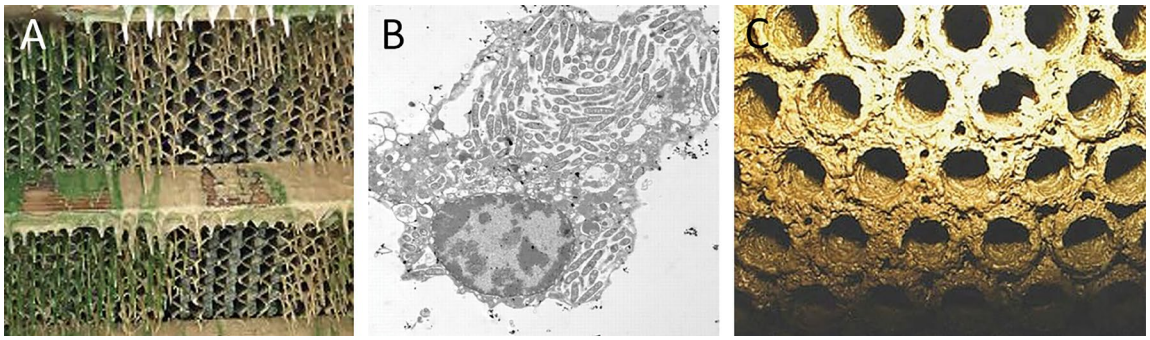


Figure 1.3. (A) Algal growth in the cooling tower packing (https://amsainc.com). (B) Microscopic image of *Acanthamoeba polyphaga*, protist host, infected by *Legionella pneumophila* (Molmeret et al. 2005). (C) Biofouling in heat exchanger (K.A. Selby).

Adhesion of bacteria to the immerged surfaces of cooling systems consequently initiates the formation of biofilms (Figure 1.3.C). This process is facilitated by large surface areas available in the packing materials, piping system, heat exchangers, and basin (Di Pippo et al. 2018). The surface-attached bacterial cells embed themselves within a self-produced matrix composed of extracellular polymeric substances (EPS). The main components of EPS include polysaccharides, proteins, nucleic acids and other biopolymers such as glycoproteins or amyloids whose inter and intramolecular interactions form a complex 3D network (Seviour et al. 2019). It provides mechanical stability, nutrient sorption, and protect the microbial communities from environmental stresses such as predation and disinfectants. The structural integrity and high density of organic compounds makes conventional chemical dosages

inefficient against established biofilms. Main consequences of biofilm formation are the loss of heat transfer efficiency, clogging and microbiologically influenced corrosion. In the latter, microbes induce corrosion of metal surfaces either via direct or indirect mechanisms (Jia et al. 2019). For instance, sulphate accumulation in cooling water can lead to growth of sulphate-reducing bacteria. These bacteria are involved in the corrosion of metal equipment used in cooling systems, through the secretion of a corrosive metabolite (Balamurugan et al. 2011, Rao et al. 2005). Other types such as iron-oxidising bacteria, iron-reducing bacteria or acid-producing bacteria are also frequently involved in metal corrosion.

1.3.2. Recent insights on the microbiome of cooling systems

Despite being a well-established process for decades, open recirculating cooling systems have only recently been the focus of several full-scale studies aiming at characterizing their microbiomes (Di Gregorio et al. 2017, Llewellyn et al. 2017, Paranjape et al. 2020b, Pereira et al. 2017, Tsao et al. 2019). This rising interest is principally motivated by the emergence of new microbial techniques such as next generation amplicon sequencing for overall microbial community characterization or quantitative polymerase chain reaction (qPCR) for the quantification of targeted microbial groups or species, allowing collection of valuable data that could not be gathered earlier. These studies explore populations of bacteria and protists, factors influencing their selection, correlations between the presence of specific members and their potential interactions, with often a closer look at pathogenic bacteria. The difficulties faced in accessing the surfaces of an operating system often results in neglecting the investigation of biofilm communities. Therefore, the majority of articles discusses planktonic or sessile populations directly analysed from the water phase, with the exception of a few studies (Di Gregorio et al. 2017, Hauer 2010, Wang et al. 2013). Three aspects are discussed in the following sections: cooling water microbiome, biofilm microbiome and microbial interactions with emphasis on *L. pneumophila*.

1.3.2.1. Water phase

Literature mainly focuses on cooling systems fed with freshwater, from sources varying between surface water, ground water and drinking water. It has been observed that the overall bacterial community closely resembles that of the make-up water, with core microbiome belonging to the taxonomic phyla Actinobacteria, Bacteroidetes, Cyanobacteria, Proteobacteria (mostly beta subdivision) and Verrucomicrobia. The same observation was made for protists community members, with the regularly found supergroups Alveolata, Amoeboza, Rhizaria and Excavata. Scientists reported that the composition of the microbiome of cooling waters is primarily influenced by the microbiome from the feed water, drawing this conclusion either by the presence or dominance of these recurrent groups (Tsao et al. 2019) or by the convergence in cooling water communities located in the same geographic area (Paranjape et al. 2020b). Looking at lower taxonomic ranks, results suggest that the community structures are further shaped by conditions specific to each cooling system (Tsao et al. 2019), linked to disinfection, operational parameters, materials, or external conditions in the direct vicinity (precipitations, agricultural fields) affecting the water quality (Gomez-Alvarez et al. 2012, Ji et al. 2015). This is supported by temporal variations non-representative from common freshwater environments, which makes the predictions and

studies of interactions highly challenging (Pereira et al. 2017). Evaporative cooling systems fed with seawater are less used and have been less investigated. Their low COC implies a short retention time which, assumedly, leads to a lower degree of differentiation from the feed water than it is the case for freshwater-fed systems.

The recurrence of biofilm-forming or biofilm-associated taxa is another common reported feature of cooling water community composition (Hauer 2010, Pereira et al. 2017, Tsao et al. 2019, Wang et al. 2013). Their presence and even dominance in some cooling waters are an indicator of biofilm accumulation on the surfaces of the systems, being released into the water phase (Figure 1.4) (Pereira et al. 2017).

1.3.2.2. Biofilms

The large majority of bacterial cells in engineered water systems are attached to surfaces, with numbers reaching up to 4 logarithmic units higher than planktonic cells (Di Pippo et al. 2018). Despite recurrence of biofilm taxa in the water phase, water analyses give very limited amount of information on biofilm composition and extent in cooling systems (Wang et al. 2013). *Acidovorax sp.* and members of the *Sphingomonadaceae* family such as *Sphingomonas sp.* or *Sphingopyxis sp.*, are believed to be pioneers in the initial phase of biofilm formation due to their adaptation to a wide range of conditions, ability to produce surface-adhesion proteins and increased resistance to chlorine (Balkwill et al. 2006, Di Gregorio et al. 2017, Romani et al. 2019, Wang et al. 2013). Among regulation processes involved in the initiation and maturation of biofilms, levels of intracellular second-messenger c-di-GMP and quorum sensing are believed to be crucial (Di Pippo et al. 2018). C-di-GMP regulates bacterial adhesion and dispersal by controlling the motile-sessile transition within bacterial cells. Cells then use quorum sensing to communicate within the biofilm, with the secretion of signal molecules affecting gene expression to induce different phenotypes, e.g. linked to EPS production (Boyd and O'Toole 2012, Whiteley et al. 2017). In addition, abiotic factors strongly affect the selection of communities populating biofilms. Sunlight plays a major role with promotion of phototrophic organisms such as Cyanobacteria, diatoms and green algae in areas of high exposure (Hauer 2010). Confocal microscopy imaging of cooling tower biofilms revealed patchy and stratified arrangement of bacterial communities, associated with phototrophic prokaryotes and eukaryotes, all embedded within an EPS matrix (Di Gregorio et al. 2017). Bulk water temperatures, surface temperatures and surface material also affect the selection of microbial populations, notably associated with MIC. Common species populating an established biofilm belong mainly to Alpha and Betaproteobacteria, Cyanobacteria, Actinobacteria and Bacteroidetes (Di Gregorio et al. 2017, Hauer 2010, Wang et al. 2013). Biofilm therefore represent a favourable habitat providing the microbial populations with ecological advantages compared to planktonic organisms (Di Gregorio et al. 2017) and allowing the establishment of synergistic microbial consortia (Seviour et al. 2019). Protist species play an important role among these consortia with their involvement in the spread of opportunistic pathogenic species (Flemming et al. 2016, Parry 2004). While biocide dosages are, in most cooling systems, able to maintain low abundance of pathogenic genera, some genera are still frequently identified. The most recurrent ones, *Acinetobacter*, *Aeromonas*, *Legionella*, *Mycobacterium*, *Pseudomonas* and *Sphingomonas*, are either associated to biofilm formation or to survival in protozoan hosts (Pereira et al. 2017, Thomas

et al. 2010, Wingender and Flemming 2011). Biofilms therefore increase the survival and persistence of pathogens by protecting its population from stress conditions and providing an adapted environment to potential hosts.

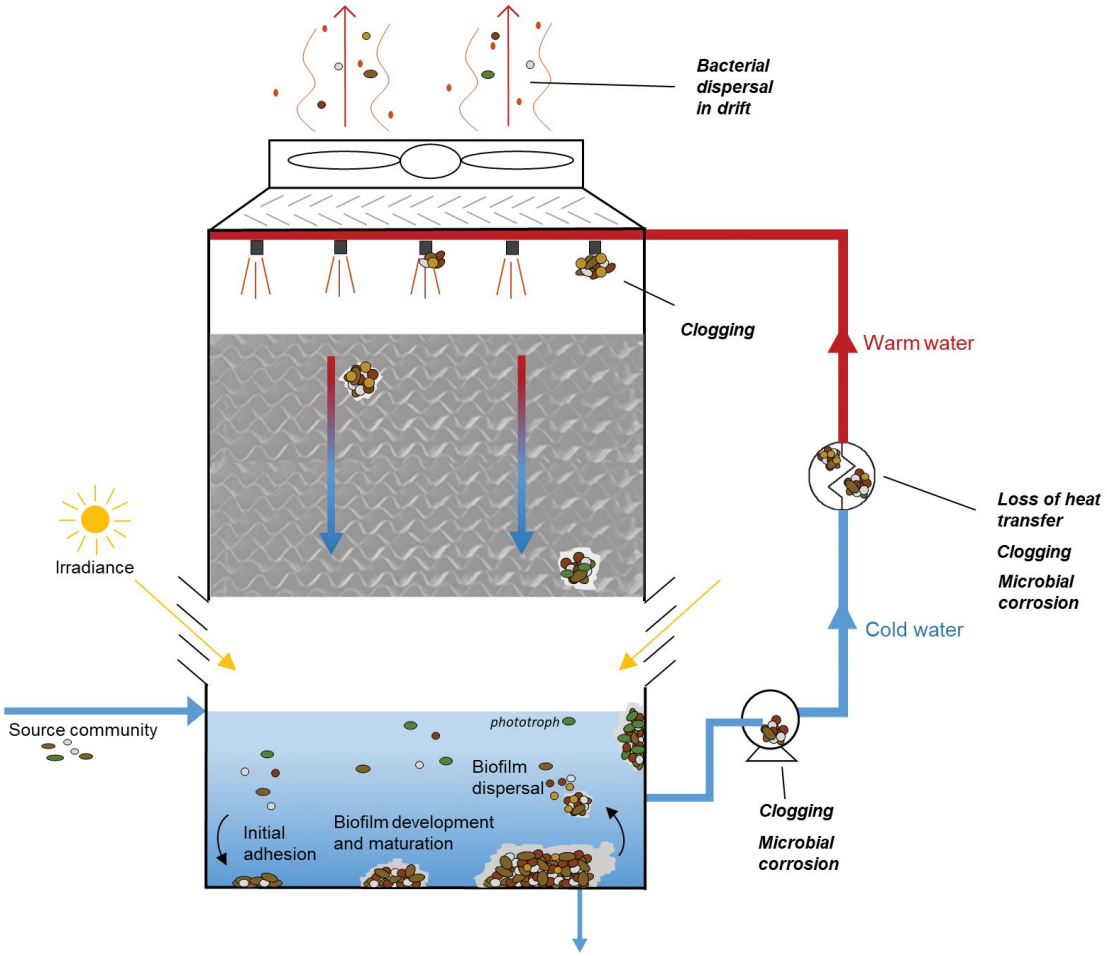


Figure 1.4. Proposed scheme for biofilm formation and associated disturbances in cooling tower systems. Based on (Di Gregorio et al. 2017).

1.3.2.3. Microbial interactions and *Legionella pneumophila*

Abiotic factors and synergy between organisms play critical roles on the colonization of evaporative cooling systems and on the pathogenicity of the water. Studies of interactions are therefore of interest when investigating the occurrence of harmful organisms, especially *L. pneumophila*. The use of linear discriminant tools applied to metagenomic analyses revealed correlations between the presence of pathogens and other bacterial community members or between pathogens and protists, providing valuable information on the environmental conditions promoting their growth and suggesting beneficial or detrimental associations between members (Koide et al. 2014, Paranjape et al. 2020a, Paranjape et al. 2020b). Indeed, non-opportunistic bacteria are known to serve as food source for potential protozoan hosts cells or as work force for the formation of an adapted habitat through the production of EPS, indirectly promoting the growth of opportunistic bacterial pathogens. On the other hand, some bacterial species are able to kill host cells through secretion of toxic compounds, as it has been shown for *Pseudomonas aeruginosa* (Matz et al. 2008), or other adaptive mechanisms acquired throughout bacterial evolution (Matz and Kjelleberg 2005). Different

chlorine dosages and frequencies were shown to oppositely affect the abundance of *Pseudomonas* and *Legionella* genera in waters from several cooling systems (Paranjape et al. 2020b). Continuous high residual chlorine (> 0.3 ppm) was efficient in limiting *Legionella sp.*, while intermittent dosages promoted its growth. The opposite trend was observed for *Pseudomonas sp*. Differences in required contact time with the biocide or loss in alpha diversity at high dosage reducing the diversity of host cells for *Legionella* are possible explanations for these observed divergences (Llewellyn et al. 2017). *Pseudomonas* species have also been shown to directly inhibit *L. pneumophila* by secreting diffusible compounds (Corre et al. 2019). Direct and indirect interactions among bacteria and between bacteria and protists are believed to be more diverse than currently identified, and are of importance when considering the spread of opportunistic bacterial pathogen. *L. pneumophila* has been so far associated with 14 types of protozoa, some belonging to *Vermamoeba*, *Hartmanella*, *Acanthamoeba* or *Naegleria* genera (Wadowsky et al. 1988). The list remains non exhaustive and this large spectrum of protozoan hosts makes the monitoring and control of the pathogen very difficult, especially in multi-species biofilms as it is the case in cooling systems. More information on *L. pneumophila* is provided in Chapter 5.

1.3.3. Emerging biofouling control strategies

As mechanical cleaning is hardly achievable in cooling towers and heat exchangers, biofouling control methods rely mainly on the dosage of biocidal products. Biocides are effective by direct contact with individual bacterial cells, but they have little action on established biofilms as their penetration through the EPS is limited by a reaction-diffusion phenomenon (Chen and Stewart 1996). Dispersants and emulsifiers are dosed in combination to help disrupt the EPS matrix and expose the biofilm cells to the biocide (Cloete et al. 1998). Hereafter is a non-exhaustive list of complementary and alternative solutions to (i) eliminate grown biofilms with curative methods, or (ii) inhibit their growth with preventive methods (Figure 1.5).

1.3.3.1. Curative methods

Chemical-based methods for biofilm removal include the development of greener, non-toxic and non-bioaccumulating bio-dispersants such as polyglycosides. These products reduce the impacts on the direct environment and biodegrade at a faster rate than conventional chemicals while maintaining an acceptable biofilm removal percentage (Di Pippo et al. 2017). Increasing knowledge on EPS chemical composition and structural network provides guidance for the development of more efficient biofilm removal products (Seviour et al. 2019). Substantial progress related to the development of enzymatic agents is being made and shows promising results through the cleavage of proteins and polysaccharides. While targeting key components of the EPS matrix, the specificity of these cleaning enzymes is however restricting their biofilm solubilization efficiency (Di Pippo et al. 2018, Kim et al. 2013). Intensive research on the topic is ongoing in order to find molecules able to effectively and entirely dissolve the EPS matrix of complex multi-species biofilms.

Physical methods to mitigate biofouling have also been developed. Some conventional techniques such as the recirculating sponge rubber balls or the brush and cage system for the

cleaning of heat exchanger tubes are still applied (Bott 1998). Other techniques, using magnetic fields or ultrasounds, have recently emerged and shown promising results as biofilm disruptive strategies (Li et al. 2019). In general, the utilisation of nonchemical treatment devices as sole method for biological control is often proven insufficient (Vidic et al. 2010) and are mainly considered as complementary technologies to chemicals rather than alternatives. Their use has the advantage of reducing the dosage of required chemicals and improving biofilm removal.

1.3.3.2. Preventive methods

Techniques preventing the growth and adhesion of organisms in cooling systems are in general preferred over curative methods. They can be pre-treatments, side-stream treatments, alteration of surface composition, physical or chemical methods.

Pre-treatments are aiming at removing elements from the water that are necessary for microbial growth. Methods are primarily focused on organic matter removal by chemical or physical mechanisms, as carbon is generally the primary growth limiting compounds (Nguyen et al. 2012). Commonly applied make-up water treatments include coagulation and flocculation followed by sand filters or ultrafiltration (Pinel et al. 2020a). In some cases, activated carbon can also be applied. While these techniques manage to remove a large fraction of organics, the remaining organic matter which gets concentrated in the cooling system can still cause biofouling. Therefore, other compounds have recently been the target of biofouling prevention. Commercially available technologies are used to reduce phosphate from the make-up water to low levels, and efforts are made towards the development of non-phosphorous chemicals against scaling formation, to avoid the enhancement of microbial growth by polyphosphate and phosphonate antiscalants. Softening of the water has shown to affect bacterial adhesion and biofilm mechanical properties due to the cross-linking ability of divalent cations (Hijnen et al. 2016, Körstgens et al. 2001). Biofilm inhibition by calcium removal has however not been reported in cooling systems. Aside from pre-treatment, some side-treatments are using biological processes to lower the concentration of nutrients. It is the case with inline biofilters built as a side-stream of the recirculating water (Bott 1998, Daamen et al. 2000).

Surface designs and compositions inspired by aquatic organisms led to the synthesis of thin polymeric layers preventing protein adsorption and to the imitation of surface roughness and wettability using lithography (Di Pippo et al. 2018). These surface designs can slow down the build-up of biofilm in critical parts of the systems like heat exchangers, spray nozzles or packing materials. Coatings with slow releasing rates of antimicrobial agents and amphiphilic layers with hydrolytic enzymes have also been developed to prevent bioadhesion and biofilm formation (Ciriminna et al. 2015).

Finally, physical approaches have shown promising results for the mitigation of microbial growth in cooling water. Some examples are anti-algae ultrasonic treatments causing disruption of cells by cavitation (Park et al. 2017), the use of ultraviolet light damaging cells' DNA (Chang and Lin 2016), or hydrodynamic cavitation as a side-stream treatment (Gaines et al. 2007).

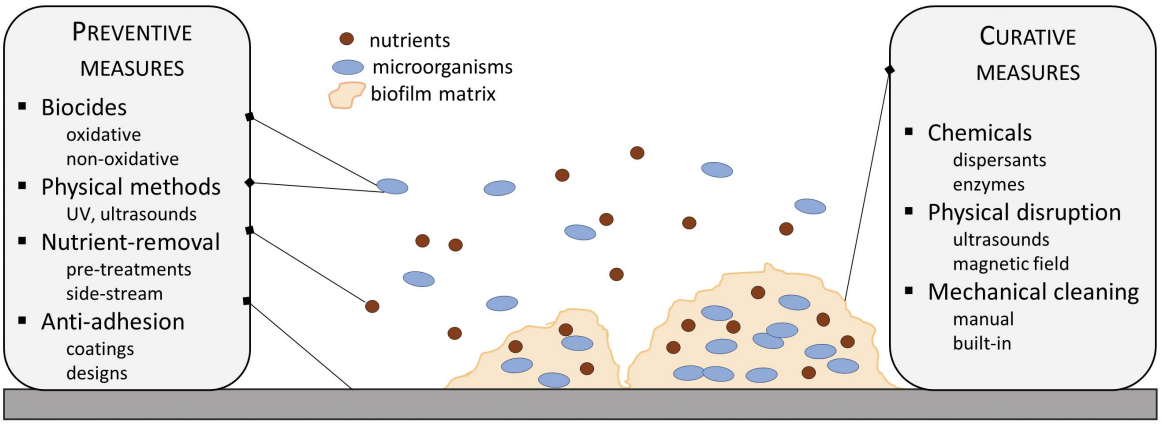


Figure 1.5. Overview of methods for biofouling control in cooling water systems involving preventive measures focused on the water quality and surfaces, and curative measures focused on established biofilms.

The implementation of the above-mentioned techniques depends on several factors, including the source water quality, design of the cooling tower, discharge requirements and economic feasibility.

1.3.4. Main research gaps

Despite some valuable insights provided by the characterization of cooling water microbiome, there is no clear methodology applied to quantify the spatial and temporal changes in microbial communities. The use of 16S and 18S rRNA gene amplicon sequencing alone can lead to a biased interpretation of relative abundances and difficulties to determine factors driving the changes in microbial community structure and associated biological processes and conversions.

The dosage of chlorine in cooling water systems can lead to selection of more resistant bacterial species, either by direct or indirect factors. *Pseudomonas* have for example low inactivation rate (1log) at free chlorine concentration of 0.5 ppm, common in cooling water. Cysts of *Acanthamoeba sp.*, host of *L. pneumophila*, can sustain concentrations of up to 5 ppm chlorine and protect the pathogen from disinfection. The identification and study of pathogenic and non-pathogenic biocide resistant bacteria in cooling water systems have not yet been thoroughly carried out (Luo et al. 2021) but are of importance to understand their involvement in human health and biofouling.

An important knowledge gap remains in assessing the impact of external factors on cooling water quality, such as rain water, particles and pollens or low air quality, and consequently on biofouling. Large volumes of air are continuously passing through cooling towers and coming in contact with the water (Milosavljevic and Heikkilä 2001). For instance, a cooling tower located nearby agricultural fields is likely to result in an increased nutrient content of the water. Such phenomena might undermine the efficiency of pre-treatments and necessitate to focus our efforts towards in-line mitigation methods, such as side-stream water treatments or physical biofilm prevention technologies. The high air-water contact could also cause contamination of the cooling water by an airborne microbiome. Investigation of these

transfers is particularly important when considering the spread of pathogenic bacteria from cooling towers to cooling towers in highly industrial or urban areas.

Development of side-stream nutrient removal methods should be promoted in the prevention of biofouling. The brackish nature of recirculating water and presence of salt is a challenge to overcome but this type of treatments have significant advantages on water quality. They can indeed counteract water contamination by external conditions and could reach higher efficiencies than pre-treatments by treating water with higher concentrations induced by evaporation.

For established biofilms, development of efficient alternative chemicals depends first on an accurate characterization of biofilms, with identification of the structural EPS compounds and their interactions. A multidisciplinary approach is necessary to reach a full understanding of its complex network. Variations in sessile communities within a same cooling water system likely result in changes in EPS matrix in different parts of the process, as EPS composition has been shown to reflect its microbial population (Flemming et al. 2007). Investigation of these changes, between the cooling tower basin, packing material and heat exchanger units would also be of interest.

1.3.5. Relevance of research at different scales

In this thesis, we studied systems at different scales, with high variations in cooling capacity: (i) full-scale (25MW, 2600 m^3/h), (ii) pilot-scale (10-15kW, 1 and 1.2 m^3/h), and (iii) lab-scale (< 100 W, 0.01 m^3/h).

Full-scale measurement campaigns are necessary for getting a clear picture of the simultaneous processes involved in cooling system fouling and microbial dynamics, and identify the critical points requiring investigation at smaller scale. The main restriction lies within the fact that full-scale studies are performed on systems already undergoing a continuous treatment procedure, due to the risks taken in the absence of it. There is a lack of information on the interactions and overall fouling behaviours when no treatment is applied. This absence of negative control makes challenging the development and assessment of alternative biofouling mitigation methods. Laboratory-scale experiments are essential to discriminate the effects of single factors and to bring answers to fundamental questions emerging from current full-scale operation. Investigations and testing of alternative operation, before implementation at full-scale, then require the use of pilot set-ups. Pilots have the advantage of mimicking full-scale conditions by being subjected to external factors as well as process-related parameters, not always considered at laboratory-scale. They provide good prediction of the behaviour in full-scale and easier access to areas for sampling, with reduced consequences in case of wrongly controlled microbiome or extensive corrosion and scaling. When pilot-scale testing provides successful results, methods can be implemented at full-scale.

1.4. THESIS SCOPE AND OUTLINE

The research described in this thesis explores two aspects linked to microbial growth in evaporative cooling systems (Figure 1.6). The first focuses on the characterization of factors governing the microbial selection and biofilm composition, addressed in chapters 2, 3 and 4. The second investigates new approaches for prevention of biofouling and *L. pneumophila* growth, described in chapters 4 and 5.

Chapter 2 characterizes a full-scale cooling water system, from the water source to the cooling water discharge, in terms of bacterial community members and their abundances. This study introduces the use of a mass balance to evaluate the impact of continuous chlorine disinfection on each bacterial group, allowing the assessment of disinfection's efficiency and the identification of less vulnerable organisms.

Chapter 3 describes the development of a laboratory-scale plate heat exchanger module with the aim to systematically investigate the impact of surface and bulk temperatures on biofilm composition i.e., matrix and active biomass.

Chapter 4 assesses phosphorus limitation as a biofouling prevention method. The impact of nutrient availability on the extent of active biomass and EPS production was explored in a pilot facility consisting in 3 parallel cooling systems. A closer look was given to the presence of sialic acid in an additional study on the collected biofilms.

Chapter 5 investigates the potential of alkaline cooling system operation as an alternative to disinfection for the control of *L. pneumophila* and associated protists. Experiments were performed in a pilot-scale system to take the environmental conditions into account and more accurately simulate full-scale processes.

Chapter 6 summarizes the main research findings, discusses the remaining knowledge gaps and suggests directions for future research, from both fundamental and industrial point of views.

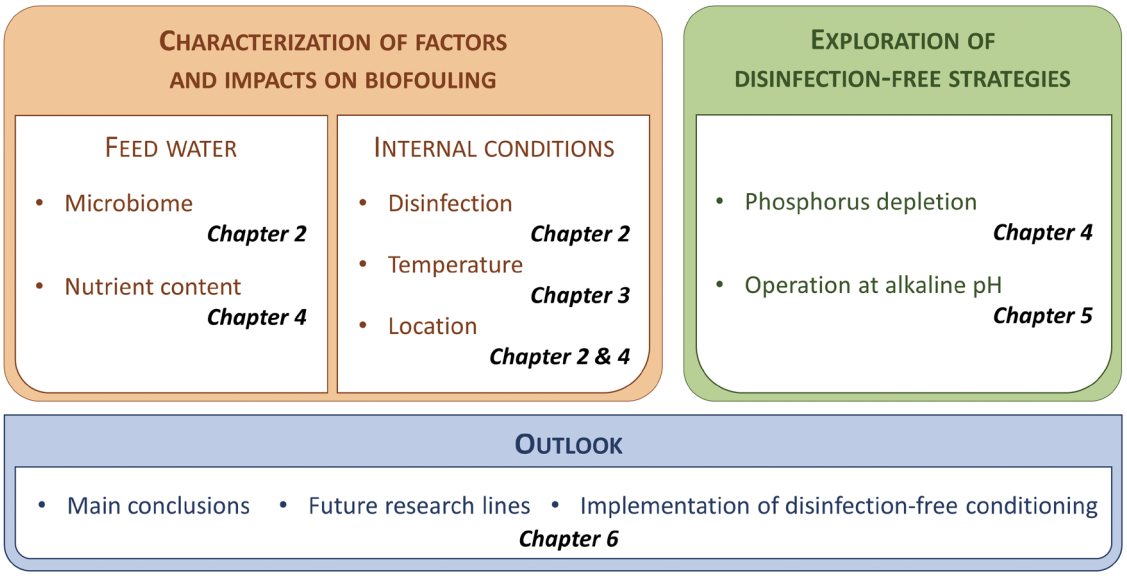
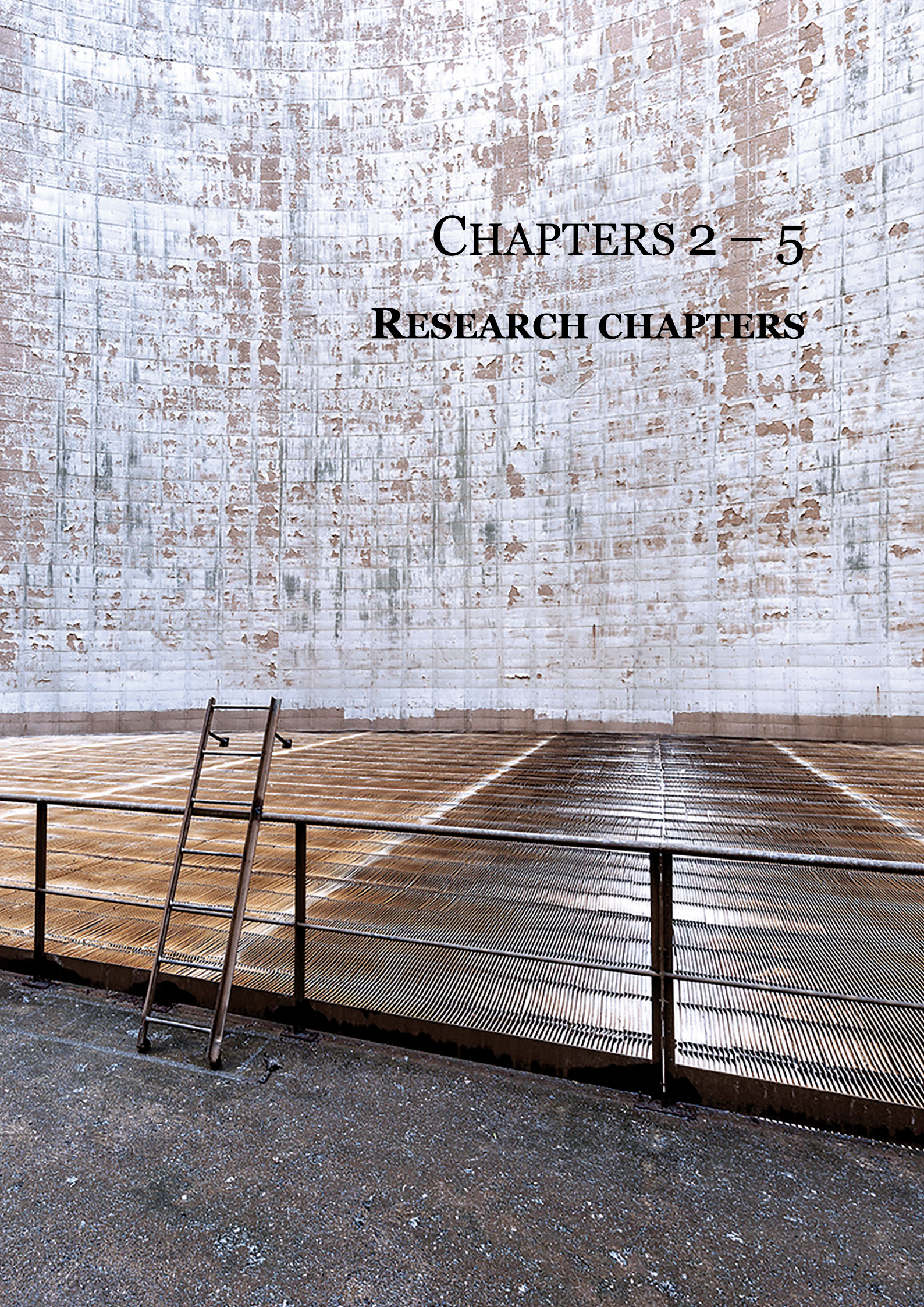


Figure 1.6. Structural overview of the thesis investigating different aspects of biofouling during laboratory-scale, pilot-scale, and full-scale studies.



Chapters 2 – 5

Research chapters

CHAPTER 2

BACTERIAL COMMUNITY DYNAMICS AND DISINFECTION IMPACT IN COOLING WATER SYSTEMS

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Supplementary figures and tables are available at the following link:
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ABSTRACT

Understanding the bacterial dynamics in cooling systems is imperative for the assessment of disinfection efficiency and management of microbial risks linked to aerosol formation. The objective of this study was to evaluate the impact of feed water on the cooling water bacterial microbiome and investigate the survival ability of its members when exposed to continuous chlorine disinfection. Water from an industrial cooling water system (2600 m^3/h) was collected over a 5-month period at 3 locations along the feed water line and 3 locations in the cooling tower. Adenosine triphosphate (ATP) measurements suggested that the average ATP-per-cell in the cooling tower evolved independently from the average ATP-per-cell in the feed water. Flow cytometry and 16S rRNA gene amplicon sequencing were then combined to quantify the bacterial dynamics in the whole system. A mass balance based equation was established to determine net growth and net decay of the cooling tower bacterial communities in order to evaluate the impact of continuous chlorination (0.35 to 0.41 mg Cl_2/L residual chlorine). The results indicated that cooling system main community members were determined by the input feed water microbiome and the bacterial community structure was further shaped by varying decay rates of the microorganisms. Notably, the order *Obscuribacterales* showed to be growing in the cooling tower in the presence of residual chlorine up to 0.4 mg Cl_2/L, with a recurrent net growth of 260 ± 95 %, taking into account the impact of the concentration factor. This conclusion was only possible thanks to the systematic analysis described in this paper and generates discussion about the resistance of *Obscuribacterales* to residual chlorine. The described mass balance approach provides a high level of understanding on bacterial dynamics and should be considered for future characterization studies of cooling systems in which accurate investigation of microbiome changes is essential.

2.1. INTRODUCTION

The use of open recirculating cooling systems is crucial to cool down full-scale industrial processes. In the Netherlands alone, the number of evaporative cooling towers is estimated to be around 4000 (RIVM 2018). Cooling water constitutes the highest water withdrawal in several European countries and represent a major source for Legionnaires' disease (European Commission 2018, Llewellyn et al. 2017).

In order to avoid the proliferation of harmful species such as *Legionella pneumophila* and to reduce the risk of biofilm formation and microbial corrosion in the system, the daily operation of cooling towers usually includes continuous or shock dosage of biocide. The most common biocide, chlorine, is often added as sodium hypochlorite (NaOCl) or chlorine dioxide (ClO_2). Despite continuous chlorine dosage, prior research has shown that active bacteria can still be present in cooling tower systems (Chien et al. 2013, Helmi et al. 2018) and outbreaks keep occurring (ECDC 2019), bringing into question the efficacy of this method in managing risks linked to *Legionella*. Chlorine efficiency can decline by a low-quality feed water or side-reaction with organic compounds concentrating in the cooling system (Hua et al. 2015). A few case studies have reported the identification of bacterial communities and microbial interactions in chlorinated cooling towers (Di Gregorio et al. 2017, Paranjape et al. 2020b, Pereira et al. 2017, Tsao et al. 2019). Feed water has been considered as a major factor affecting cooling water microbial communities based on results from 18 cooling towers from 6 geographic locations and using local water sources (Paranjape et al. 2020b). Another paper pointed out the recurrence of biofilm-forming taxa in cooling tower basins (Tsao et al. 2019). However, most studies simply report the observed communities without quantifying the contribution of the feed water microbiome or evaluating the impact of biocides on decay or resistance of microorganisms in the cooling water.

The complexity of open recirculating cooling systems makes the study of their microbiome at laboratory-scale and the collection of representative results challenging (Chien et al. 2013). Therefore, the available knowledge on the microbial dynamics in cooling towers is still limited. Investigating the behaviour of the bacterial populations in long-term operated full-scale systems is a necessary step in identifying the factors shaping the community compositions and understanding how biocidal treatments impact the cooling system microbial communities.

In this study we combined flow cytometry, adenosine triphosphate (ATP) and 16S rRNA gene amplicon sequencing analyses of water samples from an industrial full-scale cooling water system (2600 m^3/h). Data collected from the feed line (3 locations) and from the cooling tower (3 locations) over a 5-month period was used to: (i) characterize the microbiome along the system, (ii) evaluate the impact of feed water on cooling water bacterial dynamics, (iii) detect bacteria potentially resistant to continuous chlorine dosage. The approach provides qualitative and quantitative information on the temporal and spatial bacterial changes in the cooling water system and on the disinfection efficiency by chlorine dosage.

2.2. MATERIALS AND METHODS

2.2.1. Full-scale system

The studied system is located in Zeeland, the south-west region of the Netherlands. As shown in Figure 2.1, it consists in two main parts: the feed water line and the open recirculating cooling system. The pre-treatment of the feed water is performed at the Braakman water treatment plant of Evides Industriewater before being distributed to the induced draft cooling tower situated on the site of an industrial gases company. The freshwater originates from two main sources, Belgian polder water and surface water from the Biesbosch. Pre-treatment involves the dosage of an iron-based coagulant, then use of bandfilter, microstrainer, rapid sand filter for removal of suspended solids and dosage of sodium hypochlorite as a biocide. The pipe distance of the distribution line connecting both sites is approximately 8 km. The cooling tower has a capacity of 25 MW and is maintained at a concentration factor (i.e. ratio between basin and feed water conductivities) of around 5 based on online conductivity measurements. When the conductivity in the basin exceeds 3500 µS/cm, the discharge valve opens and part of the concentrated water is replaced by new feed water. The system recirculates on average 2600 m^3/h of cooling water and operates at a temperature difference of 6 to 8 °C between the inlet and outlet of the heat exchanger line, location points D and E respectively (Figure 2.1). The chemical dosages include mild steel and copper corrosion inhibitors (containing respectively phosphoric acid and sodium tolyltriazole), $CaCO_3$ scale inhibitor (phosphinosuccinic oligomer) and dispersant (high stress polymer). Online monitoring of the water parameters is achieved by sensors located in the cooling tower and allows adjustment of the dosages by an automatic controller. pH is stabilized at 7.5 with sulfuric acid and oxidation-reduction potential (ORP) is maintained between 550 and 600 mV with sodium hypochlorite dosage.

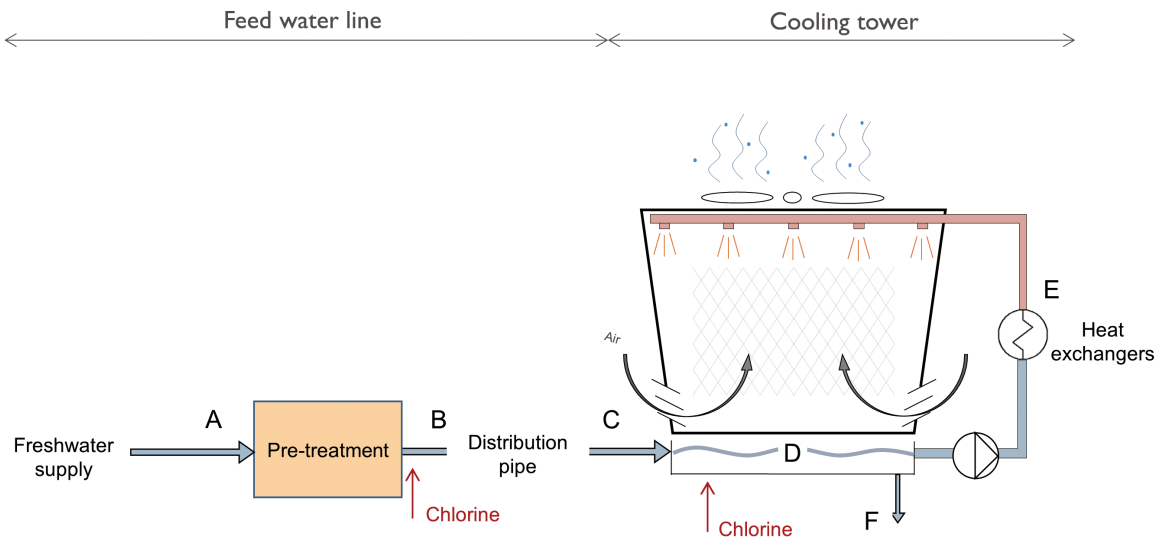

Figure 2.1. Simplified scheme of the cooling water system. Capital letters indicate the sampling locations: pre-treatment inlet (A), pre-treatment outlet (B), cooling tower feed (C), cooling tower basin (D), cooling tower heat-exchanger outlet (E) and cooling tower discharge (F). Cooling tower cold and warm waters are represented by blue and red backgrounds.

Sodium hypochlorite was therefore dosed at two locations in the entire studied system. The first location was before distribution of the feed water to the cooling tower, before sampling point B (Figure 2.1). The second location was in the basin of the cooling tower, point D. The full-scale cooling tower was continuously operated under stable conditions since the last cleaning, approximately 2 years before the sampling campaign. No biofilm growth was visually observed in the cooling tower basin and packing material.

2.2.2. Water sampling

Water samples from 3 locations along the feed water line and 3 locations in the cooling system were studied by monthly analyses over a period of 5 months. The labels A to F indicate the sampling points (Figure 2.1): pre-treatment inlet (A), pre-treatment outlet (B), cooling tower feed (C), cooling tower basin (D), cooling tower heat-exchanger outlet (E) and cooling tower discharge (F). The samples were collected in 1 L sterile glass bottles containing a solution of 10 mM (1 % v/v) sodium thiosulfate for ATP, flow cytometry and microbial community analysis to neutralize residual chlorine before transportation. ATP and flow cytometry measurements for total and intact bacterial cell numbers were performed within 4 hours after sampling. 400 mL of water was filtered through 0.22 µm pore size and 33 mm diameter sterile PVDF syringe filter (Merck, Germany) and the filter membrane was frozen at -20 °C for later DNA extraction. Samples for trace metal analyses were collected in 15 mL metal-free centrifuge tubes (VWR, USA), filtered through 0.45 µm pore size and 33 mm diameter sterile PVDF syringe filters (Merck, Germany), diluted 10 times with ultrapure water and treated with nitric acid (1 % v/v) prior to the ICP-MS analyses. All samples were kept on ice between sampling and processing.

2.2.3. Water quality analyses

The following water quality parameters were measured (Table 2.1): pH (C6010, Consort, Belgium), conductivity (C3010, Consort, Belgium), temperature (PT100 temperature sensor, Testo BV, Nederland) and residual chlorine (Controller Dulcometer, Prominent, Germany and Lovibond PC22/GB, Tintometer, Germany) at the dates of the sampling. Total organic carbon (TOC) was analysed after filtration through 0.45 µm pore size sterile PVDF syringe filters (TOC analyser model TOC-L CSH, Shimadzu Corporation, Japan). The range of ammonia concentration was reported by the feed water supplier. Nitrate, sulphate and phosphate concentrations were measured by spectrophotometry (DR 3900, Hach, USA).

Table 2.1. Main water parameters along the cooling water system (Figure 2.1) over the 5-month period. Square brackets [ ] indicate the range of values containing the data from all months. Abbreviation n.d. indicates that the value was not determined at this specific location.

Parameter	A	B	C	D	E	F
pH	[7.8-8.2]	[7.9-8.4]	[7.7-8.4]	[7.6-7.8]	[7.5-7.7]	[7.5-7.8]
Conductivity (µS/cm)	[600-750]	[600-750]	[600-750]	[3000-3500]	[3000-3500]	[3000-3500]
Temperature (°C)	[5.2-20.2]	n.d.	n.d.	[15-20]	[20-25]	[15-20]

Parameter	A	B	C	D	E	F
Residual chlorine (mg/L)	n.d.	[0.32 - 0.42]	[0.02 - 0.20]	[0.35 - 0.41]	n.d.	n.d.
TOC (mg/L)	[4.1-11.5]	[4.8-11.5]	[4.8-11.1]	[34.1-53.6]	[35.7-55.4]	[36.1-52.2]
Ammonia (mg/L)	n.d.	n.d.	[0.005-0.05]	n.d.	n.d.	n.d.
Nitrate (mg/L)	[5.8-8.9]	[6.2-8.9]	[5.8-9.3]	[32-40]	[33-41]	[33-43]
Sulphate (mg/L)	[44-55]	[44-54]	[46-54]	[672-769]	[700-816]	[697-805]
Phosphate (mg/L)	< 1.5	<1.5	<1.5	[6.0-8.1]	[6.6-8.4]	[6.9-8.7]

2.2.4. Experimental analyses

2.2.4.1. ATP measurements

ATP measurements were carried out to quantify the intracellular and extracellular ATP concentration in the water samples. The intracellular ATP measurements were performed according to the supplier's instructions. 20 mL of water sample were filtered through a 0.22 µm sterile nylon syringe filter. 5 mL of lysis reagent were processed through the filter. 100 µL of the lysis solution containing ATP was mixed to 100 µL of reconstituted Water-Glo reagent (Water-Glo Substrate mixed with Water-Glo Reconstitution Buffer). After 40 s waiting time, light intensity was measured with 2 s integration time by a Glomax 20/20 luminometer (Promega Benelux BV, Netherlands). A provided ATP standard in lysis buffer (1000 pg ATP/mL) was used to convert light units into ATP concentration. For extracellular ATP measurements, 100 µL of the filtered water sample was mixed to 100 µL of reconstituted Water-Glo reagent and analysed identically as intracellular ATP. A calibration curve was initially obtained from successive dilutions of ATP standard in ultrapure water to convert light units into ATP concentration. All samples were analysed in duplicate.

2.2.4.2. Flow cytometry measurements

All collected samples were diluted by a factor 10 in ultrapure water prior to the flow cytometry analyses. The staining protocol and flow cytometry analyses were based on previous papers (Hammes et al. 2008, Prest et al. 2013). Two staining solutions were prepared from stock solutions of SYBR Green I (10000 x conc., Invitrogen, USA) and propidium iodide (30 mM in DMSO, Invitrogen, USA). A working solution of 10 µL SYBR Green I in 1 mL of 10 mM Tris buffer pH 8 (solution SG) was used to stain the bacterial cells. For the intact cells, 10 µL of SYBR Green I stock solution was mixed with 20 µL of propidium iodide stock solution in 1 mL of 10 mM Tris buffer pH 8 (solution SGPI). 5 µL of SG for total cell count (TCC), or SGPI for intact cell count (ICC) was added to 495 µL sample. The final concentration of the stains in the samples were 1.96 µM for SYBR Green I and 6 µM for propidium iodide. After stain addition, the samples were mixed for 3 s, incubated in the dark at 37 °C for 10 min and mixed again. The measurements were performed with a BD Accuri C6 flow cytometer (BD Biosciences, Belgium) equipped with a 50 mW laser emitting at a fixed wavelength of 488 nm. Fluorescence intensity was collected at Fl1 = 533 ± 30 nm, Fl3 >

670 nm. Analyses were performed at a flow rate of 66 µL/min on 50 µL of sample volume with a threshold value of 700 on FL1. Gating strategy and data collection were performed as previously described (Prest et al. 2013) with a BD Accuri C6 software.

2.2.4.3. DNA extraction and 16S rRNA gene amplicon sequencing

After filtration of 400 mL of sample, the 0.22 µm pore size and 33 mm diameter membrane filters were recovered from the syringe filter units (Merck, Germany) and were preserved at -20 °C until processing. The genomic DNA was extracted using the DNeasy UltraClean Microbial Kit (Qiagen, Netherlands). Extraction was done following company's standard protocol with addition of an alternative lysis step. This included a combination of 5 min of heat (65 °C) followed by 5 min of bead-beating on the filters for cell disruption on a Mini-Beadbeater-24 (Biospec, USA). The quality of the extracted DNA was evaluated by (i) performing gel electrophoresis to control that the DNA was not degraded and that the molecular weight was superior to 1 kb, and (ii) checking that the target size of 465 bp was obtained after quantitative polymerase chain reaction (qPCR) amplification. Samples were sent to Novogene Ltd. (Hongkong, China) for amplicon sequencing of the V3-4 region of the 16S-rRNA gene (position 341-806) on an Illumina paired-end platform. The raw sequences were quality filtered, checked for chimera and operational taxonomic units (OTUs) were generated based on 97 % similarities using the software Mothur. The alignment and taxonomic classifications were performed using the SILVA database. DOI of the raw sequencing data: 10.4121/uuid:4d56b031-ebba-4ec6-a0e0-f185031d1ebe. The abundances of potentially pathogenic genera, obtained from the drinking water quality guidelines (WHO 2011), have been calculated and added in supplementary materials (Table S3).

2.2.4.4. Alpha and beta diversity analyses

Diversity analyses were performed on the OTUs generated after the sequencing and their relative abundances in the samples. Alpha diversity indices Shannon and Chao were calculated in Mothur and are available in supplementary materials (Table S2). Shared and non-shared OTUs as well as the corresponding number of sequences were obtained from the venn command in Mothur and Venn diagrams were constructed using the software Venn Diagram Plotter. Beta diversity measurement of the samples was assessed with principal coordinates analysis (PCoA) in Mothur using unweighted UniFrac distance matrix. Analysis was performed with subsampling parameter of 30276 sequences, corresponding to the sample with the lowest number of sequences. PCoA results provided the phylogenetic relatedness between the bacterial communities retrieved from the different sampling locations and at different sampling times.

2.2.4.5. ICP-MS analyses

Potassium (K39) was used to evaluate the water flows in the system as it is assumed that no loss of the element would occur in evaporation or precipitation. Moreover, the chemicals added to the cooling tower do not contain potassium. In one of the 5 months (November), analysis of K39 was not available. The concentration of strontium (Sr88) was used as a substitute element for the mass balance calculation for identical reasons as stated for

potassium. Water samples were filtered with 0.45 µm pore size and 33 mm diameter sterile PVDF syringe filters (Merck, Germany), diluted 10 times with ultrapure water and treated with nitric acid (1 % v/v) prior to the analyses. The element was analysed by inductively coupled plasma mass spectrometry (ICP-MS PlasmaQuant, Analytik Jena, Germany). In the analysis method, an Ar flow of 9.0 L/min was used for the plasma, with an auxiliary flow of 1.4 L/min and nebulizer flow of 1.1 L/min. The R/F was set to 1.3 kW. Measurements were performed in no gas mode.

In the calculation described in section 2.2.5., the concentration factor was based on the ratio of K39 (or Sr88) concentration in the cooling tower water by the concentration in the feed water for each sampling date (Table 2.2).

Table 2.2. ICP-MS analyses from the feed and the cooling tower water for each month. The second values (±) represent the standard deviation of three measurements of the same water sample. Potassium K39 (or strontium Sr88 in November) was used to calculate the concentration factor in the cooling tower.

	September	**October**	**November**	**December**
Reference element	K39	K39	Sr88	K39
Concentration in feed water (mg/L)	8.98 ± 0.05	6.58 ± 0.02	0.204 ± 0.001 -Sr88-	8.6 ± 0.2
Concentration in cooling water (mg/L)	50.4 ± 0.6	43.5 ± 0.4	0.94 ± 0.01 -Sr88-	30.1 ± 0.3
Concentration factor C	**5.6 ± 0.1**	**6.6 ± 0.2**	**4.6 ± 0.1**	**3.5 ± 0.1**

2.2.5. Mass balance based equation for assessment of net decay or growth of taxonomic orders

Taking into account the addition of bacterial cells by the feed water and the concentration factor estimated by the potassium (or strontium) measurements, and supposing that all cells remain intact in the cooling tower, a theoretical concentration can be obtained for each taxonomic order in the cooling water:

$$[X]_{CT.Th} = [X]_F * C = \%X_F * TCC_F * C \quad (1)$$

with $[X]_{CT\,/\,F}$: concentration of organism X in the cooling tower (theoretical) or in the feed; C: concentration factor; $\%X_F$: relative abundance of organism X in the feed; TCC_F: total cell count in the feed

An approximation of the real concentration can be determined separately:

$$[X]_{CT} = \%X_{CT} * TCC_{CT} \quad (2)$$

with $\%X_{CT}$: relative abundance of organism X in the cooling tower; TCC_{CT}: total cell count in the cooling tower

The ratio of both numbers calculated using equations (1) and (2) is used to determine growth or decay of the bacterial order:

$$R = \frac{[X]_{CT}}{[X]_{CT.Th}} \quad (3)$$

R > 100 % : growth

R < 100 % : decay

R = 100 % : cell retention (no net decay nor growth)

2.3. RESULTS

Water samples were collected from 3 locations in the feed line (A, B and C) and 3 locations in the cooling tower (D, E and F) (Figure 2.1) over a 5-month period. Cell counts, ATP and next generation sequencing analysis was performed on all samples to investigate the spatial and temporal changes in bacterial microbiome. A closer look was taken at the cooling tower feed (C) and the cooling tower samples (D, E and F) to assess the impact of chlorine dosage on the main bacterial communities composing the cooling tower water (Figure 2.1). The net decay or net growth of specific orders of bacteria were estimated from a mass balance based equation taking into account the concentration factor.

2.3.1. Water composition

Water parameters collected by online monitoring or with handheld measurement devices are reported in Table 2.1. Despite some small variations in pH, conductivity and temperature in the feed line, the cooling tower parameters were kept stable. The samples had a pH between 7.7 and 8.4 in the feed water line (A, B and C). In the cooling tower (D, E and F) the pH was between 7.5 and 7.8 due to the dosage of sulphuric acid to limit scaling and improve sodium hypochlorite disinfection efficiency (Figure 2.1). The conductivity in the feed line varied between 600 and 750 µS/cm over time while the conductivity in the cooling tower was maintained between 3000 and 3500 µS/cm. TOC concentrations reached between 34.1 and 55.4 mg/L in the cooling tower due to the concentration factor induced by the evaporation. Residual chlorine concentrations were the highest in location B and D reaching up to 0.42 and 0.41 mg Cl_2/L respectively due to the dosage of sodium hypochlorite at these locations. Lower values in C indicate a decay in residual chlorine along the distribution system. Chlorine concentrations in E and F were not quantified but were expected to contain similar concentration of residual chlorine as D, as a result of the high water recirculation rate and continuous dosage. Ammonia concentration was not analysed in the cooling tower but the feed water supplier reported a low concentration in the incoming water, between 0.005 and 0.05 mg/L. Formation of monochloramine in the cooling water cannot be excluded but is assumed to have minor impact on the residual chlorine concentration measured. The temperature was highly variable in the feed water, gradually decreasing from 20.2 to 5.2 °C during the sampling campaign from August to December 2017. On the contrary, the cooling water temperature was kept between 15 and 25 °C due to the continuous water recirculation through the heat exchangers.

The results from the ICP-MS analyses, with the corresponding concentration factor, are shown in Table 2.2. The concentration factor varied between 3.5 ± 0.1 and 6.6 ± 0.2.

2.3.2. Bacterial cell number and ATP measurements

2.3.2.1. Spatial changes in the system

Flow cytometry was applied to measure the total and intact cell counts and ATP measurements were performed to quantify the bacterial intracellular and extracellular ATP concentrations in the water samples. Figure 2.2 shows the results of the analyses performed in September. The results from the other months revealed the same trends despite some variations in absolute values. The corresponding figures for all monthly samples are available in the supplementary materials (Figures S1 and S2).

Sodium hypochlorite was dosed at two locations in the system. The first location was before distribution of the feed water to the cooling tower, before sampling point B (Figure 2.1). The second location was in the basin of the cooling tower, point D. The intact cell counts and intracellular ATP at the inlet of the feed line (A) were the highest from all samples, with [0.4-1.5] x 10^6 cells/mL (Figure 2.2.a, Figures S1 and S2) and [80-700] pg ATP/mL respectively (Figure 2.2.b, Figures S1 and S2). The intact cell counts then showed a strong decrease in the concentration range between sampling points A and B, where the pre-treatment steps and first NaOCl dosage were applied. The reduction varied between 79 and 98 % in intact cell count depending on the sampling month. The intracellular ATP measurements indicated a similar trend, with a decrease of 85 to 98 % between sampling points A and B.

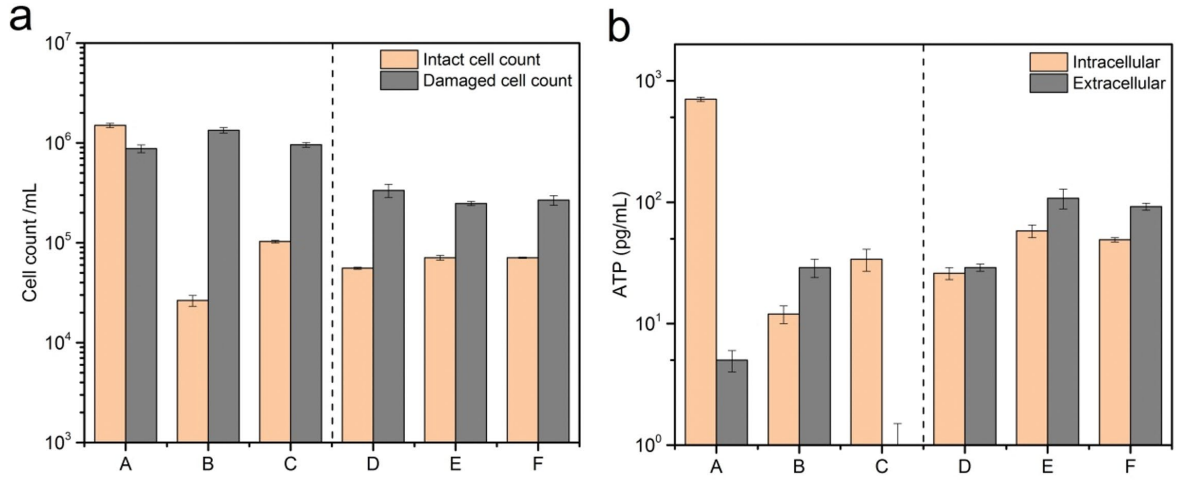

Figure 2.2. Intact and damaged cell counts (a) and ATP measurements (b) in September 2017. The letters A to F correspond to the coded sampling locations shown in Figure 2.1. Results from the other months are available in supplementary material (Figures S1 and S2). The error bars indicate the standard deviation on duplicate measurements.

In August and September, an increase of [6-7] x 10^4 in intact cells per mL was measured along the distribution pipe between points B and C. Higher feed water temperature in this period of the year could have caused regrowth in the distribution line. The opposite trend was observed in November and December, with a loss of intact cells and intracellular ATP.

No strong difference in the intact cell count and intracellular ATP was noticeable between the feed water in point C and the cooling water in points D, E and F despite continuous sodium hypochlorite dosage in the basin of the cooling tower. The chlorine dosage did not

neutralize all cells but it helped to prevent further increase in cell concentration considering the concentration factor (Table 2.2) and the water retention time in the system. The 3 samples collected in the cooling tower (D, E and F) showed comparable results for each sampling date although point E always exhibited a slightly higher microbial contents of [1-2] x 10^4 intact cells/mL.

2.3.2.2. Evolution of ATP-per-cell in time

The ATP data and intact cell counts were used to identify the spatial and temporal evolution in average ATP-per-cell value. The amount of intracellular ATP per intact cell count was calculated for each sample and the average values for feed and cooling water samples were plotted in Figure 2.3, together with the feed water temperature on each sampling day. The feed water samples showed a strong decrease in ATP per cell between August and November from 4.1 x 10^{-16} to 6.2 x 10^{-17} g ATP/cell and stabilised between November and December. The feed water temperatures also decreased in this period from 20.2 to 5.2 ˚C. The amount of ATP per intact cell inside the cooling tower was more stable over the observation period (between 2.7 x 10^{-16} and 6.8 x 10^{-16} g ATP/cell). The results suggest that (i) the average ATP-per-cell in the cooling tower evolved independently from the average ATP-per-cell in the feed water; (ii) the feed water is more affected by the changes in environmental conditions than the cooling water.

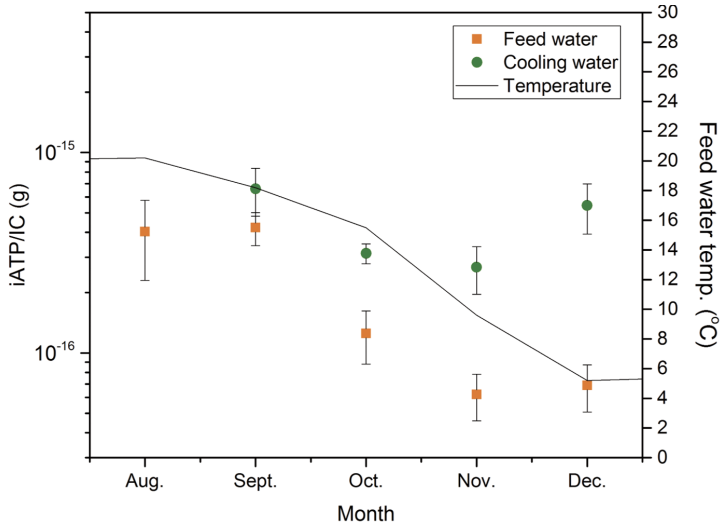

Figure 2.3. Ratio of intracellular ATP (iATP) per unit of intact cells (IC) as a measure of average ATP-per-cell over the 5-month period. The black line indicates the variation of temperature in the feed water line over time. The error bars of the data points for the feed and cooling water represent the standard deviation on the 3 feed samples (A, B and C) and the 3 cooling water samples (D, E and F).

The divergence between the intracellular ATP per cell in the feed water and in the cooling tower water could be explained by the better controlled conditions in the cooling tower or by different bacterial communities populating the two sections of the system.

2.3.3. Taxonomic diversity and relative abundances

The changes in bacterial composition in the feed and cooling water was monitored by 16S rRNA gene amplicon sequencing. The impact of the cooling tower conditions and the

continuous chlorine dosage on the microbiome were evaluated. The relative abundances of potentially pathogenic genera are available in the supplementary materials (Table S3). The *Legionella* genus represented only 0.28 % of the total number of reads from all samples and did not show an increase in relative abundance between the feed and the cooling system. The bacterial community in the feed water was dominated by *SAR_clade* (37 ± 19 %) and *Frankiales* (16 ± 8 %) orders (Figure 2.4.a and Figures S3 to S5), from the phyla *Proteobacteria* and *Actinobacteria* respectively. Only the sample from October diverged by a strong increase in *Sphingomonadales* after the pre-treatment steps (B) of 20 % (Figure S4). A less abundant but recurrent bacterial order was *Betaproteobacteriales* (6 ± 3 %). *SAR_clade* and *Frankiales* abundances dramatically dropped in the cooling tower (2 ± 2 %). The main taxonomic orders common to all cooling tower samples (locations D, E and F) were *Caulobacterales* (28 ± 12 %) and *Obscuribacterales* (18 ± 8 %), followed by *Rhizobiales* (6 ± 2 %), *Sphingomonadales* (6 ± 5 %), and *Betaproteobacteriales* (5 ± 2 %). The venn diagrams shown on Figure 2.4.b and Figures S3 to S5 indicate that the taxa shared between the feed water (C) and the basin of the cooling tower (D) accounted for 85 to 94 % of the total sequences depending on the month. In addition, the cooling tower shows less diversity than the feed water with a lower number of taxa. Hence despite the strong switch observed in relative abundance of microorganisms, the cooling tower main bacterial members were already present in the feed.

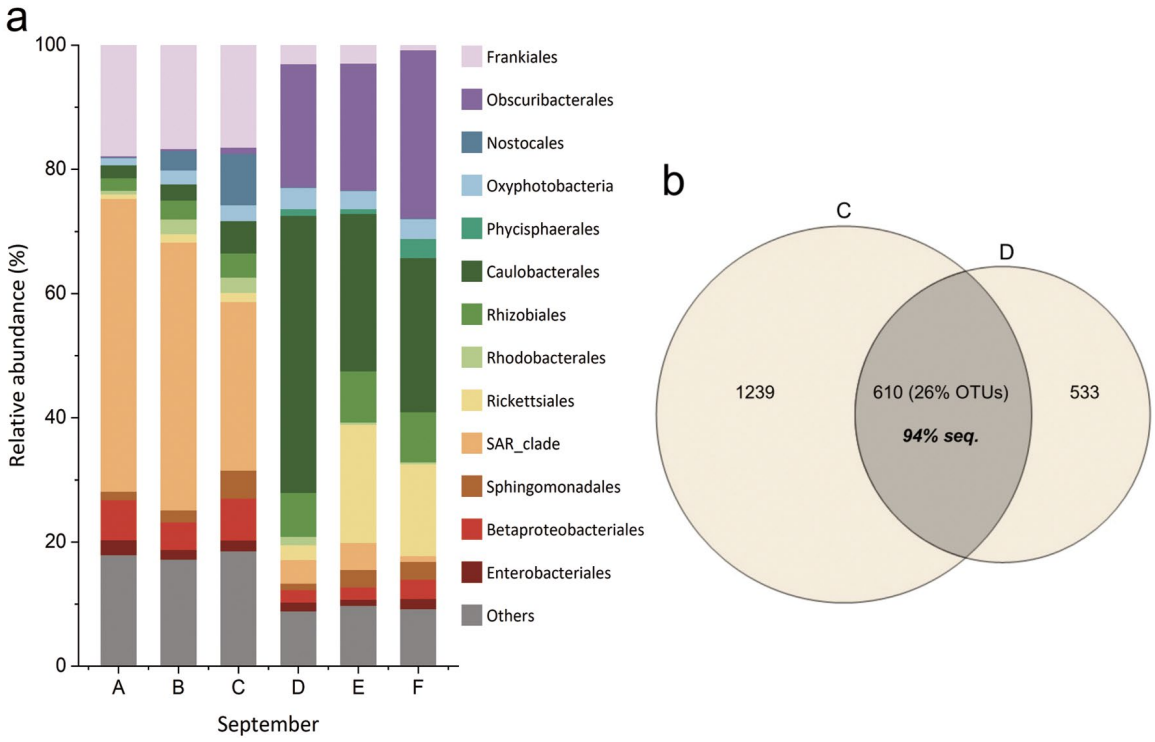

Figure 2.4. Relative abundance of bacterial order in samples of the cooling water system in September 2017 (a) and Venn diagram of cooling tower feed water C and basin water D (b). Relative abundance was generated for each OTU but displayed at the order taxonomic rank to improve visualization of the changes. "Others" contains orders of less than 1% relative abundance. b indicates the shared OTUs between samples C and D (Figure 2.1), respectively cooling tower feed water and basin water, and the percentage of total sequences (bold) included in the shared OTUs. The data from the other months are available in supplementary material (Figures S3, S4 and S5).

When comparing all collected samples, only 0.2 % of the total number of OTUs (33 OTUs) were shared between all locations over the 5 months. However, the 33 shared OTUs accounted for 46 % of the total sequence reads. These common taxa representing a large fraction of the total sequences constitutes the core microbiome of our system.

Principal coordinates analysis (Figure 2.5) shows the phylogenetic relatedness between communities of all samples based on the generated OTUs, illustrating the changes in bacterial community structures in time and at the different locations. The clusters represented with plain and dotted circles reveal structural divergence between the feed and the cooling water samples for each month. Greater distances are observable between the feed water and cooling tower samples than between the 3 samples from each section. In addition, the feed water samples clustered more closely than cooling water samples. The outlier observed in August and corresponding to the basin sample (location D) shows strong similarities with the feed water structure, suggesting that the feed water valve was open at the time of the sampling. In the same figure, the monthly clusters formed by the feed water samples do not seem to converge suggesting temporal variations in bacterial community structure in the water source, upstream from the studied system. The chart with letters corresponding to each location is available in supplementary materials (Figure S6).

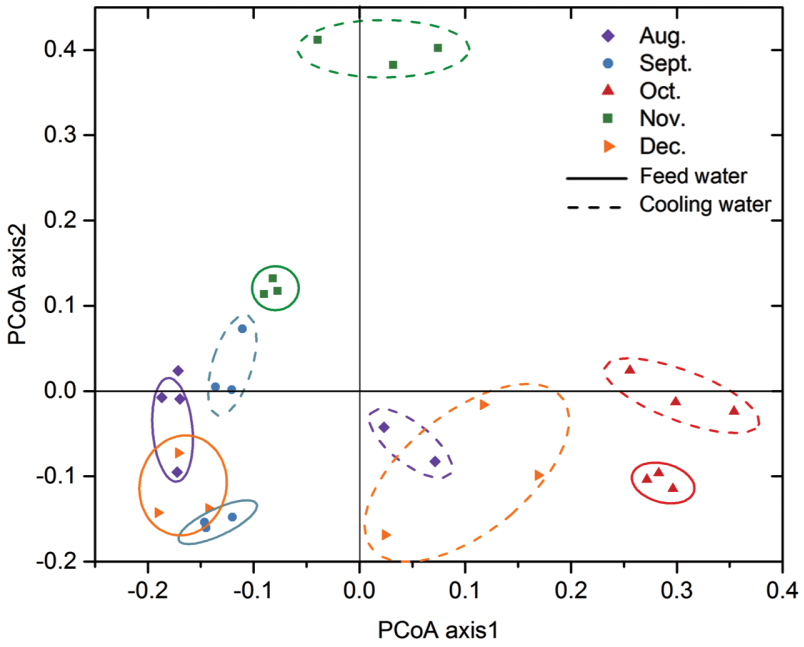

Figure 2.5. Principal coordinates analysis (PCoA) representing relatedness between bacterial communities of the cooling water system based on phylogenetic tree. The plain circles and dotted circles reveal the clusters respectively formed by the feed water samples (locations A, B and C) and by the cooling water samples (location C, D and E).

To summarize, the sequencing results suggest that the cooling tower bacterial composition is continuously determined by the feed water microbiome but is further shaped by the process conditions.

2.3.4. Growth and decay of bacterial orders in the cooling tower

The strong change in relative abundance of the main bacterial members between the feed water and the cooling system may be due to differential growth or differential decay of the feed water community members when accessing the basin of the cooling system. The sequencing data refers to all intracellular DNA, both from viable and non-viable bacteria. To estimate which community members show a net growth or decay, the relative abundances were combined with the total cell counts in a mass balance based equation. A similar approach has been implemented in the past with flocculent sludge and anaerobic digestion systems (Mei et al. 2016, Saunders et al. 2015, Winkler et al. 2012). The taxonomic rank order was chosen for visualization of the changes in microbiome and identify trends over the sampling period, as no specific functionalities were investigated.

The results presented in Figure 2.6 show the growth and decay of taxonomic orders in relative (Figure 2.6.a) and absolute numbers (Figure 2.6.b). The data indicates that the majority of the bacterial groups are decaying with less than 10 % remaining cells (less than 2 % for the *Frankiales* and *SAR11_clade*). Among the dominating organisms from the feed water and the cooling water, only the taxonomic order *Obscuribacterales* showed considerable and recurrent increase in the cooling tower microbiome (260 ± 95 %) (Figure 2.6.a) corresponding to an absolute increase of 10^4 to 10^5 cells/mL (Figure 2.6.b) depending on the month. The strong increase in 2 main OTUs within the *Obscuribacterales* order were causing this rise. *Caulobacterales* (48 ± 30 %) showed a slower decay than *Rhizobiales* (9 ± 3 %) and *Sphingomonadales* (8 ± 7 %). A further look at the most abundant OTUs of these main taxonomic orders revealed that all main genera contributed to the decay of the orders.

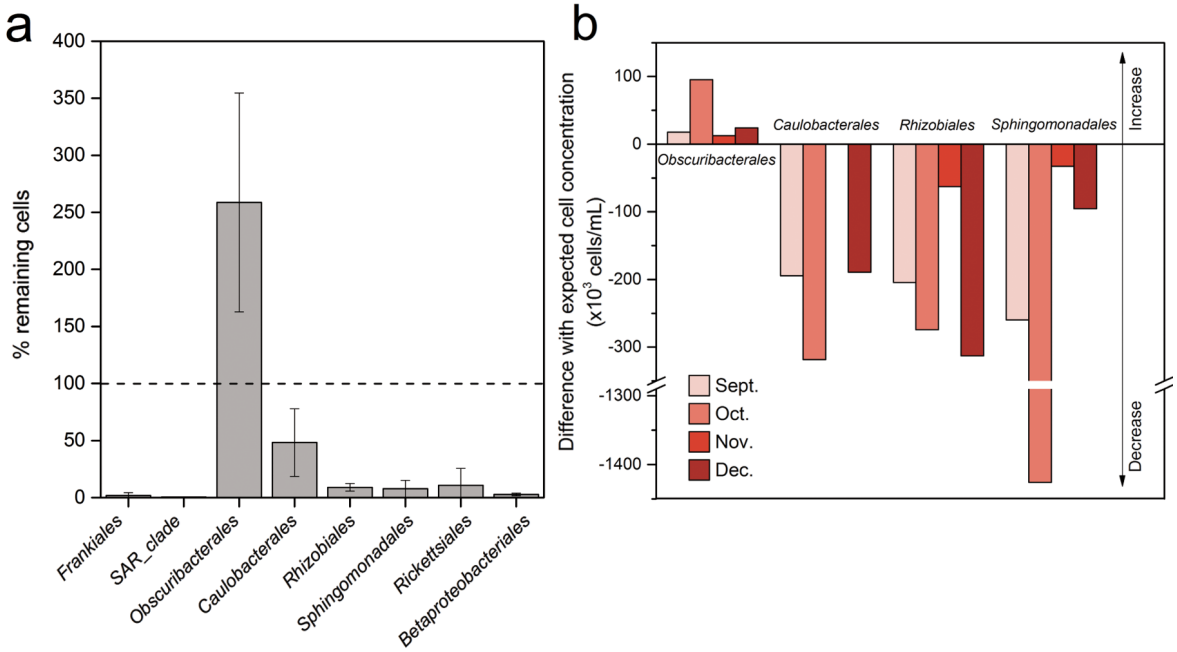

Figure 2.6. Changes in bacterial orders in the cooling water system, expressed in percentage (a) and absolute numbers (b). a shows the percentage of total remaining cells for each main taxonomic orders in the cooling tower compared to the input from the feed water. Values above 100% indicate growth. The error bars represent the standard deviation from the monthly samples. b represents the absolute deviation to the expected cell concentration for each month. Positive values indicate growth, negative values indicate decay. The 4 more recurrent taxonomic orders from the cooling water samples are represented in graph b.

Quantification of the changes in microbial structures suggest different decay rates and adaptability of individual bacterial groups under continuous chlorine dosage in the recirculating cooling water (from 0.35 to 0.41 mg Cl_2/L, Table 2.2). Only the *Obscuribacterales* order was able to significantly increase in cell number in the cooling tower despite the high residual chlorine content of the water. The absolute increase in cells, taking into account the impact of the concentration factor, could only be determined by the use of our mass balance based approach combining flow cytometry and sequencing data.

2.4. DISCUSSION

2.4.1. Commonly applied residual chlorine concentration does not allow complete inactivation of cooling water bacteria

The use of surface water as feed water for cooling tower operation requires a powerful biofouling inhibitor to avoid pathogen outbreaks and biofilm formation. The high bactericidal efficiency and competitive cost of sodium hypochlorite makes it the most common inhibitor in cooling processes (Frayne 1999). Chlorine dosage in the studied cooling water system:

(i) reduced by up to 98 % the active bacterial biomass concentration in water when dosed in the feed line (Figure 2.2), with a negligible impact on the relative abundance of the microbial community members in the surface water feeding the cooling tower (Figure 2.4.a and 2.5);

(ii) limited the bacterial growth in the cooling tower water caused by the increase in nutrient content (Table 2.1) and retention time (Figure 2.2).

Continuous dosage of biocide prevented the growth of pathogenic bacteria, including *Legionella*. However, it was not effective to entirely inactivate the bacterial cells in the water even at residual chlorine concentrations up to 0.4 mg Cl_2/L. Resistance of some specific organisms to relatively high residual chlorine concentration (0.6 mg Cl_2/L) has been recently described (Song et al. 2019, Sun et al. 2013) and previous studies on cooling tower reported that a continuous free chlorine residual of 1 mg Cl_2/L was optimal to achieve recommended biological control criteria (10^4 CFU/mL) with NaOCl (Chien et al. 2013, Helmi et al. 2018). However, concentrations higher than 0.5 mg Cl_2/L is usually not a feasible option due to the corrosiveness of the chemical, side-reaction with other inhibitors and formation of disinfection by-products (Boulay and Edwards 2001, Hsieh et al. 2010, Padhi et al. 2019). The high TOC concentration in the cooling tower, up to 55 mg/L, may have led to the reaction of organic matter with the sodium hypochlorite dosed in the basin.

As an additional point, the quantification of intact cells at different locations in the cooling system indicated a higher concentration of viable cells after the heat exchanger, suggesting cell growth or release from biofilms in that section of the process. The sampling location is therefore of importance for evaluating the microbiome of a cooling system. In the studied cooling water, 16S results showed low abundance of potentially-pathogenic genus (Table S3). However, the observation that the chlorine dosage does not completely prevent microbial growth in the heat exchanger could be a concern for other cooling systems since the water

from the outlet of the heat exchanger line is sprayed on top of the column. This is also the point where pathogens might be spread in the surroundings. To reduce the risks of outbreak, chlorine dosage could be performed right before the spray nozzles instead of inside the cooling tower basin.

2.4.2. Main cooling tower bacterial members originated from the feed water microbiome but the process conditions further influenced the bacterial community structure

A substantial core microbiome was shared between all collected samples (46 % of the total sequence reads was contained in 33 OTUs). The shared taxa between the feed water and the cooling tower basin water on each month accounted for 85 to 94 % of the sequences (Figure 2.4.b) supporting the idea that the planktonic population present in the cooling tower originated mainly from the feed water. The remaining fraction of microorganisms could have originated from different sources such as the ambient air and particles, precipitations or biofilm growth. Rain and cloud waters can contain up to 10^6 bacterial cells/mL (Hu et al. 2018) and the air to water ratio can reach up to 1.5 m^3/L in a cooling tower (Keshtkar 2017), potentially affecting the water community composition of these open recirculating systems.

Although the main cooling tower bacterial members originated from the feed water, a strong switch in relative abundance of the microbial community members was observed including a change of the dominant microorganisms (Figures 2.4.a and 2.5). Main taxonomic orders for the feed water, *LD12* subclade of *SAR_11* (*Alphaproteobacteria*) and *Hgcl* subclade of *Frankiales* (*Actinobacteria*), are common groups described in freshwater reservoirs and brackish waters (Henson et al. 2018, Llirós et al. 2014, Salcher et al. 2011) but not recurrent in cooling towers. Cooling water bacterial communities, on the contrary, were dominated by the recurrent orders *Obscuribacterales* (*Melainabacteria*) and *Caulobacterales* (*Alphaproteobacteria*) followed by *Sphingomonadales* and *Rhizobiales* (*Alphaproteobacteria*). A decrease of *Actinobacteria* and rise of *Proteobacteria* have been previously observed in cooling water (Paranjape et al. 2020b), corresponding to our findings with the strong decline in *Frankiales* and suggesting a high sensitivity of these organisms to the changes in environmental conditions.

This shift in relative abundance can be linked to several environmental factors, the main one being continuous disinfection. Continuous chlorine dosage affects bacterial community structure depending on the tolerance of the bacterial species to the biocide or their presence in protective biofilms. Even though no deposit was visible in the cooling tower basin, *Caulobacterales*, *Sphingomonadales* and *Rhizobiales* are bacterial orders frequently encountered in biofilms (Farenhorst et al. 2017, Pang and Liu 2007) and post disinfection samples (Gomez-Alvarez et al. 2012, Shaw et al. 2015) of drinking water distribution systems. The observed *Caulobacterales* family *Hyphomonadaceae* is known as prosthecate bacteria producing polysaccharide-based adhesin, called holdfast, allowing surface colonization (Abraham and Rohde 2014, Dang and Lovell 2015, Nagaraj et al. 2017). Some *Sphingomonas* species have also been linked to biofilm initiation through production of water-soluble polysaccharides and surface-adhesion proteins (Bereschenko et al. 2011, Pollock and Armentrout 1999) or have shown higher chlorine tolerance (Sun et al. 2013, Wang et al.

2019), both leading to deteriorations of membrane processes (Bereschenko et al. 2010, de Vries et al. 2019). To a lesser extent, chlorine-resistance properties of the *Rhizobiales* order have been discussed in a drinking water distribution system (Wang et al. 2018). The biofilms located on the inner surfaces of these systems constitutes the main reservoirs for bacterial regrowth (Berry et al. 2006). A recent study also reported the presence of these community members in cooling tower biofilms (Di Gregorio et al. 2017), suggesting that detached biofilm fragments likely influence cooling water bacterial composition. Chlorine disinfection can also explain the lower diversity in OTUs encountered in the cooling tower water compared to the feed water (Figure 2.4.b and Figures S3 to S5).

To summarize, most cooling tower community members originated from the feed water microbiome but the process conditions further influenced the bacterial community structure.

2.4.3. Cooling tower community structure is mainly shaped by differences in decay rate of populations caused by residual chlorine

Since 16S rRNA gene amplicon sequencing does not differentiate between live, dormant and damaged cells (Li et al. 2017), the association of relative abundance with total cell count can help quantify the abundance of bacterial groups and identify growth or decay of specific taxonomic orders. Combination of flow cytometry with 16S rRNA gene amplicon sequencing has previously been investigated for drinking water monitoring (Prest et al. 2014) and showed to be a promising method for quantification of bacterial communities in water systems. We applied the same method in this study, combined with a mass balance, to assess the impact of chlorine dosage on each bacterial order composing the cooling tower. To our knowledge, this is the first paper that describes net growth and decay of cooling tower microorganisms.

Based on our results, all dominant feed and cooling water microorganisms showed strong net decay with exception of the *Obscuribacterales* order. In the case of *Caulobacterales*, *Sphingomonadales* and *Rhizobiales*, the high relative abundance in the cooling tower resulted from a slower decay compared to the other microbial community members. Importantly, the potentially-pathogenic bacterial group *Legionella* showed negligible abundance in all samples (Table S3) and demonstrated decay in the studied cooling tower, indicating that risks of outbreak were prevented. A control study without disinfection would be able to confirm that chlorine is causing these microbial community changes but operation of a full-scale cooling tower without biocide dosage is not feasible due to the threat of pathogenic bacteria.

We can conclude from our study that the decay rate of the different bacterial orders is the main factor shaping the cooling water microbiome, and not the growth of selected bacterial groups. This finding implies that the feed water acts as a continuous inoculant, highly impacting the cooling tower populations, when chlorine is used for disinfection. The importance of the feed water composition has been observed by studying the similarities in bacterial members between multiple cooling towers based on their geographic locations (Paranjape et al. 2020b) but has never been thoroughly investigated.

2.4.4. The bacterial group *Obscuribacterales* succeeded in growing in the presence of residual chlorine

As indicated previously, the only microorganism group showing a net growth in the cooling system was the order *Obscuribacterales*. *Obscuribacterales* belong to the phylum *Melainabacteria*, a recently discovered phylum closely related to the *Cyanobacteria* but consisting of non-photosynthetic members (Bruno et al. 2018, Di Rienzi et al. 2013). Although described as a strictly fermentative bacterial phylum, a newly identified species has been shown to possess the ability for fermentation as well as aerobic and anaerobic respiration with complete respiratory chain (Soo et al. 2014). To our knowledge, the order *Obscuribacterales* has been rarely observed in other engineered water systems and never described in biofilms. It has however been identified in cooling tower studies by (Pereira et al. 2017) and (Paranjape et al. 2020b). In the latter, *Obscuribacterales* was present in 7 out of 18 evaporative cooling towers and represented a substantive portion of the total bacterial community (between 10 and 30 % relative abundance) in 2 of them. Daily and continuous disinfections were applied and residual chlorine concentrations of 0.32 and 0.27 mg Cl_2/L were reached in these 2 systems. Those results are in line with our finding and would suggest that dominance of *Obscuribacterales* is favoured by the presence of continuous high residual dosage. However, the explanation for the chlorine resistance is so far unknown, due to the lack of information available on the microorganisms and biocide's mechanism. Chlorine disinfection does not require specific target sites to exert a cytotoxic effect. Higher resistance of cells to chlorine is believed to result from reduced cell membrane permeability, or formation of a mucopolysaccharide outer layer (Bower and Daeschel 1999). Further characterization would therefore be necessary to understand the metabolism and physiology of the bacteria. In order to avoid chlorine-resistant organisms to develop, a complementary disinfection method could be considered depending on the resistance mechanism, such as UV or quaternary ammonium compounds (Phe et al. 2009, Sun et al. 2013, Rahmani et al. 2016). In addition to cell resistance, the formation of cell aggregates can also provide protection against external stress factors including biocides (Mir et al. 1997). Aggregates have not been observed during the study but their presence should not be excluded.

2.4.5. The described mass balance approach allows better characterization of cooling water bacterial changes

The use of a mass balance in combination with flow cytometry and sequencing data provided a higher level of understanding of the bacterial dynamics. Full-scale studies often omit to describe the feed water bacterial composition, which is essential information when discussing bacterial changes in chlorinated cooling water systems. Ignoring the feed water microbiome can lead to data misinterpretation, e.g. by assuming that all main cooling water taxa became accustomed to the process conditions and disinfection. In this study we included the feed water composition to identify the factors shaping the bacterial communities in the cooling tower. The studied cooling tower did not show an increase of potentially pathogenic bacterial groups (Table S3). However, the method would be able to detect an outbreak. Specifically, if *Legionella* continuously shows net growth, this would indicate the necessity of implementing alternative disinfection methods.

In addition, approximation of the net growth or net decay of microorganisms is highly valuable to detect chlorine resistant microorganisms. With this approach we could prove that the increase in *Obscuribacterales* was not only relative to the other communities nor due to the effect of concentration in the cooling tower, but resulted from the absolute growth of this microorganism group in the system containing residual chlorine. Application of this method to a large cooling tower dataset can identify recurrent and growing bacterial groups, and help further optimisation and evaluation of disinfection technique.

Limitations are however present when quantifying absolute abundances based on genomic analyses (Kleiner et al. 2017), specifically regarding the limited resolution (Poretsky et al. 2014) of the method and taxonomic profiling (Sczyrba et al. 2017). The real net growth and net decay can therefore show some deviations from the calculated values. Improvements can be made in the future by (i) taking into account copy numbers in the mass balance when the organisms are better identified and (ii) sampling and analysing the cooling tower surface to identify attached microbial communities and assess their potential impact on the microbial changes in the water phase.

2.5. CONCLUSIONS

The microbiome characterization of a full-scale industrial cooling tower (2600 m^3/h) - with residual chlorine between 0.35 and 0.41 mg-Cl_2/L and concentration factor between 3.5 and 6.6 - and comparison of bacterial community structures between the water feeding the cooling tower and the cooling tower water led to the following conclusions:

- The bacterial community composition in the cooling tower is driven by the feed water community composition. However, the structure of the bacterial community is governed by differences in decay rate of the bacterial groups in presence of residual chlorine. The characterization of feed water bacterial composition is therefore of great importance when studying microbiome dynamics in cooling towers.
- The taxonomic order *Obscuribacterales* was able to grow in the cooling system despite the presence of up to 0.41 mg-Cl_2/L residual chlorine, suggesting potential chlorine-resistance ability of this bacterial group. This taxonomic order is not commonly found in biofilms - as opposed to the other dominant organisms found in the cooling water - but has been previously observed in cooling tower studies in presence of residual chlorine.
- The proposed approach based on mass balance combining flow cytometry and 16S rRNA gene amplicon sequencing is a useful tool to determine net growth and decay of microorganisms and to assess biocide impacts on cooling tower communities.

This study points out the importance of full-scale data for identification of organisms with higher ability to survive disinfection. The establishment of a database from industrial scale cooling towers would contribute to determine recurrence of specific organisms in these processes. A better understanding of the biological processes and microbiome changes could be used to predict and develop strategies to control pathogen outbreaks.

CHAPTER 3

ASSESSMENT OF THE IMPACT OF TEMPERATURE ON BIOFILM COMPOSITION WITH A LABORATORY HEAT EXCHANGER MODULE

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ABSTRACT

Temperature change over the length of heat exchangers might be an important factor affecting biofouling. This research aimed at assessing the impact of temperature on biofilm accumulation and composition with respect to bacterial community and EPS. Two identical laboratory-scale plate heat exchanger modules were developed and tested. Tap water supplemented with nutrients was fed to the two modules to enhance biofilm formation. One "reference" module was kept at 20.0 ± 1.4 °C and one "heated" module was operated with a counter-flow hot water stream resulting in a bulk water gradient from 20 to 27 °C. Biofilms were grown during 40 days, sampled, and characterized using 16S rRNA gene amplicon sequencing, EPS extraction, FTIR, protein and polysaccharide quantifications. The experiments were performed in consecutive triplicate. Monitoring of heat transfer resistance in the heated module displayed a replicable biofilm growth profile. The module was shown suitable to study the impact of temperature on biofouling formation. Biofilm analyses revealed: (i) comparable amounts of biofilms and EPS yield in the reference and heated modules, (ii) a significantly different protein to polysaccharide ratio in the EPS of the reference (5.4 ± 1.0 %) and heated modules (7.8 ± 2.1 %), caused by a relatively lower extracellular sugar production at elevated temperatures, and (iii) a strong shift in bacterial community composition with increasing temperature. The outcomes of the study therefore suggest that heat induces a change in biofilm bacterial community members and EPS composition, which should be taken into consideration when investigating heat exchanger biofouling and cleaning strategies. Research potential and optimization of the heat exchanger modules are discussed.

3.1. INTRODUCTION

Heat exchangers constitute a crucial equipment part of cooling systems, where it is used to transfer the heat from a primary fluid to cold water (Hill et al. 2013). Biofilm formation in heat exchangers can have strong negative impacts on process efficiency and is therefore considered a problematic challenge faced in numerous industrial sites. The dependence of industrial processes to heat exchangers makes the interruption of operation very difficult and, in many cases, requires continuous operation for several years before cleaning or replacement of parts can be performed (European Commission 2001). Despite the use of biocides and dispersants, biofilms still develop over long periods of time and considerably reduce heat transfer efficiency. Additionally, they can serve as a protective habitat for pathogenic bacteria or bacteria involved in microbiologically influenced corrosion, causing irreversible damage to the equipment (Balamurugan et al. 2011, Rao et al. 2005). These detrimental consequences strongly affect the capital and operational costs of the heat exchange installations (Bott 1995, Melo et al. 2012).

Early investigations on biofilm resistance to heat transfer revealed a thermal conductivity of biofilm of 0.6 W/m.K (Characklis et al. 1981), comparable to water, which is not surprising since water forms the major mass fraction of a biofilm (Cooksey 1992). Many studies have used this key parameter for monitoring of biofilm growth and assessment of control methods in heat exchanger experiments (Chang et al. 2017, García et al. 2016, Janknecht and Melo 2003). Tian et al. (2012) used this approach to evaluate the impact of SiO_2 particles inclusion on biofilm structures and heat transfer resistance. It was also used by Chang et al. (2017) to investigate the efficiency of thermal shock on biofilm growth inhibition.

A potential important aspect for biofilm growth is the presence of a temperature gradient over the length of the heat exchanger affecting the bulk water temperature. Bacterial communities composing the biofilm and producing extracellular polymeric substances (EPS) necessary for the biofilm integrity are subjected to this temperature change and may adapt. There is, however, limited literature available on the effect of a temperature gradient on biofilm composition since most research studies were performed under isothermal conditions (Ahmad et al. 2021, Farhat et al. 2016, Obana et al. 2020). Among the few studies considering the impact of thermal gradient, Yang et al. (2013) have shown that co-current or counter-current configurations of heat exchangers cause different fouling behaviours. Undoubtedly, change in temperature exerts a strong influence on the extend and physical characteristics of biofilms. Due to these observed changes, it is reasonable to assume that variations in the bacterial communities and extracellular compounds also occur along heat exchangers. Characterizing the variations in biofilm composition is of importance to identify or predict which parts of heat exchangers are more detrimentally affected by the biofilm, and to optimize cleaning methods accordingly.

Laboratory scale heat exchanger set-ups have been designed in the past (Abu-Zaid 2000, Ling and Lund 1978, Xu et al. 2020) to predict the impact of operational variables on biofouling rates for full-scale applications. These laboratory elements are, however, not suitable for biofilm collection and composition analyses, often destructive. A compact plate heat exchanger module, inspired from a well-established membrane fouling simulator

(Vrouwenvelder et al. 2007), was therefore designed and built to enable the growth of biofilms of significant thickness and characterization of its components. In this chapter, we describe (i) the development of this new single-plate heat exchanger module for biofilm laboratory investigations, (ii) its implementation to study the effect of temperature on the bacterial community and EPS composition of biofilms, and (iii) the potential applications and limitations of the module in biofilm characterization studies.

3.2. MATERIALS AND METHODS

3.2.1. Laboratory heat exchanger module

The heat exchanger modules were made of two identical polyvinyl chloride (PVC) elements, fitting each other, and separated by a single metal plate (Figure 3.1). PVC connectors built in each side of the channels allowed connections to tubing. Diffusers were included in the geometry of the channels to provide a homogeneously distributed flow. O-rings were placed on each side of the metal plate to tightly maintain the plate and separate the water from each channel. Corrosion-resistant metal, Hastelloy C-22, was used for the metal plate. L × W × H dimensions were as follows: 189 × 34 × 8 mm^3 for the channels and 189 × 34 × 1 mm^3 for the plate. Two identical modules were produced by STT Products BV and operated simultaneously during our experiments. The technical drawing of the modules is provided in the supplementary material (Figure S1).

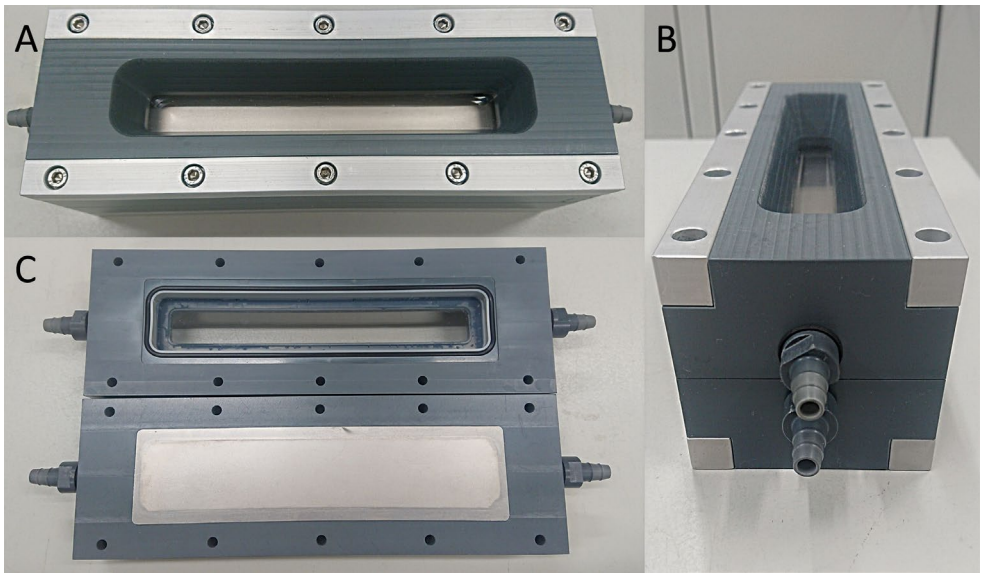

Figure 3.1. Heat exchanger module observed from the top side (A), from the left side (B) and view of the inside of the module with a rubber O-ring and metal plate (C).

3.2.2. Experimental design

In all experiments, one module was operated in single-pass flow without heating, denoted as "reference module", and a second module was operated in counter-flow with continuous heating, denoted as "heated module". The cold channel of each module was fed with tap water at a temperature of 20.0 ± 1.4 ˚C and a flow rate of 0.18 L/min. A nutrient solution

composed of sodium acetate trihydrate ($CH_3COONa{\cdot}3H_2O$; for a 'C' source), sodium nitrate ($NaNO_3$; for a 'N' source) and sodium phosphate monobasic monohydrate ($NaH_2PO_4{\cdot}H_2O$; for a 'P' source) was dosed to enhance biofilm growth. The concentrations in elements C, N and P were maintained at 500, 100 and 50 µg/L respectively in the feed water, corresponding to a mass ratio C:N:P of 100:20:10 (Siddiqui et al. 2017). The second channel of the heated module only was operated with recirculating water at 50 °C at a flow rate of 1.8 L/min, without nutrient dosage. The full laboratory set-up is illustrated in Figure 3.2. Biofilms were grown over a period of 40 days. The experiments were performed in triplicate, denoted as "exp. 1, 2 and 3".

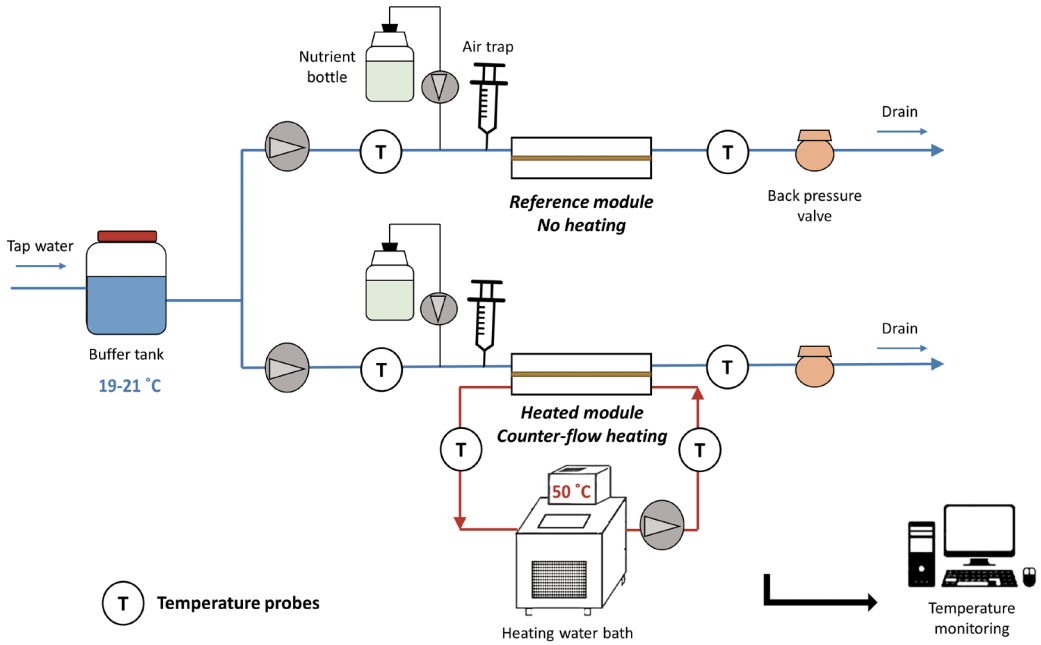

Figure 3.2. Schematic diagram of the heat exchanger set-up in operation.

3.2.3. Temperature monitoring

Platinum resistance thermometers of high-precision (CTP5000, WIKA, Germany) were located inside the inlet and outlet tubing of the channels, with a 10-cm deep immersion. Temperature probes were calibrated and showed 0.04 °C accuracy. All probes were connected to a display unit (CTR2000, WIKA, Germany) and data were recorded online via a data logging system on a local computer.

Heat transferred (Q) to the cold channel in module 2 was calculated with equation (1):

$$Q = m * Cp * dT \quad (1)$$

m: mass flow rate of water (L/s)

Cp: heat capacity of water - 4184 J/(kg.K)

With $dT = T_{out} - T_{in}$

The overall heat transfer coefficient (U) was approximated with equation (2):

$$U = \frac{Q}{A * LMTD} \quad (2)$$

A: area of the plate

$LMTD$: counter-current logarithmic mean temperature difference

With $LMTD = \frac{(T_{h.in}-T_{c.out})-(T_{h.out}-T_{c.in})}{LN((T_{h.in}-T_{c.out})/(T_{h.out}-T_{c.in}))}$

U_{ref} was calculated as average U over a period of 1 day, without dosages of nutrients. U_{exp} was monitored during the experiment to approximated the resistance (R) to heat transfer caused by the biofilm:

$$R = \frac{1}{U_{exp}} - \frac{1}{U_{ref}} \quad (3)$$

The approximated thickness (t) of the biofilm was then obtained with equation (4):

$$t = R * \lambda \quad (4)$$

with λ: thermal conductivity of biofilms (0.6 W/m.K)

3.2.4. Biofilm collection

At the end of each experiment, both modules were carefully drained and disassembled to recover the biofilms from the surface of the plates. 1 × 2 cm^2 areas were sampled from the inlet and the outlet and preserved at -20 °C before DNA extraction and 16S rRNA gene amplicon sequencing. The remaining biofilms were scraped from the plates, frozen at -80 °C and lyophilized before EPS extraction.

3.2.5. DNA extraction and 16S rRNA gene amplicon sequencing

The genomic DNA was extracted using the DNeasy UltraClean Microbial Kit (Qiagen, Netherlands). Extraction was done following company's standard protocol with addition of an alternative lysis step. This included a combination of 5 min of heat (65 °C) followed by 5 min of bead-beating on the filters for cell disruption on a Mini-Beadbeater-24 (Biospec, USA). Samples were sent to Novogene Ltd. (Hongkong, China) for amplicon sequencing of the V3-4 region of the 16S-rRNA gene (position 341–806) on an Illumina paired-end platform. The raw data was processed with the software Mothur v.1.40.5. Raw sequences were quality filtered, aligned, checked for chimera and operational taxonomic units (OTUs) were generated based on 97% similarities after removal of singletons. The alignment and taxonomic classifications were performed using the SILVA database. Beta diversity measurement was assessed with principal coordinate analysis (PCoA) in Mothur v.1.40.5 using the thetaYC distance matrix. The spatial separations visualized in the PCoA are used to compare similarities and dissimilarities between samples.

3.2.6. EPS extraction

The extraction of EPS was performed on the freeze-dried raw deposits. EPS was extracted at 80 °C in alkaline conditions, following a method previously described (Felz et al. 2016, Pinel et al. 2020b), and lyophilized. The freeze-dried EPS samples were kept in a dry environment before further analyses.

3.2.7. Fourier Transform Infrared Spectroscopy (FTIR)

FTIR spectra of the freeze-dried extracted EPS samples were carried out on a FTIR Spectrophotometer (Perkin-Elmer, Shelton, CT, USA) at room temperature, with a wavenumber range from 700 cm^{-1} to 4000 cm^{-1}. Resolution of 4 cm^{-1} and accumulation of 8 scans were applied on each sample. FTIR spectra were baseline-corrected and a min-max normalization was applied with respect to the amide I peaks (between 1700–1600 cm^{-1}) with Matlab R2018b software.

3.2.8. Polysaccharide quantification

Polysaccharides were quantified following a method developed by (Dubois et al. 1956). In short, the freeze-dried EPS extracts were diluted in a sodium hydroxide solution (0.01 M in ultrapure water) to a final concentration of 1000 mg/L. For the standards, a sugar mixture of 1000 mg/L was prepared with equal amounts of fucose, rhamnose, galactose, glucose, xylose, mannose and ribose. Sugars were selected due to their previous detection in bacterial EPS (Felz et al. 2019). The sugar mixture was diluted to concentrations of 0, 10, 25, 50, 75, 100, and 200 mg/L in order to establish the calibration curve. 200 µL of each sample or standards were pipetted in a glass reaction tube, followed by 200 µL of 5% w/v phenol solution and 1000 µL of 95% sulphuric acid. Tubes were vortexed, left at room temperature for 20 min and vortexed again before analyses. The absorbance of the samples and standards were measured at 482 nm with a spectrophotometer (DR3900 Hach, USA).

3.2.9. Protein quantification

The freeze-dried EPS extracts were diluted in a sodium hydroxide solution (0.01 M in ultrapure water) to a final concentration of 250 mg/L. Total protein concentrations were determined with the BCA assay using the Protein Quantification Kit (Interchim, France). The standards were prepared by dilution of a bovin serum albuvine (BSA) solution to concentrations of 0, 5, 10, 50, 100, 200, 350, and 500 mg/L. Reagents were added to the standards and samples according to manufacturer's instruction in a 96-well plate. The plate was shaken for 30 s and incubated in the dark at room temperature for 2 h before analysis. Absorbance was measured at 562 nm by a plate reader (Infinite M200 PRO, Tecan, Switzerland).

3.3. RESULTS

3.3.1. Operating parameters

The reference module was operated with the cold channel only, without heating. Inlet and outlet measurements indicated negligible variation of the bulk water temperature over the channel length (Table 3.1). The cold channel of the heated module was operated with the same flow rate, feed source and nutrient dosage as the reference module throughout the experiments.

Table 3.1. Operational parameters monitored during the operation of the modules, with the interval (±) representing the range of variations. Indicated values combine the data of all experiments.

Module	Temperature hot channel (˚C)		Temperature cold channel (˚C)		Flow rate hot channel (L/h)	Flow rate cold channel (L/h)	Heat loss (%)
	Inlet	Outlet	Inlet	Outlet			
Reference	r.t.*	r.t.*	20.0 ± 1.4	20.1 ± 1.2	0	11.4	approx. 0
Heated	50.4 ± 0.2	49.3 ± 0.2	20.1 ± 1.3	27.3 ± 1.3	108	11.4	27 ± 3

* r.t.: room temperature

Water heated at 50 ˚C was recirculating in counter-flow through a second channel (Figure 3.2), along which a decrease of 1 ˚C was measured. The heated flow caused a heat transfer through the metal plate, increasing the bulk water temperature of the cold channel from 20.1 ± 1.3 ˚C to 27.3 ± 1.3 ˚C between the inlet and outlet. When no biofilm was present in the heated module, the calculated overall heat transfer coefficient reached 660 ± 40 W/m².˚C, which was used as the reference value for the later calculation of the biofilm heat resistance. Biofilm growth caused a reduction of the temperature difference between inlet and outlet by 1.6 ± 0.2 ˚C in the cold channel of the heated module. Collection of temperature data along the heated system allowed the monitoring of the biofilm thickness forming on the surface of the metal plate.

3.3.2. Monitoring of biofilm growth in the heated module

Approximated biofilm thicknesses calculated from the temperature measurements of the heated module are shown in Figure 3.3. Exp. 1 revealed disturbances due to intermittent air bubbles entering the system causing fluctuations in temperature. This anomaly was fixed in exp. 2 and exp. 3. Despite slight differences in profile, all experiments show a similar growth curve with a final thickness between 200 and 250 µm after 40 days of operation. Reproducibility of the study was achieved in terms of biofilm development in the heated heat exchanger module.

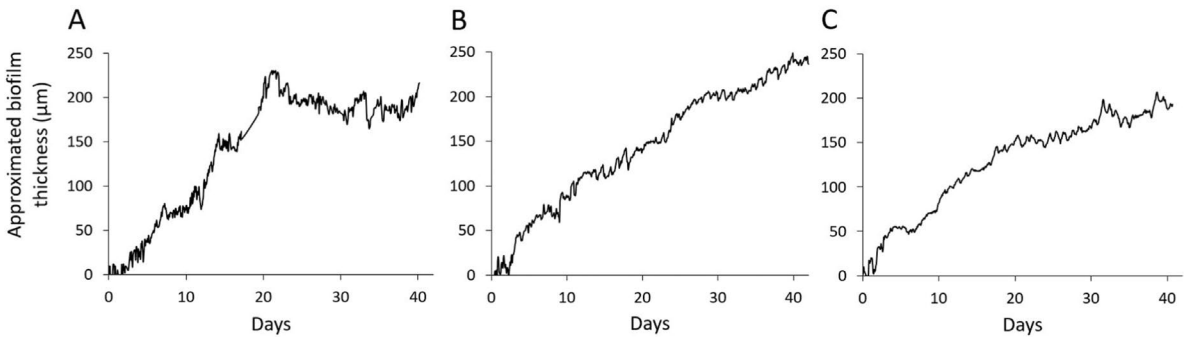

Figure 3.3. Approximated thickness of the biofilms monitored in the heated module over the duration of exp. 1 (A), exp. 2 (B), and exp. 3 (C).

3.3.3. Biofilm characterization

3.3.3.1. Bacterial community structure along the heat exchanger

Accumulated biofilms were collected from the inlet and outlet of each module to evaluate the changes in bacterial communities along the heated and reference systems, and assess the effect of a temperature gradient. Dissimilarities between the structure of bacterial communities are illustrated in Figure 3.4. Although a deviation is visible for a set of data points at coordinates (0.22, -0.64), corresponding to exp. 3, inlet and outlet of the reference module showed high similarity in each experiment. On the contrary, inlet and outlet of the heated module experienced a strong change in bacterial community structure in each experiment, represented in Figure 3.4 by dashed arrows. Therefore, changes observed along the metal plate in absence of heating were negligible compared to the changes that occurred under heated condition. The temperature gradient along the metal plate thus resulted in a change in biofilm composition in terms of bacterial community members and abundances in the heated module.

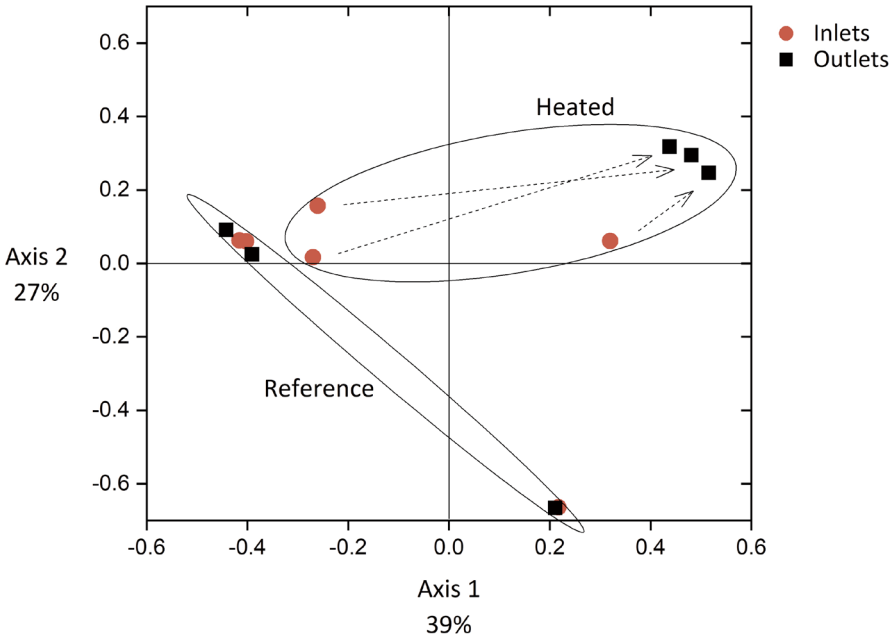

Figure 3.4. Principal coordinate analysis (PCoA) of biofilm bacterial community structures from the heated and reference modules in the three experiments. The bigger the distance between data points, the stronger the dissimilarity in community structures, based on the presence and abundance of operational taxonomic units. Dashed arrows connect inlet and outlet of each module.

Interestingly, outlet biofilm samples of the heated module from the three experiments converge to a similar structure. Taxonomic identification indicated a significant increase in *Cupriavidus respiraculi* abundance, reaching 52, 79 and 58 % in the heated outlet biofilms of exp. 1, 2 and 3 respectively (Figure S2). Results suggest the loss in diversity of main community members at elevated temperatures, with the selection of *Cupriavidus respiraculi*.

3.3.3.2. EPS amounts

The total biofilm from each metal plate was collected to obtain sufficient EPS for further characterization. This allowed the comparison of the biofilm composition of the reference module to the biofilm composition of the heated module but not the evaluation of changes along the plates. The relative low amount of EPS per plate did not allow for sampling different areas of the plate separately. The average freeze-dried deposit collected in the reference module reached 10.8 ± 3.0 mg with an extracted EPS yield of 52 ± 2 %. These values were not significantly different (p-values > 0.05) to the heated module, with 11.2 ± 2.6 mg and 57 ± 8 % of EPS yield. In conclusion, similar amounts of accumulated biofilm and EPS were found in all systems.

3.3.3.3. EPS characterization

The FTIR spectra of the extracted EPS samples display the characteristic carbohydrate band at 940-1200 cm^{-1} (Boleij et al. 2019, Zhu et al. 2012) and protein band at 1500-1700 cm^{-1} with amide I and amide II corresponding peaks (Barth 2007). The comparison of spectra indicates high similarity in molecular vibrations with a large overlapping fraction of the curves of the extracted EPS from the heated and reference modules. Only the carbohydrate peak does not overlap (* in Figure 3.5), with a striking divergence between both conditions. A lower absorbance intensity is observed in the EPS grown under heated conditions, suggesting that a relatively lower sugar content of the EPS was produced at elevated temperature.

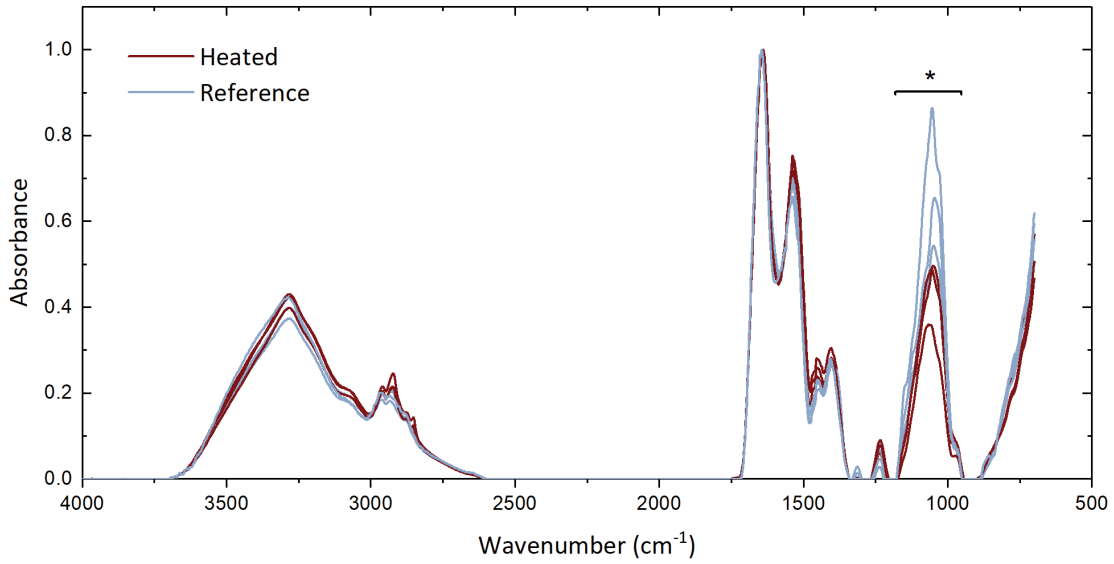

Figure 3.5. FTIR of the extracted EPS of the biofilm samples collected on the heated and reference modules. The symbol * indicates the band associated to carbohydrates at 940-1200 cm^{-1}, showing a strong divergence between the heated and reference conditions.

The quantitative analyses of protein and polysaccharide contents in the extracted EPS samples led to the results shown in Figure 3.6.A. The percentage of proteins remained quite stable with variations between 48 and 54 % of the total EPS, while polysaccharides constituted between 5 and 13 % of the total EPS. As observed with the FTIR analyses, the

reference EPS seemed to contain a higher amount of polysaccharide than the EPS from the heated module, especially in exp. 2 and 3. The ratio of proteins to polysaccharides was therefore calculated for each module and the values from both tested conditions were compared. Despite a partial overlap in standard deviations, the statistical analysis indicates a significant difference (p-value < 0.05) between the conditions, with a higher PN/PS ratio in the EPS extracted from the heated biofilm, 7.8 ± 2.1 % compared to 5.4 ± 1.0 % in the EPS extracted from the reference biofilm (Figure 3.6.B).

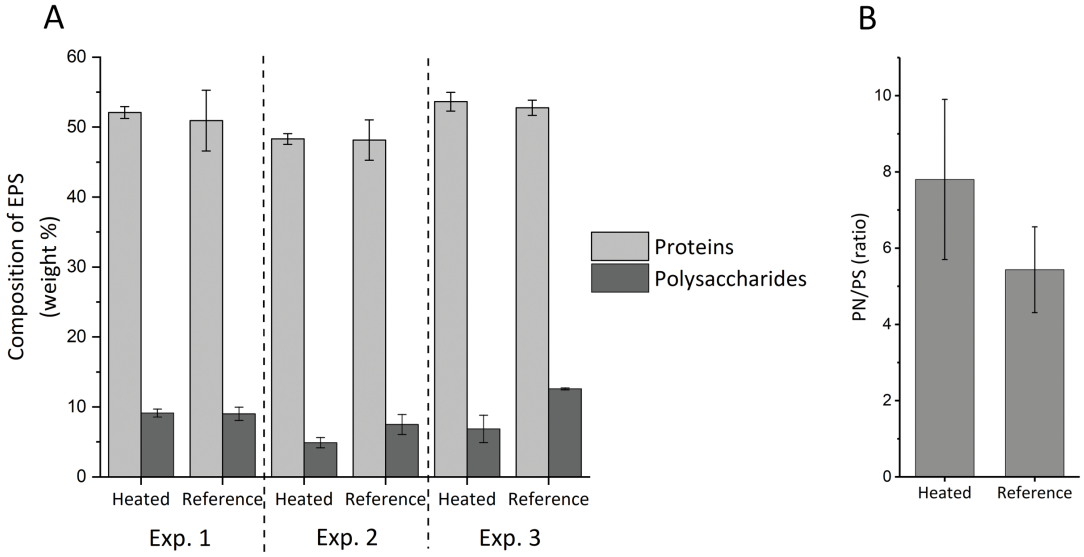

Figure 3.6. Composition of EPS in weight percentages of polysaccharides and proteins in the heated and reference modules (A) and protein-to-polysaccharide (PN/PS) ratio under both conditions, averaged over the triplicate experiments (B). The error bars indicate the standard deviations.

In summary, despite similarity in amounts of accumulated EPS in the reference and heated modules, EPS characterization reveals a small but significant change of protein to polysaccharide ratio caused by a relatively lower extracellular sugar production at elevated temperatures.

3.4. DISCUSSION

3.4.1. Impact of temperature on biofilm composition

The triplicate experiments performed with the newly designed modules led to reproducible results regarding biofilm growth profile (Figure 3.3) and EPS characterization (Figures 3.5 and 3.6). Only the bacterial community structures showed a deviation in exp. 3 compared to exp. 1 and 2 (Figure 3.4), likely caused by the effect of external factors affecting the tap water planktonic community. Variation in external temperatures and overall water use in the building could have affected the bacterial community structure (Ling et al. 2018, Prest et al. 2016a, Zlatanović et al. 2017) since the experiments were performed sequentially. Despite this deviation, conclusions could be drawn from the results. They are further discussed below.

Experimental analyses of the biofilms grown in the reference and heated modules revealed (i) variations in biofilm community structures caused by the temperature increase along the

plate heat exchanger, (ii) comparable amounts of accumulated EPS, and (iii) a relatively lower production of extracellular polysaccharides at elevated temperature by the biofilm members.

Abundant members of our study include bacterial groups frequently found in drinking water (Figure S2), such as *Piscinibacter* genus (Vandermaesen et al. 2017), *Aquabacterium* genus populating biofilm with optimal growth at 20 °C (Manz et al. 2015) or *Variovorax* genus (Lee et al. 2010). Their high abundance was particularly marked in the reference biofilms, without heating. Selection of the species *Cupriavidus respiraculi* occurred in the biofilm grown in the heated module (Figure S2). *Cupriavidus respiraculi* is a gram-negative, obligate aerobe bacterium which has been observed to grow at 28, 32 and 37 °C (Vandamme and Coenye 2004, Vaneechoutte et al. 2004). The species was isolated from patients with cystic fibrosis suggesting that they are well adapted for growth at physiological temperatures. Dominance of *Cupriavidus* over the drinking water biofilm groups with increasing temperature is an indication that the temperature change faced in heat exchangers leads to biofilm bacterial community compositions diverging from the feed water source communities. It is also important to note that the processes of detachment and dispersion or reattachment of biofilm fragments (Kaplan 2010), observed in mature biofilms, did not seem to have affected the community structure of the heated biofilm in the 40-day studies.

These results reveal that the temperature gradient in heat exchangers causes the selection of different biofilm bacterial groups along the system, and can therefore cause damages of varying nature and magnitude along the process e.g. regarding the extent of biofouling or microbial corrosion (Rao et al. 2009). The effect of heat gradient should therefore be taken into account and samples should be collected along the system when characterizing biofilms from heat exchangers.

Analyses showed that the amounts of biofilms and accumulated EPS were not significantly different between the reference and the heated biofilms. It is surprising, since more severe biofouling is usually observed at higher temperatures (Baek et al. 2011, Chamberland et al. 2019). Felz et al. (2016) has shown that EPS yield can be strongly affected by the method of EPS extraction. However, the comparable protein content from both extracted EPS (Figure 3.6) does not suggest disparities in cell lysis by the alkaline extraction. Biofilm growth and EPS production in the heated biofilm might be limited by the carbon supply or by the wide temperature range along the plate and through the thickness of the biofilm. For a better assessment, the exact temperatures at the surface of the plate and through the biofilm should be monitored in future studies.

The quantitative measurements of proteins and sugars revealed a lower sugar content of extracted EPS from the heated biofilms (Figure 3.6), which is in line with the lower band intensity in the region 940-1200 cm^{-1} observed in the FTIR analyses (Figure 3.5). The change in temperature therefore induced variations in the produced extracellular compounds. These changes in EPS composition along heated surfaces can affect the efficiency of biofilm removal products such as enzymatic treatments. Studies at larger scale and after long periods of heat exchanger operations are needed for comparison with our laboratory results. Sampling and EPS analyses from a fouled full-scale heat exchanger surface with wide temperature gradient would be of high interest to evaluate the extend of such variations and the implications from an industrial point of view.

3.4.2. Advantages and limitations of the heat exchanger laboratory module

The purpose of the developed lab-scale heat exchanger module is to reproducibly investigate the impact of a continuous temperature gradient on biofilm formation, in terms of active biomass and EPS amount and composition. It was therefore designed according to characteristics required for carrying out the research study. Its laboratory-suited size makes it easy to assemble and disassemble and allows a flexible use of the device in various locations. The height of the channel was chosen so that it can sustain the growth of a thick biofilm without causing a build-up in pressure or significant variation in fluid velocity which could eventually affect the biofilm development e.g. through increase in shear rate (Casey et al. 2000, Paul et al. 2012). Stable conditions could thus be maintained during the operating period. The material used for the metal plate (hastelloy C-22) was selected for its anti-corrosion properties and to allow an optimal heat transfer from the hot to cold channel of the module – hastelloy has a thermal conductivity of 11.1 W/m.K - while the external case was made of PVC to limit heat loss. In addition, the plate can easily be replaced to test different materials, which can broaden the spectrum of potential studies to (microbiologically influenced) corrosion investigations.

There are, however, some limitations to the heat exchanger set-up. A further developed version of the module should include online measurements of plate surface temperatures and investigation of the temperature profile over the height of the biofilm. The temperature at the surface of the plate is generally higher than the bulk temperature and is affected by the boundary layer, which could be approximated by modelling of the system. Oxygen profile probes would also provide information on the oxygen diffusion through the layer. Design could be enhanced by using material able to handle higher temperatures than PVC can resist, while maintaining an acceptable heat loss. For temperatures higher than 50 °C, it is recommended to change material to thermoplastics with high heat deflection temperature such as polyethersulfones (PES). Lastly, some loss of biofilm was observed during the drainage of the cold channels leading to a small loss in biofilm materials. The handling of this step could be improved in order to reach a full recovery of the grown biofilm.

3.4.3. Potential applications in future research

When operated over a sufficiently long period of time, the system can be used to perform studies on biofilm properties and compositions that would not be possible with biofilms of a few µm-thickness. The testing of a broader range of temperatures and the use of additional chemical characterisation methods of EPS would build on the results compiled in this study. In addition to the preliminary results described in this research, we also propose some study lines of interest, which could be tested with the laboratory plate heat exchanger module.

Regarding the characterization of the microbiome, variations can be assessed along the temperature gradient, as introduced in this study. Investigation of the bacterial community changes could also be performed across the thickness of the biofilm (Almstrand et al. 2013), by collecting layers of the biofilm formed between the metal plate and the bulk water. The differences in main bacterial group, their physiology and interactions would provide a deeper understanding of how the biofilm is able to cope with the significant changes in conditions,

not only linked to the diffusion of nutrient and oxygen (Stewart 2003) but also the effect of thermal dissipation.

The applied monitoring provides an approximated thickness, assuming a flat and homogeneous structure of the biofilm over the plate surface. Flow profile, temperature and nutrient load can, however, affect the growth and the distribution of the biofilm (Recupido et al. 2020). In addition, it has been seen in pure-culture biofilm that temperature can cause a regulation of EPS production inducing morphological changes of the biofilm (Obana et al. 2020). To evaluate this assumption accurately, the temperature monitoring should be combined with measurement of the thickness. For more in-depth investigation of the physical structure, *in-situ* imaging of the biofilm would provide valuable information on the biofilm development and morphology (e.g. roughness) when subjected to a thermal gradient, in a similar way as it was performed for different types of flow (Wagner et al. 2010).

Due to the adjustability of the module, the use of metal plate prone to corrosion such as copper or carbon steel can be explored. Microbiologically influenced corrosion causes serious damages in full-scale plants (Sharma 2014), and is very challenging to control due to the combination of processes involved in the corrosion mechanisms (Little et al. 2020). The module can for example be used for the testing of metals or innovative coatings, and assessment of their ability to withstand microbial corrosion under different temperatures.

In short, the heat exchanger can be used for a multitude of research purposes. The fact that thick biofilms can be grown in the module and collected in substantial amounts allow the use of multiples biofilm analyses for in-depth characterization of its composition.

3.5. CONCLUSIONS

A laboratory-scale plate heat exchanger module for the assessment of the impact of temperature gradient on biofilm composition was built, tested and applied to a preliminary biofilm investigation.

The online monitoring and analyses showed that:

- the developed plate heat exchanger was able to monitor online biofilm growth by measuring its resistance to heat transfer.
- the laboratory module is suitable for a large range of applications related to the effect of a thermal field, such as bacterial community identification through the height of the biofilm, composition and morphology of EPS at various temperatures, or investigations of (microbiologically influenced) corrosion.

The uniqueness of the module lies in its suitability to sustain and monitor extensive biofilm formation with negligible effect on flow properties, and to allow collection of sufficient biofilm material to perform destructive analyses of composition. Some suggestions based on the exploratory study were proposed for further improvement of the module.

Exploratory study on the effect of temperature gradient revealed:

- comparable amounts of biofilm and accumulated EPS formed in the non-heated and heated systems over the 40-day experiments.
- differences in proteins-to-polysaccharides ratio in EPS caused by the thermal field, with a lower production of polysaccharides at elevated temperature.
- differences in biofilm bacterial groups resulting from the temperature change at the surface of the plate.

Supplementary information chapter 3

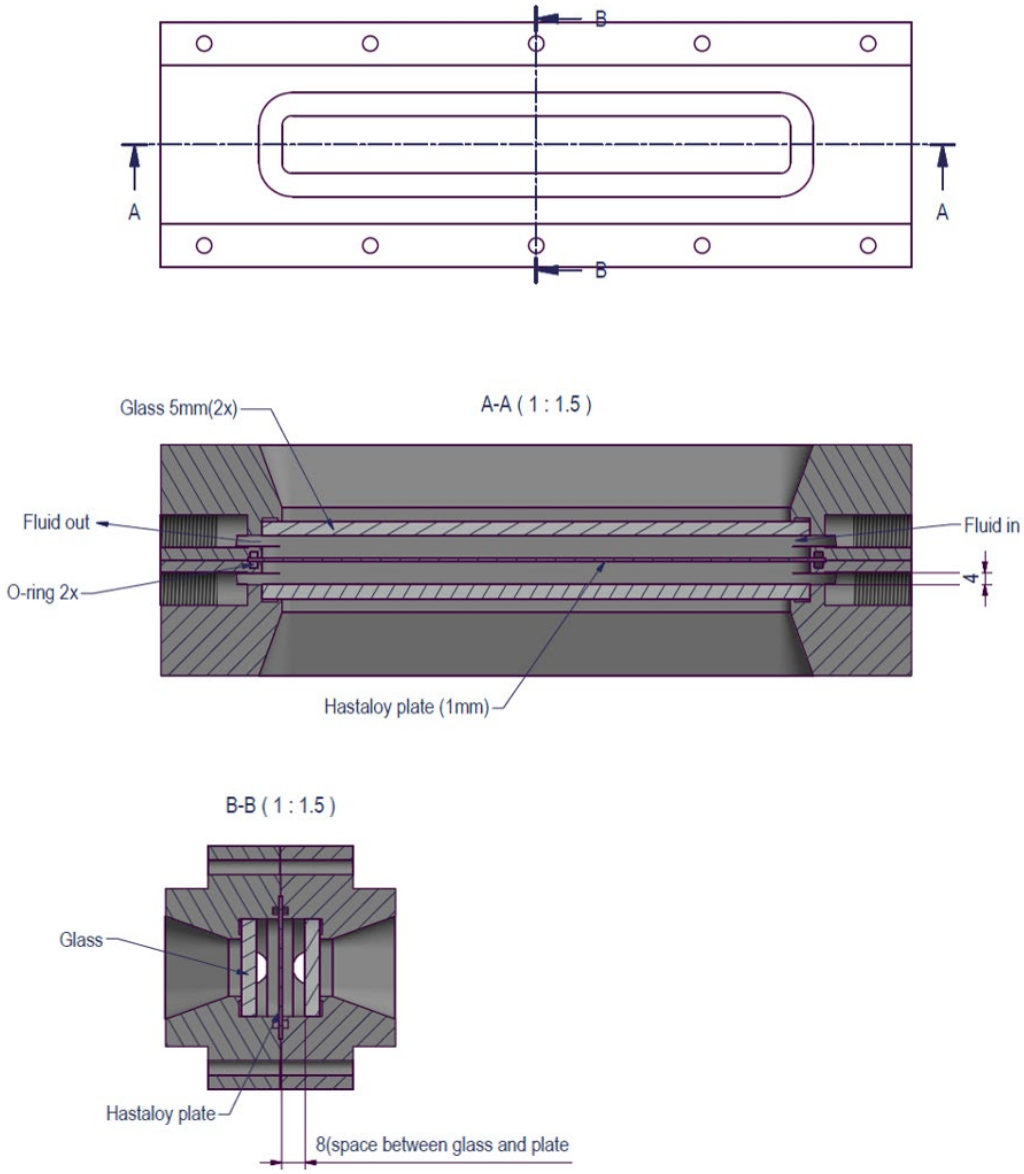

Figure S1. Technical drawings of the heat exchanger module showing the top/bottom view (top), the longitudinal cross-section (middle) and the transverse cross-section (bottom).

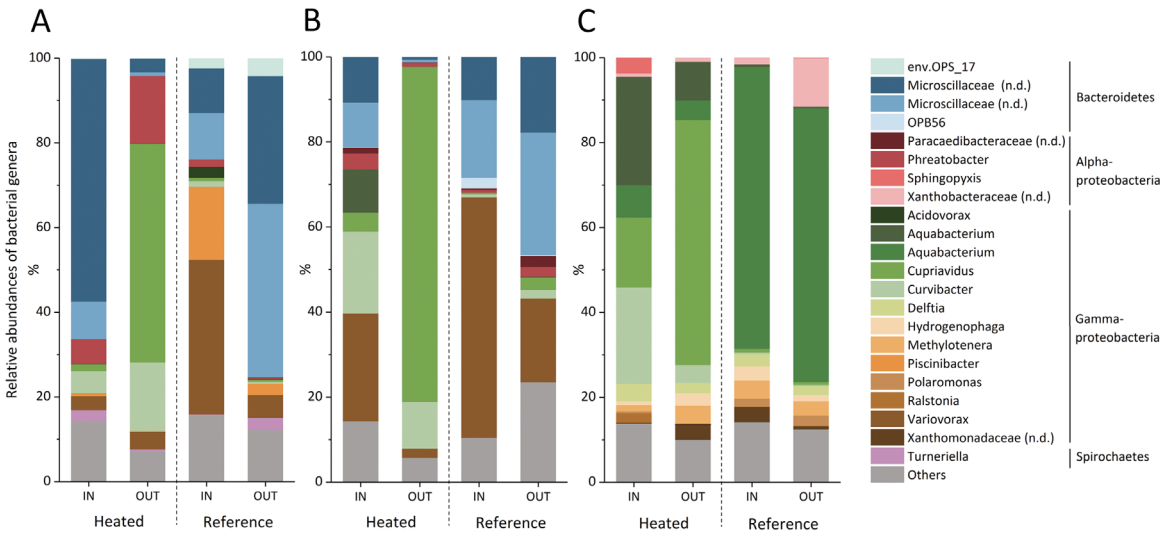

Figure S2. Relative abundances of bacterial genera in biofilm samples collected from the reference and heated modules in exp. 1 (A), exp. 2 (B), and exp. 3 (C). Phyla and classes of Proteobacteria are indicated at the right of the legend. Experiments were performed in triplicate.

CHAPTER 4

EFFECT OF PHOSPHATE AVAILABILITY ON BIOFILM FORMATION IN COOLING TOWERS

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Supplementary figures and tables are available at the following link: https://doi.org/10.1080/08927014.2020.1815011

ABSTRACT

Phosphate limitation has been suggested as a preventive method against biofilms. P-limited feed water was studied as a preventive strategy against biofouling in cooling systems. Three pilot-scale open recirculating systems were operated in parallel for five weeks. Seawater reverse osmosis (SWRO) permeate was fed to the cooling towers (i) without supplementation (reference), (ii) with supplementation of biodegradable carbon (P-limited) and (iii) with supplementation of all nutrients (non-P-limited). The P-limited water contained ≤ 10 µg o-PO_4/L. Investigation of the basins and coupons showed that P-limited water (i) did not prevent biofilm formation and (ii) resulted in a higher volume of organic matter per unit of active biomass compared to the other conditions. Exposure to external conditions and cycle of concentrations were likely factors that allowed a P concentration sufficient to cause extensive biofouling despite being the limiting compound. In conclusion, phosphate limitation in cooling water is not a suitable strategy for biofouling control in cooling systems.

4.1. INTRODUCTION

Tackling biofouling is a major challenge in the operation of open recirculating cooling systems. Biofilms forming on the inner surfaces of heat exchangers, pipes, and basin increase heat transfer resistance and provide a protective environment for microbial communities particularly involved in microbiologically influenced corrosion of metal surfaces or disease outbreaks (Rao et al. 2009, Türetgen et al. 2007). The generated loss in cooling efficiency, occasional pipe clogging and material deterioration lead to early replacement of process parts, intense cleanings and increased chemical dosages, therefore resulting in high capital and operating costs for the industry (Melo et al. 2010).

The standard biofouling control methods consist of dosage of biocides such as sodium hypochlorite, chlorine dioxide or bromine in the cooling systems (Al-Bloushi et al. 2018, Pinel et al. 2020a). Though these chemicals considerably slow down the build-up of biofilms by deactivating planktonic bacterial cells, they cannot completely prevent biofilm formation and have little curative action. Studies have shown that biocide efficiencies strongly depend on transport limitations arising from reaction-diffusion phenomena within a biofilm (Chen and Stewart 1996, De Beer et al. 1994, Xu et al. 1996). Extracellular polymeric substances (EPS) are essential compounds that form the biofilm matrix and react with these chemicals, limiting the deactivation of the embedded active cells (Xue et al. 2013). In addition, a high concentration of oxidative biocides is known to induce corrosion of metal surfaces such as plumbing parts and heat exchangers (Edwards and Dudi 2004).

Previous studies have suggested that the removal of essential nutrients can constitute a preventive measure against biofilm formation in industrial processes such as membrane filtration systems (Vrouwenvelder et al. 2010) and cooling towers (Meesters et al. 2003). Organic carbon is generally the limiting compound for microbial growth in freshwater. Removal of assimilable organic carbon (AOC) is often applied to inhibit the build-up of biofilm (Daamen et al. 2000, Visvanathan et al. 2003). Different AOC thresholds have been proposed to prevent issues linked to bacterial regrowth in unchlorinated systems: 50 µg/L, similar to groundwater concentration (Bradford et al. 1994), 10 µg/L in drinking water systems (van der Kooij et al. 1989) or even 1 µg/L against biofouling of spiral wound membrane (Hijnen et al. 2009). Reducing AOC to such low levels is very challenging with conventional techniques such as biological filtration or activated carbon adsorption (van der Kooij et al. 1989, Nguyen et al. 2012). Importantly, microbial growth also depends on the availability of phosphorus in water (Lehtola et al. 2002, Miettinen et al. 1997). Phosphate (P)-limitation has been shown to control biofouling in reverse osmosis (RO) membrane systems in the presence of high organic concentration (Kim et al. 2014, Vrouwenvelder et al. 2010) suggesting P-removal as a suitable pre-treatment for industrial water processes. No literature is, however, available on the effectiveness of P-limitation in controlling biofouling in cooling water systems. A few studies have investigated the microbiome of biofilms from cooling systems (Balamurugan et al. 2011, Di Gregorio et al. 2017, Tsao et al. 2019, Wang et al. 2013), mainly focusing on MIC and impact of disinfection, but the effect of nutrient limitations and sample location remains unexplored. In general, further investigation of the effect of nutrient limitations in cooling water would be highly valuable to the industrial field as it guides the selection of feed water, pre-treatment, and chemical dosage strategies.

The choice of feed water used in cooling systems depends mostly on the water sources available at the geographic locations. In Europe, most cooling towers are operated with freshwater (e.g. ground or surface water), causing less damage to the process than seawater, which is frequently used, for example, in the Middle-East (Al-Bloushi et al. 2018). In this study, seawater reverse osmosis (SWRO) permeate was selected as a reference feed water for three parallel pilot-scale cooling towers due to its ultra-low content of elements and nutrients required for microbial growth: RO permeate water has one of the highest water qualities achievable. The pilot set-up used in this study was located on an industrial site allowed to work under field conditions in terms of operational parameters, process design, climate (e.g. humidity, temperature, wind), air quality, and presence of particles. Contrary to a laboratory-controlled experiment, a pilot study evaluates the efficacy of biofouling control strategies with more representativeness. For five weeks the three pilot-scale cooling towers were monitored and subjected to SWRO permeate supplemented with different nutrient dosages to (i) investigate the impact of limiting nutrients (C and P) on biofilm formation, (ii) evaluate the changes in biofilm composition throughout the system and (iii) assess the potential of low-nutrient content feed water against biofouling in cooling systems.

4.2. MATERIALS AND METHODS

4.2.1. Pilot set-up

4.2.1.1. System operation

The pilot facility is located at the central utility plant of King Abdullah University of Science and Technology (KAUST) in Saudi Arabia. It consists of three identical parallel counter flow induced draft cooling systems. The systems are shown in Figure 4.1, and the detailed information was described in a previous study (Al-Bloushi et al. 2017). Each cooling tower unit has a total volume of 62 L, and a cooling capacity of 15 kW. SWRO permeate produced at the desalination plant in KAUST (Belila et al. 2016) was used as feed water. The SWRO permeate was carried through a 40 m long plastic pipe to a buffer tank of 7 m^3. Three connected pipelines at the bottom of the buffer tank allowed the distribution of feed water to the three pilot cooling towers. Feed water valves were triggered when the water levels in the basin dropped, resulting in average flows between 25 and 35 L/h depending on external temperature and humidity. Continuous blowdown water flows were set at 5 L/h, and maintained a cycle of concentration between 5 and 7. Temperature differences along the heat exchangers were kept between 6 and 10 ˚C with a cold temperature between 27 and 30 ˚C in the basin and a hot temperature between 32 and 37 ˚C after the heat exchanger. The operation of the pilot was controlled automatically with an online system monitoring the following parameters: flow rate, temperature and pH. In addition, the pH and conductivity of the collected water samples were measured manually using a pH meter (Cyberscan pH6000, Eutech, USA) and a conductivity meter (ProfiLine Cond3310, WTW, Germany). The pH values remained between 8.0 and 8.3, and conductivity values varied between 6 and 8 mS/cm in all cooling towers along the experiment. Cleaning of the systems was performed prior to the experiment as follow: (i) recirculation of sodium hydroxide solution 0.1 M during

for 2 h; (ii) flushing with SWRO permeate, (iii) recirculation of hydrochloric acid solution at pH 2 for 2 h; (iv) flushing with SWRO permeate.

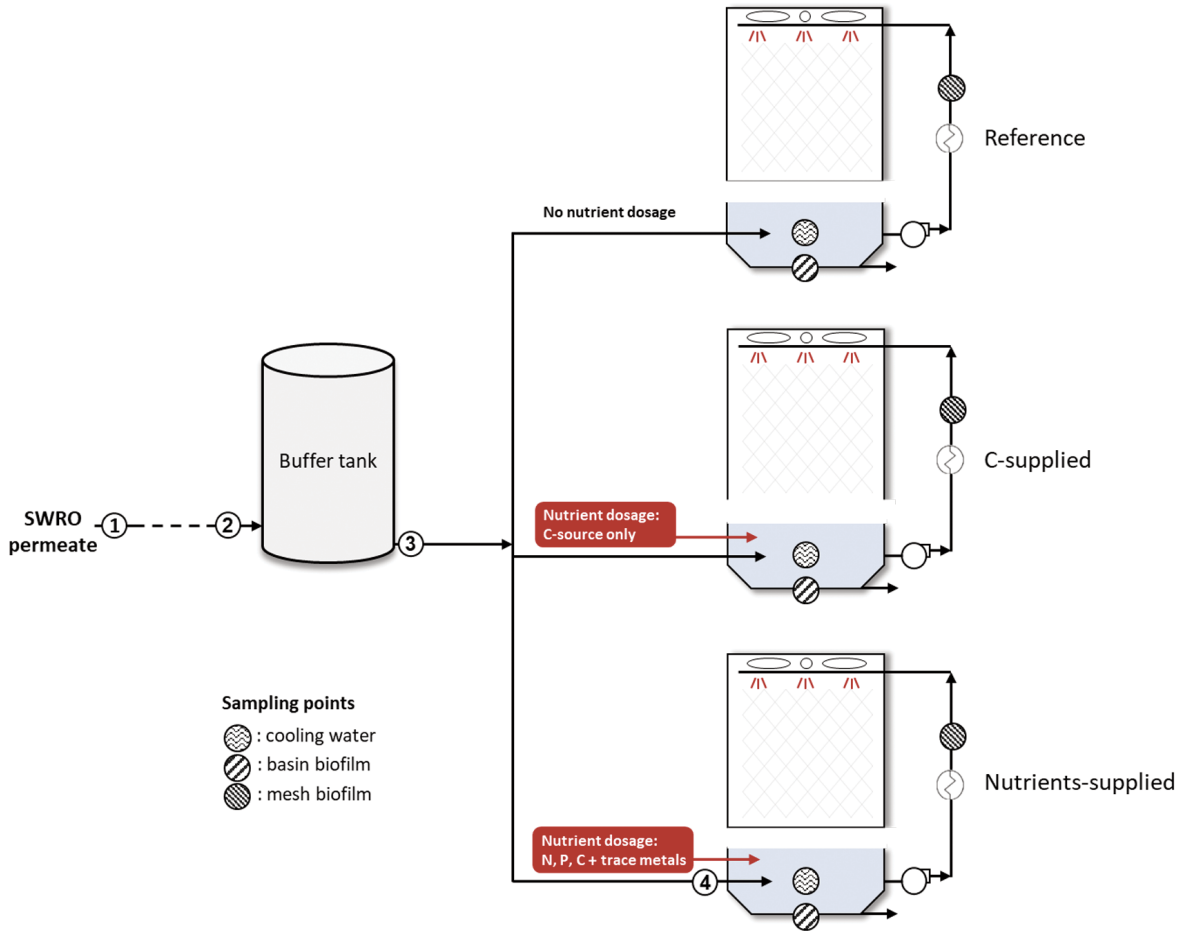

Figure 4.1. Scheme of the pilot installation. SWRO permeate was used as source water. Acetate (C) was dosed in the C-supplied cooling tower and nitrate, phosphate and acetate (N, P, C) were dosed in the Nutrients-supplied cooling tower. In addition to the cooling tower samples, the water from locations 1 to 4 was collected for a water quality test.

4.2.1.2. Nutrient dosages

Nutrients were added to the cooling tower basins to avoid bacterial growth inside the feed water pipelines. The following conditions were applied: (i) reference: without the addition of nutrient; (ii) C-supplied: with a concentration of organic carbon of 500 µg/Lfeed water; (iii) Nutrients-supplied: with concentrations of carbon, nitrogen and phosphorus (C, N, P) of 500, 100, and 50 µg/Lfeed water respectively and trace metals (composition described in Table S1). The following sources were used for the C, N, P nutrients: sodium acetate trihydrate ($CH_3COONa{\cdot}3H_2O$; for 'C' source), sodium nitrate ($NaNO_3$; for 'N' source) and sodium phosphate monobasic monohydrate ($NaH_2PO_4{\cdot}H_2O$; for 'P' source); all purchased in analytical grade from Sigma Aldrich. Tables 4.1 and S1 summarize the compositions and dosing amounts of the nutrient solutions in each cooling tower unit. All the nutrient solutions were adjusted to pH 11 with the addition of NaOH prior to dosage, and the solutions were refreshed every two days to avoid bacterial growth in the nutrient solution containers.

Table 4.1. Nutrient dosages applied in the cooling towers.

Dosage	Reference	P-limited	Non-P-limited
Main elements	-	C	C, N, P
Compounds	-	Acetate	Acetate, Nitrate, Phosphate
Concentration in feed (µg/L)	-	500	500, 100, 50
Trace metals	No	No	Yes (see Table S1)

4.2.2. Water quality analyses

Total phosphorus was analysed by inductively coupled plasma atomic emission spectroscopy (ICP-OES, Varian 720-ES, USA). Standard curve was made with a phosphorus standard solution. 10 mL of water samples and standard solutions (20-500 ppb) which were made using a phosphorus standard solution (Inorganic Ventures, USA) were prepared by adding 1 % (v/v, final concentration) nitric acid. Total phosphorus concentration was calculated based on the standard curve. Orthophosphate concentration was measured with a low detection auto analyser using a colorimetric based method (SEAL AutoAnalyser 3 HR, Seal Analytical, UK) following the proposed protocol by Murphy and Riley (1962). Total organic carbon (TOC) (Table 4.2) was analysed using a TOC analyser (TOC-L CSH, Shimadzu Corporation, Japan) after filtration through 0.45 µm pore size sterile PVDF syringe filters. Chemical oxygen demand (COD) was measured using a Hach TNT plus COD test kit and measured with a Hach spectrophotometer (DR 3900, Hach, USA). Trace metal concentrations were analysed by inductively coupled plasma mass spectrometry (ICP-MS) (Agilent 7500CX, Agilent, USA).

Table 4.2. Analyses of water parameters.

Parameter	Unit	Concentrations in feedwater before nutrient dosages
Total phosphorus	µg/L	<20
Orthophosphate	µg/L	<10
TOC	µg/L	<0.3
COD	µg/L	<2

4.2.3. Bacterial growth potential

4.2.3.1. Identification of limiting nutrient in cooling waters

The methodology applied for determining the limiting nutrient has been derived from Prest et al. (2016b). Bacterial growth potential tests were performed in sterile containers. Recirculating water samples from the three cooling towers were collected onsite and immediately filtered through a 0.45 µm pore-size sterile nylon syringe filter (Sartorius, Germany) to avoid predation by higher organisms such as protozoa. Each sample was split in ten aliquots of 30 mL. The same nutrient compounds dosed in the cooling towers were added to the aliquots as followed: no addition ('Blank'), 1 mg-N + trace elements ('N+M'), 1 mg-P ('P'), 2 mg-C ('C'), 2 mg-C + 1 mg-N + 1 mg-P + trace elements ('All'). The test was performed in duplicate (n=2). The aliquots were incubated at 37 °C in the dark with no shaking.

Bacterial growth was monitored daily using a BD AccuriTM C6 flow cytometry (BD Biosciences, USA) by staining with SYBR Green I (10,000 ×; Invitrogen, USA). The final concentration of SYBR Green I in the samples was 1.96 µM. The staining protocol and flow cytometry analysis are described in literature (Hammes et al. 2008, Prest et al. 2013). The incubation period lasted five days, after which the stationary phase was reached in all aliquots. The net growth was calculated by subtracting the cell count on day 0 from the cell count on day 5 for each growth test.

4.2.3.2. Growth potential of the feed water

The glass vessels used in this incubation test were soaked overnight in 0.2 M HCl solution, rinsed with deionized water and placed in an oven at 450 °C for 4 h to remove AOC residues. Samples of 100 mL from the sampling locations 1 to 4 shown in Figure 4.1 (1: SWRO permeate, 2: buffer tank inlet, 3: buffer tank outlet, 4: cooling tower feed) were collected and incubated in the dark at 37 °C for five days. Total cell numbers were monitored using a BD AccuriTM C6 flow cytometry (BD Biosciences, USA). Staining protocol with SYBR Green I (10000 ×; Invitrogen, USA) and flow cytometry analyses were described in previous papers (Hammes et al. 2008, Prest et al. 2013).

4.2.4. Collection of deposit samples

Deposits consisting of biofilm and sediment accumulation were collected from the recirculation line and basin of each cooling tower unit (Figure 4.1) at the end of the experiment after the water was drained from the system. Pieces of 4 cm × 4 cm of deposit from each basin floor were collected for quantification of dry-ash, adenosine triphosphate (ATP) and chlorophyll, EPS extractions, and 16S/18S rRNA gene amplicon sequencing. To sample each recirculation line, three stainless steel mesh coupons (Imotron, Belgium) were inserted in a corrosion rack prior to the start of the experiment. The corrosion racks were located after the heat exchangers, in the heated water stream of each CT. The coupons were collected after the 5-week experiment to analyse the accumulated material in terms of wet weight, ATP quantification and 16S/18S rRNA gene amplicon sequencing.

4.2.5. Biofilm analytical methods

4.2.5.1. Dry-ash

The wet weight of fresh deposit samples was measured using a high precision analytical balance. For the dry mass, basin deposit samples were spread over a disposable aluminium foil cup to allow optimal contact with air. The samples were heated at 80 °C until no further water loss was measured. Dried samples were then transferred to crucibles, and the ash-free dry weight for the accumulated organic content was obtained after dry ashing at 500 °C for 4 h. The measurements were performed in triplicate.

4.2.5.2. ATP measurement

For each cooling system, one mesh coupon and one piece of fresh basin deposit (4 cm × 4 cm) were collected in 50 mL sterile centrifuge tubes (Greiner) containing 30 mL of NaCl 0.85 %.

The tubes were vortexed at maximal speed for 5 min and sonicated using an ultrasonic homogenizer (Qsonica sonicators, USA) for 1 min with the following settings: output power 15 W and frequency 20 kHz. The homogenized solutions were subsequently filtered through a 0.45 µm pore-size sterile filter (Sartorius, Germany). ATP measurements were performed in duplicate with an ATP analyser (AdvanceTM, Celsis, USA) according to the manufacturer's protocols.

4.2.5.3. EPS extraction

Triplicate deposit samples from the cooling tower basins were frozen at -80 °C and lyophilized (Alpha 1-4 LDplus, Martin Christ, Germany) prior to the extraction. EPS were extracted at high temperature in alkaline conditions, following a method adapted from (Felz et al. 2016). In brief, 0.5 g of freeze-dried samples were stirred in 50 mL of 0.1 mol/L NaOH at 80 °C for 30 min. The containers were placed in ice water for 3 min to stop the extraction. Cooled mixtures were centrifuged at 3,300 × g at 4 °C for 30 min. The supernatants were dialyzed overnight (SnakeSkin 3.5K MW, Thermo Fisher Scientific, USA), frozen at -80 °C and lyophilized.

4.2.5.4. Quantification of phosphorus in EPS

For phosphorus quantification, 3 mg of lyophilized EPS were solubilized in 5 mL of ultrapure water. Total phosphorus content was determined with a HACH spectrophotometer (DR3900, Hach Lange, USA) using the Hach Lange LCK 349 cuvette test kit (Hach, USA).

4.2.5.5. Functional groups of EPS

The Fourier transform infra-red (FTIR) spectrum of the lyophilized EPS was performed on a FTIR Spectrophotometer (Perkin-Elmer, USA) at room temperature, with a wavenumber range from 500 cm^{-1} to 4000 cm^{-1}. Resolution of 4 cm^{-1} and accumulation of 8 scans were applied on each sample.

4.2.5.6. Chlorophyll measurements

Quantification of chlorophyll a and b (chlorophyll a+b) was conducted following the proposed protocol from Caesar et al. (2018). In brief, each basin biofilm sample was dried at 60 °C for 24 h before analysis. 1 g dry weight of sample was placed in a 15 mL screw-cap glass vials, followed by a spatula tip of CaCO3 and 6 mL of dimethyl sulfoxide (DMSO) (Sigma Aldrich). The vial was placed in a water bath at 65 °C for 90 min. After the first extraction cycle, the supernatant was transferred to a new vial. Another 6 mL DMSO was added to the initial vial containing the biofilm and a second extraction was performed at 65 °C for 90 min. The supernatants from the first and second extractions were combined and centrifuged for 10 min at 3,000 × g. The absorbance was measured using a UV-vis spectrophotometer (Lambda 45, Perkin-Elmer) at 648, 665, and 700 nm. The equations for the calculation of the amount of chlorophyll a and b are described elsewhere (Caesar et al. 2018).

4.2.6. Microbial communities in water and deposit samples

2.4 L of cooling water samples were collected and filtered on 0.2 µm pore sized mixed cellulose esters membrane (GSWP04700; Millipore, USA). Deposits from one mesh coupon and from the basin floor of each cooling system were collected and frozen at -20 °C until DNA extraction. Genomic DNA was extracted using a DNeasy PowerWater kit (Qiagen, Germany) according to the manufacturer's instruction. As an additional step, samples in the lysis buffer were heated at 65 °C for 10 min. DNA concentration was quantified using a Qubit dsDNA high sensitivity (HS) or Qubit broad range (BR) assay kit (Life Technologies, USA). The extracted DNA was stored at -80 °C until further analysis. Eukaryotic communities were analysed by DNASense ApS in Aalborg with 18S rRNA gene amplicon sequencing targeting the eukaryotic variable region V4 (position 571-980). The following forward and reverse tailed primers were used: [571F] 5'-GCCGCGGTAATTCCAGCTC-3' and [980R] 5'-CYTTCGYYCTTGATTRA-3'. Bacterial communities were analysed by DNASense ApS with amplicon sequencing of the 16S rRNA gene targeting the variable region V4 (position 515-806). The following forward and reverse tailed primers were used: [515F] 5'-GTGYCAGCMGCCGCGGTAA-3' and [806R] 5'-GGACTACNVGGGTWTCTAAT-3'. Samples were paired-end sequenced (2 × 300 bp) on an Illumina MiSeq instrument using the MiSeq Reagent kit v3 (Illumina). > 10 % PhiX Control v3 Library was added as a spike-in to overcome low nucleotide diversity. The reads were trimmed for quality, clustered and operational taxonomic unit (OTU) were generated based on 97 % similarities using USEARCH. Taxonomy was assigned using QIIME, with a confidence of 0.8, and the SILVA database. Biological duplicates of the basin deposit and cooling water of each cooling tower were analysed. The DOI of the raw sequencing data is indicated in supplementary materials. The bacterial and eukaryotic communities are described in Figures 4.6, and in supplementary Figures S5, S6 and Tables S2, S3. Principal component analyses (PCA) comparing bacterial community structures and eukaryotic community structures were performed on the generated OTUs and their abundance with the Bray-Curtis matrix using the software Mothur v.1.40.5. PCA charts displayed in Figures 4.7 and S8 indicates high similarity in community structures of samples clustering together.

4.3. RESULTS

4.3.1. Determination of the limiting nutrient in the cooling tower waters

Growth potential tests were used to identify the bacterial growth limiting compound in each cooling system (Figure 4.2). A similar approach has been applied for determining the limiting nutrients in drinking water distribution networks (Prest et al. 2016b). In the reference cooling tower and cooling tower supplied with carbon (Figure 4.2.a and 4.2.b), higher net bacterial growth occurred with the addition of phosphorus (P) to the water aliquots (5.9 ± 0.4 × 10^6 cells/mL and 8.1 ± 0.4 × 10^6 cells/mL respectively). In contrast, the addition of nitrogen with trace metals (N+M) and carbon (C) did not promote bacterial growth. The growth of microorganisms in the reference and the carbon supplied cooling towers was, therefore, primarily limited by phosphorus. In the cooling tower enriched with all nutrients (Figure 4.2.c), the high initial concentration of C, N and P supplied with the feed water caused a

significant net cell growth in the blank aliquots (5.7 ± 0.7 × 10^6 cells/mL). Further addition of N+M and P had a negligible impact on cell growth compared to the addition of C source (21.2 ± 1.8 × 10^6 cells/mL), indicating that organic carbon was the primary limiting compound. Cooling towers were therefore operated with different limiting compounds, either carbon or phosphorus. These differences could affect the selection and metabolism of the microorganisms, and thereby the biofouling potential in the processes.

In the remaining part of the manuscript, the cooling tower (i) not supplied in nutrient is denoted as 'Reference'; (ii) supplied with only carbon is denoted as 'P-limited' and (iii) enriched in all nutrients is denoted as 'Non-P-limited'.

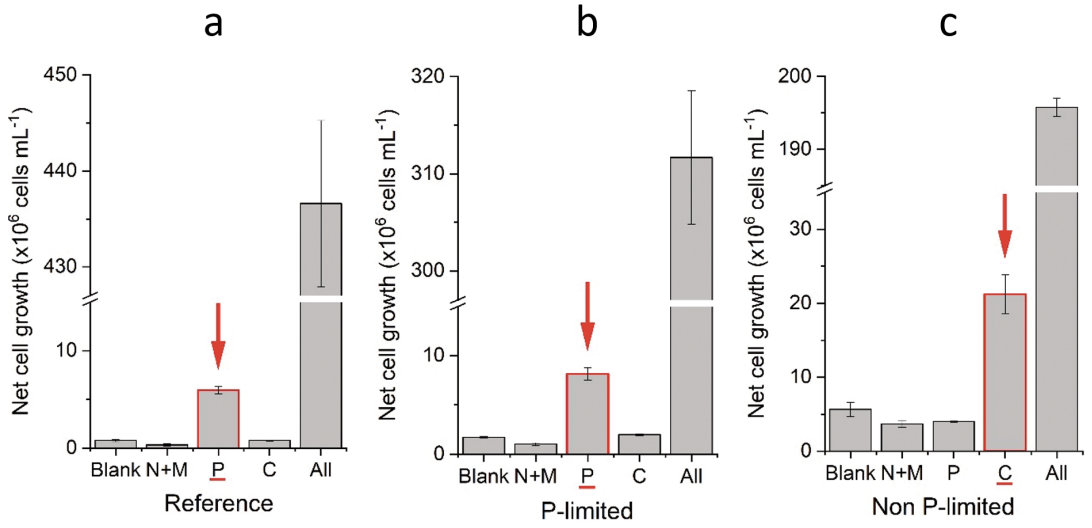

Figure 4.2. Determination of the limiting nutrient in the three cooling tower systems supplied with (a) RO permeate only, denoted as "Reference"; (b) RO permeate enriched in carbon source, denoted as "P-limited"; and (c) RO permeate enriched in all nutrients, denoted as "Non P-limited". Elements were added in cooling water samples as follows: no addition (Blank), nitrogen + trace metals addition (N+M), phosphorus (P), carbon (C), all nutrients (All). The error bars indicate the error on duplicate samples for each condition. The red arrows indicate the limiting nutrient in the corresponding cooling tower: phosphorus in (a) and (b), and carbon in (c).

4.3.2. Deposit formation in the cooling towers

4.3.2.1. Macroscopic description

Deposits were collected from the basin floor, and the mesh coupons were retrieved from the recirculation line after five weeks of operation. The visual appearance of the accumulated material in the basin (Figure 4.3) strongly varied between the three cooling systems. The reference cooling system displayed limited growth, mostly accumulation of sediment forming a sludge-like layer. In the P-limited cooling system, fed with carbon enriched feed water, an extensive deposit layer of 3 to 5 mm thick was formed with high structural integrity, homogeneously spread and covering the entire floor of the basin. Contrary to the others, the P-limited layer could be removed from the surface as one piece when manually taken out. The non-P-limited cooling system showed extensive and non-homogeneous deposit on the bottom surface with visible growth of phototrophic organisms. Results from the dry-ashing showed that the deposits from all basins contained between 40 and 60 mg/cm^2 of sediment,

mainly silica from sand particles carried by the wind or sandstorms, and other inorganic compounds.

Figure 4.3. Deposits in the basin of the reference (a), P-limited (b) and non-P-limited (c) cooling towers at the end of the 5-week experiment.

The mesh coupons collected from the recirculation lines had a similar appearance, with brown deposits suggesting a combination of biofilm and sediment (Figure S2). Accumulated matter on the mesh coupons from the P-limited and non-P-limited cooling systems were considerably higher than on the coupons from the reference system. Even so, the presence of accumulated material in the reference system was unexpected since RO permeate was used as feed water with no additional dosage.

Overall, the changes in nutrient dosages caused different morphologies and varied thickness of biofilm layers in the cooling tower basins. The presence of sediments confirmed the high sensitivity of cooling tower systems to the accumulation of materials introduced by the air flow.

4.3.2.2. Compositions of basin biofilms

In the reference cooling tower, 3.22 ± 0.69 mg/cm^2 of organic matter (Figure 4.4.a) had accumulated on the basin floor. In the cooling tower operated with carbon enriched feed water, P-limited, the amount of accumulated organic matter was 7.30 ± 0.38 mg/cm^2 while the nutrient containing feed water, non-P-limited, resulted in 6.14 ± 1.49 mg/cm^2 accumulation. P-limited and non-P-limited cooling towers showed significantly higher organic matter accumulation than the reference (p-values < 0.01 based on Z-score values). However, the difference between P-limited and non-P-limited was not significant (p-value > 0.05). The larger standard deviation illustrates the uneven biofilm layer of the non-P-limited cooling tower basin. ATP quantification indicated a very low active biomass concentration in the reference and P-limited biofilms (< 10 ng/g organic matter) as compared to the non-P-limited samples (2.6 ± 0.1 × 10^3 ng/g organic matter) (Figure 4.4.b). The results suggest that under phosphate limitation, microbial growth was indeed limited, but the abundant accumulation of organic matter still occurred in the presence of AOC. The percentage of extracted EPS in the different samples, however, were all very similar to values ranging between 20 and 30 % (w/w) of the organic matter (Figure 4.4.c). A relatively higher phosphorus content (Figure 4.4.d) was observed in the EPS of the non-P-limited cooling tower (0.62 ± 0.02 % compared to 0.37 ± 0.06 % and 0.35 ± 0.01 % in the reference and P-limited).

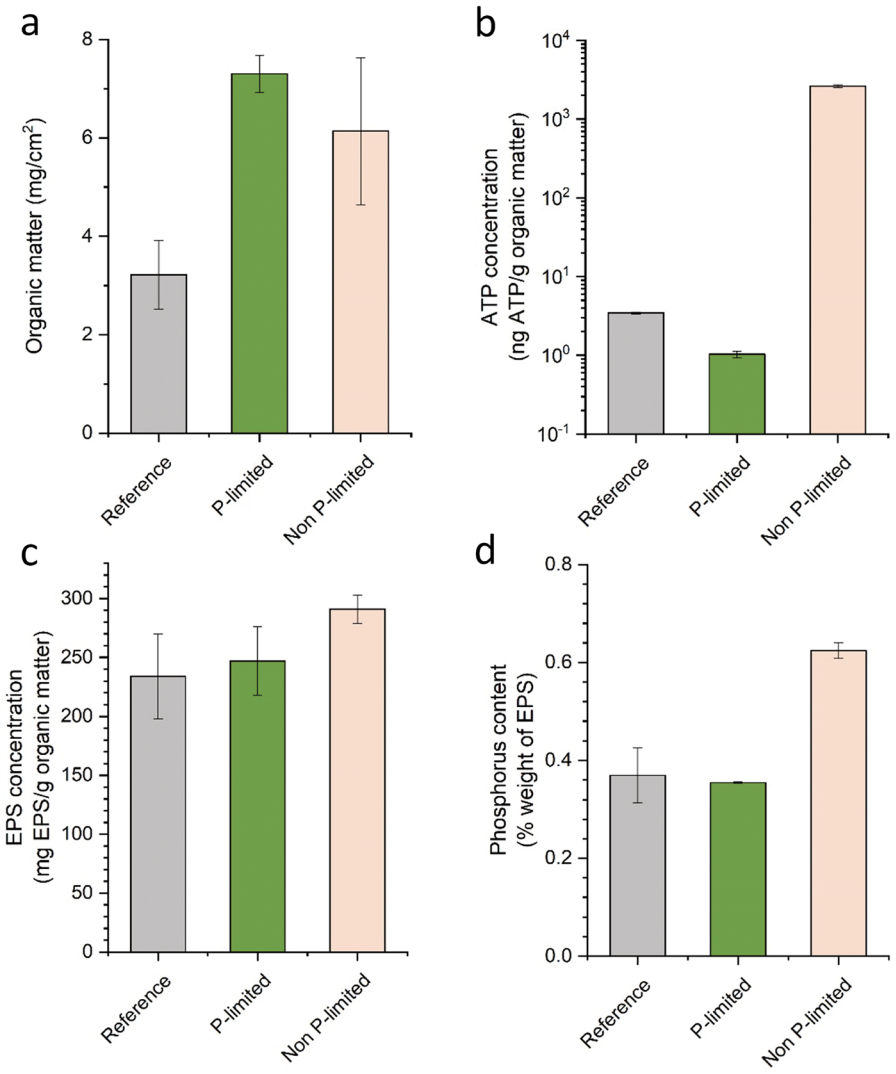

Figure 4.4. Amounts of organic matter (a), ATP concentrations (b), EPS concentrations (c) and phosphorus contents in EPS (d) in the basin deposits.

Fourier transform infrared spectroscopy spectra (Figure S3) of the extracted EPS display the characteristic carbohydrate band at 940-1200 cm^{-1} (Boleij et al. 2019, Zhu et al. 2012) and protein band at 1500-1700 cm^{-1} with amide I and amide II corresponding peaks (Barth 2007). The ratio of the intensity of the protein band compared to the carbohydrate band is lower in the reference and P-limited samples than in the non-P-limited sample, indicating a preference for carbohydrate production rather than protein when phosphorus is limited. In addition, chlorophyll a and b measurements (Figure S4) correlate with visual observation with a considerably higher concentration in the non-P-limited conditions than in the reference and P-limited ones (respectively 40, 3 and 19 µg/g basin deposit).

4.3.2.3. Compositions of deposit layers on the mesh coupons

Deposit mass and ATP were also quantified from the mesh coupons placed in the recirculation pipe (Figure 4.5). The mesh coupon from the reference system reached only 28 ± 3 mg/cm^2 of accumulated material, significantly less than on the coupons from the P-limited and non-P-limited systems (p-values < 0.0001), which had accumulated 50 ± 5 mg/cm^2 and

49 ± 4 mg/cm² respectively. The mesh coupon deposit from the non-P-limited cooling system contained a significantly higher active biomass concentration (43 ng ATP/g wet deposit) than deposits from the reference and P-limited systems (5 and 2 ng ATP/g wet deposit).

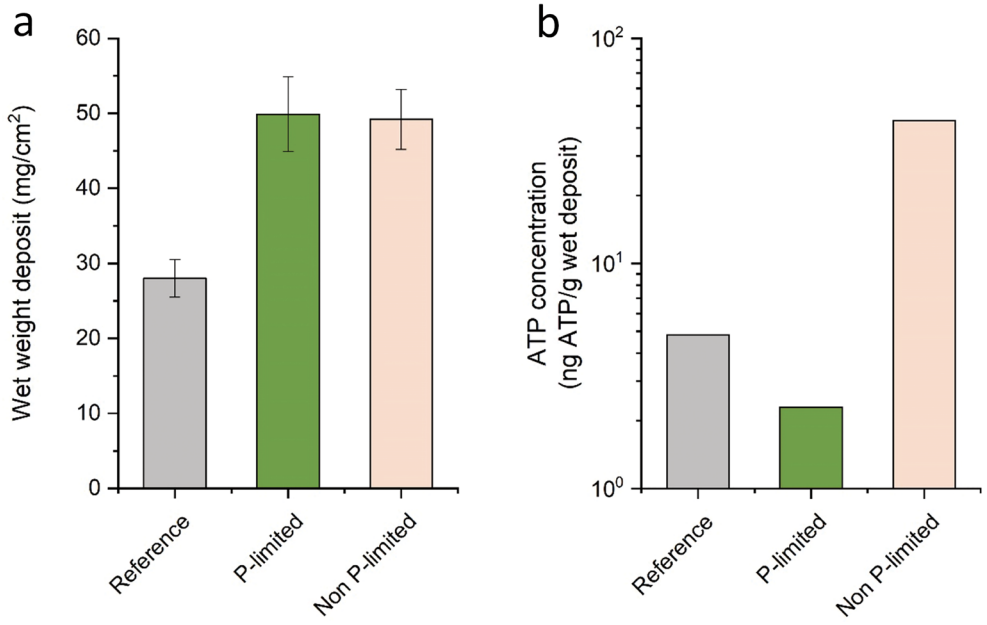

Figure 4.5. Deposit wet weights (a) and ATP concentrations (b) on the mesh coupons located in the recirculation line, after the heat exchanger.

The results for biomass growth in the cooling tower basins and on the coupons in the recirculation pipe are congruent, suggesting that biofouling occurs consistently along the process, i.e. basin and recirculation pipe.

4.3.3. Dynamics of microbial community

Bacterial community and diversity analyses (Figures 4.6 and 4.7) provided more in-depth details on the collected biomass and allowed to visualize changes depending on location in the system and nutrient availability.

The bacterial community composition at the phylum level (Figure 4.6) showed similarity based on the locations of the samples. Cyanobacteria had relatively high abundances in the basin deposits (23, 36 and 43% of 16S rRNA gene reads in the reference, P-limited and non-P-limited cooling towers respectively) due to the exposure to the sunlight during daytime. They were in lower abundances in the water samples (4, 7 and 15 %) and negligible in the mesh deposits located in the dark (< 2 %). Proteobacteria was clearly the main water sample phylum with 66, 69 and 60% while Bacteroidetes, Planctomycetes and Proteobacteria were more evenly represented in the mesh deposits.

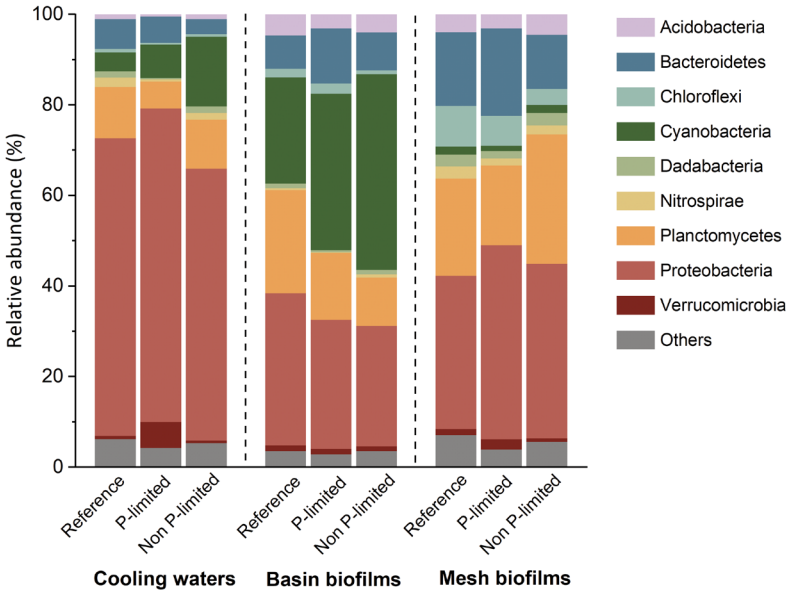

Figure 4.6. Relative abundance of bacterial communities at the phylum level in the cooling water and deposit samples (in percentage of 16S rRNA gene reads). Abundance of Cyanobacteria constitutes the main difference between basin and mesh biofilms, while cooling waters are dominated by the Proteobacteria phylum. "Others" contains bacterial phyla of less than 1% abundance in all samples.

Principal component analysis (Figure 4.7) displays the similarity in bacterial community structures of samples based on the generated OTUs and their abundances. The principal components PC1 and PC2 showed 29.3 and 19.5 % of the variation, respectively. In each cooling system, the water, mesh and basin biofilm samples diverge from each other, confirming that a bacterial selection occurs within the biofilms due to the local environmental conditions (e.g. exposure to the sunlight, temperature), resulting in a distinct microbiome from the water phase. Interestingly, the limitation in phosphate seems to have caused an additional distinction. The plot shows clustering between the reference and non-P-limited samples but divergence of the P-limited samples.

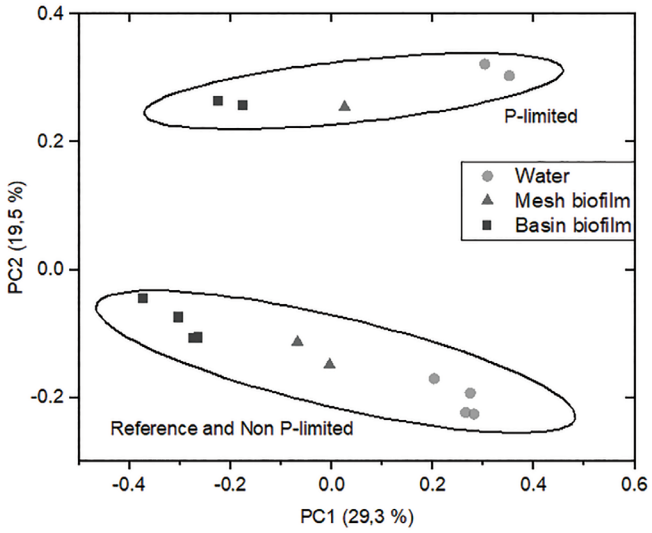

Figure 4.7. Principal component analysis (PCA) comparing the bacterial community structure of the waters, mesh biofilms and basin biofilms. The circles indicate that the P-limited cooling tower samples diverge from the reference and non-P-limited cooling towers. Additionally, the three sampling locations diverge from each other. Waters and basin biofilms were analysed in duplicate.

P-limitation caused a switch in bacterial community structure while the enrichment in all nutrients (C, N, and P) promoted bacterial growth but did not seem to strongly affect the community structure when compared to the reference.

4.3.4. Deterioration in water quality along the feed water line

Due to the observation of biofilm accumulation in the reference system operated with SWRO permeate, it was decided to investigate the stability of the feed water quality along the line between RO installation and cooling tower. Measured water quality parameters – total organic carbon (TOC), chemical oxygen demand (COD) and total phosphorus concentrations – were all below the quantification limit (Table 4.2). A bacterial incubation test was performed to determine the potential impact of the pipes and buffer tank on the feed water quality before supply to the cooling towers.

Samples from the feed water line were collected at four locations according to the numbering in Figure 4.1: SWRO permeate, buffer tank inlet, buffer tank outlet and cooling tower feed. Each sample was incubated in the laboratory, and total bacterial cell count was monitored over five incubation days. As shown in Figure 4.8, samples already displayed higher cell counts on collection day (day 0) in the buffer tank outlet and in the cooling tower feed (61.9 ± 0.7 × 10^3 and 90.3 ± 0.6 × 10^3 cells/mL) compared to the buffer tank inlet (3.2 ± 0.1 × 10^3 cells/mL). The difference in total cell numbers between the inlet and outlet of the buffer tank can be attributed to the retention time of around one day of the RO permeate in the tank. After five days of incubation of the samples, higher cell count was reached in the last sampling location, cooling tower feed (2.7 × 10^5 cell/mL).

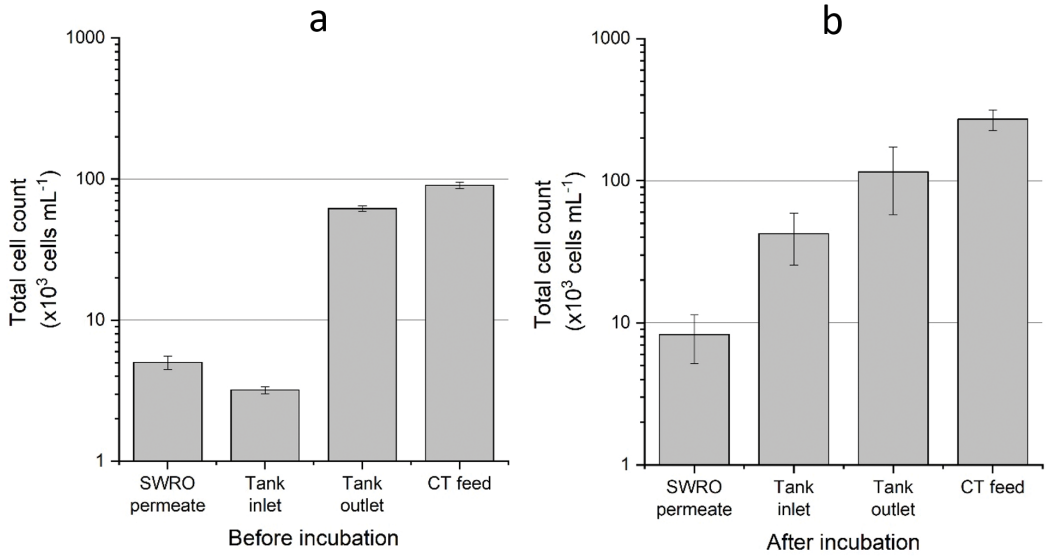

Figure 4.8. Cell count before (a) and after (b) incubation of water samples along the feed water pipe. Samples were collected from (1) the SWRO permeate, (2) the buffer tank inlet, (3) the buffer tank outlet, and (4) the cooling tower feed as shown in Figure 4.1. The daily total cell counts are shown in Figure S8.

These results indicate that the pipe and buffer tank of the feed water line contributed to cell growth through organic carbon leaching from the surface materials to the water phase. Even though the tank and pipes were extensively cleaned before the start of the experiments, the high-quality SWRO permeate was highly deteriorated by the installation and surrounding

environments (e.g. temperature, wind, sunlight). As a result, no conclusion can be drawn on the efficiency of SWRO permeate as feed water for cooling systems due to quality deterioration. Still, the study illustrates the difficulty of maintaining clean installations at an industrial scale to avoid any contamination.

4.4. DISCUSSION

4.4.1. P-limitation restricts microorganism growth but not biofilm formation in cooling tower systems

A goal of this study was to investigate the efficiency of phosphate limitation as a preventive method for biofouling control in cooling systems. The applied short-term approach, based on nutrient dosages in feed water, has been shown to be suitable for prediction of long-term biofouling (Sanawar et al. 2017) and has been used to study membrane filtration systems (Sanawar et al. 2018, Siddiqui et al. 2017). The pilot-scale experiment showed that: (i) active biomass growth in the cooling system was strongly inhibited under P-limited condition compared to conditions with additional phosphate supplied (Figures 4.4 and 4.5); (ii) the overall accumulation of organic matter was similar when all nutrients were supplied or under P-limited conditions with only organic carbon supplied (Figure 4.4); (iii) P-limitation caused a shift in bacterial community structure (Figure 4.7) and seemed to promote the production of carbohydrates over proteins in the extracellular matrix by microorganisms and to result in a more homogeneous biofilm (Figure 4.3).

Phosphorus limitation of the cooling water affected microbial growth and composition of EPS but was not efficient against biofouling in the presence of high AOC. The availability in carbon was, therefore, the main factor determining the extent of biofilm growth.

The necessity of phosphorus for microbial growth has been extensively described in the literature (He et al. 2018, Holtan et al. 1988, Smith and Prairie 2004). The molar ratio between C:N:P in microbial biomass has been approximated to 100:20:1.7 (Tchobanoglous and Burton 1991). At low enough concentration, phosphorus can then become a limiting nutrient for cell growth. According to mixed and pure culture studies, phosphate limitation has been shown to cause selection of microbial communities (Keinänen et al. 2002, Samaddar et al. 2019) and to induce changes in cell physiology - such as degradation of polyphosphate or exchange of phosphorus-free membrane lipids - by affecting their metabolism (Romano et al. 2015).

There are, however, diverging opinions regarding the impact of low phosphorus on biofilm formation. Some authors report a strongly inhibited biofilm formation under P-limited conditions (Kim et al. 2014, Vrouwenvelder et al. 2010). Other authors indicate a higher EPS production by cells resulting in low populated but extensive and homogeneous biofilm with gel-like structure (Desmond et al. 2018, Fang et al. 2009, Hoa et al. 2003, Li et al. 2016). In the studies that performed phosphate quantification, concentrations in the P-limited conditions were reported below detection limit of the applied method, the lowest being 0.01 mg-P/L. It is, therefore, a possibility that the phosphate concentrations in the studies from Vrouwenvelder et al. (2010) and Kim et al. (2014) were lower than in the studies where abundant biofilms were produced although absolute values are lacking. Our results support

previous observations that P-limitation inhibits cell growth but causes higher accumulated organic matter per active biomass unit. In other words, reaching P-limited conditions is not enough to avoid biofouling when there is still AOC in the system. Essentially complete absence of phosphate would be needed for biofouling prevention, which is difficult to achieve in open systems exposed to external conditions as it is the case with cooling towers.

Under P-limitation, the transition to a more carbohydrate-dominated EPS structure was observed, compared to non-P-limited conditions (Figure S3). Native biofilm, i.e. during initial and irreversible attachment, is likely based on a high fraction of proteins due to the essential role of cell surface proteins in the attachment to the substrate, the motility and the surface colonization (Fong and Yildiz 2015). Nonetheless, when P is the primarily limited compound, a shift in carbon metabolism can occur during biofilm maturation, and polysaccharide synthesis can be seen as an overflow metabolism induced by growth limitation (Hessen and Anderson 2008, Russell and Cook 1995). Similar observations have been made in studies of drinking water and wastewater bacterial biofilms (Fang et al. 2009, Hoa et al. 2003) and in diatoms (Brembu et al. 2017). When cell division becomes hindered, the synthesis of proteins and membrane lipids is strongly reduced as cells promote the production of extracellular polysaccharides over the synthesis of cell components and energy production.

Regarding the yield of extracted EPS, one would expect a higher EPS content of the accumulated organic matter in the P-limited sample compared to the non-P-limited sample since active biomass was strongly inhibited. It is important to note that limitations remain on the efficiency of the EPS extraction method. The choice was made to use an alkaline extraction at high temperature to improve the solubilisation of the structural EPS matrix and collect a substantial fraction of the extracellular polymers (Felz et al. 2016). However, such extreme conditions can also cause loss of cell integrity and release of intracellular compounds (Seviour et al. 2019) while not extracting the totality of the matrix components. The higher phosphorus content in the non-P-limited extracted EPS might be caused by a higher extracellular DNA concentration and also possible release of intracellular DNA occurring with cell lysis. The presence of intracellular material and integrity of the cells with this EPS extraction method should be further investigated.

4.4.2. Comparison of biofilm microbial composition along the cooling tower system

Biofilms from full-scale cooling systems are generally collected from the wall of the cooling tower basins, which is the most accessible location of the system and does not require interruption of operation. Our pilot study is the first describing analyses of microbial communities from different locations in the recirculation line of a non-disinfected cooling system.

The results from basin and mesh biofilms for each of the tested conditions were congruent regarding the extent of accumulated organic matter per active biomass (Figures 4.4 and 4.5). Yet, these results showed: (i) divergence of bacterial communities compared to the water sample (Figure 4.7), and (ii) variations in terms of phototrophic organisms' content and structure of the deposit (Figures 4.3 and S4).

Previous investigations of full-scale water processes and distribution networks have shown divergences of planktonic bacterial communities with sessile communities and pointed out a higher richness in biofilm species (Di Gregorio et al. 2017, Keinänen et al. 2002, Wang et al. 2013). Changes are usually attributed to the attachment abilities of the microorganisms during the colonization phase. Indeed, bacteria able to produce adhesion proteins and pili or containing lipopolysaccharides on the outer layers would be favoured and initiate biofilm formation (Conrad 2012, Hori and Matsumoto 2010, Walker et al. 2004). The planktonic microorganisms therefore play the role of inoculant from which bacteria selectively attach to the substratum. The attached bacterial community further evolves independently from the water microbiome. The principal component analysis supports this assumption with the biofilm community structures from two locations in the system - basin and recirculation line after heat exchanger – deviating from the water community structure. As described in other reports, the water community majorly constituted of Proteobacteria (Tsao et al. 2019, Wang et al. 2013), while bacterial phyla were more evenly represented in biofilm communities. In addition, the divergence between the community structures of the basin and mesh biofilms for each condition suggests that the biofilm communities vary along the system and that samples from the basin biofilm only are not representative of other locations e.g. heat exchangers. The deviation is partially driven by the presence of phototrophic organisms (Figures 4.6 and S4) in the basin following intense light exposure (Di Gregorio et al. 2017) and can be amplified by additional factors such as the difference in temperature and substratum (Hancock et al. 2011). The presence of phototrophs may worsen biofilm development through fixation of CO_2 from the atmosphere contributing to the accumulation of organic matter (Roeselers et al. 2008, Rossi and De Philippis 2015). Phototrophic mats have been largely described as multilayer ecosystems (Guerrero et al. 2002, Roeselers et al. 2007, Ward et al. 1998). Such structures were observed in the basins of the cooling towers, especially under P-limited condition with a homogeneous surface layer and non-P-limited condition with more disparate biofilm arrangement (Figure 4.3). These findings point out the importance of sampling location when studying biofilm microbiome in a cooling system.

4.4.3. Implications of the study for industrial cooling tower systems

As described previously, phosphorus removal from the feed water could have an undesirable effect and aggravate biofilm formation if the remaining concentration is not low enough. Its limitation has been shown to induce a greater EPS production by the cells and to result in a homogenous structural deposit, detrimental for full-scale processes. Total removal of phosphorus from a water source is hardly achievable, and phosphorus-based corrosion inhibitors would have to be replaced by phosphorus-free products for the protection of the heat exchangers (Sandu et al. 2016, Wang Jun et al. 2016). The intensive contact between water, ambient air and particles in the cooling tower as well as the evaporation are all factors that can also raise the element concentration in the system (Katra et al. 2016, Mahowald et al. 2008). Following the outcome of this study, removal of AOC seems to be a better approach than phosphorus removal to delay the formation of biofilm.

A growth potential measurement of the feed water was carried out to evaluate the SWRO permeate quality along the line. This approach has been previously applied to evaluate biological stability of waters (Farhat et al. 2018a, Farhat et al. 2018b, Prest et al. 2016b).

Results showed a leaching of nutrient along the pipes and reservoir, resulting in higher bacterial cell growth (Figure 4.8) and therefore compromising the assessment of SWRO permeate as cooling tower feed water. This study was exposed to some difficulties related to the industrial scale of the set-up that are not usually considered during laboratory scale experiments, that is to say the impact of the onsite distribution system and effect of external conditions. A conclusion could not be made on the suitability of SWRO permeate as cooling water, but advices and suggestions for future research can be drawn from the obtained results. Our data illustrate the low feasibility of maintaining a sufficiently high feed water quality against biofouling in industrial cooling systems. As cooling towers are long-established systems, their used feed water lines impact the quality of the water. For use as feed water, the RO installation should be located directly next to the cooling water system to have negligible contact time with the pipe surfaces, or the feed water line should be replaced prior to the change of water supply and made of non-leaching materials.

4.5. CONCLUSIONS

This pilot-scale study on the impact of nutrient availability on biofilm formation combined with an efficiency assessment of seawater reverse osmosis permeate as feed water in open recirculating cooling towers showed that:

- P-limitation restricts the growth of microorganisms but not biofilm formation in cooling systems.
- this study underlines the significant risks of (i) water quality deterioration by the feed water line and (ii) contamination from the environment of cooling water in open recirculating cooling systems.
- higher C:P ratio affects the bacterial community structure and seems to promote the production of carbohydrates over proteins.
- in each system, biofilms from the basin and coupons in the recirculation pipe diverge from each other and from the water phase in terms of bacterial community structure. The biofilm microbiome, therefore, develops independently from the planktonic microbiome and adapts to local conditions.

These research outcomes provide a basis for understanding biofilm growth factors in open recirculating cooling systems and contributes to the industrial field in the selection of feed water type and pre-treatments. Evaluation of RO permeate as feed water to avoid biofouling requires further investigation and may be addressed in future studies.

CHAPTER 4 - ADDITIONAL

SIALIC ACIDS: AN IMPORTANT FAMILY OF CARBOHYDRATES OVERLOOKED IN ENVIRONMENTAL BIOFILMS

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Supplementary figures and tables are available at the following link: https://doi.org/10.3390/app10217694

ABSTRACT

Sialic acids in the structural matrix of biofilms developing in engineered water systems constitute a potential target in the battle against biofouling. This report focuses specifically on the presence of sialic acids as part of the EPS of biofilms forming in cooling towers and the potential effect of nutrient starvation on sialic acid presence and abundance. Two cooling water compositions were compared in parallel pilot-scale cooling towers, one poor in nutrients and one enriched in nutrients. Fresh deposits from the two cooling towers were collected after a 5-week operation period. 16S rRNA gene amplicon sequencing, extracellular polymeric substances (EPS) extractions and analyses by Fourier transform infrared spectroscopy (FTIR) and high-resolution mass spectrometry (MS) were performed. The results of MS analyses showed the presence of pseudaminic/legionaminic acids (Pse/Leg) and 2-keto-3-deoxy-D-glycero-D-galacto-nononic acid (KDN) in both biofilm EPS samples. FTIR measurements showed the characteristic vibration of sialic acid-like compounds ν(C=O)OH in the nutrient poor sample exclusively. Our findings, combined with other recent studies, suggest that bacterial sialic acids are common compounds in environmental biofilms. Additionally, the conservation of sialic acid production pathways under nutrient starvation highlights their importance as constituents of the EPS. Further in-depth studies are necessary to understand the role of sialic acids in the structural cohesion and protection of environmental biofilm layer.

4.6. INTRODUCTION

Biofilm formation is highly detrimental to the daily operation and lifetime of engineered water processes, such as heat exchangers and membrane filtration systems. Commonly applied chemical cleanings of fouled units do not manage to recover the initial efficiency of the systems. This is often due to a lack of understanding of the extracellular polymeric substances (EPS) matrix formed by the microorganisms (Seviour et al. 2019), eventually resulting in the use of non-adapted chemicals (e.g. surfactants or acid and base cleaning products).

Biofilm formation is facilitated by the production of EPS, consisting of carbohydrates, proteins, nucleic acids, and other biopolymers (Seviour et al. 2019). Carbohydrates produced by the microorganisms exhibit great diversity. Within those carbohydrates, sialic acids, a subset of the nonulosonic acid family comprising more than 50 structurally distinct acidic carbohydrates, have been proposed to play an important role in biofilm formation related to human bacterial infections (Trappetti et al. 2009). For example, these nine-carbon acidic sugars can act as growth and adhesion factors for some periodontal organisms, both for colonization of host surfaces and interactions with other oral dwelling bacteria within a biofilm (Stafford et al. 2012). Since it was widely believed that the ability of bacteria to biosynthesize sialic acids was predominantly found in pathogenic and commensal species (Angata and Varki 2002), little study has been performed on environmental biofilms.

Sialic acids have very recently been reported in water treatment systems as part of the EPS of seawater-adapted granular sludge (de Graaff et al. 2019). In addition, a newly established mass spectrometric approach by Kleikamp et al. (2020) revealed their wide-spread occurrence in non-pathogenic species. These recent discoveries raise interesting questions regarding the occurrence of sialic acids in the biofouling layer of engineered water systems. Investigation of sialic acids in samples from water processes such as cooling towers would contribute to a better understanding of the biofilm composition and encourage further in-depth studies on their functions as a component of the extracellular matrix.

Furthermore, sialic acids are present on the surface of all vertebrate cells where they terminate glycan chains and are exposed to many interactions with the surrounding environment (Lewis et al. 2009). In granular activated sludge, they were found both on bacterial surface-related structure and in the EPS of granular activated sludge, i.e. they are located within the space between the cells and their environment (de Graaff et al. 2019). In this respect, it is reasonable to assume that changes in the surrounding environment may also have an impact on sialic acid production. Exploring the potential impact of environmental stresses (e.g. nutrient starvation) on their diversity and abundance in environmental biofilms would provide significant insights on their protective role and relevance in the matrix.

For this study, biofilm samples were collected at the end of a 5-week experiment from pilot-scale cooling tower systems operated in parallel. The full experiment has been previously described in Chapter 4, investigating the efficiency of phosphate limitation as a biofouling control method for cooling towers. In the present report, sialic acid identification and relative abundance were assessed from the biofilms obtained from two of the cooling towers supplied with seawater reverse osmosis permeate - the first without addition of nutrients (nutrient

poor), primarily limited by phosphorus, and the second with addition of nutrients (nutrient enriched). The objectives were to (i) evaluate the presence of sialic acids in EPS of environmental biofilms and estimate the potential for sialic acid production by the biofilm bacterial communities, (ii) investigate the impact of nutrient poor condition on the presence and abundance of sialic acids, and (iii) explore the implications of the findings related to engineered water processes prone to biofouling.

4.7. MATERIALS AND METHODS

4.7.1. Pilot set-up

The cooling tower pilot facility is located at the King Abdullah University of Science and Technology (KAUST) in Saudi Arabia. The process and operating parameters have been described in Chapter 4. Two of the parallel cooling towers were considered in this study. In short, seawater reverse osmosis (SWRO) permeate produced by the nearby desalination plant (Belila et al. 2016) was used as feed water during the 5-week experiment. Cycles of concentration were maintained between 5 and 7, with cooling water conductivities varying between 6 and 8 mS/cm and pH between 8.0 and 8.3. The temperature of the water in the cooling tower basins varied between 27 and 30 °C, depending on external climate factors. Biofilms were developed under the following conditions: (i) poor in nutrients, i.e. SWRO permeate without additional nutrients as feed water, and (ii) enriched in nutrients, i.e. SWRO permeate with addition of 0.5:0.1:0.05 (C:N:P) mg/L and trace metals as feed water. Concentrations were chosen as described in Chapter 4. The C, N and P sources used in the enriched nutrient condition were as follows: sodium acetate trihydrate ($CH_3COONa \cdot 3H_2O$), sodium nitrate ($NaNO_3$) and monosodium phosphate monohydrate ($NaH_2PO_4 \cdot H_2O$), all purchased from Sigma Aldrich.

4.7.2. Collection of samples

The collected deposits are the same as described in Chapter 4. Deposits were sampled from the basin surface of the cooling towers after drainage of the two basins at the end of 5 consecutive weeks of stable operation. Pieces of 4 cm × 4 cm were collected in 50 mL sterile Greiner tubes. Locations of the samples were arbitrary selected but repeated identically in the cooling towers. Two of the pieces from each investigated cooling tower – nutrient poor or nutrient enriched - were used for the analyses described below.

4.7.3. Microscopic pictures

Deposit samples from the cooling tower basins were frozen at -80 °C upon collection and lyophilized at -50 °C and 0.05 mbar (Alpha 1-4 LDplus, Martin Christ, Germany). Microscopic pictures of the freeze-dried raw deposits were taken with a Zeiss AxioPlan 2 imaging microscope (Carl Zeiss, Germany). Samples were observed with normal light and all images were obtained with a 400× total magnification.

4.7.4. EPS extraction

Extraction of EPS was performed on the freeze-dried raw deposits. EPS was extracted at 80 °C in alkaline conditions, following the method described in Chapter 4, and lyophilized. The freeze-dried EPS samples were kept in a dry environment before further analyses.

4.7.5. Fourier transform infrared spectroscopy (FTIR)

The Fourier transform infra-red (FTIR) spectra of the freeze-dried raw deposits and extracted EPS samples were performed on a FTIR Spectrophotometer (Perkin-Elmer, Shelton, USA) at room temperature, with a wavenumber range from 500 cm^{-1} to 4000 cm^{-1}. Resolution of 4 cm^{-1} and accumulation of 8 scans were applied on each sample.

4.7.6. High-resolution mass spectrometry (MS)

Freeze-dried extracted EPS samples were analyzed for sialic acids using a recently established untargeted high-resolution mass spectrometric approach, described by Kleikamp et al. (2020). Sialic acid peaks from KDN and Pse/Leg identified by mass and characteristic fragment ions were integrated using Thermo Xcalibur Qualbrowser. Observed and theoretical masses, C-9 marker fragments and water loss peaks are provided as supplementary information (Table S1). Analyses were performed in duplicates. A statistical analysis was conducted on the sialic acid peak areas at 95 % confidence level based on *Z*-scores. Variations in abundances with *p*-values higher than 0.05 were considered statistically insignificant.

4.7.7. DNA extraction and 16S rRNA gene amplicon sequencing

The raw deposit samples from the basin surface were frozen at -20 °C upon collection and until DNA extraction. The genomic DNA of 0.5 g of sample was extracted using the DNeasy PowerWater kit (Qiagen, Germany) according to the manufacturer's instruction. DNA concentration was quantified using a Qubit dsDNA high sensitivity (HS) or Qubit broad range (BR) assay kit (Life Technologies, USA). The extracted DNA was stored at -80 °C until further analysis. The DNA extracts were sent to DNASense ApS (Aalborg, Denmark) for amplicon sequencing of the 16S rRNA gene targeting the variable region V4 (position 515-806). Samples were paired-end sequenced (2 × 300 bp) on an Illumina MiSeq instrument. The reads were trimmed for quality, clustered and OTU abundances were generated based on 97 % similarities using USEARCH. Taxonomy was assigned using the RDP classifier (Wang et al. 2007) as implemented in the parallel_assign_taxonomy_rdp.py script in QIIME (Knirel et al. 2003), with a confidence of 0.8 and the SILVA database. OTUs were generated based on a subsampling parameter of 10000 sequences per sample. Extraction and analyses were performed in duplicates. Alpha and beta diversity analyses are available in Supplementary materials (Table S3 and Figure S1).

4.7.8. Basic local alignment search tool (BLAST)

The Basic Local Alignment Search Tool (BLAST) was accessed on the NCBI website. Reference protein sequences of CMP-pseudaminic acid (Pse), CMP-legionaminic acid (Leg)

and CMP-2-keto-3-deoxy-D-glycero-D-galacto-nononic acid (KDN) synthetases (Schoenhofen et al. 2009, Wang et al. 2008) were blasted against the available NCBI databases sequences of the main bacterial groups, families or genera, present in the biofilms. The compared sequences were considered having a significant match when the E-value was inferior to 10^{-15}.

4.8. RESULTS AND DISCUSSION

4.8.1. Sialic acids are present in environmental biofilms

Biofilms developed at the bottom of the cooling tower basins - under (i) nutrient poor and (ii) nutrient enriched conditions. Figure 4.9 shows pictures of the biofilms before collection (A1 and B1) and microscopic images of the collected samples (A2 and B2). Both biofilm samples contained a complex ecosystem within the matrix, including both algae and bacteria. Visual observations revealed a less extensive biofilm formed under nutrient poor conditions with lower algae growth than in the nutrient enriched biofilm.

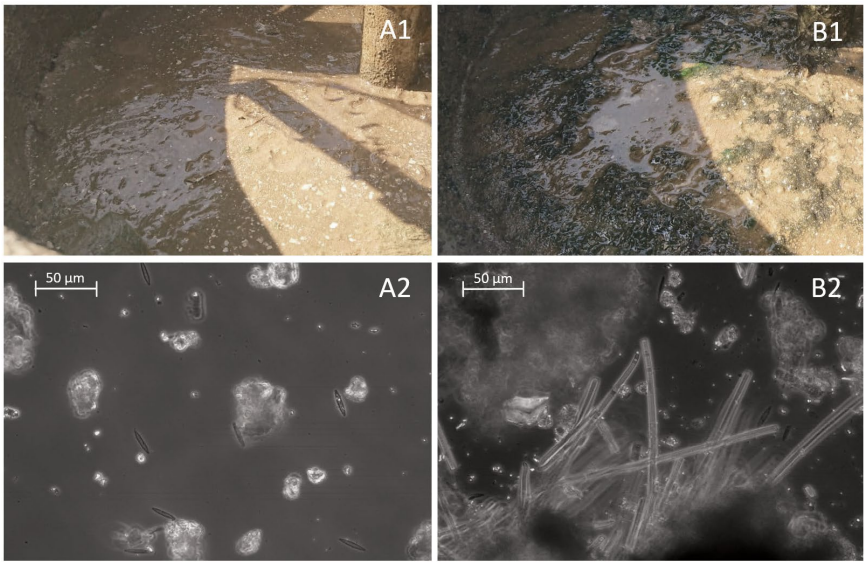

Figure 4.9. Biofilm deposit in the basins of the cooling tower fed with RO permeate poor in nutrients (A1) and the cooling tower fed with RO permeate enriched in nutrients (B1) and associated microscopic pictures (A2 and B2).

16S rRNA gene amplicon sequencing analyses revealed large fractions of Proteobacteria: 32 and 19 %, Cyanobacteria: 25 and 57 %, and Planctomycetes: 21 and 7 % in the nutrient poor and nutrient enriched biofilms respectively. Relative abundances of the main families are shown in Figure 4.10. *Microscillaceae*, *Leptolyngbyaceae* and an undefined Nostocales family were the most represented groups in both samples.

Due to the presence of a large fraction of inorganic particles (e.g. sand) accumulated in the biofilm, EPS was extracted to eliminate the inorganic background and most intracellular compounds. The presence of sialic acids and their relative abundance in the extracted biofilm EPS samples are displayed in Figure 4.11. Sialic acids with the compositions consistent with Pse/Leg, which are indistinguishable by mass spectrometry only, and KDN, or a related

desamino form of a molecule consistent with the neuraminic acid composition, were present in both biofilm EPS samples. KDN was reported to be part of glycoconjugates, including glycolipids, glycoproteins, and capsular polysaccharides, at the terminate position of the glycan chain. Pse/Leg have been mainly found as constituents of heteropolysaccharides, as internal carbohydrate (Knirel et al. 2003). Their charged group allows both sialic acids to engage in cross-linking, potentially protecting the biofilm matrix.

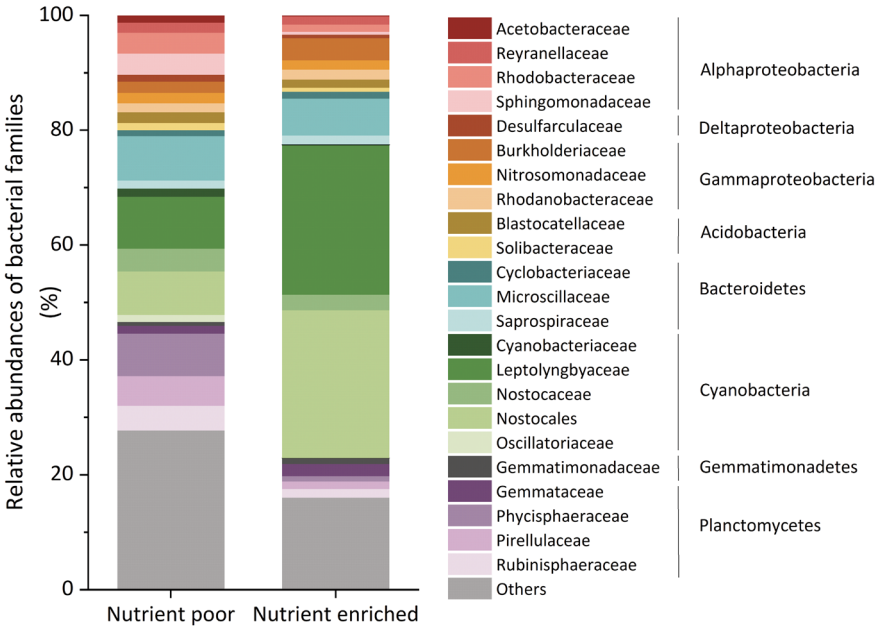

Figure 4.10. Relative abundance of the main bacterial families in the biofilm samples grown under nutrient poor and nutrient enriched conditions with corresponding phyla and class of Proteobacteria. "Others" contains bacterial families of less than 1 % abundance in both samples.

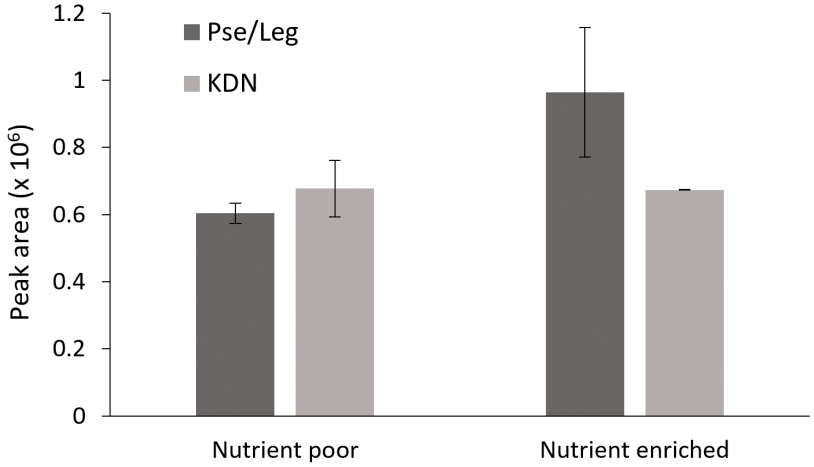

Figure 4.11. Observed mass spectrometric peak areas of sialic acids in nutrient enriched and nutrient poor biofilm EPS. The error bars represent the minimum and maximum values. Abundances of both sialic acids were not significantly different between the samples (p-values > 0.05).

Sialyltransferases are key enzymes in the biosynthesis of sialic acid-containing oligosaccharides and glycoconjugates (Boleij et al. 2020). They catalyse the transfer reaction of a sialic acid residue to the glycoconjugates. Presence of these compounds in the extracellular matrix indicates that some bacterial members of the biofilms are able to produce

transferases. Reference protein sequences of the transferases were compared to the most abundant bacterial groups in the biofilms in order to approximately evaluate the potential of sialic acid production by the bacterial community. Since *Campylobacter jejuni* has characterized pathways for biosynthesis of sialic acids (e.g. Leg and Pse), sialyltransferases in these pathways were used as reference protein sequences (Schoenhofen et al. 2009). The reference sequence for KDN was obtained from Wang et al. (2008). A BLAST analysis was then performed to compare the reference sequences of the enzymes CMP-Pse, CMP-Leg and CMP-KDN synthetases with the NCBI database sequences of the identified bacterial groups (Figure 4.10). Significant matches (E-value < 10^{-15}) are shown in Table 4.3 for the main families and corresponding genera identified in the biofilm samples. A large part of the genera present in both biofilm samples includes species that possess the genes responsible for the sialic acid residue transfer to the glycoconjugates, suggesting that sialic acids can be produced by these microorganisms.

Table 4.3. Results of the BLAST analysis comparing the enzymes CMP-Pse, CMP-Leg and CMP-KDN synthetases to the available NCBI database sequences of the bacterial groups in the biofilms (identified by 16S rRNA gene amplicon sequencing). The main families (> 1 %) and identified genera in these families, all present in both biofilm samples, were blasted. Significant matches (E-value < 10^{-15}) are indicated by a green background, no match or insignificant matches (E-value > 10^{-15}) by a red background.

Family	Genus	CMP-Pse synthetase	CMP-Leg synthetase	CMP-KDN synthetase
Acetobacteraceae				
	Roseomonas			
Blastocatellaceae				
Burkholderiaceae				
	Hydrogenophaga			
	Candidimonas			
	Aquabacterium			
	Limnobacter			
	Noviherbaspirillum			
	Ideonella			
	Azohydromonas			
Cyanobacteriaceae				
	Annamia HOs24			
	Geminocystis			
Cyclobacteriaceae				
	Marinoscillum			
	Ekhidna			
	Algoriphagus			
	Imperialibacter			
Desulfarculaceae				
Gemmataceae				
	Gemmata			
	Fimbriiglobus			
Gemmatimonadaceae				
	Gemmatimonas			
Leptolyngbyaceae				
	Leptolyngbya			
Microscillaceae				
Nitrosomonadaceae				

Family	Genus	CMP-Pse synthetase	CMP-Leg synthetase	CMP-KDN synthetase
Nostocaceae				
	Stigonema			
	Nostoc			
	Chlorogloeopsis			
Nostocales (undefined)				
	Microseira wollei			
Oscillatoriaceae				
	Oscillatoria			
Phycisphaeraceae				
Pirellulaceae				
	Blastopirellula			
	Bythopirellula			
Reyranellaceae				
Rhodanobacteraceae				
	Aquimonas			
Rhodobacteraceae				
	Rhodobacter			
Rubinisphaeraceae				
	Planctomicrobium			
	SH-PL14			
Saprospiraceae				
	Phaeodactylibacter			
Solibacteraceae				
Sphingomonadaceae				
	Porphyrobacter			
	Sphingopyxis			
	Blastomonas			
	Erythrobacter			
	Sphingobium			
	Altererythrobacter			
	Sphingomonas			

In short, the mass spectrometric measurements showed that diverse sialic acids were produced in the two environmental biofilms collected from the cooling towers. Results of the BLAST analysis, combined to observations from earlier studies, indicate that a wide range of microorganisms have the ability to produce these compounds. This confirms that sialic acid-containing glycoconjugates are more widely found in environmental biofilms than previously thought (Boleij et al. 2020, Harduin-Lepers et al. 2005, Kleikamp et al. 2020, Lewis et al. 2009, Wagstaff et al. 2018).

4.8.2. Nutrient availability did not significantly influence the production of sialic acids in the environmental biofilms

Due to the exposure of sialic acid molecules to the surrounding environment, both as terminal position of the glycan chain on vertebrate cell surfaces (Lewis et al. 2009) and as constituent of the EPS of granular sludge (de Graaff et al. 2019), it was assumed that changes in the direct environment would impact sialic acid production. The influence of nutrient availability - nutrient poor and nutrient enriched conditions in this study - was, however, not reflected by

the MS results of the biofilm EPS extracts. Two different types of sialic acids, Pse/Leg and KDN (methylated), were detected in both the nutrient poor and nutrient enriched biofilm samples (Figure 4.11). Their abundances were not significantly different between the samples (p-values > 0.05 based on Z-scores). The fact that sialic acid production is maintained under nutrient starvation suggests that these carbohydrates do not only contribute to biofilm formation but might also have value under stress conditions to maintain protection and structure of the biofilm. First of all, the acidic functional group and the ability of forming ionic bond with multivalent cations such as Ca^{2+} (Ganss et al. 1993), suggest the involvement of the molecules in complex network and contribution to the structural integrity of the overall biofilm. Secondly, sialic acids are not only produced and used by microorganisms to colonize host surfaces, but are also produced and released in the EPS to protect the biofilm and microbiome from environmental stresses (Badr et al. 2015, Boleij et al. 2020, Di Dato et al. 2019, de Graaff et al. 2019). A recent study on granular sludge has shown that sialic acids, due to their terminal position and large size with nine carbons, were able to protect the granules from enzymatic degradation (de Graaff et al. 2019). Other systems have shown similar behavior, such as the CMP-sialic acid transporter which is upregulated in diatoms under silica-limited conditions. This transporter transfers CMP-sialic acid from the cytosol into Golgi vesicles where glycosyltransferases are activated and produce sialylated glycolipids that can act as defensive molecules. Therefore, this upregulation indicates that diatom cells are coping with starvation by accumulating defensive molecules (Di Dato et al. 2019). Another example are cancer cells, which react to nutrient-deprived condition by using sialic acids to maintain cell surface glycosylation, through complementary mechanisms (Badr et al. 2015).

The FTIR analysis shows a sharp peak around 1738 cm^{-1} and a shoulder peak at 1730 cm^{-1} in the nutrient poor EPS spectrum, which are absent from the nutrient enriched EPS spectrum (Figure 4.12). The shoulder peak at 1730 cm^{-1} has been previously associated to the alpha-keto aldonic acid structure in sialic acids (de Graaff et al. 2019). The presence of this peak in the FTIR analysis of the nutrient poor EPS might indicate additional production of other ulosonic acid variants (e.g. 7 or 8 carbon sugars) at larger quantities potentially induced by nutrient starvation, or increased exposure of the observed nine-carbon sugars. Correlation between FTIR and MS analyses needs to be further investigated.

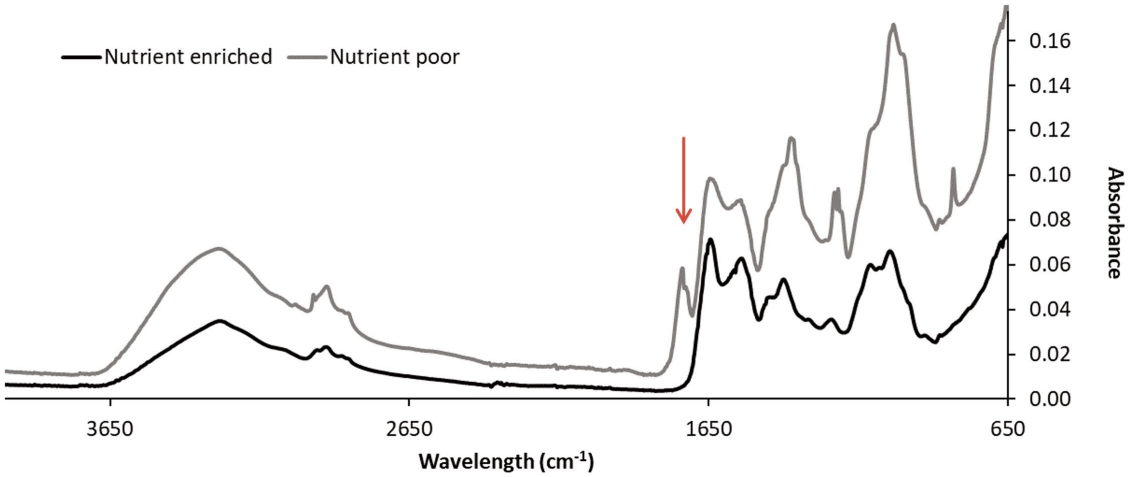

Figure 4.12. FTIR of the extracted EPS of the biofilms. The arrow shows the peak at 1730 cm^{-1} associated with the ν(C=O)OH of sialic acids.

To summarize, the change in nutrient availability did not affect the types and abundances of sialic acids produced by the bacterial community. The conservation of energetically costly production pathways of sialic acids under nutrient starvation highlights their importance as a constituent of EPS and raises questions on their function in environmental biofilms.

4.8.3. Relevance to industrial processes and future considerations

Removal of biofilms in industrial processes and from medical devices, specially grown over a long period of time, is very challenging with conventional chemical cleaning methods (e.g. surfactant and chlorine dosages or acid and base cleanings). Thanks to increasing awareness of the role of EPS on biofilm integrity and efforts in understanding its composition, alternative biofilm removal methods are being developed (Kaur et al. 2020, Stiefel et al. 2016, Xu et al. 2017). The use of enzymes able to cleave the sugar chains and proteins of the extracellular matrix have shown promising results in the medical field for solubilizing the biofilm and preventing the spread of infections (Saggu et al. 2019, Stiefel et al. 2016). However, enzymatic treatments face limitations when used on environmental biofilms due to a lack of substrate specific enzymes and heterogeneous mixture of extracellular molecules (Nahar et al. 2018).

A study on the efficiency of acylase I and proteinase K on removal of biofilms from reverse osmosis membranes revealed that the enzymes were ineffective on some fractions of the EPS and should be used with additional EPS degrading agents to improve cleaning (Kim et al. 2013). Their analyses showed that the treatments removed humic-like substances but did not affect protein-like molecules. This is likely caused by the presence of functional groups protecting the macromolecules from degradation. In this context, sialic acids might be protecting the matrix from such enzymatic agents, as it is suggested in recent research (Boleij et al. 2020, de Graaff et al. 2019) and supported by our preliminary results. Cleavage of the molecule would weaken the matrix and provide exposure of the glycoproteins to enzymatic hydrolysis. Additionally, the combination with emerging physical biofilm disruptive strategies, such as magnetic fields or ultrasounds (Li et al. 2019, Hotrum et al. 2015), could significantly improve the efficiency of the cleaning. The partial breakups and detachments of the biofilm would eventually allow a better penetration of biofilm degrading agents.

Hence, potential combination of sialidases with established enzymatic agents and with biofilm disrupting technologies needs to be further investigated as sialic acids might constitute a key compound in biofilm stability.

CHAPTER 5

EFFICIENT COOLING TOWER OPERATION AT ALKALINE pH FOR THE CONTROL OF *LEGIONELLA PNEUMOPHILA* AND OTHER PATHOGENIC GENERA

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ABSTRACT

Efficient control of pathogenic bacteria, specifically *Legionella pneumophila*, is one of the main concerns when operating industrial cooling towers. Common practices to limit proliferation involves use of disinfectants, leading to formation of disinfection by-product and increase in water corrosiveness. A disinfectant-free *Legionella* control method would make the industry more environmentally friendly. A pilot-scale cooling tower (1 m^3/h) operated with demineralized water was used to investigate the potential of high-pH conditioning as a disinfectant-free alternative for control of *L. pneumophila* and other pathogens. One control experiment was performed under standard full-scale operation involving sodium hypochlorite dosage. Thereafter 3 alkaline pHs of the cooling water were tested: 9.0, 9.4 and 9.6. The tests lasted between 25 and 35 days. The cooling water from the basins were analysed for total cell count by flow cytometry, *L. pneumophila* concentration by plate count and occasional qPCR analyses targeting the *mip*-gene, bacterial and eukaryotic community analyses with 16S and 18S rRNA gene amplicon sequencing, relative abundance of eukaryotic to prokaryotic DNA by qPCR of the 16S and 18S rRNA gene. The *L. pneumophila* analyses showed considerable growth at pH 9.0 and pH 9.4 but was maintained below detection limit (< 100 CFU/L) at pH 9.6 without disinfection. Interestingly, the results correlated with the overall abundance of protozoa in the water samples but not directly with the relative abundance of specific reported protozoan hosts of *Legionella*. The pathogenicity based on 16S rRNA gene amplicon sequencing of the cooling water DNA decreased with increasing pH with a strong decline between pH 9.0 and pH 9.4, from 7.1 % to 1.6 % of relative abundance of pathogenic genera respectively. A strong shift in microbiome was observed between each tested pH and reproducibility of the experiment at pH 9.6 was confirmed with a duplicate test lasting 80 days. High-pH conditioning ≥ 9.6 is therefore considered as an efficient disinfectant-free cooling tower operation for control of pathogenicity, including *L. pneumophila*.

5.1. INTRODUCTION

Evaporative cooling towers are prone to the growth of *Legionella pneumophila*, a bacterial pathogen which induces a severe form of pneumonia, named Legionellosis, when inhaled (Prussin II et al. 2017, Ricketts et al. 2012). Together with other artificial water systems such as shower plumbing, swimming pools or wastewater treatment plants, they represent important sources of outbreaks due to the formation of aerosols spreading to the surrounding environment (Bartram et al. 2007). Evaporative cooling towers are the most often confirmed sources of Legionellosis outbreaks and can contaminate large numbers of people as their aerosols can spread over several kilometres (Paschke et al. 2019). Number of cases of Legionellosis in the European Union has increased over the last decade, causing at least 450 yearly death (Beauté et al. 2017), and is expected to worsen due to increasing number of cooling towers and climate change (Paschke et al. 2019).

Current methods to control pathogen growth and ensure safety of people consists in continuous or shock dosage of disinfectants, often oxidative, such as sodium hypochlorite, chlorine dioxide, or bromine (Bartram et al. 2007). Despite the biocides, outbreaks of *L. pneumophila* still occur due to an inappropriate balance in the complex chemical conditioning and delayed response caused by sparse and indirect plate count monitoring (Bentham 2000). In the Netherlands, *L. pneumophila* plate count is the standard monitoring method, with a detection limit of 100 CFU/L. While the Drinking Water Act requires response actions to be taken when the concentration exceeds 100 CFU/L, the Environmental Protection Act that applies to evaporative cooling towers does not specify any threshold (National Academies of Sciences 2020). The industrial areas can therefore implement their own risk management plan, often using a 10^4 CFU/L threshold according to technical guidelines (ESCMID 2017).

Growth of *L. pneumophila* in water systems has been linked to presence of protist organisms. Past studies have shown that this pathogenic species performs intracellular replication within the vacuoles of amoebas and ciliated protozoan cells, where it obtains required nutrients for growth, and later evades the hosts via host cell lysis (Lau and Ashbolt 2009, Wadowsky et al. 1988). Protozoan species such as *Vermamoeba*, *Acanthamoeba* and *Naegleria* have been reported to constitute reservoirs allowing proliferation of *L. pneumophila* in nutrient poor waters, such as process water or drinking water (Wadowsky et al. 1988). In addition, this interaction provides protection of the pathogen towards sudden changes in environmental conditions and chlorine due the ability of the host to switch to a cyst form (Kilvington and Price 1990).

Past research has been focusing on complementary or alternative methods to disinfection for cooling tower operation by altering system conditions in order to prevent pathogen growth and also prevent formation of disinfection by-products that are discharged in the environment. As *L. pneumophila* grows at temperature between 20 and 45 °C, Kusnetsov et al. (1997) showed that a recirculating water temperature below 20 °C allowed to maintain *L. pneumophila* concentration below 1000 CFU/L. However, operating with a cooling water temperature kept below 20 °C is unlikely in most industrial sites. Alternatively, application of alkaline pH has been suggested in past studies (Brouse et al. 2017, States et al. 1987) and

applied on some industrial sites but no thorough assessment of the effect of alkaline pH conditioning on the overall cooling tower microbiome and pathogen presence has been previously described. It has been reported from laboratory studies that *L. pneumophila* can grow in a pH range of 5.5 to 9.2 (Wadowsky et al. 1988) but there is still a lack of literature available above that range. In general, a strong knowledge gap remains on the survival ability of *L. pneumophila* and other pathogens at alkaline pH, i.e. above 9.0.

In this pilot cooling tower study, demineralized water at elevated pH was used as cooling water with the aim of controlling growth of *L. pneumophila* and other potential pathogens, without involving use of disinfectants. More precisely, the goals of this investigation were to (i) identify an optimal pH above which *L. pneumophila* growth is prevented, (ii) characterize the changes in overall microbiome caused by the increase in alkalinity, with focus on potential pathogenic genera, and (iii) discuss advantages and disadvantages of this approach in term of industrial applicability. The use of a pilot-scale facility was preferred to a laboratory-scale set-up as it allowed to accurately control operational parameters without excluding the impact of environmental conditions linked to external temperatures, precipitations and air quality, which are crucial factors in the microbiome dynamics.

5.2. MATERIALS AND METHODS

5.2.1. Cooling tower installation

5.2.1.1. Pilot description

One pilot-scale evaporative cooling system was built in a mobile container located in Terneuzen, Netherlands, at the time of the study. Suspended materials were initially removed from the inlet water by a candle filter. The packing fill of the cooling tower was composed of HDPE pall rings. The system was operated with a basin volume of 1 m^3 and a recirculation flow of 1 m^3/h. An automatic valve allowed the refill of the basin. Heating was performed by a secondary water loop warmed up by a water boiler and recirculating through 6 steel-based heat exchangers mounted in series. Cooling efficiency was controlled by automatic adjustment of the blower speed. The water temperatures were maintained at 25 °C in the cold section of the cooling system and simultaneously at 35 °C in the hot section of the same system. When maximal conductivity was reached, a valve triggered the discharge of concentrated cooling water.

5.2.1.2. Online monitoring

pH (Orbisint CPS11D, Endress+Hauser, Switzerland), conductivity (Condumax CLS21D, Endress+Hauser, Switzerland), temperatures (Thermophant T TTR31, Endress+Hauser, Switzerland) and free chlorine concentration (Analyzer AMI Codes-II CC, Swan, Switzerland) were monitored online.

5.2.1.3. Conditionings

Conditionings and corresponding physical parameters are described in Table 5.1. Conditions were tested consecutively. The control condition (C) simulated the conventional full-scale

operation of cooling tower, with a conductivity of 3500 µS/cm corresponding to a cycle of concentration of 5 of the feed surface water. Dosage of antiscalant and corrosion inhibitors were applied manually. Sulphuric acid for pH control at 7.5 and sodium hypochlorite for disinfection (Brenntag, Switzerland) were added automatically. The conductivity was maintained via discharge of concentrated cooling water and automatic refill with fresh surface water. In all alkaline conditions (pH 9.0, pH 9.4, pH 9.6(a) and pH 9.6(b)), the high purity of the demineralized feed water resulted in a negligible increase in conductivity. No discharge was thus applied. In the first tested alkaline condition, pH 9.0 was reached naturally and sodium silicate was dosed as corrosion inhibitor. In the other tested conditions, pH was increased by manual addition of sodium hydroxide (Brenntag, Switzerland) to pH 9.4 or sodium hydroxide combined to sodium bicarbonate (Brenntag, Switzerland) to pH 9.6. In pH 9.6(b), 120 g of sodium chloride (Esco, Germany) was added to the basin of 1 m^3 to approach the composition of reverse osmosis permeate. The comparison of pH 9.6(a) and pH 9.6(b) showed negligible impact of sodium chloride on the growth of *L. pneumophila* and overall microbiology. pH 9.6(a) and pH 9.6(b) were therefore considered as duplicate tests in this study. In general, conditions were tested for 25 to 35 days. pH 9.0 was prematurely stopped after 21 days due to the high *L. pneumophila* cell count. pH 9.6(b) was operated for 80 days as a long-term validation experiment.

Table 5.1. Description of the main operational parameters and chemical dosages for each of the tested conditions.

Code	Feed water	Final conductivity	Final pH	Days	Chemicals	Dosages
Control C	Surface water	3400 - 3600	7.5	27	Sodium tolyltriazole 40-60 % Phosphoric acid, dispersant and phosphinosuccinic oligomer Polypropylene glycol 20-30 % Sulphuric acid Sodium hypochlorite	2 mg/L 90 mg/L 2 mg/L to pH 7.5 2 mg-Cl_2/L
pH 9.0		280 - 300	9.0	21	Sodium silicate	60 mg-Si/L
pH 9.4		1050 - 1200	9.42	35	Sodium hydroxide Film-forming corrosion inhibitor	280 mg/L 1 g/L
pH 9.6(a)	Demin. water	2700 - 2800	9.57	34	Sodium hydroxide Sodium bicarbonate Film-forming corrosion inhibitor	200 mg/L 2.8 g/L 1 g/L
pH 9.6(b)		2450 - 2600	9.59	80	Sodium hydroxide Sodium bicarbonate Film-forming corrosion inhibitor Sodium chloride	200 mg/L 2.8 g/L 1 g/L 0.12 g/L

The cooling system was emptied and a subsequent two-step chemical cleaning was performed between each experiment. The first cleaning step involved the recirculation of demineralized water and sodium hypochlorite at a concentration of 30 ppm active chlorine for one hour,

which was then discharged. The second cleaning step involved the recirculation of demineralized water with sulfuric acid dosage to pH 2 for one hour. Chemical solutions were flushed away with fresh demineralized water before the next experiment.

5.2.2. Experimental analyses

5.2.2.1. Sampling

Water samples from the cold channel of the recirculation line were collected in sterile glass bottles and kept at 4 °C until analyses. Flow cytometry, plate count and quantitative polymerase chain reaction (qPCR) measurements were performed within 24 hours from the sampling time. For *L. pneumophila* quantification, Aqualab Zuid laboratory followed the standard NEN-EN-ISO/IEC 17025:2017. For 16S and 18S rRNA sequencing analyses, 400 mL of water was filtered through 0.22 µm pore size and 33 mm diameter sterile PVDF syringe filters (Merck, Germany) and the filter membranes containing the biomass were kept at -20 °C for later DNA extraction. Biological duplicates were analysed at the end of each testing period.

5.2.2.2. Total cell count

Samples were diluted by a factor 10 (control C) or 100 (pH 9.0, pH 9.4, pH 9.6(a) and pH 9.6(b)) with ultrapure water prior to the flow cytometry measurements. Preparation of the stock solution of SYBR Green I and staining protocol are described elsewhere (Pinel et al. 2020a). Analyses were performed on a BD Accuri C6 flow cytometer (BD Biosciences, Belgium) with a flow rate of 66 µL/min on 50 µL of sample. The fluorescence intensity was collected at Fl1 = 533 ± 30 nm and Fl3 > 670 nm with a threshold of 700 on FL1. The applied gating strategy was based on a previous paper (Prest et al. 2013).

5.2.2.3. *Legionella pneumophila* quantification

L. pneumophila was quantified by both indirect and direct methods with plate count and qPCR. Water samples were analysed by Aqualab Zuid laboratory, Werkendam, Netherlands.

***Legionella pneumophila* plate count**. Enumeration of colony-forming units of *Legionella* was based on the protocol described in the standard ISO 11731:2017. In short, 1 mL of the water samples was spread on GVPC (glycine vancomycin polymyxin cycloheximide) agar plates. The plates were subsequently incubated at 36 °C. After 10 days of incubation, the plates were examined for colonies matching the *Legionella* species colonies. *L. pneumophila* colonies were then identified by MALDITOF MS (matrix-assisted laser desorption/ionization time-of-flight mass spectrometry) analysis, according to the formerly developed method (Dilger et al. 2016) used at Aqualab Zuid. Measurements were performed every 3 days in average.

Quantitative PCR. qPCR quantification of the number of gene copies of *L. pneumophila* was performed following the method described in NEN 6254:2012, targeting the *mip* gene (macrophage infectivity potentiator surface protein). The forward primer LpneuF 5'-CCGATGCCACATCATTAGC -3', reverse primer LpneuR 5'-CCAATTGAGCGCCACTCATAG -3' and probe LpneuP 5'- 6-carboxyfluorescein

[FAM]TGCCTTTAGCCATTGCTTCCG-BHQ1 -3′ were used for the analysis. *Taq* polymerase was activated at 95°C for 3 min, and was followed by 43 cycles of denaturation at 95°C for 20 s and annealing/extension at 60°C for 60 s.

5.2.2.4. DNA extraction and sequencing

The membrane filters containing the biomass collected from the water samples were recovered from the syringe filter units (Merck, Germany) for further processing. The genomic DNA was extracted using the DNeasy UltraClean Microbial Kit (Qiagen, Netherlands). The company's standard method was followed during the extraction, with an additional lysis step. The lysis step consisted of 5 min of heating at 65 °C and 5 min of bead-beating on a Mini-Beadbeater-24 (Biospec, USA) for cell disruption of the collected biomass. The quality of the extracted DNA was assessed by gel electrophoresis and size check (465 bp) after qPCR amplification. Samples were sent to Novogene Ltd. (Hongkong, China) for amplicon sequencing of the V4 region (position 528-706) of the 18S-rRNA gene and the V3-4 region (position 341-806) of the 16S-rRNA gene on an Illumina paired-end platform. The raw 18S-rRNA gene sequences were processed by Novogene Ltd. (Hongkong, China) using QIIME software. Chimeric sequences were detected with UCHIME and removed. Operational taxonomic units (OTUs) were generated using UPARSE. Potential protozoan hosts for *L. pneumophila* were identified based on a previous study (Boamah et al. 2017). The raw 16S-rRNA gene sequences were processed with the software Mothur v.1.40.5 for quality filtering, alignment, chimera check and taxonomic classification. Generation of OTUs were performed based on 97 % similarities between the representative sequences after removal of singletons. The alignment and taxonomic classifications were performed using the SILVA database. Potentially pathogenic bacterial genera were identified based on the list available in the drinking water quality guidelines (WHO 2011). The main representative OTU sequences were compared to the RefSeq NCBI database using the Basic Local Alignment Search Tool (BLAST) for species identification. Results from the duplicate samples are shown in Figures S1, S2 and S3. The DOI of the raw sequencing data is available in supplementary material.

5.2.2.5. Beta diversity analysis

Diversity analyses were performed on the bacterial OTUs generated after the 16S-rRNA gene amplicon sequencing and their relative abundances in the samples. Beta diversity measurement was assessed with principal component analysis (PCA) in Mothur v.1.40.5 using the thetaYC distance matrix. The spatial separations visualized in the PCA provide information on the similarity between samples.

5.2.2.6. Abundance of prokaryotes and eukaryotes

qPCR was performed to determine the absolute abundance of prokaryotes and eukaryotes by quantifying the number of copies of the 16S and 18S genes respectively. The 16S DNA standards were commercially obtained. A product template for the 18S DNA was initially created by PCR to be used as standard. The template was based on the DNA extracted from the alkaline test pH 9.6(b). The DreamTaq Green PCR Master Mix (ThermoScientific, USA)

was combined to the forward primer Euk1F 5'- ACC-TGG-TTG-ATC-CTG-CCA-GT -3', reverse primer Euk563R 5'- ACC-AGA-CTT-GCC-CTC-C -3' and pH 9.6(b) DNA extract according to manufacturer's specifications. PCR was performed with the following settings: 30 cycles of 30 s at 95 °C, 40 s at 55 °C and 40 s at 72 °C. The size of the amplified DNA fragments was assessed by agarose gel electrophoresis. Amplified DNA was purified following the Monarch DNA Gel Extraction Kit (BioLabs, UK). A final DNA concentration of 10.7 ng/L was measured with a Qubit fluorometer (Invitrogen, USA).

Serial dilution from 10^{-3} ng DNA/µL to 10^{-8} ng DNA/µL were used to generate the standard curves. qPCR reaction mix were composed of 0.2 µL of each primer at a concentration of 50 µM, 10 µL of IQTM SYBR® Green Supermix (Bio-Rad, USA), 7.6 µL of nuclease-free water (Sigma-Aldrich, UK) and 2 µL of the extracted DNA from the cooling water samples. The primer sequences, thermal profiles and qPCR efficiencies are described in Table S1.

5.3. RESULTS

5.3.1. Total cell counts

Total bacterial cell concentration in the cooling water was measured by flow cytometry throughout the testing periods. Figure 5.1 shows the cell number reached at the end of each experiment, averaged over the last 5 days. The control C, subjected to chlorine disinfection, had the lowest bacterial cell number with 6.6 (± 1.5) × 10^7 cells/L. Chlorine disinfection applied in the control experiment was efficient in maintaining a low cell count. The alkaline conditions, without biocide dosage, reached 3.2 (± 0.5) × 10^9 cells/L at pH 9.0, 1.7 (± 0.1) × 10^{10} cells/L at pH 9.4, 1.4 (± 0.4) × 10^{10} cells/L and 5.2 (± 0.4) × 10^9 cells/L at pH 9.6 (a) and (b) respectively.

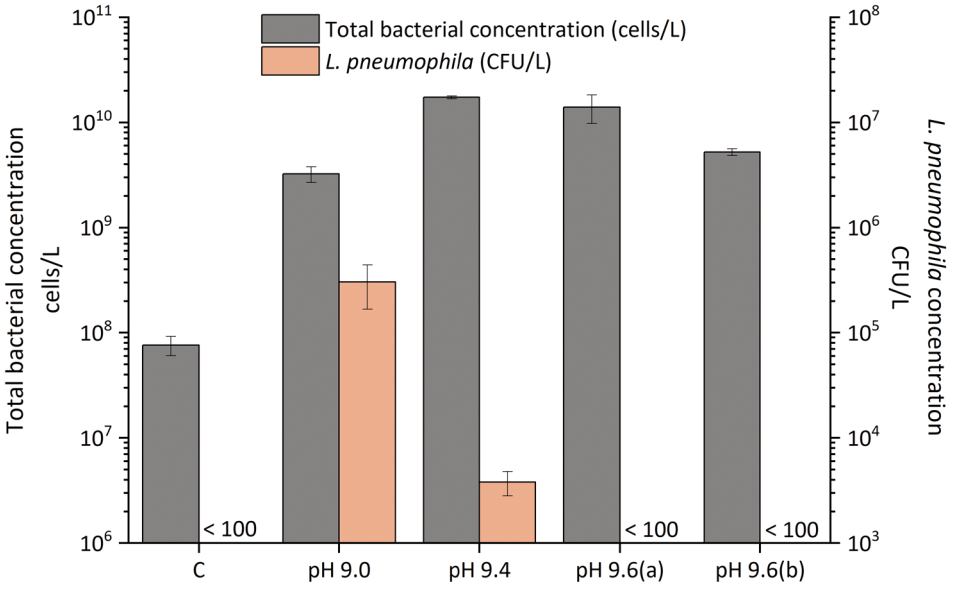


Figure 5.1. Bacterial cell counts reached in the cooling waters at the end of the experiments (Table 5.1) and concentrations of *Legionella pneumophila* in cell forming units over the 5 last days of each experiment. The control experiment C and alkaline conditioning at pH 9.6 had a *L. pneumophila* concentration below detection limit (100 CFU/L).

5.3.2. Impact of increasing pH on *Legionella pneumophila* growth

The *L. pneumophila* plate count results for the 5 pilot tests are also shown in Figure 5.1. The control test (C) performed under the conventional full-scale operating conditions, at pH 7.5 with sodium hypochlorite dosage, maintained a *L. pneumophila* concentration below the detection limit of 100 CFU/L during the entire 27 days of operation. Tests at pH 9.0 and pH 9.4 exceeded the tolerated limit of 10^3 CFU/L set on the industrial site, with 3.0 (± 1.4) × 10^5 and 3.8 (± 1.0) × 10^3 CFU/L respectively at the end of the experiments. qPCR measurements confirmed the presence of *L. pneumophila* with values reaching up to 4.4 × 10^5 and 950 gene copies/L at pH 9.0 and pH 9.4 respectively. Demineralized water at pH 9.0 seems to constitute a suitable environment for the growth of *L. pneumophila* in cooling towers. The ability of the pathogen to also grow at pH 9.4 was unexpected and therefore indicates that pH 9.4 is not alkaline enough to prevent proliferation. The duplicates at pH 9.6 both maintained *L. pneumophila* at very low or undetectable levels during the duration of the experiments, i.e. 34 and 80 days respectively. pH 9.6(a) reached 200 CFU/L on day 19 and 100 CFU/L on day 21 while pH 9.6(b) reached 100 CFU/L on days 14 and 35. In the other plate count analyses (87 %), the concentration was below quantification limit (<100 CFU/L) while all qPCR measurements were below quantification (<375 gene copies/L). The results demonstrate that a pH of or above 9.6 constitutes an efficient conditioning for the control of *L. pneumophila* in open recirculating cooling tower, alternatively to biocide dosage.

5.3.3. Relative abundance of 18S (eukaryotic) to 16S (prokaryotic) gene copies

The absolute quantification of the 18S and 16S gene copies by qPCR allowed an estimation of the proportion of eukaryotic DNA to prokaryotic DNA in the samples. The ratio of 18S to 16S gene copies is displayed in Figure 5.2. All samples contained a substantially lower amount of eukaryotic gene copies compared to prokaryotic gene copies, between 1 log and 3 log fewer. Control C at pH 7.5 and tests at pH 9.4 and pH 9.6 (a) and (b) display a ratio of 18S:16S gene copies of 1.7 (± 0.3) × 10^{-3}, 0.9 (± 0.2) × 10^{-3}, 1.4 (± 0.5) × 10^{-3} and 3.5 (± 2.2) × 10^{-3} respectively.

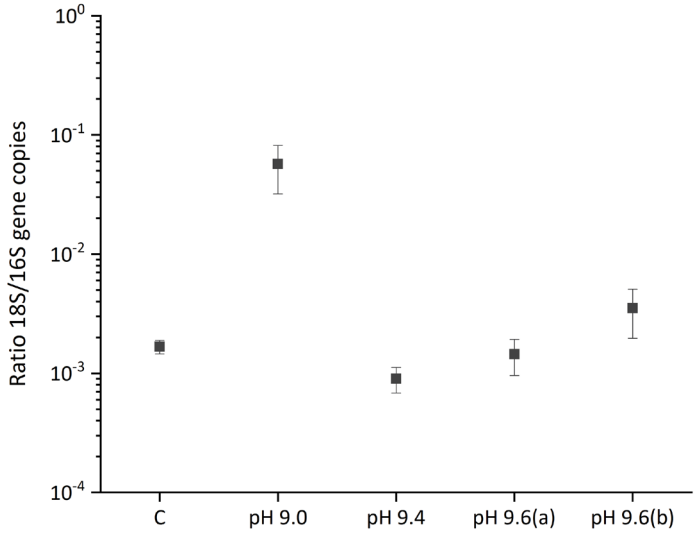


Figure 5.2. Ratio of the number of gene copies of 18S to 16S in the cooling water samples illustrating a higher relative abundance of eukaryotic DNA to prokaryotic DNA at pH 9.0 compared to the other tested conditions.

At pH 9.0, however, the ratio strongly diverges from the other samples with 5.7 (± 3.5) × 10^{-2} indicating a relatively higher concentration of eukaryotic DNA per prokaryotic DNA with more than 1 log difference when compared to the other tested conditions. The greater relative fraction of eukaryotic DNA at pH 9.0 may suggest a higher availability of potential hosts for *L. pneumophila* to multiply intracellularly.

5.3.4. Presence of potential protozoan hosts of *Legionella pneumophila*

Identification of the eukaryotic community members was performed with 18S rRNA gene sequencing of the DNA extracted from the cooling water samples to assess the presence of potential hosts. Figure 5.3.A shows the relative abundances of the main eukaryotic kingdoms. Protista and Chromista kingdoms accounted for the majority of the reads in the control C and at pH 9.0 and pH 9.4 with 43 (± 10) %, 62 (± 5) % and 58 (± 8) % respectively. The duplicate tests at pH 9.6 show only 2 (± 1) and 3 (± 1) % of Protista and Chromista abundances, with dominance of the kingdom Fungi. The combination of the results from Figures 5.2 and 5.3.A therefore suggests a limited availability of hosts for *L. pneumophila* at pH 9.6 due to the low occurrence of protists. The composition and structure of the protist communities diverged strongly (Figure 5.3.B) between the tested conditions. The test at pH 9.0, with the highest *L. pneumophila* concentration and eukaryotic to prokaryotic DNA ratio, was largely dominated by the genera *Spumella* from the phylum *Ochrophyta* and *Vorticella* from the phylum *Ciliophora*. These genera were also identified in the other tested conditions in lower abundances.

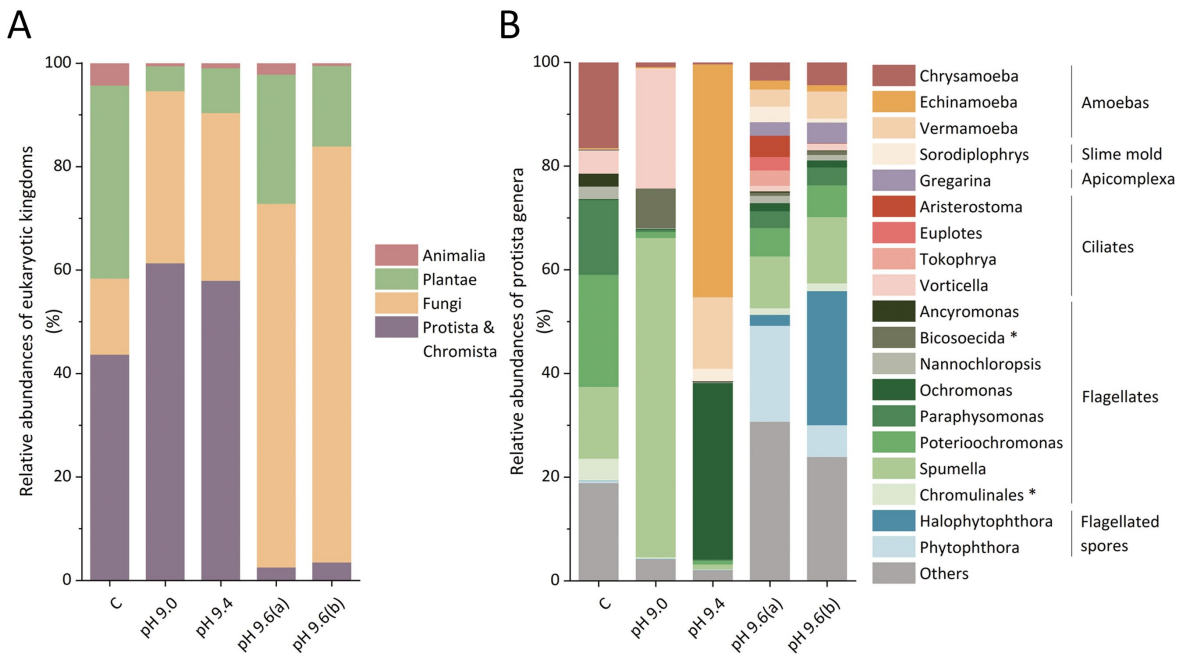


Figure 5.3. Relative abundances of eukaryotic kingdoms (A) and composition of the kingdoms Protista and Chromista at the genus taxonomic level (B) in the cooling water samples analysed with 18S rRNA gene amplicon sequencing. The category "Others" accounts for the operational taxonomic units of relative abundance lower than 2 %. The symbol * indicates the order taxonomic level when the genus was not identified. Duplicate data are shown in supplementary material (Figures S1 and S2).

The duplicate tests at pH 9.6 show high similarity with each other regarding the protist community structures, dominated by *Spumella, Phytophthora* and *Halophytophthora*. *Halophytophthora* is a reclassified genus previously assigned to *Phytophthora*, but regrouping marine species (Cooke et al. 2000). As the only difference in the operation of the duplicate tests was the dosage of NaCl, the higher salt concentration in pH 9.6(b) likely justifies the increase in relative abundance of *Halophytophthora*.

The protist organisms present in the water samples were then compared to experimentally defined hosts and potential hosts of *L. pneumophila* (Boamah et al. 2017). Their presence among the protist community of each test can be visualized in Table 5.2. The control condition showed a negligible abundance of known hosts representing only 0.1 % of the total eukaryotic reads. A similarly low number was obtained at pH 9.0 with 0.2 % despite the high concentration of *L. pneumophila* measured, suggesting the presence of non-identified *Legionella* hosts. The two commonly found *L. pneumophila* hosts *Echinamoeba exudans* and *Vermamoeba vermiformis* added up to 59 % of the protist community and 33 % of the whole eukaryotic community at pH 9.4. They also constituted an important fraction of the protists at pH 9.6, 5 % and 6 % respectively, but representing only 0.1 % and 0.2 % of the eukaryotic communities due to the low abundance of protists at this pH.

Table 5.2. Relative abundances of potential protozoan hosts in the water samples based on the 18S rRNA gene amplicon sequencing results.

Potential hosts (% of protist reads)	C	pH 9.0	pH 9.4	pH 9.6(a)	pH 9.6(b)
Acanthamoeba_unidentified *	0.01%	-	-	-	-
Echinamoeba exudans *	0.14%	0.23%	44.93%	1.73%	1.20%
Vermamoeba vermiformis *	0.15%	0.17%	13.81%	3.29%	5.20%
Aspidisca_unidentified	0.02%	-	-	0.02%	0.02%
Colpoda_unidentified	-	0.01%	0.02%	0.15%	0.26%
Euglypha rotunda	-	0.01%	0.01%	0.04%	0.01%
% of total protist reads	**0.3%**	**0.4%**	**58.8%**	**5.2%**	**6.7%**
% of total eukaryotic reads	**0.1%**	**0.2%**	**32.8%**	**0.1%**	**0.2%**

* experimentally defined host

5.3.5. Presence of potentially pathogenic bacteria under different alkaline pH

The abundance of potentially pathogenic bacterial genera, based on the drinking water quality guideline (WHO 2011), are listed in Figure 5.4. The control C at pH 7.5 and test at pH 9.0 show a greatly higher total relative abundance of potential pathogens (7.1 ± 0.9 % and 5.7 ± 0.3 % of the total reads) compared to the tests at pH 9.4 and pH 9.6 (1.6 ± 0.1 %, 1.3 ± 0.1 % and 0.2 ± 0.1 %). *Pseudomonas* and *Enterobacter* are the most abundant in the control test, representing 4.2 ± 0.1 % and 1.8 ± 0.3 % respectively, while *Bulkhoderia* and *Legionella* are dominant at pH 9.0, with 1.5 ± 0.6 % and 1.4 ± 0.1 %, with also considerable abundance of *Enterobacter* (1.3 ± 0.8 %). The increase from pH 9.0 to pH 9.4 therefore considerably reduced the potential pathogenicity of the cooling water based on the results from the 16S rRNA gene amplicon sequencing.

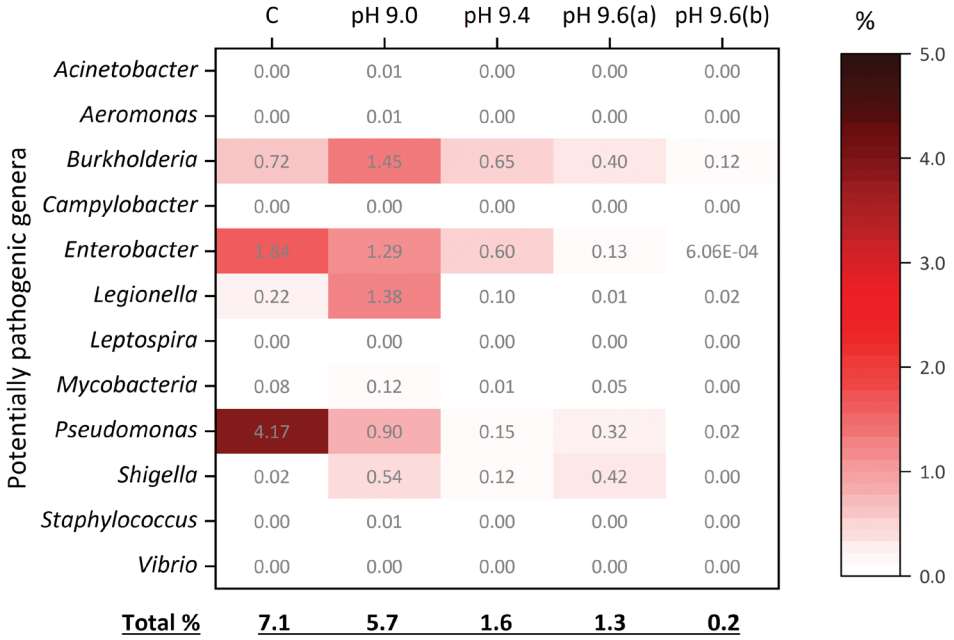


Figure 5.4. Heatmap of the potentially pathogenic genera obtained from 16S rRNA gene amplicon sequencing of the cooling water DNA samples. The concentration is expressed in % of the bacterial relative abundance.

5.3.6. Overall bacterial community analyses

Figure 5.5 shows the relative abundance of the main bacterial genera in the cooling water samples. Variations in microbial community structure can be observed between the different tests with a switch in the main bacterial members. At the phylum level, the main difference originated from the promotion of *Bacteroidetes* at alkaline pH. While it accounted for 3 ± 1 % of the relative abundance in control C, it reached 38 ± 6 % and 62 ± 1 % at pH 9.6. The remaining abundance was mainly represented by *Alpha* and *Gammaproteobacteria*. At the genus level, *Sphingobium* was dominating in the control C at 44 ± 8 % but was present in negligible abundance at alkaline pH with less than 0.5 %. pH 9.0 and pH 9.4 show a more distributed relative abundance among genera, with the main members being *Sediminibacterium* at pH 9.0 (22 ± 4 %) and *Sphingopyxis* and *Flavobacterium* at pH 9.4 (28 ± 6 % and 16 ± 6 % respectively). The duplicates at pH 9.6, however, display similarity in main community members. The genus *Mongoliitalea* from the family *Cyclobacteriaceae* dominated both cooling waters with 35 ± 5 % and 60 ± 1 % although (a) contained also large abundances of *Phenylobacterium* (21 ± 6 %) and *Aliihoeflea* (13 ± 3 %) genera. The blast of the OTU representative sequence corresponding to the *Mongoliitalea* genus resulted in 99.56 % similarity in identity with the species *Mongoliitalea lutea*, a facultative alkaliphilic and halotolerant bacterial species collected from haloalkaline lakes. The other 26 main OTUs whose genera are displayed in Figure 5.5 were also compared to the database. Among those, the representative sequences of the members labelled with the symbol * showed over 99 % similarity with sequences of known alkaliphilic or alkalitolerant species i.e. with optimum pH ≥ 9.0 or optimum pH < 9.0 but able to grow at higher values. The sum of the relative abundances of these OTUs was calculated for each experimental test. While confirmed alkalitolerant organisms accounted for only 9 % of the relative abundance of the main

members in control C, their abundance increased with increasing pH: 28 % at pH 9.0, 56 % at pH 9.4, 69 % and 78 % at pH 9.6 for (a) and (b) respectively. As expected, the higher fraction of identified alkaliphilic bacteria at pH 9.4 and 9.6 implies that neutrophilic bacteria were outcompeted.

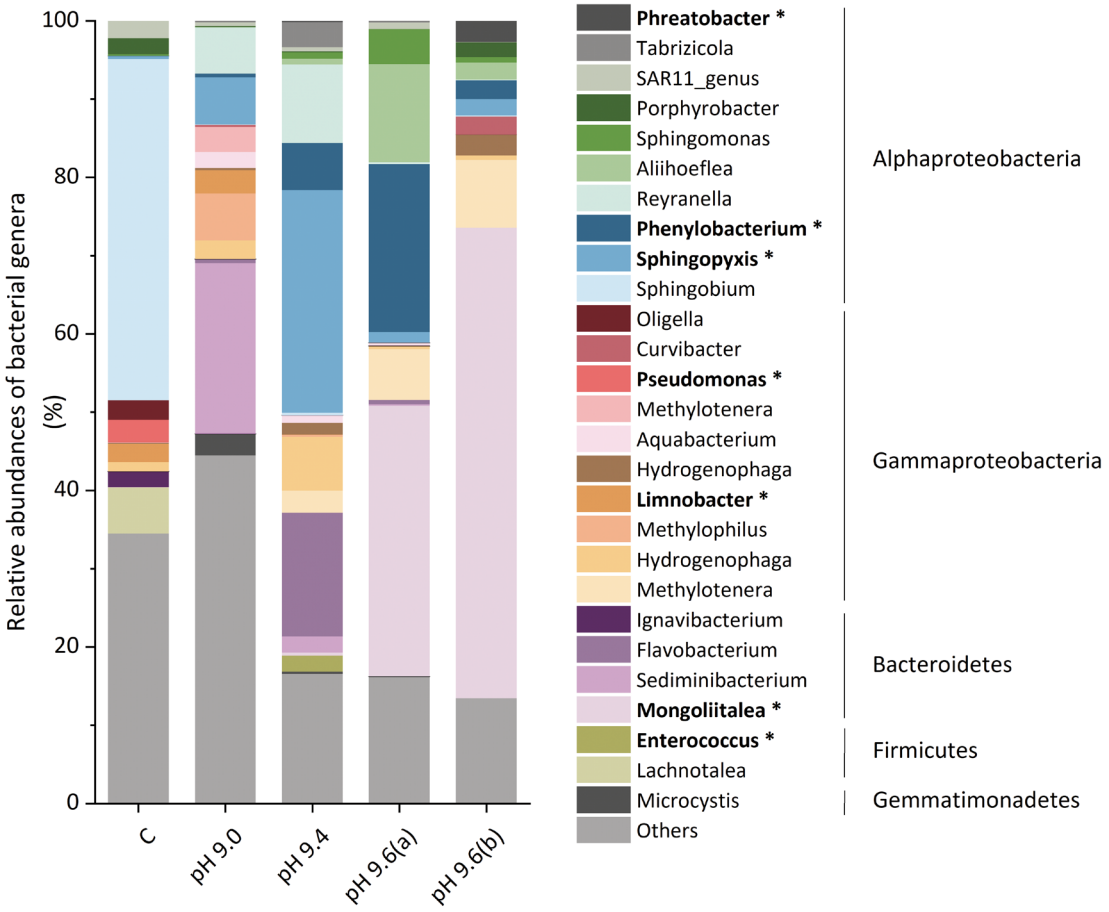


Figure 5.5. Composition of the bacterial communities at the genus taxonomic level obtained from 16S rRNA amplicon sequencing of the cooling water DNA samples. The category "Others" accounts for the operational taxonomic units of relative abundance lower than 2 %. The symbol * indicates alkalitolerant and alkaliphilic microorganisms i.e. whose representative sequence corresponds - with more than 99 % similarity in identity - to a species able to grow at pH above 9. Duplicate data are shown in supplementary material (Figure S3).

The principal component analysis in Figure 5.6 represents the similarities between samples taking into account the members of the communities and their relative abundances. The bacterial communities at alkaline pH all diverge from the control C at pH 7.5. Among the tested conditions, samples from pH 9.6(a) and pH 9.6(b) form a close cluster on the chart validating the reproducibility of the result for the conditioning at pH 9.6.

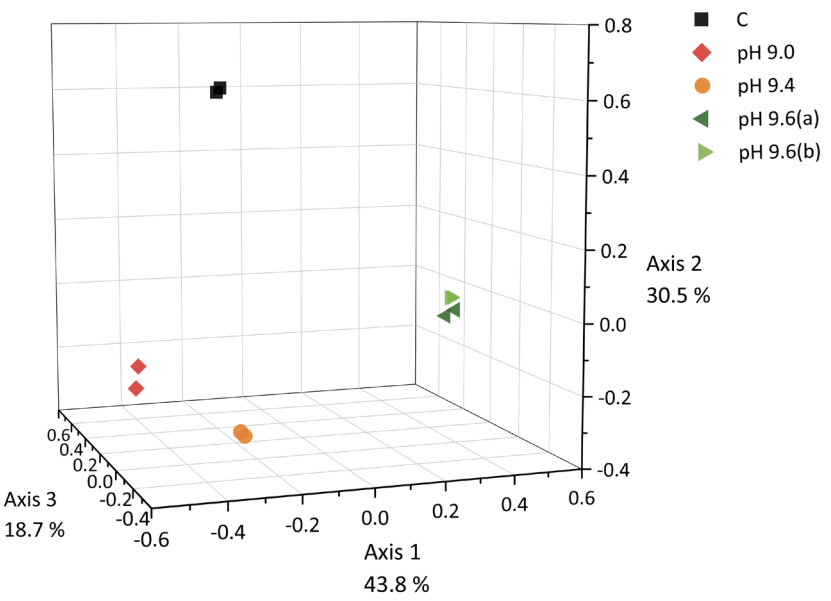


Figure 5.6. Principal component analysis (PCA) of bacterial community structures of the cooling water samples based on operational taxonomic units. The bigger the distance between data points, the stronger the dissimilarity in community structures. Duplicate samples are shown for each condition.

5.4. DISCUSSION

5.4.1. Cooling tower operation at pH ≥ 9.6 efficiently prevents growth of *Legionella pneumophila*

Previous research based on a survey of 40 cooling towers operated in a pH ranging from 6.9 to 9.1 reported higher *L. pneumophila* densities at pH between 8.4 and 9.1 (Yamamoto et al. 1992). An earlier laboratory-controlled pH batch experiment on cooling water samples showed, however, a better correlation of the pathogen growth with neutral pH values (States et al. 1987). These disparities in results point out the significant impact of operational conditions, water sources and surrounding environment on the growth of *L. pneumophila* in full-scale cooling systems. According to the available literature, *L. pneumophila* has been shown to grow at pH up to 9.2 but was not able to multiply from pH 10 (Brouse et al. 2017, Ohno et al. 2003, Wadowsky et al. 1988). An important lack of information remained about the survival of the pathogen within the pH range from 9.2 to 10. Our study explored this gap and revealed that *L. pneumophila* can survive and grow at pH 9.4 although at a much lower concentration than at pH 9.0. Most importantly, *L. pneumophila* was unable to grow at pH 9.6.

Multiplication of *Legionella* in engineered water systems requires presence of host protozoan cells (Lau and Ashbolt 2009, Wadowsky et al. 1988). The amount and nature of nutrients in cooling water is generally too limited for *L. pneumophila* multiplication as a free-living organism since it requires a complex medium to grow, including compounds like amino acids as carbon and energy source (Sauer et al. 2005, Tesh and Miller 1981, Tesh et al. 1983) or fatty acids (Fonseca and Swanson 2014). The intracellular components of some amoebas such as *Acanthamoeba*, *Vermamoeba* and *Naegleria sp.* (Jjemba et al. 2015, Lau and Ashbolt 2009, Molofsky and Swanson 2004) and ciliated protozoa (Caicedo et al. 2018, Fields et al. 1984)

therefore constitute an appropriate reservoir for the growth of the pathogen. When encountering extreme conditions such as chlorine disinfection, protozoa go through a mechanism of encystment to protect themselves from the hostile environment (Kilvington and Price 1990). Contrarily to their vegetative trophozoite form, cysts of protozoa are in a dormant state and do not proliferate but their reinforced cell wall constitutes a strong protection for the intracellular pathogens.

Our study reflects a positive correlation between the abundance in protozoa (Figure 5.2 and 5.3.A) and *L. pneumophila* counts (Figure 5.1), as was also reported in the cooling tower survey by Yamamoto et al. (1992). The dominance of protozoa at pH 9.0 and 9.4 and the higher eukaryotic to prokaryotic DNA ratio at pH 9.0 suggest the presence of unicellular trophozoite reservoirs to sustain the observed *L. pneumophila* growth in these two tested conditions. In the duplicate test runs at pH 9.6, a poor abundance of protists was observed concomitant with a good control of *L. pneumophila* growth. The relative abundance of potential hosts (Figure 5.3.B) in our samples - based on a suggested list derived from laboratory experiments and environmental analyses (Boamah et al. 2017) – did, however, not show a clear correlation. At pH 9.0, the *L. pneumophila* counts were substantial but previously reported hosts were present only in minor abundance contrarily to pH 9.4 (Table 5.2). *Spumella* (flagellate) and *Vorticella* (ciliate), the main genera at pH 9.0, have not been reported as hosts but the limited knowledge available on the specificity of *L. pneumophila* for intracellular replication (Guerrieri et al. 2005) as well as the non-exhaustive list of cultivated natural hosts cannot exclude their involvement. In the experiments at pH 9.6, the fraction of the protist abundance of reported hosts (5.2 and 6.6 %) represented a negligible fraction of the total abundance of eukaryotes. In fact, the eukaryotic communities were mainly represented by fungi. Our study revealed a substantial impact of the pH increase from 9.4 to 9.6 on the limitation of protists and a direct correlation with the inhibition of *L. pneumophila* growth.

5.4.2. Increased pH leads to a strong bacterial community shift with lower pathogenicity

A strong shift in bacterial community was observed between the control experiment at pH 7.5 with chlorine disinfection and each of the tested alkaline pH conditions with no disinfection. *Proteobacteria* dominated the control C with *Sphingobium sp.*, a recurrent organism in drinking water and cooling systems subjected to chlorination due to their oligotrophic nature and ability to grow in biofilm mode (Paranjape et al. 2020b, Shaw et al. 2015, Vaz-Moreira et al. 2011). *Pseudomonas* is also a common genus encountered in cooling systems under disinfection (Paranjape et al. 2020b). Bacterial community composition and relatively low total cell count in the control experiment therefore reproduced the environment of a full-scale system under standard operation. Regarding alkaline conditions, the abundance of *Bacteroidetes* was previously shown to positively correlate with increasing pH (Ganzert et al. 2014, Lauber et al. 2009) as it is the case in our study. *Mongoliitalea Lutea* (*Cyclobacteriaceae, Bacteroidete*) is a facultative alkaliphile able to develop at pH between 6.5 and 12.0 with an optimum at pH 9.0 (Yang et al. 2012). However, it was in our study only present in negligible abundance at pH 9.4 while it was the dominant bacterium at pH 9.6. Our data suggests that the pH increase from 7.5 (C) to 9.6 gradually limited the growth of

neutrophiles and promoted alkalitolerant and alkaliphilic species such as *Mongoliitalea Lutea*. Driving pH away from neutral value requires bacteria to adjust their proton motive force to maintain a cytoplasmic pH in the neutral range (Krulwich et al. 2011). In too alkaline conditions, the proton concentration in the surrounding water is not sufficient and causes the cell to decay while alkaliphile organisms rely on alternative membrane processes such as additional antiporters and coupled membrane transporters using sodium motive force (Padan et al. 2005). Most pathogenic bacteria show optimum growth at physiological pH which allows them to infect and proliferate within the body (Kim and Ndegwa 2018) but will decay when facing extreme conditions. The decrease in relative abundance of potentially pathogenic bacteria that we observed correlated with this assumption, with a strong decline from 7.1 % to 1.6 % between pH 7.5 and pH 9.4. Limitations remain regarding the use of amplicon sequencing for the assessment of pathogenicity, related to the limited resolution of the technique, the varying gene copy number per organism, and the lack of data available on pathogenic microorganisms. To accurately assess the levels of pathogens, alternative methods should be applied such as qPCR analyses of the known species which will provide absolute concentrations for the comparison of tested conditions. Yet, the observed loss of overall potential pathogenicity of the water at elevated pH, based on abundance of genera, suggests an extra advantage of alkaline cooling tower conditioning.

5.4.3. Implications for industry

In the presented study, the pilot cooling tower was operated at different alkaline pHs and demineralized water was selected as feed water. The suggested conditioning does not require use of disinfectant and maintains a simpler chemistry compared to standard practice, with the use of less chemicals while controlling the pathogenicity of the water. It also anticipates a potential increase in regulations which could be expected in the coming years regarding the discharge of disinfection by-products to the environment.

The use of demineralized water or reverse osmosis permeate has several advantages. The very low content in minerals prevents scaling formation on the surface of the cooling unit thus no dosage of antiscalant – often contributing to a higher nutrient content of the water - is required. The limited concentration in organic compounds considerably restricts the formation of biofilm and therefore the proliferation of protozoa and associated bacteria. Last but not least, the high water quality allows to operate the cooling tower with almost zero discharge, thus maintaining a stable cooling water chemistry which ensue limited dosage of base (NaOH) for alkaline pH control. However, important parts of the process i.e. pumps, spray nozzles and heat exchangers will be prone to corrosion due to the nature of the water. Corrosion is a major drawback shortening the lifetime of the exchangers, leading to system deficiencies and providing space for microbial communities to grow. This phenomenon needs to be tackled with alternative material choices or addition of inhibitors effective at high pH and not contributing to the nutrient and mineral content of the water.

For cooling towers already operating with other water sources such as surface water or ground water, the switch to high feed water quality is not always possible. Economic feasibility needs to be assessed for each specific case, considering that reverse osmosis permeate or demineralized water can cost between 0.5 and 1 euros higher per m^3 than surface

water. If the original water source is kept, removal of divalent ions (calcium, magnesium) from the feed water is necessary to lower the hardness and switch to alkaline pH without a substantial increase in scaling potential (Hensley 1985, Matson and Harris 1979). Corrosiveness is also a major challenge with softened water, especially due to the high chloride concentration, and needs to be addressed to avoid deterioration of the heat exchangers. Finally, attention should to be paid to the pre-existence of biofilm in the system and a deep cleaning would be required to avoid contamination when converting to alkaline conditioning.

5.4.4. Recommendations for further research

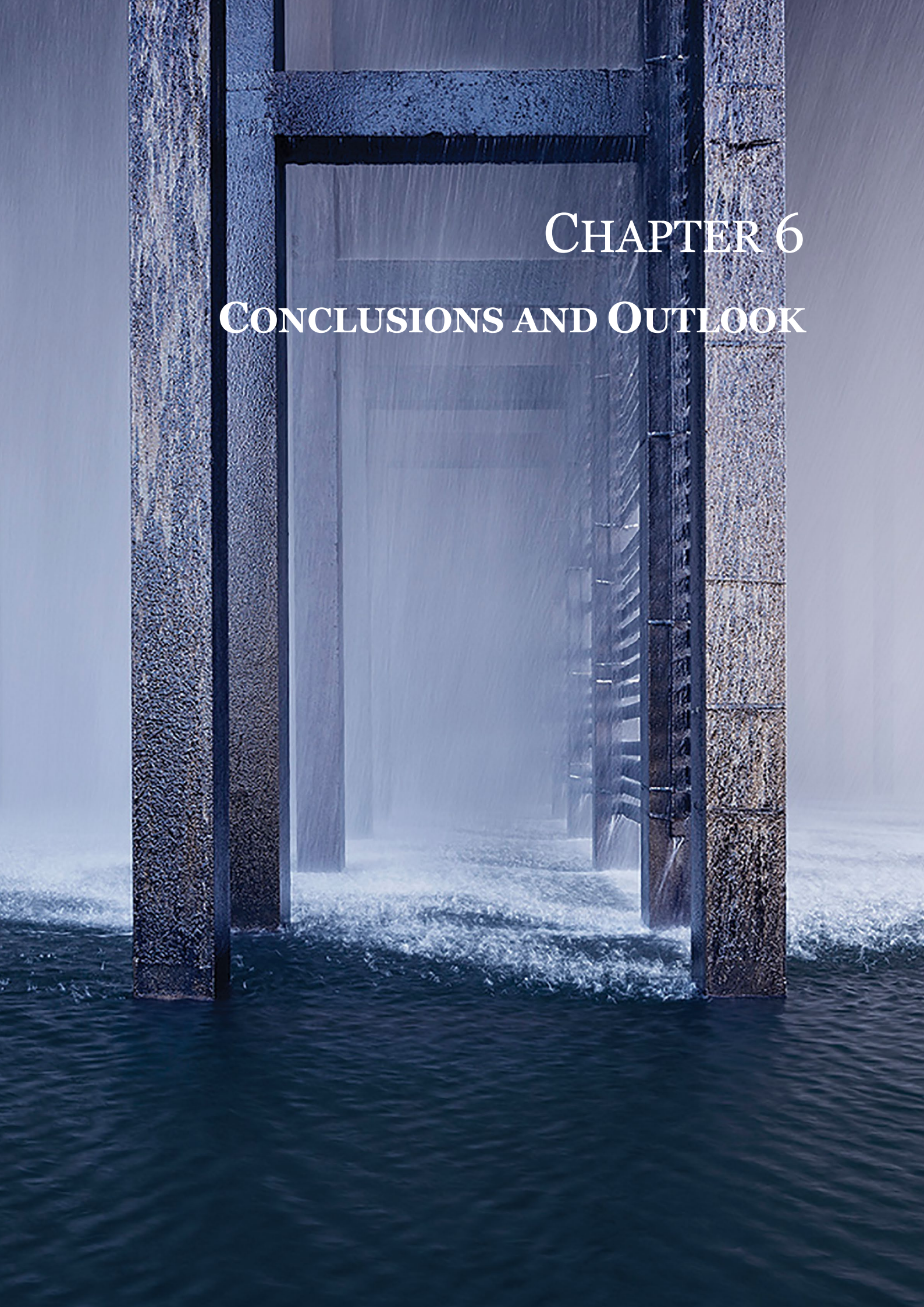
Past studies suggested that the infection of protozoa by *Legionella* mainly occurs in biofilms (Abdel-Nour et al. 2013, Murga et al. 2001) which provides a more stable environment to multiply e.g. on the wall of the cooling tower basins or packing fill surface. Although a biofilm is not expected to have formed in significant amount during the experimental periods and with the high water quality used, this phenomena can contribute to *L. pneumophila* proliferation. Further research investigating biofilm development and composition in terms of bacterial and eukaryotic communities at alkaline pH would help understand the biological interactions, the biofouling potential and potential for *Legionella* to develop inside an alkaline biofilm.

Overall, the operation of cooling towers at pH equal or above 9.6 without disinfectant dosage represents an attractive alternative to the conventional practices. The comparison of these results to the microbiome of full-scale cooling towers operating at high pH on the long-term is important to validate the industrial applicability.

5.5. CONCLUSIONS

The investigation of alkaline conditioning in a pilot-scale cooling tower (1 m^3/h) - operated with demineralized water as feed water and without addition of disinfectant - for the control of *Legionella pneumophila* and other potentially pathogenic genera showed that:

- Growth of *L. pneumophila* was prevented in the cooling tower maintaining a pH ≥ 9.6 without biocide dosage. *L. pneumophila* was, however, still able to grow at pH up to 9.4.
- The overall suspected pathogenicity of the cooling water microbial community declined with increasing alkalinity. Divergence of the alkaline operating pH from physiological pH and dominance of the bacterial community by alkaliphilic microorganisms suggests a reduced biological health threat to the operators and surrounding population when in contact with aerosols.
- Operating at pH ≥ 9.6 is a more sustainable and simpler cooling tower conditioning than dosing disinfection chemicals. It anticipates potential future regulations, further restricting discharge of disinfection by-products. Nonetheless, this alternative pathogen control method requires attention in regard to scaling and corrosion potential and choice of materials.



Chapter 6

Conclusions and Outlook

This thesis aimed at providing a better understanding of biofouling in evaporative cooling systems and investigated different directions for the prevention and mitigation of microbial growth, with a focus on *Legionella pneumophila*, and biofilm development. The findings presented in the research chapters contribute to the current knowledge in the field of cooling and were used to suggest an alternative approach to conventional operation. They also lead to new questions, opening the way to further investigations. Both are summarized in this section.

6.1. GENERAL CONCLUSIONS

The characterization of factors involved in the microbial growth and biofilm composition in open recirculating cooling systems showed the following:

- A mass balance approach combining flow cytometry and 16S rRNA gene amplicon sequencing was established and allowed the assessment of bacterial community changes between feed water and cooling water (**Chapter 2**).
- Application of the above-mentioned method to a cooling system operated with continuous chlorine dosage indicated that the difference in decay rate of bacterial members was the main factor for bacterial group selection in cooling water (**Chapter 2**).
- The temperature change along heat exchanger affects the biofilm, in terms of bacterial community structure and composition of extracellular polymeric substances (EPSs) (**Chapter 3**).
- Biofilm microbiome develops independently from planktonic microbiome and adapts to local conditions in the cooling system (**Chapter 3** and **4**).
- The presence of sialic acids in the extracellular matrix of biofilms collected from cooling systems suggests an essential role of these compounds in structural cohesion or biofilm protection (**Chapter 4-additional**).

Investigation of approaches for microbial growth control in pilot evaporative cooling systems revealed that:

- Cooling system operation at pH 9.6 efficiently prevented *L. pneumophila* growth and reduced the overall pathogenicity of the water compared to more neutral pH (**Chapter 5**).
- Phosphorus limitation in the cooling water restricted the growth of microorganisms in the biofilm but did not limit biofilm formation (**Chapter 4**).
- A well-maintained feedwater line is of critical importance when using extensively treated feed water, to avoid water quality deterioration (**Chapter 4**).

In addition, a high importance was given to onsite investigations and experiments in this project in order to collect results representative of large-scale cooling systems. Open recirculating cooling systems have a complexity that is difficult to represent at laboratory scale. Pilot set-ups have the advantage to be open to the outside environment and subjected to varying conditions which can also affect biofouling, such as precipitation, air quality or presence of particles. Applied research at pilot-scale should be promoted, as it is a valuable

step in the development of alternative conditionings with efficient assessment of potential industrial application.

6.2. FURTHER RESEARCH LINES AND RECOMMENDATIONS

The findings described in this thesis represent a solid basis for future investigations and implementation of new operational strategies. Some recommendations are summarized below.

The mass balance approach for quantification of bacterial changes established in Chapter 2 should be applied to future studies of cooling water microbiome. Results would provide valuable information on selection of bacterial groups and avoid misinterpretation of data sets based on relative abundances. If the mass balance method indicates growth of specific bacterial groups associated to pathogens, risks of outbreak can be anticipated and early mitigation actions can be implemented. In addition, application of this mass balance to a large number of cooling towers can help identify redundant organisms surviving chlorinated environment. These organisms are of interest in the study of chlorine resistance.

The bacterial order Obscuribacterales was the only bacterial group consistently showing net increase in our full-scale cooling tower study (Chapter 2) operated with continuous residual chlorine. This group has been observed in other full-scale cooling towers subjected to oxidative disinfection (Paranjape et al. 2020b, Pereira et al. 2017). Lower taxonomic ranks were not identified, but investigation of the species corresponding to the obtained representative sequences could lead to identification of chlorine resistant organisms. Their characterization and the study of their morphological and physiological features, e.g. related to membrane permeability or presence of an outer layer, would provide insight on their survival ability in such a harsh environment.

Nutrient availability, location in the system and temperature were shown to affect biofilm composition (Chapters 3 and 4). While the applied analysing techniques provided valuable insights on the communities and EPS compositions in cooling systems, they only allowed for partial characterization of biofilm layers. Efforts are made to better identify the components of EPS matrix and their functions (Boleij et al. 2019, Seviour et al. 2019). In general, a better understanding of the chemical composition of the EPS matrix can help in the development of efficient biofilm removal techniques.

Chapter 4 showed heavy pollution of the cooling systems by sand particles carried by the wind and accumulating in the cooling tower basins as well as on the surfaces. Chapter 2 revealed that 21 % of the taxa shared between feed water and chlorinated cooling water of the studied cooling tower accounted for 92 % of the total sequences (average values). It is reasonable to assume that a part of the remaining sequences identified in the cooling water - and represented by a large number of OTUs - result from inoculation of the water with airborne bacteria. Both studies expose the strong influence of weather conditions, particles or air contaminants on the quality of cooling water. Development of methods for the assessment of these factors, mainly in term of microbial composition and nutrient contents, would help characterizing the extent of these impacts on cooling water quality. For systems prone to contamination from the environment, the focus should be oriented towards the

implementation of treatments incorporated in the recirculation line of the cooling towers. There have been some previous studies on the removal of nutrients by internal treatment of the cooling water, for example via sand filter used as side stream biofilter (Bott 1998, Daamen et al. 2000). Some technologies are commercialized. The challenges faced by internal treatment units, or side-stream units are mainly related to the cooling water chemistry e.g. salt content or presence of dispersant.

Chapter 5 introduced an alternative method for the control of *L. pneumophila* by applying alkaline conditions in the cooling water circuit. The water analyses recorded colony forming units of the pathogenic species at pH 9.4 which exceeded the limit defined by the plant, while at pH 9.6 the concentrations remained very low or below detection. Although *L. pneumophila* seemed to be well controlled by the alkaline conditions, it is unclear if this is due to negative impact of alkaline conditions on its protozoan hosts or a direct negative impact on the organism itself. Since the use of alkaline conditions to prevent the growth and spread of pathogenic bacteria from cooling towers is a very attractive mode of operation, the response of *L. pneumophila* and infected hosts to an increase in alkalinity is worth exploring. Following the previous point, biofilm formation in long-term operated cooling system, with the water quality and operational parameters suggested in Chapter 5, should also be investigated.

In Europe, the gold standard method for *L. pneumophila* monitoring remains plate counting, using a selective medium (ESCMID 2017, European Commission 2001). This culture-based technique is indirect and requires at least 7 days of incubation. As an alternative, qPCR analyses do not require incubation and provide a result within hours. The use of this direct method would be particularly valuable in the detection of a source of legionellosis outbreak and in the monitoring of cooling water stability, allowing fast implementation of control measures when necessary (European Commission 2015). Yearly *L. pneumophila* analyses are declared sufficient by the guidelines applying to industrial cooling towers (Oesterholt 2013). However, in an unstable system, growth of the pathogen can occur within days (Chapter 5) and represent a health risk to the surrounding area if not detected and managed fast enough. A higher frequency of monitoring, e.g. monthly, is advised so that mitigation measures can be taken rapidly, and would be facilitated by qPCR. The lack of correlation between qPCR and standard plate count is however a bottleneck, and leads to difficulties in interpretation of qPCR results with regard to health risk (Whiley and Taylor 2016). As all available guidelines express ranges of concentration in colony forming units, not in genome units, qPCR results cannot be used for compliance assessment at the moment (ESCMID 2017). Efforts should be put in standardizing qPCR protocols for routine monitoring of *L. pneumophila* with defining ranges of acceptable levels. In general, the cooling water sector would highly benefit from the establishment of a direct detection method in legislations.

As indicated in Chapter 5, no threshold for the level of *L. pneumophila* in open recirculating cooling towers is stated in the Dutch Environmental protection act (VROM 2007). The lack of strict regulations surrounding the management of *L. pneumophila* in open recirculating cooling towers lies in the fact that the maximum acceptable concentration of the pathogen in cooling water, with regard to human health, is not known and varies for each case (European Commission 2001). Clear cause and effect relationship between cooling water and

outbreak of Legionellosis is hard to establish, in contrast to other water facilities where direct contact occurs with the population such as drinking water systems or swimming pools (ESCMID 2017). The development of tools for risk assessments of *L. pneumophila* from cooling tower would be valuable to understand the impact of contaminated aerosol on surrounding population and to determine more accurately maximum acceptable concentrations for open cooling systems.

6.3. PROPOSED APPROACH FOR BIOFOULING CONTROL IN COOLING SYSTEMS

The management of cooling systems is mainly performed with extensive dosage of disinfectants (Chapter 1) with the goal to maintain a very low level of microorganisms in the water phase, specifically pathogens, and to restrict the formation of biofilms. Dosage of disinfectant is indeed necessary in cooling systems with conventional operation due to the often low feed water quality and to the optimal environment the operating parameters create for microbial growth. However, microbial control doesn't necessarily require extensive disinfection when the water quality and operating conditions are optimised. Based on literature and on our research findings, another approach is proposed that combines alternative treatments and adjusted operational conditions.

L. pneumophila is a very adaptable and resilient pathogenic species. It has been repeatedly detected in cooling water, wastewater and also in oligotrophic environments, such as drinking water distribution systems (Paranjape et al. 2020b, Wadowsky et al. 1988). Pre-treatment efforts are therefore not considered sufficient to ensure the prevention of *L. pneumophila* growth in cooling systems in absence of disinfection. The CFU levels indicated in guidelines can be rapidly reached if no additional measures are taken (Oesterholt 2013), eventually leading to a risk of Legionellosis outbreak. Chapter 5 considered pH as a parameter of interest to tackle pathogenic bacterial groups. A pH value of 9.6 or higher was determined to be unsuitable for the development of *L. pneumophila* specifically, and reduced the relative abundance of potential pathogenic groups. Operation of industrial cooling systems at pH above 9.6 hence represents a viable alternative to disinfection in the prevention of risks linked to bacterial pathogens.

With regard to inhibition of biofilm formation, effective pre-treatments for nutrient removal are necessary when no disinfectants are dosed in the system. Since organic matter is often abundant in natural water sources in contrast to other compounds (Zularisam et al. 2006), depletion of other nutrients has been investigated. However, we have shown in Chapter 4 that phosphorus depletion promoted EPS production by the cells and therefore enhanced biofouling, despite being the limiting nutrient for cell growth. Due to the open design of cooling towers, nitrogen removal is not a reasonable option as nitrogen from the air can be the nitrogen source. The removal of multivalent cations such as calcium has shown to affect bacterial adhesion and biofilm mechanical properties (Hijnen et al. 2016, van der Waal and van der Sluis 2012, Körstgens et al. 2001), but reaching low enough concentrations to observe biofilm inhibition in cooling water is practically unrealistic. For industrial implementation of an alternative conditioning, and in view of the current treatment technologies as well as characteristics of cooling systems, removal of organic matter still constitutes the preferred target to effectively slow down the build-up of biofilms and reduce planktonic growth.

High quality water with low organic carbon content, combined with alkaline operation of the cooling system, represent a reliable and implementable solution against biofouling without necessitating continuous disinfection nor modification of the system's design. As stated in Chapter 5 and developed in the following paragraph, the use of feed water such as reverse osmosis (RO) permeate or demineralized water is recommended since it already contains negligible amount of nutrients needed for the microbial community populating the cooling system. The cooling system can then be operated at high cycles of concentration (> 20). However, this type of feed water is rarely directly available on industrial sites, or its use might not be economically favourable.

6.4. CONSIDERATIONS FOR FULL-SCALE INTEGRATION

Economic feasibility of the implementation of the aforementioned approach for cooling tower operation and long-term benefits should be assessed for each industrial case specifically. Several factors need to be taken into account such as water quality available, location, and cooling tower size.

When RO permeate water quality can be supplied onsite directly, the cost for the consumption of water necessary for the operation of the cooling system needs to be evaluated. In the case that such water quality is not available, membrane treatment steps can be implemented in the feed water line requiring capital investment and operational expenses varying according to the source water and required water flow. Nevertheless, the use of extensively treated feed water has significant advantages leading to reduction in cooling system operational costs, lower health risk associated to pathogens and anticipation of future regulations, e.g. linked to the formation of disinfection by-products. While conventional cooling towers operate with a blowdown flow rate of around 20 % of the feed water flow rate, the alternative strategy can operate with negligible discharge (1-2%). Associated blowdown treatment costs or onsite treatments for the reuse of the blowdown water on the industrial site will therefore not be necessary with the alkaline conditioning. Regarding the use of chemicals, conventional treatment includes continuous dosages of antiscalants (phosphorus-based compounds and copolymers), corrosion inhibitors, disinfectant and dispersant to compensate for their degradation in the system and partial loss in the blowdown. With high quality water, dosages of antiscalants, disinfectant and dispersant are not required. Bases are dosed to reach the optimal pH, mainly at the start of operation due to the near absence of blowdown water, and to compensate for CO_2 absorption. Corrosion inhibitor is still necessary, mostly to counteract the effect of chloride ions, and should be effective at elevated pHs. These lower chemical requirements eventually lead to a simpler and more stable operation of cooling systems.

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CURRICULUM VITAE

Ingrid Pinel was born on the 18th of February 1993 and grew up in Normandie, France. She always had an affinity for scientific disciplines and therefore decided to start her studies in chemistry in 2010. In 2012, after obtaining a 2-year diploma of general chemistry at the Université de Rouen, she moved to the south of France to follow an MSc degree at the Ecole Nationale Supérieure de Chimie de Montpellier. She graduated in 2016 with specialization in environmental chemistry.

Between 2014 and 2015, she took a gap year of internships to explore different sectors and professions. During this year, a 6-month placement on the optimization of reversal electrodialysis for the reuse of industrial wastewater triggered her interest for the water field. She then performed her MSc end-project at the Technische Universiteit Delft on reverse osmosis membranes for drinking water production. The project was aiming at assessing efficiency of chemicals (DBNPA and urea) against the build-up of biofilms. This is where she got introduced to the mysteries of microbiology.

Late 2016, the opportunity to perform a PhD together with TU Delft and Evides Industriewater B.V. on cooling water system optimization arose. The goals of the project focused on increasing the understanding of cooling system microbiology and the factors influencing it, and exploring disinfectant-free strategies for the control of biofilms and pathogens.

After her PhD, she started working as an R&D engineer at Lenntech B.V. on the treatment of wastewater streams and recovery of substances of interest.

List of publications

Pinel, I.S.M., Hankinson, P.M., Moed, D.H., Wyseure, L.J., Vrouwenvelder, J.S. and van Loosdrecht, M.C.M. (2021) Efficient cooling tower operation at alkaline pH for the control of *Legionella pneumophila* and other pathogenic genera. *Water Research*, 117047.

Pinel, I.S.M., Kleikamp, H.B.C., Pabst, M., Vrouwenvelder, J.S., van Loosdrecht, M.C.M. and Lin, Y. (2020) Sialic Acids: An Important Family of Carbohydrates Overlooked in Environmental Biofilms. *Applied Sciences* 10(21).

Pinel, I.S.M., Kim, L.H., Proença Borges, V.R., Farhat, N.M., Witkamp, G.-J., van Loosdrecht, M.C.M. and Vrouwenvelder, J.S. (2020) Effect of phosphate availability on biofilm formation in cooling towers. *Biofouling* 36(7), 800-815.

Pinel, I.S.M., Moed, D.H., Vrouwenvelder, J.S. and van Loosdrecht, M.C.M. (2020) Bacterial community dynamics and disinfection impact in cooling water systems. *Water Research* 172, 115505.

Sanawar, H., **Pinel, I.**, Farhat, N., Bucs, S.S., Zlopasa, J., Kruithof, J., Witkamp, G., van Loosdrecht, M.C.M., Vrouwenvelder, J.S. (2018) Enhanced biofilm solubilization by urea in reverse osmosis membrane systems. *Water research X* 1:100004.

Siddiqui, A., **Pinel, I.**, Prest, E., Bucs, S., van Loosdrecht, M., Kruithof, J. and Vrouwenvelder, J.S. (2017) Application of DBNPA dosage for biofouling control in spiral wound membrane systems. *Desalination and Water Treatment* 68, 12–22.

Bisselink, R., de Schepper, W., Trampé, J., van den Broek, W., **Pinel, I.**, Krutko, A. and Groot, N. (2016) Mild desalination demo pilot: New normalization approach to effectively evaluate electrodialysis reversal technology. *Water Resources and Industry* 14, 18-25.

Conference contributions

Disinfection & Disinfection By-Products Conference, Beijing, China. (2018) Impact of chlorine disinfection on the microbial communities of a full-scale cooling water systems. *Oral presentation*.

Vakantiecursus Delft, Netherlands. (2018) Evolution of microbial communities in a cooling water system. *Oral presentation*.

Young Water Professionals Conference, Ghent, Belgium. (2017) Biofouling characterization and control in wet cooling systems. *Poster presentation*.

Heat Exchanger Fouling & Cleaning Conference, Madrid, Spain. (2017) Biofouling characterization in wet cooling towers and impact on heat exchangers. *Poster presentation*.