Impact of marine organisms on the functioning of a SWRO desalination plant

By

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THESIS SUMMARY

Water resources are being pushed to their limit under the pressure of an ever-increasing global population, industrial and agriculture needs, and climate change. More than half of the global population will live in water stressed locations by 2025. Alternate water sources such as seawater reverse osmosis (SWRO) desalination are being used to offset the limited resources available. SWRO desalination is a pressure driven system which allows for the production of potable water through the removal of salts via a reverse osmosis (RO) membrane. A major limitation of SWRO is the fouling of the RO membranes. While pre-treatment of the intake seawater through a variety of physical and chemical methods reduces the fouling load in the water, an inflow of microorganisms, nutrients, organic and inorganic compounds still reach the RO membrane. This has a detrimental effect on the longevity of the membrane and on the production of potable water. The overarching aim of this research thesis was to assess the presence and taxonomy of prokaryotic and eukaryotic organisms within the Penneshaw SWRO desalination plant and their association with fouling.

The identification of prokaryotic and eukaryotic organisms was undertaken on the intake seawater and the SWRO feed tank water of the pre-treatment system at Penneshaw. Similarly, identification was carried out on 1st and 2nd stage membranes that were operational for 2- and 4- years. While the pre-treatment system in the Penneshaw SWRO desalination plant removed both prokaryotic and eukaryotic organisms from the water, those organisms <0.5 µm in size where able to pass through all pre-treatment systems. Ultimately, forming adaptive communities that flourished in their niche environment including on the SWRO membranes. Biological fouling parameters also examined in this study revealed that the pre-treatment pipeline in Penneshaw was limited in its ability to remove fouling precursors.

In addition, the efficacy of the Penneshaw pre-treatment pipeline was examined via the formation of protobiofilms. Along the pre-treatment pipeline of Penneshaw, larger fouling precursors were removed, however smaller fragments of precursors could by-pass the pre-treatments system, or were formed as a by-product of the system. As a result, protobiofilms are formed and providing nutrient hot spots for the subsequent colonisation by both prokaryotic and eukaryotic organisms. The colonising organisms were consistent with those identified on the fouled 1st and 2nd stage membranes that were autopsied.

Furthermore, the fouling potential of known biofouling organisms *Pseudoalteromonas* sp. and *Pseudomonas* sp. under different conditions was also evaluated. The synthesis of EPS and the excretion of eDNA by *Pseudoalteromonas* sp. was evaluated as they are components with have been recognised in influence the formation of biofilms. Whereas, the production of fouling pre-cursor of transparent exopolymer particles (TEP) by *Pseudomonas* sp. was quantified under different operating conditions.

Overall, the studies give insights into the organisms and components associated with biofouling within the Penneshaw SWRO desalination plant. The work also highlights how vulnerable desalination plants are to fouling if the pre-treatment systems are limited in their effectiveness. As the need for alternate water sources increases, knowledge of the impact that organisms within the desalination systems have could be invaluable for the development of future plants.

DECLARATION

I certify that this thesis does not incorporate without acknowledgment any material previously submitted for a degree or diploma in any university; and that to the best of my knowledge and belief it does not contain any material previously published or written by another person except where due reference is made in the text.

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ABSTRACTS

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CHAPTER 1: Introduction

1.1 Background Information

The United Nations General Assembly in 2010 openly acknowledged that access to water and sanitation is a basic human right. An essential element of society, potable water provides health security and support for sustainable socio-economic growth. Access to safe drinking water is not only essential to human health and well-being but also sanitation and hygiene, thereby reducing the exposure to preventable diseases and health risks. Currently, approximately 2 billion people only have access to a contaminated water source, with estimates that water stressed regions will affect half the world's population by 2025 (World Health Organization, 2019). Compounding the challenge of providing sustainable potable water is climate change, an increasing global population, rapid urbanisation, and agricultural requirements. To combat these challenges alternate sources for the production of safe drinking water is essential.

The production of potable water via the desalination of seawater and brackish water is an attractive alternate method due to advances in membrane technologies (Qasim et al., 2019). Desalination plants are now frequently used throughout many of the water scarce areas such as the Eastern Mediterranean, the Middle East including Saudi Arabia, United Arab Emirates, Kuwait, Bahrain, and Qatar, Australia, Tunisia, United States of America, Singapore, and the Maldives. Reverse osmosis desalination is considered to be the most efficient method for production of potable water. A semi-permeable membrane within a pressure driven system rejects dissolved components within the feed water source based on size, charge, and membrane interactions (Malaeb and Ayoub, 2011). A major limiting factor within reverse osmosis (RO) desalination is fouling. The development of fouling on the RO membranes contributes to the decline in permeate flux and a decrease in salt rejection reducing the efficiency of the system and potable water output (Matin et al., 2011; Jamieson and Leterme, 2021). The type of fouling experienced by RO membranes is influenced by the feed water and is generally

categorised as inorganic fouling, organic fouling, particulate and colloidal fouling, and microbial fouling (Jamieson and Leterme, 2021). Pre-treatment within the desalination system assist in reducing the quantity of foulants reaching the RO membranes. However, it has been recognised that biofouling components cannot be removed by pre-treatment alone (Matin et al., 2011; Balzano et al., 2015c).

Biofouling is generally considered to be 'the unwanted accumulation of organisms on a surface' (Flemming, 2020). Within a desalination plant, biofouling is considered a sequential process (Nagaraj et al., 2018; Jamieson and Leterme, 2021). First is the formation of a preconditioning layer on the membrane surface, consisting of microgels such as transparent exopolymer particles (TEP) known as "protobiofilm" because of their highly colonised state. Followed by the adherence of planktonic cells onto the preconditioned membrane and their growth due to the constant inflow of nutrients (Nagaraj et al., 2018; Jamieson and Leterme, 2021). Eventually, the organisms colonise the surface and excrete extracellular polysaccharides (EPS) in the form of a biofilm matrix (Anwar et al., 2020; Jamieson and Leterme, 2020). The biofouling of membranes is regulated by the available concentration of nutrients, growth rate of organisms inhabiting the biofilm, the structural stability of the biofilms and the shear force applied to the biofilm (Anwar et al., 2020).

1.1.1. Marine microbes

The largest and most diverse microbial habitat on the planet is the ocean, however the activity and function of many of its inhabitants is mostly unknown. With a majority of the microbes living in communities which are interactive and highly organised, these are known to be adaptable, complex, and challenging to analyse. Marine microbes include archaea, eukaryotic, and prokaryotic organisms. Archaea are abundant and ubiquitous within the marine environment (Santoro et al., 2019). Consisting of four phylogenetic groups that are physiologically and ecologically distinct: Thaumarchaeota,

Euryarchaeota, Pontarchaea and MGIV (Santoro et al., 2019). However, they are a significant part of the marine food web and the biogeochemical cycle, where they contribute via ammonia oxidation to the nitrogen and carbon cycle (Santoro et al., 2019). The domain of eukaryotes comprises of phytoplankton, which are extremely abundant in all the marine layers (Davies et al., 2016; Karlusich et al., 2020). They are known to be a core component of the marine food web and an important member of the biogeochemical cycle (Karlusich et al., 2020). With roles in oxygen generation, elemental nutrient recycling and organic biomass production from atmospheric CO_2 removal critical for the biogeochemical cycle (Karlusich et al., 2020). Further, they have been identified to be useful indicators of climate change and water quality deterioration, owing to their high growth rates and sensitivity to changes in environmental conditions (Davies et al., 2016; Basu and MacKey, 2018). Prokaryotes are divided into two domains: bacteria including virus, and archaea. Bacteria are highly abundant with bacterial cells estimated to be 3.6×10^{29} within the marine environment (Dash et al., 2013). While bacteria are ubiquitous in the ocean, their taxonomic dispersion is still highly dependent on location and depth, and influenced by environmental factors (Dash et al., 2013). A leading problem in marine research is the identification of the bacteria, even with the advancement of metagenomic and amplicon sequencing techniques, as most cannot be assigned to known species (Dash et al., 2013). Viruses are estimated to be 15-fold more abundant than both bacteria and archaea, though their contribution to the prokaryotic biomass is relatively small (Suttle, 2007). The abundance of viruses is linked to the prosperity and production of the prokaryotes in any ecosystem. This is also reflected in the marine environment with a reduction in the viral abundance in open and deeper waters (Suttle, 2007). One of the most challenging aspects of marine virus research is the ability to find techniques which allow for accurate, and reproduceable results (Suttle, 2007). Of note, is the roles that archaea, eukaryotic and prokaryotic organisms have in the biogeochemical cycle. Resulting in the production,

either actively or passively, of extracellular polymeric substances such as transparent exopolymer particles (Mühlenbruch et al., 2018; Arnosti et al., 2021)

1.1.2. Transparent exopolymer particles (TEP)

TEP are widely found in marine and freshwater environments and have frequently been associated with membrane fouling as well as the formation and development of marine biofilms (Bar-Zeev et al., 2015; Meng et al., 2020). The formation of TEP can be spontaneous, through the aggregation of dissolved precursor substances. Environmental parameters including turbulence, ion density and inorganic colloid concentrations along with the type and concentration of precursors, influence the particles formation (Passow, 2002a; Meng et al., 2020). Within the current literature, the interpretations on the size of TEP as well as its size distribution vary and are influenced by filtration separation (Meng et al., 2013). Alldredge et al. (1993) first reported TEP to range from 3 to 100 µm, however it is typically considered to be >0.4 µm. Particles below that range (e.g., 0.05 – 0.4 µm in size) have been identified within desalination plants, although these are generally classified as a colloidal TEP or TEP precursors (Villacorte et al., 2009a, 2009b). These transparent gel-like particles are deformable and appear in many different shapes, such as amorphous blobs, clouds, sheets, filaments, or clumps (Linares et al., 2012; Bar-Zeev et al., 2015). TEP have unique characteristics due to their large size range, high abundance, and high viscosity, thereby enhancing the accumulation of non-sticky particles (Berman et al., 2011; Bar-Zeev et al., 2015).

TEP's ability to be stained with alcian blue indicates that they predominantly consist of acidic polysaccharides. Although the chemical composition of the particles is subject to species and location, they largely contain sugars including fucose, rhamnose and arabinose (Passow, 2002b). This means that TEP is formed from organisms' excretions released as dissolved organic matter into the water.

TEP's acidity results from the presence of sulfate half ester groups ($R\text{-}OSO_3\text{-}$) known to be excreted by marine organisms especially diatoms (Passow, 2002a). Other materials such as lipid, amino acids, trace elements as well as heavy metals are known have been associated with TEP due to their high binding affinity (Passow, 2002b). Of equal importance is the high carbon content that the particles have. In some cases, the carbon content has been estimated to be similar to that of phytoplankton, leading to the suggestion that TEP provides a significant contribution to the carbon pool in the marine environment (Engel and Passow, 2001; Mari et al., 2017).

The large surface area of TEP, combined with their high viscosity and negative charge, allows for the absorption of organic molecules and trace metals from the surrounding environment, thereby creating favourable conditions for the colonisation of microorganisms (Berman et al., 2011; Meng et al., 2020). Thus far, the colonisation of TEP have been quantified via DAPI, SYBR green or SYTO9 staining and flow cytometry (Mari and Kiørboe, 1996; Berman and Parparova, 2010; Bar-Zeev et al., 2012). The attachment of bacteria to TEP is dependent on the chemical composition of the particles, with the percentage of attachment ranging from 5 -10% (Meiners et al., 2004; Busch et al., 2017). Similarly, the proportion of bacterial colonisation is related to the size of the TEP (Mari and Kiørboe, 1996; Busch et al., 2017). Within water treatment plants, TEP serve as 'hot spots' of heavily colonised highly viscous particles which facilitates the easy attachment to membrane surfaces (Bar-Zeev et al., 2012). Bacteria are the dominant colonisers of marine particles, consisting of 80 – 90% of microbial population identified (Gram et al., 2002). Molecular analysis has allowed the identification of α- and β- Proteobacteria classes (as well as Flavobacterium and Bacteroides genera) as the organisms primarily colonising TEP (Gram et al., 2002).

1.1.3. Extracellular polysaccharides (EPS)

EPS is primarily formed of metabolites produced during cell growth, such as polysaccharides, proteins, lipids, humic elements, and extracellular DNA (eDNA). The nature of the EPS components such as concentration, charge, specificity etc. influences the matrix architecture (Flemming and Wingender, 2010). Furthermore, the EPS matrix shapes the internal space within the biofilm amongst the cells influencing the lifestyle within the biofilm (Flemming et al., 2016). The polymeric structure of the EPS on the membrane subsequently changes the physical-chemistry properties of the membrane surface such as the roughness, hydrophilicity, zeta potential, and surface energy. This can potentially create favourable conditions for the settlement of more organisms and the deposition of organic material. A stable and intact bio-layer of biofilm eventually covers the surface of the membrane whether through direct deposition or biofilm detachment and relocation. The adhesion of biofilms to the membrane is enhanced by the EPS matrix in addition to providing a barrier from chemical cleaning agents biocidal components (Anwar et al., 2020).

1.1.4. Extracellular DNA (eDNA)

DNA molecules have been recognised as an essential component within the extracellular environment. eDNA is found in many different natural environments, such as Rhizospheres, soil and aquatic ecosystems, the human body, and biofilms (Nagler et al., 2018). Within biofilms, numerous microorganisms release eDNA and it is considered to be a prevalent characteristic (Vorkapic et al., 2016). Within biofilms a number of functions have been proposed for eDNA including (i) an essential structural component to the EPS matrix to provide stability to the biofilm (Steinberger et al., 2002; Lappann et al., 2010), (ii) the promoting factor for the formation of the biofilm and the fabrication of the EPS matrix (Whitchurch et al., 2002; Seper et al., 2011), and (iii) a source of genes during horizontal

gene transfer (Molin and Tolker-Nielsen, 2003). Other microorganisms use eDNA as a source for reparation of damaged DNA (de Aldecoa et al., 2017), as well as a potential source of nutrients during starvation inverals (Finkel and Kolter, 2001; de Aldecoa et al., 2017). These different functions are regulated via networks linked to Quorum sensing and community behaviours (Vorkapic et al., 2016). The release of eDNA is species dependent in most cases, however it is excreted either via autolysis or active secretion systems, although release via extracellular membrane vesicles is also an option (de Aldecoa et al., 2017). The multiple functions and roles that eDNA has in biofilms make it a relevant molecule in understanding the biofilm lifestyle but also as a relevant target for the disruption of biofilm development.

1.2 Scope of the thesis

Despite the recent advances in membrane technology and novel pre-treatment technologies, the control of biofouling within desalination plants is no closer to being prevented. Not only are biofouling precursors still being able to traverse the length of pre-treatment systems and reaggregate into fouling hotspots, but they are also being colonised by microorganisms small enough to bypass all pre-treatment systems. These eventually settle on the seawater reverse osmosis (SWRO) membrane and form a biofilm on the surface, resulting in poor water production and negatively influencing the life span of the SWRO membrane. The adeptness and persistence of fouling organisms within desalination plants continues to be a burden and is an area of increasing concern both industriously and economically. The efficacy of producing potable is diminished over time, leading to the introduction of chemical treatments and/or replacement membranes. Identifying the eukaryotic and prokaryotic organisms that evade, colonise and proliferate on the pre-treatment measures and/or the RO membranes will contribute to the understanding of the biofouling potential of SWRO desalination

plants. The aim of this study was to examine the communities of marine microbes and their biofouling potential in a SWRO desalination plant. The Penneshaw SWRO desalination plant (Kangaroo Island, South Australia) was utilised for all the studies presented within this thesis. Each research chapter examines a specific aspect of the Penneshaw desalination plant, the influence of the communities and their biofouling potential. As well highlighting potential improvements to the pre-treatment system within the plant.

Chapter 2 reviews the published literature in recent years in relation to biofouling of SWRO desalination plants. It assesses the formation of biofilms and the contributing factors to biofouling. It also discusses the developmental stages of biofouling and the repercussions on the SWRO membranes.

Chapter 3 explores the prokaryotic communities associated with the Penneshaw SWRO desalination plant (Kangaroo Island, South Australia). Insights into communities associated with the pre-treatment system at the intake seawater and the SWRO feed tank water were determined over a 12-month period. 1st and 2nd stage membranes. These membranes were operational for two and four years and were autopsied for biological parameters as well as community structure. The diversity of the prokaryotic communities along the pre-treatment system are described and the core organisms were assessed to determine their fouling influence on the SWRO membranes. Biological biofouling components are characterised and measured on the autopsied SWRO membranes.

Chapter 4 examines the eukaryotic communities associated with the Penneshaw SWRO desalination plant (Kangaroo Island, South Australia). It represents the first comprehensive study of eukaryotic communities along a SWRO desalination pre-treatment system. The intake seawater and the SWRO feed tank water communities of the Penneshaw pre-treatment system were determined over a 12-month period. Similarly, the communities associated with membranes from the 1st and 2nd

stage of the SWRO unit that were operational for two and four years were identified. The eukaryotic diversity at each location is described and the fouling influence of the eukaryotic core communities is evaluated in regard to the SWRO membranes.

Chapter 5 investigates the formation of aggregates within the Penneshaw SWRO desalination plant (Kangaroo Island, South Australia). Aggregates were formed in intake seawater and SWRO feed tank water of the Penneshaw pre-treatment system via the aggregation of fouling precursors. The prokaryotic and eukaryotic communities attached to the protobiofilms are described and fouling potential of the communities analysed using the SWRO membrane in chapter 3.

Chapter 6 sought to examine the biofouling potential of a Penneshaw SWRO desalination plant (Kangaroo Island, South Australia) isolated *Pseudomonas* sp.. SWRO feed tank water was used for the formation of biofilms within static experiments with bacteria cultured for identification. The production of the biofouling pre-cursor TEP by *Pseudomonas* sp. was quantified during static and cross-flow laboratory experiments.

Chapter 7 explores the production of EPS and eDNA by *Pseudoalteromonas* sp. isolated from the Penneshaw SWRO desalination plant (Kangaroo Island, South Australia) SWRO feed tank water. Within laboratory conditions eDNA and the fractional components of EPS were extracted over time from *Pseudoalteromonas* sp in both the planktonic and biofilm state.

The different range of experimental techniques and analysis presented in this thesis have provided new knowledge on the contribution that not only communities but also fouling precursors within the Penneshaw SWRO desalination plant have on biofouling of SWRO membranes. This work also highlights the impact that ineffective pre-treatment systems have within the desalination plants and their contribution to membrane fouling. Collectively, the results reported here have advanced our

knowledge into the complex nature of biofouling and the *in-situ* conditions that promote fouling on SWRO membranes.

CHAPTER 2: Influences and impacts of biofouling in SWRO desalination plants

2.1 Preface

This chapter is closely based on the accepted manuscript of an article published by Taylor & Francis by Jamieson, T., and Leterme, S. C. (2021) Influences and impacts of biofouling in SWRO desalination plants. *Critical Reviews in Environmental Science and Technology*, 51:12, 1281-1301, DOI: 10.1080/10643389.2020.1757937.

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Outline your (the student's) contribution to the publication:

Conceptualisation, data curation, formal analysis, writing, editing and revision of the manuscript, final manuscript.

☒ I confirm that the details above are an accurate record of the student's contribution to the work.

Name of Co-Author 1: Sophie Leterme Signed: Sophie Leterme Digitally signed by Sophie Leterme Date: 2021.06.02 18:37:23 +09'30' Date: 02/06/21

2.2 Abstract

The ability to produce fresh potable water is an ever-growing challenge, especially with an increase in drought conditions worldwide. Due to its capacity to treat different types of water, reverse osmosis (RO) technology is an increasingly popular solution to the water shortage problem. The major restriction associated with the treatment of water by RO technology is the fouling of the RO membrane, in particular through biofouling. Membrane fouling is a multifaceted problem that causes an increase in operating pressure, frequent cleaning, and limited membrane lifespan. The current paper summarizes the impact of biofouling of RO membranes used in seawater desalination plants. Following a brief introduction, the elements that contribute to biofouling are discussed: biofilm formation, role of extracellular polymeric substances (EPS), marine environment, developmental phases of biofouling. Following this, is a section on the implications of membrane biofouling especially permeate flux and salt rejection. The final section focuses on the new phenomenon of compression and hydraulic resistance of biofilms. Lastly, considerations on future research requirements on biofouling and its control in seawater reverse osmosis (SWRO) membrane systems are presented at the end of the article.

2.3 Introduction

Water scarcity is a growing problem worldwide, with the demand escalating due to increasing population, uneven water distribution and rigorous quality regulations. Future water shortages are being recognised as a significant problem since much of the global economy relies upon sustainable high-quality water (Lee and Kim, 2011; Matin et al., 2011). A vast majority of the water that is accessible to us for direct consumption is not consumable due to its salinity level; therefore, developing

alternative technologies in an effort to provide water resources is a continual challenge. A commonly used clean water technology is RO membrane desalination (Goh et al., 2017).

SWRO desalination is considered to be one of the most cost-effective methods of producing potable water. The desalination process involves the feeding of water under high pressure across a semi-permeable membrane to reject salts, organic and biological matter, including bacteria and viruses, and obtaining fresh water (Lee and Kim, 2011; Matin et al., 2011).

RO is a membrane-based pressure driven system wherein the membrane separates unwanted components from the feed water to obtain a pure product. SWRO desalination uses a semi-permeable membrane to reject salt while allowing the selective transport of water through the membrane to produce clean water. Worldwide reliance on RO is increasing due to its versatility and continuous technological improvements, that have resulted in cost reductions and increased energy efficiency (Harif et al., 2011; Lee and Kim, 2011; Goh et al., 2017). In particular, RO allows for the removal of large amounts of dissolved solids, organics, colloidal matter, and microorganisms via a semi-permeable membrane (Lee and Kim, 2011; Matin et al., 2011). Due to the high rejection feature and the efficiency of the membranes, SWRO is considered to be the simplest and most cost-effective method of freshwater production in comparison to other separation methods such as distillation, solvent extraction, ion exchange and adsorption (Lee and Kim, 2011; Matin et al., 2011; Farahbakhsh et al., 2017; Goh et al., 2017).

While RO technology has many applications, membrane fouling is a significant shortcoming that limits the efficiency of the RO process. Even after seawater pre-treatment and cross flowing within the RO system, aquatic organisms and organic compounds still enter the RO module therefore allowing for the dynamic process of colonisation and growth on the membrane to cause biological fouling (Flemming, 2002; Ivnitsky et al., 2007; Komlenic, 2010; Kochkodan and Sharma, 2012). There are many

different types of fouling i.e., inorganic fouling, particles/colloids, organic fouling, and biofouling (Flemming, 1997; Reverter et al., 2001; Fujiwara and Matsuyama, 2008; Bartman et al., 2011).

Fouling is the reversible, or irreversible, attachment of organic or inorganic particles to the surface of the membrane (Hong and Elimelech, 1997; Belfer et al., 1998). Several factors including intake water quality and pre-treatment measures drive RO membrane fouling. For example, pre-treatment barriers such as micro-filtration (MF) and ultra-filtration are commonly used in SWRO desalination plants to reduce the amount of foulants reaching the RO membrane, inducing biofoulants (Rapenne et al., 2007; Mo et al., 2008). Biofilm formation on the RO membrane creates adverse effects on the operation of desalination plant resulting in a decline in salt rejection, water quality and flux and an increase in operating pressure (Herzberg et al., 2009; Berman et al., 2011; Harif et al., 2011).

This paper aims at summarizing the globally significant topic of membrane biofouling within saltwater reverse osmosis desalination plants based on existing literature. The review starts with the formation of biofilms and elements that contribute to the biofouling of RO membranes. Subsequently, the developmental phases of biofouling are discussed with a focus on a new paradigm. The implications that biofouling has on membranes, especially permeate flux and salt rejection, are also reviewed. Next, a new phenomenon will be examined, the compression and hydraulic resistance in biofilms. Finally, we reflect on the differences between biofilms formed naturally and under pressure before presenting our considerations on future research requirements on biofouling and its control in SWRO membrane systems.

2.4 Elements contributing to the biofouling of reverse osmosis membranes

Within pelagic ecosystems, key processes such as flux, cycling and sedimentation of elements and energy have been extensively studied over the years. Rich in populations, these plankton

organisms consist of bacteria, archaea, algae, protozoa, and multicellular zooplankton (Hays et al., 2005). Most oceanic pelagic systems are nutrient poor stratified systems in which picoplankton are the dominant component of the planktonic biomass (Berglund et al., 2007). Prokaryotes make up a major component of the picoplankton biomass in marine environments and are an integral component of the microbial food web (Sherr and Sherr, 1988; Kiørboe et al., 1990; Sommer et al., 2002). This microbial loop is an essential link between dissolved organic matter (DOM) and the higher trophic levels and is made available when metabolised by bacteria. Therefore, the role of microbes within the pelagic region is to regulate the energy flow through the foodweb, thereby limiting the export of biomass towards the benthos.

Marine microbes have the specificity to produce extracellular polysaccharides in the form of transparent exopolymer particles (TEP) that are species specific and dependent on surrounding environmental (growth) conditions (Gordon, 1970; Alldredge et al., 1993; Passow and Alldredge, 1994; Simon et al., 2002; Verdugo et al., 2004; Li et al., 2016c; Taucher et al., 2018). TEP are deformable, gel like transparent particles that appear in many forms, such as amorphous blobs, clouds, sheets, filaments, or clumps (Chin et al., 1998; Verdugo et al., 2004; Linares et al., 2012). In the marine environment, they have been found to range in size from micro- up to milli- metre scales but can also span several centimetres through the formation of dense networks (Azetsu-Scott and Passow, 2004). TEP can be formed spontaneously from the aggregation/encounter of dissolved precursor substances and will differ depending on the type and concentration of precursors present in the water (Passow, 2002b; Thuy et al., 2015). TEP can also be produced from colloidal material through the breakdown of algal aggregates by bacteria (Kiørboe and Hansen, 1993; Hansen et al., 1995; Passow and Alldredge, 1995a; Grossart et al., 1997; Mari and Burd, 1998; Engel, 2000). TEP, which consist of EPS with the addition of high surface-active polymers, are likely to have a role in coating surfaces and providing a nutritious substrate for bacteria and other microorganisms to colonise (Bar-Zeev et al., 2012). In the

marine environment, TEP serve as "hot spots" of intense microbial and chemical activity within the water column facilitating the attachment of planktonic TEP to surfaces (Berman et al., 2011). When attached to surfaces such as biofilms or macroaggregates, marine microbes have the capability to produce exopolysaccharides in large amounts (Decho, 1990; Costerton et al., 1995; Heissenberger et al., 1996; Stoderegger and Herndl, 1998). However, when non-aggregated in the water column, they are also reported to produce TEP.

Those aggregates formed by microorganisms in the pelagic realm are commonly called marine snow. Marine snow is regarded as aggregates, of 0.5 mm or larger in diameter, which are highly diverse in origin, morphology and characteristics within marine environments (Alldredge and Silver, 1988; Silver et al., 1998; Simon et al., 2002; Burd and Jackson, 2009; Iversen and Ploug, 2010). The structural components of aggregates therefore vary from fragile, porous, loosely associated smaller particles and organisms to those that are extremely cohesive, robust, and gelatinous in structure (Alldredge and Silver, 1988; Taucher et al., 2018). Marine snow is primarily formed from algae, inorganic particles, zooplankton feeding structures, faecal pellets, and detritus (Alldredge and Gotschalk, 1988; Alldredge and Gotschalk, 1990; Hansen et al., 1996; Alldredge et al., 1998). The formation of marine snow via physical aggregation is enhanced through two biological-mediated pathways (Alldredge and Silver, 1988). First, via the production of sticky mucus, exopolymers or products of cell lysis which increase the probability of colliding particles attaching, and also through the probability for potential collision resulting from biological alteration of the size and surface characteristics of the particles (Alldredge and Silver, 1988; Jackson, 1990; Riebesell, 1991; Burd, 2013; Taucher et al., 2018). Marine snow can be seen as macromolecular structures containing bacterial biofilms associated with the suspended particles (Gupta et al., 2016).They frequently contain higher concentrations of organic and inorganic particles than that of the surrounding environment (Shanks and Trent, 1979; Prézelin and Alldredge, 1983; Kiørboe and Jackson, 2001; Grossart et al., 2003a), often resulting in heavy colonisation by

heterotrophic bacteria (Alldredge and Youngbluth, 1985; Alldredge et al., 1986; Simon et al., 2002; Grossart et al., 2007; Vojvoda et al., 2014; Thiele et al., 2015; Ivančič et al., 2018; Duret et al., 2019). Polysaccharides, excreted by bacteria, produce a sticky medium consisting of gel like particles, which provides further structure to the aggregates together with the colloids and organic gels and organic matter (Alldredge et al., 1993; Jackson, 1995; Long and Azam, 2001; Passow, 2002a). Living and lysed cells in the majority of natural environments excrete extracellular polymeric material (Passow, 2002b). Dissolved organic matter is removed from the surrounding environment by attached bacteria and converted to particulate matter through extracellular excretion (Alldredge and Silver, 1988).

Much like the colonization of surfaces, the colonization of aggregates by bacteria is complex and occurs in several steps. First, bacteria will attach loosely to the aggregate, but the attachment will gradually increase until cells are permanently attached, and growth rates dominate over attachment (Grossart et al., 2003b). Fast swimming bacteria will encounter an aggregate in about <1 day (Kiørboe et al., 2002), but also non-motile bacteria collide with aggregates in lower frequency. Subsequently the total cell numbers on the aggregate increase and the bacterial community becomes established like during the formation of bacteria biofilms on inert surfaces. Although no studies have been conducted to assess the contribution of marine aggregations in fouling of the SWRO membranes, it is known that pre-treatment of the feed water has limitations and not all precursors can be removed. The limited removal of TEP (marine snow) from seawater via pre-treatments increases the biofouling potential (Balzano et al., 2015c).

2.5 Developmental phases of biofouling

The predominant contributors to biofilm formation on RO membranes are microbial communities and nutrients. The adherence of microbes to the surface of the membrane and the

continual growth of aggregates thus result in the formation of biofilms (Lee et al., 2015; Jiang et al., 2017; Nagaraj et al., 2018).

A new paradigm was proposed by Bar-Zeev et al., (2012) in the role that TEP has alongside the stages of the "classic" formation of biofilm. TEP are often found in marine environment and play a role in the formation and development of marine biofilms (Bar-Zeev et al., 2009; Berman et al., 2011; Bar-Zeev et al., 2015; Nagaraj et al., 2018). Within the desalination process, high levels of potential biofilm forming TEP were found to be reaching the RO membrane (Bar-Zeev et al., 2009; Le Lan et al., 2015).

The formation of biofilm on the RO membrane involves several key phases:

(I) Conditioning of the membrane surface

(II) Attachment of microorganisms

(III) Formation of biofilm matrix

(IV) Establishment of mature biofilm

(V) Biofilm stability and reduction

Organic and inorganic particles present in the water adhere to the membrane surface forming a nutrient rich 'conditioning film' (Phase I). It is during this phase that TEP precursors absorb to the surface of the membrane subsequently producing a patchy, thin negatively charged conditioning layer (Jain and Bhosle, 2009; Hwang et al., 2013; Khan et al., 2013a; Li et al., 2016c). Subsequently, increasing the attachment of bacteria through hydrophobic interactions and hydrogen bonding (Hwang et al., 2013). 'Protobiofilm' aggregates of free-floating microgels potentially heavily colonised by bacteria, can also adhere to surfaces simultaneously with TEP precursors (Bar-Zeev et al., 2012). Once the protobiofilm is attached, it exhibits all the traits common to a mature biofilm and can expedite the development of biofilms. The initial conditioning of the membrane surface allows microorganisms to adhere due to the nutrient rich environment thereon (Phase II). Van der Waals, hydrophobic and hydrogen bonding allow bacteria are able to overcome electrostatic repulsive forces and reversible

attachment of the surface coating (Redman et al., 2004; Hori and Matsumoto, 2010). During this phase the application of weak shear forces still has the ability to reverse the attachment to the membrane. The production of EPS by the attached bacteria changes their attachment from reversible to irreversible (Phase III; Dunne, 2002; Hori and Matsumoto, 2010). Simultaneously, micro-gel patches of carbon rich TEP are covering and attaching to the membrane. The EPS layer produced adsorbs the TEP, which then becomes a structural component of the matrix (Barnes et al., 2014; Bar-Zeev et al., 2015). Thereby, increasing the attachment of bacteria and micro-particles through a more favourable organic content, elasticity, and roughness of the membrane surface (Bar-Zeev et al., 2012). The surface and the polysaccharides within the EPS interact through hydrophobic interactions, dipole-dipole forces, and hydrogen bonding (Dunne, 2002; Hori and Matsumoto, 2010). Organic molecules, colloidal particles, suspended solids, and other microorganism cells are captured over time and used to build a thicker matrix layer resulting in a higher permeate flux resistance (Phase IV; Stoodley et al., 2002;, Hall-Stoodley et al., 2004). An equilibrium is finally established due to the availability and quantity of the nutrients / food sources also the production of by-products from the bacterial growth process and their subsequent removal and, additionally, the turbulence produced by the cross flow on the surface of the membrane and space limitations (Phase V; Stoodley et al., 2002; Hall-Stoodley et al., 2004; Gupta et al., 2016). The formation of biofouling of SWRO membranes results in well documented consequences, which can have a detrimental impact on the productivity of the desalination plant (Jiang et al., 2017).

2.6 Within-system implications of biofouling

The formation of permanent biofilms on RO membranes within a desalination plant is the result of a combination of factors such as the presence of microorganisms within the water, the availability

of nutrients and the flow of water through the membrane (Herzberg and Elimelech, 2007; Mo et al., 2008; Nagaraj et al., 2018). This results in a negative impact on the performance of the system through a decline in the water flux (including permeate), as well as an increase in the amount of seawater rejected, energy requirements, system pressure and also the potential of damage to the RO membrane. The impact of all these leads to a decrease in the quality of freshwater produced (Komlenic, 2010; Matin et al., 2011; Katebian and Jiang, 2013; Goh et al., 2018).

2.6.1 Permeate flux decline

Permeate flux decline is directly impacted by the development of biofilm on the RO membranes whether the development is rapid or gradual (Matin et al., 2011; Lee et al., 2015). Two phases of flux decline have been noted; the initial decline coincides with the early phases of biofilm development especially the attachment and proliferation on the surface of the membrane (Kim et al., 2019). The second phase displays a slow decline and has been found to correspond to the final phases in the biofilm development in which equilibrium is developed between biofilm growth, EPS production and biofilm loss (Matin et al., 2011; Li et al., 2016c; Kim et al., 2019).

Hydraulic resistance is a direct result of the presence of biofilm and increased osmotic pressure is the result of concentration polarization as a consequence of the complicated biofilm structure (Herzberg and Elimelech, 2007; Chong et al., 2008; Herzberg et al., 2009; Kwan et al., 2015; Ferrando et al., 2017; Kim et al., 2019). Another factor causing a decline in flux is the resistance of the biofilm due to its EPS matrix. Kwan et al., (2015) proposed that the resulting shape of the biofilm, due to the pressure exerted upon it, changes the characteristics of the biofilm, and thereby causes a greater decline in the flux in RO systems.

2.6.2 Salt rejection

A phenomenon known as 'concentration polarization' is often seen in pressure driven separation processes such as RO desalination (Hoek and Elimelech, 2003; Herzberg and Elimelech, 2007; Herzberg and Elimelech, 2008). The passage of water through the membrane, leads dissolved substrates to accumulate in the feed water resulting in a steady increase in solute concentration in the RO permeate water. The formation of the EPS matrix contributes to the suppression of dissolved solutes mixing at the membrane surface thereby leading to an enhanced concentration polarization (Hoek and Elimelech, 2003; Herzberg and Elimelech, 2007). An increase in the transport of solute material through the membrane is the result of an increase in ionic activity at the surface.

A decline in the rejection of salt in the pressure driven separation system commonly seen in desalination plants is also associated with the growth of biofilm on the membrane (Herzberg and Elimelech, 2007; Radu et al., 2012; Ferrando et al., 2017; Siebdrath et al., 2019). A study conducted by Herzberg et al., (2009) found that the leading cause of an increase in salt passage was the microorganisms within the biofilm and that EPS played only a minor role. However, Ferrando et al., (2017) observed that salt passage increased in parallel with the production of the *Pseudomonas aeruginosa* EPS component Psl, thereby establishing that EPS is a critical factor in not only concentration polarization but also in membrane performance. Within cellulose acetate membrane systems biodegradation has also been associated with the decrease in the rejection of salt (Beverly et al., 2000; Murphy et al., 2001). Siebdrath et al., (2019) investigated biofouling formation in a long channel membrane test cell pilot plant. While salt rejection decline was evident in all the test cells in series, the decline in the lead cell was the result of biofouling, whereas the decline seen in the tail test cell was a result of high flux decline. However, further insights into the negative impact of biofilms within desalination plants has led to the consideration of biofilm resistance as a concern.

2.7 Compression and hydraulic resistance of biofilm

The consolidation of biofilms is a process that has recently been defined, implying that under dynamic conditions there is possible restructuring of the biofilm. Compaction is the mechanism contributing to the realignment of the biofilm structure when under high shear force that was proposed by Casey (2007). When compacted, the biofilm increases in density but its porosity decreases, resulting in a decline of biofilm thickness. Biofilm relaxation has the opposite effect resulting in an increase in porosity and, thereby, biofilm thickness. However, it is not yet fully understood how this biofilm behaviour and the resulting impact affect performance loss within desalination plants. The effect of differing fluid velocities on biofilm density was investigated by Ohl et al., (2004). Increasing the flow velocity sees the biofilms become more compact, and the density of the biofilm decreased with the reduction in flow velocity. The thickness of the biofilm also determines the time needed to see an impact of the flow velocity, the thicker the biofilm the greater the adaption time.

Currently there are limited studies mechanically exploring the compressibility of biofilms. Körstgens et al., (2001) investigated the elasticity and yield strength of *P. aeruginosa* biofilms using a film rheometer. Whereas, Paramonova et al. (2007) and Paramonova et al., (2009) used low-load compression testing on various bacteria. While these studies provided new reproducible insights into measuring biofilm compressibility, they do not adequately reflect the biofouling parameters. Dreszer et al. (2013) investigated the role of biofilm resistance on the performance of MF membranes through different flux and cross flow of drinking water. However, while increased biofilm resistance is seen when the flux increases, the biofilm can be returned to the original resistance when the flux is also reverted back. Dreszer et al. (2013) also found that the biofilm had only a small role in the transmembrane resistance due to the intrinsic membrane resistance. They thus suggested that in RO

desalination systems, biofilms are likely to enhance biofouling through concentration polarization and feed channel pressure drop rather than pure biofilms. They also proposed that it is the tortuosity of the EPS matrix that leads to the failure of the water to penetrate the matrix. Further, Dreszer et al. (2014) undertook an *in-situ* experiment examining the hydraulic resistance in drinking water biofilms and the performance parameters of the MF membrane. Over time, with an increase of biofilm thickness, the resistance of the biofilm increased, resulting in a pressure drop within the system. However, the original resistance of the biofilm could not be completely restored with the decrease of flux as evident in their previous study. Dreszer et al. (2014) also found that the shape of the biofilm changes as a result of the flux upon it; as the flux increases the biofilm becomes more compact whereas at a lower permeate flux the biofilm relaxes. The compaction and morphology of the biofilm also have role in the biofouling of the system, as does the thickness. It is possible to estimate the effects of compression through biofilm modelling. A study conducted by Laspidou et al., (2014) determined that compression of the biofilm 'closed' the channels within resulting in a more rigid biofilm. *In-situ* work by Linares et al. (2015) determined how flux variations influence biofilm compaction and relaxation. They found that compaction of the biofilm was seen rapidly after an increase in flux, and although when the original flux was restored the thickness of the biofilm increased, the biofilm was not returned to its initial state. The changes in the biofilm parameters were influenced by the hydraulic conditions leading to a compressed, stiff biofilm, with an increased hydraulic resistance which resulted in a loss of membrane performance. The use of optical coherence tomography did not allow for the observation of 'closed' channels as predicted by Laspidou et al. (2014), however, the biofilms did increase in stiffness after compression.

Derlon et al. (2016) explored the influence that the composition of the biofilm had on hydraulic resistance in gravity-driven membrane ultra-filtration systems. The impact of inorganic materials and cells present within the biofilm was determined to be limited due to the minor fraction they

represented in the total biofilm volume. However, hydraulic resistance was impacted by biofilms in which EPS was a major component. Derlon et al. (2016) also found that the biofilms, while compressible, exhibited two responses to a change in transmembrane pressure. The first response was immediate with the biofilm relaxing or compressing, upon a sudden change in pressure leading to a change in hydraulic resistance, although the compression was found to be entirely reversible under these conditions. Derlon et al. (2016) proposes that these changes are driven the mechanical properties of the biofilm. The second response was a long-term response in which the permeability of the biofilm reduced over time, increasing hydraulic resistance. Derlon et al. (2016) theorises that being under constant pressure causes the biofilm to restructure the internal architecture. Desmond et al. (2018) proposed that EPS of different composition would influence hydraulic resistance in biofilms with a gravity driven membrane ultrafiltration system. In particular, Desmond et al. (2018) found that the biofilm thickness is relative to the morphology of the EPS, and to the hydraulic resistance. Biofilms which display a homogeneous morphology, containing increased concentrations of polysaccharides and eDNA, resulted in the production of dense structures with reduced permeability, hence, they had a higher hydraulic resistance (Desmond et al., 2018) due to the close-range electrostatic interactions. The heterogeneous morphology of biofilms was the product of lower concentrations of not only polysaccharides but also eDNA. With a low concentration of polysaccharides and eDNA thereby limiting the electrostatic interactions with the structure that are required for the formation of a dense biofilm.

2.8 Reflection on natural biofilm formation

The prevalence of biofilms in nature allows microorganisms to survive unforgiving environments while also supporting significant ecological and biogeochemical functions (Dang and

Lovell, 2016; Azeredo et al., 2017). Within the industrial and medical industries biofilms are considered to have a negative impact, in particular the ability to resist antimicrobial agents (Lewis, 2001; Azeredo et al., 2017). However, whether being formed on rocks or medical implants, the development of biofilms is largely consistent. Indeed, biofilms consist of sessile microorganisms contained within a heterogeneous matrix of EPS, which attaches irreversibly to a solid surface. These cells differ from free-living cells of the same species in terms of growth rate and gene expression as they have an altered phenotype (Donlan and Costerton, 2002; Bryers and Ratner, 2004). Physical, chemical, and biological processes are involved in guiding the formation of biofilm. Biofilms are considered to be one of the most robust forms of life, and have a strong structure ensuring from the development of EPS layers by the microorganism (Flemming, 2002; Hall-Stoodley et al., 2004). A sequence of consecutive stages leads to the development of biofilms such as the initial reversible attachment of microorganism, irreversible attachment of pioneer microorganisms, maturation of biofilm stage I, maturation of biofilm stage II and dispersion (Sauer et al., 2002; Stoodley et al., 2002; Gupta et al., 2016).

The initial stage of formation of biofilms is heavily dependent upon the transport and attachment of planktonic bacteria by physical, chemical and/or biological factors such as pilli, or flagella, to a solid-liquid interface (Maric and Vranes, 2007; Armbruster and Parsek, 2018). Biofilm formation is also reliant upon other elements such as surface roughness, hydrodynamics and the characteristics of the surrounding water including pH, nutrient content, ionic strength and multivalent cations biofilms (Vieira et al., 1993; Millsap et al., 1997; Davies et al., 1998; Stoodley et al., 1999; Sauer and Camper, 2001; Donlan, 2002; Allison, 2003; Parsek and Greenberg, 2005; Chae et al., 2006; Herzberg and Elimelech, 2007; Patel et al., 2007; Simões et al., 2007; Oulahal et al., 2008; Simões et al., 2008). Electrostatic and hydrophobic interactions thus play an important role in attachment as microbes approach the surface (Bos et al., 1999; Kang et al., 2004; Redman et al., 2004; Walker et al., 2004;

Schneider et al., 2005). Flagella, fimbriae and pili appendages overcome the repulsive forces between the cells and the surface, resulting in a change from reversible to irreversible attachment to the surface during the second stage of biofilm formation (Kumar and Anand, 1998; Garrett et al., 2008; Tribedi and Sil, 2014; O'Toole and Wong, 2016; Armbruster and Parsek, 2018). During the third stage of biofilm development, the organisms start to produce biofilm specific genes as a result of communication through autoinducer signals (Davies et al., 1998; Vasudevan, 2014; Gupta et al., 2016). A strategy for growth and maturation of the biofilm is the production of EPS, which are responsible for the composition, structure and mechanical stability of the matrix that binds microbes and particulate materials together and to surfaces (Stoodley et al., 2002; Flemming et al., 2007; Simões et al., 2010; Colvin et al., 2011; Franklin et al., 2011).

The composition of EPS is more complex than only polysaccharides; proteins, glycoproteins, glycolipids, fibrous proteins, and even extracellular DNA (eDNA) are also found (Whitchurch et al., 2002; Flemming et al., 2007; Herzberg et al., 2009; Gloag et al., 2013; Erskine et al., 2018). The production and composition of EPS is dependent on a number of metabolic processes including growth phase changes, cell lysis, active secretions, macromolecules released from the cell surface and also environmental interactions such as composition, light, pH, and temperature (Otero and Vincenzini, 2003; Eboigbodin and Biggs, 2008; Quintelas et al., 2011). The composition of EPS also influences the interactions that microorganisms have with the surrounding environment (Dragoš and Kovács, 2017). Biofilm structural integrity has recently been attributed to fibre-forming proteins which also provide a degree of protection from predation (Blanco et al., 2012; Romero and Kolter, 2014; Taglialegna et al., 2016; Vidakovic et al., 2018), while the stability of biofilms is shaped by interactions of the polysaccharide chains within EPS (Higgins and Novak, 1997; Donot et al, 2012). The environment within the biofilm is thus controlled by EPS as it regulates the porosity, density, water content, charge, sorption properties, hydrophobicity, and mechanical stability (Flemming et al., 2007; Flemming and

Wingender, 2010). The physical and chemical properties of EPS at different growth stages are particularly important since EPS determines the ability of the microorganisms or aggregates to trap charged contaminant colloids, adhere to a substrate, and resist external forces. The formation of a biofilm allows bacteria a greater defence against desiccation, predation and EPS allows for the capture of nutrients and other minerals that are essential to the survival of the organisms making the biofilm (Flemming and Wingender, 2010; Rasamiravaka et al., 2015). During this formation period, the biofilm increases in layers and thickness. A major factor shaping the maturation of the biofilm is the physiological cooperation of microbial communities. (Costerton et al., 1995; Strassmann and Queller, 2011; van Gestel et al., 2015; Nadell et al., 2016). Indeed, auto-aggregation and micro-colony formation of the attached cells, as well as the production of EPS ultimately condition the microenvironment within the biofilm (Costerton et al., 1995; Herzberg and Elimelech, 2007). The development of the biofilm is thus dependent on the response of the bacteria (i.e. growth) to the micro-environmental conditions allowing for the development of a complex mature biofilm (Costerton et al., 1995; Davey and O' Toole, 2000; Nadell et al., 2016). The physiological cooperation is in turn achieved by channels permeating the biofilm and acting as a circulatory system allowing the microorganisms to exchange water, nutrients, enzymes, signals and to dispose of potentially toxic metabolites (Costerton et al., 1995; Davey and O' Toole, 2000; Harrison and Buckling, 2009; Boyle et al., 2013; Drescher et al., 2014; Nadell et al., 2016; Pollak et al., 2016; Dragoš and Kovács, 2017; Dragoš et al., 2018). Acting as a collective also allows the group to influence the surrounding local environment to their own benefit. In the fifth and final stage the biofilm disperses through shedding releasing the sessile organisms (Hall-Stoodley et al., 2004). Within the biofilm, the microorganism community disrupts the matrix stabilizing polysaccharides through the production of various saccharolytic enzymes releasing the residing bacteria. To enable translocation, the expression of

flagella proteins is upregulated prior to release allowing for the spreading of the biofilm through the colonisation of new surfaces (Gupta et al., 2016).

While the process of basic biofilm formation provides insights into the formation of biofilms on RO membranes, however, other factors influence biofouling:

- The availability of biofilm precursors in the seawater
- The formation of aggregates within the desalination system.
- The inflow of nutrients to the biofilm.
- The pressure of the system driving aggregates, bacteria, and nutrients into the RO membrane.

2.9 Conclusions and future area of study

Biofouling is a widely recognised issue in membrane-based systems, including desalination plants. The RO membrane within saltwater desalination plants are one of the components that is significantly affected by biofouling. The inflow of live biofilm forming bacteria, organics and nutrients onto the RO membrane allows for growth and proliferation of biofilms. The fouling of membranes has detrimental consequences including increased hydraulic resistance, enhanced concentration polarization, decreased salt rejection and decreased membrane permeability. As the membrane becomes further fouled, the performance deteriorates impacting not only the life span of the membrane but also the production of water and the systems energy requirements consequently increasing fiscal requirements. Hence, the development of methods to reduce the impact that biofouling has on RO membranes is of the utmost importance.

Biofilms are found to be ubiquitous in most environments. The basic stages in the formation of biofilms are mostly well understood, however, there is still some debate about the determining

factors such as genetic or environmental influences that contribute to biofilm formation. Being embedded in a matrix of EPS provides structure and protection to the biofilm, the viscoelastic nature of EPS allows them to adjust to different environmental conditions. The marine environment, and thereby the feed water, has a substantial role in biofouling. As the in-flow contains all the components that makes biofouling possible and deposits them on the RO membrane. It is the DOM and the micro and macro aggregates that consist of, or contain, TEP that are the initiators of the biofouling, thereby, changing the process of biofilm formation on the membranes. The negative impact results in biofilm development on the RO membranes, reducing the ability of seawater to pass through. Both microorganism and EPS have been attributed to a decline in the permeate flux, but only microorganisms influence the decrease of salt rejection with EPS only participating slightly. Biofilm compaction is seen under increased permeate flux and influences the structure of the biofilm. The biofilm becomes compressed and stiff as a result and over time hydraulic resistance is increased leading to reduced membrane performance.

With many different aspects involved in biofouling, improving control strategies is essential to maintain a cost -effective desalination plant; TEP is recognised as a key component in biofouling and should be focused on when researching improved ways to control biofouling. Based on the current research to date (Bar-Zeev et al., 2012; Linares et al., 2015; Derlon et al., 2016; Li et al., 2016c; Ferrando et al., 2017; Desmond et al., 2018; Kim et al., 2019; Siebdrath et al., 2019) recommendations for future research are as follow:

- Better understanding of the interactions between the membrane, TEP and microorganisms. There is limited knowledge on the chemical and physical interactions that occur at the microbial level. While TEP has been identified as a critical component in the development of biofilms there is still a lack of information on the chemical bonds developed between the membranes and TEP as well as

between the microorganisms and TEP. Novel membrane surfaces that would change the state or properties of TEP could allow for the effective control of biofilm development on surfaces.

- Improved knowledge of the physicochemical nature of TEP. Due to the complex nature of TEP, not much is known of the physicochemical characteristics such as solubility or melting points. This type of information is needed to provide new insights not only for reducing the biofilm via novel cleaning treatments, but also in devising pre-treatments that could remove or reduce the amount in the feed water.
- Obtaining more information on the formation and morphology of biofilms within pressure driven systems, is essential, to determine if the biofilm model is relevant. Additionally, further knowledge of the compression and relaxation of biofilms especially under cross-flow velocity and permeate flux would greatly enhance treatment capabilities.
- Increased investigations into the viscoelasticity of EPS, since it has been determined that it is responsible an increase in hydraulic resistance and membrane performance, is critical. Indeed, the viscoelastic nature of EPS and its deformability need to be better understood if we want to develop better performing membranes.

CHAPTER 3: Survival of the fittest: Prokaryotic communities within a SWRO desalination plant

3.1 Preface

This chapter is closely based on the accepted manuscript of an article published by Elsevier Jamieson, T., Balzano, S., Le Lan, C., Kildea, T., Ellis, A.V., Brown, M., H., and Leterme, S. C. (2021) Survival of the fittest: Prokaryotic communities within a SWRO desalination plant. *Desalination*, 514:15, 115152, DOI: 10.1016/j.desal.2021.115152

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Writing and editing 80 %

Outline your (the student's) contribution to the publication:

Conceptualisation, Methodology, Formal analysis, Investigation, Data curation, Writing - Original draft, Writing - editing, revision of the manuscript, and final manuscript.

☐ I confirm that the details above are an accurate record of the student's contribution to the work.

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3.2 Abstract

Water scarcity is known to affect 40% of the global population. By 2030, it is estimated that 700 million people will potentially become displaced due to drought. Seawater reverse osmosis (SWRO) desalination is recognised as one of the most cost effective and efficient methods to produce freshwater. However, biofouling of the SWRO membranes is detrimental to the efficacy of the desalination plants. In this study, the bacterial community composition within the pre-treatment system at Penneshaw SWRO desalination plant (Kangaroo Island, Australia) and the biological fouling parameters on the SWRO membranes were characterised. Membrane autopsies were undertaken on stages 1 and 2 positioned membranes that were operational for two and four years. Results showed that the pre-treatment system allowed for the removal of microorganisms from the water, however niche communities were able to establish and proliferate within the plant due to environmental adaption. The communities associated with the SWRO membranes were stable and had the ability to flourish on the membranes within biofilms. This study provides insights into the community structure within the pre-treatment system of the desalination plant, as well as on the SWRO membranes and examines how they impact on the performance of the plant.

3.3 Introduction

Water paucity is not only experienced in arid and semi-arid areas of the world but has become a growing problem worldwide. Increasing population, uneven water distribution, aquatic pollution, water exploitation and rigorous quality regulations have all led to a high demand for alternative technologies for water production and purification. Clean water technologies such as ultra-filtration (UF) and reverse osmosis (RO) are most commonly used as they produce high quality water through the use of membrane treatments. Today, RO is the most frequently utilized membrane technology for

the purification of water. However, membrane fouling is a significant shortcoming that limits the efficiency of the process, increasing operating costs and reducing the quality of the water produced due the presence of inorganic and organic compounds, colloidal material and microorganisms (Resosudarmo et al., 2013; Tian et al., 2013; Zhang et al., 2016). While there are processes in place to reduce inorganic fouling, the prevention and control of microorganism attachment and subsequent growth on the membranes is difficult to accomplish (Al-Ahmad et al., 2000). Pre-treatments such as micro-filtration (MF) and UF are common in RO desalination plants to reduce the amount of foulants reaching the RO membrane (Mo et al., 2008; Kavitha et al., 2019). The addition of disinfectants and/or chemical agent dosing are the most common techniques used to reduce membrane biofouling. However, these treatments only provide temporary resolution as colonization of the membrane by microorganisms is inevitable (Bereschenko et al., 2008). Biofilm formation on these membranes impacts negatively on the operation of desalination plant, resulting in a decline in salt rejection, water quality and flux and an increase in operating pressure (Flemming, 2002; Herzberg et al., 2009; Berman et al., 2011; Harif et al., 2011).

With biofouling influencing plant production and costs, more effective methods of prevention and control are required. However, the origin of biofouling is indeterminate. Several studies have focused on the biofilm communities, especially bacteria, within water treatment plants to determine the origin of biofouling (Pang and Liu, 2007; Bereschenko et al., 2011; Chiellini et al., 2012). These studies are based on culture-dependant methods which are limited in providing insights into real conditions as a large portion of the microorganisms are uncultivable. Alternatively, molecular methods allow for a more precise description of the microbial diversity and abundance. Thus far, studies using rRNA gene clone libraries to identify biofilm communities indicate that α-proteobacteria are the predominant class in microbial communities on membrane surfaces (Chen et al., 2004; Pang and Liu,

2007; Bereschenko et al., 2011; Zhang et al., 2011; Ayache et al., 2013; Khan et al., 2013b; Khan et al., 2014).

Here, this study describes the composition of microorganisms within the seawater reverse osmosis (SWRO) feed water and on associated membranes within a SWRO desalination plant using next generation sequencing technology. The SWRO membranes (stages 1 and 2) were removed after 2- and 4-years use. Comparison between the positions and two time periods was performed to determine the dominant organisms associated with membrane biofouling within a SWRO desalination plant. The putative functions of these microorganisms were identified in order to better understand their role in the biofilm. Moreover, the potential effects of feedwater quality on biofilm development on the SWRO membranes was investigated over time.

3.4 Materials and Methods

3.4.1 Description of the Penneshaw SWRO desalination plant

The SWRO desalination plant in Penneshaw, Kangaroo Island, South Australia, Australia has been in operation since 1999 with a nominal output of 300 $kL.day^{-1}$, at a 40% recovery rate (Dixon et al., 2012). A diagram of the installation at Penneshaw is shown in Figure 3.1. The screened seawater intake pipe (10 cm and 0.5 mm pore size) is situated 220 m offshore at a minimum depth of 8 m. In the pre-treatment section, the seawater is treated with sulfuric acid weekly for scaling control (intake seawater pH = 8.08 ± 0.20, after acid treatment pH = 6.88 ± 0.27). A medium-pressure ultraviolet (MP-UV) system pre-treats the seawater prior to filtration via sand filters. Each sand filter contains filter coal (0.9-1.1 mm size; 300 mm depth), quartz sand (0.45-0.55 mm size; 500 mm depth), garnet sand (0.3 mm size; 200 mm depth) and graded gravel (500 mm depth). Further filtration of the seawater is undertaken through 3 x 15 µm and 3 x 5 µm cartridge filters before it enters the SWRO

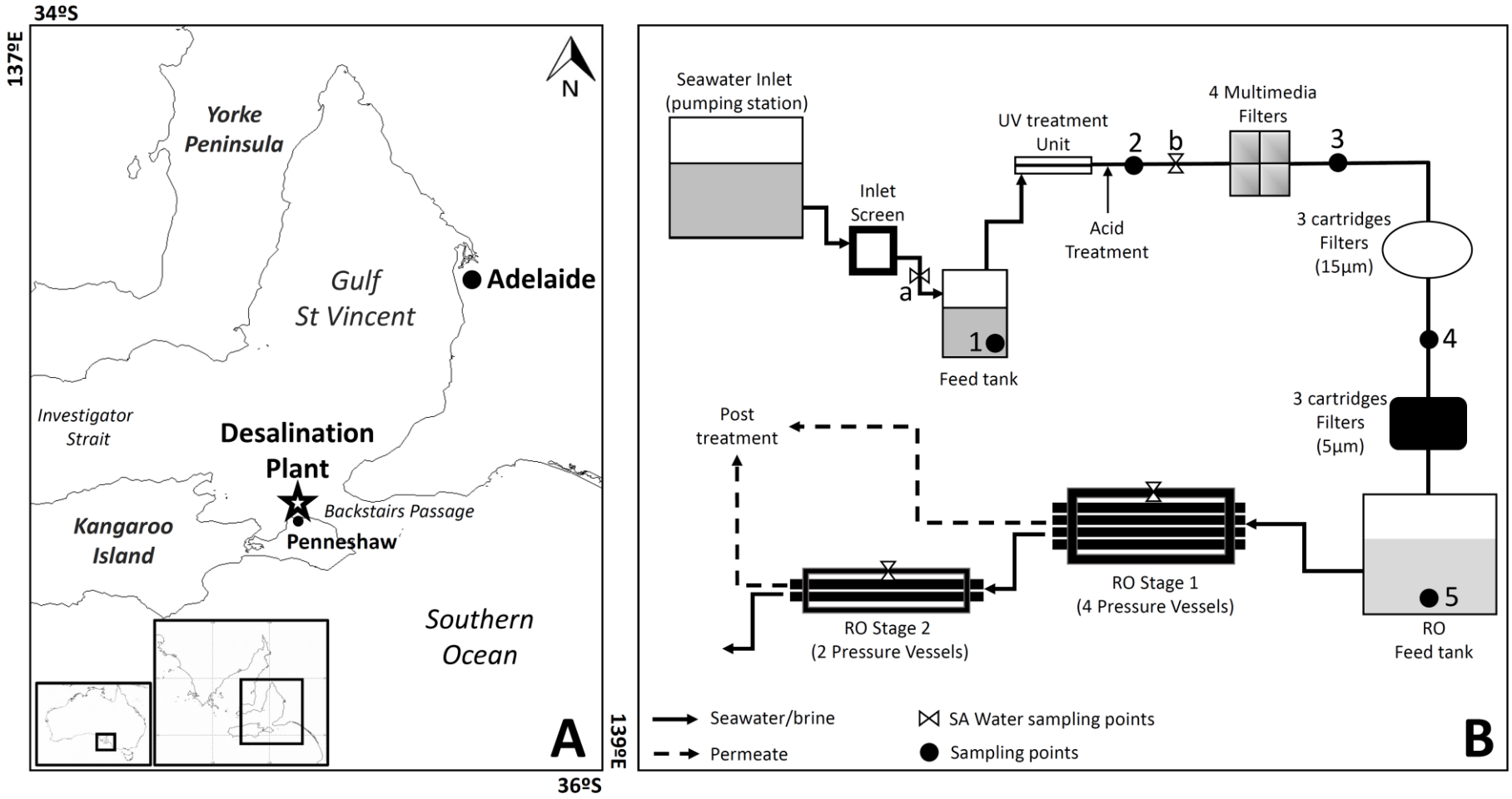

Figure 3.1: (A) Location of the Penneshaw SWRO desalination plant and (B) Schematic diagram of the Penneshaw SWRO desalination plant. Numbers indicate the different sampling points: (1) Intake seawater, (2) Post MP-UV and acid treatment (3) post sand filter treatment (4) post cartridge filter treatment (5) SWRO feed tank water. The letters indicate the SA Water sampling points: (a) intake seawater and (b) intake seawater after acid treatment.

feed tank. The flow rate of the seawater through the system is predominately 8.4 $L.s^{-1}$ (17 $L/(m^2\ h$; LMH) under a pressure of 5800 to 6000 kPa (58 – 60 bar; measured at stage 1). Backwash occurs every 48 hrs for each multimedia filter for 420 – 510 s at a flow of ~ 16 L/s, followed by a forward rinse of raw seawater for 300 s at 5 L/s. Ancillary data (S1) were provided by SA Water for sites a and b (Figure 3.1), the operator of the Penneshaw SWRO desalination plant. These data included the pH of seawater after sulfuric acid treatment.

The SWRO unit compartment has a single framework containing 12 pressure vessels, where each vessel contains 4 membranes (Figure 3.1) The vessels are organised in a unit of 3 high by 4 wide. The membranes are spiral wound thin-film composite SWRO polyamide membranes (FILMTEC™ SW30HRLE-440i) with an active surface area of 41 m^2 and a permeate flow rate of 30.2 m^3/day. The membranes obtained for this project were installed on the 19th August 2010 and removed on the day of sampling, the 1st September 2014. Membranes that were damaged (via irreversible scaling or mechanical damage) were discarded and not to be used in this study. Four fouled SWRO membranes were provided by SA Water for autopsy: a membrane from each stage of the SWRO unit (1st stage and 2nd stage) and which had been in service for two and four years. During this period no chemical cleaning of the plant or the membranes was undertaken apart from the sulfuric acid addition.

3.4.2 Water sampling sites

Within the desalination plant, seawater was collected between December 2012 and November 2013 from five different pre-treatment sites to assess water quality (salinity, pH, temperature, and Chlorophyll a), transparent exopolymer particles (TEP) and phytoplankton counts (Balzano et al. 2015c). Two of these sites: Site 1 located prior to MP-UV treatment (intake seawater) and Site 5 within the SWRO water feed tank located post-5 μm cartridge filters (Figure. 3.1) were sampled 5 times over

a 13 months period (December 2012, March 2013, July 2013, and November 2013) for molecular analyses. At these sites, 120 L of seawater was concentrated by tangential flow filtration (Marie et al. 2010) to 2 L and pre-filtered using 10 µm cellulose filters before being filtered through 0.22 mm pore size Sterivex units (Millipore). Samples were processed according to Balzano et al. (2015c).

3.4.3 Foulant removal and DNA extraction

Samples (1 cm x 10 cm) were collected from the feed, middle and end leaves that make up each of the membrane. The foulant was scraped from the membrane sample using a sterile scalpel and resuspended in 1 mL of tangential flow filtered seawater. Nucleic acids were extracted from the foulant using MPBio Lysing Matrix M tubes in combination with the FastPrep-24^{TM} 5^{G} (MPBio) sample preparation system. To the foulant, 250 µL of lysis buffer and 250 µL of proteinase K were added prior to mixing by vortex and overnight incubation at 56 °C. Samples were processed twice for 40 s at a speed setting of 6 in the FastPrep-24^{TM} 5^{G} (MPBio) before incubation for 60 min at 56 °C. This process was repeated 8 times prior to DNA extraction. The aqueous phase was then removed, and nucleic acids were extracted using a modified protocol from Real Genomics HiYield^{TM} DNA extraction kit (Real Biotech Corporation, Taiwan).

3.4.4 PCR and sequencing

Amplification of the prokaryotic V1-V2 region of the 16S rRNA gene was undertaken using the universal primers 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 338R (5′-TGCTGCCTCCCGTAGGAGT-3′) (Hamady et al., 2008). Primers were modified to include an A-adaptor (5′-CCATCTCATCCCTGCGTGTCTCCGACTCAG-3′) and a sample specific, 11 bp barcode, to the 5′ - end of

the forward primer, and a P1-adaptor (5′-CCTCTCTATGGGCAGTCGGTGAT-3′) to the 5′-end of the reverse primer for Ion Torrent next generation sequencing. Polymerase chain reactions (PCR) were performed in a total volume of 50 µL, containing approximately 1 ng.µL^{-1} of template DNA, 2 U Q5 Hot Start High-Fidelity DNA Polymerase (New England Biolabs), 1 x Q5 reaction buffer (New England Biolabs), 1 µL of complementary primers, 2.5 mM of deoxynucleotide triphosphates (Promega) and MilliQ water. The PCR consisted of an initial denaturation step at 98 °C for 1 min, 14 cycles of 30 s at 98 °C, 30 s at 60-72 °C and 30 s at 72 °C, followed by 21 cycles of 30 s at 98 °C and 1 min at 72 °C. The PCR products were purified using a Wizard SV Gel and PCR clean-up system (Promega). Amplicons were sequenced by The Australian Cancer Research Foundation Biomolecular Resource Facility using an Ion Torrent Personal Genome Machine provided with a 318 chip (Life Technology) and adapted for a maximum read length of 400 bp.

3.4.5 Bioinformatic analysis

The Ion Torrent platform sequence data were analysed using Mothur (Schloss et al., 2009). A lower Phred quality threshold (20) was used to filter the reads, in comparison to other sequencing platforms (i.e., 25) as the Ion Torrent have been found to underestimate the real base accuracy (Bragg et al., 2013).

Reads <200 bp, with a Phred quality <20 over a 50 bp sliding window (Frank-Fahle et al., 2014), with one or more nucleotide mismatches for the forward primer (Behnke et al., 2011) and >8 homopolymers were removed from the dataset. Forward primer sequences and barcodes were removed, and reads were trimmed to 250 bp in length. The UCHIME algorithm was implemented to identify and remove chimeric sequences and singletons (Edgar et al., 2011; Guillou et al., 2013). Distinct Operational Taxonomic Units (OTUs) were determined using the UCLUST algorithm based on a 97%

similarity. A representative set of samples was randomly selected from the data set to compare diversity. The SILVA database (release 132) was applied to infer taxonomic affiliation of the OTUs employing the UCLUST algorithm (Edgar, 2010).

3.4.6 Transparent exopolymer particle quantification

To quantify the TEP present within different sections of the SWRO membrane, three pieces of membrane (1 cm x 10 cm) were removed from the feed, middle and end positions and stored in 0.2 µm filtered seawater at -20 °C. The membrane samples were thawed at room temperature prior to being sonicated for 2 min at 50/50 Hz (Heat system Ultrasonic processor Model XL2020) to assist with the detachment of the biofilm. The biofilm was scraped from the membranes into a sterile petri dish using a sterile scalpel. To remove any remaining biofilm the membrane was rinsed with 0.2 µm filtered seawater. The biofilm was then filtered onto a 0.4 µm Millipore polycarbonate filter under gentle vacuum conditions, as described by Passow and Alldredge (1995b). Samples were stored at -20 °C and analysed as described by Balzano et al. (2015c). Briefly, the filter were centrifuged for 30 minutes at 3,200 x *g* before the filters were discarded. The samples were recentrifuged, supernatant removed and 0.02% w/v alcian blue (2 mL) in 0.06% v/v acetic acid were added to the resuspended pellet. The supernatant was removed after centrifugation before washing the pellet in 2 mL of distilled water. Next, 4 mL of 80% v/v sulfuric acid was added to the resuspended pellet, followed by an incubation at room temperature for 2 hours. The solutions resuspended followed by centrifugation at 3,200 x *g* for 20 minutes. The supernatant absorption was measured at 787 nm using a FLUOstar Omega spectrophotometer (BMG Labtech, Australia). Xanthan gum (Sigma-Aldrich, Australia) suspended in ethanol were used to calibrate the TEP values (Claquin et al., 2008). The relative fluorescence of the TEP were converted to µg equivalent of xanthan gum L^{-1} (µg Xeq. L^{-1}).

3.4.7 Attenuated total reflectance and Fourier transform infrared spectroscopy

Samples were collected from the feed, middle, and end leaves of each of the membranes and freeze dried. The samples were analysed using a PerkinElmer Spectrum 400 Fourier transform infrared spectroscopy (FTIR) instrument equipped with an attenuated total reflectance (ATR) module to enable recording of the ATR-FTIR spectrum. The ATR crystal was zinc selenide (ZnSe) with an angle of total reflection of 45° at 1000 cm^{-1} and a depth of penetration (path-length) of 6 µm. The ZnSe crystal had a refractive index of 2.4 and a 3-bounce system was used for measurements, using 128 scans between 4,000 and 650 cm^{-1} with a resolution of 4 cm^{-1} in absorbance mode, with background (i.e., air) subtraction.

3.4.8 Scanning electron microscopy

Three samples (1 cm x 10 cm) were collected from each of the feed, middle and end leaves of each of the membranes before being fixed and dehydrated as per the protocol of Lee et al. (2010). Prior to examination, the samples were mounted onto carbon-tape coated SEM stubs before being sputter coated with platinum (15 nm) and observed using a FEI Inspect F50 SEM operating at 20 kV.

3.4.9 Data analysis

The following statistical analyses were performed for the 16S rRNA sequencing data unless noted. All data were transformed using Log+1 before undertaking Bray-Curtis similarity to calculate a similarity matrix between the prokaryote communities. The data was analysed by Principle Coordinate

Analysis (PCoA) and SIMPER using the Primer7-PERMANOVA Software (version 7.0.13). Differential abundances between microorganism communities were compared using the DESeq2 package (version 1.29.4; Love et al., 2014) for R Software (version 4.0.0). Core microbiota were identified for prokaryotic organisms within the water samples as well as the membrane samples. To identify the core, the variable, and the unique taxa among the intake water, the RO feed tank water and the membrane samples, Venn diagrams were created with the online tool access through https://bioinfogp.cnb.csic.es/tools/venny/. The functional prediction of genes in the water and membrane microbiota was acquired from web-based software Piphillin (Iwai et al., 2016) based on the relative abundance of the OTU table (taxonomy was assigned using the Silva database 132). The function prediction matrix was clustered and categorized utilising the Kyoto Encyclopaedia of Genes and Genomes (KEGG) orthologs (KOs) and pathways. TEP data were analysed by one-way ANOVA using the SPSS Software (25.0.0.2). Microsoft excel was used to create the abundance graphics. Circular heatmaps were created using the circlize package (version 0.4.11; Gu et al., 2014) for R Software (version 4.0.3).

3.5 Results

3.5.1 Prokaryotic community structure

In this study, we investigated the prokaryotic organisms present in the intake seawater as well as in the SWRO feed tank of the Penneshaw desalination plant (Figure. 3.1). PCoA based on Bray-Curtis distance ordination demonstrated dissimilarities in the prokaryotic community composition between the seawater intake and the SWRO feed tank water samples (Figure. 3.2A.). The dissimilarities in the seasonal community structure, between the intake seawater and the SWRO feed tank water, is seen along the principle coordinate PCO1. While a clear separation of the seawater intake seawater and the

SWRO feed tank water is evident along the principle coordinate PCO2 (permanova p=<0.05), displaying the effect of pre-treatment within the desalination system. Other factors such as SWRO feed tank water pH, and temperature have an impact in influencing not only the prokaryotic diversity within the SWRO desalination plant but also the pre-treatment systems efficacy. The pH of the intake seawater was ~7.89 with variation observed throughout the four-year period where the lowest pH was 7.40 and highest was 8.20. After the addition of sulfuric acid, the pH of the intake water was lower at ~6.85. The highest pH documented after acid treatment was 8.10 and the lowest was 6.5. While variations in the pH were evident it was relatively stable throughout the four-year period. During the sampling period the pH at Site 1 displayed variation over time while the pH at sites 2 -5 were quite stable (Figure 3.3).

We also investigated the fouling communities on the SWRO membranes from stages 1 and 2 after two- and four-year's service within the Penneshaw desalination plant. PCoA based on Bray-Curtis distance ordination demonstrated dissimilarities in the prokaryotic community composition between stages 1 and 2 SWRO membranes after two- and four- years' service (Figure 3.2B). The dissimilarities in the community structure between the membranes of two- and four- years' service is seen along the principle coordinate PCO1 (permanova p=<0.05). While a clear separation between the stages 1 and 2 positioning of the SWRO membranes is evident along the principle coordinate PCO2 (permanova p=<0.05). These results suggest that PCO1 might represent the time that the SWRO membranes were operational within the system. In contrast, PCO2 might represent the degree of fouling on each membrane. However, due to the small sample size of the present study the conclusions presented would benefit from more data points.

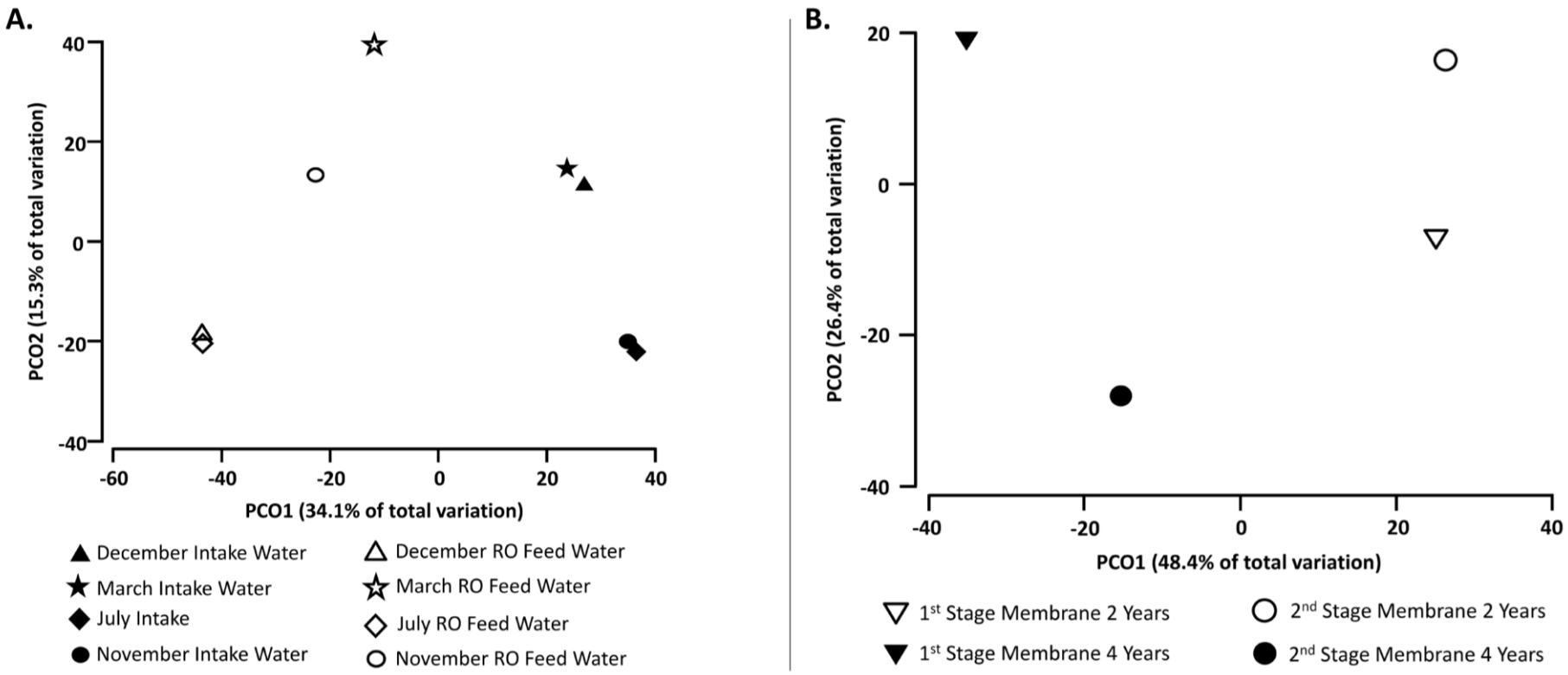

Figure 3.2: Principal coordinate analysis (PCoA) based on Bray-Curtis distance ordination displaying the differences in the prokaryotic composition between (A) the intake seawater and SWRO feed tank water samples (permanova p=0.05), and (B) the 2-year and 4-year membranes in the lead and lag position (permanova p=0.05). The total variability is explained by the two PCoA axes, with the ordination of water samples (A) explaining 49.4% of the seasonal and pre-treatment variability observed in the samples and (B) explaining 74.8% of the variability observed in the membranes as well as the ordination of membranes.

3.5.2 Taxonomic diversity

Within the intake seawater and the SWRO feed tank water samples (December 2012, March 2013, July 2013, and November 2013), 31 bacterial taxa were identified. The dominant phyla within the intake seawater were Cyanobacteria (47%), Proteobacteria (42%) and Bacteroidetes (4%; Figure 3.4A). Within the SWRO feed tank, the phyla Proteobacteria was the most dominant (50%) followed by Cyanobacteria (17%), Bacteriodetes (14%) and Patescribacteria (10%; Figure 3.4A). Some phyla were only found in one of the seawater samples: Tenericutes in the intake seawater, and Armatimonadetes, BRC1, Deinococcus-Thermus, Entotheonellaeota, Eremiobacterota, Omnitrophicaeota, and PAUC34f in the RO feed tank water. Many of these organisms showed significant differences ($p < 0.05$) in abundance between the intake seawater and the SWRO feed water across the duration of the study (Figure 3.5A) with an overall dissimilarity of 89.6% between the intake seawater and the SWRO feed tank water (SIMPER) driven by the orders of Candidatus Peribacteria, Coxiellales, Chitinophagales, Acidithiobacillales and Candidatus Peregrinibacteria. However, the SIMPER analysis showed that the dissimilarity in December (92.6%), March (83.1%), July (94.2%) and November (85.8%) between the intake seawater and the SWRO feed tank water was influenced predominately by the orders of Synechococcales and Rhodobacterales.

Of the 31 bacterial taxa identified in the intake water and the SWRO feed tank water samples, 20 were also observed in the SWRO membrane samples: Acidobacteria, Actinobacteria, Bacteroidetes Calditrichaeota, Chloroflexi, Cyanobacteria, Dadabacteria, Dependentiae, Firmicutes, Fusobacteria, Gemmatimonadetes, Lentisphaerae, Nitrospirae, Patescibacteria, Planctomycetes, Proteobacteria, Spirochaetes, Tenericutes and WPS-2 (Figure 3.4B). As opposed to the seawater communities, the phyla Proteobacteria was the most dominant on the membranes (52%), followed by Actinobacteria (33%), and Dependentiae (3%). The phylum Nitrospirae was only identified on the 2-year stage 1

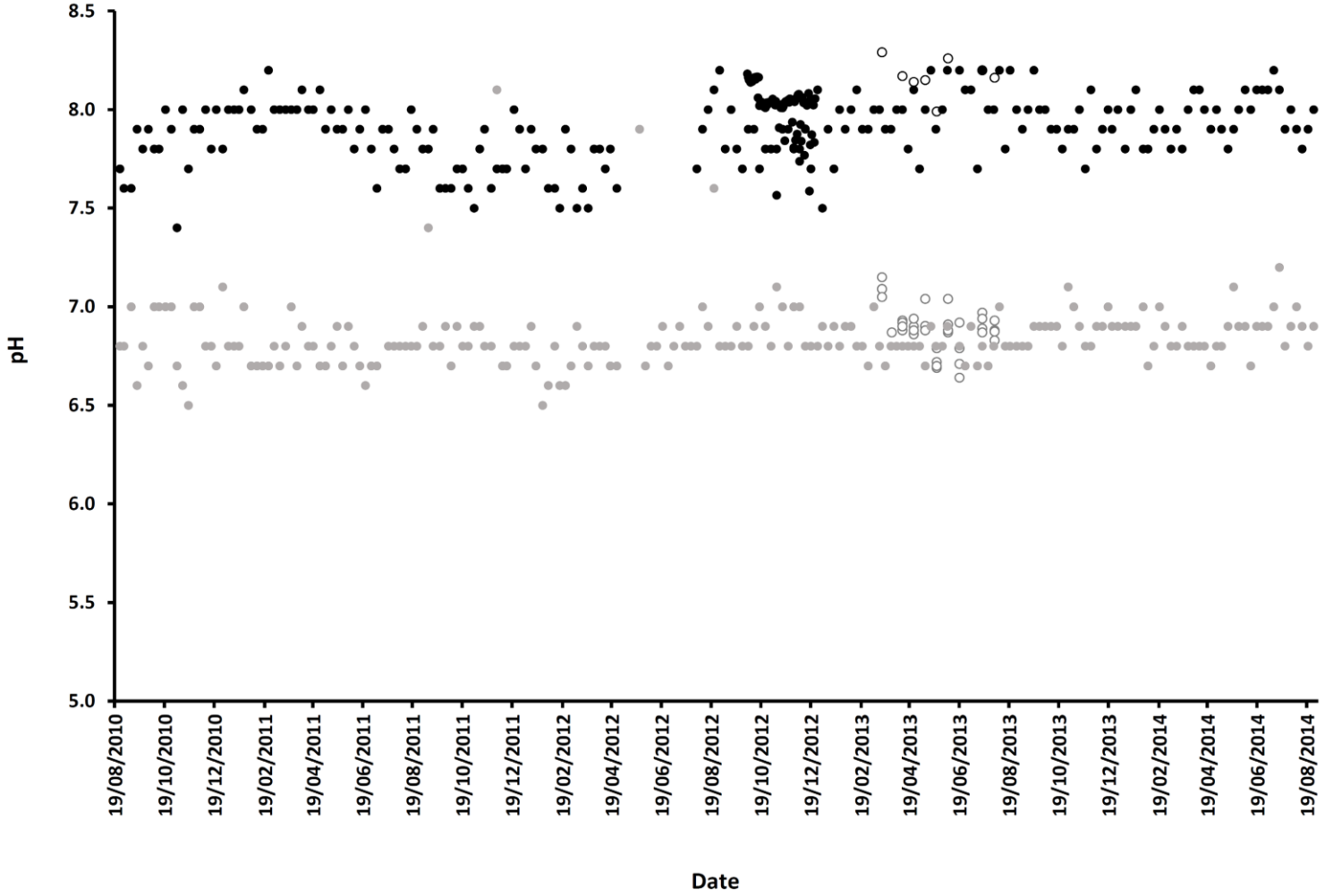

Figure 3.3: The pH at Site 1 (White dot with black outline) and Site 2-5 (White dot with grey outline) during the sampling months of December 2012, March, July and November 2013 and the pH at Site a (Black dot with black outline) and Site b (Grey dot with grey outline) for duration of the operational life of the SWRO membranes.

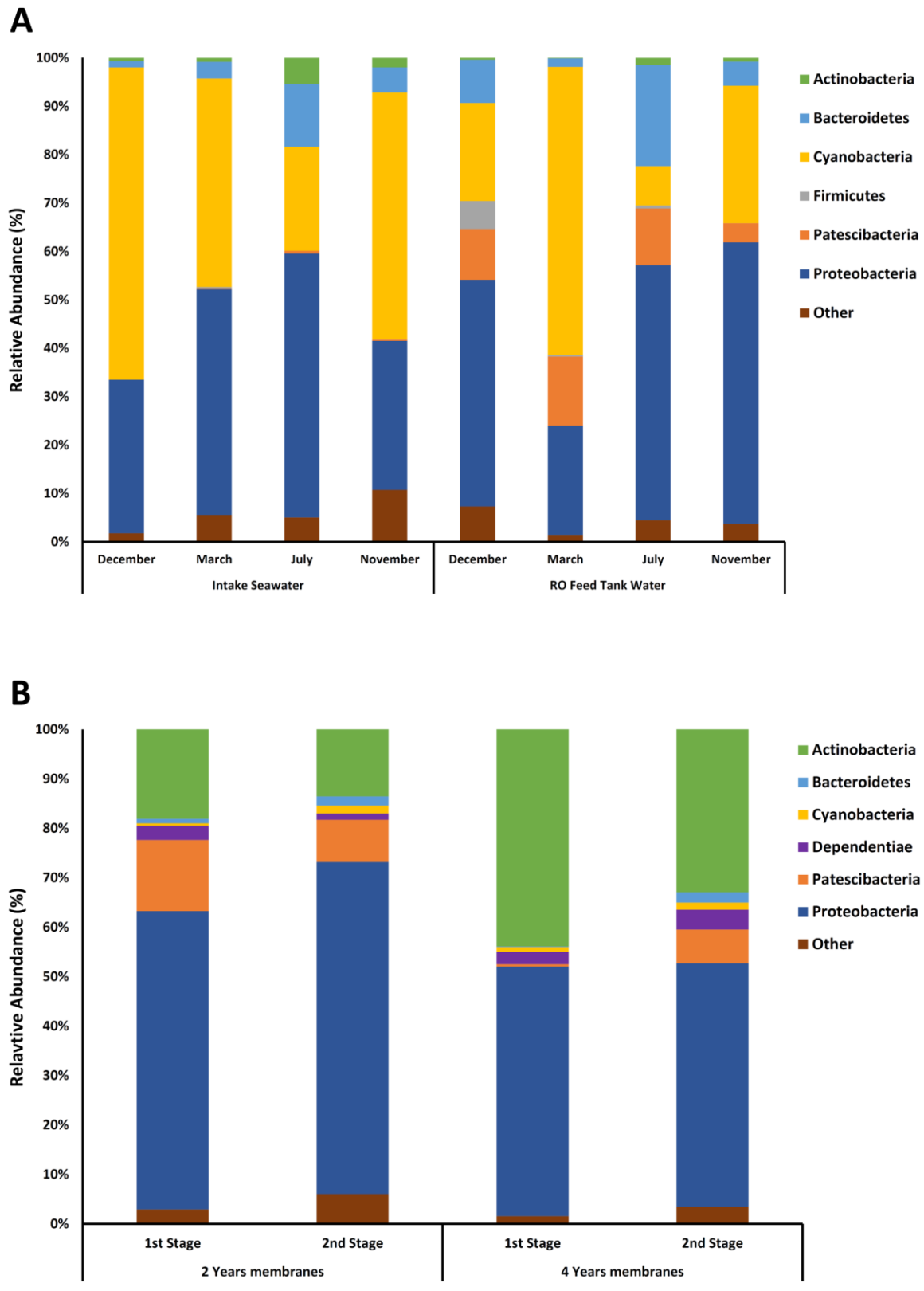

Figure 3.4: The relative abundance composition of the phylum taxonomy of the prokaryotic communities (A) of the intake seawater and the SWRO feed tank water and (B) on the 2- and 4-year SWRO membranes from composite samples.

membrane, while the phyla Lentisphaerae and Tenericutes, were only identified on the 2-year stage 2 SWRO membrane. The phyla Fusobacteria and Calditrichaeota were only found on the 4-year stage 1 membrane. The average dissimilarity between the SWRO membranes (61.7%) is much lower than that of the water samples (SIMPER) and is driven by different taxonomical orders: Solirubrobacterales, Corynebacteriales, and Kordiimonadales. The community of prokaryotes of the SWRO feed water was compared to that of the membranes. Significant differences ($p < 0.05$) in read abundance were observed between SWRO membranes and SWRO feed water for 22% of genera (Figure 3.5B). With 16% of genera exhibiting a greater read abundance on the SWRO membrane comparted to the SWRO feed tank water, 6% were instead more abundant in the SWRO feed tank water and 78% did not exhibit significant differences between the SWRO membrane and the feed water.

3.5.3 Core and unique operational taxonomic units

The organisms considered to be essential to the function of their communities can be labelled as "common core microorganisms". The presence of these organisms, identified within all assemblages of a particular ecosystem, allows for the characterization of a "healthy" community and thus the impact assessment of any perturbation (Shade and Handelsman, 2012). In this study, the common core of prokaryotes within the intake water was analysed using the OTU data obtained from sequencing and are presented in Figure 3.6A. The core OTU is represented by the areas which overlap in a Venn diagram (Oliveros, 2007). Three different groups were used to classify the OTUs: core OTUs (identified in all sampling months), variable OTUs (identified in two or more months but not all) and unique OTUs (identified in only one month). Of the 2,585 prokaryote OTUs obtained from the intake seawater 3.3% were core OTUs, 27.6% were variable OTUs and 69.1% were found to be unique OTUs. The core OTUs were affiliated to six classes within four phyla, namely Actinobacteria, α-Proteobacteria, Bacteroidia,

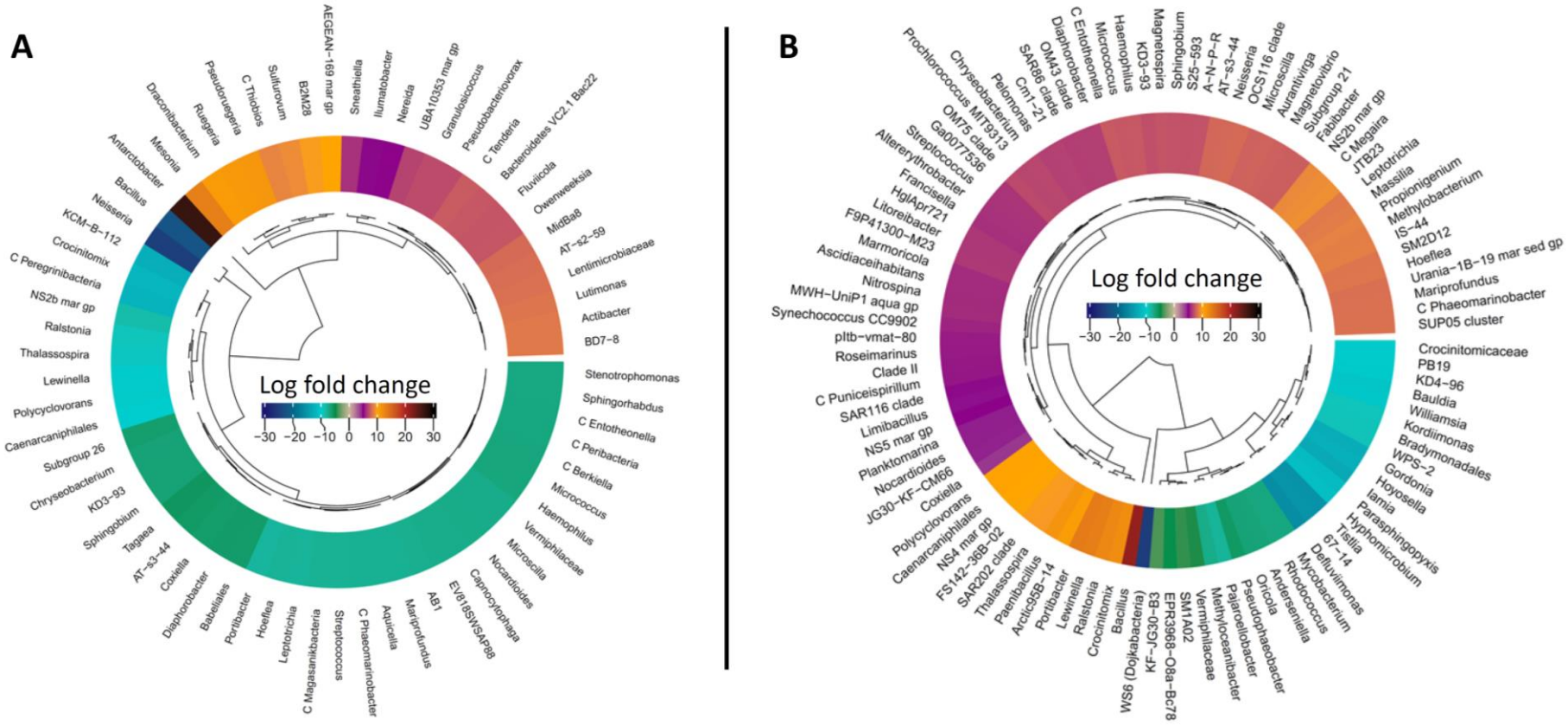

Figure 3.5: Circular heatmap representation of the log fold changes of the prokaryotic organisms at the genus level which showed significant difference ($p < 0.005$) between (A) the intake seawater and the SWRO feed tank water (B) the SWRO feed tank water and the SWRO membranes. Dendrogram represents the hierarchical clustering of the column based on the Euclidean distance with complete linkage clustering (Gu et al., 2014 and 2016). Shades of colour represent the log fold change increase and decrease (see colour scale).

ε-Proteobacteria, γ-Proteobacteria, and Oxyphotobacteria. The SWRO feed tank water showed different results (Figure 3.6B) with 9,426 prokaryote OTUs obtained, of which 58.5% are unique, 37.4% are variable and 4.1% core. The core OTUs consisted of twenty classes within thirteen phyla. These core OTU classes included Acidimicrobiia, Actinobacteria, α-Proteobacteria, Anaerolineae, Babeliae, Bacilli, Bacteroidia, Dehalococcoidia, ε-Proteobacteria, Entotheonellia, γ-Proteobacteria, Gracilibacteria, Lentisphaeria, Melainabacteria, Nitrospinia, Oxyphotobacteria, and Phycisphaerae.

Of the 2,415 prokaryote OTUs obtained from the SWRO membranes of stages 1 and 2 after 2- and 4-years operation, 70.4% were unique OTUs, 23.1% were variable OTUs and 6.5% were Core OTU's (Figure 3.6C). The core OTUs included twelve classes within eight phyla. The core OTU classes were found to be Acidimicrobiia, Actinobacteria, α-proteobacteria, Anaerolineae, Babeliae, Bacteroidia, ε-proteobacteria, γ-proteobacteria, Gracilibacteria, Oxyphotobacteria, Phycisphaerae and Thermoleophilia.

3.5.4 Functional gene predictions

To evaluate which lifestyle attributes are advantageous to the prokaryotes within biofilm on the membrane Piphillin was used to predict the function at genus level. The KEGG functional pathways predicted for the identified prokaryotic species were compared. No significant difference in the pathways was found between the 2-year and 4-year SWRO membranes (data not shown). The 2-year stage 1 and 2 SWRO membranes, as well as the 4-year 1st stage and 2nd membranes, displayed no significant difference in the pathways. Implying that the diversity and the lifestyle of the organism across all membrane positions and durations are comparatively constant.

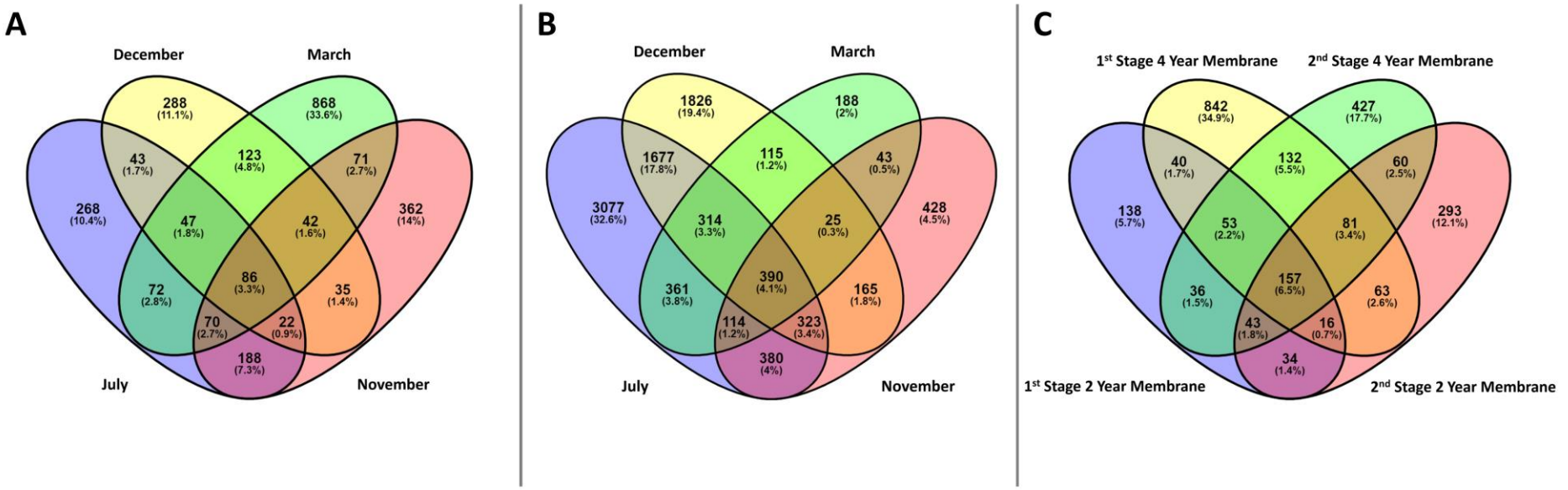

Figure 3.6: Venn diagram displaying the overlap between the prokaryotic communities in the (A) intake seawater and (B) SWRO feed tank water over a 12-month period (samples taken each quarter), and on the (C) 2- and 4- membranes in the 1st and 2nd stage positions. Core OTUs, identified in all sampling sites; variable OTUs identified in two or more sites but not all; unique OTUs, identified in only one site.

The predicted functional KEGG pathways of the SWRO feed water organisms were also compared to those found on the membranes (Table 3.1). In total, fifteen pathways were identified to be significantly (P <0.05) different. The genes encoded for the pathways such as "Amino Acid Metabolism", "Lipid Metabolism", and "Membrane Transport" were predicted to be less abundant within the organisms on the membranes than the SWRO feed tank. Whereas the encoded genes for the pathways such as "Biosynthesis of other secondary metabolites", "Nucleotide Metabolism", "Glycan biosynthesis and metabolism", "Metabolism of cofactors and vitamins" and "Energy metabolism were predicated to have increased in the organisms on the membranes indicating that there is a shift in prokaryotic diversity and lifestyle from the SWRO feed tank to SWRO membranes. However, it should be noted that the prediction algorithm is heavily influenced by the availability of reference genomes.

3.5.5 Autopsy of the SWRO membranes

Overall, the TEP concentration was significantly higher (p < 0.05) on the membrane in service for 4 years compared to the 2-years membrane. For the 2-years membranes, the TEP concentration of stage 1 (783.77 ± 58.19 μg.Xg.L^{-1}.m^{-2}) was significantly higher than that found on stage 2 (459.01 ± 48.34 μg.Xg.L^{-1}.m^{-2}). Whereas, for the 4-years membranes, the concentration of TEP between the stage 1 membrane (1,055.9 ± 46.93 μg.Xg.L^{-1}.m^{-2}) was comparable (p > 0.05) to that of the stage 2 membrane (1,013.96 ± 27.67 μg.Xg.L^{-1}.m^{-2}). This is reflected on the SEM images (Figure 3.7).

The SEM imaging of the fouled membrane provided insights into the fouling layer development. Images collected from the stage 1 and 2 membranes present a linear timeline of fouling within the Penneshaw SWRO desalination plant (Figure 3.7). The pristine membrane surface shows ridged and valley structures (Figure 3.7E). The stage 1 2-year old membrane (Figure 3.7A) shows cake-like layers

Table 3.1: Log fold change difference in the predicted KEGG pathway of the SWRO feed tank water compared the SWRO membranes.

Predicted KEGG Pathway	*Log fold change*
Amino Acid Metabolism	-0.19
Biosynthesis of other secondary metabolites	0.19
Cellular community	-0.59
Energy metabolism	0.46
Folding, sorting and degradation	0.40
Glycan biosynthesis and metabolism	0.32
Lipid metabolism	-0.26
Membrane transport	-0.29
Metabolism of cofactors and vitamins	0.43
Nucleotide Metabolism	0.30
Replication and repair	0.43
Signal transduction	-0.47
Transcription	0.65
Translation	0.46
Xenobiotics biodegration and metabolism	-0.71

Table 3.2: ATR-FTIR spectral vibrations and peak assignments of the functional groups within the biofouling on the lead and lag membranes in Penneshaw desalination plant after 2- and 4-years of service.

Functional group/Structure	2-year lead	2-year lag	4-year lead	4-year lag
ν(N-H), ν(O-H)/polypeptides released by cell lysis	3299	3340	3286	3289
ν(C-H)/lipids	2924	2925	2923 2853	2923
C=C, COO^-; N-H bending (amide II), amine deformation, ν(C=0), N-C=O (amide I)/ polypeptides released by cell lysis, protein	1643	1643	1635 1542	1636 1585 1544
CH^2, CH^3, C-O, COO^-, proteins			1454 1410	
CH^2, CH^3, COO^- bending, proteins			1378	
OH, C-O			1237	
-C-O alcohol, C-O-C/ presence of sugar; -CO stretching of polysaccharide, polysaccharides substances	1079	1080 1014	1038	1104 1074 1038 1014

Note: ATR-FTIR spectral vibrations and peak assignments (Wavenumber [cm^{-1}])

of fouling with biofilm matrix, bacteria and aggregates visible on the SWRO membrane. The stage 2 2-year old membrane (Figure 3.7B) show a cake-like fouling and a mature biofilm matrix embedding aggregates and bacteria, although the membrane is not entirely fouled. The stage 1 4-year old membrane (Figure 3.7C) display layers of fouling with polymeric substrates visible. The stage 2 4-year SWRO membrane (Figure 3.7D) exhibits a scale-like fouling on the membrane with microorganisms present within aggregates, no biofilm matrix is visible. A cake-like layer with aggregations is also visible on some parts of that membrane.

ATR-FTIR was undertaken to ascertain the functional groups in the foulants on SWRO membranes in stages 1 and 2 after 2- and 4-years of operation (Table 3.2). The peaks of the FTIR spectrum indicate functionality characteristics of polypeptides and proteins released during the lysis of cells as well as lipids and polysaccharides substances. These results are typical of biofilms and of biofilm forming bacteria (Quiles et al., 2010; Li et al., 2016a; Quiles et al., 2016).

3.5.6 Membrane performance

Data provided by SA Water allowed for the analysis of the membrane performance during their use in the SWRO desalination plant. Following guidelines from the SWRO membrane manufacturer, data for salt rejection, permeate flow and resistivity were analysed. As the data were provided for the complete 1st stage and 2nd stage modules, we do not have information on individual membrane performance. However, it is evident from these results (Figure 3.3) that the membranes of stage 1 and 2 had constant salt rejection as shown by the R^2 values of 0.00005 and 0.0184, respectively (Figure 3.8), however the permeate flux decreased over time for both stages as shown by the R^2 values of 0.7282 and 0.7157, respectively (Figure 3.9). Membrane productivity is very sensitive to changes in feedwater temperature and as water temperature increases, water flux should increase almost linearly

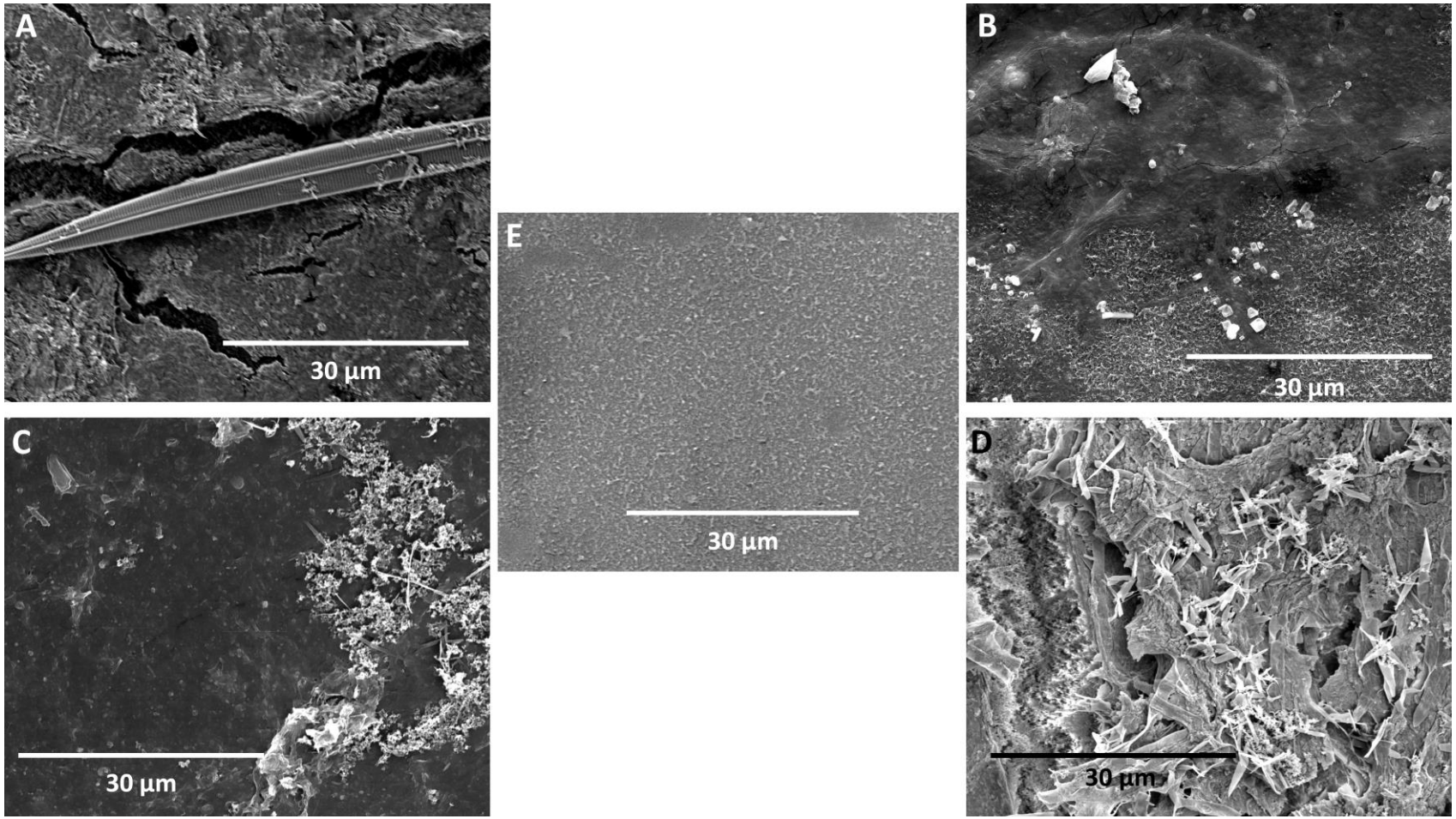

Figure 3.7: Scanning electron microscope images of the fouled SWRO membranes in the (A) stage 1 position after 2-years of service, (B) stage 2 position after 2-years of service, (C) stage 1 position after 4-years of service and, (D) stage 2 position after 4-years of service, (E) pristine membrane surface.

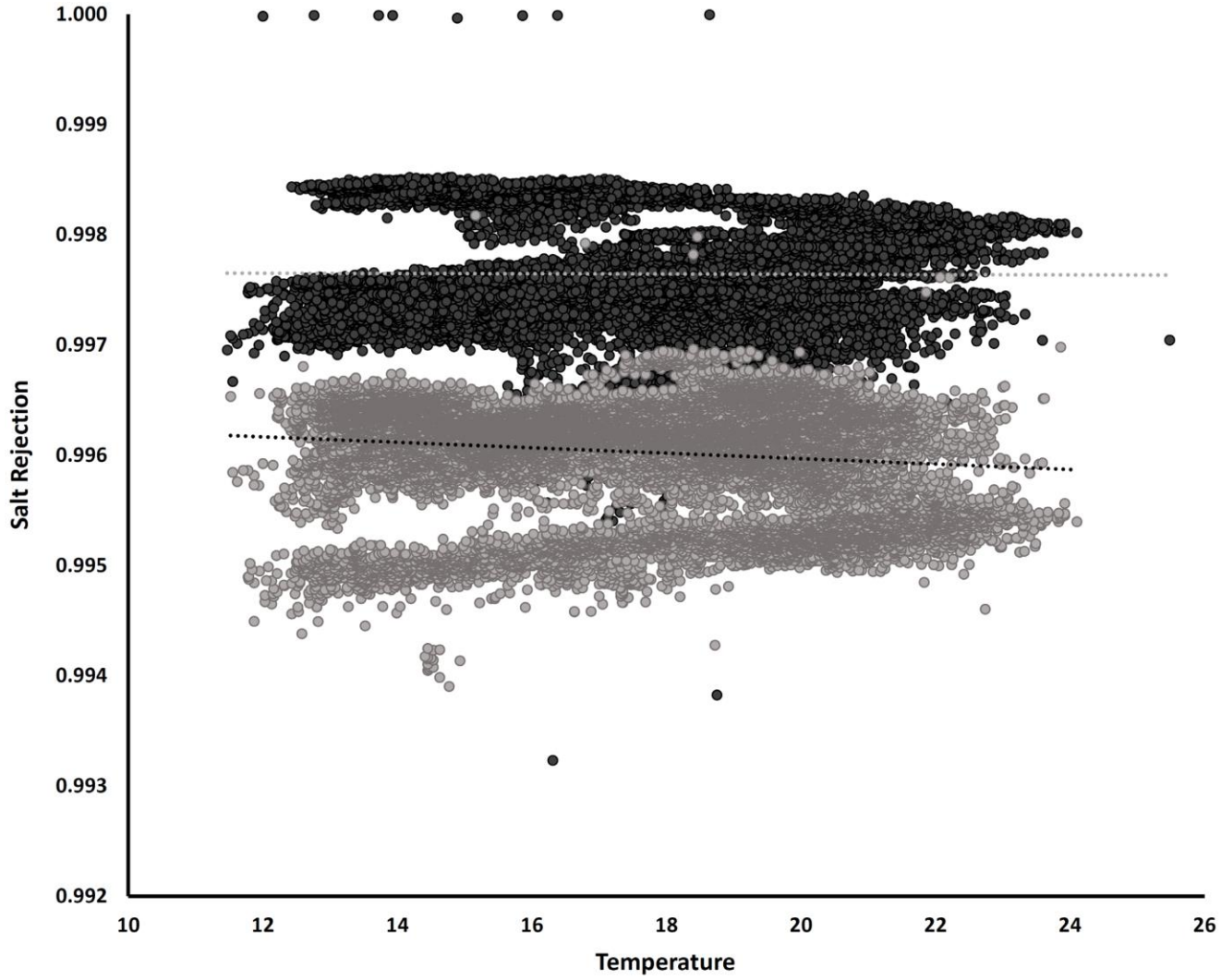

Figure 3.8: The effect of the SWRO feed water temperature on the salt rejection of the SWRO membranes in the 1st (Grey with black outline; linear trendline y = -1E-06x = 0.9977, R2 = 5E-05) and 2nd (Grey with grey outline; y = -2E-05x + 0.9965, R2 = 0.0184) stage modules.

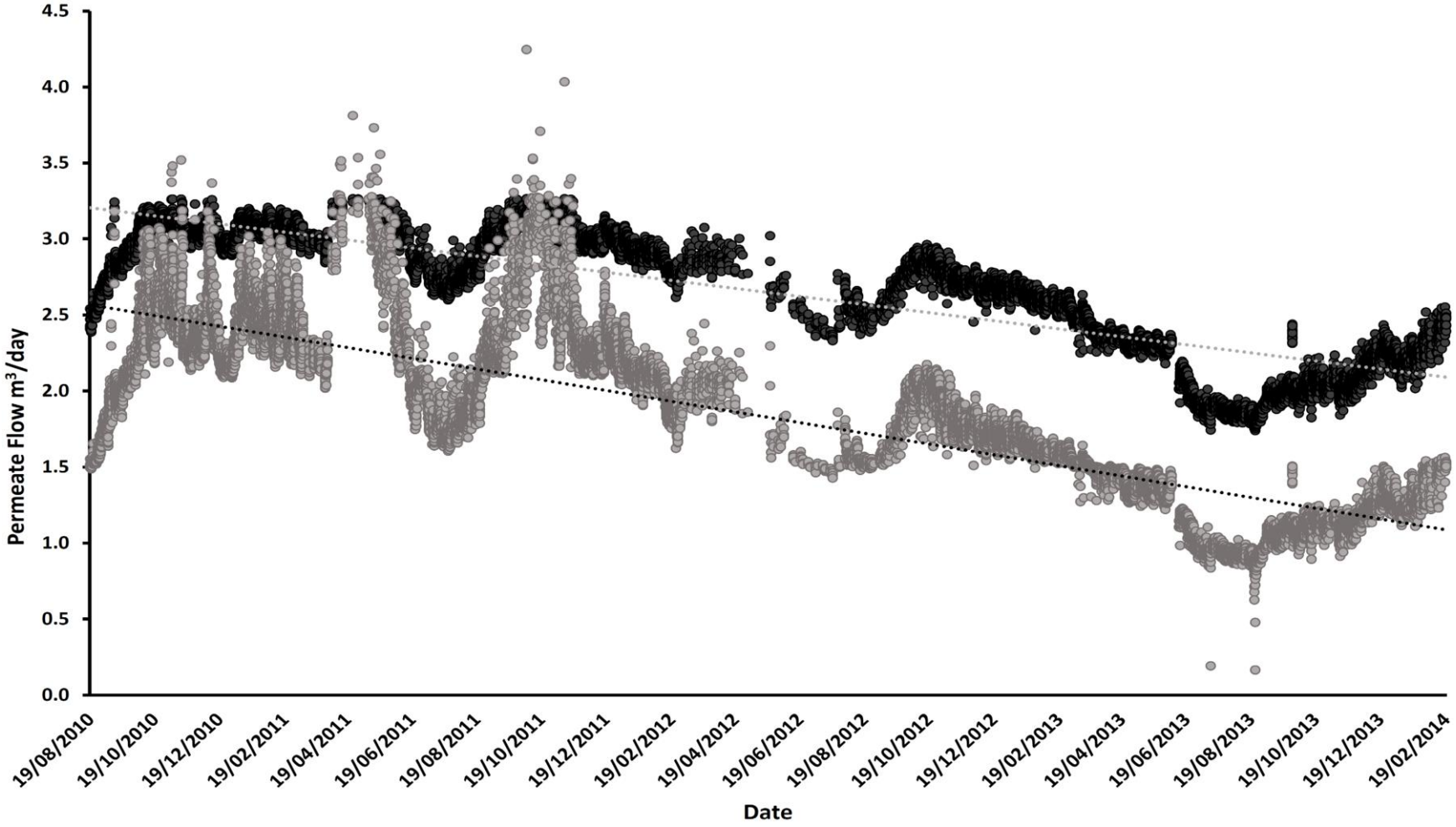

Figure 3.9: The permeate flux of the 1st (Grey with black outline; linear trendline y = -0.0009x + 38.389, R^2 = 0.7282) and 2nd (Grey with grey outline; y = -0.0012x + 49.264, R^2 = 0.7157) stages over time.

as the diffusion rates of water through the membrane gets higher (DOW 2019). Here, this is only visible for stage 2 membrane as stages as shown by the R^2 values of 0.0414 and 0.5405, respectively (Figure 3.10). Overall, 1st stage membrane shows a lower performance compared to stage 2, likely due to biofouling.

3.6 Discussion

World-wide water scarcity has determined the need for alternate potable water sources, with SWRO desalination recognised as an effective and efficient method. A key problem with SWRO membranes is the development of fouling. This widely recognised challenge results in a loss of permeability, increasing energy consumption and decreasing the life of the membrane.

3.6.1 Community dynamics of microorganisms in intake seawater and SWRO feed tank water

The Penneshaw SWRO desalination plant is host to a high microbial diversity with distinct communities present in the intake seawater and the SWRO feed water. Seasonal variation of marine microbes is a well-recognised predictable pattern within the marine environment. Indeed, eutrophic, and oligotrophic events are experienced throughout the year by those organisms which are dependent upon nutrients within the oceans (Bunse and Pinhassi, 2017; Ward et al., 2017; Mestre et al., 2020). Within the Penneshaw SWRO desalination plant, seasonal fluctuation of nutrients, microorganisms, and phytoplankton has been previously described (Balzano et al., 2015b; Balzano et al. 2015c). Here, we observed that the intake water was dominated by Cyanobacteria, Proteobacteria

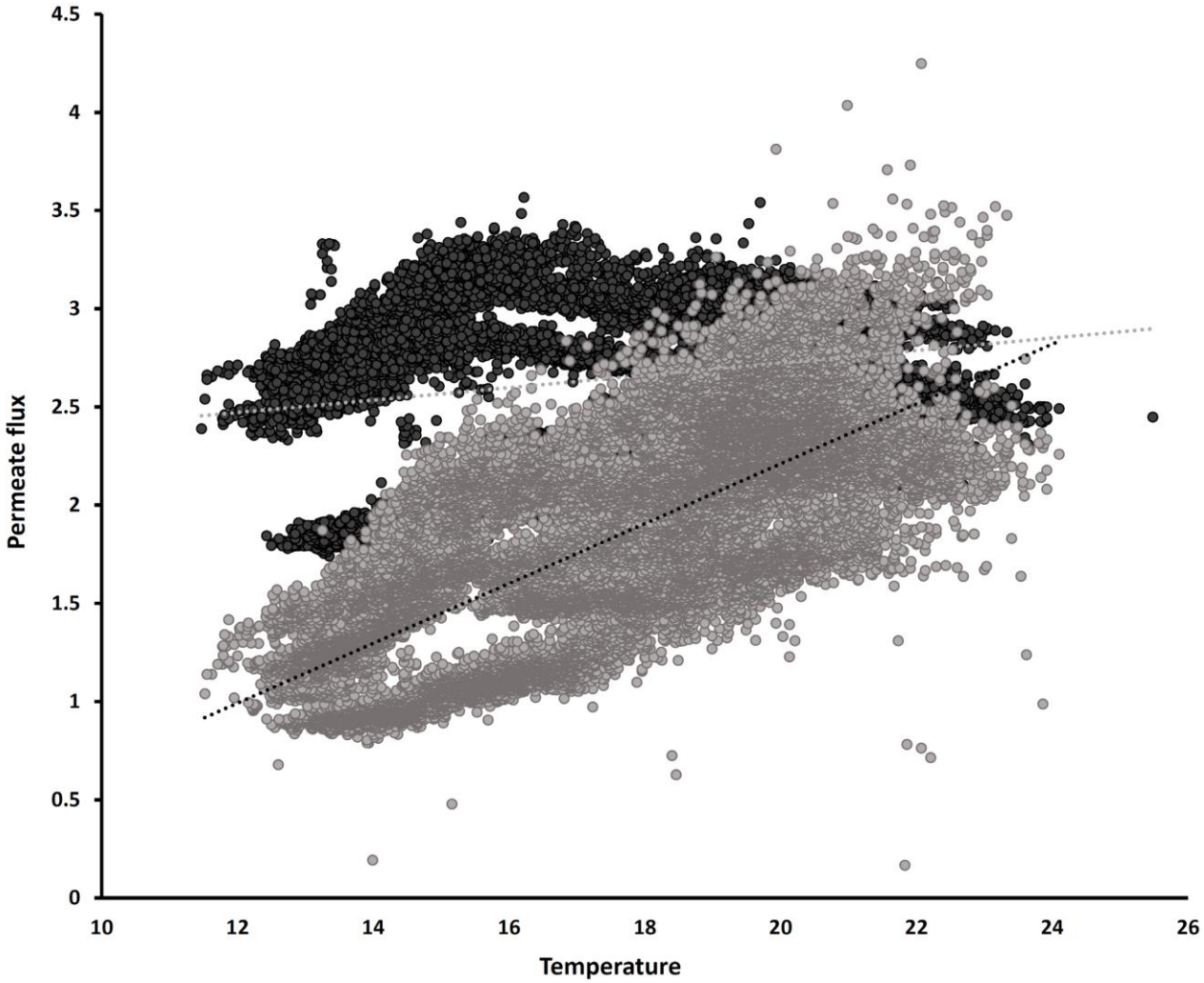

Figure 3.10: The effect of the SWRO feed water temperature on the SWRO membrane flux of the membranes in the 1st (Grey with black outline; linear trendline y = 0.0316x + 2.0921, R2 = 0.0414) and 2nd (Grey with grey outline; y = 0.1523x – 0.8355, R2 = 0.5405) stage modules.

and Bacteroides which are amongst the most abundant phyla within marine environments (Cram et al., 2015; Bunse and Pinhassi, 2017), while the SWRO feed tank water was also dominated by while the SWRO feed tank water was also dominated by Patescibacteria. These prokaryotic communities are similar to those described in other SWRO desalination plants (Belila et al., 2016; Levi et al., 2016; Li et al., 2017; Nagaraj et al., 2017). Surviving in an oligotrophic environment is challenging for any bacteria due to the need to increase their nutrient absorption rate, however, the phylum Patescibacteria adapts well (Sowell et al., 2009; Taubert et al., 2019). The Patescibacteria phylum consists of highly diverse ultra-small bacteria (~0.3 μm) with a compact cell size (Brown et al., 2015; Luef et al., 2015; Goh et al., 2019). A small cell size with a large amount of storage space is advantageous within oligotrophic environments as the gathering of nutrients is prioritised (Sowell et al., 2009; Herrmann et al., 2019). Due to Patescribacteria reducing the size of their genome to decrease their nutrient requirements, they are then reliant on scavenging the required compounds they are unable to create for metabolic functions such as amino acid or nucleotide biosynthesis (Castelle et al., 2018; Herrmann et al., 2019). Consequently, it has been suggested that their condensed size compels them to develop symbiotic relationships with other prokaryotic and/or eukaryotic organisms (Lemos et al., 2019). The phyla of Tenericutes was identified in the intake water during the summer (December 2012) and on the 2nd stage SWRO membranes after two years of operation. Tenericutes have been found in an expansive range of diverse environments including water and biofilm samples from the ocean within the spring and summer seasons (Suh et al., 2015; Antunes et al., 2020). Tenericutes is a phyla that is comprised of small (< 0.2 μm) "wall-less bacteria" as they lack the peptidoglycan cell wall which confers the rigidity of the shape (Trachtenberg, 2005; Vermassen et al., 2019; Wang et al., 2020). Within the marine environment Tenericutes demonstrate adaptive flexibility (Wang et al., 2020). As a consequence, this easy misshapenness combined with the small size would allow them to bypass a majority of the pre-treatment systems within a pressurised SWRO desalination plant.

Pre-treatment systems have long been associated with improved microbial loads; however, our results show that only selective bacterial groups are removed thereby creating a dynamic shift in community composition within the system (Manes et al., 2011a; Chun et al., 2012;). This is evident when looking at the diversity present within the core OTUs of the SWRO feed tank in comparison to the intake water (Figure 3.6). Due to the constant inflow of nutrients, pre-treatment systems can create niche environments for the proliferation of adaptable bacteria, potential becoming a reservoir for diverse microorganisms (Bae et al., 2014).

To survive in a competitive environment, bacteria must rapidly adapt to changes such as shifts in temperature and nutrient concentrations (Tollerson and Ibba, 2020). A study by Parter et al. (2007) determined that if the environment surrounding the bacteria was stable, fewer metabolic functions would be required, causing a down-regulation of those not required. Whereas variability within an environment promotes the up-regulation of pathways to provide stability in an unstable environment. The up-regulation of the biosynthesis of other secondary metabolites pathways can indicate selective advantages for the organisms as they have a role in regulating interactions with other organisms and the surrounding environment (Straight et al., 2007; Osbourn, 2010;).

3.6.2 Community dynamics of microorganisms on RO membranes

The fouling of SWRO membranes has detrimental consequences for the production of potable water (Jamieson and Leterme, 2021). Indeed, the impact of biofouling on SWRO membranes results in permeate flux decline, hydraulic resistance and concentration polarization leading to a loss of salt rejection (Jamieson and Leterme, 2021). Here, we observed a decreased in permeate flux over time and a decrease in salt rejection, except for the 2nd stage membranes which showed constant salt rejection throughout the operation. The accumulation of EPS/TEP and microorganisms on the

membrane surface has been well recognised as the leading cause of biofouling. Biofouling is influenced by environmental factors and pre-treatment systems within the desalination plant. However, pre-treatment has been recognised as the ideal method to prevent biofouling through the removal of organic, inorganic, and biological components (Maddah and Chogle, 2017). While the pre-treatment systems within the Penneshaw SWRO desalination plant, especially the multimedia filtration system, are effective in reducing the microbial load (Balzano et al., 2015c), the inflow of even limited amounts of microorganisms after pre-treatment can have a profound effect on SWRO membranes through proliferation and biofilm formation (Maddah and Chogle, 2017). The ability for microorganisms to colonise aggregates post pre-treatment provides them with nutrient hot spots allowing for their multiplication (Unpublished data).

In this study, the SWRO membranes from stages 1 and 2 were studied after 2- and 4-years of operational service. Autopsies on the membranes are commonly used to determine and understand the fouling characteristics within the system. The 2- and 4-year stage 1 membranes were noticeably more fouled than the stage 2 membranes. These results are consistent with earlier studies in seawater and wastewater reclamation plants (Raffin et al., 2012; Khan et al., 2014; Tang et al., 2016; Nagaraj et al., 2017; Zheng et al., 2018). The variation in water quality along the feed channel influences the fouling characteristic diversity displayed on the membranes. The fouling observed on the membranes was consistent with the TEP quantification results (Figure 3.7). Indeed, polymeric substances and aggregates which are visible on the membranes from both stages after 4-years resulted in TEP quantifications that were significantly higher than those in either stages 1 and 2 after two-years of service. Previous studies have shown that pre-treatment systems can reduce the load of TEP within the plant, however low amounts still reach the SWRO membrane (Balzano et al., 2015c; Villacorte et al., 2009a; Meng et al., 2020). Consequently, it has been found that the relationship between the rate of fouling on SWRO membranes is strongly correlated to concentration of TEP found within the plant.

Here, the order of magnitude of TEP for the 2nd stage membranes was similar to that of the seawater within the desalination plant (Balzano et al., 2015c). Due to its flexible nature, TEP has the ability to pass through pores much smaller than its size, especially under pressure (Villacorte et al., 2009a). This is considered to have a substantial impact on not only the severity of the fouling, but also the clogging of membrane pores (Nagaraj et al., 2018). TEP is recognised as a significant precursor in biofilm formation within the marine environment (Bar-Zeev et al., 2012). Previous studies have shown the presence of TEP accumulation on fouled SWRO membranes, although it remains unclear as to the whether it is solely the result of the presence of TEP in the feed water or if biofouling organisms, contributes to this accumulation. The presence of TEP within the desalination system provides bacteria with a nutrient source, allowing for their proliferation on the membrane (Li et al., 2016b).

Proteobacteria are commonly the most dominant organisms identified not only within the feed water, but also on fouled membranes (Nagaraj et al., 2018). α- and γ-proteobacteria, as well as Actinobacteria are frequently found on fouled membranes worldwide (Nagaraj et al., 2017; Yu et al., 2017; Zheng et al., 2018; Tong et al., 2020). Moreover, previous studies have noted that both α- and γ- proteobacteria increased in their relative abundance after pre-treatment (Belila et al., 2016; Levi et al., 2016). Within biofilms, the primary colonizers often belong to the class of α-proteobacteria (Pang and Liu, 2007; Bereschenko et al., 2010; Al Ashhab et al., 2014). Organisms commonly recognised on fouled RO membranes, as well as in drinking water systems, are also known for their ability to form biofilms (Douterelo et al., 2017; Nagaraj et al., 2018; Sanchez, 2018).

Two α-proteobacteria orders that were identified in all water and membrane samples and are known for their biofouling capabilities are Sphingomonas and Caulobacterales. These α-proteobacteria orders have been identified as organisms which are not only initial colonizers of biofilms but also have the ability to produce unique and individual EPS/TEP structures (Bereschenko

et al., 2010; Berne et al., 2013; Gutman et al., 2014; Hernando-Perez et al., 2018). This unique ability to change the structure of the EPS/TEP allows the organisms to counterattack the conditions and treatments within the desalination plants. As a consequence, the physical-chemical properties such as the hydrophilicity, zeta potential, roughness of the membrane surface can be changed (Gutman et al., 2014; Anwar et al., 2020), potentially, leading to further settlement of organic matter and accumulation of microorganisms to further spread the biofilm (Anwar et al., 2020).

The functional gene predictions across all the membranes were determined to be consistent. It has been well established that surface-associated bacteria express physiological and phenotypic traits that are different from those in a planktonic state (Stewart and Franklin, 2008). It has also been suggested that over the course of biofilm formation the cells undergo a range of phenotypic switches, however these may be temporary (Stewart and Franklin, 2008; de la Fuente-Nunez et al., 2013). Within the biofilm, as the bacteria respond to their surrounding environment, concentration gradients, diffusional processes, signalling compounds and waste, results in a heterogeneous structure. Subsequently, the cells within the biofilm are physiologically distant from planktonic bacteria but also from each other (Stewart and Franklin, 2008).

3.6.3 Potential influence of feed water on the SWRO membranes and control strategies

The complexity of the SWRO membrane environment has an impact on the community composition seen within the desalination plant and the SWRO membrane. Complexity of the ecosystem promotes the coexistence of organisms creating niches and increasing the diversity within the communities (Smith et al., 2014). The inflow of water populated with a diverse microorganism community are not the only factor influencing the formation of fouling on the SWRO membranes (Antunes et al., 2019). The inflow of nutrients and biofilm precursors such as TEP also creates an environment ideal for

microbial growth and biofouling (Jamieson and Leterme, 2020; Meng et a. 2020). For example, Proteobacteria have the ability to dominate SWRO biofilms as they are small enough (0.22 - 2 μm) to bypass all the pre-treatment systems within the plant. With TEP providing a conditioning layer on the membrane this provides an attractive environment for bacteria to proliferate and develop biofilms. The biofilm and the SWRO membrane also provide a substrate for settlement and growth for other organisms such as diatoms, dinoflagellates, and ciliates. Between diatoms and bacteria, a mutualistic relationship develops due to complimentary lifestyles (Landoulsi et al., 2011).

The fouling of SWRO membranes will continue to reduce the quality and quantity of potable water produced if the current conventional pre-treatment systems are not improved. Adaptation of novel treatments to complement the current system would allow for longevity of the SWRO membranes, for example, the introduction of an additional pre-treatment method such as microfiltration membranes. The development of microfiltration membranes with silver nanoparticles on the surface (after 10 months ~90% of Ag remained) has shown success in reducing the number of viable organisms attached to the membrane surface (Linhares et al., 2020). Microfiltration membranes have the ability to remove most bacteria with pore sizes of 0.1 – 10 μm. The addition of silver to the microfiltration membrane provides a biocidal effect when in contact with the bacteria (Linhares et al., 2020), thereby, reducing the quantity of viable cells that would end up on the SWRO membranes overtime. A novel treatment for the reduction of fouling precursors and organisms within desalination plants is the use of a coagulation/flocculation treatment. The introduction of nanochitosan-grafted (CPAM-g-NCS) flocculants were found to have greater success than organic flocculants in the coagulation of particles (Chen et al., 2020). In addition, the CPAM-g-NCS demonstrated antibacterial properties and was efficient in the sterilisation through the disruption of the cells (Chen et al., 2020). The use of the dual-function flocculant could provide an effective and efficient pre-treatment process within SWRO desalination plants.

The introduction of novel treatments to remove the smaller sized particles and cells from the conventional pre-treatment system could reduce fouling on the SWRO membrane. In addition, the removal of the cartridge filters from the pre-treatment system could also be beneficial as they have been found to exacerbate fouling pre-cursors within desalination plants (Bar-Zeev et al., 2009; Balzano et al., 2015c; Fortunato et al., 2020). The colonisation of cartridge filters by bacteria is well characterised and while the organisms are highly diverse thus far, they are seen to have relatively little impact on SWRO fouling (Belila et al., 2016; Zhang et al., 2011; Nagaraj et al., 2019). Ultimately, cartridge filters provide a unique ecosystem within the desalination plant for the proliferation of organisms and perpetuation of fouling pre-cursors. Any replacement or modification to this treatment option would be a win for a reduction in fouling potential.

3.7 Conclusion

Water within desalination systems undergoes multiple pre-treatment stages in an attempt to reduce or remove biofouling potential elements. Assessment of the fouling potential of the feedwater combined with autopsies of fouled membranes can provide important information on the fouling structure within a plant. The key results obtained from our study in the Penneshaw SWRO desalination plant are consistent and complementary of each other:

- Niche environments exist within the Penneshaw SWRO desalination plant along the pre-treatment stages. Adaptive success of microorganisms to their surrounding environment permits the formation of dominant groups to form in each environment.
- The SEM analysis displays a cake-like layer on the Stage 1 membranes with particulate matter embedded within polymeric substrates, whereas the lag membranes contain an amorphous matrix unevenly covering the membrane with particulate matter embedded within.

- Quantification of TEP on the fouled membranes determined that the membrane in the Stage 1 position was impacted more by TEP than the lag. The membranes that were in service for 4-years had more TEP than those in service for 2-years.
- Identification of functional groups using ATR-FTIR spectroscopy were consistent with proteins and polysaccharides which would suggest that they are a product of biofouling.
- The niche community structure on the membranes is stable and, diverse, but also dependent on the membrane position and year of service reflecting the water flow quality within the system.

This study provides insights into the influence that the pre-treatment systems have on membrane fouling, as well as information on the unique environment that each membrane provides for fouling organisms. Future considerations should focus on the fouling timeline especially in regard to the fouling of all the membranes within each vessel within the SWRO compartment. Targeted approaches for the removal of TEP as well as TEP pre-cursors within water treatment systems needs to be established.

3.8 Acknowledgements

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CHAPTER 4: Eukaryotic diversity within a seawater reverse osmosis desalination plant and its impact on fouling

4.1 Preface

This chapter is closely based on the manuscript of an article under review by Elsevier Jamieson, T., Balzano, S., Kildea, T., Ellis, A.V., Brown, M., H., and Leterme, S. C. Eukaryotic diversity within a seawater reverse osmosis desalination plant and its impact on fouling. *Desalination*.

3. **Full publication Details** Eukaryotic diversity within a seawater reverse osmosis desalination plant and its impact on fouling

Section of the thesis where the publication is referred to Chapter 4

Student's Contribution to the publication:

Research Design 80 %

Data Collection and analysis 85 %

Writing and editing 75 %

Outline your (the student's) contribution to the publication:

Conceptualisation, Methodology, Formal analysis, Investigation, Data curation, Writing - Original draft, Writing - editing, revision of the manuscript, and final manuscript

☑ I confirm that the details above are an accurate record of the student's contribution to the work.

Name of Co-Author 1: Sergio Balzano Signed: Sergio Balzano Digitally signed by Sergio Balzano DN: cn=Sergio Balzano, o=Stazione Zoologica Anton dohrn, ou=Bluebiotech, email=sergio.balzano@szn.it, c=IT Date: 2021.06.01 16:33:15 +02'00' Date: 01/06/21

☑ I confirm that the details above are an accurate record of the student's contribution to the work

Name of Co-Author 2: Sophie Leterme Signed: Sophie Leterme Digitally signed by Sophie Leterme Date: 2021.06.02 18:38:56 +09'30' Date: 02/06/21

4.2 Abstract

The majority of research into biofouling focusses on prokaryotic organisms, with very little exploration undertaken, thus far, on the eukaryotic organisms within seawater reverse osmosis (SWRO) desalination plants. To this end, the aim of this study was to explore the structure of the eukaryotic community in the intake seawater, the reverse osmosis feed tank water and on the polyamide thin-film composite SWRO membranes of the Penneshaw Desalination Plant (Kangaroo Island, South Australia, Australia). The eukaryotic communities were characterised through 18S rRNA sequencing. The intake seawater was dominated by the eukaryotic supergroups Archaeplastida, Opisthokonta and SAR while an increased diversity was observed in the SWRO feed tank water with supergroups Archaeplastida, Centrohelea, Cryptophyta, Excavata, Haptophyta, Opisthokonta, Picozoa and SAR observed. Representatives from Archaeplastida and SAR supergroups were also found on the SWRO membranes along with Hacrobia and Opisthokonta. These supergroups represent multiple different life strategies including autotrophy, heterotrophy and mixotrophy providing the ability to adapt and survive in extreme environments such as a desalination plant. Further research should be undertaken to determine if the presence of eukaryotic organisms could be used as a means to reduce the fouling load on SWRO membranes.

4.3 Introduction

Effective potable water production is essential as water scarcity is ever increasing. Expanding populations, uneven water distribution, pollution, water exploitation and rigorous quality regulations contribute to the growing world-wide water problem (Anis et al., 2019a; Qasim et al., 2019). The World Health Organization (WHO) has predicted that more than half the world-wide population will be living in water-stressed environments by 2025. The demand for alternative technologies for the production

and purification of water has therefore never been greater. Today, reverse osmosis (RO) technologies are the leading producers of potable water throughout the world (Valavala et al., 2011). Seawater reverse osmosis (SWRO) desalination feed water is pressurised allowing water to be passed through a semi-permeable SWRO membrane while rejecting salt. But, a substantial shortcoming of this system is membrane fouling, resulting in a reduction in the efficiency of the process, increasing operating costs and reducing the quality of the water produced (Subramani and Jacangelo, 2015). The major mechanism of fouling on SWRO membranes is surface fouling due to inorganic and organic compounds, colloidal material and microorganism found within the feedwater (Goh et al., 2018). The pre-treatment system within the desalination plant is imperative in reducing the fouling load before the SWRO membrane. These systems allow for the reduction in organic and inorganic foulants as well as the prevention and control of microorganism attachment and consequent growth on the membrane surface (Al-Ahmad et al., 2000; Kavitha et al., 2019). Due to the variation in composition of the source water of the desalination plants factors such as industrial discharge, temperature, and depth of the intake water, as well as ocean currents and concentration of algae, complete removal is difficult to accomplish. Pre-treatment systems within SWRO plants are generally categorised into two approaches: physical and chemical processes. The physical method reduces the quantity of particulate matter within the water through screens and filters, whereas the chemical method uses scale inhibitors, coagulants, and disinfectants (Kavitha et al., 2019). Desalination plants which use RO technology commonly use micro-filtration (MF) and ultra-filtration (UF) to reduce the fouling capacity of the water (Anis et al., 2019a). However, it is inevitable that microorganisms will colonise the SWRO membrane as the pre-treatment systems only provide a temporary solution (Bereschenko et al., 2008). The inflow of nutrients as well as the presence of microorganisms and the pressure of the water through the membrane create the perfect storm for the formation of biofilms on the membranes. This results in poor desalination plant performance; an increase in water rejection, energy requirement and

system pressure as well as a decline in water flux and the potential damage to the membrane (Jamieson and Leterme, 2021).

Production and costs within the SWRO plant are influenced by biofouling, necessitating effective and efficient methods of prevention and control. Most often, biofouling studies focus on the bacteria within the water treatment plant to determine the origin of the fouling (Pang and Liu, 2007; Bereschenko et al., 2011; Chiellini et al., 2012;). However, eukaryotic organisms have also been identified within water treatment systems (van Lieverloo et al., 2004; Hageskal et al., 2009; Valster et al., 2009; Loret and Greub, 2010; Pereira et al., 2010; Belila et al., 2017). Biofilms typically harbour a range of eukaryotic organisms as well as bacteria and can also provide shelter and protection for potentially pathogenic organisms (Kuiper et al., 2004; Logares et al., 2008). Previous studies have also shown that eukaryotic organisms such as Amphipods, insect larvae, nematodes and copepods isolated from water distribution systems contain the potentially pathogenic opportunistic bacteria *Aeromonas*, *Enterococcus*, *Pseudomonas*, *Acinetobacter,* and *Staphylococcus* spp. (Wolmarans et al., 2005). Despite the vast quantities of eukaryotic organisms found within water distribution systems, WHO does not regard them as a public health concern due to limited documentation between their association with waterborne infections (Bichai et al., 2011).

As some of the eukaryotic organisms are heterotrophic or mixotrophic, predominantly feeding on bacteria, fungi, and algae, they potentially have an essential part in shaping the *in situ* microbial community composition within desalination plants. Despite this, the main focus of biofouling studies is usually on the prokaryotic organisms within desalination plants either via next generation sequencing or culture techniques (Pang and Liu, 2007; Bereschenko et al., 2011; Chiellini et al., 2012; Jamieson et al., 2016; Nagaraj et al., 2017). However, several studies have assessed eukaryotic diversity

in seawater, surface water, and ground water as well as biofilms, providing some background knowledge in natural environments.

The objective of this study was thus to assess the diversity of microbial eukaryotes in the SWRO feedwater tank and within the SWRO membrane-associated biofilms within a SWRO desalination plant. Seawater intake and SWRO feedwater were sampled monthly over a 12-month period and the polyamide thin-film composite SWRO membranes were removed after 2- and 4-years of operation from the 1st and 2nd stage positions. Comparison between biofilms developed at the different time periods and in the different positions, was performed to ascertain the dominant group of organisms associated in the membrane biofouling of a SWRO desalination plant. Moreover, further comparisons were made to investigate the potential effects of feedwater quality on the biofilm development on the SWRO membranes over time.

4.4 Materials and Methods

4.4.1 Description of the Penneshaw SWRO desalination plant

The SWRO desalination plant in Penneshaw, Kangaroo Island, South Australia, Australia, has been previously described in Jamieson et al., (2021). It has been in operation since 1999 with a nominal output of 300 $kL.day^{-1}$ at a 40% recovery rate (Dixon et al., 2012). The plant has a pre-treatment system that consists of a screened seawater intake pipe (10 cm and 0.5 mm pore size; Figure 4.1(1)). Treatment is with sulphuric acid weekly (intake water pH = 8.08 ± 0.20, after acid treatment pH = 6.88 ± 0.27), followed by a medium-pressure ultraviolet (MP-UV; Figure 4.1(2)) system. Before multimedia filtration (Figure 4.1(3)) containing filter coal (0.9-1.1 mm size; 300 mm depth), quartz sand (0.45-0.55 mm size; 500 mm depth), garnet sand (0.3 mm size; 200 mm depth) and graded gravel (500 mm depth) is

undertaken. Cartridge filtration was carried out prior to the seawater entering the SWRO feed tank (3 x 15 µm; Figure 4.1(4) and 3 x 5 µm; Figure 4.1(5)) cartridge filters. Throughout the system the flow rate of the seawater was approximately 8.4 $L.s^{-1}$.

The SWRO unit comprised of 12 pressure vessels organised as three high and four wide in a single frame (Figure 4.1f). The membranes, of which four are contained in each vessel, are SWRO membranes made of polyamide which are a spiral wound thin-film composite (FILMTEC™ SW30HRLE-440i) with an active surface area of 41 m^2. In this membrane autopsy study, SA Water provided four fouled SWRO membranes. These were membranes from the stages 1 and 2 positions of the pressure vessel after 2- and 4-years of service.

4.4.2 Water sampling sites

Seawater was collected from two different pre-treatment sites within the desalination plant between December 2012 and November 2013. The first collection point (Site 1) was at the intake seawater, prior to the addition of sulphuric acid, and the second collection point (Site 2) was in the SWRO feed water tank, after all pre-treatments (Figure 4.1). At these sites, 120 L of seawater was concentrated by tangential flow filtration (Marie et al. 2010) to 2 L and pre-filtered using 10 µm cellulose filters before being filtered through 0.45 µm pore size Sterivex units (Millipore). Samples were processed according to Balzano et al. (2015c).

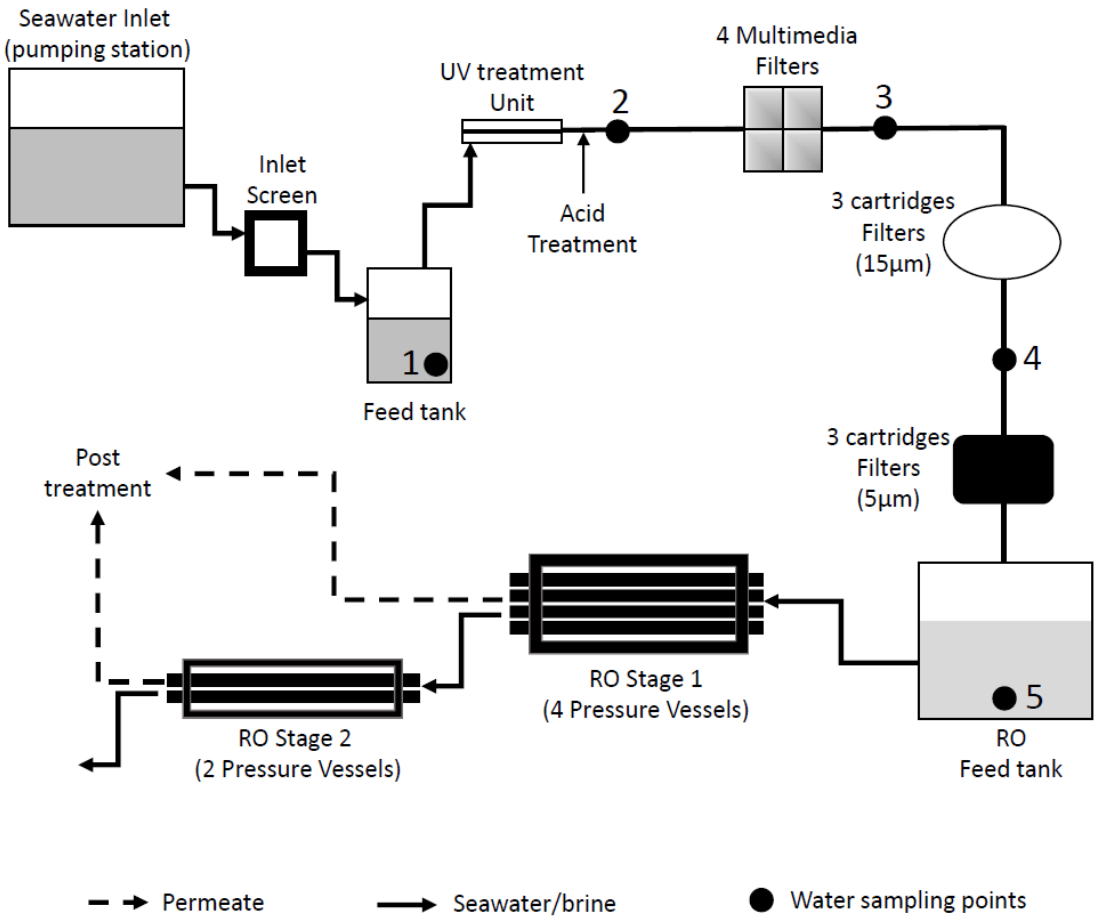

Figure 4.1: Schematic diagram of the Penneshaw SWRO desalination plant. Numbers indicate the different sampling points: (1) Intake seawater screened with 10 cm and 0.5 mm pore sizes, (2) after the medium pressure ultraviolet system, (3) after the multimedia medium pressure filter contains filter coal, quartz sand, garnet sand and graded gravel, (4) after the 15 µm cartridge filters and (5) after the 5 µm cartridge filters, SWRO feed water.

4.4.3 Foulant removal

Polyamide thin-film composite SWRO membranes were collected from the Penneshaw desalination plant to take into account different positions within the system (stages 1 and 2) as well as period of use (2- or 4-years). Samples (1 x 10 cm) were taken from the different membrane leaves that make up the membrane. The foulant was scraped from each membrane sample and resuspended in 1 mL of tangential flow filtered seawater.

4.4.4 PCR and sequencing

DNA extraction was conducted as described in Jamieson et al. (2021). Amplification of the eukaryotic V4 region of the 18S rRNA gene were undertaken using the universal primer 528F (5'-GCGGTAATTCCAGCTCCAA-3') and a reverse primer 706R (5'-AATCCRAGAATTTCACCTCT-3') (Cheung et al., 2010). Primers were modified to include an A-adaptor (5'-CCATCTCATCCCTGCGTGTCTCCGACTCAG-3') and a sample specific, 11 bp barcode, to the 5'-end of the forward primer, and a P1-adaptor (5'-CCTCTCTATGGGCAGTCGGTGAT-3') to the 5'-end of the reverse primer for Ion Torrent next generation sequencing. Polymerase chain reactions (PCR) were performed as described in Jamieson et al. (2021). Amplicons were sequenced by The Australian Cancer Research Foundation Biomolecular Resource Facility using an Ion Torrent Personal Genome Machine fitted with a 318 chip (Life Technology) and adapted for a maximum read length of 400 bp.

4.4.5 Bioinformatic analysis

The Ion Torrent platform sequence data were analysed using Mothur (Schloss et al., 2009). A lower Phred quality threshold (20) was used to filter the reads, in comparison to other sequencing platforms

(i.e., 25) as the Ion Torrent has been found to underestimate the real base accuracy (Bragg et al., 2013). Reads data were cleaned as described in Jamieson et al. (in press). The Protist Ribosomal Database (Guillou et al., 2013) was used to infer taxonomic affiliation of the operational taxonomic units (OTUs) employing the UCLUST algorithm (Edgar, 2010).

4.4.6 Data analysis

The following statistical analyses was performed for the 18S rRNA sequencing data unless described otherwise. All data were transformed using Log+1 before undertaking Bray-Curtis similarity to calculate a similarity matrix between the eukaryotic communities. The data were analysed by Principle Coordinate Analysis (PCoA), Non-metric Multi-Dimensional Scaling (nMDS), Pielou's evenness, similarity percentage (SIMPER) tests and similarity profile (SIMPROF) tests using the Primer7-PERMANOVA Software (version 7.0.13).

Differential abundances between microorganism communities were compared using the DESeq2 package (version 1.29.4; Love et al., 2014) for R Software (version 4.0.0). Core microbiota were identified for eukaryotic organisms within the water samples as well as the SWRO membrane samples. To identify the core, the variable, and the unique taxa among the intake water, the SWRO feed tank water and the SWRO membrane samples, Venn diagrams were created with the online tool access through https://bioinfogp.cnb.csic.es/tools/venny/. Microsoft excel was used to create the abundance graphics. Circular heatmaps were created using the circlize package (version 0.4.11; Gu et al., 2014) for R Software (version 4.0.3).

4.5 Results

4.5.1 Eukaryotic community structure

In this study, we investigated the eukaryotic organisms present in the seawater intake, as well as in the SWRO feed tank, of the Penneshaw desalination plant, South Australia. PCoA, based on Jaccard distance ordination, displayed dissimilarities in the community composition of the eukaryotic organisms between the intake seawater and the SWRO feed tank water samples (Figure 4.2A.). The dissimilarities in the seasonal communities of the intake seawater and the SWRO feed tank water (permanova $p = <0.05$) were observed along the principal coordinate PCO1. Additionally, along the principal coordinate PCO2, the effect of the pre-treatment system within the Penneshaw desalination plant was seen in the distinct separation of the intake seawater and the SWRO feed tank water. This highlights the differences in community structure between the two water types and the effect the pre-treatment system had on the communities.

The eukaryotic organisms present on the SWRO membrane were also investigated from stages 1 and 2 after 2- and 4-years of service, within the Penneshaw desalination plant. The eukaryotic community composition demonstrated differences in the PCoA, based on Jaccard distance ordination (Figure 4.2B.). The dissimilarities in the eukaryotic community structure between the membrane positions (permanova = $p<0.05$) within the system were seen along the principal coordinate PCO1. Furthermore, along the principal coordinate PCO2 the evident separation potentially represents the fouling on the membranes within the system between the 2- and 4-year membranes (permanova = $p<0.05$). Thus, from the differences highlighted on the PCoA, it is evident that the eukaryotic communities present on the membranes have evolved with time.

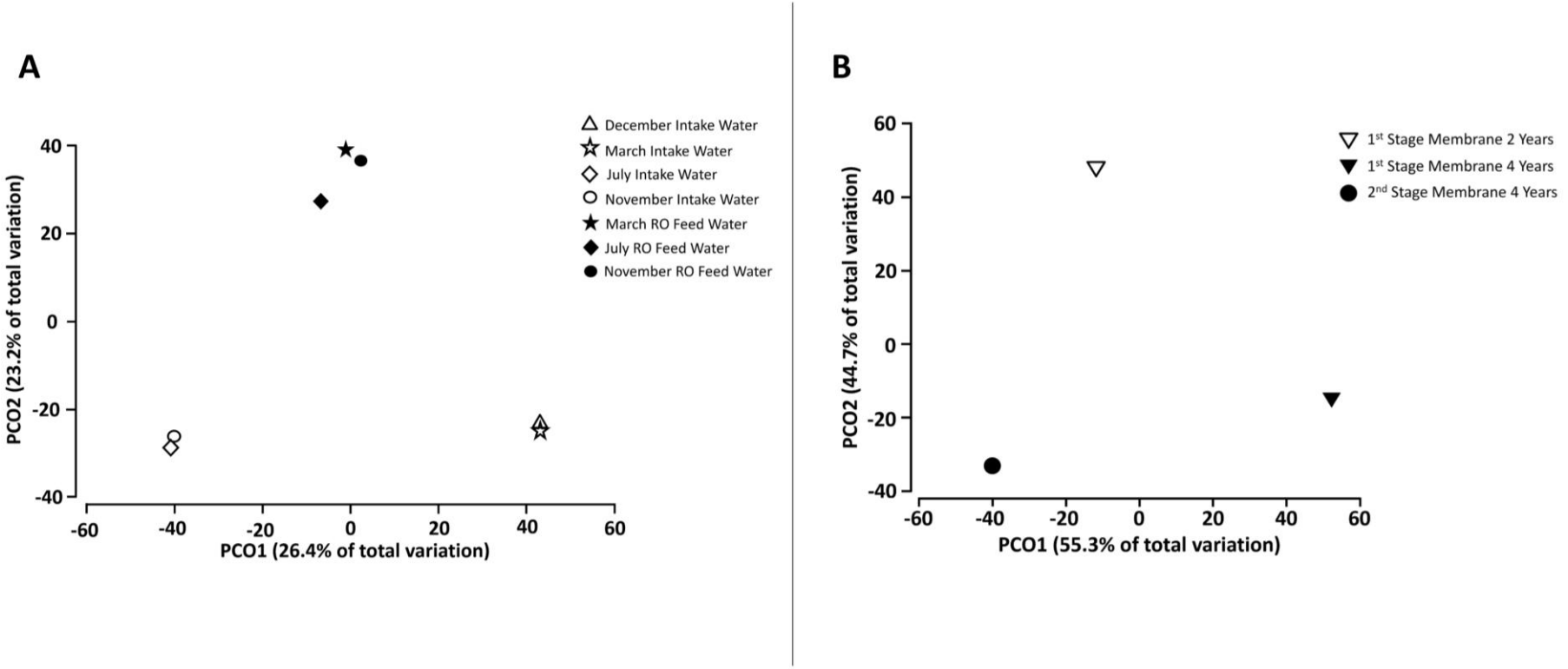

Figure 4.2: Principal coordinate analysis (PCoA) based on Bray-Curtis distance ordination displaying the differences in the eukaryotic composition between (A) the intake seawater and SWRO feed tank water samples (permanova = $p<0.05$), and (B) the 2-year and 4-year membranes (permanova = $p<0.05$) in the 1st and 2nd stage position (permanova = $p<0.05$). The total variability is explained by the two PCoA axes, with the ordination of water samples (A) explaining 49.4% of the seasonal and pre-treatment variability observed in the samples and (B) explaining 74.8% of the variability observed in the membranes as well as the ordination of membranes.

4.5.2 Taxonomic diversity

Within the water samples (intake seawater and the SWRO feed tank water), nine distinct eukaryotic taxa were identified: Archaeplastida (green and red algae), Centrohelida (amoeboid), Crytophyta, Excavata, Amoebozoa, Haptophyta, Opisthokonta (Fungi, Ischthyosporea), Picoza and SAR (Cercozoa, ciliates, dinoflagellates, Labyrinthulids, Omycetes, diatoms), in line with the revised eukaryotic classification put forward by Adl et al. (2012). The supergroup of Archaeplastida (45.3%) was the most dominant in the intake seawater, followed by Opisthokonta (17.9%) and SAR (9.9%). Within the SWRO feed tank water, SAR (61.2%) was dominant followed by Archaeplastida (35.8%; Figure 4.3A.). The groups Excavata, Haptophyta and Picoza were only found in the SWRO feed tank water. Pielou's evenness values showed that the diversity in OTU varied between months, with a higher diversity in Winter and Spring within the intake seawater and the SWRO feed tank water samples (Table 4.1). Some of these organisms showed significant differences ($p < 0.05$) in abundance between the intake seawater and the SWRO feed water across the duration of the study (Figure 4.4A). No data were shown for the SWRO feed tank in December as no 18S rRNA region was amplified due to the low levels of rDNA extracted.

Analyses of the SWRO membrane surface identified four distinct eukaryotic taxa; Archaeplastida, Centrohelida, Opisthokonta, and SAR. The supergroup of Archaeplastida was dominant (63%) followed by Opisthokonta (12%; Figure 4.3B.). The taxa Centrohelida was only found on the 2-year Stage 1 membrane and the 4-year Stage 2 membrane. Pielou's evenness values indicated that OTU abundances across the stages 1 and 2 membranes after 2- and 4-years of service were approximately equally distributed (Table 4.1). No 18S rRNA region was amplified from the 2-year Stage 2 membrane due to low levels of rDNA extracted.

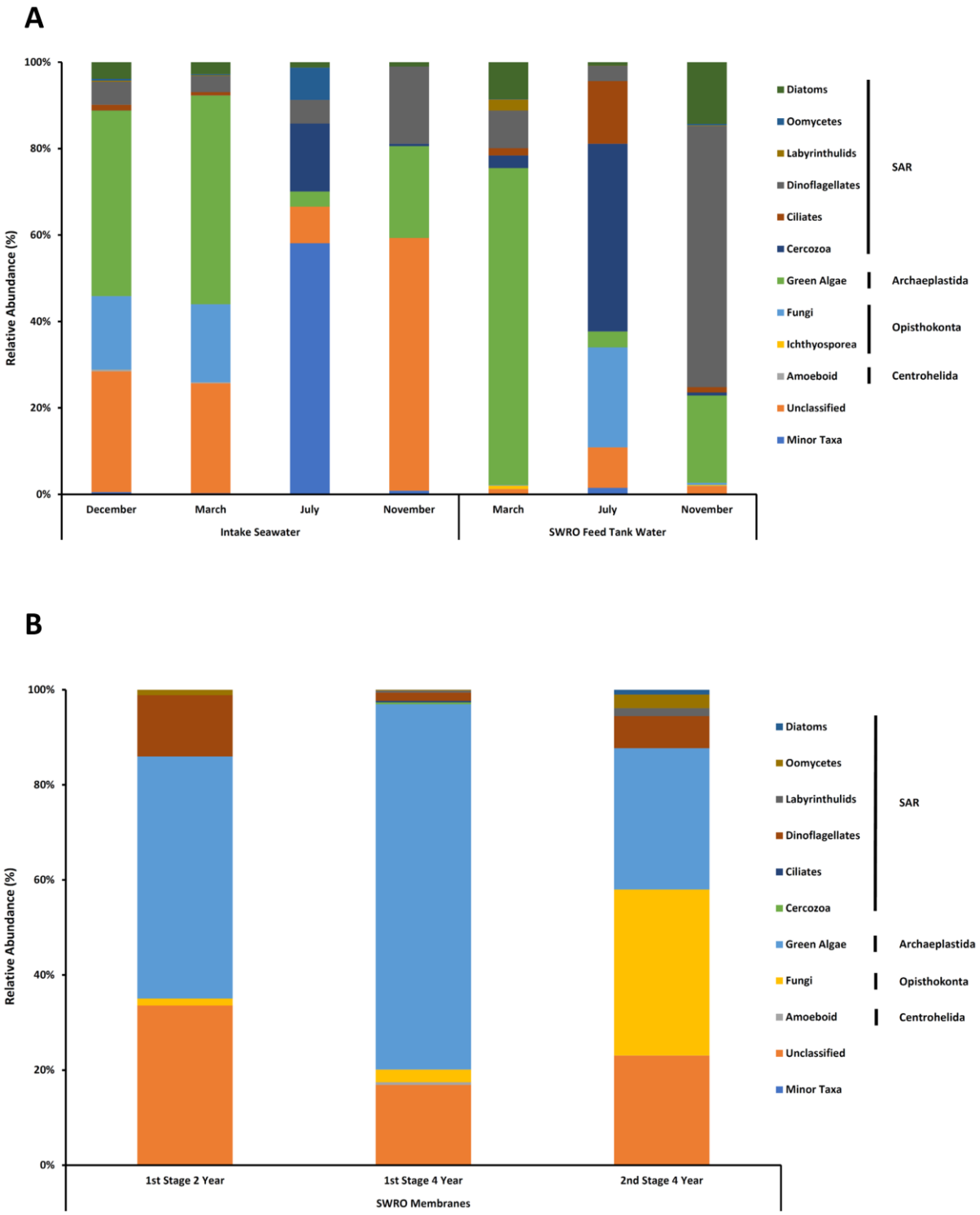

Figure 4.3: The relative abundance composition of the taxonomy of the eukaryotic communities (A) of the intake seawater and the SWRO feed tank water and (B) on the SWRO membranes.

Table 4.1: Pielou's evenness of the intake water and SWRO feed tank water sites within the pre-treatment system at Penneshaw SWRO desalination plant throughout 2012-2013. As well as the membranes in the lead and lag position after 2-year and 4-year operation within the plant.

Sampling Site	*Pielou's evenness*
Intake Seawater December	0.31
Intake Seawater March	0.28
Intake Seawater July	0.53
Intake Seawater November	0.65
SWRO Feed tank water March	0.31
SWRO Feed tank water July	0.64
SWRO Feed tank water November	0.49
2-year 1st Stage SWRO membrane	0.42
4-year 1st Stage SWRO membrane	0.64
4-year 2nd Stage SWRO membrane	0.34

4.5.3 Core and operational taxonomic units

The common core microorganisms identified within all assemblages of a particular ecosystem are considered to be essential to the function of the communities within, allowing for the characterization of a "healthy" community and thus the impact of any perturbation (Shade and Handelsman, 2012). The core OTUs of the intake seawater are represented by the areas which overlap in a Venn diagram (Oliveros, 2015). Three different groups were used to classify the OTUs: core OTUs (identified in all sampling sites), variable OTUs (identified in multiple sites but not all) and unique OTUs (identified in only one site). Of the 2,141 eukaryote OTUs obtained from the intake seawater, 2 (0.1%) were considered core OTUs, 655 (30.5%) were considered variable OTUs and 1,484 (69.3%) were considered unique OTUs (Figure 4.5A). The core OTUs are dominated by the class of Chloropicophyceae and Dinophyceae.

The SWRO feed tank water showed different results (Figure 4.5B) with 2,567 eukaryote OTUs obtained, of which 81 (3.2%) were considered core OTUs, 387 (15.1%) were considered variable OTUs and 2,099 (81.7%) are considered unique OTUs. The core OTUs consisted of twenty classes within thirteen phyla. The core OTU classes were found to be Bacillariophyceae, Chlorodendrophyceae, Dinophyceae, Dothideomycetes, Labyrinthulea, Malasseziomycetes, Mamiellophyceae, Oligohymenophorea, Pyramimonadophyceae, Sordariomycetes, and Thecofilosea.

Of the 304 eukaryote OTUs obtained from the 2-year as well as the 4-year Stage 1 and 2 membranes, 16 (5.3%) were considered core OTUs, 62 (20.4%) were considered variable OTUs and 226 (74.3%) were considered unique OTUs (Figure 4.5C). The core OTUs consisted of five classes within five phyla. The core OTUs are dominated by the class of Chloropicophyceae, Dinophyceae, Peronosporea, Sordariomycetes and Trebouxiophyceae.

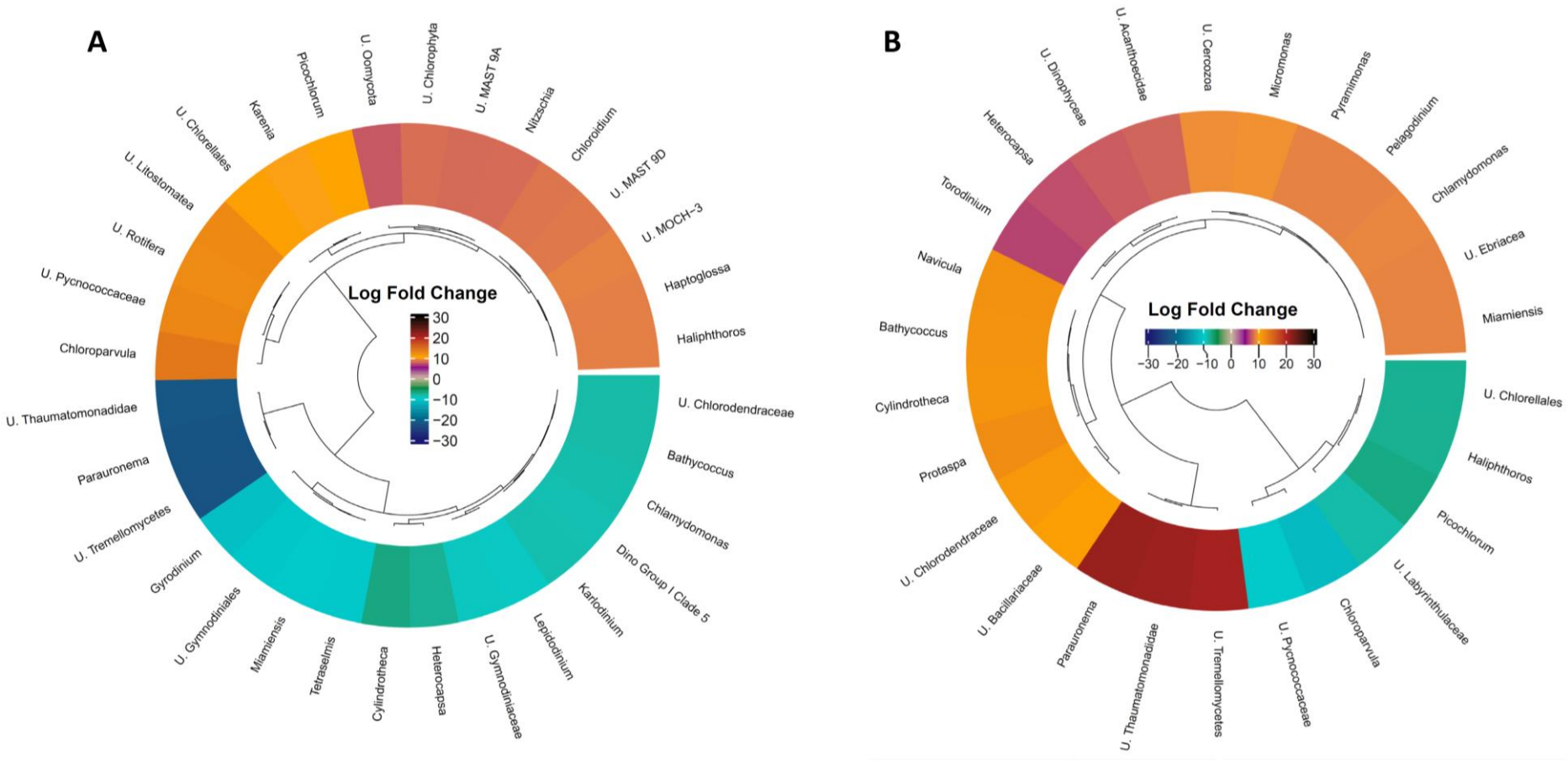

Figure 4.4: Circular heatmap representation of the significant differences ($p < 0.005$) in log fold changes of the eukaryotic organisms at the genus level. Between (A) the intake seawater and the SWRO feed tank water (B) the SWRO feed tank water and the SWRO membranes. Shades of colour represent the log fold change increase and decrease (see colour scale).

4.5.4 Phytoplankton community structure

Analysis of the phytoplankton community was also undertaken within the water samples with a focus on the two main classes, dinoflagellates and ciliates, and diatoms. Diatoms, as they are recognised as the major biofouling agents of marine systems (Molino and Wetherbee, 2008; Landoulsi et al., 2011), were anticipated to be found in the Penneshaw Desalination Plant and were analysed separately from the dinoflagellates and ciliates.

A nMDS based on Bray Curtis ordination was obtained for the phytoplankton communities in the intake seawater, the SWRO feed tank water, and the SWRO membranes. Hierarchical cluster analysis was used to define the group clustering which was present in the nMDS on which (SIMPROF) tests were performed (Figure 4.6). The CLUSTER analysis, together with the SIMPROF test, identified 3 groups of samples (coloured in red) which are statistically distinct. This method, together with the SIMPER analysis, determined that the dissimilarity between the diatom communities in the intake seawater and the SWRO feed water was 96.24%. The main contributors of the dissimilarity were due to an increase in abundance from the genera of *Cylindrotheca*, *Navicula*, *Minutocellus*, and *Pseudo-Nitzschia* in the feed tank. Conversely, the genera of *Minidiscus*, *Delphineis*, and *Nitzschia* decreased in abundance. The diatom community (OTU abundance at genus level) of the intake seawater was compared to that of the SWRO feed tank. Significant differences ($p < 0.05$) were observed over time for the abundance of the diatom genera: *Cylindrotheca* (log fold change -3.2), *Navicula* (log fold change 2.5), *Minidiscus* (log fold change 8.2), and *Delphineis* (log fold change 7.9). SIMPER analysis determined that the dissimilarity between the dinoflagellate and ciliate communities in the intake seawater and the SWRO feed water was 96.38%. The genus of Uronema, Miamiensis and Gyrodinium were the main contributors to the dissimilarity due to an increase in abundance in the SWRO feed

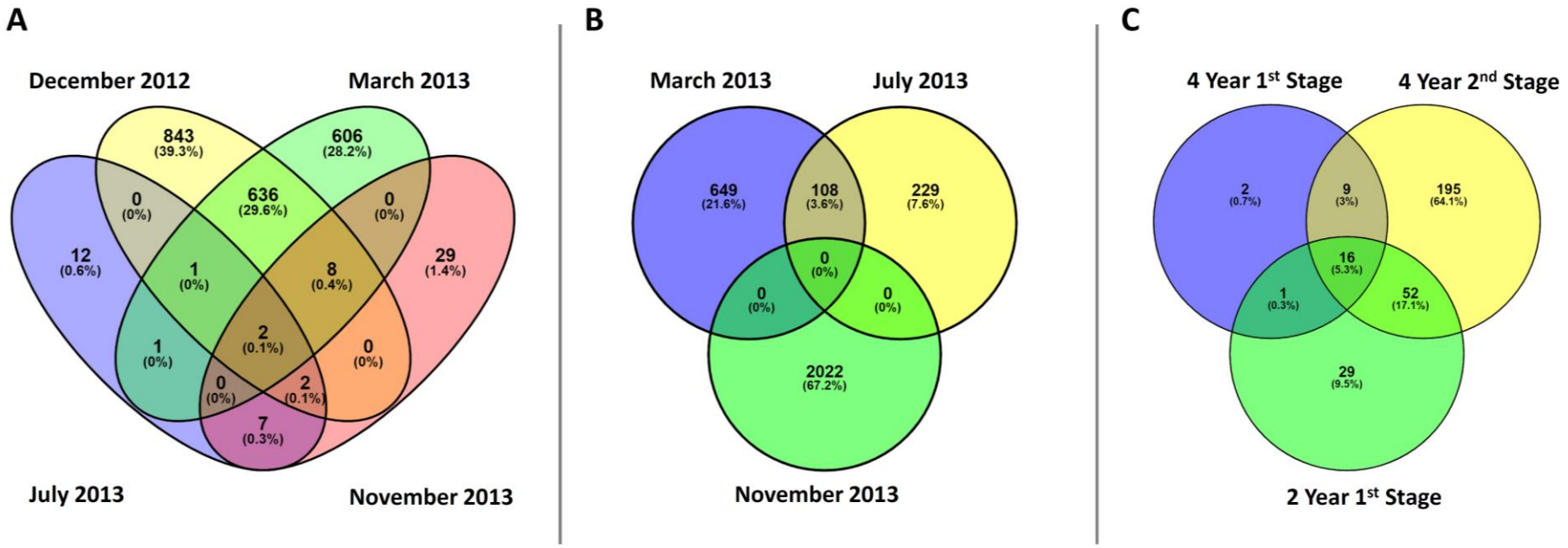

Figure 4.5: Venn diagram displaying the overlap between the eukaryotic communities in the (A) intake seawater and (B) SWRO feed tank water over a 12-month period (samples taken each quarter), and on the (C) 2- and 4-year membranes in the Stage 1 and 2 positions. Core OTUs, identified in all sampling sites; variable OTUs identified in two or more sites but not all; unique OTUs, identified in only one site.

tank water. The dinoflagellate and ciliate community (OTU abundance at genus level) of the intake seawater was compared to that of the SWRO feed tank. The abundance of the genera *Parauronema* and *Woloszynskia* decreased significantly (log fold change -25.6 and -6, respectively), whereas that of *Karenia* increased (log-fold change 10.9) from the intake seawater to the SWRO feed tank.

4.5.5 Influence of feed tank water on the SWRO membranes

The eukaryotic community (OTU abundance at genus level) of the SWRO feed tank water was compared to that on the SWRO membranes. The relative abundances of some eukaryotes identified in the SWRO feed tank water were significantly different ($p < 0.05$) from that on the SWRO membranes across the study period (Figure 4.4B). No significant difference was found for dinoflagellates, ciliates, and diatoms, between the SWRO feed tank water and the SWRO membranes.

4.6 Discussion

There are limited studies focusing on the eukaryotic communities and the role that they have in water treatment plants. Here, we explored the eukaryotic communities found within the intake seawater of the Penneshaw SWRO desalination plant in South Australia, as well as the SWRO feed tank water. We also undertook autopsies of SWRO membranes from the stages 1 and 2 position in the desalination plant, which were in service for 2- and 4-years. Results revealed a highly diverse and variable community not only within the intake seawater but also the SWRO feed tank water which was somewhat mirrored in the biofouling found on the SWRO membranes used in the plant.

Seasonal variation of eukaryotes within the marine environment has been well established within estuarine ecosystems (Balzano et al. 2015b) as well as large oceans (de Vargas et al., 2015; Tragin et

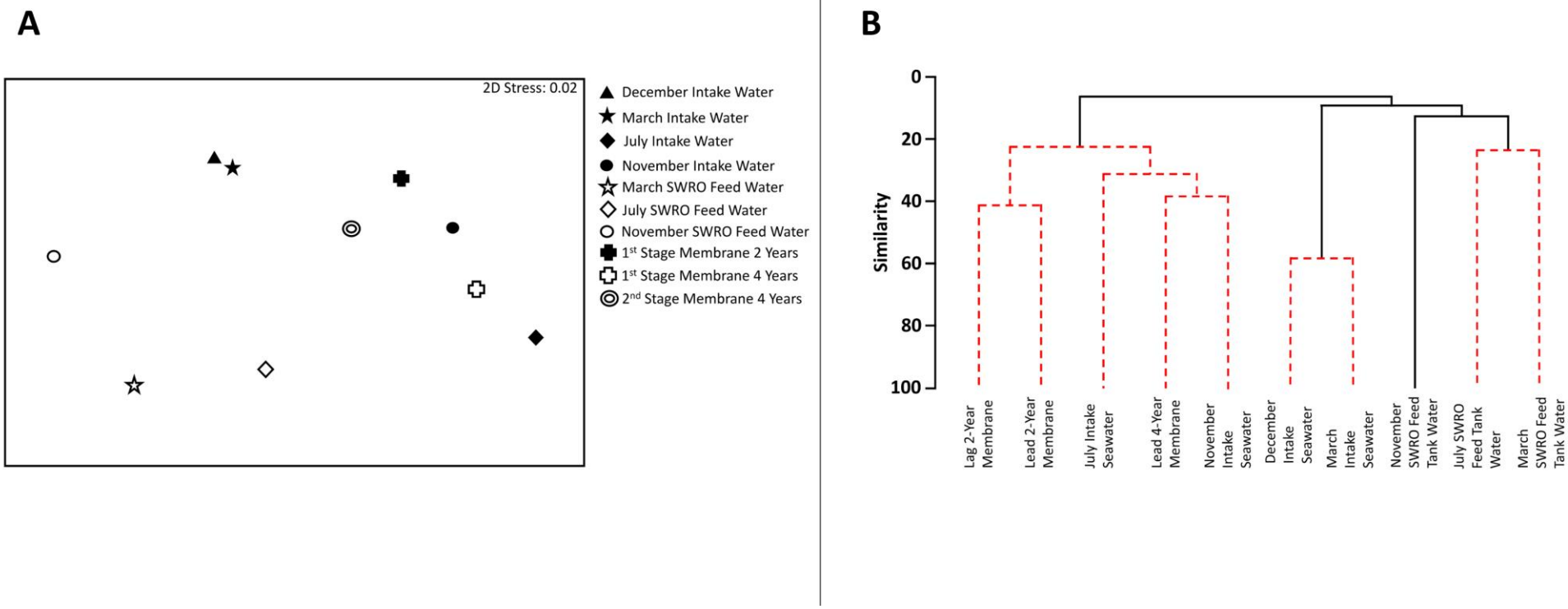

Figure 4.6: nMDS site plot (A) based on Bray-Curtis distance ordination displaying the differences in the composition of the diatoms, dinoflagellates and ciliates within the Penneshaw desalination plant (Kangaroo Island, Australia) showing clusters identified by SIMPROF tests on dendrograms from hierarchical clustering (B). The CLUSTER analysis, together with the SIMPROF test, identified 3 groups of samples which are statistically distinct. The dashed red lines indicate at which similarity levels the clusters are grouped together by the SIMPROF test.

al., 2018), with eutrophic and oligotrophic events experienced by nutrient-dependant organisms throughout the year (Balzano et al., 2015a; Hemraj et al., 2017; Kataoka et al., 2017; Hernandez-Ruiz et al., 2018). Seasonal fluctuations, nutrient availability, microorganisms, and phytoplankton found within the Penneshaw desalination plant have been previously described (Balzano et al. 2015c). The eukaryotic communities described in this work are consistent with those previously determined within a SWRO desalination plant in the Kingdom of Saudi Arabia (Belila et al., 2017).

The supergroup Archaeplastida is comprised of green algae, red algae and glaucophytes organisms (Simon et al., 2009). Likewise, the SAR supergroup consisting of Stramenopila, Alveolata and Rhizaria groups incorporates a vast array of eukaryotes including ciliates, diatoms, and amoebae (Burki et al., 2007). These supergroups represent multiple different life strategies including autotrophy, heterotrophy, mixotrophy and parasitism (Grattepanche et al., 2018), thereby providing the ability to adapt and survive in extreme environments.

Due to the sensitivity of the SWRO membranes to chemical contaminants, the main goal of the pre-treatment systems is to ensure the compatibility of the feed water with the SWRO membrane (Sutzkover-Gutman and Hasson, 2010). However, the efficacy of the pre-treatment components is difficult to control due to the changing nature of the intake seawater (Prihasto et al., 2009). Previous studies in Thuwal, the Kingdom of Saudi Arabia (Belila et al., 2017), Paris, France (Thomas et al., 2008), and Kangaroo Island, Australia (Balzano et al., 2015c), have shown that within the water pre-treatment system of the treatment plant there is an effective reduction of eukaryotic organisms compared to the feed water. Balzano et al. (2015c) showed that the Penneshaw SWRO desalination plant successfully removes 95% of the large eukaryotic organisms and 86% of picoeukaryotic organisms found within the plant using pre-treatment. However, here we established that the diversity of eukaryotic organisms underwent spatial diversification throughout the pre-treatment system of the SWRO desalination

plant, with an increased diversity observed within the SWRO feed tank. Increased diversity within ecosystems has been linked to the population's health and ability to adapt to the surrounding environments. Therefore, as a result of both competition and cooperation within the multispecies community a stable ecosystem develops (Zengler and Zaramela, 2018). This is especially evident within biofilms which are largely heterogeneous environments in which the interaction between intra- and inter species are essential for resource exchange (Rendueles and Ghigo, 2015). This results in more resilient biofilm communities which might be harder to control and/or eradicate.

Fouling of SWRO membranes within desalination plants can potentially result in detrimental consequences in potable water production (Jamieson and Leterme, 2021). Biofouling impacts on multiple facets, such as permeate flux decline, hydraulic resistance, and concentration polarization, all leading in a loss of salt rejection (Jamieson and Leterme, 2021). The leading cause of biofouling has been recognised as the accumulation of extracellular polymeric substances/transparent exopolymer substances (EPS/TEP) and organisms on the membrane surface. Biotic and abiotic factors influence the production of EPS/TEP in eukaryotic organisms. Algae (diatoms, dinoflagellates, and green algae) are known to produce EPS under conditions of stress such as increased temperature, salinity, heavy metals, and nutrient limitations (Thornton, 2002; Aguilera et al., 2008; Vanucci et al., 2010; Vidyarathna and Graneli, 2012; Shetty et al., 2019). Within desalination plants, pre-treatment has often been identified as a crucial method to prevent biofouling by the removal of organic, inorganic, and biological components (Maddah and Chogle, 2017). Within the Penneshaw desalination plant, the pre-treatment system has been effective in reducing the eukaryotic load by two orders of magnitude, especially using the multimedia filtration system (Balzano et al., 2015c). Although the eukaryotic load within Penneshaw is lower in comparison to the prokaryotic organisms (Balzano et al., 2015c). Still, even a reduced level of organisms after pre-treatment can have a profound effect on the SWRO membrane through the formation and proliferation of biofilms on the surface (Maddah and Chogle,

2017). Indeed, eukaryotic organisms have been found to inhabit aggregates formed post pre-treatment providing them with nutrient hot spots for proliferation (unpublished).

The SWRO feed tank water of Penneshaw desalination plant is populated with eukaryotic organisms many of which are known for their biofilm forming capabilities. Diatoms have been recognised to colonise submerged surfaces within marine environments (Cooksey and Wigglesworthcooksey, 1995; Landoulsi et al., 2011). Interestingly, pennate diatoms have been observed to initially colonise surfaces followed by centric diatoms (Patil and Anil, 2005). A conditioning layer of glycoproteins allows the diatoms to bind to receptors thus starting a chain reaction for the secretion of EPS (Cooksey and Wigglesworthcooksey, 1995; Landoulsi et al., 2011). The production of EPS by the diatoms allows them to adhere to both hydrophilic and hydrophobic surfaces (Landoulsi et al., 2011). Fungi have also been recognised for their ability to produce EPS as well as form biofilms. The capability of fungi to communicate via quorum sensing allows them to proliferate under any conditions either in a planktonic form or a biofilm (Donlan, 2001; Kernien et al., 2018). Biofilms in their heterogeneity create physicochemical micro-zones due to the metabolism of organisms thus creating severe gradients of products and substrates (Stewart and Franklin, 2008), while establishing a homeostatic environment in ever changing conditions.

In this work, the stages 1 and 2 SWRO membranes were studied after 2- and 4-years of service within the Penneshaw desalination plant. Commonly, to determine and understand the fouling characteristics within a system, autopsies are performed on SWRO membranes. The 2- and 4-year membranes in the Stage 1 position were noticeably fouled with the Stage 2 membranes exhibiting a lower degree of fouling. These results are consistent with previous studies of SWRO desalination plants in the Kingdom of Saudi Arabia (Belila et al., 2017), as well as water treatment plants in France (Poitelon et al., 2009), USA (Buse et al., 2013) and China (Lin et al., 2014). A major influence on the

diversity of the fouling present on the SWRO membranes is the variation within the water quality along the feed channel. The membrane fouling observed was consistent with previous SWRO membrane autopsy studies (Jeong et al., 2016; Ruiz-Garcia et al., 2018; Fortunato et al., 2020).

Picophytoplankton (0.2 – 2 µm cell diameter) biomass is often dominated by cyanobacteria, and within the marine food web they have an important role especially in oligotrophic environments (Monier et al., 2016; Otero-Ferrer et al., 2018; Sozer et al., 2021). Conversely, within the marine ecosystem they are known to inhabit a wide variety of areas, although adaption to the environmental conditions influences their distribution (Vannier et al., 2016). Here, one of the core components of the communities of the intake seawater and fouled membranes was the clade Chloropicophyceae. The species within the clade are small in diameter <5 µm. This small size is advantageous for the organisms as it enables nutrient uptake efficiency due to a higher surface area to volume ratio, as well as decreasing predation and increasing buoyancy. Balzano et al. (2015c) determined that the pre-treatment systems within the Penneshaw SWRO desalination plant only removed approx. 86% of the picoeukaryotic community, including the class Chlorophyta, throughout the sampling period. Seasonality influences the size and shape of the phytoplankton community with smaller cells sizes and elongated shapes present during the winter months allowed more cells to pass through the pre-treatment systems compared to the summer months.

Some of the major plankton groups within the oceans are diatoms, dinoflagellates, and ciliates; a group that is morphologically diverse and adaptive of most environments. Here, dinoflagellates were found in both the intake seawater and the SWRO feed tank water. Dinoflagellate feeding diversity is very broad therefore, heterotrophic, mixotrophic, autotrophic, parasitic, and predatory lifestyles are apparent (Smayda and Reynolds, 2003). Together with endophytic, symbiotic, and parasitic relationships, dinoflagellates have the ability to survive and flourish under challenging conditions. As

a result, dinoflagellates have developed adaptive strategies to overcome habitat disturbances and nutrient stress allowing them to exploit favourable conditions and remain competitive (Smayda and Reynolds, 2003). With adaptivity crucial to the success of the dinoflagellate community composition, the diversity that is seen in the intake seawater and the SWRO feed tank water is not unexpected. Surviving the oligotrophic environment and the continual pressure and water movement within the desalination plant would challenge any organism, however, the dinoflagellate genus *Gyrodinium* has adapted well to the environment. Here, *Gyrodinium* was present along the pre-treatment system, with a significantly higher abundance in the SWRO feed tank water. *Gyrodinium* is part of the "unarmoured" dinoflagellates in which a theca is lacking allowing for their easy deformation (Rene et al., 2015). This feature would allow *Gyrodinium* organisms to pass easily through the pre-treatment systems within the desalination plant. Balzano et al. (2015c) identified *Gyrodinium* within the Penneshaw SWRO desalination plant at all the sampling sites although it was found to be most abundant prior to the multimedia pre-treatment system, whereas in this study it was identified within the SWRO feed tank water.

One group of Eukaryote that was not expected to be found post pre-treatment due to their size range was the ciliates. Ciliate cell sizes range from 10 µm to 1-2 mm with immense variation in ecological strategies (Lynn, 2008). Ciliate lifestyles range from free-living to symbiosis and from heterotrophy to mixotrophy, while having an expansive range of prey types (Lynn, 2008). Ciliates are comprehensively integrated into the marine ecosystem as evident in the formation of symbiotic relationships with many different organisms including fish, invertebrates, and prokaryotes (Lynn, 2008). At the Penneshaw desalination plant, only one ciliate species, *Mesodinium rubrum,* was identified in the SWRO feed tank water during March 2013. *M. rubrum* has acquired the ability to perform photosynthesis through the sequestration of plastids from various phytoplankton species (Johnson et al., 2016). They have long

been associated with the formation of non-toxic harmful algal blooms colouring the surrounding water red (Johnson et al., 2013; Kang et al., 2013; Lips and Lips, 2017).

The most abundant class of phytoplankton in the ocean are diatoms, considered to be the most diverse and abundant siliceous marine organisms, as well as being ecologically crucial to primary productivity (Finkel et al., 2005; Treguer and De La Rocha, 2013). Characteristically, they are single-celled photosynthetic algae enclosed in a silicon dioxide wall. Typically, the distribution of diatoms is driven by ocean physics and nutrient availability, in addition to predator, pathogen and parasite interactions. The versatility of diatoms allows them to occupy niche environments and a competitiveness that allows them to outcompete other phytoplankton. Throughout the world's oceans the genus *Pseudo-Nitzschia* is extensively distributed (Trainer et al., 2012), although, their growth and distribution are impacted by a number of environmental factors such as salinity, photoperiod, nutrient concentrations, pH, and wind (Lundholm et al., 2004; Thessen et al., 2005; Fehling et al., 2006; Thessen et al., 2009; Louw et al., 2017; Louw et al., 2018). As pennate diatoms *Pseudo-Nitzschia* presents with longitudinal symmetry, a distinguishing attribute, however, is their ability to form chains (Hasle, 1994). The shape of *Pseudo-Nitzschia* may allow it to be driven through any pre-treatment systems within a desalination plant. Their pointed shape has the ability to potentially damage SWRO membranes. Here, *Pseudo-Nitzschia* was found only within the SWRO feed tank water during the summer months (March & November).

One group that we expected to find in the SWRO feed tank water, was picophytoplankton as their size range is mainly smaller than 5 μm. Oligotrophic environments such as the Gulf Saint Vincent and the waters surrounding Kangaroo Island are often dominated by picophytoplankton organisms who have an important role in primary production (Vaulot et al., 2008; Shi et al., 2009). The picophytoplankton genus of *Minutocellus* which is <3 μm in diameter and is fusiform shaped would pass with ease

through any of the pre-treatment systems within the Penneshaw desalination plant. Indeed, *Minutocellus* was identified in the SWRO feed tank water throughout the sampling period. The small size of the *Minutocellus* genus is advantageous on account of a low sinking rate, efficient acquisition of nutrients and light utilization efficiency (Raven et al., 2005).

The complexity of the habitat is likely more influential in driving the diversity of species and abundance on SWRO membranes then the feed tank water (Smith et al., 2014). The "insurance hypothesis" advocates that protection is obtained in unstable environments through diversity within biofilms (McCann, 2000). It is through the subpopulations' ability to thrive under conditions imposed that complex environments are sustainable (McCann, 2000). In biofilms this diversity can occur rapidly and can influence different functions including motility, required nutrients, secreted product production, the morphology of the colonies and biofilm phenotypes (Boles et al., 2004).

4.7 Conclusion

Pre-treatment systems within desalination plants endeavour to reduce the load of biofouling elements before the SWRO membrane. Information on the fouling composition of the water as well as the membranes is essential to combat the ever-present fouling problem. The key results obtained from our study are consistent and complementary to previous work focussing on prokaryotic organisms. Within the Penneshaw SWRO desalination plant, the spatial and temporal composition clearly demonstrates the intricate dynamics of the eukaryotic organism communities of the intake seawater and the SWRO feed tank water. The niche community structure on the membranes is diverse but stable, yet also dependent on membrane position and duration of service, reflecting the quality of the intake water within the desalination plant. Moreover, the spatial differences are elucidated between

the SWRO feed tank water and the SWRO biofouling communities reflecting the adaptive success of eukaryotic organisms to the surrounding environment.

This study provides insights into the eukaryotic organisms found within a SWRO desalination plant, as well as information on the impact of pre-treatment on the communities, in addition to the potential contribution of the organisms on the fouling of the membranes. It emphasizes that not only prokaryotic organisms contribute to the fouling in SWRO desalination plants but also eukaryotic organisms. Ultimately, it demonstrates that approaches to further target the reduction/removal of organisms sized <5 µm are needed. The development of techniques to utilise the grazing potential of organisms such as copepods, microflagellates, ciliates, and amoebae on the SWRO membranes would create a sustainable biofouling treatment system for the removal of prokaryotic and eukaryotic organisms.

4.8 Acknowledgements

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CHAPTER 5: Can aggregate attached organisms influence the fouling in a SWRO desalination plant?

5.1 Preface

This chapter is closely based on the accepted manuscript of an article published by MDPI Jamieson, T., Whiley H., Gascooke, J.R., and Leterme, S. C. (2022) Can Aggregate-Associated Organisms Influence the Fouling in a SWRO Desalination Plant? *Microorganisms*, 10:4, 682, DOI: 10.3390/microorganisms10040682

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Outline your (the student's) contribution to the publication:

Conceptualisation, Methodology, Formal analysis, Investigation, Data curation, Writing - Original draft, Writing - editing, revision of the manuscript, and final manuscript.

☑ I confirm that the details above are an accurate record of the student's contribution to the work.

Name of Co-Author 1: Sophie Leterme Signed: Sophie Leterme Digitally signed by Sophie Leterme Date: 2021.06.02 18:39:27 +09'30' Date: 02/06/21

5.2 Abstract

This pilot study investigates the formation of aggregates within a desalination plant before and after pre-treatment, as well as their potential impact on fouling. The objectives are to provide an understanding into the biofouling potential of the feed water within a seawater reverse osmosis (SWRO) desalination plant due to the limited removal of fouling precursors. Intake seawater and SWRO feed water was collected from the Penneshaw desalination plant (Kangaroo Island, South Australia). Magnetic beads were introduced into the water and stirred overnight before the aggregates were collected via a magnetic field. 16S and 18S rRNA was extracted from the water samples and the aggregates and sequenced. Pre-treatment systems, multimedia filters, 15 and 5 µm cartridge filters, within the plant remove medium to large size precursors and organisms, however, smaller size particles progress through the plant, allowing for the formation of aggregates. These become hot spots for microbes due to their nutrient gradients, facilitating the formation of niche environments supporting the proliferation of those organisms. Aggregate associated organisms are consistent with those identified on fouled SWRO membranes. This study examines for the first time the factors supporting the formation of aggregates within a desalination system, their microbial communities, and their biofouling potential.

5.3 Introduction

Oceanic microorganisms can secrete a diverse array of large molecules, collectively called extracellular polymeric substances (EPS; Decho and Gutierrez, 2017). While EPS are believed to be the precursors of biofilm formation, in open-water environments they contribute to the formation of organic colloids, and larger aggregations of cells, called particulate organic matter (POM) or 'marine snow'. POM, a source of carbon and nutrients to heterotrophic microorganisms, is essential for the transport of

elements and energy towards the deep ocean and is the main method for the removal of carbon from surface waters (Pelve et al., 2017; Poff et al., 2021). POMs harbour a diverse and complex disparity of inorganic particles and, can be regarded as microhabitats due to the large amount of autotrophic and heterotrophic organisms found within (Poff et al., 2021). POM's microbial community abundances can reach up to two orders of magnitude higher than the surrounding seawater environment (Grossart et al., 2007). The high microbial activity of POM-associated (PA) bacteria is reflected by their enhanced cell-specific rates of polymer hydrolysis and substrate uptake relative to the free-living (FL) bacteria in the surrounding water (de Carvalho, 2018). In their studies, Milici et al. (2017) showed remarkable taxonomic differences between PA and FL bacteria in the deep Southern Ocean water masses. PA-bacterial communities had high numbers of polymer-degrading bacteria such as Flavobacteria, γ-proteobacteria, Planctomycetes and Verrucomicrobia, whereas the FL bacterial communities had high numbers of α-protebacteria (Milici et al., 2016; Mestre et al., 2017; Mestre et al., 2020). The PA communities are commonly found in marine biofilms especially as biofilm initiators (Lee et al., 2016; Rampadarath et al., 2017; Pollet et al., 2018; Antunes et al., 2020; Caruso, 2020;). In particular, γ-proteobacteria perform an important role within marine biofilms especially through their capability for polysaccharide biodegradation and cellulose metabolism (Edwards et al., 2010; Lee et al., 2016; Gobet et al., 2018). It can also dominate the initial phase of biofilm formation (Rampadarath et al. 2017). However, the FL community of α-proteobacteria have also been known to dominate all stages of biofilms (Gao et al., 2012; Antunes et al., 2020; Caruso, 2020).

Much like the colonization of surfaces, the colonization of aggregates by bacteria is complex and occurs in several steps. First, bacteria will attach loosely to the aggregate. This attachment will gradually increase until cells are permanently attached, then, growth rates of the attached bacteria will drive the colonization over attachment (Grossart et al., 2003). Fast moving bacteria will encounter an aggregate in about <1 day (Kiørboe et al., 2002), and non-motile bacteria will collide with

aggregates, at a lower frequency, due to the motion of the liquid they are in. Eventually, the total number of cells on the aggregate will increase, and the bacterial community becomes established much like during the formation of bacteria biofilms on inert surfaces. Biofilm formation is an impediment for many water treatment infrastructures, such as desalination plants as membrane biofouling is considered to be a major contributor to the increase in production costs (Qasim et al., 2019). Biofouling of the SWRO membrane is often described as the accumulation of complex sessile microbial communities, which are surrounded by an impenetrable, heterogeneous matrix of EPS primarily comprised of polysaccharides and proteins (Jamieson and Leterme, 2021). To date there has been limited research assessing the contribution of marine aggregates to the fouling of seawater reverse osmosis (SWRO) membranes *in situ*, it is known that not all biofilm precursors can be removed by pre-treatment (Balzano et al., 2015c). Recent studies have focused on the role of transparent exopolymer particles (TEP; marine snow pre-cursor) as potential precursors of SWRO membrane biofouling (Zhang et al., 2020; Blazyte et al., 2021; Meng et al., 2021).

The limited removal of TEP from seawater via pre-treatments increases the biofouling potential (Balzano et al., 2015c). Bar-Zeev et al. (2012) proposed a new paradigm stating that TEP plays a critical role alongside the "traditional" stages of biofilm formation and introduced the term "protobiofilm" to characterise TEP showing extensive microbial outgrowth and colonization. TEP are often found in marine environments and play a role in the formation and development of marine biofilms (Bar-Zeev et al., 2015; Jamieson and Leterme, 2020). Within the desalination process, high levels of potential biofilm forming TEP have been found to be reach the SWRO membrane (Bar-Zeev et al., 2009). Bar-Zeev et al. (2015) highlight that a better understanding of TEP formation pathways, size spectrum, chemical nature and bacteria interactions could instigate new pre-treatment methods for their efficient removal as well as novel cleaning strategies following attachment to a membrane surface.

The production of fresh water via desalination has been extensively recognized as a valuable solution to ensure water security (Darre and Toor, 2018). This is especially true in drought affected areas and is increasing important as global water shortages are predicted to be further exacerbated through climate change (Mekonnen and Hoekstra, 2016; Damania et al., 2017). SWRO is a reliable and efficient process, enabling the separation of salts and water molecules through a semi-permeable membrane due to a pressure gradient (Darre and Toor, 2018). SWRO is considered the simplest and most cost-effective method for potable water production due to low energy consumption and reduced production costs (Nassrullah et al., 2020). Established biofilms, due to the complex nature of EPS, have been found to be impervious to oxidizing agents and biocides, making the extrication of biofilms problematic (Malaeb and Ayoub, 2011; Matin et al., 2011). Pre-treatment systems are thus essential in SWRO facilities to moderate organic and inorganic fouling of the RO membranes. Multimedia filtration as well as cartridge filtration are frequently part of the coagulation/flocculation steps found in most pre-treatment systems (Prihasto et al., 2009; Anis et al., 2019b).

Previous studies have examined the fouling potential of feedwater and the impact that it has on biofouling of SWRO membranes both in laboratory settings and in pilot scale systems. With RO membrane biofouling monitored over time (Bereschenko et al., 2010; Khan et al., 2013b; Kim et al., 2014b). Other studies focused on the microbial communities of the cartridge filters and the SWRO membranes, or on the validity of pre-treatment methods on the permeate communities' post-treatment within desalination systems (Bereschenko et al., 2008; Manes et al., 2011a & b; Zhang et al., 2011; Chun et al., 2012; Belila et al., 2016; Levi et al., 2016; Nagaraj et al., 2017; Nagaraj et al., 2019; Benladghem et al., 2020). In this study, we explore the formation and composition of aggregates within a SWRO desalination system pre- and post-treatment, and their influence in biofouling. Aggregates were formed in water collected from a SWRO desalination plant pre- and post-treatment. Comparison of the microbial composition of the aggregates was performed to ascertain the

organisms associated in biofouling within the plant. Moreover, further comparisons were made looking at the size and composition of the aggregates in order to investigate how they could influence biofilm development on the SWRO membranes. The findings from this study will inform future strategies aimed at controlling the formation of aggregates to reduce membrane fouling.

5.4 Materials and Methods

5.4.1 Sampling sites

The Penneshaw SWRO desalination plant has a capacity of 3×10^5 L per day and has been described in detail in previous studies (Dixon et al., 2012). Seawater from a depth of 6 m is pumped from the coastal waters north of Kangaroo Island (South Australia) at a site located 200 m from the Penneshaw desalination plant (Figure 5.1) and enters the system through two pre-filtration screens (10 cm and 0.5 mm pore sizes, respectively). This is then followed by the pre-treatment system which includes an MP-UV disinfection unit, four parallel multimedia filters (gravel, garnet, sand and coal with grain size ranging from 0.3 to 10 mm), and two consecutive sets of three cartridge filters each with a pore size of 15 µm and 5 µm, respectively. The flow rate through the system is typically 8.4 $L.s^{-1}$ after which the seawater enters the SWRO feed tank.

The Penneshaw SWRO unit is a single framework compartment comprising of 12 pressure vessels, each containing 4 membranes. The SWRO membrane are spiral wound thin-film composite of

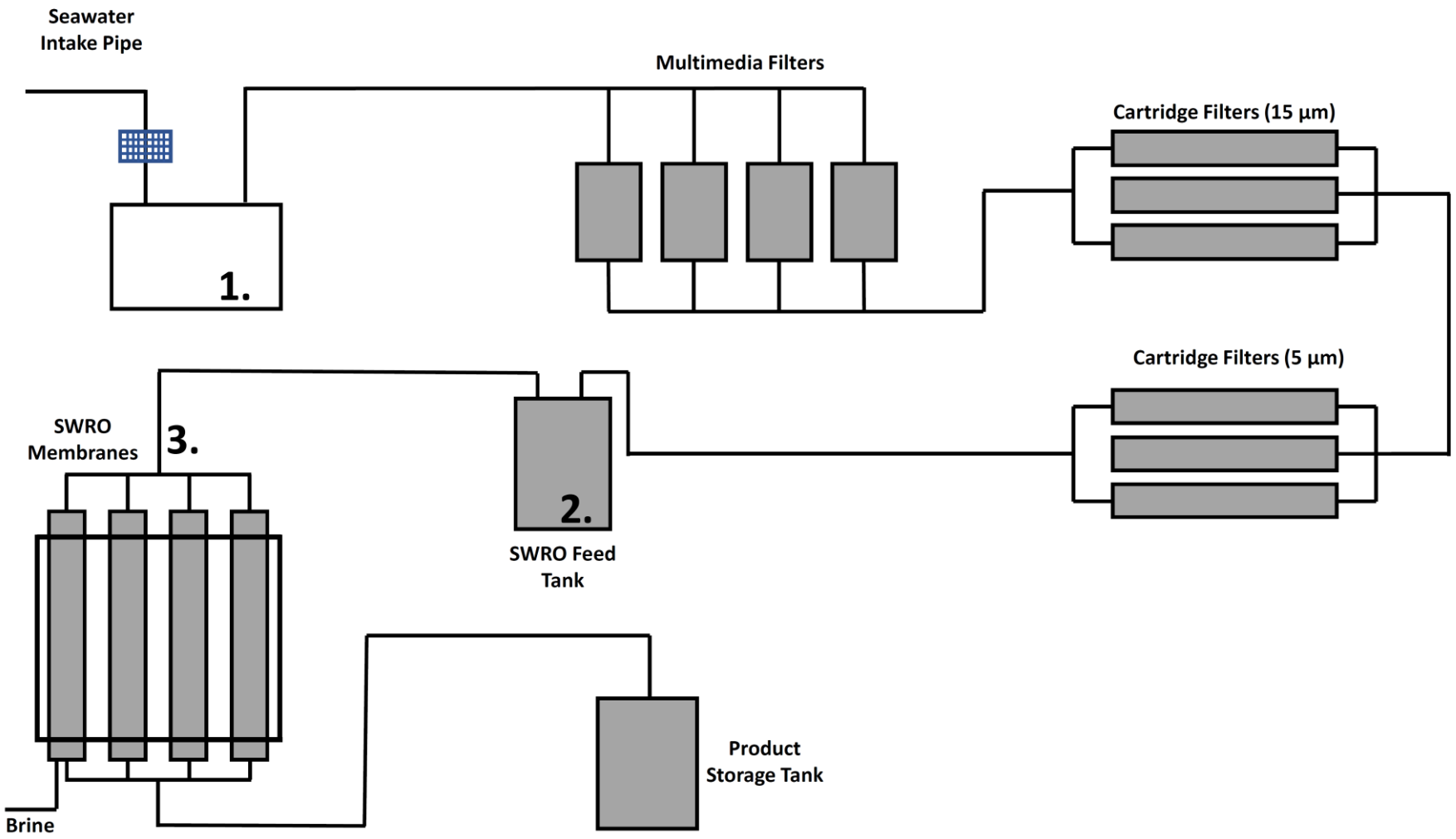

Figure 5.1: Schematic diagram of the Penneshaw SWRO desalination plant. Numbers indicate the different sampling points: (1) Intake seawater, (2) SWRO feed water and (3) SWRO membranes.

polyamide (FILMTEC^{TM} SW30HRLE-440i), with and an active surface area of 41 m^2. Four fouled membranes from the 1st stage and 2nd stage position after 2- and 4- years- service were used in this study.

Seawater was collected at two sampling points within the desalination plant: (1) intake seawater,located prior to any treatment, and (2) pre-treated seawater, within the SWRO feed tank located directly after the cartridge filters and before the SWRO membranes. Composite samples were collected in 2 L grabs every 30 minutes until a total volume of 20 L was collected. Samples were stored in 20 L white opaque carboys and kept on ice during transportation to the laboratory at Flinders University.

Membranes obtained for this project were installed on the 19th August 2010 and removed on the day of sampling, the 1st September 2014. In total, four fouled SWRO membranes were provided by SA Water for an autopsy study: a membrane from each stage of the SWRO unit (1st stage and 2nd stage) and which had been in service for two and four years (Jamieson et al., in press). During this period no chemical cleaning of the plant or the membranes was undertaken apart from the sulfuric acid addition.

5.4.2 Formation of aggregates

In this experiment, aggregates were produced in a 20 L clear carboy (Nalgene) using collected water. Microspheres (BioMag Carboxl; Bang Laboratories Inc) were added to the carboy at a concentration of 2.5 x 10^5 particles mL^{-1}, following the protocols of Mari et al. (2012). Briefly, the seawater was placed on an orbital shaker to stir at 200 rpm overnight at ambient temperature (20 °C). Aggregates were isolated using the magnetic properties of the microspheres. The seawater was filtered through a magnetic field (2 x 27 mega gauss oersteds) consisting of a tissue culture flask encased by two

magnets (Bang Laboratories Inc) held together by a rubber band while in an ice bath. The seawater inflow tube was placed at the bottom of the flask and the output tube was at the top. A peristaltic pump (MasterFlex, Cole Parmer) maintained an optimal flow of 25 mL min^{-1} to separate the microspheres from the water. Samples were collected from the initial seawater (before magnetic separation), inside the tissue culture flask and in the filtrate seawater (after magnetic separation).

5.4.3 TEP analysis of aggregates

Under vacuum aggregates were filtered onto 0.4 μm polycarbonate filters (Merck Millipore Ltd). The filters were then stained with alcian blue for 20 minutes before being washed with sterile seawater. Each filter was placed face down on a cover slip before being submerged in liquid nitrogen. The filter was then removed from the coverslip before being examined using a Nikon Eclipse T*i*2 inverted microscope.

5.4.4 Structural analysis of aggregates

Aggregates were filtered (100 mL) onto 0.4 μm polycarbonate filters (Merck Millipore Ltd) under gentle vacuum before being cut into 1 cm^2 samples. The samples were then fixed and dehydrated following the previously described protocol of Lee et al. (2010). Each sample was mounted onto studs using carbon-tape and sputter coated with platinum (15 nm) before being observed using a FEI Inspect F50 Scanning Electron Microscope (SEM) with images obtained at 5 kV. The energy dispersive X-ray (EDX) spectroscopic analysis was conducted at 10 kV for 2000 seconds.

5.4.5 Extracellular DNA of aggregates

Aggregates were filtered onto 0.4 µm polycarbonate filters (Merck Millipore Ltd) under low vacuum. The filters were then stained with PicoGreen™ (Invitrogen) and examined using a Nikon Eclipse T*i*2 inverted fluorescence microscope.

5.4.6 DNA Extraction, sequencing, and bioinformatics

In order to identify the bacterial strains associated to TEP, nucleic acids were extracted from the water and the aggregates using MPBio Lysing Matrix M tubes in combination with FastPrep-24™ 5^{G} (MPBio) sample preparation system. Extractions were performed by the addition of 250 µl of lysis buffer and 250 µl of proteinase K, mixing by vortex before overnight incubation at 56 °C. Samples were lysed for 40 seconds at 6 m s^{-1} before 8 incubations at 56 °C for 60 minutes prior to DNA extraction. The aqueous phase was then removed, and nucleic acids were extracted using a modified protocol from Real Genomics HiYield™ DNA extraction kit (Real Biotech Corporation, Taiwan).

Amplification of the prokaryotic V1-2 region of the 16S rRNA gene was undertaken using the universal primer 27F and a reverse primer 338R. Amplification of the eukaryotic V4 region of the 18S rRNA gene was undertaken using the universal primer 528F and a reverse primer 706R. The primers were modified to include an A-adaptor and a sample specific, 11 bp barcode, to the 5'- end of the forward primer, and a P1-adaptor to the 5'-end of the reverse primer for Ion Torrent next generation sequencing. PCR reactions were performed on a total volume of 50 µl, containing approximately 1 ng μL^{-1} of template DNA, 2 U Q5 Hot Start High-Fidelity DNA Polymerase (New England Biolabs), 1 x Q5 reaction buffer (New England Biolabs), 1 µl of complementary primers, 2.5 mM of deoxynucleotide triphosphates (dNTP) (Promega) and MQ water. PCR consisted of an initial denaturation at 98 °C for 1 min, 14 cycles

of 30 s at 98 °C, 30 s at 60-72 °C and 30 s at 72 °C, followed by 21 cycles of 30 s at 98 °C, 1 min at 72 °C. PCR products were purified using a Wizard SV Gel and PCR clean-up system (Promega). Amplicons were sequenced by The Australian Cancer Research Foundation (ACRF) Biomolecular Resource Facility (BRF) using an Ion Torrent Personal Genome Machine (PGM) provided with a 318 chip (Life Technology) and adapted for a maximum read length of 400 bp. From the intake seawater aggregates no 18S rRNA region was amplified due to low levels of rDNA extracted.

The Ion Torrent platform sequence data was analysed using Mothur (Schloss et al., 2009). A lower phred quality threshold (20) was used to filter the reads, in comparison to other sequencing platforms (i.e. 25) as Ion Torrent have been found to underestimate the real base accuracy. Reads shorter than 200 bp, with a phred quality below 20 over a 50 bp sliding window, with one or more nucleotide mismatches for the forward primer and more than 8 homopolymers were removed from the dataset. Forward primer sequences and barcodes were removed, and reads were trimmed to 250 bp in length. The UCHIME algorithm was used to identify and remove chimeric sequences. The UCLUST algorithm was used to identify and remove singletons. Distinct Operational Taxonomic Units (OTUs) were determined using the UCLUST algorithm based on a 97% similarity. A representative set of samples were randomly selected from the data set to compare diversity. The SILVA (version 132) and the Protist Ribosomal Database (version 4.12.0; Guillou et al., 2012) was used to infer taxonomic affiliation of the OTUs using the UCLUST algorithm.

5.4.7 Data analysis

The following statistical analyses were performed for both 16S and 18S rRNA sequencing data unless noted. All data was transformed using Log+1 before undertaking Bray-Curtis similarity and Jaccard distance to calculate similarity matrices between the prokaryote and eukaryote communities,

respectively. The data were then analysed by Principle Coordinate Analysis (PCoA) using Primer7 (version 7.0.13). Differential abundance between two microorganism communities (intake water vs SWRO Feed tank water, and intake water aggregates vs SWRO feed tank water aggregates) were compared using the DESeq2 package (version 1.29.4; Love et al., 2014) using R (version 4.0.0). To identify the core, the variable, and the unique taxa among the water samples and the aggregates, Venn diagrams were created with the online tool access through https://bioinfogp.cnb.csic.es/tools/venny/. The functional prediction of genes of the water and aggregate microbiota was acquired from web-based software Piphillin (Iwai et al., 2016) based on the relative abundance of the OTU table (taxonomy was assigned with Silva database 132). Piphillin is a tool that assists with the prediction of metabolic profiles by mapping 16S sequences to known reference genomes: the KEGG pathways. The function prediction matrix was clustered and categorized utilising the Kyoto Encyclopaedia of Genes and Genomes (KEGG) orthologs (KOs) and pathways. Microsoft Excel was used to create the abundance graphs.

5.5 Results

5.5.1 Intake seawater and SWRO feed tank water community structure

In this study, we investigated the planktonic and aggregate-attached communities present in the intake seawater as well as in the SWRO feed tank water of the Penneshaw desalination system. Based on the 3,106 bacteria OTUs identified in the water and/or the aggregates after sequencing, dissimilarities in the prokaryotic community composition between the intake seawater and the SWRO feed tank water samples were identified using a PCoA. The separation along the principle coordinate PCO1 displays the dissimilarities in the prokaryotic community structure between the intake seawater and the SWRO feed tank. Whereas, along the principle coordinate PCO2 a lesser separation is evident

between water samples and aggregate communities (Figure 5.2A). Similarly, differences in eukaryotic community structure were identified between the intake seawater and the SWRO feed tank using the 1,208 eukaryote OTUs identified after sequencing (Figure 5.2B).

To further our understanding of the differences between water and aggregate communities, core OTUs were identified using Venn diagrams. These core OTUs are considered to be core

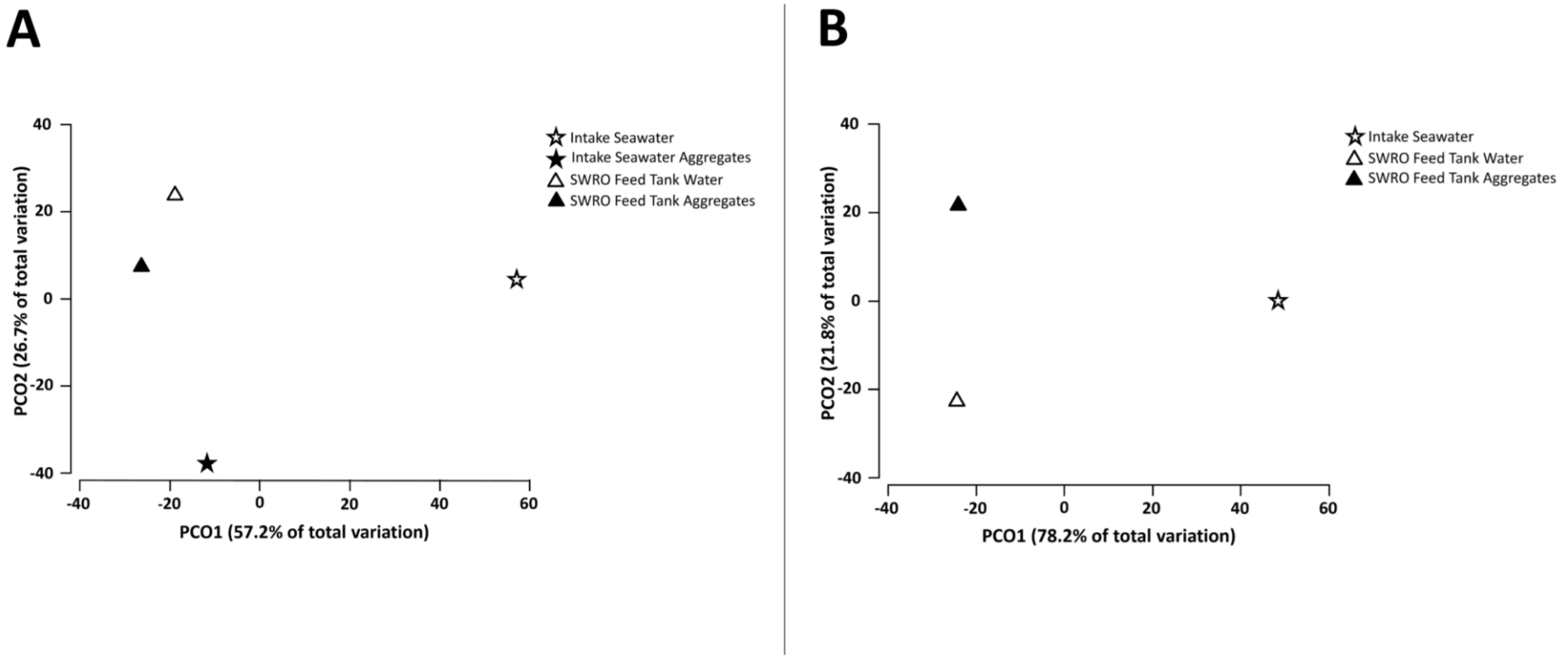

Figure 5.2: Principal coordinate analysis (PCoA) based on Bray-Curtis distance ordination displaying the differences in the intake seawater and the SWRO feed tank water in (A) the prokaryotic communities in the planktonic and aggregate attached samples, and (B) the eukaryotic communities in the planktonic and aggregate attached samples. The total variability is explained by the two PCoA axes, with the ordination of water samples (A) explaining 83.9% of the attachment and pre-treatment variability observed in the samples and (B) explaining 100% of the variability observed in the pre-treatment and the attachment of the samples.

microorganisms essential to the function of the population, thus allowing for the representation of a "healthy" population and the influence or effect of any impediment (Shade and Handelsman, 2012). Three different groups were identified: Core OTUs (identified in all sampling sites), Variable OTUs (identified in multiple sites but not all), Unique OTUs (identified in only one site). Of the 3,106 bacteria OTUs identified in the water and/or the aggregates, 1,705 (54.9%) are considered unique OTUs, 1,331 (40.9%) are considered Variable OTUs and 129 (4.2%) are considered Core OTU's (Figure 5.3A). The core OTUs consisted of seven classes within four phyla. The core OTU classes were found to be Actinobacteria, Bacilli, Gracilibacteria, α-proteobacteria, β-proteobacteria, ε-proteobacteria and γ-proteobacteria. Of the 1,208 eukaryote OTUs identified in the water and/or the aggregates: 667 (55.2%) are considered unique OTUs, 344 (28.5%) are considered Variable OTUs and 197 (16.3%) are considered Core OTUs (Figure 5.3B). The core OTUs are dominated by the class of Ascomycota and Basidiomycota and Chlorophyta.

5.5.2 Water community composition

13 bacterial taxa were identified in the water samples: Acidobacteria, Actinobacteria, Bacteroidetes, Chloroflexi, Cyanobacteria, Firmicutes, Fusobacteria, Gemmatimonadetes, Marinimicrobia, Patescibacteria, Proteobacteria, and Synergistetes (Figure 5.4A). The phyla Proteobacteria was the most dominant (69.25%) followed by Actinobacteria (20.2%), Epsilonbacteraeota (6.2%) and Firmicutes (3%). The phyla Cyanobacteria, Marinimicrobia, Acidobacteria and Verrucomicrobia were only found in the intake seawater, while, the phyla Chloroflexi, and Gemmatimonadetes were only identified in the SWRO feed tank water. Pielou's evenness values indicate that OTU abundances within the seawater samples were highly diverse but similar across samples (Table 5.1). SIMPER analysis determined a significant dissimilarity between the prokaryotic communities in the intake water and the SWRO feed

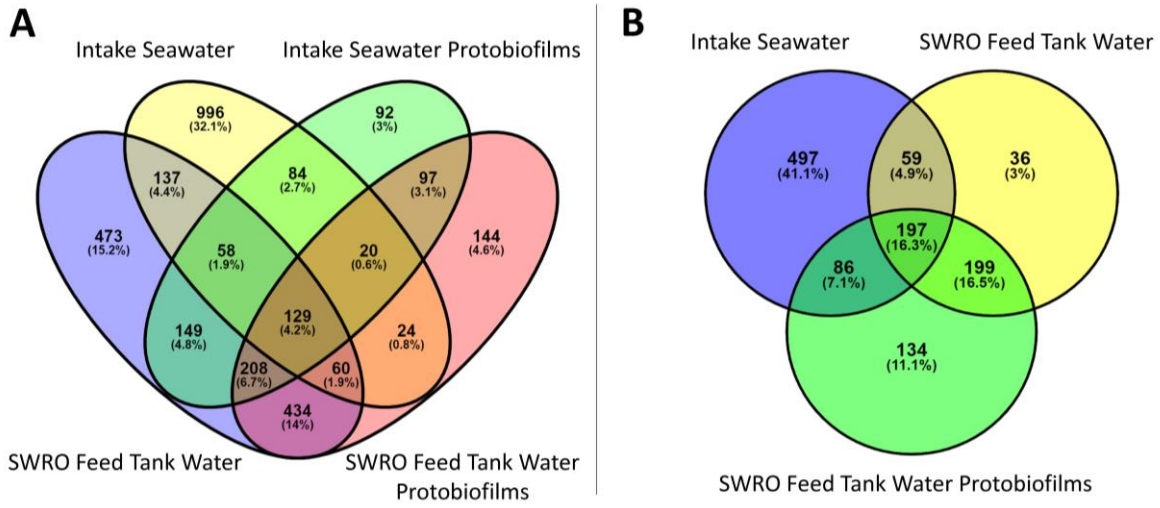

Figure 5.3: Venn diagram displaying the overlap between the (A) prokaryotic communities and (B) the eukaryotic communities in the water and aggregates. Core OTUs, identified in all sampling sites; variable OTUs identified in two or more sites but not all; unique OTUs, identified in only one site.

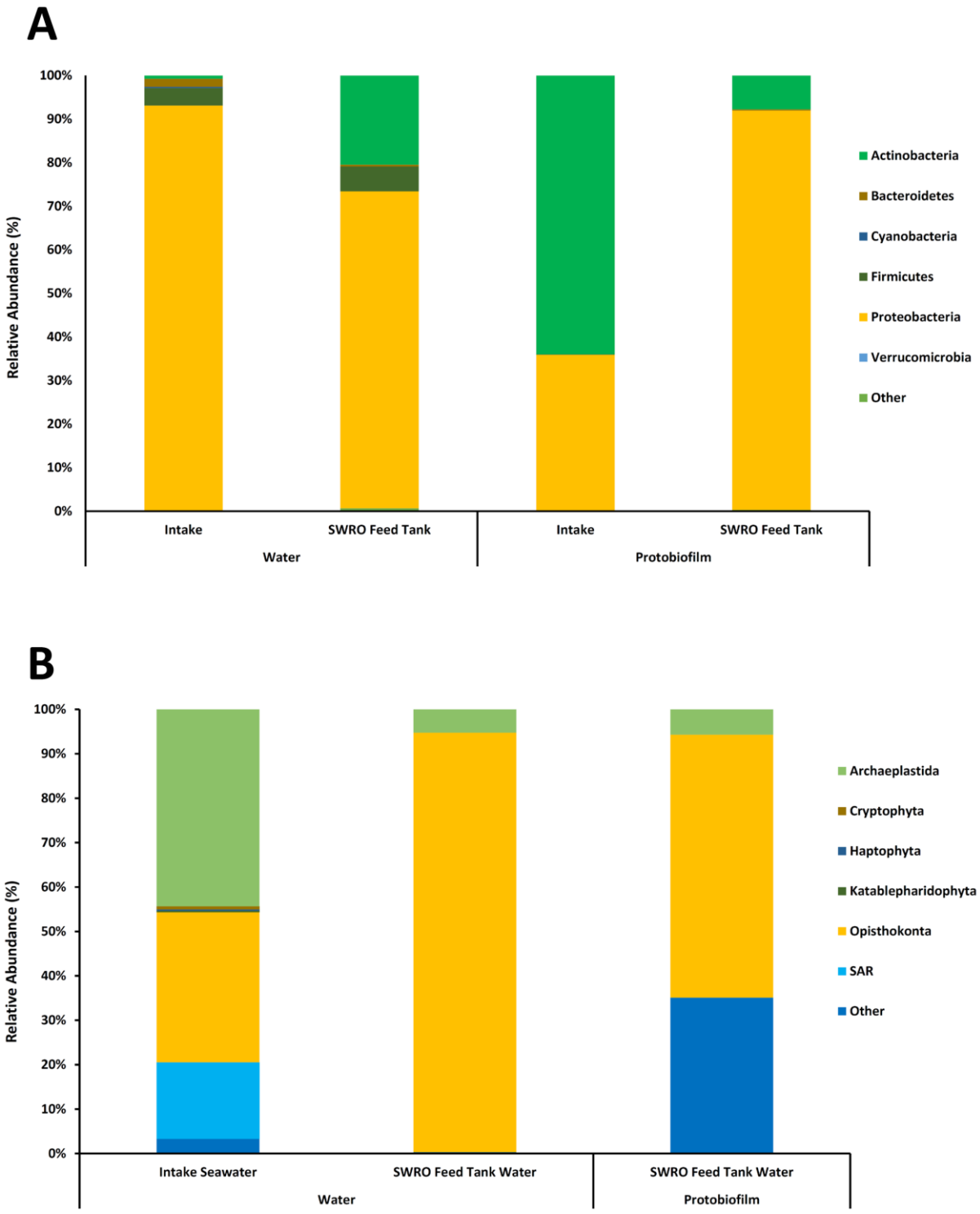

Figure 5.4: The relative abundance composition of the phylum taxonomy of the intake seawater and SWRO feed tank water samples (A) of the bacteria communities of the water samples and aggregates and (B) of the eukaryotic communities of the water samples and aggregates.

Table 5.1: Pielou's evenness values of the prokaryotic and eukaryotic organisms in the water, aggregates and the biofouled membranes analysed in the present study.

Sampling Site	*Prokaryotes Pielou's evenness*	*Eukaryotes Pielou's evenness*
Intake Seawater	0.96	0.97
Intake Seawater Aggregate	0.96	
SWRO Feed Tank Water	0.96	0.96
SWRO Feed Tank Water Aggregate	0.96	0.97
2-years 1st Stage SWRO membrane	0.64	0.93
2-years 2nd Stage SWRO membrane	0.58	
4-years 1st Stage SWRO membrane	0.51	0.96
4-years 2nd Stage SWRO membrane	0.60	0.93

water was 82.57%. The dissimilarity was due to a significant increase in *Pseudomonas*, *Aeromonas*, *Streptococcus*, *Rahnella*, *Cedecea*, *Stenotrophomonas*, *Cutibactium*, and *Staphylococcus* in the SWRO feed tank water, and to a significant decrease in *Effusibacillus*, and *Pseudoalteromonas*.

Six eukaryotic taxa were identified in the water samples: Archaeplastida, Opisthokonta, Cryptophyta, Haptophyta, Katablepharidaceae and SAR, in line with the revised Eukaryotic classification put forward by Adl et al. (2012). The supergroup of Opisthokonta was the most dominant (Intake water 53.8%, SWRO feed tank water 96.10%) followed by Archaeplastida (Intake water 43.87%, SWRO feed tank water 3.10%; Figure 5.4B). The groups Cryptophyta, Haptophyta and Katablepharidaceae were only found in the intake seawater. Pielou's evenness values indicate that OTU abundances within the seawater samples were highly diverse but similar across samples (Table 5.1). SIMPER analysis determined a significant dissimilarity between the eukaryotic communities in the intake seawater and the SWRO feed tank water of 66.68%. This was due to a significant increase in abundance of the two Opisthokonta classes of Sordariomycetes and Exobasidiomycetes.

5.5.3 Aggregate-associated community composition

Nine bacterial taxa were detected in the aggregates: Actinobacteria, Bacteroidetes, Cyanobacteria, Epsilonbacteraeota, Firmicutes, Fusobacteria, Patescribacteria, Proteobacteria and Synergistetes (Figure 5.4A.). The phylum Proteobacteria dominated the community (63.7%) followed by Actinobacteria (35.9%). The phyla Cyanobacteria and Synergistetes were only found in the intake aggregates, whereas phyla Patescibacteria and Fusobacteria were only found in the SWRO feed aggregates. Pielou's evenness indicates that OTU abundances within the aggregates were highly diverse and equally distributed (Table 5.1). SIMPER analysis determined that the significant dissimilarity between the prokaryotic communities in the intake water and the SWRO feed tank water

aggregates was 59.14%. This was due to a significant increase in the γ-proteobacteria genus *Cutibactium*, *Delftia, Serratia*, *Rahnella*, and *Cedecea* in the SWRO feed tank water. While a significant decrease in the γ-proteobacteria genera Pseudomonas, α-proteobacteria genera *Altererythrobacter*, and Actinobacteria genera *Cornebacterium* in the SWRO feed tank water, also contributed to the dissimilarity. The functional prediction of genes of the water and aggregate bacteria was acquired from web-based software Piphillin. Several pathways i.e., amino acid metabolism, carbohydrate metabolism, folding, sorting and degradation, metabolism of cofactors and vitamins, biosynthesis of other secondary metabolites and glycan biosynthesis and metabolism were identified to be significantly higher (p <0.05) in the SWRO feed aggregates compared to the intake seawater aggregates (Table 5.2).

Three eukaryotic taxa were detected in the aggregates formed in the SWRO feed tank water: Archaeplastida, Opisthokonta, and SAR. The supergroup of Opisthokonta dominated the community (92.6%) followed by Archaeplastida (5.8%) (Figure 5.4B). Pielou's evenness indicates that OTU abundances within the aggregate sample was highly diverse (Table 5.2). From the intake seawater aggregates no 18S rRNA region was amplified due to low levels of rDNA extracted.

5.5.4 Aggregate composition, size, and fouling potential

To observe the role of TEP in the formation of the aggregate, acidic alcian blue stain was applied. TEP particles are recognised by the ability of the acidic polysaccharides to be stained by a low pH alcian blue stain reacting to the presence of anionic carboxyl and half-ester-sulfate groups (Bar-Zeev et al., 2015). The aggregates observed under x40 microscopy formed from the intake seawater were of a viscous nature in which the magnetic beads were apparent (Figure 5.5A & B). The alcian blue staining shows the presence of TEP particles in many of the aggregates however, it is

Table 5.2: The elemental composition and the proposed biological and chemical components of the aggregate formed within the Penneshaw SWRO desalination plant intake seawater and SWRO feed tank water analysed in the present study.

	Intake Seawater Aggregate 1	*Intake Seawater Aggregate 2*	*SWRO Feed Tank Aggregate 1*	*SWRO Feed Tank Aggregate 2*
Chemical Elements	C, N, O, Fe, Mg, Al, Si, S, Cl, Ca	C, N, O, Fe, Mg, Al, Si, Cl, K, Ca	C, N, O, Fe, Na, Al, Ca, K, Cl, S	C, N, O, Fe, Na, Al, Si, Cl, K, Ca, Cr, Ni
Proposed Biological & Chemical Components	Shell/Bone Diatom Aluminosilicate Iron Oxide Calcium Silicate Polysaccharide	Polysaccharide Aluminosilicate Salt – CaCl2	Salt – KCl, NaCl Sulfate Iron Oxide Calcium Polysaccharide	Stainless steel Salt – NaCl, KCl, CaCl2 Tentative – CaSO4 Iron Oxide Polysaccharide Silica

not formed only from TEP particles. The aggregates formed in the SWRO feed tank water are of a gelatinous nature in which the magnetic beads can be observed. Alcian blue staining of the aggregate is apparent and the complete aggregate is not stained. (Figure 5.6A & B).

PicoGeen was used to visualise the extracellular DNA (eDNA) in the aggregates. PicoGreen is a fluorescent nucleic acid stain for double-stranded DNA. The staining of the aggregates formed in the intake seawater for eDNA displayed two distinct sizes of fluoresced cells (Figure 5.7A & B). The larger brighter cells could be attributed to bacteria within the aggregates with the smaller sized particles could denote a diffusion of the eDNA into the EPS surrounding the aggregates. The aggregate sample formed in the SWRO feed tank water display as uniformity in the coverage of eDNA with more bacteria cells visible in the aggregates. Similarly, there is also the smaller sized particles evident surrounding the bacteria cells (Figure 5.7C & D).

Scanning electron microscopy (SEM) and energy-dispersive X-ray analysis (EDX) were applied to the aggregates to determine their structure and chemical composition. This technique gives an overall mapping of the aggregates by analysing near-surface elements and estimating their elemental proportion at different positions by moving the electron beam across the aggregates. The aggregates formed within the intake seawater were robust in structure with a large amount of debris attached (Figure 5.8A & C). In contrast, those formed within the SWRO feed tank water presented a more viscous structure with limited debris (Figure 5.8E & G). SEM-EDX analysis of the aggregates showed that their elemental composition was similar for the intake water (Figure 5.8B & D) and the SWRO feed tank water (Figure 5.8F & H). The presence of carbon, nitrogen, iron, sodium, magnesium, aluminium, silicon, sulphur, chlorine, potassium, chromium, nickel, and calcium was detected in the aggregates in varying concentrations (Table 5.2.). The aggregates analysed showed that the chemical composition of the structures is complex and variable. The aggregate formed in the intake.

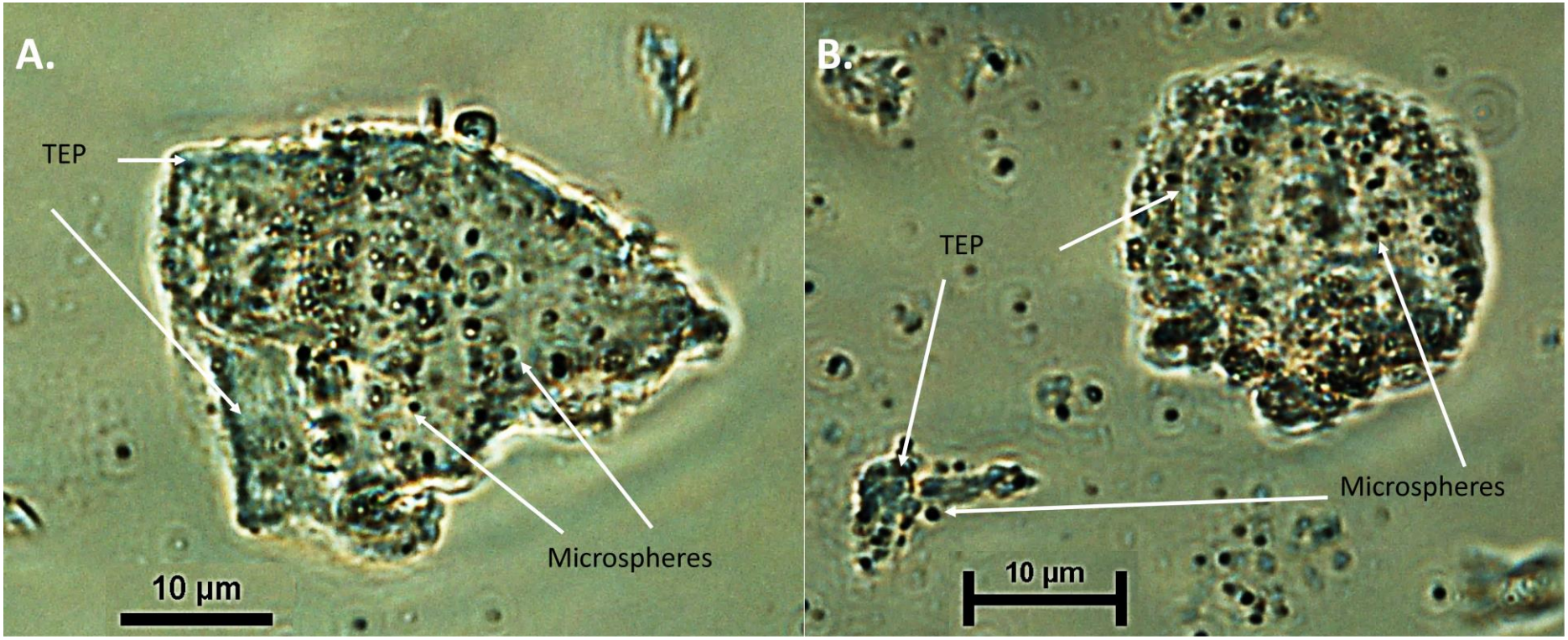

Figure 5.5: Microscopy of Alcian blue stained aggregate samples formed in the Penneshaw desalination plant. (A & B) Aggregate formed in the intake seawater.

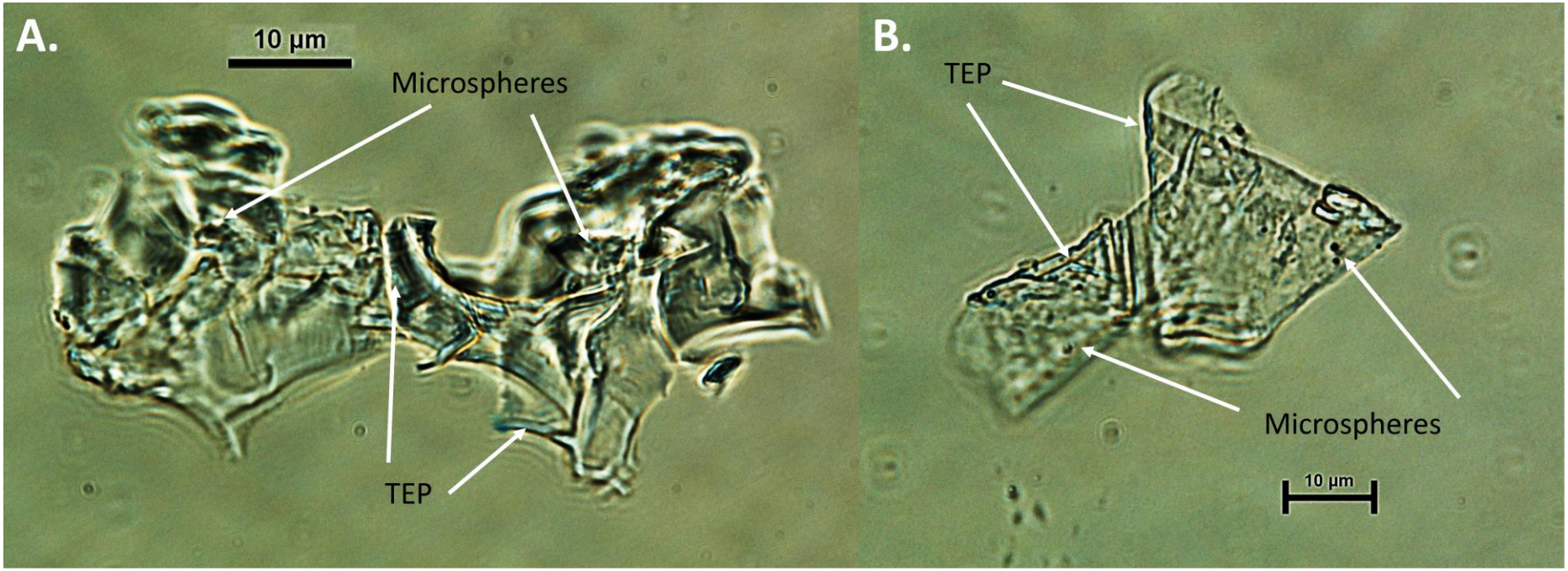

Figure 5.6: Microscopy of Alcian blue stained aggregate samples formed in the Penneshaw desalination plant. (A & B) Aggregate formed in the SWRO feed tank water

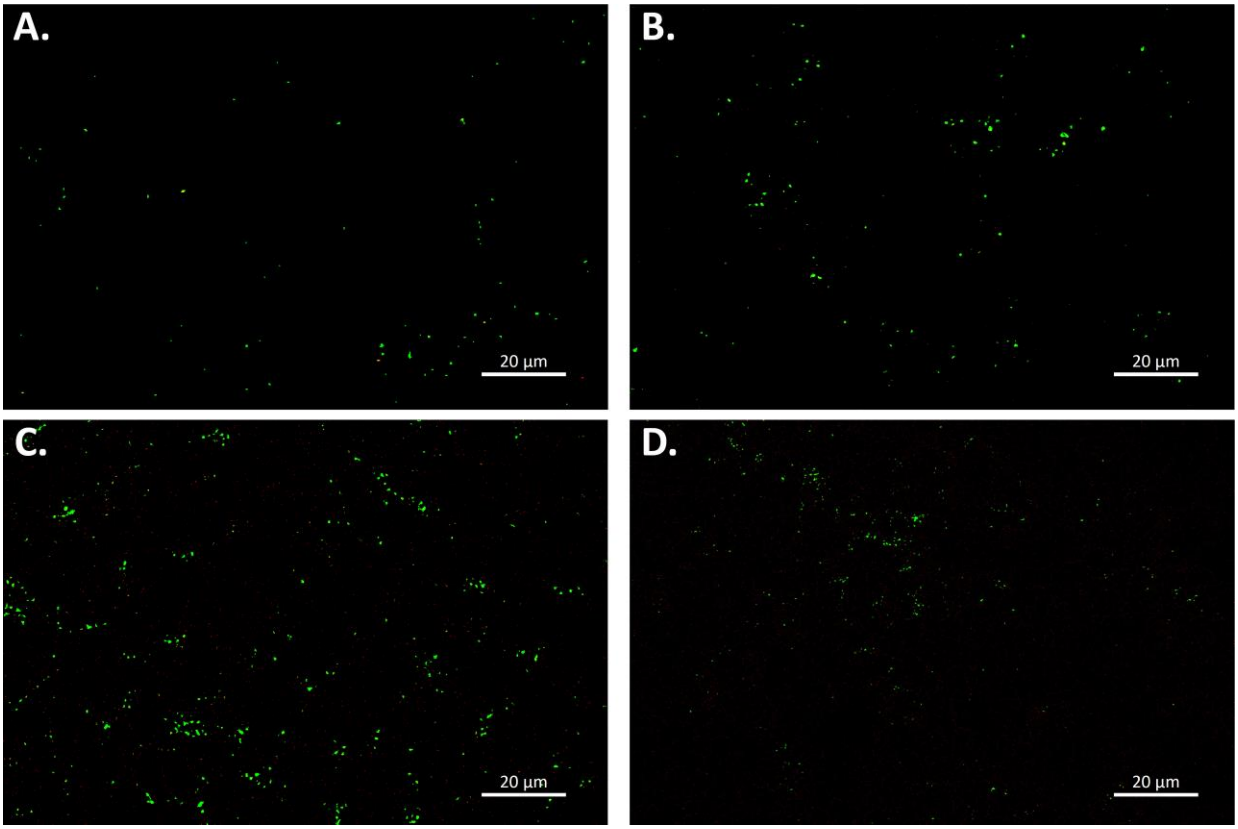

Figure 5.7: PicoGreen staining of extracellular DNA in the aggregates formed in Penneshaw SWRO desalination

seawater contained relatively low abundances of carbon in comparison to those formed in the SWRO feed tank water which contained moderate amounts. Conversely, the intake seawater aggregates contained moderate amounts of oxygen whereas, those formed in the SWRO feed tank water displayed only low abundances. The elements chromium and nickel were only found to be present in the SWRO feed tank aggregate.

In order to assess the fouling potential of the aggregate communities, these were compared to the communities found on fouled membranes extracted from the Penneshaw desalination plant (Jamieson et al., 2021) using Venn diagrams. Here, OTUs were compared at family level. Of the 239 prokaryote OTUs found in the aggregates and the SWRO membrane after 2-years' service, 87 (51%) are considered unique OTUs, 78 (40%) are considered to be variable OTUs and 15 (9%) are considered to be essential OTUs (Figure 5.9A). The OTUs considered to be essential were dominated by the families of the Proteobacteria phylum including *Sphingomonadaceae*, *Rhodobacteraceae*, *Parvularculaceae*, *Legionellaceae*, *Parvibaculaceae*, *Xanthobacteraceae*, SAR116 clade, *Burkholderiaceae*, *Pseudomonadaceae*. As well as *Propionibacteriaceae* from the Actinobacteria phylum and *Flavobacteriaceae* from the Bacteroidetes phylum. From the aggregates and the 4-year-old SWRO membranes, 213 prokaryote OTUs were analysed, of which, 90 (51%) are considered unique OTUs, 74 (42%) are considered variable OTUs and 13 (7%) are considered essential OTUs (Figure 5.9B). The essential OTUs are dominated by the families of the phylum Proteobacteria: *Rhodobacteraceae*, *Sphingomonadaceae*, *Parvularculaceae*, *Parvibaculaceae*, *Legionellaceae*, *Xanthobacteraceae*, PS1 clade, *Burkholderiaceae*, PS1 clade and SAR116 clade. As well as *Propionibacteriaceae* from the phylum Actinobacteria, and *Flavobacteriaceae* from the Bacteroidetes phylum.

The aggregates and the 1st stage 2-year-old SWRO membrane consisted of 28 eukaryote OTUs at the class level (Figure 5.9C), of which 25(%) are considered unique OTUs, and 3(%) are considered to

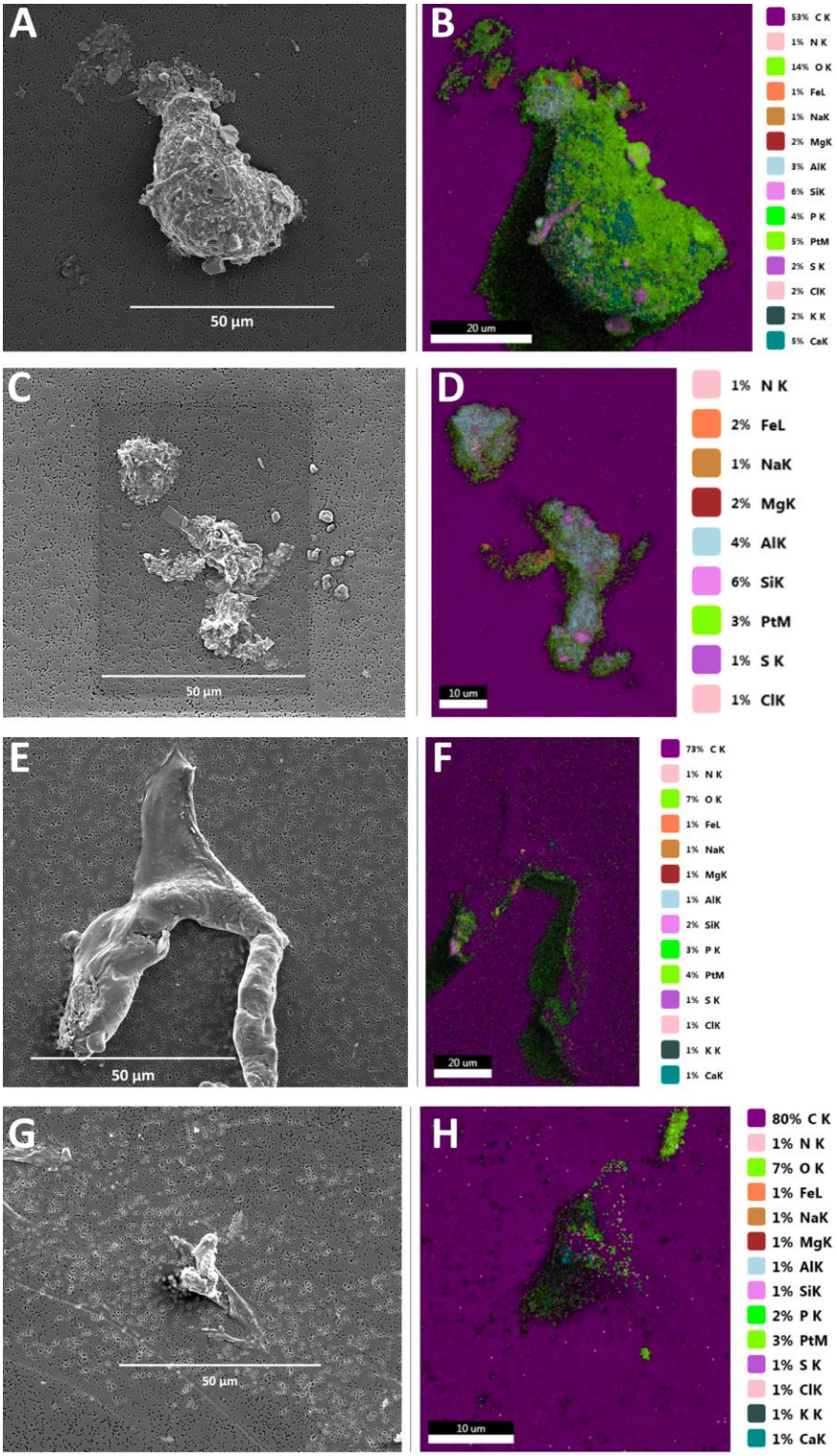

Figure 5.8: Scanning electron microscope images of an aggregates created within the intake water (A & C) and the corresponding energy dispersive X-ray (EDX) spectroscopic analysis (B & D). A aggregate formed within the RO feed tank water (E & G) alongside the EDX spectroscopic analysis of the aggregate (F & H).

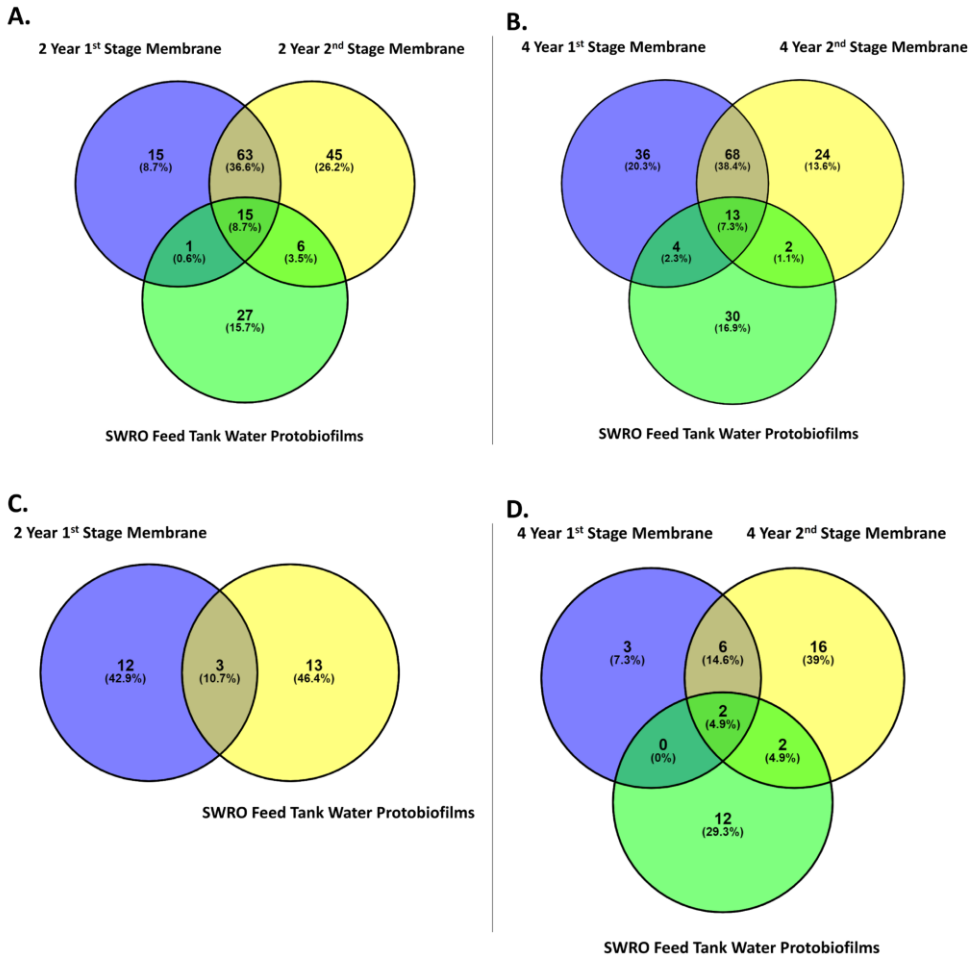

Figure 5.9: Venn diagram displaying the overlap between the communities of the aggregates and the 1st and 2nd stage membranes. (A) prokaryotic communities in the SWRO feed tank water aggregates and the 1st stage and 2nd stage membrane after two years' service and (B) the prokaryotic communities in the SWRO feed tank water aggregates and the 1st stage and 2nd stage membranes after four years of service. (C) the eukaryotic communities in the SWRO feed tank water aggregates and the 1st stage membrane after two years' service and (D) the eukaryotic communities of the SWRO feed tank water aggregates and 1st stage and 2nd stage membranes after four years of service. Core OTUs, identified in all sampling sites; variable OTUs identified in two or more sites but not all; unique OTUs, identified in only one site.

be essential OTUs. These were dominated by the classes of Sordariomycetes and Dothideomycetes, of the super group Opisthokonta, and of the Stramenopiles supergroup Dinophyceae. Of the 37 eukaryote OTUs found in aggregates and 4-year-old SWRO membranes, 23 (%) are considered unique OTUs, 9 (42%) are considered variable OTUs and 2 (7%) are considered essential OTUs (Figure 5.9D). The essential OTUs are dominated by the classes of Sordariomycetes and Dinophyceae.

5.6 Discussion

Within water treatment plants it is widely recognised that pre-treatment systems are essential for the efficient production of potable water. This novel study looks at the microbial composition and the biofouling potential of aggregates formed within a SWRO desalination plant. A study conducted by Balzano et al. (2015c) established that the use of pre-treatment especially multimedia filtration within the desalination system had the ability to reduce the microbial biomass by one order of magnitude, thereby, affecting change within the planktonic prokaryotic and eukaryotic community composition within the desalination plant. However, pre-treatment systems also create niche environments thereby producing conditions that are favourable for development of aggregates and the proliferation of organisms.

5.6.1 Prokaryotic communities in water

Seasonal fluctuations of nutrients, microorganisms and phytoplankton have been previously described within the Penneshaw SWRO desalination plant (Balzano et al., 2015b; Balzano et al., 2015c). This results in a highly diverse yet unique microbial community within the intake seawater and the SWRO feed tank water. The composition of the prokaryotic community observed in the intake

seawater and in the SWRO feed tank was consistent with that previously observed in SWRO desalination plants globally (Belila et al., 2016; Levi et al., 2016; Li et al., 2017; Nagaraj et al., 2017). Here, it was observed that Proteobacteria and Actinobacteria were the dominant phyla within the intake seawater and the SWRO feed tank water. Proteobacteria, and Actinobacteria, along with Bacteroidetes, Cyanobacteria, and Verrucomicribia are amongst the most abundant phyla within the marine environments (Ward and Bora, 2006; Freitas et al., 2012; Subramani and Aalbersberg, 2012). Verrucomicribia, in particular, is a polymer-degrading bacterium commonly associated to marine POM.

Within water treatment systems, the classes of Proteobacteria are often the most dominant organism identified not only within the intake water but also on fouled membranes (Lee et al., 2009). Proteobacteria classes of α- and γ-proteobacteria, as well as the class of Actinobactiera, are commonly found within the intake water of desalination plant (Belila et al., 2016; Levi et al., 2016). Furthermore, both α- and γ- proteobacteria abundance can increase after pre-treatment, (Belila et al., 2016; Levi et al., 2016) and could be the result of the development of organic compound layers within some of the components of the pre-treatment systems such as cartridge filters (Manes et al., 2011b; Chun et al., 2012). In general, α-proteobacteria are often considered to be the primary colonizers within biofilms (Pang and Liu, 2007; Bereschenko et al., 2010; Al Ashhab et al., 2014) whereas, β-proteobacteria are more commonly associated with fouled membranes within desalination plants as they have a key role in mature biofilm development (Miura et al., 2007; Al Ashhab et al., 2014).

5.6.2 Eukaryotic communities in water

The eukaryotic communities identified within the intake seawater and SWRO feed waters are similar to those present in marine ecosystems (Worden et al., 2015; Belila et al., 2016;). Both phototrophic

and heterotrophic eukaryotes have an important role within the marine environment, especially in primary production, respiration, and their role in the microbial loop (Heywood et al., 2011). The eukaryotic communities observed in the water samples is also consistent with those previously observed in SWRO desalination plants (Belila et al., 2016). For example, fungi were present in both the intake seawater and the SWRO feed tank water however, it is the classes of Sordariomycetes and Exobasidiomycetes that drive the diversity between the water samples. While the role and impact of fungi within biofilms of water treatment systems is in the initial stages of research, the formation of biofilms by fungi, especially those developed by *Aspergillus fumigatus* and *Candida albicans*, are well documented (Doggett, 2000; Lynch and Robertson, 2008; Siqueira et al., 2011; Ramage et al., 2012; Sheppard and Howell, 2016).

The green algae family members of the class Chlorophyta often dominate the picoplankton biomass and they have an important role in the marine food web (Monier et al., 2016). However, they are known to inhabit a wide variety of marine ecosystem; although their distribution is influenced by their ability to adapt to environmental conditions (Vannier et al., 2016). *Ostreococcus* was present in the water samples as well as in the SWRO feed tank aggregates. The genera of *Ostreococcus* is within the pico size fraction of eukaryotes (<2 – 3 µm diameter) and is a unicellular, non-flagellated green alga (Derelle et al., 2006). Due to a large surface area to volume ratio, *Ostreococcus* is known for its rapid growth in oligotrophic environments (Derelle et al., 2006; Cardol et al., 2008). In addition, *Ostreococcus* has been shown to thrive under low irradiances (Cardol et al., 2008), as encountered within the SWRO system. The *Bathycoccus* genera was present within the intake seawater as well as the SWRO feed tank water aggregate. The *Bathycoccus* genera is a widespread oceanic green alga (Vannier et al., 2016), which ranges in size from 1 – 2 µm, the cells have no flagella but are covered in a spider web pattern of scales (Joli et al., 2017). The relatively small size of the *Ostreococcus* and *Bathycoccus* (< 2 µm) would allow for them to pass freely through the cartridge filters (pore size 15

μm and 5 μm) within the Penneshaw desalination plant. Their ability to adapt to nutrient gradients within oligotrophic environments would also be advantageous for their survival within the desalination system (Derelle et al., 2006; Palenik et al., 2007; Simmons et al., 2016).

5.6.3 Aggregate communities

A novel aspect of this study is the examination of the aggregate-attached organisms within the formed microenvironments. Due to the niche environments created within the Penneshaw SWRO desalination plant the attachment of organisms is a selective process reflected in the decreased diversity of the inhabitants. The presence of Cyanobacteria only within the intake aggregates is not unexpected as they are known as oxygenic photosynthetic prokaryotes (Soo et al., 2017) which requires the use of light to generate CO_2 from water (Fischer et al., 2016). Identified within the RO feed tank aggregates, Patescribacteria have the ability to succeed in oligotrophic environments. In addition to their ultra-small cell size, this would enable it to pass through the pre-treatment structures and flourish within the water treatment system (Tian et al., 2020). On the other hand, Fusobacteria observed in both the intake seawater and the SWRO feed tank water possess a tapered rod shape. This would allow it to enter the SWRO feed tank as it would readily fit through the different pore sizes of the cartridge filter (Brennan and Garrett, 2019). In addition, Fusobacteria are known to have the ability to co-aggregate with many bacteria (Zilm and Rogers, 2007). The colonisation of aggregates in any environment is multifaceted and relies heavily upon numerous factors including, the microorganism's motility, ability to attach or detach, growth, mortality, the dynamics of the environment, organism interactions and communication (Kiørboe, 2003). The nutrient richness of the aggregates contributes to its colonization by microorganism communities (Wörner et al., 2000).

The colonisation of the aggregates with Burkholderiales, and Sphingomonadales microbes is not surprising as they are often identified within aqueous environments as well as being associated with particle attachment (Eloe et al., 2011; Vongphayloth et al., 2012; De Corte et al., 2014; Duret et al., 2019). Of particular note is the common association that these organisms have with biofilms and biofouling (Bereschenko et al., 2010; Al Ashhab et al., 2014; Zodrow et al., 2014). For example, β-proteobacteria have long been associated with biofilms especially as a class that contains organisms that can pioneer biofilm formation. The success of the betaproteobacteria has been attributed to the ability of its cells to co-aggregate (Rickard et al., 2000). Burkholderiales have also been categorised as second colonisers of biofilms preferring a pre-developed biofilm to adhere and grow upon (Belgini et al., 2014). Finally, Sphingomonadales are known to colonize aggregates where they breakdown the polymer-rich substrates and later release them into the surrounding environment (Schweitzer et al., 2001). Within the SWRO feed tank, the broken-down aggregate substrates could serve as hot spots to promote biofilm growth on SWRO membranes (Berman et al., 2011; Meng et al., 2013). While in our study we see a reduction in the colonization of aggregates by Burkholderiaceae, Janthinobacterium and Sphingomonadales this would indicate that they are not reliant upon the colonization of aggregates to initiate the formation of biofilms that they are known for (Siboni et al., 2007; Bereschenko et al., 2010; Luo et al., 2017).

The taxonomic diversity of the eukaryotic organisms within the SWRO feed tank water and SWRO feed tank aggregates was very similar, mainly due to the abundance of planktonic and aggregate-bound Ascomycota and Basidiomycota organisms. The class of Ascomycota and Basidiomycota form the subkingdom of Dikarya, which is principally made of fungi and is often observed in marine environments (Le Calvez et al., 2009; Jebaraj et al., 2010; Edgcomb et al., 2011; Gladfelter et al., 2019). The adaption of fungi to life in anaerobic and partially anaerobic environments through cellular and genomic adaptions allows them to flourish in any environment (Richards et al., 2011). The presence

of Fungi (92.51%) within the aggregates is not unusual as fungi are commonly identified in the marine environments (Rédou et al., 2015; Richards et al., 2015; Hassett and Gradinger, 2016; Tisthammer et al., 2016). The diversity of fungi allows for these ubiquitous organisms to not only survive in marine and freshwater environments but to also perform key roles in the biogeochemical cycling and the production of secondary metabolites (Berbee et al., 2017). Bochdansky et al. (2017) determined that within the marine snow particles, the contribution of fungal cells was similar to that of the prokaryotic cells, and they have been known to dominate cells counts compared with eukaryotic cells. This suggests that within aggregates, fungi have a saprophytic or symbiotic lifestyle that relies on other prokaryotic cells.

The Opisthokonta is a large supergroup of eukaryotes including metazoans, fungi, choanoflagellates, amoeboids and sporozoan protists. These organisms are phagotrophic or osmotrophic. The nutrient rich substrate of the formed (see section 3.4) aggregates potentially provides the optimum environment for the osmotropic lifestyle of these eukaryotic organisms (Bochdansky et al., 2017).

5.6.4 Fouling potential of aggregates

SEM-EDX analysis was applied to the aggregates of the intake water and the SWRO feed tank water. Similar chemical elements were found in fouled cartridge filters and SWRO membranes from within a commercial desalination plant (Chun et al., 2012), suggesting that the cartridge filters may trap some of the aggregates during the pre-treatment process. This trapping would create favourable conditions for microorganisms to flourish. However, due to the pressure within the system, the aggregates which are trapped in the cartridge filter could breakdown and pass through the pores allowing the particles to hypothetically coagulate further downstream. The formation of TEP within environments is through the coagulation of dissolved organic matter (Verdugo et al., 2004). As a consequence, the nature of

TEP is highly viscous and has been reported to be 2-4 magnitudes higher than any other particle (Bar-Zeev et al., 2015; Meng et al., 2020). Thereby, ensuring a role in the aggregation/sedimentation process within the marine environment (Verdugo et al., 2004). The polymer network that forms the TEP particle is negatively charged, thus, absorbing surrounding organic molecules and trace metal (Meng et al., 2020). Subsequently, the three-dimensional structure of TEP particles results in a large surface area providing an environment with an abundance of nutrients (Meng et al., 2020). Previous research has shown that within freshwater and seawater 0.5-25% of bacteria are attached to TEP particles (Passow, 2002b; Berman and Parparova, 2010).

The PA communities were compared to those found in SWRO fouling to assess the fouling potential of the aggregates within a desalination plant. With the aggregate-associated communities contributing to 10. 7% of the communities identified on the 1st stage membrane after two years of operation. While 4.9% of organisms were found to be consistent with the communities found on the 1st stage and 2nd stage membranes after four years of operation. The core OTUs are consistent with those identified on fouled SWRO membranes but also are known to form biofilms. Many of the core OTUs are ubiquitous in water treatment and distribution systems, as they have the ability to survive extreme conditions (Li et al., 2019). The essential OTUs especially Sphingomonadaceae, Rhodobacteraceae, Legionellaceae, Burkholderiaceae, and Pseudomonadaceae are commonly associated with biofilms and/or biofouling (Dang and Lovell, 2002; Hwang et al., 2012; Bereschenko et al., 2010; Abu Khweek and Amer, 2018). *Sphingomonas* has been identified as having a unique role in the fouling of SWRO membranes especially in the formation of the initial biofilms (Bereschenko et al., 2010). They have also been recognised for its ability to survive high concentration of chlorine which is directly linked to the production of EPS (Wang et al., 2019; Zhu et al., 2020). The Rhodobacteriaceae family are abundant within the marine environments and are often found to the be the primary colonisers within biofilms on submerged surfaces as well as water treatment systems (Dang and Lovell,

2002; Chao et al., 2015;). Rhodobacteria are known to contain gene transfer agents (GTA) a particle which allows the transfer of fragments of genome DNA to be transferred to other cells (Fogg, 2019). While the focus of HGT is on the survival of cells this is not the case with GTA as it is not selective of the fragments transferred (Fogg, 2019). Allowing for the potential to not only disseminate virulence and antimicrobial resistance genes but also to force the evolution of bacteria (Fogg, 2019). Biofilm development in species within the Burkhoderiaceae family has been found to be positively correlated to the quantity of eDNA from living cells (Pakkulnan et al., 2019). eDNA is essential for the attachment of cells as well as during the development of the biofilm (Whitchurch et al., 2002; Montanaro et al., 2011; Okshevsky and Meyer, 2015; Panlilio and Rice, 2021;).

5.6.5 Future considerations

The ability for particles to come together within a SWRO desalination plant after pre-treatment would suggest that the current methods of removal are both inadequate and ineffective. The conventional pre-treatment system within the Penneshaw SWRO desalination is limited, however the adaption of novel treatments alongside the conventional pre-treatment system enhance the quality and quantity of potable water produced. Vertical wells (subsurface intake systems) have been successful in effectively improving the quality of the intake water of various desalination plants worldwide. The transfer aquifer reduces the fouling and biofouling constitutions before the seawater enters the well (Dehwah et al., 2015b; Dehwah and Missimer, 2016). Another novel pre-treatment method that has success in reducing the biofouling potential of the water within the desalination system is that of the granular activated carbon (GAC) biofilters. Studies have shown that GAC biofilters were more effective in the removal of low molecular weight organics in the system than microfilters and ultra-filtration membranes. As well as reducing fouling precursors such as TEP and assimilable

organic carbon (Naidu et al., 2013). Coagulation is another novel method that is providing promising results in reducing the fouling potential of organisms. The addition of liquid ferrate even at low levels was effective in the reduction of fouling precursors as well as the reduction of algal and bacteria cells within the feed seawater (Alshahri et al., 2019; Alshahri et al., 2021). Thereby, reducing the prospect of rapid fouling/biofouling on the SWRO membrane.

5.7 Conclusion

This study is the first to investigate the formation of aggregates within a SWRO desalination plant, examine the microbial community of the aggregates, as well as to investigate the role they may have on membrane fouling. The prevalence of polysaccharide precursors within desalination plants has been established, as has the colonization of aggregates within the water column. Even though the water within a desalination plant undergoes multiple pre-treatment steps, the pressure driven SWRO system creates the perfect environment for the formation and inhabitation of aggregates. The pre-treatment systems removes larger particles, flocculation and microorganisms, yet the smaller fragments have the ability to come together to form aggregates further in the system. These aggregates are a hot spot for nutrients and enable the formation of niche communities within. Evidence suggests that within these hot spots, the transfer of genes allows the attached microorganisms a competitive edge to survive in such an oligotrophic environment where they are able to persist in the developed biofilms. Future work should focus on the whether the removal of aggregates from the system reduces biofouling within SWRO desalination plants. The introduction of smaller pore size within the cartridge filters to remove the <5 µm organisms, or the introduction of another pre-treatment system such as coagulation prior to the SWRO membrane (i.e., after the feed tank) may help to further reduce the biofouling precursors reaching the SWRO membrane. A well as

the efficacity of current pre-treatment methods in reducing the load of biofouling precursors material and organisms.

5.8 Acknowledgements

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CHAPTER 6: The impact of bacteria in the production of transparent exopolymer particles in biofouled reverse osmosis membranes

6.1 Preface

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Research Design 70 %

Data Collection and analysis 80 %

Writing and editing 75 %

Outline your (the student's) contribution to the publication:

Conceptualisation, Methodology, Formal analysis, Investigation, Data curation, Writing - Original draft, Writing - editing, revision of the manuscript, and final manuscript.

☑ I confirm that the details above are an accurate record of the student's contribution to the work.

Name of Co-Author 1: Sergio Balzano Signed: Sergio Balzano Digitally signed by Sergio Balzano DN: cn=Sergio Balzano, o=Stazione Zoologica Anton dohrn, ou=Bluebiotech, email=sergio.balzano@szn.it, c=IT Date: 2021.06.01 16:34:05 +02'00' Date: 01/06/21

☑ I confirm that the details above are an accurate record of the student's contribution to the work

Name of Co-Author 2: Sophie Leterme Signed: Sophie Leterme Digitally signed by Sophie Leterme Date: 2021.06.02 18:39:48 +09'30' Date: 02/06/21

6.2 Abstract

Biofouling of seawater reverse osmosis (SWRO) membranes represents one of the leading causes of performance deterioration in the desalination industry. The purpose of this investigation was to identify and evaluate the biofouling potential of the microbial communities present in a reverse osmosis (RO) feed tank. As an example, water from the RO feed tank of the Penneshaw desalination plant (Kangaroo Island, South Australia) was used in static and laboratory-based cross-flow biofilm formation experiments. The 16S clone library showed that α-proteobacteria and γ-proteobacteria accounted for nearly 80% of the classes cultured from indigenous biofilms formed. *Pseudomonas* sp. was identified and isolated for further static and laboratory-based cross-flow biofouling experiments. Nutrient depletion over the progression of the static experiment influenced the production of transparent exopolymer particles (TEPs) by the bacteria *Pseudomonas* sp.. The microbial community composition and flow impacted the amount of TEP produced by *Pseudomonas* sp. throughout the laboratory-based cross-flow experiment. Overall, this study provides insights into the phenomenon of biofouling by assessing the production of biofouling precursors from one of the main genera of biofilm-forming bacteria, allowing for the development of a targeted approach to treat the problem.

6.3 Introduction

Throughout the world, the use of seawater desalination is expanding in response to climate change and associated increasing temperatures, desertification, and drought (Greenlee et al., 2009). Current water shortages are further exacerbated due to the stress of an increasing population, uneven water distribution and stringent water quality regulations (Greenlee et al., 2009).

Desalination plants are extensively recognized as an effective treatment of seawater and/or brackish water to produce fresh water, especially with the advances made in membrane materials and

components (Harif et al., 2011). Thus, RO seawater desalination is considered the simplest and most cost effective method of freshwater production in comparison to other separation methods such as distillation, extraction, ion-exchange and adsorption (Matin et al., 2011). However, seawater reverse osmosis (SWRO) systems are prone to clogging and biofilm formation on the RO membrane. Membrane fouling still occurs even after seawater pre-treatment and cross-flowing within the RO system (Komlenic, 2010). This results in a negative impact on the performance of the system through a decline in the water flux as well as an increase in the amount of seawater rejected, energy requirement and system pressure (Harif et al., 2011; Lee and Kim, 2011; Katebian and Jiang, 2013).

The control of biofilm formation is a complicated and controversial process involving the reduction of microorganisms within the RO water, monitoring strategies, and controlling factors such as nutrient concentrations and physico-chemical interactions between microorganism and membrane particles (Al-Juboori et al., 2012). In particular, bacteria are highly abundant organisms in aquatic habitats and can take part in the biofouling process (Borlee et al., 2010).

The inflow of live biofilm forming bacteria, organics and nutrients onto the RO membrane allows for growth and proliferation of the bacteria leading to biofouling (Manes et al., 2011b). The accumulation of nutrients from the water and metabolites produced by bacteria such as extracellular polymeric substances (EPS), proteins, and lipids further allow microorganisms to adhere and grow on the membrane surface (Katebian and Jiang, 2013).

Biofilms consist of sessile microbial cells contained within a heterogeneous matrix of EPS, which attach irreversibly to a solid surface. These cells differ from free-living cells of the same species in terms of growth rate and gene expression as they have an altered phenotype (Donlan and Costerton, 2002). The physical and chemical processes that are involved in the early formation of a biofilm are not well understood. However, a sequence of processes is thought to lead to the formation of a biofilm such

as a) the adsorption of organic and inorganic particles on the surface, b) attachment of pioneer microorganisms, c) growth and reproduction of primary colonisers and d) maturation of the biofilm matrix (Bar-Zeev et al., 2012).

Transparent exopolymer particles (TEPs) are often found in the marine environment and play a role in the formation and development of marine biofilms (Bar-Zeev et al., 2009). They are deformable, gel like transparent particles that appear in many forms, such as amorphous blobs, clouds, sheets, filaments, or clumps (Linares et al., 2012). TEPs can be formed spontaneously from the aggregation of dissolved precursor substances, which is controlled by environmental parameters such as turbulence, ion density and concentration of inorganic colloids as well as by the type and concentration of precursors present in the water (Passow, 2002b). In the marine environment, TEPs serve as "hot spots" of intense microbial and chemical activity within the water column facilitating the attachment of planktonic TEPs to surfaces (Berman et al., 2011). Within the desalination process, high levels of potential biofilm forming TEPs have been found to reach the RO membrane (Bar-Zeev et al., 2009).

EPS, a main component of TEPs, is produced by phytoplankton and bacteria (Passow and Alldredge, 1994). EPS production has been found to be species specific and dependent on surrounding growth conditions (Simon et al., 2002). When attached to surfaces such as biofilms, bacteria produce EPS in large amounts (Stoderegger and Herndl, 1999). In contrast, when in a planktonic state within the water column, bacteria produce TEP (Stoderegger and Herndl, 1999). However, the role of bacteria in the production of TEPs is not yet known due to the close association between phytoplankton and bacteria when experiments are conducted *in situ* (Simon et al., 2002).

Biofilms have been strongly implicated in the biofouling of the SWRO membranes present in desalination plants. However, only very small portions of biofouling microbes have been identified thus far. As the microbial community composition changes seasonally, so do the conditions that

influence biofouling. Therefore, the present study aims to fill this gap in knowledge by identifying the composition, diversity and biofouling potential of the cultivable microbial communities present after seawater pre-treatments but before the RO process (i.e., RO feed tank water) within a desalination plant. This study thus identifies the bacteria likely to be involved in biofilm formation on the SWRO membranes. In particular, the bacteria *Pseudomonas* sp. was isolated from RO feed tank water and tested.

6.4 Materials and Methods

6.4.1 Study site

Seawater samples used in this study were obtained from the RO feed tank of the desalination plant at Penneshaw on Kangaroo Island (South Australia). Samples from the RO feed tank were collected in 20 L white opaque carboys and kept on ice during transportation to the laboratory where they were stored at 4 °C in the dark to minimize changes in the water properties (i.e., nutrients and microbial content).

6.4.2 Biofilm formation from RO feed tank water

6.4.2.1 Preparation of seawater from the RO feed tank

To remove all bacteria and viruses from the water collected from Penneshaw desalination, the RO feed water was filtered. It was first filtered through a 100 KDa hydrosart cartridge (Sartorius Stedim Biotech) using Vivaflow 200 tangential flow filtration (TFF) (Sartorius Stedim Biotech) in combination with a Masterflex L/S peristaltic tubing pump (Cole Parmer, Chatswood, Australia) to remove bacteria. Additional filtration to remove viruses was conducted using Vivaflow TFF (Sartorius Stedim Biotech)

with a Masterflex L/S peristaltic tubing pump (Cole Parmer, Chatswood, Australia) through a 10 KDa polyethersulfone (PES) cartridge (Sartorius Stedim Biotech).

6.4.2.2 Static experimental setup

Flat sheets of polyamide thin-film composite (TFC) seawater reverse osmosis membranes FILMTEC™ SW30HR (DOW, California, USA) similar to those used in the RO unit at Penneshaw were used for this experiment. TFC membranes were sterilized with 80% v/v isopropanol and then washed with sterile Milli-Q water (18.2 Ω cm). To investigate the sequential formation of biofilm over time the TFC membranes were incubated in RO feed tank water under static conditions. Five 1 L containers were filled with RO feed tank water in which seven TFC membranes were placed. Four containers were incubated in the dark one of which contained sterile RO feed tank water (15 min at 121 °C), the remaining container was under a 12:12 hour light/dark cycle. The RO feed tank water in each container was replaced every three days and assessed for microbial abundance.

The seven membranes in the containers were dedicated to a specific incubation period (i.e., 14, 28 or 56 days; Table 6.1). At the end of the incubation periods of 14, 28 and 56 days, one membrane was removed from each container for bacteria isolation and a second membrane was removed to analyse the amount of TEP accumulated in the biofilm formed on the membrane. Those membranes were then then replaced by a clean membrane (Table 6.1).

6.4.2.3 Isolation of biofouling microbial communities

Upon the removal of the membrane from the incubation container, the biofilm was removed via scraping with a scalpel and resuspended in 1 mL of autoclaved raw seawater. Dilutions of 1:10, 1:50

Table 6.1: Time periods (days) of removal and replacement of each SWRO TFC membrane used in static experiments and the analysis to be carried out on each. The periods of renewal of water is also indicated.

Periods (days)	14	28	56
Membrane 1 – isolation of bacteria	x		
Membrane 2 – TEP	x		
Membrane 3 – isolation of bacteria		x	
Membrane 4 – TEP		x	
Membrane 5 – isolation of bacteria			x
Membrane 6 – TEP			x
Renewal of water	x	x	x

and 1:100 in sterile seawater were spread plated onto either Luria-Bertani (LB) agar or nutrient agar and incubated at 20 °C in a temperature cycling chamber (Labec, Australia).

Five different single colonies were patched on solid media and incubated at 20 °C in a temperature cycling chamber. Individual colonies were inoculated into 5 mL of the sterile liquid phase of the same medium and incubated as previously described.

6.4.2.4 DNA extraction

A modified protocol from Real Genomics HiYield™ DNA extraction kit (Real Biotech Corporation, Taiwan) was used to extract DNA. Samples (10 mL) were centrifuged (4000 rpm/10 min/RT) and the supernatant removed. The pellet was resuspended in 200 µL Lysozyme buffer (20 mg/mL lysozyme), transferred to a 1.5 mL microfuge tube and incubated at RT for 15 min. The cells were lysed by the addition of 200 µL of GB Buffer from the kit by vortexing and the subsequent incubation for 15 min at 70 °C. Ethanol (200 µL) was then added to precipitate DNA and samples were transferred to a GB column. The columns were then centrifuged (13,500 rpm/5 min/RT) and the filtrate containing the lysate mixture discarded. The DNA bound to the membrane of the column was then purified by adding 400 µL W1 buffer, centrifuging as above, then 600 µL wash buffer was added and centrifuged again. An additional centrifugation was carried out to remove residual ethanol, which may otherwise interfere with the following elution step. DNA was then eluted from the column by addition of 100 µL of preheated elution buffer, 20 min incubation at RT and centrifugation as above. DNA concentration was estimated using a Thermo Scientific NanoDropTM 1000 spectrophotometer (Biolab)

6.4.2.5 Identification of biofouling microbial communities

Amplification of the 16S rRNA regions from the genomic DNA was undertaken with one pair of universal primers for bacteria: CC (5'CAGACTCCTACGGGAGGCAGC3') and CD (5'CTTGTGCGGGCCCCCGTCAATTC3') (Rudi et al., 1997). For the PCR, a 25 µL volume, containing approximately 1 ng/µL of genomic DNA, 2.5 µL 2.5 mM of deoxynucleotide triphosphates (dNTP) (Promega), 1 µL of complementary primer to the 3' and 5' ends of the 16S region to be amplified, 0.25 µL of Hot Start Q5 polymerase and 5 µL of 10X Q5 reaction buffer. PCR conditions were as follows: initial denaturing step of 1 min at 98 °C, 30 cycles of a denaturing step of 30 sec at 98 °C, annealing step of 35 s at 53 °C, and an extension step of 35 s at 72 °C, followed by a final extension step of 72 °C for 3 min. The PCR products were subsequently purified using a Wizard SV Gel and PCR clean-up system (Promega). The taxonomic identification of the sequences was then inferred using Basic Local Alignment Search Tool (BLAST) available from the National Centre for Biotechnology Information (NCBI). ClustalW application within Bioedit software (Ibis Biosciences) was used to align the sequences. NJ and Maximum Likelihood (ML) phylogenetic trees were constructed using Mega5 software (Tamura et al., 2011).

6.4.2.6 TEP analysis

Determination of TEP was carried out following previously published methods (Passow and Alldredge, 1995b; Claquin et al., 2008).

6.4.3 Assessment of TEP production by Pseudomonas sp. under static conditions

6.4.3.1 Static experimental setup

Pseudomonas sp. culture was prepared in LB broth before being washed with RO feed tank water, previously filtered via tangential flow filtration (TFF) (see section 6.4.2.1) and inoculated in the dark into 3 replicates of TFF filtered RO feed tank water (Nalgene carboy; 5 L). The controls for the experiment were (i) another inoculation of 1000 mL of culture into a 5 L carboy containing TFF filtered RO feed tank water and incubated in the light and (ii) a sterile control of a 5L carboy containing only TFF filtered RO feed tank water incubated in the dark.

6.4.3.2 Growth monitoring of *Pseudomonas* sp.

Samples (1 mL) were collected daily in triplicates from each carboy and used for flow cytometry analysis in order to monitor the growth of *Pseudomonas* sp. (Marie et al. 2001).

6.4.3.3 TEP analysis

Samples (10 mL) were collected daily in triplicate from each carboy and analyzed for TEPs, following previously published methods (Passow and Alldredge, 1995b; Claquin et al., 2008).

6.4.3.4 Nutrient analysis

Daily samples (10 mL) for nutrient analysis were taken in triplicate from each carboy and filtered through 0.45 µm bonnet syringe Minisart filters (Sartorius Stedim, Australia). Filtrates were then stored at -20 °C until analysis. Analyses of all chemical concentrations were measured simultaneously and carried out following published methods (Hansen and Koroleff, 2007), using a Lachat Quickchem Flow

Injection Analyser (FIA). Samples were thawed on ice and approximately 7 mL from each replicate were injected in the FIA in duplicate for a total of 6 replicates per sample. The detection limits were 40 nM for dissolved silica species, 70 nM for ammonia, 30 nM for orthophosphate and 70 nM for nitrate/nitrite; the method was calibrated using standard solutions prepared in 0.6 M sodium chloride, corresponding to the a seawater salinity of 35 practical salinity units (PSU).

6.4.4 Assessment of TEP production by Pseudomonas sp. under cross-flow conditions

6.4.4.1 Bacterial strain and media

Pseudomonas sp. was used as an inoculum for an overnight culture grown in 250 mL of autoclaved raw seawater. This overnight culture was diluted in 5 L of TFF filtered raw seawater to be used as the inoculum for the laboratory-based cross-flow experiment.

6.4.4.2 Laboratory-based cross-flow system

A laboratory scale SWRO test unit comprising of six membrane cells (Sterlitech CF042, Sterlitech), a high pressure pump (Hydra-Cell, Wanner Engineering), a feed water reservoir and a data acquisition system (PC interfaced) was used to acquire the permeate flow rate (see Supplementary Information for cleaning protocol). Flat sheets of polyamide TFC SWRO FILMTEC^{TM} SW30HR (DOW, California, USA) were used in the system.

6.4.4.3 Cleaning of the laboratory-based cross-flow system

Prior to the to the insertion of the RO membrane the entire cross-flow system was disinfected and

cleaned to remove any trace organic matter by the following steps: [1] Circulation of 0.5% hypochlorite at 400 psi for 15 min, [2] circulation of deionized water at 400 psi for 15 min, [3] insertion of membranes into each cross-flow cell for cleaning purposes and the circulation of 0.5% hypochlorite at 400 psi for 1 h, [4] circulation of deionized water at 400 psi for 1 h, [5] removal of membranes from within the cross-flow cells before 5 mM EDTA was added inside the cross-flow cell to incubate overnight, and [6] repetition of step 1 followed by repetition of step 2 (Modified from Herzberg and Elimelech, 2007).

6.4.4.4 Biofouling protocol using a laboratory-based cross-flow system

Six TFC SWRO membranes were incubated for 1 h in 100% isopropanol followed by sterilization in 80% isopropanol for 1 h before being washed with sterile Milli-Q water for 1 h. Sterile TFC SWRO membranes were then placed in each of the 6 cells of the laboratory-based cross-flow system. *Pseudomonas* sp. (5 L) (4755701.9 ± 250015.9 Cell.mL^{-1}) was added to the TFF filtered raw seawater (35 L) within the reservoir tank of the laboratory-based cross-flow system. The bacteria were circulated within the system at a pressure of 400 psi for 8 h at approximately 20 °C (kept at this temperature over the duration of the experiment). Samples (10 mL) for microbial communities, temperature, pH and salinity were taken daily to monitor the experimental conditions.

6.4.4.5 TEP analysis

Samples (10 mL) were collected from the reservoir tank of the laboratory-based cross-flow system hourly and analyzed following previously published methods (Passow and Alldredge, 1995b; Claquin et al., 2008).

6.4.5 Statistical analysis

All environmental and bacterial abundance data were tested for normality using Shapiro-Wilks tests computed with the R statistical package. However, due to the data not being of normal distribution, non-parametric tests were applied to determine correlations (Spearman's rank correlation coefficient) and for the comparison for mean (Kruskal-Wallis / Wilcoxon rank sum test).

6.5 Results

6.5.1 Diversity of culturable bacteria

Biofilms formed on SWRO membranes submerged in RO feed tank water and incubated under static conditions were analysed for biofouling microorganisms. Phylogenetic analysis based on the 16S region from bacteria isolated from the biofilm sample revealed that the majority of the isolated strains belonged to the α-Proteobacteria (39%), γ-Proteobacteria (38%) and Actinobacteria (22%) classes. Moreover, 1% of the strains belonged to Flavobacteria (*Muricauda* sp.) or to Bacilli (*Staphilococcus* sp.) lineages (Figure 6.1). α-Proteobacteria included 13 strains which could not be identified at the genus level and *Celeribacter* sp. (9 strains) whereas *Alteromonas* spp., *Pseudoalteromonas* sp., *Marinomonas* sp. and *Pseudomonas* sp. were the main genera found in the γ-Proteobacteria class. Finally, Actinobacteria comprised of 8 genera including *Microbacterium* sp. and *Micrococcus* sp..

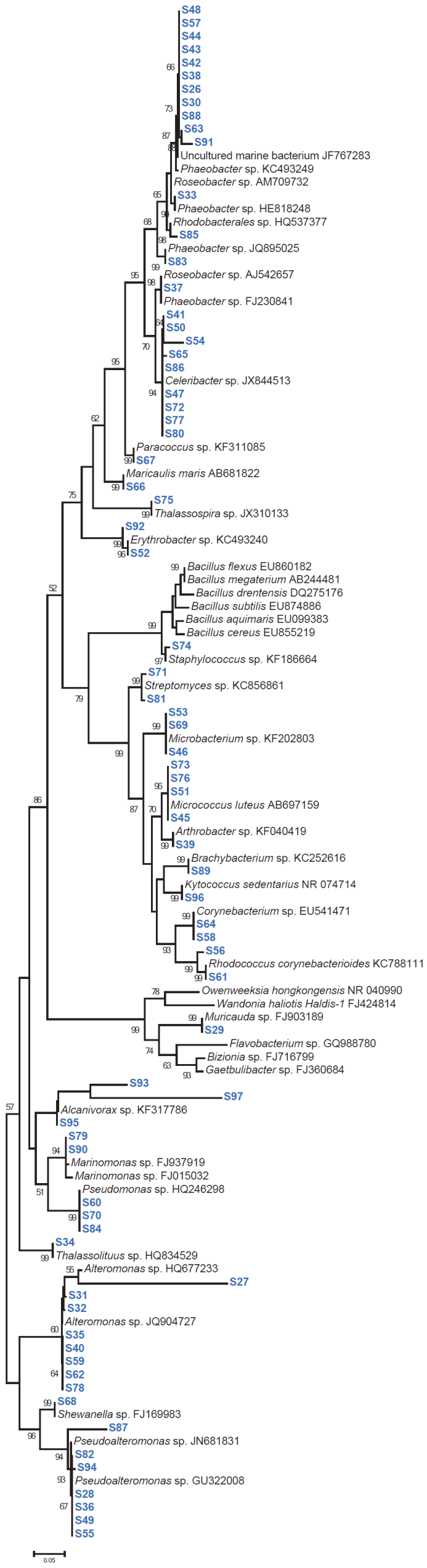

Figure 6.1: Maximum likelihood 16S rDNA phylogenetic tree showing the phylogenetic relationships between the bacteria isolated in the present study. The branch length corresponds to the number of substitutions per site and the percentage of likelihood for the taxa to be clustered together is shown next to the branches. The analysis involved 116 nucleotide sequences for a total of 489 positions.

6.5.2 Assessment of TEP production by the indigenous bacteria community and nutrient concentrations

The concentration of TEP present on the SWRO membranes was assessed over three static incubation periods (14, 21 and 56 days; Figure 6.2). The TEP production significantly increased between the 14-day to 28-day incubations (Kruskal-Wallis, $p<0.05$) (T_{14d}: 2124.8 ± 100.3 Xg.µg.L^{-1} and T_{28d}: 2953.9 ± 169.6 Xg.µg.L^{-1}) and then remained consistent between the 28-days and 56-days incubation (T_{28d}: 2953.9 ± 169.6 Xg.µg.L^{-1} and T_{56d}: 2636.6 ± 415.3 Xg.µg.L^{-1}).

6.5.3 Static experimental conditions

Exponential growth of *Pseudomonas* sp. was evident as well as daily variations in TEP production (Figure 6.3). An inverse correlation was apparent between population growth and the production of TEP (population $\rho = -0.371$, $p < 0.05$). However, nutrients were negatively correlated to TEP (phosphate $\rho = -0.466$, $p < 0.05$, nitrate $\rho = -0.364$, $p < 0.05$; Figure 6.4) suggesting that the production of TEP is influenced by the nutrients that are available in solution.

6.5.4 Cross-flow experimental conditions

Laboratory-based cross-flow experiments are the closest mimicry of what happens to the water circulated within a desalination plant system. Here, a mono-culture of *Pseudomonas* sp. isolated from RO feed tank water was circulated within a laboratory-based cross-flow system at a pressure of 400 psi for 8 h at approximately 20 °C. A significant correlation between *Pseudomonas* sp. and the TEP in the reservoir water of the laboratory-based cross-flow system was apparent ($\rho = -0.595$, $p < 0.05$) (Figure 6.5).

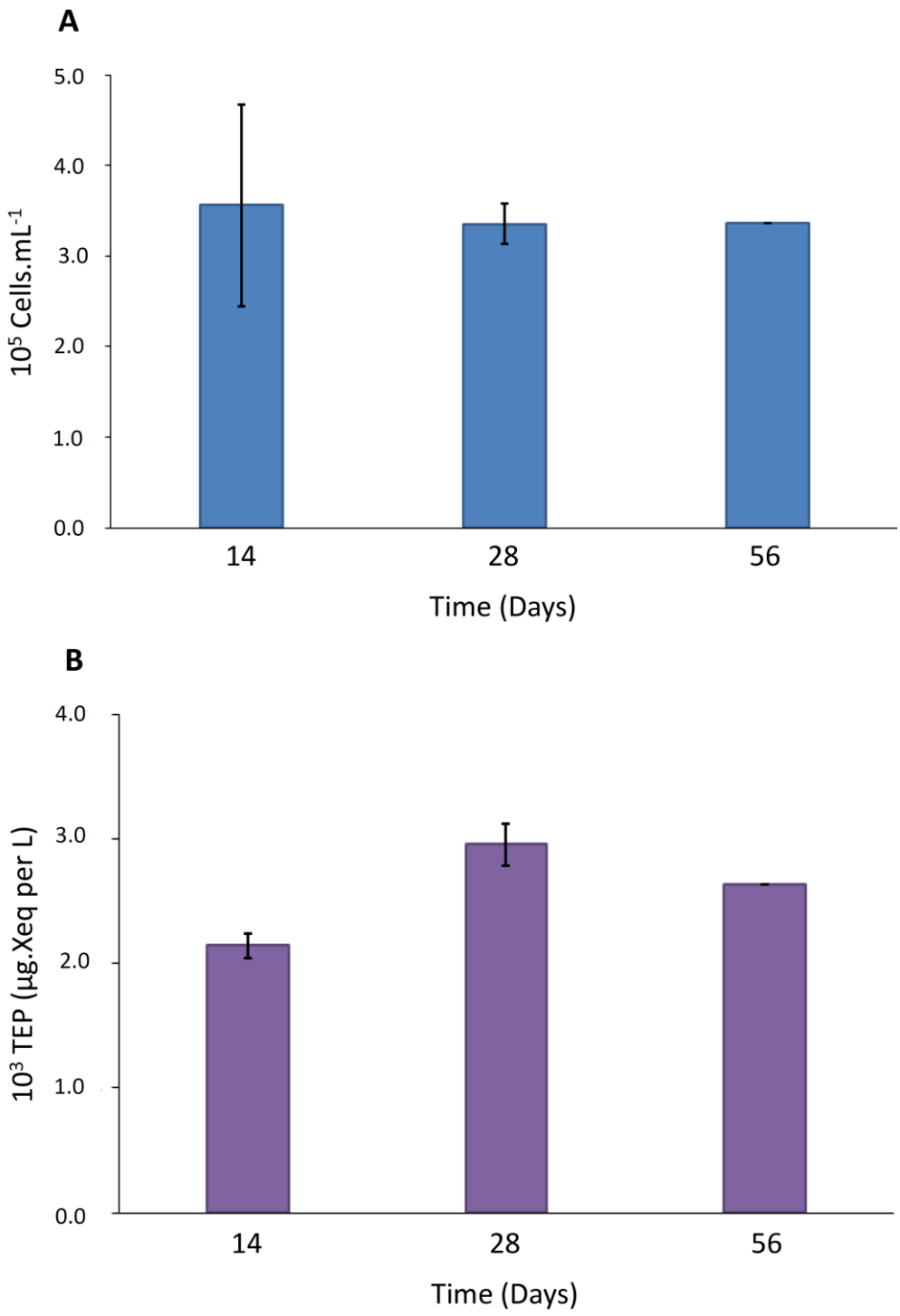

Figure 6.2: (A) Average indigenous bacterial abundance determined by flow cytometry during incubation periods 14, 28, and 56 days under static conditions and (B) TEP concentrations measured from the biofilms formed on the SWRO membranes after incubation periods of 14, 28, and 56 days under static conditions. A significant different in TEP production was observed between days 14-day to 28-day (Kruskal-Wallis, $p<0.05$).

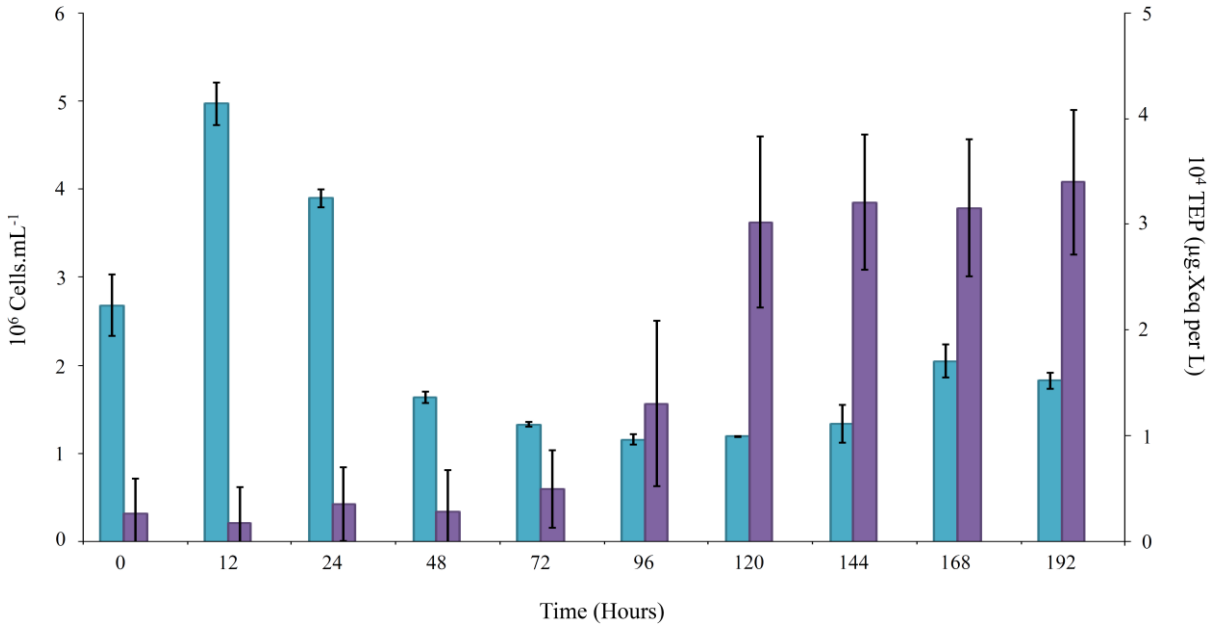

Figure 6.3: Fluctuations in *Pseudomonas* sp. population (blue) and TEP production (purple) overtime during static conditions.

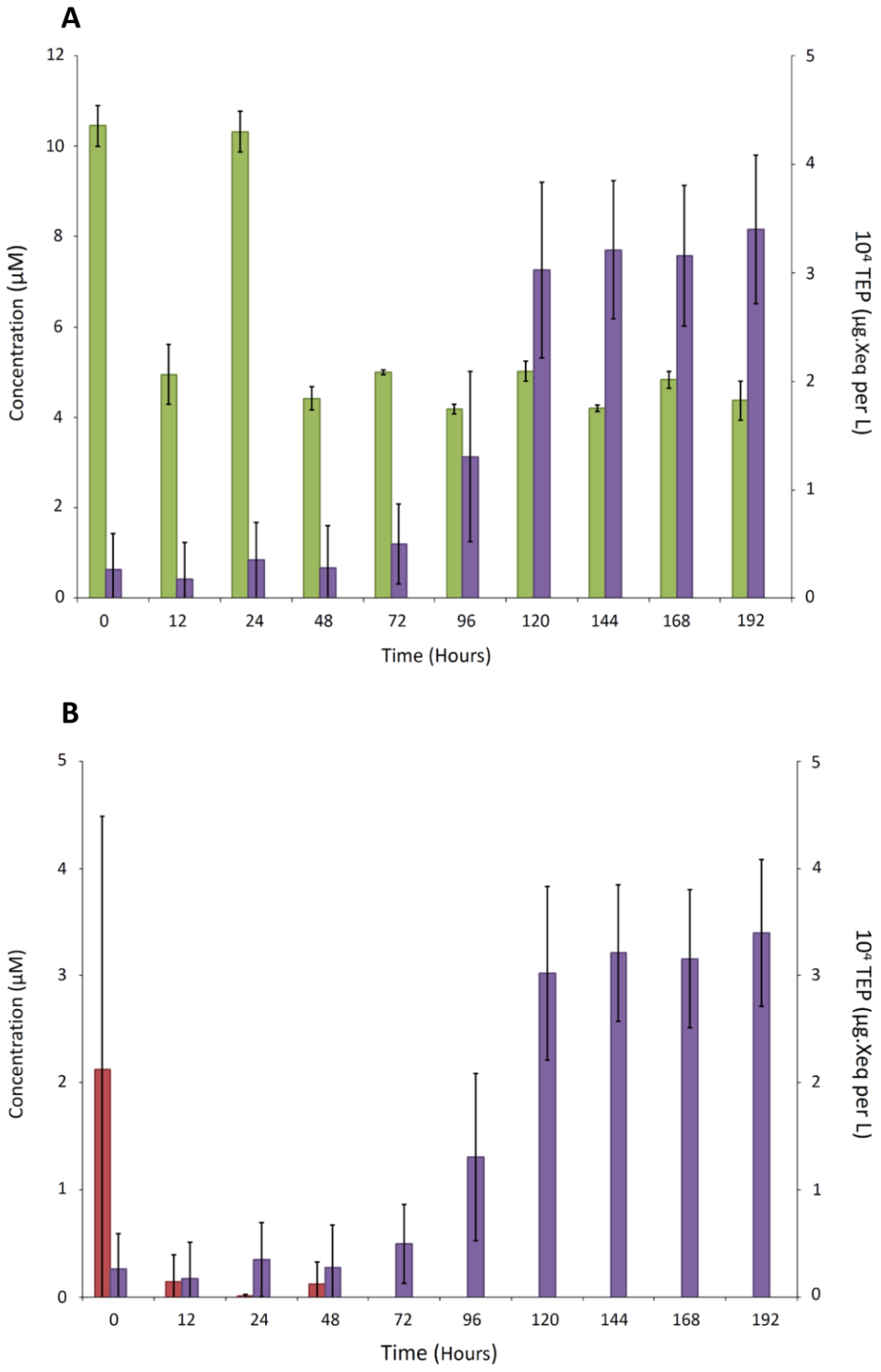

Figure 6.4: (A) Fluctuations in phosphate (green) and TEP production (purple; $p < 0.05$) overtime during static conditions and (B) Fluctuations in nitrogen (red) and TEP production (purple; $p < 0.05$) overtime during static conditions.

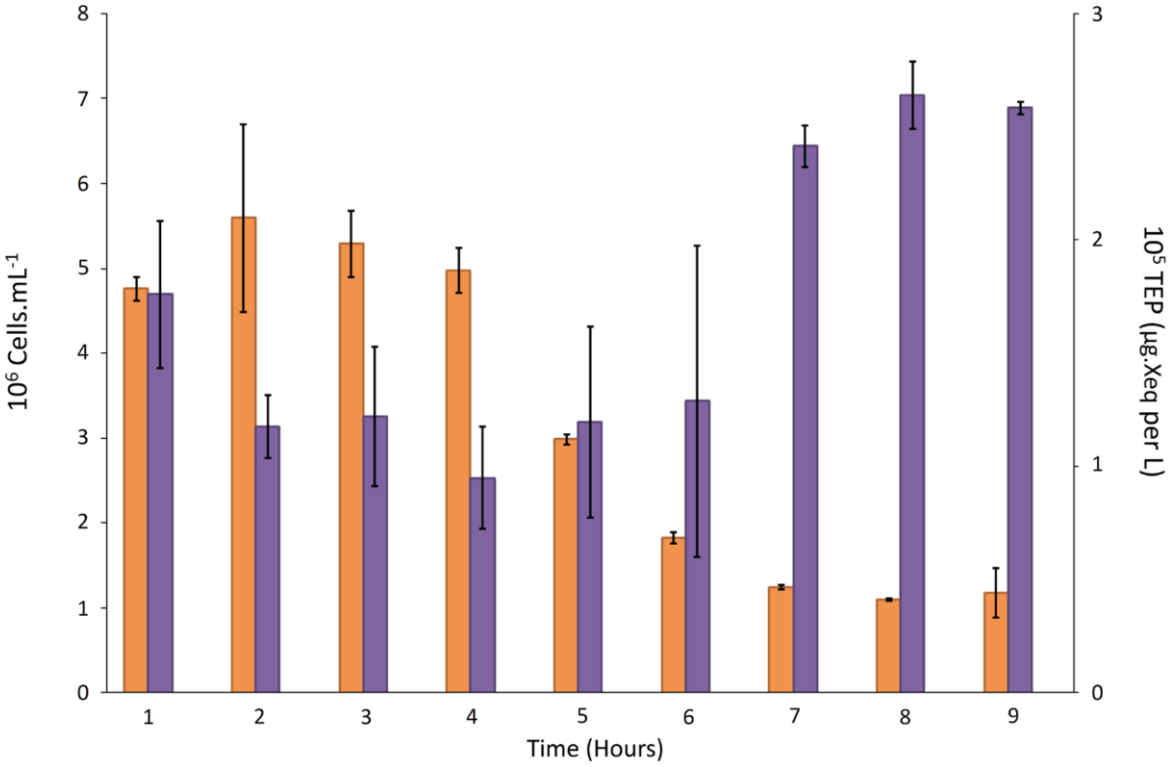

Figure. 6.5: Fluctuations in the planktonic *Pseudomonas* sp. population (orange) and TEP production (purple) overtime during the laboratory-based cross-flow experiments.

6.6 Discussion

As a result of the recognition of biofouling as a leading cause of system inefficiency within SWRO desalination plants, considerable efforts have been made to elucidate details about the mechanisms involved and the significance of TEP in biofouling (Flemming et al., 1997; Al-Ahmad et al., 2000; Flemming, 2002; Liao et al., 2004; Kumar et al., 2006; Lee et al., 2009; Komlenic, 2010; Krivorot et al., 2011; Matin et al., 2011; Al-Juboori and Yusaf, 2012; Bar-Zeev et al., 2012; Nguyen et al., 2012; Haung et al., 2013). Here, biofilms were formed on SWRO membranes using RO feed tank water and showed that the prevailing cultivable phylum was Proteobacteria (>70%) and the α-Proteobacteria class dominated the samples (Figure 6.1). These results are in agreement with Chen et al. (2004), Zhang et al. (2011) and Ayache et al. (2013) although the ratio of α- and γ-Proteobacteria varies between the studies. It has been suggested that the α-Proteobacteria class are present in larger quantities in mature biofilms and replace β-Proteobacteria which are generally thought to be instrumental in initial biofilm development (Bereschenko et al., 2011).

TEPs also play an important part of biofilm formation within aquatic environments (Bar-Zeev et al., 2009; Berman et al., 2011; Bar-Zeev et al., 2012), facilitating and accelerating biofilm development (Bar-Zeev et al., 2012). In particular, TEPs have a role in the conditioning of surfaces by creating a more favourable environment for the attachment of planktonic cells and the proceeding biofilm that is developed (Berman and Holenberg, 2005; Bar-Zeev et al., 2009; Berman et al., 2011). In addition, the concentration of TEPs produced by the biofilm suggests that production reflects the growth stages of the biofilm from the initial adherence of bacteria to the membrane, resulting in low levels of TEPs which increase over time as the biofilm expands. This increase in TEP production, due to an increase in the abundance of bacteria, has been seen in Mediterranean lakes (de Vicente et al., 2010) and also the Mediterranean Sea (Ortega-Retuerta et al., 2010). While these studies were conducted on

planktonic bacteria the assumption could still stand as a reduction in organic matter results in the increased production of TEPs (Ortega-Retuerta et al., 2010).

Here, the volume of TEPs generated by *Pseudomonas* sp. during the laboratory-based cross-flow experiment, in comparison to that produced during the static experiment, suggests that it was the result of various stimuli such as microbial composition and flow throughout the cross-flow system. Our study corroborates findings from Passow (2002a) who showed that indigenous bacteria under flow conditions produced a significant amount of TEPs in comparison to that produced under static conditions. In particular, they showed that shear and turbulent conditions impacted on the TEP production. This is in contrast to the findings of Stogeregger and Herndl (1999) who observed a greater production of TEPs by bacteria in a stagnant environment (such as in a static experiment). Further, Radic et al. (2003), observed no difference in the generation of TEP under static or turbulent conditions.

Microorganisms are constantly subject to the environment and their ability to sense and respond accordingly is therefore essential to their survival (Wai et al., 1999). In response to nutrient starvation, or limitation, bacteria adapt to the environment through a number of different activities and in an attempt to maintain viability they may adopt a more resistant state (Wai et al., 1999; Seshasayee et al., 2006). Prior to nutrient starvation bacteria are well dispersed; however, it has been observed that during nutrient limited conditions there is increased adhesion and surface hydrophobicity (Kjelleberg and Hermansson 1984; Sanin et al., 2003). In addition, limitation of nutrients such as carbon, nitrogen and phosphorous within aquatic ecosystems has been found to affect not only bacterial growth and EPS production but also biomass (Farjalla et al., 2002, Graneli et al., 2004; Jansson et al., 2006). Moreover, phosphate deprivation can result in the production of larger quantities of EPS in comparison to eutrophic environments (Sutherland, 1999; Looijesteijn et al., 2000; Myszka and Czaczyk, 2009; Kim et al., 2014a). The production of large amounts of EPS has thus been suggested as

a survival mechanism with the matrix being an effective strategy to trap nutrients from the surrounding environment (Dunne 2002). Under continuing starvation conditions Myszka and Czaczyk (2009) found that *P. aeruginosa* had a high level of EPS output and produced the highest amount of EPS after an incubation period of 120 h.

In conclusion, this study demonstrates the importance of TEP production by microorganisms in the fouling process within desalination plants. Our results indicate that in a planktonic state within the natural environment the production of TEP is relatively controlled, in particular by the availability of nutrients, however, within the desalination system microbial composition and turbulence determine the generation of TEP. This shows that direct approaches such as multimedia filters and coagulation, as well as indirect approaches such as subsurface intake systems, need to be undertaken in order to reduce the biofouling capacity of the microorganisms present within the RO feed tank and make the system more economical.

6.7 Acknowledgements

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CHAPTER 7: eDNA excretion and extracellular polysaccharide structure in biofouling associated bacterium *Pseudoalteromonas* sp.

7.1 Abstract

Extracellular polysaccharides (EPS) are an essential component of biofilms and the detrimental source in fouling. This exploratory study provides information on the eDNA excretion and EPS composition of *Pseudoalteromonas* sp. in a planktonic setup. It also investigates the structure of EPS as well as the relationship between EPS and extracellular DNA (eDNA) as potential anti-fouling targets. The bacteria *Pseudoalteromonas* sp. was previously isolated from seawater reverse osmosis membrane biofouling. EPS and eDNA samples were collected every 12 hours and the EPS was fractionated into soluble and tightly bound samples. eDNA excretion was assessed using PicoGreen fluorescent probe spectrophotometrically. The EPS fractions were analysed using ^{1}H Nuclear magnetic resonance (NMR) spectroscopy and attenuated total reflectance and fourier transform infrared (FTIR) spectroscopy. The growth of *Pseudoalteromonas* sp. was significantly correlated to the secretion of eDNA over time, as well as the synthesis of soluble EPS. The ^{1}H NMR and FTIR analysis determined that the EPS fractions consisted of polysaccharides, proteins, carbohydrates, acids, and lipids. With the soluble EPS composition also consisting of amino and fatty acids, while the composition of the tightly bound EPS additionally contained carboxylic acids. New anti-fouling treatments could target the carboxylic acids components of the tightly bound EPS.

7.2 Introduction

Biofilm formation on surfaces exposed to the marine environment is frequently regarded as the first step in the biofouling fouling process. Biofouling is considered to be the unwanted accumulation of organisms on a surface, with the ecological and economic ramifications impacting multiple industries such as marine transport, aquaculture, defence, and fisheries, worldwide (Callow and Callow, 2002; de Carvalho, 2018; Bannister et al., 2019). The accumulation of organisms on submerged surfaces is

considered to be a complex multi-stage process (Caruso, 2020). The surface of submerged materials rapidly accumulates organic and inorganic compounds creating a pre-conditioning layer of favourable conditions for colonisation (Bar-Zeev et al., 2012). The physical and chemical properties of the substrate influence the colonising organisms (Dang and Lovell, 2016; de Carvalho, 2018). As primary colonisers, bacteria are considered to have an important role in influencing subsequent organisms settling (Dang and Lovell, 2016; Caruso, 2020). Once bacteria have settled, a variety of organisms gradually colonise the surface including diatoms, fungi, protists, and protozoa (Callow and Callow, 2002; de Carvalho, 2018). Additional factors, including environmental interactions, topographical interactions, and the cycling of surrounding nutrient and organic matter also influence the organisms (Caruso, 2020). These biofilms potentially consist of both prokaryotic and eukaryotic organisms within microcolonies enclosed in an extracellular matrix (Allison, 2003).

The extracellular matrix is an essential yet complex component of the biofilm, providing structure and stability to the encased populations (Davey and O'Toole, 2000; Sutherland, 2001; Flemming and Wingender, 2010). The matrix composition is influenced by a number of different factors including community composition as well as the surrounding physico-chemical environment (Sutherland, 2001). Water is the largest component of the matrix (approx. 97%) and includes varying amounts of extracellular polysaccharides (EPS; 1-2%), glycoproteins and proteins (1-2%), nucleic acids (1-2%), lipids, phospholipids, and ions (Sutherland, 2001). EPS is a common matrix element of essentially all biofilms, although the fraction of EPS present within the matrix is trivial. It has nevertheless been determined that EPS is a key structural component of the biofilm complex in which it provides the framework (Whitchurch et al., 2002; Flemming and Wingender, 2010). Although, it is interactions between polysaccharides and other molecules that allow for the structure to maintain its integrity (Sutherland, 2001). Variation is observed in the polysaccharides of biofilm matrices due to the chemical composition and physical properties of the monomer units, the nature of the glycosidic

linkages and the various organic and inorganic substitutions present (Sutherland, 2001; Flemming and Wingender, 2010;).

The presence of extracellular DNA (eDNA) within the biofilm matrix was first thought to be the result of cell lysis due to the amount accumulated (Flemming and Wingender, 2010). However, it has been established that it has an essential role in the adhesion of the matrix to surfaces (Flemming and Wingender, 2010; Montanaro et al., 2011). It is during the irreversible phase of attachment throughout biofilm formation that eDNA has the most impact. The eDNA allows the cells to overcome the repulsive forces of the surface through the creation of bridging structures from the cells to the surface (Okshevsky and Meyer, 2015). In addition, eDNA has a critical role in the stability of the biofilm structure however, this is only temporary (Montanaro et al., 2011). While the amount of eDNA accumulated in the matrix is species specific the significance of the role that eDNA has in the stability of the structure is not (Okshevsky and Meyer, 2015).

The present exploratory study describes the eDNA and EPS associated to the growth of *Pseudoaltermonas* sp. in a planktonic state within a laboratory setup. *Pseudoaltermonas* sp. are ubiquitous within the marine environments and are highly significant to the ecosystem (Holmstrom and Kjelleberg, 1999; Bernbom et al., 2011). They are known to form biofilms and are regularly identified in biofouling on seawater reverse osmosis (SWRO) membranes (Saravanan et al., 2006; Chun et al., 2012; Nagaraj et al., 2019; Parrilli et al., 2021; Jamieson et al., 2021). *Pseudoalteromonas* sp. was isolated from static biofilms formed in SWRO feed tank water from the Penneshaw desalination plant (Kangaroo Island, South Australia). It allowed for the characterisation of fractional components of the EPS produced, providing a comparative analysis of the soluble, and tightly bound EPS fractions as well as the excretion of eDNA over time. Therefore, this current study aims to provide information on potential new anti-fouling targets to combat the detrimental impact of fouling.

7.3 Materials and Methods

7.3.1 Bacterial culture

Pseudoalteromonas sp. was isolated (see section 6.4.23) from static biofilms formed on flat sheet RO membranes FILMTEC™ SW30HR (DOW, California, USA) immersed in SWRO feed tank water from the Penneshaw desalination plant (Kangaroo Island, South Australia; Jamieson et al., 2016). The *Pseudoalteromonas* sp. culture was grown in a modified Luria-Bertani (LB) broth (Table 7.1) and kept at -80 °C between experiment. The strain was always cultured until post exponential phase prior to starting with the experimental setup.

7.3.2 Experimental setup

To investigate the excretion of planktonic eDNA and EPS synthesis by *Pseudoalteromonas* sp. over time, replicates were incubated in shaking conditions in a modified LB broth. Four containers were filled with 1.3 litres of seawater LB broth. Each replicate was inoculated at a concentration of approx. 0.01 at 600 nm (OD_{600}) of planktonic culture. The containers were incubated at 26 °C in the dark on a shaking platform at 150 rpm.

7.3.3 Growth curve

For the first 24 hours, 1 mL of culture was collected every hour and optical density (OD) was measured at 600 nm using a spectrophotometer (Beckman CU640). Subsequently, samples were collected every 12 hours until late lag/early death phase was observed.

Table 7.1: Luria-Bertani (LB) broth constituents and concentrations added to seawater to create a modified LB broth.

Constituents	**Concentration**
Tryptone	1% (w/v)
Yeast Extract	0.5% (w/v)

7.3.4 eDNA

Samples (1 mL) were collected from each container every 12 hours for the first 48 hours, samples were then collected every 24 hrs until 240 hrs. eDNA was quantified following previously described methods of Allesen-Holm et al., (2006) & Tang et. al., (2013). Briefly, after collection the samples were centrifuged at 13, 500 rpm for 10 mins. The supernatant was collected and a 2:1 volume of 96% EtOH was added before being stored at -20 °C until processing. Upon thawing the samples, they were centrifuged at 4, 500 rpm for 60 mins. Samples were washed with ice cold 70% EtOH with the pellet dried at 43 °C for <3 mins. The pellet was resuspended in 40 µl of TE buffer by vortexing. The pellet was solubilised overnight at 4 °C. Samples were then analysed following the Quant-iT PicoGreen dsDNA (Invitrogen) manufacturer guidelines.

7.3.5 Isolation of soluble-EPS, loosely bound-EPS & tightly bound-EPS

Planktonic culture (10 mL) was collected every 12 hrs to isolate the soluble, loosely bound and tightly bound EPS. The samples were sonicated at 20 kHz and 40 W for 30 seconds before being centrifuged at 2000g at 4 °C for 15 mins. The supernatant was filtered (0.22 µm), dialyzed (6-8 kDa) and freeze dried to obtain the soluble EPS sample. Some samples were damaged during the process and were thus discarded from further analysis. The pellet was resuspended in TFF filtered seawater (Jamieson et al., 2016) with the addition of formamide (37%) before being incubated at 4 °C shaking for 60 mins. Followed by centrifugation at 5000 g at 4 °C for 15 mins. The supernatant was filtered (0.22 µm), dialyzed (6-8 kDa) and freeze dried to obtain the loosely bound EPS sample. The pellet was resuspended in extraction buffer (10 mL; 2 mM Na2HPO4.12H_2O, 4 mM $NaH_2PO_4.H_2O$, 9 mM NaCl, 1 mM KCl, pH 7) prior to the addition of 1M NaOH (5 mL). The samples were incubated at 4 °C for 120

mins before centrifugation at 10, 000 g for 15 mins. The supernatant was filtered (0.22 µm), dialyzed (6-8 kDa) and freeze dried to obtain the tightly bound EPS sample.

7.3.6 Nuclear magnetic resonance (NMR) spectroscopy

NMR analysis was undertaken on the EPS fractions in D_2O (99.9%). The ^{1}H-NMR spectra were obtained using a 400 MHz Bruker Avance NMR spectrometer (Bruker, Wissembourg, France). Data was analysed using Bruker TopSpin 4.0.7.

7.3.7 Attenuated total reflectance and Fourier transform infrared spectroscopy

The IR spectra of the freeze-dried EPS samples (soluble, & tightly-bound) was obtained using an attenuated total reflectance (ATR) module equipped PerkinElmer Spectrum 400 Fourier transform infrared spectroscopy (FTIR) instrument to record the ATR-FTIR spectrum. The zinc selenide (ZnSe) ATR crystal had a total reflection angle of 45° at 1000 cm^{-1} and penetration depth of 6 µm (path-length). The refractive index of the ZnSe crystal was 2.4 and a 3-bounce system was used for measurements, using 10 scans between 4,000 and 600 cm^{-1} with a resolution of 4 cm^{-1} in absorbance mode, with background (i.e., air) subtraction.

7.3.8 Data analysis

The experimental data is presented as the mean value ± standard error which were calculated from triplicate experiments using Microsoft Excel. Pearson's correlation and cross correlations function were tested using the SPSS Software (25.0.0.2). Microsoft Excel was used to create the line graphs.

7.4 Results and Discussion

7.4.1 The excretion of eDNA by Pseudoalteromonas sp.

Within the supernatant of the planktonic culture of *Pseudoalteromonas* sp., the concentration of eDNA was determined over time. The eDNA was positively correlated to the growth of *Pseudoalteromonas* sp. (cross-correlation function (ccf), $R=0.852$, $p<0.05$; Figure 7.1A) with a lag of 12 hours, indicating that the excretion of eDNA might be influenced by the growth of the *Pseudoalteromonas* sp. over time. This corroborated our hypothesis that the eDNA present in the culture had been actively secreted from the *Pseudoalteromonas* sp. cells. The concentration of eDNA after 12 hours was 38.78 ng mL^{-1}, at the start of the exponential growth phase. An increase in the concentration of eDNA was observed throughout the exponential phase, with a peak concentration at the end of the exponential phase and early death stage (Figure 7.1A). These findings are consistent with the pattern of eDNA accumulation reported in previous studies on environmental single species cultures (Heijstra et al., 2009; Suzuki et al., 2009; Tang et al., 2013) where, there is accumulation of eDNA produced over time. However, studies conducted on the isolates *Pseudomonas aeruginosa* and *Streptococcus mutans*, found that the accumulation of eDNA peaked at the early stationary phase followed by a decline (Petersen et al., 2005; Allesen-Holm et al., 2006). Here, the peak concentration of eDNA was determined to be later, with 568.75 ng mL^{-1} measured at 408 hours, the beginning of the death phase. The concentration of eDNA in the *Pseudoalteromonas* sp. culture was generally higher than those in other studies, however, the modified LB culture media may have had a part in the increase in eDNA excretion. The *Pseudoalteromonas* sp. used within this study was isolated from

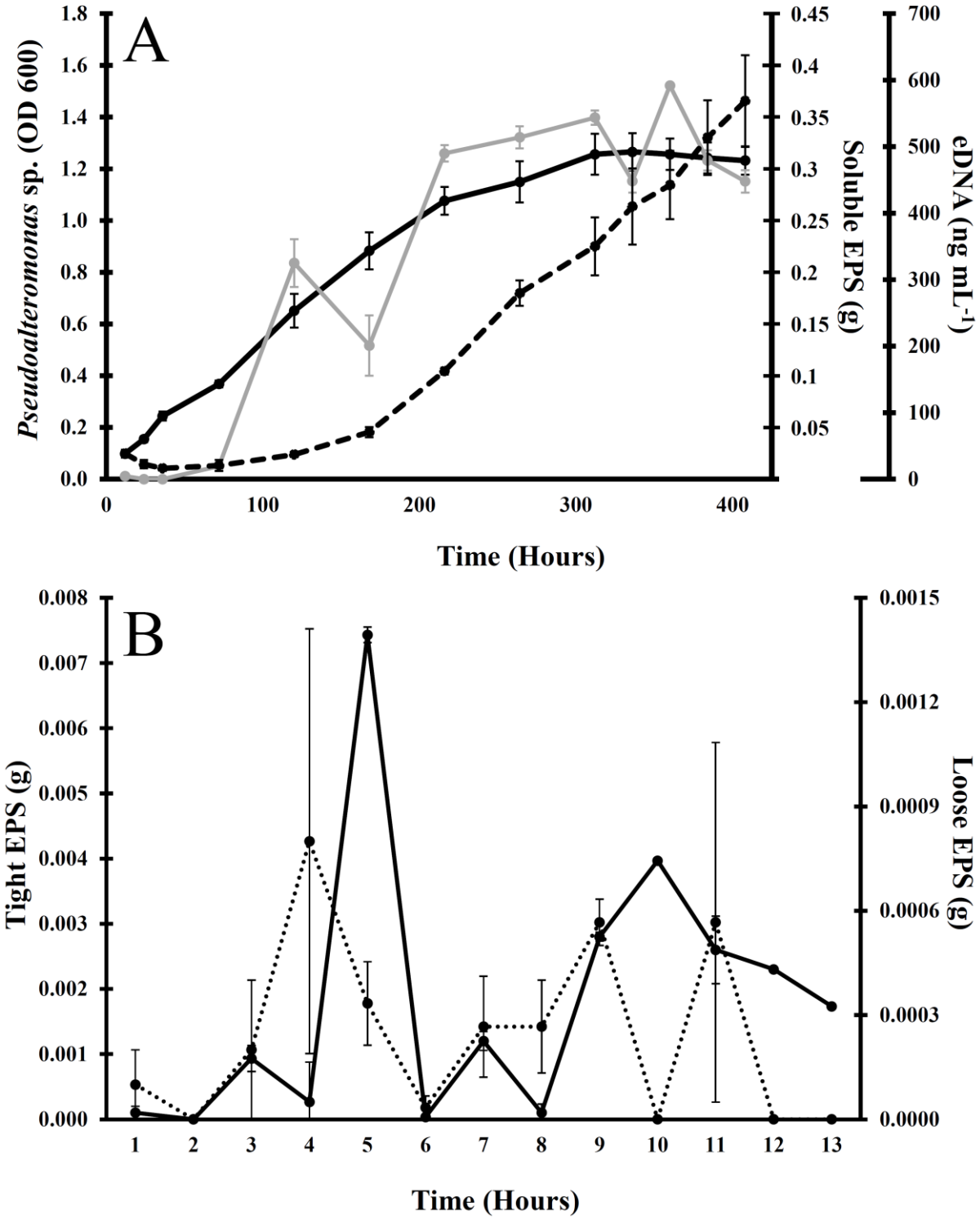

Figure 7.1: (A) The growth of *Pseudoalteromonas* sp. cells (black), the excretion of eDNA (dashed black) and the synthesis of soluble EPS (grey) throughout the experimental duration. (B) Synthesis of tight (black) and loose (dotted line) EPS throughout the experimental duration.

pre-treated seawater within the Penneshaw desalination plant (Jamieson et al., 2016) which is considered to be an oligotrophic environment. As this study was conducted in LB media, which is nutrient rich, this may have influenced the excretion of eDNA. Indeed, it has been shown that environmental conditions influence eDNA excretion, and the role it plays in the initial attachment and formation of biofilms by *Pseudoalteromonas* sp. (Ricciardelli et al., 2019). Studies involving eDNA in oligotrophic and eutrophic habitats have demonstrated that there is a marked increase in the amount of eDNA produced within eutrophic ecosystem compared to those considered to be oligotrophic (de Aldecoa et al., 2017).

7.4.2 The synthesis of EPS by Pseudoalteromonas sp.

7.4.2.1 EPS synthesis

The concentration of the various EPS fractions (i.e. soluble, loose, and tight) was determined over time within the supernatant of the planktonic culture of *Pseudoalteromonas* sp.. The EPS was collected every 12 hours and extracted into soluble EPS, and tightly bound EPS. The dry weight of the EPS mass showed that the largest amount of soluble EPS (0.0504 grams) was isolated early in the experiment (120 hours). No correlation was found between the synthesis of soluble EPS to the production of tightly bound EPS over time (Figure 7.1B). Soluble EPS are considered to be polymers that are actively produced by bacteria (Laspidou and Rittmann, 2002). The soluble fraction of EPS should only weakly bound to the cells (Flemming et al., 1999), while the tightly bound EPS should be active polymers and/or capsular EPS with characteristics similar to a gel, and robust elasticity (Jia et al., 2017). Here, the soluble EPS followed along the temporal scale of the *Pseudoalteromonas* sp. growth as well as the eDNA excretion (Figure 7.1A). The soluble EPS was negatively correlated to the secretion of eDNA with a lag of 12 hours (ccf, $R=-0.671$, $p<0.05$). As both the secretion of eDNA and

the production of EPS are active processes which rely on external components, it takes time for the cells to generate these products so as such a lag is not surprising (de Aldecoa et al., 2017; Gupta and Diwan, 2017; Moradali and Rehm, 2020). In contrast, the soluble EPS production was negatively correlated to the growth of the *Pseudoalteromonas* sp. culture with a lag of 12 hours (ccf, R= -0.832, $p<0.05$). EPS has many varied roles upon synthesis, including as a nutrient source for carbon, nitrogen, and phosphorus compounds (Flemming and Wingender, 2010). With this in mind, we hypothesise that the negative correlation between the *Pseudoalteromonas* sp. growth and the soluble EPS is the results of the consumption of the soluble EPS by *Pseudoalteromonas* sp.. Indeed, as nutrients get depleted from the liquid media overtime this soluble EPS fraction is readily available in solution as alternative nutrient source.

The largest amount of tightly bound EPS (0.0220 grams) was extracted at 120 hours. The tightly bound EPS did not follow the same pattern as the soluble EPS. No significant correlation was determined between the *Pseudoalteromonas* sp. and the production of tightly bound EPS, similarly, eDNA was not correlated to tightly bound over time. Suggesting that the tightly bound EPS could potentially be capsule-associated EPS. Capsules are comprised of polysaccharides and have been identified as a survival strategy as they are a protective structure that encases the bacteria (Whitfield, 2006; Yother, 2011). The polysaccharide structure of the capsule is infinitely diverse depending on the bacteria (Yother, 2011). The relationship between cell growth and EPS production has previously been reported with the synthesis of EPS impacted by the life stage of the cells (Czaczyk and Myszka, 2007). The method of eDNA secretion for many bacteria are currently unknown, including for the *Pseudoalteromonas* sp. used in this study. This being the case, we have to take into consideration the possibility that a portion of the EPS and eDNA may be a result of other factors such as membrane vesicles (MV). Membrane vesicles are small spherical structures that are diverse in their shape and structural components which is regulated by a multifaceted gene network (Toyofuku et al., 2019; Cao

and Lin, 2021). Both gram negative and positive organisms have been found to release eDNA via membrane vesicles including *Helicobacter pylori*, *Pseudomonas aeruginosa*, *Streptococcus mutans* (de Aldecoa et al., 2017). As these membrane vesicles have a range of sizes from 20 to 400 nm (nanometre; 1 µm = 1000 nm), they would not be removed via filtration as the pore size is 0.20 µm (Gyorgy et al., 2011; Toyofuku et al., 2019). Research on membrane vesicles has determined that many of the components of EPS that are found in their structure include DNA (Toyofuku et al., 2019). Therefore, the development of a method that takes into account the influence of membrane vesicles or has the ability to extract them prior to EPS extraction is required.

7.4.2.2 NMR EPS characterisation

The composition of the EPS fractions from *Pseudoalteromonas* sp. was further investigated by ^{1}H NMR analysis. The downstream ^{1}H spectrum of polysaccharides generally consists of an anomeric region (δ 4.5-5.5 ppm) where the anomeric protons signals of each of the sugar residue are shown; the ring protons region (δ 3.1-4.5 ppm) and the alkyl groups region (δ 1.2-2.3 ppm). The ^{1}H spectrum of the EPS from the *Pseudoalteromonas* sp. revealed a complex and heterogeneous EPS structure. The ^{1}H NMR signals for the soluble EPS timepoints were predominately detected in the downfield regions (1-5 ppm), with some peaks found in the upfield region (6-10 ppm). NMR signals were displayed in the alkyl group region, the ring protons region, the anomeric region as well as the aromatic region, however over time chemical shift is apparent.

The soluble EPS showed multiple resonance in the ring protons region over time (Table 7.2). The tightly bound EPS displayed signals on the ^{1}H spectra primarily in the downfield region (1-5 ppm) although signals were observed in the upfield region (6-10 ppm). The tightly bound EPS produced the majority of its signals in the alkyl group region and the ring protons region, although signals were

Table 7.2: ^{1}H NMR spectral data of soluble EPS in D_2O.

Type of Proton	72 Hours	120 Hours	216 Hours	360 Hours	408 Hours
Amine/Alcohol	1.08 1.09 1.12 1.18 1.26	0.78 0.80 0.84 1.08 1.11	0.78 0.84 1.08	0.84 1.05 1.22	0.85
R – CH_3 Alkyl (methyl)	1.32		1.30		
R – CH_2 – R Alkyl (methylene)		1.31		1.31	1.23
R_3C – H Alkyl (methine)	1.59	1.57 1.94	1.93	1.81 1.85 1.93	1.95
Allylic (C is next to a pi bond)		1.82			1.83 1.86
R – ND – CO – CH_3 Acetamide		2.19	2.19	2.18	2.20
α to carbonyle (C is next to C=O)	2.29				
α – CH resonances	2.64 2.91 3.25 3.41 3.43 3.69 3.77 3.98 3.99 4.04 4.09 4.24	2.63 2.90 3.40 3.42 3.65 3.76 3.98 4.08 4.22	2.63 3.65 3.79	2.64 2.91 3.40 3.66 3.80 3.99 4.03 4.12	2.65 3.67

	4.36				
α – to fluorine (C is attached to F)	4.51				
β – anomer protons			4.65 4.75	7.24 7.96	
Amide/Phenol	6.74				
Aromatic (H is on phenyl ring)	7.17 7.26 7.28	7.27	7.27		
Amide/Phenol				8.36	

also observed in the anomeric region (Table 7.3). The tightly bound EPS of *Pseudoalteromonas* sp. displayed multiple resonance in the ring protons region over time. Detection of signals in the 3-5 ppm region of the spectrum indicates the presence of polysaccharides (Sathishkumar et al., 2021). The results of the ^{1}H NMR composition complement those of the FTIR for both the soluble and tightly bound EPS.

7.4.2.3 ATR-FTIR EPS characterisation

The composition of the functional groups of the soluble and tightly bound EPS fractions generated a spectrum of the stretching frequencies involved and the parallel biological molecules. A majority of the spectrum for both the soluble and tightly bound EPS was in the 2,000 cm^{-1} down to 600 cm^{-1} region. The FTIR spectrum is presented in Figure 7.2A confirming the composition of the soluble EPS containing polysaccharides, proteins, amino and fatty acids, and lipids (Table 7.4; Rani et al., 2018). Only the spectrum of *Pseudoalteromonas* sp. soluble EPS obtained after 216 hours of growth displayed a peak at 1738 cm^{-1} indicating the presence of carboxylic acids. The tightly bound EPS contained polysaccharides, proteins, carbohydrates, acids, and lipids (Figure 7.2B; Table 7.5). The peak for amino and fatty acids (1,405 cm^{-1}) was only visible on the FTIR spectrum for the tightly bound EPS obtained after 408 hours of growth of *Pseudoalteromonas* sp.. The tightly bound EPS displayed peaks for carboxylic acid, indicating that it was an acidic polysaccharide (Shang et al., 2013).

EPS has a diverse chemical nature with carbohydrates, proteins, DNA, and lipids varying greatly their concentration and forms (More et al., 2014; Costa et al., 2018; Di Martino, 2018). Carbohydrates are generally, the major component of EPS with uronic acid or other common substitutes determining the nature of EPS either homopolysaccharides or heteropolysaccharides (More et al., 2014). Proteins are

Table 7.3: ^{1}H NMR spectral data of tightly bound EPS in D_2O.

Type of Proton	72 Hours	120 Hours	216 Hours	360 Hours	408 Hours
Amine/Alcohol		0.82 0.95	0.79 0.80 0.81 0.82 0.83 0.85	0.82	0.81 1.22
R – CH_3 Alkyl (methyl)			0.97		
R – CH_2 – R Alkyl (methylene)					
R_3C – H Alkyl (methine)	1.83				1.82
Allylic (C is next to a pi bond)				1.83	
R – ND – CO – CH_3 Acetamide					
α to carbonyle (C is next to C=O)				2.31	
α – CH resonances		2.13 3.17		3.18 4.80	4.65 4.75
α to halogen (C is attached to Cl, Br, I)	4.64 4.64 4.64 4.65 4.66 4.73 4.74 4.75 4.76 4.77	4.64 4.65 4.66 4.74 4.75 4.75 4.76 4.76 4.77 4.80			

	4.77	4.83			
Amide/Phenol	8.37	8.36		8.38	

Table 7.4: Infrared transmittance band assignment for the soluble EPS of *Pseudoaltermonas* sp. and the suggested functional types.

Wavelength (cm^{-1})	**72 Hours**	**120 Hours**	**216 Hours**	**312 Hours**	**360 Hours**	**408 Hours**	**Functional Type**
***V*[a](O-H)**	3277	3281	3264	3280	3280	3274	O-H into polymeric compounds / polypeptides
***V*(CH_3, CH_2, CH)**	2965 2934 2881	2965 2932 2877	2917 2849	2966 2931	2964 2926 2856	2960 2920 2852	Lipids
P-H	2325	2345					
***V*(C=O)**			1738				Carboxylic Acids
Amide I: *V*(C=O, C-N)	1643	1643	1643	1633	1631	1625	Proteins (peptidic bond)
Amide II: *V*(N-H, C-N)	1546	1542	1536	1539	1553	1554	Proteins (peptidic bond)
δ[b](CH_2, CH_3)	1446	1447	1463	1449			
***V*($COOCH_3$, $-CH_2-CO-$, $-CH_2-C=C-$)**	1410	1410	1405	1405	1405	1403	Amino acids, fatty acids
***V*(O-C)**	1318 1245	1319 1242	1317 1239	1318 1243	1320 1262	1316 1243	
***V*(C-OH, C-O-C, C-C)** ***V_{as}*[c](P=O)**	1203 1075	1073	1032	1043	1045	1033	Polysaccharides Phosphodiesters
***V*(C-O, C-N)**	1006	1008	1000				
δ(C-H) ring puckering,			719				

***δ*(NH2 & N-H wagging) shifts on H-bonding**			848 927				D-Galactose-4-sulphate Glycoside link

[a]V = vibration, [b]δ = bending vibrations, [c]V_{as} = asymmetric stretching vibration.

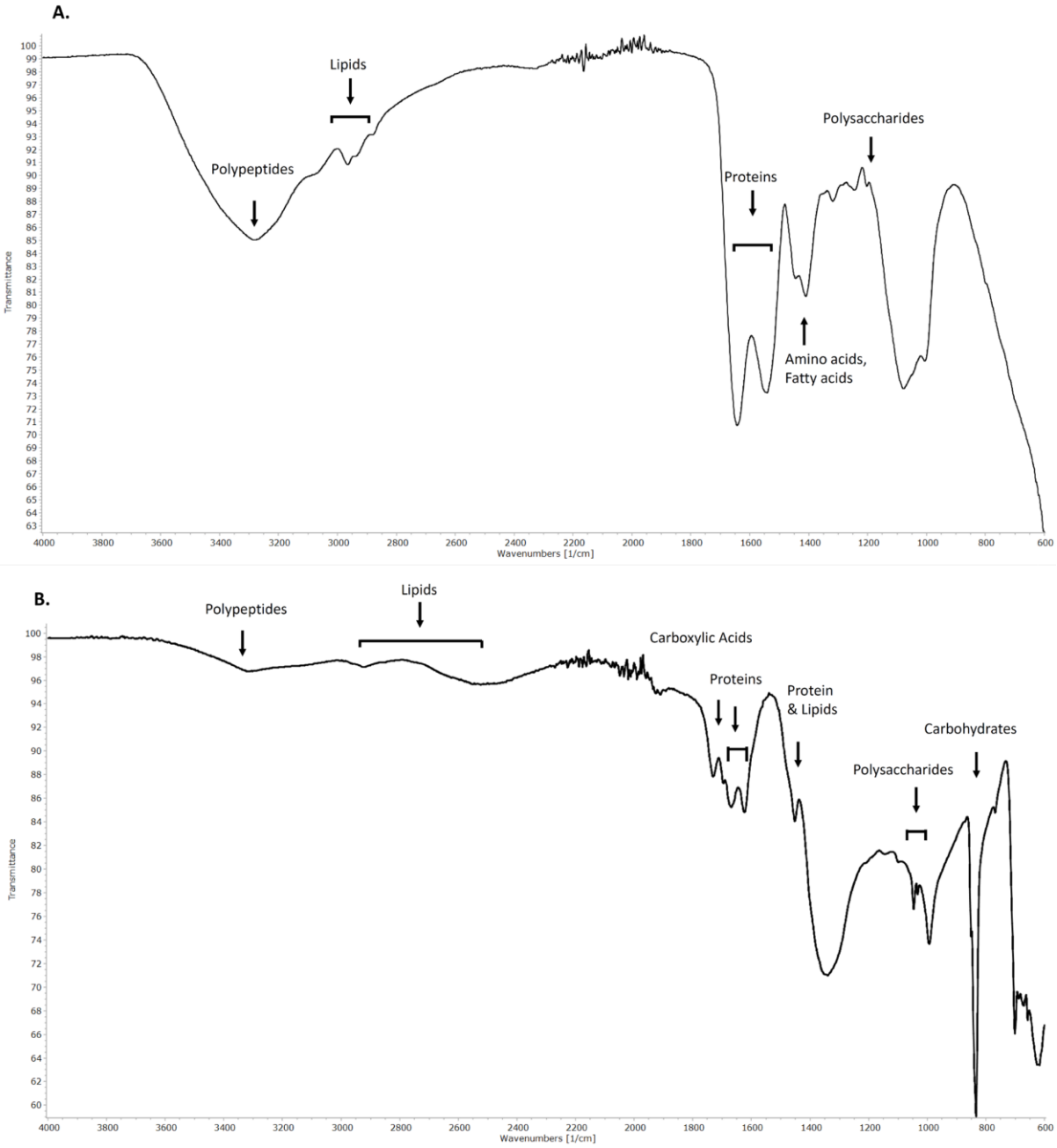

Figure 7.2: The FTIR spectra of soluble EPS (A) and tighly bound EPS (B) from collected every 12 hours *Pseudoalteromonas* sp. and the identified functional groups.

Table 7.5: Infrared transmittance band assignment for the tightly bound EPS of *Pseudoaltermonas* sp. and the proposed functional types.

Wavelength (cm^{-1})	**72 Hours**	**120 Hours**	**216 Hours**	**312 Hours**	**360 Hours**	**408 Hours**	**Functional Type**
***V*[a](O-H) H-bonded**						3357	
***V*(OH)**	3311	3324	3303	3316	3315		OH into polymeric compounds
***V*(CH_3, CH_2, CH)**	2923 2524	2921 2540	2921 2510	2917 2512	2924 2527	2920 2851	Lipids
***V_{as}*[b](C=C)**				1920			
***V*(C=O)**	1734	1729	1731	1731	1730		Carboxylic acids
Amide I: *V*(C=O, C-N)	1669	1669	1665	1668	1671		Proteins (peptidic bond)
δ(NH_2 scissoring - 1° -amines), δ(N-H - 1¡-amide) II band	1623		1628	1623		1618	Proteins (peptidic bond)
***V*($COOCH_3$, -CH_2-CO-, -CH_2-C=C-)**						1405	Amino acids, fatty acids
δ(CH_2, CH_3)	1452	1454 1366	1453	1451	1454		Protein & Lipids
δ(α-CH_3) δ(CH_2 & CH_3)			1350		1355		
δ(O-H - in-plane)	1340			1332			
***Vs*[d](C-O-C, C-C)**	1151	1044	1149	1204	1204	998	Polysaccharides

	1047		1046	1185 1136 1101 1099 1046 1032	1181 1045		
δ(=C-H & =CH_2)	993	970	994	993	998		
δ(C-H) ring puckering, *δ*(NH2 & N-H wagging) shifts on H-bonding	833 769 701	851 840 701	838 701	833 803 782 768 700	839 802 701 669	832 768 701 699	Carbohydrates
***δ*(O-H) out-of-plane *δ*(NH2 & N-H wagging) shifts on H-bonding**		671	669				
***И*(C-H_2)**	617	617	618	657 636 619	616		Polysaccharides

[a]V = vibration, [b]V_{as} = asymmetric stretching vibration, [c]δ = bending vibrations, [d]Vs = symmetric stretching vibration.

also, major component of EPS either as enzymes or structured proteins (More et al., 2014; Costa et al., 2018; Di Martino, 2018). A number of key functions of EPS have been ascertained over the years of note is the adherence to surfaces, the formation of biofilms, and as a protective layer surrounding cells (More et al., 2014). These aspects all contribute to the endurance of biofouling within water treatment systems (More et al., 2014; Nagaraj et al., 2018).

7.5 Conclusion

In this exploratory study, eDNA and EPS production of known biofilm forming bacteria *Pseudoalteromonas* sp. was assessed. The EPS fractions of soluble EPS and tightly bound EPS were separately extracted and characterised using spectroscopic methods using peak location and shifts to identify characteristics. The main outcomes are:

The growth of *Pseudoalteromonas* sp. positively influenced the excretion of eDNA over time.

The soluble EPS composition was determined to contain polysaccharides, proteins, amino and fatty acids, and lipids.

The tightly bound EPS composition contained polysaccharides, proteins, carbohydrates, acids, and lipids

This study has shown that further exploration of the EPS structure of biofouling organisms could potentially be a target for anti-fouling activity. Determination of the structure and composition of the EPS on fouled membranes could be beneficial for the development of potential antifouling methods. For example, based on the FTIR spectra unique or rare functional types identified in the structure of the different EPS fractions could be extracted or targets for potential antifouling treatments.

7.6 Acknowledgements

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CHAPTER 8: Discussion

RO desalination is the application of pressure to move water across a semi-permeable membrane to obtain potable water (Darre and Toor, 2018; Jamieson and Leterme, 2020). RO desalination plants commonly consist of five main components: (i) intake system, (ii) pre-treatment system, (iii) high pressure pumps, (iv) SWRO membranes, and (v) brine output and post treatment system (Darre and Toor, 2018). One of the most common sources of intake water is seawater, however it is a complex environment composed of organic and inorganic substrates, microorganisms, and natural organic matter (Song et al., 2017; Jamieson and Leterme, 2020). As a consequence, pre-treatment systems need to be able to reduce the fouling potential of the water prior to the SWRO membrane (Qasim et al., 2019). The pre-treatment systems commonly installed in desalination plants are considered conventional, although non-conventional methods are being introduced. Conventional pre-treatment systems most often consist of a mix of physical and chemical processes using screens, filters, disinfectants, and coagulants to improve the quality of the intake water (Kavitha et al., 2019; Figure 8.1). Although the combination of systems is dependent on water quality and fouling characteristics. Findings in chapters 3 – 5 have established that further reduction in the fouling load is required within the desalination system. Therefore, a novel pre-treatment system for the Penneshaw SWRO desalination plant that consists of a subsurface intake system, sand filtration, coagulation/flocculation, ultrafiltration, coagulation/flocculation, reverse osmosis membrane, post desalination treatment could be proposed (Figure 8.1).

Subsurface intake systems are being used globally to improve the quality of raw intake seawater in desalination plants (Rachman et al., 2014; Dehwah et al., 2015b;), and are categorised into two categories: wells (Figure 8.2) and galleries (Figure 8.3; Missimer et al., 2013). Well systems have been shown to significantly reduce a majority of the components commonly associated with fouling. As the water passes through the aquifer on the way to the well concentrations of turbidity, algae, bacteria, total organic carbon, natural organic matter, and transparent exopolymer particles

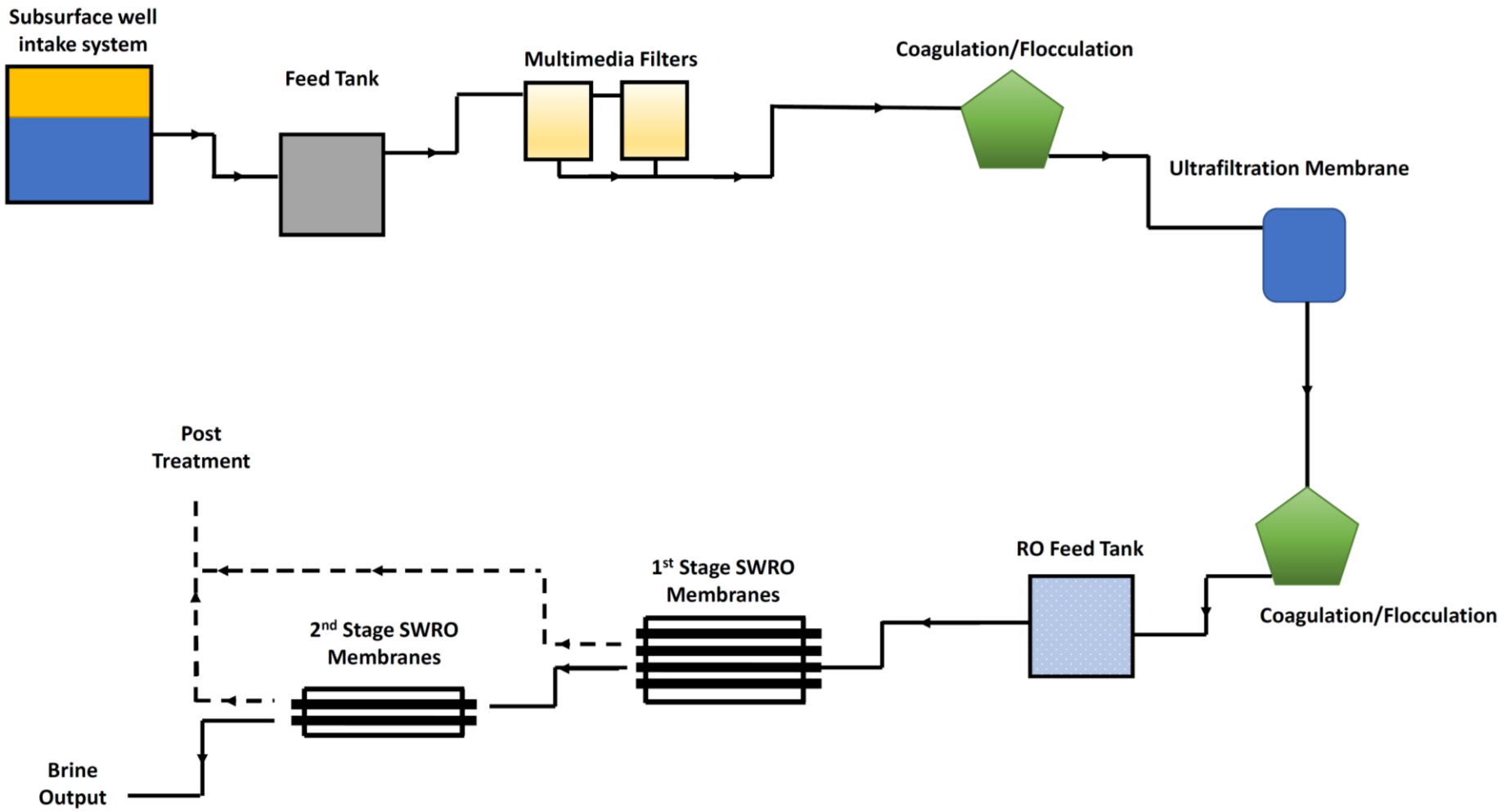

Figure 8.1: Pipeline of the novel pre-treatment system proposed for the Penneshaw SWRO desalination plant.

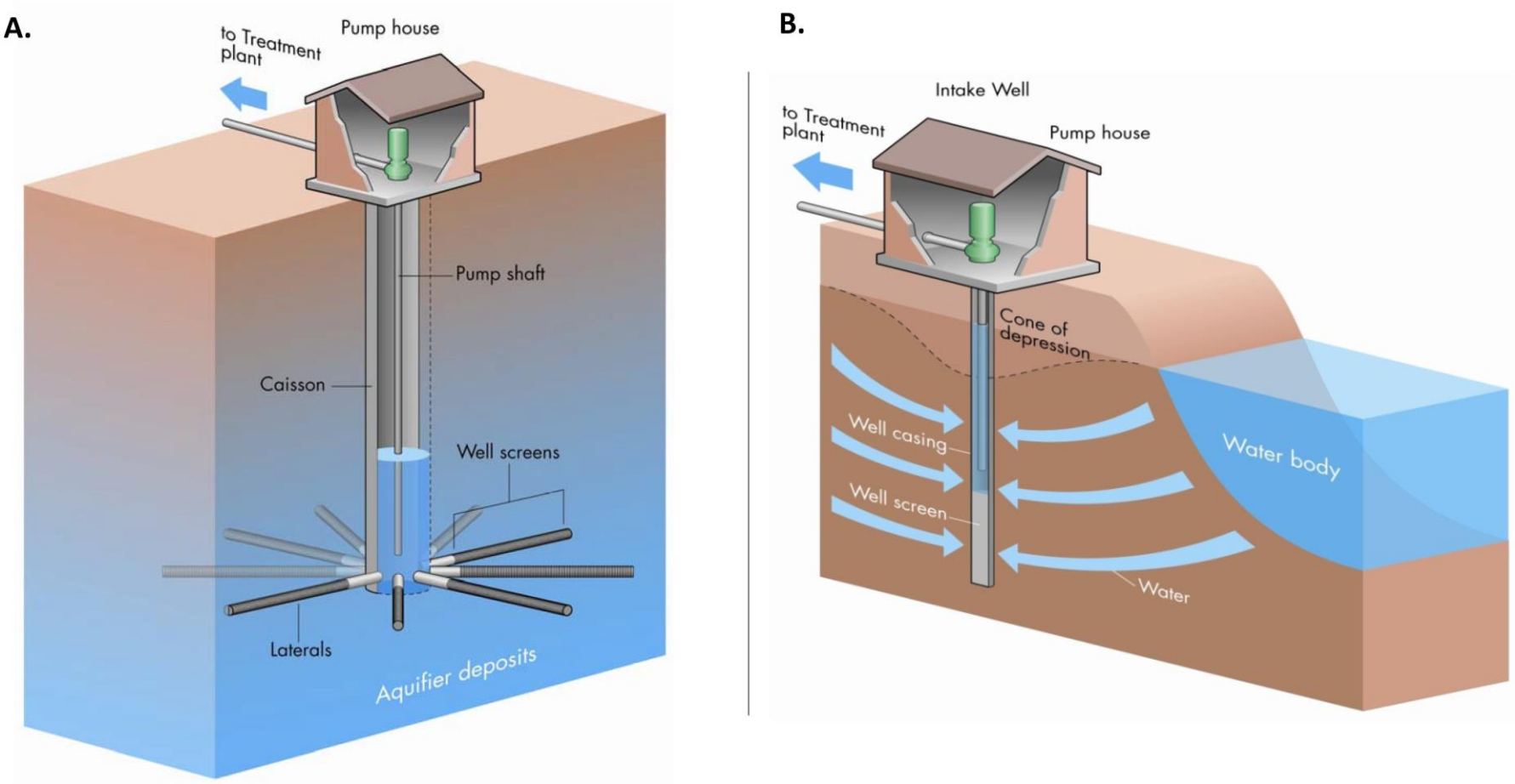

Figure 8.2: Examples of subsurface desalination intake water systems; (A) Vertical beach well intake system. (B) Horizontal (Radial) intake well intake system. (Source: Watereuse Association, 2011).

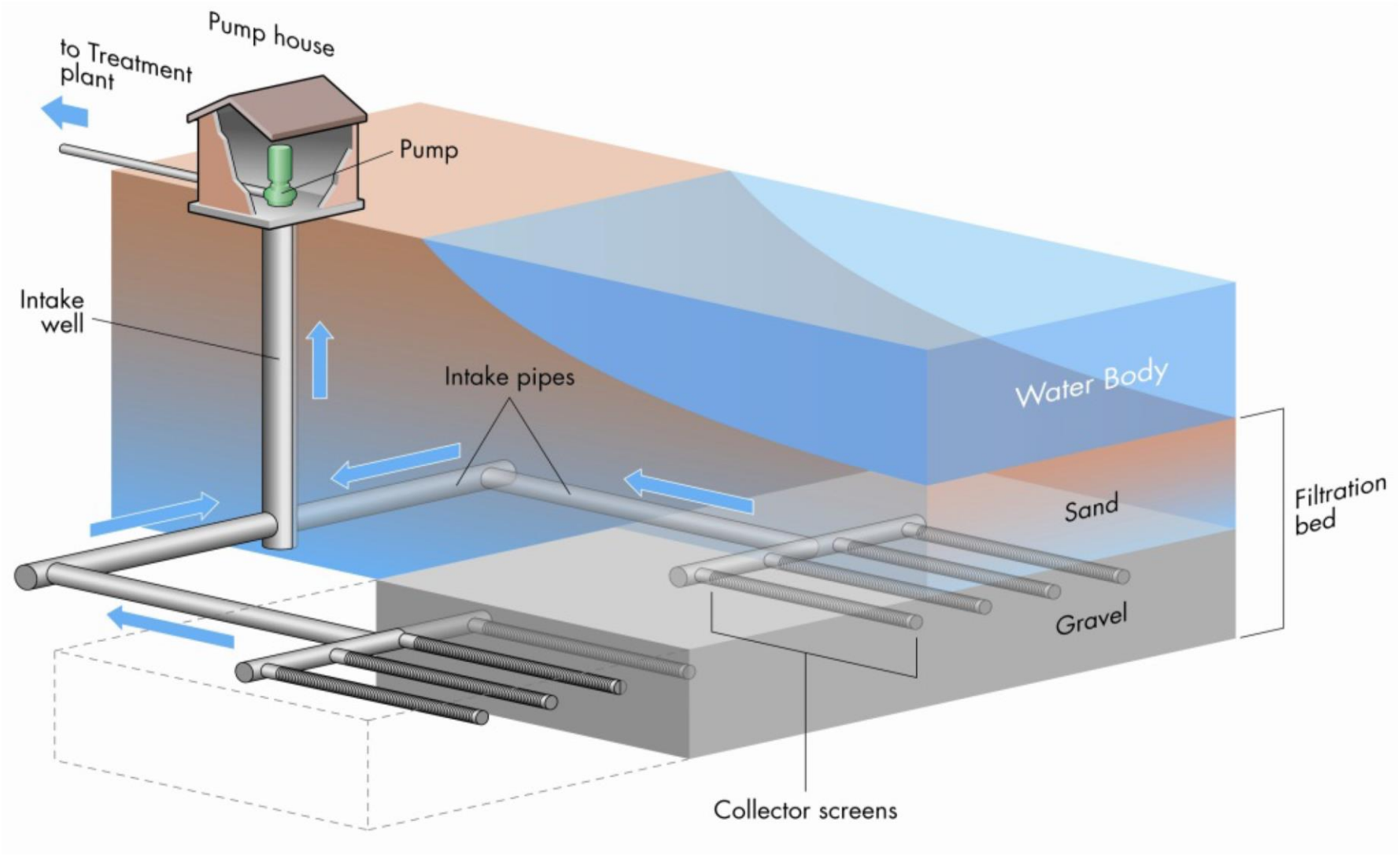

Figure 8.3: Seabed (Infiltration) gallery subsurface desalination intake water system (Source: Watereuse Association, 2011).

(TEP) are reduced (Missimer et al., 2013; Rachman et al., 2014; Dehwah et al., 2015a, 2015b; Dehwah and Missimer, 2016; Dehwah et al., 2017). Aquifer transport also has been shown to reduce the concentration of biopolymer and assimilable organic carbon (Schneider et al., 2012). Gallery intake systems are classified into two types: beach galleries and seabed galleries. Beach galleries are generally constructed underneath the intertidal section of the beach with seabed galleries assembled in the subtidal areas offshore (Maliva and Missimer, 2010; Missimer et al., 2015). Seabed galleries are similar to those of slow sand filters which are commonly found in water treatment plants (Dehwah and Missimer, 2017). The active layer determines the efficacy and removal rate of the organic matter, which generally consists of fine grain quartz sand, however location also influences the active layer choice (Rasheed et al., 2003; Wild et al., 2005; Missimer et al., 2015). A pilot study in California, using the slow sand filtration, observed relatively low amounts of fouling present on the membrane including minimal flux decline after being in operation for one year (Desormeaux et al., 2009). A key issue identified in the Penneshaw SWRO desalination plant, is the inflow of fouling precursors such as TEP as well as prokaryotic and eukaryotic organisms (see sections 3.5 and 4.5). Consequently, over time fouling builds on the SWRO membranes, decreasing salt rejection, increasing in flux decline, and increasing operating costs over time. The introduction of either a subsurface well system, or a gallery system, as opposed to the open ocean intake would deliver water with limited fouling precursors and biofouling organisms into the system thereby, making the SWRO membrane in Penneshaw less vulnerable to fouling.

The findings from the Penneshaw SWRO desalination pre-treatment and membrane autopsy study (see sections 3.5 and 4.5), in addition to the study on the formation of aggregates (see section 5.5) showed that many fouling precursors (i.e. organic and inorganic matter, TEP, and particulate matter) are still able to reach the SWRO feed tank and membranes in the Penneshaw SWRO desalination plant. Early removal of these components in the pre-treatment system could potentially result in a reduction

in necessary cleaning of each pre-treatment system or fewer pre-treatment systems along the pipeline. One method for the removal of fouling precursors is that of coagulation and flocculation. The coagulation process removes components which have a low molecular weight and as a result are not removed by gravitation force (Anis et al., 2019b). Two different types of coagulation are used within desalination plants, chemical or electro coagulation. The addition of chemicals enhances the removal of particulate and organic matter by producing larger particles via the aggregation of smaller ones (Anis et al., 2019b). Chemicals such as aluminium sulphate, ferric sulphate, ferric chloride are commonly used in the coagulation process although there are other novel coagulants on the market (Qasim et al., 2019). The coagulant chemicals typically reduce or remove the surface charge of the particles it the water lessening the repulsive interactions between the particles allowing them to bind together (Anis et al., 2019b). Studies have shown that the efficiency of the coagulant of choice would be influenced by the water quality, nevertheless the outcome is always a reduction in the fouling potential of the water (Duan et al., 2003; Peiris et al., 2013; Tabatabai et al., 2014). Flocculation, which always follows coagulation, brings together the microflocs formed in coagulation into larger particles which can be removed via sedimentation, flotation, or filtration (Qasim et al., 2019).

Sand filtration is currently installed in the Penneshaw SWRO desalination plant in the pre-treatment system and consists of filter coal (0.9-1.1 mm size; 300 mm depth), quartz sand (0.45-0.55 mm size; 500 mm depth), garnet sand (0.3 mm size; 200 mm depth) and graded gravel (500 mm depth; see section 3.4.1). Studies have observed the efficiency of sand filtration in the removal of turbidity, viruses, bacteria, protozoa, and higher organisms (Weber-Shirk and Dick, 1999; Hijnen et al., 2004). These findings are also supported by studies undertaken at Penneshaw desalination plant regarding the reduction in fouling components (Balzano et al., 2015c). Of equal importance is also the potential of the sand filters to contribute to fouling within the system. During sand filtration, water passes through the filter bed of mixed media in which the particles contact and stick to the surface of the grains or to

the materials previously deposited (Prihasto et al., 2009). This allows for the microbial biomass to accumulate on the grains/previously accumulated material, thereby creating a niche environment for cell proliferation (Campos et al., 2002). Within a desalination system such as Penneshaw, the water is being driven through the sand filters under pressure which could allow for the detachment of fouling precursors and microorganisms from the grains. As a consequence, detached TEP and prokaryotic and eukaryotic organisms are able to colonise formed aggregates further along the pre-treatment system (see section 5.5).

Ultrafiltration (UF) has been added to desalination plants globally as a pre-treatment option to reduce fouling components reaching the SWRO membrane (Badruzzaman et al., 2019). UF uses hydrostatic pressure to move liquid through semi permeable membranes based on size exclusion (Al Aani et al., 2020). It has been suggested that UF water quality is superior in comparison to conventional membrane treatments due to the smaller pore structure (Badruzzaman et al., 2019). UF has been added to the proposed novel Penneshaw desalination pipeline for its ability to remove smaller size molecules. The findings from the Penneshaw SWRO desalination plant in sections 3.5 and 4.5 identified a majority of the biofouling organisms in the pre-treatment system and on the fouled SWRO membranes to be <0.2 µm, some with a rigid cell structure and others without. Equally, fragments of TEP/EPS were able to pass through all the conventional pre-treatment systems in Penneshaw to form aggregates (see section 5.5). UF membranes have a pore diameter from 10 A to 1000 A which is small enough to remove bacteria and eukaryotic cells <0.2 µm. Some research has also proposed that UF membrane could remove most viruses form the source water (Gao et al., 2011; Gentile et al., 2018).

Although UF membranes have the ability to remove most, if not all components, within the intake seawater, they are subject to fouling like any other membranes in seawater (Shi et al., 2014). Hence why a flocculation/coagulation stage has been suggested in the novel pre-treatment pipeline, prior

and after to UF. The flocculation/coagulation stage after UF would prevent the formation of aggregates due to the pressure driving smaller fragments of TEP/EPS through the UF pores (see section 5.5). The formation of aggregates has a detrimental effect on the longevity of the SWRO membranes.

SWRO membranes are primarily made of thin film composite, they are stable under pressure and provide constant high degree of separation. These membranes consist of a selective layer as well as a support layer which provides the mechanical strength (Lim et al., 2021). Many developments in antifouling membrane technology, including surface modifications and coatings, have been studied extensively, however a balance has yet to be found between suppression of fouling and an effective water treatment option (Lim et al., 2021). Results in chapter 3 - 4 showed that the foulant on the SWRO membranes is not only precursors that have come through the pre-treatment system, but also consists of EPS produced by organisms surviving on the membrane surface due to the inflow of nutrients. Furthermore, a majority of the organisms, both prokaryotic and eukaryotic, identified on the fouled membranes (see sections 3.5 and 4.5) were <0.2 µm, signifying that they could have bypassed all the pre-treatment system within Penneshaw due to their size. Another aspect in Penneshaw, that contributes to fouling, is the ability for organisms to form and colonise aggregates after the pre-treatment system. In the proposed novel pre-treatment pipeline, prior to the SWRO membranes, the suggested pre-treatment stage should remove 100% of the organisms within the water. With this in mind, the use of a tailored SWRO membrane would be beneficial to the system. One such membrane is a thin-film composite membrane incorporated with silver nanoparticles on embedded graphene oxide quantum dots (TFN-GOQD/Ag; Yu et al., 2019). Modified thin-film composite (TFC) membranes require a balance between selectivity and permeability without compromising the rejection rate. The TFN-GOQD/Ag membrane demonstrated similar rejection rates as TFN RO membranes, furthermore, improved permeate fluxes were observed (Yu et al., 2019). The addition of Silver (Ag) proved to be an

effective antimicrobial treatment against *Escherichia coli* and *Staphylococcus aureus* (Yu et al., 2019). The antimicrobial properties of Ag have long been recognised, owing to the wide range of antibacterial capabilities against many microorganisms (Knetsch and Koole, 2011; Paladini and Pollini, 2019). A key attribute of Ag, as an effective antimicrobial, is the relatively low occurrence of development of resistance, unlike antibiotics (Paladini and Pollini, 2019). Therefore, the introduction of Ag into TFC membrane could be an effective solution against the proliferation and growth of organisms on SWRO membranes (see sections 3.5 and 4.5; Linhares et al., 2020). On the other hand, the build-up of dead cells on the membranes form a cake layer overtime, resulting in the same unfavourable outcomes as fouling on the SWRO membrane (Hoek and Elimelech, 2003). Because of this, the TFN-GOQD/Ag membrane should be used in conjunction with grazing predators.

Predation is an essential part in biofilms, influencing solute transport and increasing porosity but also instigating unique membrane fouling activities (Weitere et al., 2005; Böhme et al., 2009; Gao et al., 2010). Success of predatory grazing has been demonstrated on UF membranes as well as in gravity driven filtration systems (Derlon et al., 2012; Derlon et al., 2013; Klein et al., 2016; Chen et al., 2021). Results from the studies showed that not only was the thickness of the fouling layer reduced, but also the density subsequently increasing the permeate flux (Derlon et al., 2012; Klein et al., 2016; Chen et al., 2021). Furthermore, the use of metazoans as grazing predators was seen to have an impact on the composition of the prokaryotic and eukaryotic community on gravity driven filtration membranes (Klein et al., 2016). The results of section 4.5 showed that ciliates and amoeba are able to survive within the pressured SWRO desalination plant at Penneshaw. With this in mind, the introduction of predatory grazers on the SWRO membrane for the purpose of reducing the fouling is not unconceivable.

The novel pre-treatment system presented here (Figure 8.1) based on the findings of work undertaken at the Penneshaw SWRO desalination plant will by no means solve the problem of fouling within

Penneshaw. However, it does present a chance to potentially reduce the need for frequent clean, maintenance and ultimately replacement of the SWRO membranes. Thereby, reducing the financial requirements of the production of potable water. Nevertheless, water production technologies are no match for a frequently overlooked, yet the most influential aspect of the production of safe drinking water: public perception.

As far back as 1970, the level of acceptance by the public for recycled water has been investigated and in the more current climate desalinated water (Dolnicar et al., 2011). Shockingly, the perceptions from 1970 are very similar to current public surveys regarding the use of recycled and desalinated water (Dolnicar et al., 2011). With the Australian population very specific about the distinction between recycled and desalinated water. Essentially, most people are willing to use recycled water for outdoor activities such as watering the lawn or garden, as long as they don't have to have any contact with it. Whereas desalinated water was preferred for drinking and body contact (Dolnicar and Schäfer, 2009; Dolnicar et al., 2011). Health concerns are a major contributor to the distrust of alternative water sources but many other factors such as environmental impact attitude, positive perceptions, influence of peers and acquaintances, cultural background, religion etc... influence a person's perception of alternate water sources (Dolnicar et al., 2011). Research also identified that the Australian population were more willing to use desalinated water even though they had received relatively little information on the desalination process. It was proposed that a willingness to use desalinated water was due to not being aware of the negative environmental aspect that are commonly associated with desalinated water (Dolnicar et al., 2011). Conclusions from the public perception reports confirmed that knowledge is the key to gaining public support for alternate water sources (Dolnicar and Schäfer, 2009; Hurlimann and Dolnicar, 2010; Dolnicar et al., 2011; Fielding and Roiko, 2014; Ross et al., 2014). The delivery of the information is just as important as the message. With findings suggesting that desalination/recycled water is seen in a much more favourable light if

there is a positive perspective in the message (Dolnicar and Schäfer, 2009; Dolnicar et al., 2011). We are at a stage now where the Australian public needs to know that the use of desalinated water is not optional any longer, it is a necessity.

CHAPTER 9: References

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