Microbial Desalination

An exploratory research to assess the potential of desalination by microbial methods

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by

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Summary

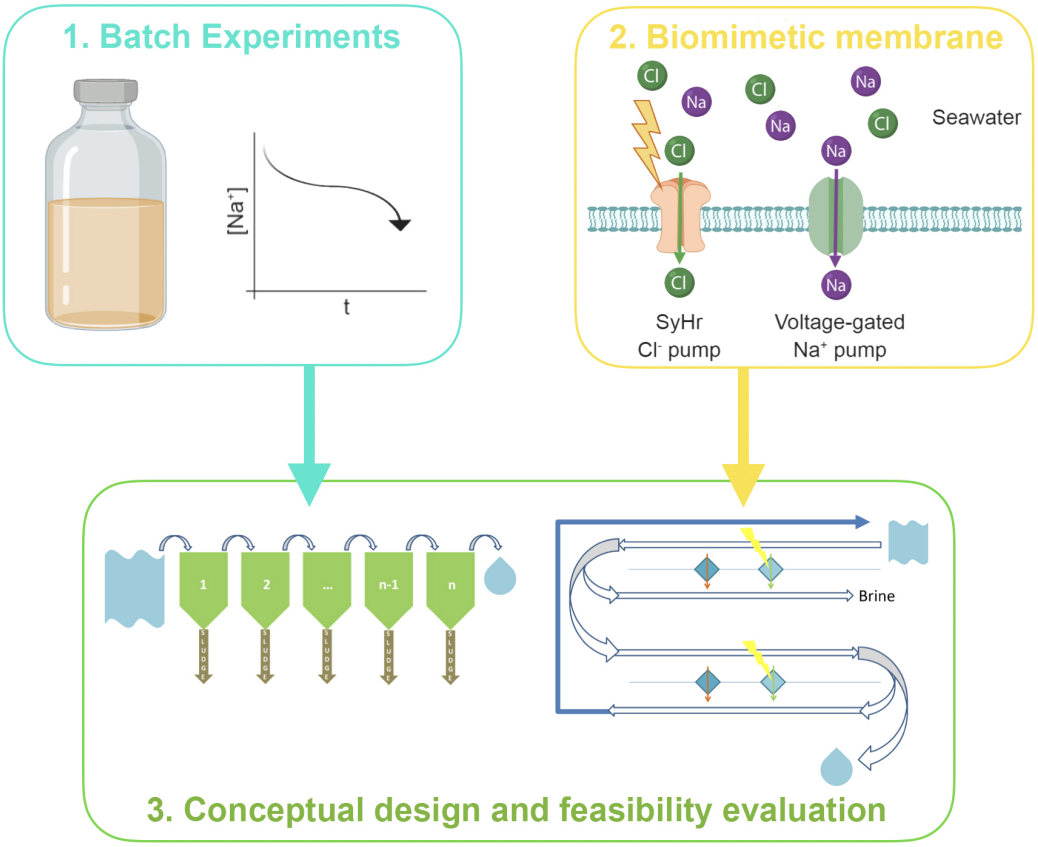

Figure 1: Graphical abstract of this research, showing the three research lines.

As freshwater resources are predicted to become more scarce in the future, desalination will become a more prevalent treatment method. Microbial desalination, defined as the use of microbial processes to remove ions from a saline solution, may have potential as a new desalination method to produce water for domestic, agricultural or industrial purposes. This research explored the theory, performance, application and feasibility of microbial desalination. It consists of three research lines, as represented in Figure 1. Firstly, the performance and optimal conditions for microbial desalination were examined in batch experiments. In the second research line, the use of microbial ion transport proteins in a biomimetic membrane was proposed and the microbial light powered transport protein SyHr was genetically designed and expressed for this purpose. Finally, two models were made which evaluate the feasibility of microbial desalination at full scale; one which compares the costs of microbial desalination in sequencing batch reactors to seawater reverse osmosis and one to calculate the recovery and required size of a biomimetic membrane as described in the second research line. Based on the results of all research lines, it was concluded that microbial desalination is be a promising new technology for desalination, which should be further developed. A summary of every research line is provided below.

1. Determining the Optimal Conditions for Microbial Desalination through Batch Experiments

Microbial desalination is the direct use of microbial processes to remove ions from a saline solution. Experiments have previously been described where microbial desalination is proposed or achieved using pure cultures or genetically modified organisms, but never using mixed cultures such as activated sludge, which is widely available in wastewater treatment plants. In this section, a series of mixed culture batch experiments are described, which were conducted with the objective of gaining knowledge on the optimal conditions for microbial desalination. Sodium profiles over time were made of experiments, in which the varying conditions were medium composition, pH control, type of biomass (PNSB enriched, activated sludge and green phototrophs), light or dark conditions, inoculation concentration (0,02 gVSS/L, 0,2 gVSS/L and 2 gVSS/L), initial sodium concentration and condition of biomass (inactive/active). In all samples except one containing a pure *Rhodopseudomonas* culture, sodium removal from the medium was observed of 20-40 %. It was found that light is not needed for desalination, chloride was also removed besides sodium, inactive biomass can still remove ions, ion exchange is not a desalination mechanism and there is no linear correlation between biomass concentration and ion removal. By fitting curves to relative sodium removal data, two parameters were obtained by which all experiments could be compared: K_{Na}, a rate constant which indicates speed of sodium removal and $R_{Na,max}$, which is the maximum percentage of sodium which is expected to be removed. The best performing desalination condition was found to be inactive mixed culture PNSB biomass, in seawater medium, without buffer and at an inoculation concentration of 2 gVSS/L. Under these conditions, $R_{Na,max}$=39% and K_{Na}=0,27. A 39% removal can be considered successful as this already lowers costs for further desalination significantly and a the K_{Na} indicates that after 1/0,27=3,7 hours, half of maximum removal is reached. A novel mechanism for ion removal is proposed, wherein ions which enter the cell are directed towards a bacterial microcompartment, a virus-like pocket within the cell, which is widespread in microorganisms and archae. The local sodium and chloride concentration here would be sufficiently high to precipitate sodium chloride or halite, which enables storage without high osmotic gradients.

2. Towards a Light Powered Biomimetic Desalination Membrane

Biomimetic membranes offer high selectivity and permeability, and have been proposed as promising for desalination purposes. In this study, a novel concept is designed for a light powered desalination membrane containing SyHr, a halorhodopsin protein, and a voltage gated sodium pump. Halorhodopsins are light gated chloride pumps. In order to lay the foundations for the construction of such a membrane, SyHr was produced within the solubilised membrane protein fraction at wet lab. To this end, firstly a SyHr DNA fragment including a his-tag was designed and synthesised. This fragment was restricted from a pUC-sp vector and successfully ligated into a Ptrc99a expression vector, with which *E.Coli* BL21 cells were transformed. Overexpression was induced using Isopropyl β-D-1-thiogalactopyranoside (IPTG), after which cells were harvested by centrifugation, passed through the cell disruptor and all membrane proteins isolated. This research lays the foundation for testing of the potential of halorhodopsins in desalination. A next study can focus on inserting the purified SyHr in a membrane to assess it's performance and stability. Although more research is needed and scalability is still a faraway station, the concept of light powered desalination with this membrane offers a novel option within the quest for desalination solutions, which could offer high selectivity, low energy cost and reduced fouling compared to seawater reverse osmosis.

3. A Model Approach to Assess the Feasibility of Microbial Desalination

The topic of microbial desalination has been researched by various groups on a laboratory scale, but feasibility analyses are currently lacking. Reverse osmosis (RO) is the prevalent method of seawater desalination at this moment, but it is not yet cost effective for world wide application. In this study, two conceptual designs for full scale microbial desalination setups were proposed, based upon the results of part 1 and 2 of this thesis. The first design entails a series of sequencing batch reactors (SBRs) for desalination using excess biomass from a wastewater treatment plant (WWTP). To evaluate feasibility of batch full scale desalination, cost comparison was made of four scenarios: seawater RO (1), three SBR steps and RO (2), seven SBR steps and RO (3) and eleven SBR steps (4). Combining SBR steps with RO has advantages, as the SBR pretreatment lowers the cost for RO and the effluent water is of high quality. The costs per m^3 were €0,95 (1), €0,91 (2), €0,86 (3) and €0,75 (4). For agricultural or industrial use, just a series of 11 SBRs (scenario 4) could be an attractive option. For drinking water, scenario 3 is optimal as the final RO step removes enough pollutants to deliver water of (near) drinking water quality. Based on the amount of available sludge, the daily capacity for microbial desalination treatment was calculated to be 22680 m^3 seawater for Harnaschpolder WWTP (Delft,

The Netherlands), so in terms of sludge this concept is feasible.
The second design entails two cross flow steps based on light powered membranes. A model was constructed with which practical feasibility was evaluated based on membrane surface required and recovery γ. A required membrane surface of 1,22 m^2 and a recovery of 68,4% were calculated, indicating practical feasibility of this technique. Brine was proposed to be concentrated up to 80,000 mg/L TDS. The difference in salinity between the brine and influent could be used for energy generation.
Although based on rough first estimates, the results from both models indicate that microbial desalination could be feasible at full scale. However, at present some knowledge gaps, such as whether microbial desalination is still effective at lower sodium concentrations and whether the used biomass is still fit for anaerobic digestion, need to be addressed before a full scale test is desirable.

List of Abbreviations and Definitions

Abbreviations

ATP Adenosine Triphosphate

BCP Block copolymer

BMC Bacterial microcompartment

CAPEX Capital expenditures or investment cost

CEMOVIS Cryo-electron microscopy of vitreous sections

EC Electroconductivity

EPS Extracellular Polymeric Substances; biopolymers produced by the organism which form a matrix on the exterior of the cell. (Wingender et al., 1999)

HEPES 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

IC Ion Chromatography

ISS Inorganic suspended solids

OPEX Operational expenditures

PNSB Purple Non-Sulphur Bacteria

RO Reverse Osmosis, a membrane filtration technique.

SBR Sequencing Batch Reactor

SyHr Synechocystis Halorhodopsin, a light-gated anion channel from the organism *Synechocystis sp.*

TDS Total dissolved solids

TEM Transmission Electron Microscopy

VSS Volatile suspended solids; amount of volatile matter present in the solid fraction.

WWTP Waste water treatment plant

Symbols

Table 1: Symbols used in this work, description and unit

Symbol	Description	Unit
K_{Na}	A rate constant which indicates initial speed of sodium sequestration	h^{-1}
R_{Na}	Sodium removal	%
HC	Hourly treatment capacity	m^3/h
PP	Payback period	years
R_{TDS}	TDS removal	%
P_E	Energy price in the Netherlands	€/KWH
CAP_{SBR}	CAPEX per SBR unit	$€/m^3$
OP_{SBR}	OPEX per SBR unit	$€/m^3$
E_{SBR}	Energy consumption per SBR	KWH/m^3
Q_x	Flow parameters	m^3/h
TDS_x	TDS concentration at certain treatment point	g/m^3
C_SyHr	Ion transport capacity of SyHr	h^{-1}
A_SyHr	Area of one SyHr protein	m^3

General Introduction

The need for new desalination methods

Global Water Stress

Life on our planet depends greatly on water. Unfortunately, due to increasing consumption from populations and industries and in some locations also climate change, freshwater demand is quickly starting to exceed supply. By 2025, 50% of the worlds population is expected to be living in a water stressed area, which causes or enhances several problems such as disease, poverty and conflict (World Health Organisation, 2019). The upcoming shortage is expected to elevate the socioeconomic status of water to be equivalent to that of oil in the future, with water rights already being traded between countries (Wahl (2013); Savenije (2002)). Just as oil, water is also being used as a power tool, for example when Israel threatened Lebanon with diverting the Wazzani river and the long-standing conflict between Pakistan and Afghanistan on the Kabul river basin (Luft (2002); Atef et al. (2019)). Besides human wellbeing and peace, the increasing water shortage has devastating implications for nature as well. It is becoming increasingly urgent that new knowledge and technologies to tackle these problems emerge from the fields of science and engineering. A solution may be found in innovative water collection and/or treatment methods, adapted to the aqueous resources of a specific area.

The distribution of global water resources: predominantly saline

A stringent shortage of water is a strange problem to have on a planet of which the surface consists of water for 70%. However, the vast majority of this water (97,5%) is saline ocean water, which is not directly potable to humans, leaving only 2,5 % freshwater. Of this water, less than one third is available currently, as 69,5 % is stored in ice caps and glaciers (Gleick, 1993). Of the 30,5% accessible freshwater, the vast majority is nonrenewable, meaning that it has accumulated slowly over time and cannot easily be regenerated naturally. This distribution of water resources is shown in Figure 2.

When examining this availability of water resources, it seems like a logical solution would be to use the abundant seawater as a water resource. The prectice of removing ions from seawater in order to produce fresh water, or less saline water, is called desalination. In recent years, treating seawater through desalination has been taken more seriously as a scalable solution to freshwater shortages. Consequently, the technologies to enable this have been further developed and use of desalination is increasing. The Global Water Intelligence (2017) reports that in 2016 80 Mm^3 of water was being desalinated every day, in comparison to 30 Mm^3 in 2013. Currently, the regions most relying on seawater desalination are Africa and the Middle East (Badruzzaman et al., 2019).

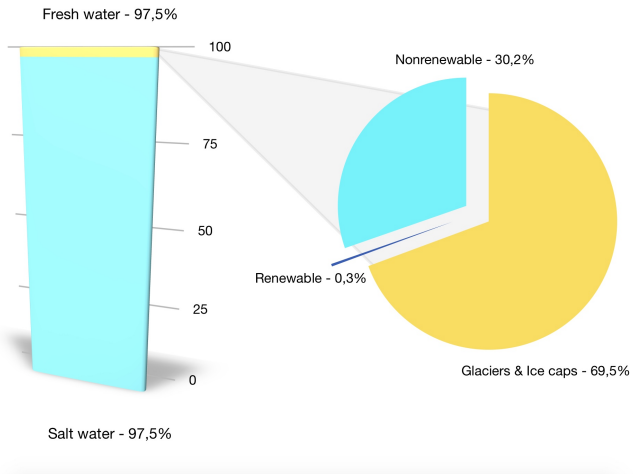

Figure 2: Distribution of water resources. Own figure based upon Gleick (1993).

Current Methods of Desalination

The two most widely applied desalination methods are (Sadrzadeh and Mohammadi, 2008):

- **Membrane based**
  Membrane based desalination methods include Reverse Osmosis, Forward Osmosis and Electrodialysis. The majority of modern desalination plants (>65%) use Reverse Osmosis (RO). In RO, a saline solution is pushed by both mechanical and osmotic pressure through a semipermeable micropore membrane. The water can be collected at the other side of the membrane. Due to the water pressure needed for RO in the case of a highly saline inflow and the additional freshwater needed to clean the membranes, this technology is seen as far from efficient for seawater at this point in time. In addition, a concentrated saline brine is formed, which must be disposed of in some way. Furthermore, in the case of highly saline solutions such as seawater, the membranes are fouled relatively quickly and must be chemically cleaned and changed often, which produces solid and liquid waste. Pretreatment of seawater before RO can help reduce fouling and energy consumption (Badruzzaman et al., 2019). Costs for RO are around €1 per m^3 and energy consumption is around 2,5 kWh/m^3 just for the RO unit, and about 3,6 kWh/m^3 including pretreatment and infrastructure (Voutchkov, 2018).

- **Thermal**
  As NaCl has a boiling temperature of more than 500 °C, and water boils at 100 °C, the salt will stay behind if one boils seawater: distillation. This technology has been around for millennia; In 1000 B.C. Greek soldiers at sea boiled seawater and caught the steam in sponges to obtain drinking water (Fairley, 1907). Modern technologies for thermal desalination are: solar distillation (SD), multieffect evaporationdistillation (MED), multistage flash distillation (MSF), thermal vapor compression (TVC) and mechanical vapor compression (MVC). Because of the high temperatures needed, distillation is very energy demanding unless solar heat/power can efficiently and reliably be used. The energy demand for thermal desalination is about 10-15 kWh/m^3, although this can be lowered by using solar or industrial heat. Therefore, costs are largely dependent on location. Without use of alternative heat flows, costs would be around €3 per m^3 (Voutchkov, 2018).

Combinations of these two methods are applied in hybrid desalination plants.

Need for new technologies

As mentioned previously, the main limitations of reverse osmosis are high energy consumption for seawater desalination due to the high TDS, production of a concentrated brine, solid waste from membrane changing and the use of chemicals to clean membranes and to avoid scaling and fouling. The main issue of thermal desalination is the large energy input needed, which is unrealistic in most geographical locations. As these solutions can not at present solve the increasing scarcity of water worldwide, it is worthwile to aspire novel methods of desalination and to research their feasibility.

Biodesalination: taking note from nature

In order to advance in desalination technology, science must take inspiration from creative angles, including processes occurring in nature. There are many natural occurences of desalination to facilitate life; after all, many organisms have been at home in saline environments for millenia without excessive energy consumption or waste production. Some fascinating examples are mangrove trees which are able to grow in coastal areas due to an extremely efficient filter system, marine birds which can drink seawater and expel brine from their nostrils using a salt-secreting gland and of course fish gills, which posess a multitude of ion pumps to either take up or extrude ions in respectively fresh- or saline water (Popp et al. (1993); Schmidt-Nielsen (1960); Evans et al. (1999)).
Studying such examples has led to the birth of the research field of biodesalination, which refers to all desalination methods which find their origins in nature. There are three main classes of biodesalination, which are listed here including examples (Taheri et al. (2016); Vullev (2011)):

- Direct application of natures desalination capacity.
  Use of green algae for desalination, microbial desalination (cells) and genetically engineered species which accumulate sodium (not yet realised)

- Biomimicry, where biological systems or their elements are considered as prototypes to be further advanced.

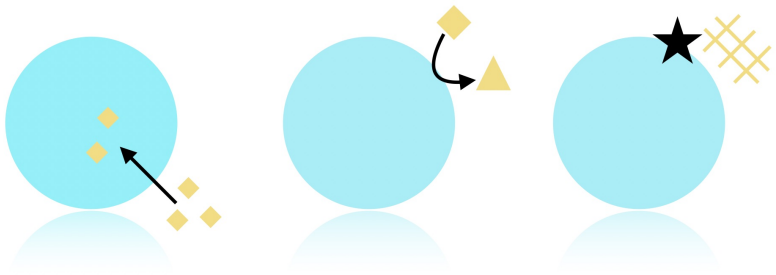

Figure 3: A graphical representation of three possible mechanisms of microbial desalination: uptake, ion exchange or precipitation. Own figure.

Use of aquaporin in artificial membranes, biological systems for sodium detection and removal (not yet realised)

- Bio-inspired desalination, where the applied engineering approach is inspired by a natural process, but constructed artificially.
  Tree-like membranes, smart polymers in forward osmosis, superhydrophobic modification for membranes, bio-inspired surfaces for desalination

Biodesalination research has yielded many interesting proposals and a first collection of exploratory research, but at this point the technologies have not yet made it to the foreground of desalination science in prominent journals, or seen any full scale trials. Furthermore, an evaluation of feasibility is often lacking.

Microbial Desalination

Definition and scope

Within the field of biodesalination, microbial desalination* is a subject which has been researched in an exploratory manner by various groups. Within this thesis, microbial desalination is defined as the direct use of microbial processes to remove ions from a saline solution. Where some, such as Amezaga et al. (2014), present a not yet executed microbial desalination concept, including scale sketches, based on the objective of microbial desalination for drinking water with genetically manipulated organisms, others such as Sasaki et al. (2017) sees it as a means to fertiliser, achieving a remarkable sodium removal of 94% using the organisms *Rhodobacter* and *Rhodovolum*. Lababpour (2017) achieve simultaneous lipid production and a reduction of electroconductivity to 43,5%, using *Spirulina*. Unfortunately, in these papers the methods section and conclusions are lacking in specific information such as biomass concentration and exact medium composition, which makes the research difficult to replicate and validate. An extensive review of published research on microbial desalination can be found in Section 1.2.

Mechanisms

Although a dozen groups have observed some kind of decrease in ion concentration (See Section 1.2), no research has been done (or at least, has been found in a literature review) which specifically researches and identifies a cellular mechanism of desalination. I have formulated three physical mechanisms of microbial desalination, which are pictured in Figure 3. The places where these three mechanisms could take place are the interior of the cell, the environment (medium/seawater) or on the exterior of the cell (around cell membrane or the EPS). EPS are biopolymers produced by the organism which form a matrix on the exterior of the cell (Wingender et al., 1999). Ions could in some way be bound to these polymers, or exhanged with other ions. It has been shown by Mishra and Jha (2009) that the amount of EPS increases with salt concentration, so perhaps the amount of ions which is encapsulated in or bound to this EPS increases as well. It could be some kind of buffer to protect the cell from salinity.
In the first mechanism, ions are taken up from the environment into the cytoplasm over the cell membrane.

*With the term microbial desalination, I refer to the direct removal of ions from a solution by microorganisms, and not to microbial desalination cells, which generate a current from biomass activity and use this to pull ions across a membrane. Although this research is also interesting, it is of a different nature than microbial desalination as cells do not bind or take up ions, and therefore not relevant to discuss extensively. Also, this technology is currently not expected to become scalable (Sevda et al. (2015); Sophia et al. (2016)).

There is a multitude of membrane channels and carriers for anions and cations which can transport ions both into and out of the cell, the presence and prevalence of which depends on species and the environment. In saline environments, these carriers and channels are hard at work to ensure the cell remains alive. Ion levels at the interior of the cell must be sufficiently low to avoid toxicity, but sufficiently high to minimise the osmotic difference between the cell and the exterior and thereby to avoid the cell shrivelling (Cambridge et al. (2017); Amtmann and Sanders (1998)). For microbial desalination, the question would be: what would be the benefit to the cell, the driving force, to take up substantial amounts of ions from the saline broth? And how can a higher uptake be stimulated using environmental biotechnology techniques?
Another possible mechanism by which a descrease in sodium and/or chloride could be seen, would be exchange of ions. This would most probably occur in the extracellular polymeric substances, the EPS. In the case of ion exchange, electrical conductivity would not decrease when sodium and chloride concentrations decrease, as the charge of sodium is replaced with the ion it is exchanged for. What is difficult to understand about this hypothesis, is that the most abundant ions by far in seawater are sodium and chloride, which do not easily react with other ions. Therefore, it may be unlikely that they replace ions such as magnesium, calcium, sulphate or iron in the EPS, or that they are taken up in the EPS.
Finally, cells may facilitate precipitation of minerals in some way. There are three methods of biological precipitation processes (Anbu et al., 2016): biologically controlled (when cellular activities are specifically aimed at precipitation), biologically influenced (passive precipitation due to interaction of the environment with the cell exterior, for example EPS) and biologically induced (when the chemical balance in the environment shifts due to cellular activity leading to oversaturation and precipitation of minerals). It is more straightforward to form salts which do not include sodium or chloride, as these salts tend to have a high saturation index and therefore are very soluble. However, the organism *Halomonas* has been reported to precipitate Halite, solid NaCl, amongst other minerals. Bacterially remediated mineralisation has also been proposed as a post-treatment for brine, to precipitate out the metals in order for the brine to be less damaging to the environment (Achal et al., 2012).

Advantages and limitations of microbial desalination

There are several advantages to microbial desalination. Firstly, as desalination could be performed in a standard, large sized reactor, this saves the use of intricate or vulnerable components such as membranes, which makes it attractive from both a financial and practical point of view. Another expected advantage of microbial desalination, is that it relies principally on chemical energy of the cell, and therefore external energy input is expected to be lower than in the case of current seawater desalination. Furthermore, no harmful chemicals are expected to be needed for microbial desalination and no non-biodegradable solid waste is produced, as is the case with RO membranes which need to be replaced.
A limitation of microbial desalination could be separation of biomass from the liquid fraction. How this will work out in practice greatly depends on the design of the desalination setup, and characteristics of the biomass such as settling rate. A large centrifuge or settling procedure should be feasible to design. Obtaining enough biomass for desalination is also important, however it may be possible to use the excess biomass from a regular water treatment facility. The question of disposal of the sludge is also relevant; perhaps the sludge can be used for biogas production as in a regular WWTP, if the exposure to salinity is not a problem. Finally, it must be researched up to which extent microorganisms can remove ions; whether desalination becomes less effective at lower sodium concentrations.

Knowledge gaps

The work which has currently been done on microbial desalination (described in detail in 1.2) is varied in experimental set-up and choice of organisms, but exclusively focused on pure cultures of one organism. Therefore, it is not yet known whether mixed cultures are capable of microbial desalination, even though there are financial and practical benefits to operating mixed cultures. The optimal conditions for microbial desalination have also not been extensively examined; the effect of choices such as inoculation concentration, medium composition and pH control on desalination performance. Furthermore, results from previous experiments have not yet been used to indicate or eliminate possible mechanisms of ion sequestration, thereby extending fundamental knowledge on this topic. In experiments where sodium is seen to decrease, the inorganic suspended solids fraction has not been reported, which would indicate where the ions have gone. Finally, most articles only report on removal of sodium, not including other ions.

Furthermore, although an increasing amount of microbial membrane proteins for ion transport is being discovered and examined, they have not yet been applied to the field of desalination in artificial membranes. Artificial membrane research for desalination is mainly based on aquaporin, which is a water pump and not an ion pump. Using a light powered ion pump such as Halorhodopsin, no external pressure would be need to be applied. The construction of such a membrane has not yet been attempted. The current state of biomimetic membrane research is described in detail in 2.2.
Up to now, microbial desalination was only examined in small scale batch or reactor experiments and focused experiments in the case of biomimetic membranes. Although this contributes to fundamental knowledge and the development of microbial desalination technologies, there has (to the knowledge of the author) not yet been a quantitative analysis of whether this concept could be competitive with seawater RO within a full scale seawater treatment line-up. Such research is relevant as it would enable a comparison to current desalination methods in terms of investment costs, energy efficiency and waste production. Also, such an analysis is valuable to assess whether microbial desalination is worth researching, or whether it is predicted to never become scalable.

Research Objectives

The overall research question for this thesis is:

"Is there potential in using biological principles to desalinate seawater?"

Three research lines were chosen in order to arrive at answering this question. A sub-objective has been assigned to all research phases, as shown in Table 2.

Table 2: Objectives for the three Research lines

Phase	Wet/dry lab	Objective
1. Investigation of microbial desalination	Wet lab	Quantifying microbial desalination performance of mixed culture under various conditions by a series of batch experiments.
2. Biomimetic Artificial Membrane	Wet lab	To develop the concept of a light driven desalination membrane containing SyHr.
3. Conceptual design of full scale system	Dry lab	To evaluate the feasibility of two scalable microbial desalination designs.

Research Approach

This chapter describes the research approach which was applied in this project and the manner in which this thesis is composed.

Research structure: from the laboratory to an engineering approach

Research phases

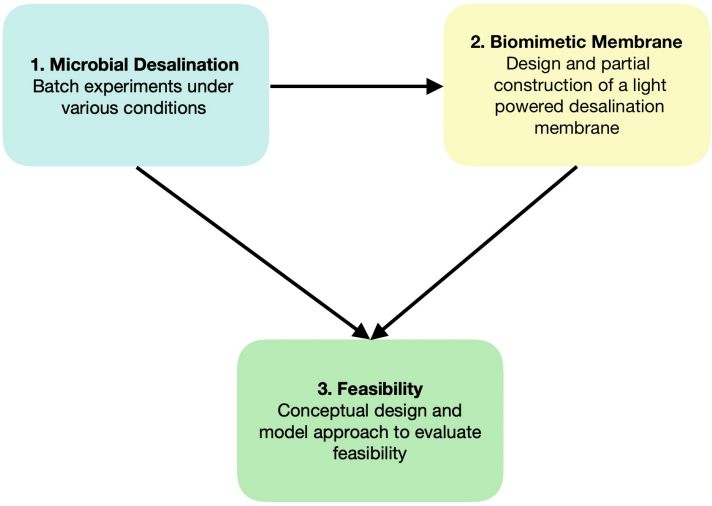

Figure 4: Research structure of this thesis, divided in three research lines

Due to the absence of fundamental knowledge within this topic and the limited prior research which was available, an exploratory research approach was chosen consisting of three research lines. The overall structure of this research approach is shown in Figure 4. Firstly, the practical process of microbial desalination was researched in a series of shake flask experiments, and the optimal conditions for ion removal were examined. As fully examining and understanding all mechanisms of microbial desalination is beyond the scope and duration of this project, it was chosen as a next step to examine ion uptake by means of a bio-inspired membrane. This is a synthetic membrane which is supplemented with cellular transport proteins. In order to do this, membrane proteins were expressed and purified. With data from both the shake flask experiments and the membrane research, two conceptual designs for a full scale microbial desalination operation could be made, with corresponding models which enabled comparison to current desalination methods. This enabled a rudimentary evaluation of the feasibility of microbial desalination for practical application.

Structure of the thesis

As the three research lines are different in background, methods and nature, it has been chosen to write a separate article for every one of them within this thesis, as is prevailing in PhD dissertations. In this manner, the topics can be introduced, described and discussed with more clarity. The thesis commences with an overall introduction into the topic of microbial desalination and the objectives of this research. Following this, the three articles are included. Chapter 1 describes the batch culture experiments which were conducted, Research line 1 in Figure 4. Chapter 2 describes research line 2; the concept of a bio-inspired desalination membrane, the purification process and the results of this research. Chapter 3 describes the model which was constructed, research line 3. The thesis ends with an overall conclusion, which discusses the main conclusions, outlook and impact of this work as a whole.

1

Determining the Optimal Conditions for Microbial Desalination through Batch Experiments

1.1. Abstract

Microbial desalination is the direct use of microbial processes to remove ions from a saline solution. Experiments have previously been described where microbial desalination is proposed or achieved using pure cultures or genetically modified organisms, but never using mixed cultures such as activated sludge, which is widely available in wastewater treatment plants. In this section, a series of mixed culture batch experiments are described, which were conducted with the objective of gaining knowledge on the optimal conditions for microbial desalination. Sodium profiles over time were made of experiments, in which the varying conditions were medium composition, pH control, type of biomass (PNSB enriched, activated sludge and green phototrophs), light or dark conditions, inoculation concentration (0,02 gVSS/L, 0,2 gVSS/L and 2 gVSS/L), initial sodium concentration and condition of biomass (inactive/active). In all samples except one containing a pure Rhodopseudomonas culture, sodium removal from the medium was observed of 20-30 %. It was found that light is not needed for desalination, chloride was also removed besides sodium, inactive biomass can still remove ions, ion exchange is not a desalination mechanism and there is no linear correlation between biomass concentration and ion removal. By fitting curves to relative sodium removal data, two parameters were obtained by which all experiments could be compared: K_{Na}, a rate constant which indicates speed of sodium removal and $R_{Na,max}$, which is the maximum percentage of sodium which is expected to be removed. The best performing desalination condition was found to be inactive mixed culture PNSB biomass, in seawater medium, without buffer and at an inoculation concentration of 2 gVSS/L. Under these conditions, $R_{Na,max}$=39% and K_{Na}=0,27. A 39% removal can be considered successful as this already lowers costs for further desalination significantly and a the K_{Na} indicates that after 1/0,27=3,7 hours, half of maximum removal is reached. A novel mechanism for ion removal is proposed, wherein ions which enter the cell are directed towards a bacterial microcompartment, which is a virus like pocket within the cell, which is widespread in microorganisms and archae. The local sodium and chloride concentration here would be sufficiently high to precipitate sodium chloride or halite, which enables storage without high osmotic gradients.

1.2. Introduction

Previous research: a variety of organisms

Practical demonstration of sodium removal Sasaki et al. (2017) have achieved a remarkable maximum sodium removal of 94 % through microbial desalination. The objective of this research was to produce fertiliser by desalinating seawater. The organisms used are *Rhodovolum* and *Rhodobacter* subsequently, with a centrifugation step in between. They hypothesise that sodium and chloride may be bound to the external cell surface as EPS, but do not propose a molecular mechanism for such binding. A desalination experiment was conducted under both light and dark conditions, which did not effect the final removal significantly. Unfortunately the concentrations of biomass are not reported and some medium components ("nutrients") are not specified, making this experiment difficult to replicate.
Desalination using *Spirulina* has been described by Lababpour (2017). *Spirulina* has been cultivated in a photo bioreactor in various brine media, leading to maximally 43,5% decrease in electroconductivity and 71% decrease in total dissolved solids. A collateral advantage of this desalination method is biomass production, as *Spirulina* can be used for biofuel production or as a food product. It was observed that after a lag phase of two days, *Spirulina* is able to grow under saline conditions. This same principle of simultaneous biomass growth and desalination has also been applied by El-Sayed and Abdel-Maguid (2010) and Gan et al. (2016), however with *Scenedesmus*, another algal species which is used for lipid production.It has been found by Gan et al. that at higher medium salinities (up to 8,8 g/L sodium chloride) ion removal was highest; around 30%, but at low salinities lipid production was higher. Growth was also lower at high salinities. El Sayed uses four different dilutions of Red Sea water (20,4 g/L sodium chloride) and finds the highest sodium removal rate (0,36 g/day) at 50% dilution. The biomass concentration approximately icreases from 1 to 2 gVSS/L during the experiment.

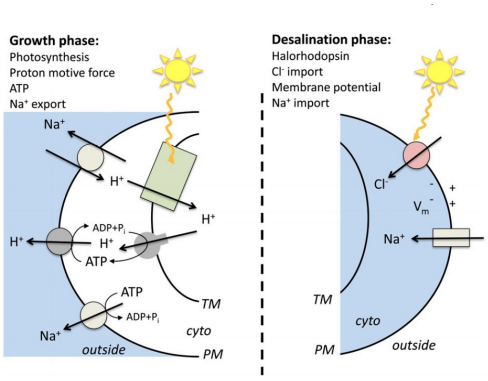

Figure 1.1: A concept for light-powered ion transport over a cell membrane: during standard cell growth, pictured left, there is active and passive Na^+ export. If cells are depleted in ATP, passive or voltage gated Na^+ import will occur until the cell is in osmotic equilibrium with the environment. When the solar powered transporter Halorhodopsin is successfully expressed in Cyanobacteria, Cl^- will be actively imported. From Amezaga et al. (2014)

Concepts featuring genetically modified organisms Amezaga et al. (2014) describe the potential of biodesalination using cyanobateria, sum up the possibilities for manipulation of environmental conditions or the cyanobacterial genome to enhance uptake of sodium and Chloride ions and propose a molecular mechanism for ion import. This mechanism is sketched in Figure 1.1. In the proposed mechanism, cyanobacterial cells are manipulated to express Halorhodopsin, a light-gated chloride importer (Engelhard et al., 2018). Under regular circumstances cells function normally and grow until a high cell density is reached. Sodium ions are exported actively from the cell. Consequently, ATP is depleted by changing the light wavelength and inducing a phosphate shortage. Active export halts. A passive sodium and chloride import will take place until equilibrium with the environment is reached. Halorhodopsin will still function in the absence of ATP, as it is light powered, so chloride will be imported if light is supplied. To maintain neutral charge, sodium will be imported too by voltage gated channels. Although it is a great achievement that this research group has actually formulated a physiological mechanism for biological desalination, this is still merely a proposal. No results or progress have been published or

otherwise made known.* Besides this, gene manipulation may not be necessary for this plan as various types of cyanobacteria express Halorhodopsin naturally (Inoue et al. (2014); Hasemi et al. (2016)). Sung et al. (2012) also propose a microbial desalination method based on Halorhodopsin. They propose to express the Hr gene in *E.coli* so the biomass will take up chloride, in order to generate energy from the change in salinity using sodium manganese oxide and polypyrrole electrodes. They report on successfully crafting these electrodes, but not on any desalination results. As these ideas require genetically modified organisms, they are expected to be unsuitable for direct use in large scale water treatment as such organisms may be harmful to humans and the natural environment.

Precipitation research There are three methods of biological precipitation processes: biologically controlled (when cellular activities are specifically aimed at precipitation), biologically influenced (passive precipitation due to interaction environment-cell exterior, for example EPS) and biologically induced (when the chemical balance in the environment shifts due to cellular activity leading to oversaturation and precipitation of minerals) (Anbu et al., 2016). If one of these precipitation mechanisms could be exploited for halite precipitation in seawater, perhaps by creating some kind of oversturated "pockets" within the cell, it would offer new perspectives for biological desalination. Arias et al. (2017a) have reviewed the application of ureolytic bacteria for biomineralisation. They have reported promising results for the removal of calcium and magnesium ions from seawater, but not sodium and chloride. Arias et al. (2017b) has reported in another study, that the halophilic organism *Halomonas* is capable of precipitating halite, the crystal form of sodium chloride, which offers perspectives for seawater treatment. 57% of the examined precipitate consisted of halite.

Settling capacity of desalination biomass A practical issue of using biomass for desalination, is separation of the saline biomass from the liquid. As EPS have been reported to increase in quantity in saline environments, it is predicted that cells may agglomerate more, making them easier to separate (Mishra and Jha, 2009). Minas et al. (2015) measured macromolecules on the surface of two algal species (*Synechococcus PCC* and *Synechocystis PCC*) under saline conditions, and modeled agglomeration of biomass using XDLVO modelling. *Synechococcus PCC* showed more proteins on the cell surface and *Synechocystis PCC* showed more polysaccharides. However, the results of the model indicate that irreversible agglomeration would not occur for either species.

Research gaps

The work described in the previous paragraph is varied in experimental set-up and choice of organisms, but exclusively focused on pure cultures of one organism. When considering microbial desalination for practical applications, this has a number of disadvantages;

- It requires sterile conditons to keep the pure culture conditions. For large scale application and non-artificial inflow, this would be expensive, inefficient and infeasible.
- Mixed cultures are more robust, as the composition of the community is ever evolving to adapt to changing circumstances. Therefore, they can generally handle more fluctuating and more extreme conditions than a pure culture.
- In mixed cultures, syntrophism and other forms of symbiosis can take place, enabling organisms to support each other(Schink and Stams, 2013). This can reduce operational costs and the amount of chemicals used.

As despite these advantages, no publications describing mixed culture microbial desalination research have been found, quantifying mixed culture desalination performance has been chosen as experimental focus for this research line.
Furthermore, the removal of other ions than sodium have not been quantified, specifically chloride. By quantifying concentrations of other ions over time, the mechanism of ion exchange can also be confirmed or excluded as a possible desalination mechanism.
Currently, there is also a lack of knowledge on how certain experimental conditions influence desalination performance. This is the case for the effect of pH control, inoculation concentration and active or inactive biomass.

*An enquiry to the contact person of this article, prof. A. Amtmann, was left unanswered

Another research gap is where the ions which are removed go, when a decrease is seen in concentration. A simple analysis of volatile and inorganic suspended solids (VSS, ISS) could clarify whether the ions are present in or around the biomass fraction.
Finally, previous works have not included an analysis which links back the desalination results to a possible mechanisms of desalination.

Research Objective

The overarching research objective of this research line is:

> **Quantifying microbial desalination performance of mixed cultures under various circumstances by a series of batch experiments.**

Three batch experiments using shake flasks were designed and performed. Table 2.1 shows these experiments with their respective research questions.

Table 1.1: Research questions for the three experiments of the batch experiment research (phase 1).

Experiment	**Objective**
Pure culture versus Mixed Culture	Are sodium ions removed? What is the pattern of removal? What is the performance of pure vs mixed culture?
Artificial seawater, types of biomass and pH control	Can different types of biomass perform desalination? Does pH control influence sodium removal? Is growth required for desalination? Are the measurements of the sodium probe correct? Are other ions than sodium removed? What is the influence of artificial seawater as medium?
Inoculation concentration and live/inactive biomass	What is the influence of inoculation concentration on sodium removal? Do cells need to be active to remove sodium? Does total electrical conductivity decrease?
Quantifying Inorganic Suspended Solids	Does biomass cause the observed sodium decrease? Is an increase of the fraction ISS/TSS seen? Are ions stored around or inside the cell?

1.3. Materials & Methods

Culturing and sampling

Infrared illumination setup Unless otherwise stated, all experiments were illuminated continuously (Gamma Breedstraler lamps, 120 W), filtered for infrared light (>700 nm).

Inoculation Medium was inoculated with biomass according to specific requirements per experiment using a syringe (BD Plastipak, 2,5 mL). Pressure shake flasks (50 or 100 mL) were capped with rubber stoppers and aluminum caps. Flushing with argon was performed for five minutes per flask to ensure an anaerobic environment.

Sampling Samples were taken daily, from which firstly the biomass concentration was determined spectrophotometrically as described above. Subsequently, samples were centrifuged (15 min, 13000 rpm), after which the supernatant was filtered with a syringe filter (Whatman, 0,45nm). Sodium concentration was determined using the Twin Sodium Meter (Laqua), of which the procedure is described below. Pellet and supernatant were frozen and stored.

Analytical methods

Considering that for some analytical tools the methodology was identical, some descriptions were adapted from the work of Kim (2018).

Optical density measurement for biomass concentration Biomass growth was analysed spectrophotometrically by measuring the absorbance (λ = 660 nm). This absorbance value was converted to a biomass concentration by means of a calibration curve, which was made using the method next described. If the value given by the spectrophotometer was above 0.500, a dilution was prepared and another measurement performed.

Determining biomass concentration by incineration Biomass dry weight was measured to translate optical density to biomass concentration (gVSS/L) using a calibration line. Samples of known volume were first centrifuged (5 min, 12,000 rpm) after which the pellet was resuspended. Subsequently the biomass was dried on glass fiber filter membrane (24 h, 105°C), which had been previously weighed after drying (1 h, 105°C). Dried samples were burned in the oven at (2h, 550°C). Residual ash mass was determined and the VSS concentration was calculated as follows:

$$VSS = \frac{(m_{dry} - m_{empty}) - (m_{ash} - m_{empty})}{V_{sample}} \tag{1.1}$$

where:
m_{dry} = Weight of dried sample in g
m_{empty} = Weight of empty filter membrane in g
m_{ash} = Residual ash weight in g
V_{sample} = Volume of liquid sample in L

Determining Na+ Concentration using a sodium meter For an instant indication of the concentration of sodium ions in solution, the Twin Sodium Meter (Laqua)[†], which uses an ion-selective membrane in combination with a conductivity meter, was used. 400 µL of sample was diluted 1:1.

Determining electroconductivity using an EC meter Electroconductivity was determined for samples using the Twin EC Meter (Laqua)[‡]. Samples were not diluted.

Ion Chromatography for multi-ion analysis Sodium, potassium, calcium, magnesium, ammonium, sulphate, phosphate and chloride were measured by means of ion chromatography (IC, Metrohm IC 883 Basic IC Plus) with a Metrosep C4 – 150/4.0 column and Metrosep RP2 Guard/3.5 guard column and as eluent 3 mM HNO_3 at 0.9 mL/min). Samples were diluted 10x and 100x in order to fit the range of the machine both for the prevalent and scarce ions in seawater.

Genetic sequencing for community analysis DNA was extracted from the supernatant of samples using a UltraClean Microbial DNA Isolation Kit (Mo Bio). Extracted gDNA samples were sent to Novogene (China) for 16s rRNA gene amplicon sequencing.

[†] https://www.specmeters.com/nutrient-management/nutrient-meters/sodium/laqua-twin-sodium-meter/
[‡] https://www.specmeters.com/nutrient-management/ph-and-ec-meters/ec/laqua-twin-ec-meter/

Media

Stevens medium A PNSB medium recipe was taken from Ouboter (2017) who adapted it from Stevens (2017). All components are listed in Table 1.2. This medium will hereafter be referred to as Stevens medium.

Table 1.2: Stevens medium composition (Ouboter, 2017)

Component	Concentration (mg/L)
$CH_3COONa \cdot 3H_2O$	914 mg
KH_2PO_4	14
K_2HPO_4	21
NH_4Cl	229
$MgSO_4 * 7H_2O$	200
$NaCl$	200 or 24000 (for saline)
$CaCl_2 * 2H_20$	50
Yeast extract (BD BactoTM, ThermoFisher)	0,100
Liquid components	
Vitamin Solution	1 mL
Trace Element Solution	1 mL
HEPES buffer	4,7 mL

Seawater medium Table 1.3 shows the theoretical concentration of the medium hereafter referred to as "seawater medium". These values were obtained by ICP-OES[§].

Table 1.3: Composition of InstantOcean artificial seawater medium, supplemented with nutrients (de Graaff et al., 2019)

Component	Concentration (mg/L)
Na^+	8995
$K+$	560
Mg^{2+}	1107
Ca^{2+}	404
Sr^{2+}	14
Cl^-	15240
SO_4^{2-}	608
$PO_4^{3-} - P$	1,3
$NO_3^- - N$	12
$SiO_3^{2-} - Si$	97
Li^+	307
Si^{4+}	370
Mo^{2+}	<0,1
Ba^{2+}	95
Ni^{2+}	82
Cr^{3+}	321,26
Al^{3+}	0,02
Cu^+ / Cu^{2+}	0,6
Zn^{2+}	0,06
Cd^{2+}	22
Pb^{2+} / Pb^{4+}	358
Ag^+	205
Ti^{3+} / Ti^{4+}	26
Liquid components	
Vitamin solution	1 mL
Trace element solution	1 mL

[§]These values were kindly supplied by Danny de Graaff

Pure culture *Rhodopseudomonas* vs mixed culture PNSB

A batch experiment was performed with two types of biomass, a pure *Rhodopseudomonas sp.* (hereafter referred to as *Rhodopseudomonas*) culture isolated by Ouboter (2017) and a mixed culture inoculum which was taken from a continuous reactor containing predominantly PNSB which was enriched by Stevens (2017) and Ligtenberg (2017) from an activated sludge sample from Harnaschpolder WWTP, The Netherlands. Stevens medium was prepared at two NaCl concentrations: saline (24 g/L) and control (0,200 g). 100 mL shake flasks of both the saline and control medium were inoculated with either *Rhodopseudomonas* or mixed PNSB culture at 0,01 gVSS/L, leading to the experimental conditions described in Table 1.4.

Table 1.4: Composition of the bottles for Experiment 1A, run in duplicate.

Inoculum	**Concentration NaCl (g/L)**
Rhodopseudomonas	0,2
Rhodopseudomonas	0,2
Mixed culture	24
Mixed culture	24

Flasks were cultured (10 days, 170 rpm, 35 °C, infrared illumination). The experiment was performed in duplicate.

Artificial seawater, types of biomass and pH control

In this experiment three types of biomass (green phototrophs, activated sludge and PNSB) were cultivated with and without a buffer for pH control. Seawater medium was used as foundation of the medium for all samples. When pH was buffer controlled, HEPES buffer was added (4,7 g/L). Under the non-pH controlled conditions, pH was set at 7 with concentrated acid (4M HCl) or base (4M NaOH). Medium composition per sample is specified below and summarised in Table 1.5. The experiment was run in duplicate.
For PNSB, two types of medium were used. In order to examine the influence of using seawater on results, one type of medium was made with all ingredients of Stevens medium, containing seawater medium instead of demi water. The second type of medium was seawater supplemented with sodium acetate (5 gCOD/L), KH_2PO_4 (0,01 g/L), K_2HPO_4 (0,01 g/L), NH_4SO_4 (0,45 g/L), $CaCO_3$ (0,5 g/L). As before, a mixed PNSB sample was taken from the continuous reactor as inoculum. The PNSB flasks were illuminated with infrared light as described in the general methods.
For green phototrophs, the medium was supplemented to select for autotrophs with KH_2PO_4 (0,01 g/L), K_2HPO_4 (0,01 g/L) $NaNO_3$ (0.1 g/L), $CaCO_3$ (0.5 g/L) and NH_4SO_4 (0,45 g/L). No organic carbon source was added. The green phototrophs were kindly supplied by Wageningen University.[¶] In addition to this, an enrichment was succesfully performed for green phototrops, which is described in Appendix D. Flasks were illuminated by a white LED light (LED breedstraler, 100 W).
For activated sludge, the artificial seawater was supplemented with sodium acetate (5 g COD/L), KH_2PO_4 (0,01 g/L), K_2HPO_4 (0,01 g/L), NH_4SO_4 (0,45 g/L), $CaCO_3$ (0,5 g/L). The activated sludge was collected two weeks prior to the experiment (Harnaschpolder WWTP, the Netherlands) and stored (4°C). Activated sludge was cultivated under dark conditions.
Shake flasks (100 mL) were inoculated as described in the general methods section. Flasks were cultured (21 days, 190 rpm, 35 °C). Samples were taken six times in the first 24 hours and once every 24 hours after that.

Table 1.5: Composition of the shake flasks for Experiment 1B, run in duplicate.

Medium	**Biomass type**	**pH control**
Seawater + 5gCOD Ac	PNSB	pH7
Seawater + 5gCOD Ac	PNSB	HEPES
Stevens + seawater	PNSB	HEPES (within Stevens medium)
Seawater + supplements	Green phototrophs	pH7
Seawater + supplements	Green phototrophs	HEPES
Seawater + 5gCOD Ac	Sludge	pH7
Seawater + 5gCOD Ac	Sludge	HEPES

[¶]This co-culture consisted in equal parts of *Chlorella vulgaris, Anabaena flosagna, Haematococcus pluvialis, Scenedesmus obliquus* and *Synechococcus elongatus*, which had been cultivated at 21 °C.

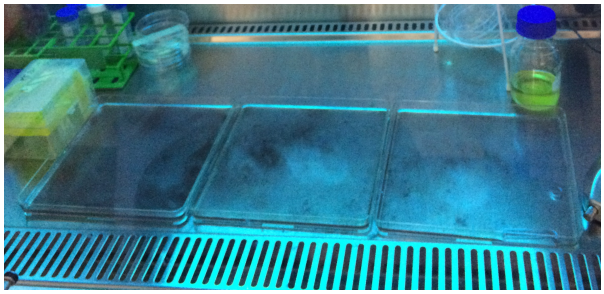

Figure 1.2: Method of UV irridiation

Inoculation concentration and active/inactive biomass

Seawater medium was supplemented with sodium acetate (5gCOD/L). The inoculum taken from the same PNSB reactor as in previous experiments. Half the biomass was inactivated by exposure to UV irradiation in large flat plates as shown in Figure 1.2 (2 h). This part of the biomass is hereafter referred to as "inactive". The other half was not subjected to any treatment, hereafter referred to as "active".
Shake flasks (50 mL) were filled with medium and inoculated with both biomass fractions (inactive and active) at three concentrations, as shown in Table 1.6. The experiment was carried out in duplicate. Flasks were cultured (200 h, 190 rpm, 35 °C, infrared illumination). Sampling frequency during the first 24 hours was five times, thereafter once every day. Electroconductivity measurements were performed without duplicates.

Table 1.6: Composition of the shake flasks, run in duplicate

Inoculation Concentration	**Inactive/active**
0,002 gVSS/L	Active
0,002 gVSS/L	Inactive
0,02 gVSS/L	Active
0,02 gVSS/L	Inactive
0,2 gVSS/L	Active
0,2 gVSS/L	Inactive

PNSB Desalination including ISS measurement

In this experiment, seawater medium supplemented as described above for PNSB culturing was inoculated with PNSB mixed culture biomass in duplicate. One blank sample was added, which contained only seawater medium without biomass. Culturing was performed as previously described (2 days, 170 rpm, 35 °C, infrared illumination). Samples were taken and analysed for sodium four times during the first 24 hours, and once a day afterwards. At the beginning and ending of the experiment, VSS and ISS were determined by incineration according to the methods described above.

Fitting of curves for sodium removal rates

Firstly, from the experimental data (sodium concentration over time), percentage removal plots were made, displaying time on the x-axis and removal of sodium in % on the y axis. The sodium concentration data points were converted to percental removal according to the following equation:

$$R_{Na,t} = \frac{[Na^+]_{t=0} - [Na^+]_t}{[Na^+]_{t=0}} \cdot 100\% \tag{1.2}$$

Wherein:
$R_{Na,t}$ = Sodium removal efficiency at a certain time point (%)
$[Na^+]_{t=0}$ = Initial sodium concentration (g/L)
$[Na^+]_t$ = Sodium concentration as measured at every time point (g/L)

As the shape of the sodium removal curves is generally one of steep initial sodium removal trailing off towards a plateau, an equation was formulated based upon Michaelis Menten kinetics:

$$R_{Na}(t) = R_{Na,max} \cdot \frac{t}{\frac{1}{k_{Na}} + t} \tag{1.3}$$

$$\lim_{t \to \infty} R_{Na}(t) = R_{Na,max} \tag{1.4}$$

Wherein:
R_{Na} = Sodium removal in (%)
$R_{Na,max}$ = Theoretical maximum sodium removal (%)
K_{Na} = Rate constant which indicates the velocity of sodium removal (h^{-1})
t = Time (h)

The meaning of the parameter k_{Na} is the following:

$$\frac{1}{K_{Na}} = t\left(\frac{1}{2} R_{Na,max}\right) \tag{1.5}$$

Or explained, K_{Na} is the inverse of the time it takes to reach half of the maximum sodium removal. The reason an inverse was chosen, is so that k_{Na} will increase when the removal is faster, which is a more intuitive way to compare sodium removal between experiments.

1.4. Results & Discussion

Pure culture *Rhodopseudomonas* versus Mixed Culture PNSB

This section describes the results of the experiment where sodium removal of *Rhodopseudomonas* and a mixed PNSB culture were compared.

Sodium concentration and biomass concentration over time The measured sodium and biomass concentrations over time for the samples in the saline (24 g/L NaCl) medium are shown in Figure 1.4a. For *Rhodopseudomonas* and the mixed PNSB culture, an initial decrease in sodium concentration after 24 hours was observed of respectively 0,4 g/L and 1,2 g/L. Considering the low biomass concentration at this point (0,01 gVSS/L to 0,1 gVSS/L from 0h to 24 h for mixed culture), an average removal of around 1,2 g/L is remarkable as it implies values of over 10 g sodium uptake per gVSS biomass. Such an an absolute removal in 24 hours is in the same range as values reported by Sasaki et al. (2017) (approximately 2g/L removal in 24 hours), although it is difficult to compare because biomass concentrations are not given in this article. In the case of *Rhodopseudomonas*, the observed removal is considered significant due to the sodium decrease which was measured being lower than the resolution of the device, which is 0,5 g/L. After 48 hours, the sodium concentrations for both *Rhodopseudomonas* and mixed culture increased with respectively 0,4 g/L and 0,8 g/L. Such a pattern is also seen in the first set of results of Sasaki et al. (2017), but later (between 3-5 days). It can be seen in Figure 1.4a that little to no growth was observed in the case of *Rhodopseudomonas*, whilst the mixed PNSB culture reached a concentration of around 0,5 gVSS/L biomass after three days. It can be concluded that under saline conditions, *Rhodopseudomonas* does not thrive. Figure 1.4b shows the sodium (a) and biomass (b) concentrations over time under the control conditions in non saline Stevens medium. For *Rhodopseudomonas* growth up to 0,2 g/L biomass was observed. A lower final mixed culture biomass concentration (0,32 gVSS/L) was observed under control conditions than under highly saline conditions. Apparently, on the balance of all the organisms present in the mixed culture, the salinity contributes to growth. The sodium concentration in this medium remains constant at a measured value of around 0,25 g/L (Figure 1.4b. The medium was composed to contain 0,2 g/L sodium chloride, which would correspond to 0,08 g/L sodium. However, a value of between 0,2-0,3 g/L sodium was measured. A plausible reason for this is that the lower precision limit of the used device is 0,23 g/L.

Community composition The community composition of the mixed culture at the beginning and end of the experiment is shown in Figure 1.3. Although initial reactor inocolum is mixed, with *Thiobaca* and *Rhodopseudomonas* as most prevalent species, it can be seen that at the end of the experiment *Rhodobacter* is the most common genus both in the case of saline and non-saline medium. Apparently culturing in shake flasks under these conditions favours *Rhodobacter*. In the case of saline medium, the dominance of *Rhodobacter* was more pronounced for both duplicates than on non-saline medium. For non-saline medium, the fraction of *Rhodopseudomonas* was lower in comparison to t=0 h, but still around a factor five larger than the saline medium cultures. *Rhodopseudomonas* being outcompeted in a saline environment corresponds with the results shown in Figure 1.4a, where the pure culture *Rhodopseudomonas* hardly grows under saline conditions. The visual appearance of the shake flasks over time can be found in Figure B.1 (Appendix B).

To conclude The research questions for this experiment were:

- Are sodium ions removed?
- What is the pattern of removal?
- What is the performance of pure vs mixed culture?

Sodium was removed; for both mixed culture duplicates a significant sodium removal of 1,2 g/L after 24 hours and a subsequent increase of 0,8 g/L after 48 hours were observed. The pattern under these conditions is of removal and release. In the case of the *Rhodopseudomonas*, no significant sodium removal was observed.

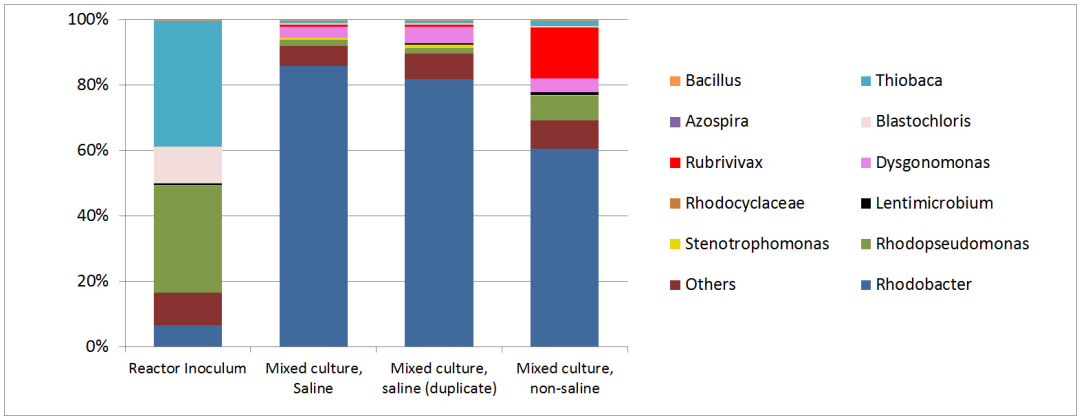

Figure 1.3: Community composition of the mixed culture in Experiment 1A, consisting of reactor inoculum at t=0 h and samples from both saline (duplicate) and non-saline shake flasks at the end of the experiment (t=220h)

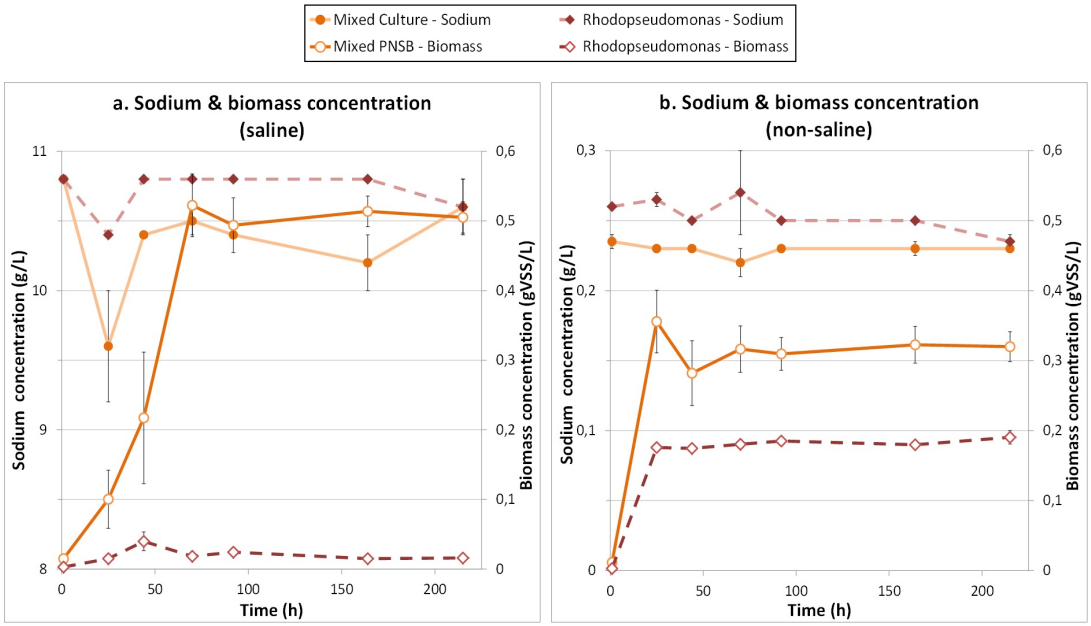

Figure 1.4: (a) Sodium and biomass concentration for mixed PNSB and *Rhodopseudomonas* cultures in saline (24 g/L NaCl) Stevens medium, based upon duplicate measurements. Spread between duplicates indicated by error bars.
(b) Sodium and biomass concentration for mixed PNSB and *Rhodopseudomonas* cultures in regular Stevens medium, based upon duplicate measurements. Spread between duplicates indicated by error bars.

Artificial Seawater, types of biomass and pH control

This section describes the results of the experiment wherein three different mixed cultures were compared for desalination capacity, with and without pH control (HEPES buffer).

Sodium concentration and biomass concentration over time Sodium concentration over time is shown in Figure 1.5a and biomass concentration in Figure 1.5b. For every type of biomass, sodium and biomass concentration are shown separately in 1.5c,d,e. It can be seen that all biomass types show an erratic sodium concentration decrease in the first 48 hours, generally followed by a more gradual decrease as time progresses.

In the case of PNSB, sodium removal of 2,3 g/L (Seawater without pH control), 2,3 g/L (Seawater with HEPES) and 1,4 g/L (Stevens medium components in seawater) was observed. The third type which shows the lowest removal, also had the lowest starting concentration of sodium. The difference in initial sodium concentration is caused by the different media, as the seawater medium was supplemented with 5 g sodium acetate. A less pronounced removal at lower initial salinity has also been observed by Gan et al. (2016). In terms of biomass concentration, the only PNSB samples which showed growth within the first 24 hours were the ones cultivated on Stevens medium components with seawater. After 48 hours, the samples in the medium without pH control started growing. The cultures in medium with HEPES buffer showed no growth for over 350 hours. A possible explanation may be that HEPES can degrade under influence of oxygen and light to form toxic hydrogen peroxide (Kirsch et al., 1998). Due to the flushing of bottles with argon, this should not have occurred sufficiently to hamper growth. Also, it is not logical that the peroxide would no longer inhibit growth after 350 hours. What is most remarkable, is that this lack of growth does not at all change the sodium uptake significantly in comparison to the other samples.

In the case of activated sludge (Figure 1.5c), sodium removal was observed to be 2 g/L (without pH control) and 2,15 g/L (HEPES). The first 350 hours, no growth was observed. The reason for this could be that under dark, anaerobic conditions one selects mainly for quite slow growing organisms such as methanogens. The mechanism for sodium removal is apprently not light driven, as removal is seen for the activated sludge samples which were cultivated under dark conditions. As previously noted for the HEPES PNSB, when there was no growth and low biomass concentration, the activated sludge was still able to remove a similar amount of biomass as other samples which were growing.

In the case of green phototrophs (Figure 1.5d), final sodium removal was 1,6 g/L (without pH control) and 1,75 (HEPES). After an initial sodium decrease comparable to the profile of sludge and PNSB, some pronounced increases and decreases in sodium concentration can be seen between 200-400 hours for all green phototroph duplicates (small error bars). The appearance of these increases and decreases may hint at some kind of cellular cycle. As the flasks were continuously illuminated, there was no light-dark cycle present to cause this. At the second sodium increase peak, the cultures had not been shaken for around 72h. This may be related but does not explain the other cycle. As shown in Figure 1.5e, all green phototroph samples took around 100 h to become acclimatised to the saline surroundings before growth was observed. Thereafter, the growth between the HEPES and pH7 cultures was comparable, although at some times large spread between duplicates was observed.

In comparison to the sodium concentration pattern in experiment 1, the sodium concentration was not observed to increase as strongly after decreasing in this experiment. This may be related to the seawater medium. The sodium chloride concentration differs from Stevens medium and there are many other ions present, which may interfere with uptake, binding or precipitation of sodium ions.

The visual appearance of the shake flasks over time can be found in Figure B.2 (Appendix B). In Appendix E, supplemental information concerning this experiment can be found, such as extended graphs up to 1005 hours, acetate concentration and community composition.

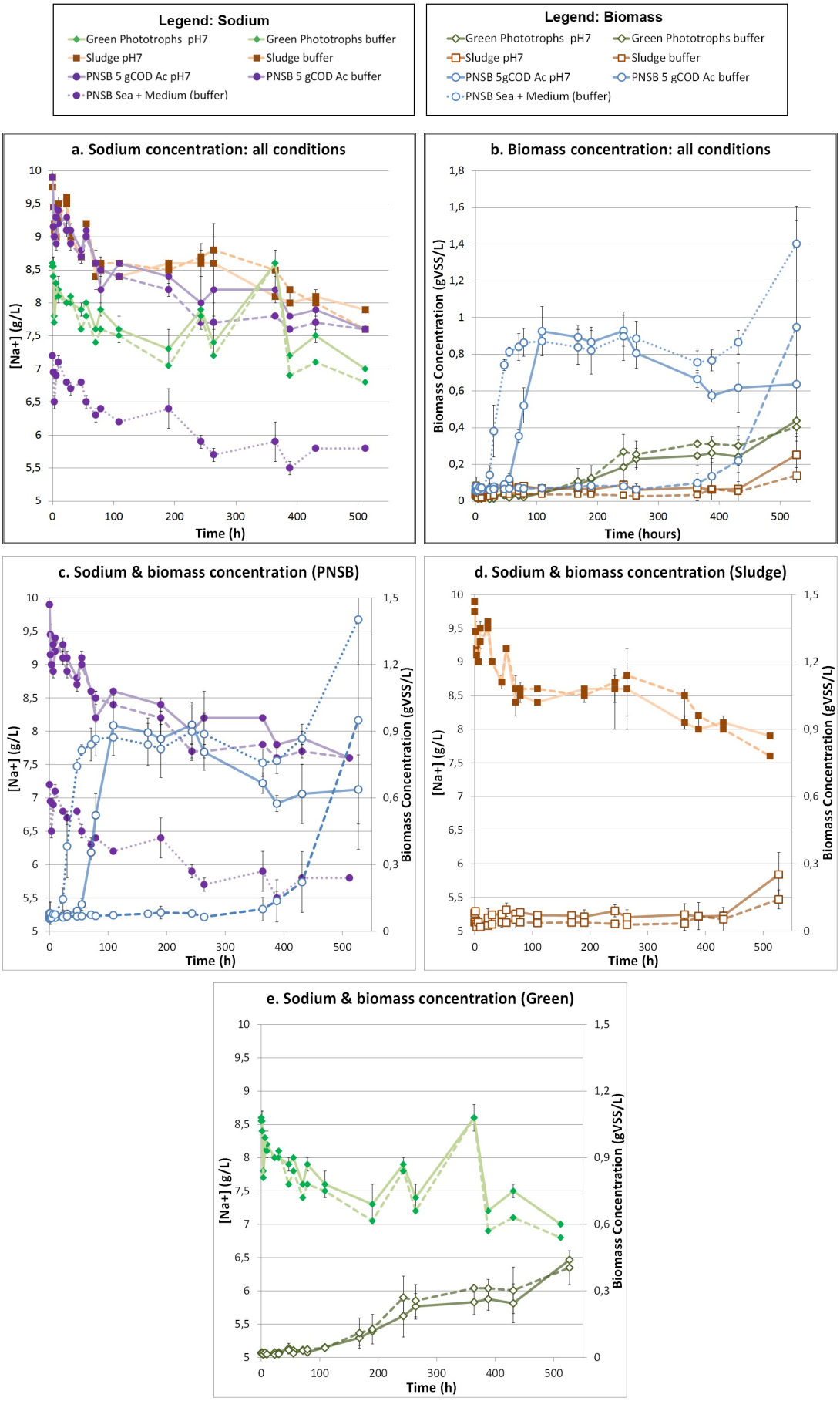

Figure 1.5: (a) Sodium concentration over time for all types of biomass, based on duplicate measurements, including error bars.
(b) Biomass concentration over time for all types of biomass, based upon duplicate measurements, including error bars.
(c) Sodium and biomass concentration over time for PNSB cultures including error bars.
(d) Sodium and biomass concentration over time for activated sludge including error bars.
(e) Sodium and biomass concentration over time for green phototrophs including error bars.
Overview of inoculum and conditions can be found in Table 1.5.

Ion chromatography results Ion chromatography results for the pH7 samples of PNSB, sludge and green phototrophs are shown in Figure 1.6a,b,c. Note that the time scale is different to Figure 1.5, so final sodium removal cannot be directly compared. Ion chromatography results confirm the pattern of the sodium probe; that sodium concentration decreases during the experiment. The total sodium concentration decrease in 110 hours was 2,81 g/L (PNSB), 3,15 g/L (sludge) and 2,5 g/L (green phototrophs). The sodium concentration starting point at 0 hours is measured as higher with IC than with the sodium probe in the case of PNSB and sludge. For green phototrophs, the concentration measured with IC is measured as equal to the sodium probe. This error may be caused by a calibration defect, or an imprecision with the standards either for IC or for the sodium probe. It is indicated by all IC plots that chloride, the most abundant anion in seawater, was also removed in considerable amounts. In all cases, the highest chloride removal is reached within the first 24 hours (PNSB: 5,04 g/L, sludge: 5,27 g/L and green phototrophs: 3,9 g/L), after which an increase occurs, like the profile of sodium in the mixed culture experiment on saline Stevens medium (Figure 1.4). Final chloride removal values are 3,06 g/L (PNSB), 4,18 g/L (sludge) and 3,67 g/L (green phototrophs). No literature has been found which provides rates or absolute amounts of microbial chloride removal to compare this observed removal with. It can be seen from Figure 1.6 that for the other ions, concentrations are several factors smaller and (except for sulphate), do not change. This indicates that ion exchange where magnesium or calcium is traded for sodium, can be eliminated as an important sodium removal mechanism. The sulphate concentration does show fluctuations. In the case of activated sludge, it had increased by 0,45 g/L at the end of the experiment, perhaps indicating an exchange or production by the organisms.

Charge balance Figure 1.6d shows the molar charge balance per liter, based on ion chromatography results for all series, combined with acetate HPLC (Appendix E) in the case of sludge and PNSB. It must be taken into account that pH was not measured at any point, so the concentration of protons and hydroxide ions (H^+ and OH^-) is unknown. For the PNSB and sludge samples, the net charge balance is positive at all time points with +0,15 mol as maximum point. This would correspond to a hydroxide concentration of 2,6 g/L to reach a zero charge balance, which is unrealistically high as it corresponds to a 13,2 pH. In the case of green phototrophs, the charge balance over time is closer to zero than for PNSB and sludge. Besides one positive spike at 6,5 h, there is a net negative charge. The minimum value is -0,055 mol charge. If compensated solely by protons, this would result in a concentration of 0,055 g/L protons, correponding to a pH of 1,3, which is generally too low to allow microbial survival and growth. The charge deficiency may be caused by other relevant ions present in solution which were not measured in ion chromatography, such as silicate, nitrate, lithium, lead, chromium or silicium, considering the composition of the artificial seawater (see table 1.3). Another possibility is that the sodium or chloride measurements are imprecise.

To conclude The research questions for this experiment were formulated as:

- Can different types of biomass perform desalination?
- Does pH control influence sodium removal?
- Is growth required for desalination?
- Are the measurements of the sodium probe correct?
- Are other ions than sodium removed?
- What is the effect of using artificial seawater as medium?

A decrease in sodium concentration was observed for all three species (mixed PNSB, activated sludge and green phototrophs). As all sludge samples showed decrease in ion concentrations, light is a prerequisite for ion removal. From the results of the sodium probe, the mixed PNSB culture performed best for sodium removal. cultures with and without HEPES buffer followed a similar sodium profile over time. Therefore, pH control was found not to influence desalination success significantly. For samples which did not show increase in biomass, sodium decreased, so growth does not seem to be a prerequisite for desalination. The general trend as measured by the sodium probe was confirmed by ion chromatography, although the starting point of sodium was measured as higher with ion chromatography. Chloride was seen to decrease. Except for that, concentrations of other ions were quite constant, which eliminates ion exchange as the driving mechanism of microbial desalination. It was

seen that in seawater medium the initial decrease in sodium concentration was not followed by an almost equal increase, as with the Stevens medium. A similar effect of using seawater was observed by Sasaki et al. (2017). Perhaps the abundant presence of other ions influences cellular export of sodium in some way, for example by increased complexity of the EPS. It may also be some other factor than the medium which caused a different effect in the experiment with Stevens medium, such as a different community composition.

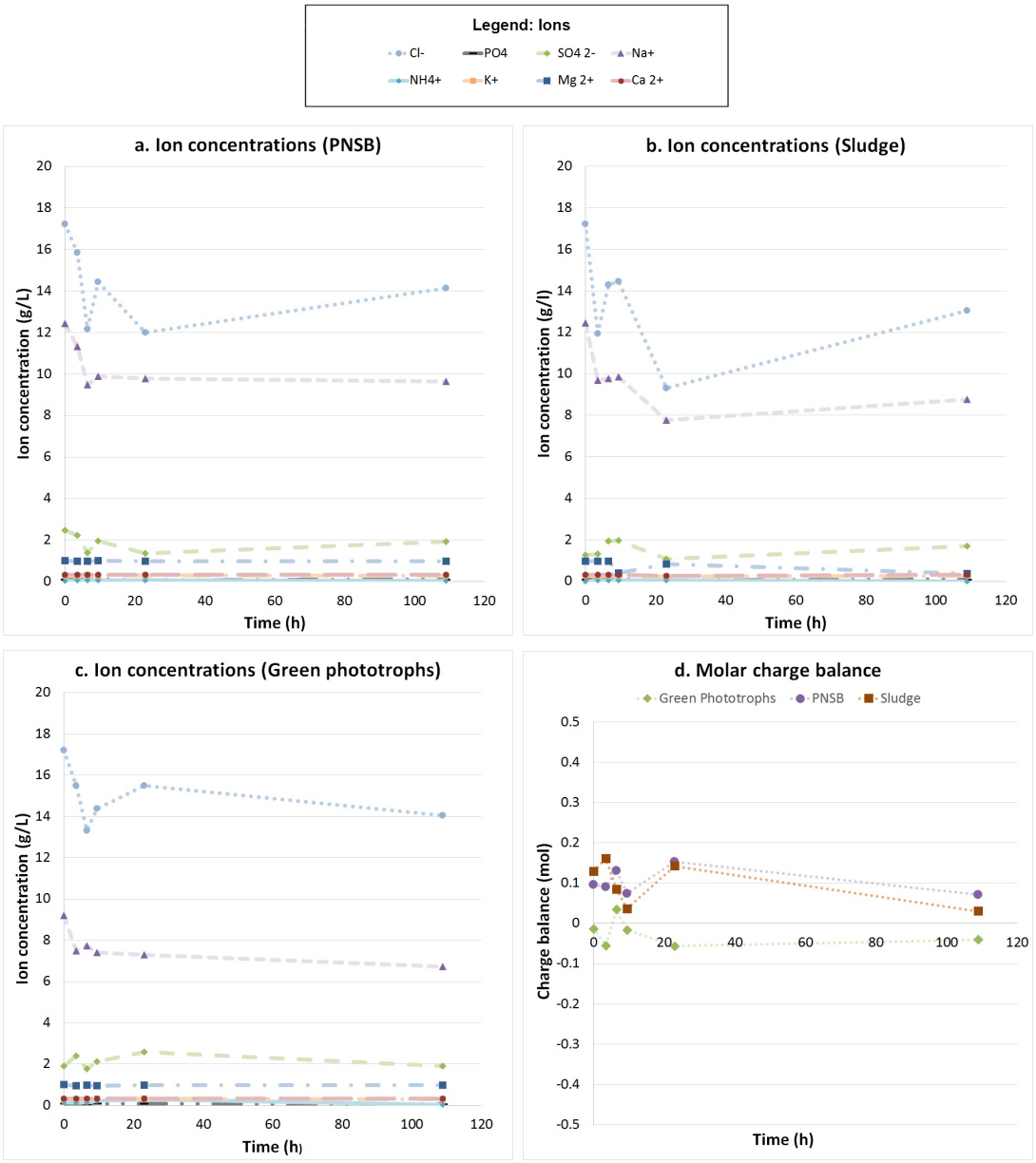

Figure 1.6: Ion chromatography results based upon single measurements of pH7 samples (a) For PNSB (b) For activated sludge and (c) For green phototrophs.
(d) Molar charge balance for the three biomass types over time.

Inoculation concentration and active/inactive biomass

This section describes the results of the experiment where desalination capacity of active and UV-inactivated biomass was compared.

Sodium and biomass concentration Sodium concentration over time is shown in Figure 1.7a. It can be seen that sodium is removed in all cases; 2,15 g/L (active, 0,02gVSS/L), 2,05 g/L (inactive, 0,02gVSS/L), 1,95 g/L (active, 0,2gVSS/L), 1,9 g/L (inactive, 0,2gVSS/L), 2,6 g/L (active, 2 gVSS/L) and 3,4 g/L (inactive, 2gVSS/L). For the duration of the experiment (120 h), sodium does not increase significantly after decreasing. The inactive biomass can only be considered truly inactive the first 24 hours after UV irradiation, after this time the small percentage of surviving cells may repair the damaged DNA. Inactive biomass, inoculated at the highest concentration (2 gVSS/L) showed the highest initial sodium decrease rate by far. The live biomass inoculated at 2 gVSS/L shows 0,8 g/L less removal. The considerable difference between inactive and active biomass at the high inoculation concentration may be explained by an active sodium export process which is halted in inactive cells, whilst a passive sodium import mechanism can still work. Such an active sodium export pump was previously described by Amtmann and Sanders (1998) and Amezaga et al. (2014). The 2gVSS/L active condition and the lower inoculation concentration samples (0,02gVSS/L and 0,2 gVSS/L) are comparable in terms of initial removal pattern, but the active 2gVSS/L do reach a slightly higher final removal. The difference in sodium concentration profiles between inoculation concentrations of 0,2 gVSS/L and 0,002 gVSS/L is small both for inactive and active biomass, even though the initial biomass concentration differs by a factor 10. In figure 1.7b, biomass concentration over time is shown for this experiment. It can be seen for all inoculation concentrations that the inactive cultures hardly grow over time whilst the active cultures do, indicating that UV irradiation was an effective method of inactivating the biomass. The visual appearance of the shake flasks over time can be found in Figure B.3 (Appendix B).

Electrical conductivity Electrical conductivity during the experiment is shown in Figure 1.7e. Electrical conductivity is seen to decrease over time, sometimes following a similar pattern as in the case of sodium concentration (see Figure 1.7a). At the end of the experiment, electrical conductivity decrease is equal to 11,1 mS/cm (active, 0,02gVSS/L), 10,8 mS/cm (inactive, 0,02gVSS/L), 13,4 mS/cm (active, 0,2gVSS/L), 10,9 mS/cm (inactive, 0,2gVSS/L), 15,6 mS/cm (active, 2 gVSS/L) and 20 mS/cm (inactive, 2gVSS/L). Decrease of electrical conductivity is another indication that ion exchange is not an important desalination mechanism.

To conclude The research questions for this experiment were formulated as:

- What is the effect of inoculation concentration on sodium removal?
- Do cells need to be active to remove sodium?
- Does total electrical conductivity decrease?

Samples, both active and inactive, with inoculation concentrations of 0,02 gVSS/L and 0,2 gVSS/L showed near comparable sodium concentration profiles over time; all removing around 2 g/L. In the case of 2 gVSS/L, a more pronounced removal of 2,6 g/L (active) and 3,4 g/L (inactive) sodium was observed, but not proportional to the 10x/100x increase in biomass concentration. Therefore, it seems like sodium may only be removed to a certain tolerable concentration for growth, and not to the maximum removal capacity of the biomass. It can be concluded that cells do not need to be active for desalination. The fact that inactive biomass is best for sodium removal suggests that an active (ATP driven) sodium export system is usually present in microorganisms, but disabled after UV exposure. Electrical conductivity was seen to decrease, which further eliminates ion exchange as a mechanism of sodium removal.

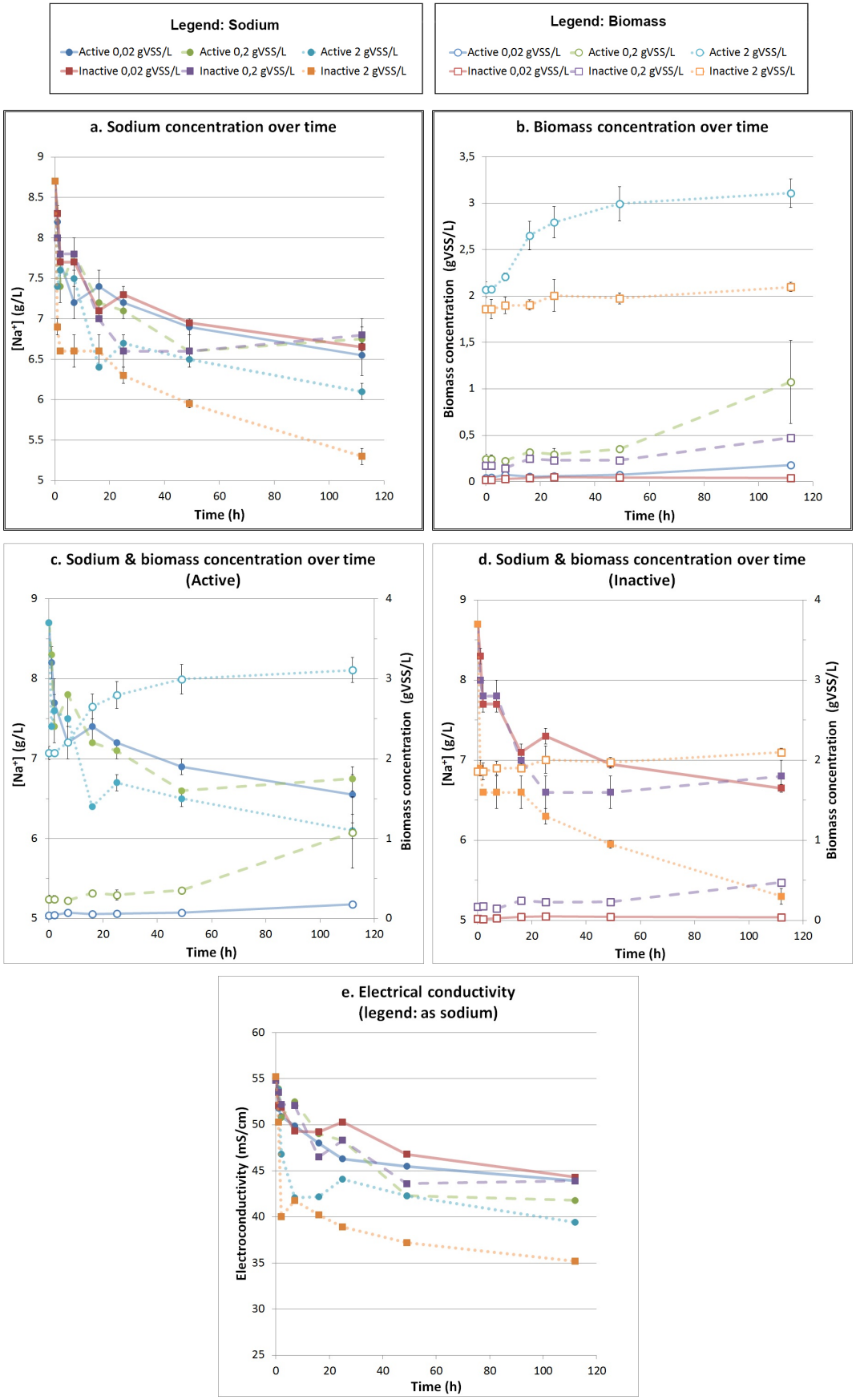

Figure 1.7: (a) Sodium concentration over time for inactive and active biomass, based upon duplicate measurements.
(b) Biomass concentration over time for inactive and active biomass, based upon duplicate measurements. (c) Sodium and biomass concentration over time for active cultures. Spread between duplicates indicated by error bars.
(d) Sodium and biomass concentration for inactive cultures. (d) Spread between duplicates indicated by error bars.
(e) Electrical conductivity over the duration of the experiment, based upon single measurements.

Quantifying Inorganic Suspended Solids

This section describes the results of an example where concentration of suspended solids was determined during a short microbial desalination batch experiment. The analysis focuses on inorganic suspended solids (ISS), which can be calculated when total suspended solids (TSS) and volatile suspended solids (VSS) are known: ISS=TSS-VSS.

Sodium and biomass concentration The sodium and biomass concentration during this experiment can be seen in Figure 1.8a. In the blank sample without biomass, sodium remained equal, within a margin of 0,3 g/L. This indicates that decreases in sodium in this and previous experiments were directly related to biomass, and do not occur by a process independent of the biomass (abiotic precipitation for example). The low VSS concentration of the blank sample indicates that no growth took place. So, the sodium results indeed correspond to a negative control without biomass.
For the PNSB samples, sodium concentration decreased in both duplicates, on average by 1,65 g/L in 60 hours. Biomass concentration remained more or less equal, decreasing by 0,04 gVSS/L on average.

Inorganic Suspended Solids The inorganic suspended solids fraction (ISS) is the ash fraction of total suspended solids, after burning at 550 °C, the inorganic compounds. In Figure 1.8b, the ISS and VSS concentrations in g/L are shown for 0 h and 50 h. During the experiment, ISS increases from 0,2 g/L (18% of TSS) to 1,2 g/L (59% of TSS). An ISS fraction of 59% is high; even in organisms which accumulate inorganic compounds such as phosphate accumulating organisms (PAO's), the ISS fraction is maximally around 40% (Weissbrodt et al., 2013).
This indicates that sodium is indeed stored within biomass or as bound precipitate, as it is present in the inorganic suspended solids fraction.

To conclude The questions for this experiment were:

- Does biomass cause the observed sodium decrease?
- Is an increase of the fraction ISS/TSS seen?
- Are ions stored around or inside the cell?

A blank seawater shake flask without biomass showed no significant decrease in sodium whilst PNSB cultures showed 1,65 g/L decrease. Therefore, it can be concluded that the decreases in sodium concentration seen in this experiment and previous experiments are directly caused by biomass.
An increase of the fraction ISS/TSS was seen from 18% to 59%, with an absolute increase of the ISS concentration of 1 g/L on average. This indicates that sodium is indeed stored in or around the biomass, ending up in the inorganic suspended solids fraction during measurement.

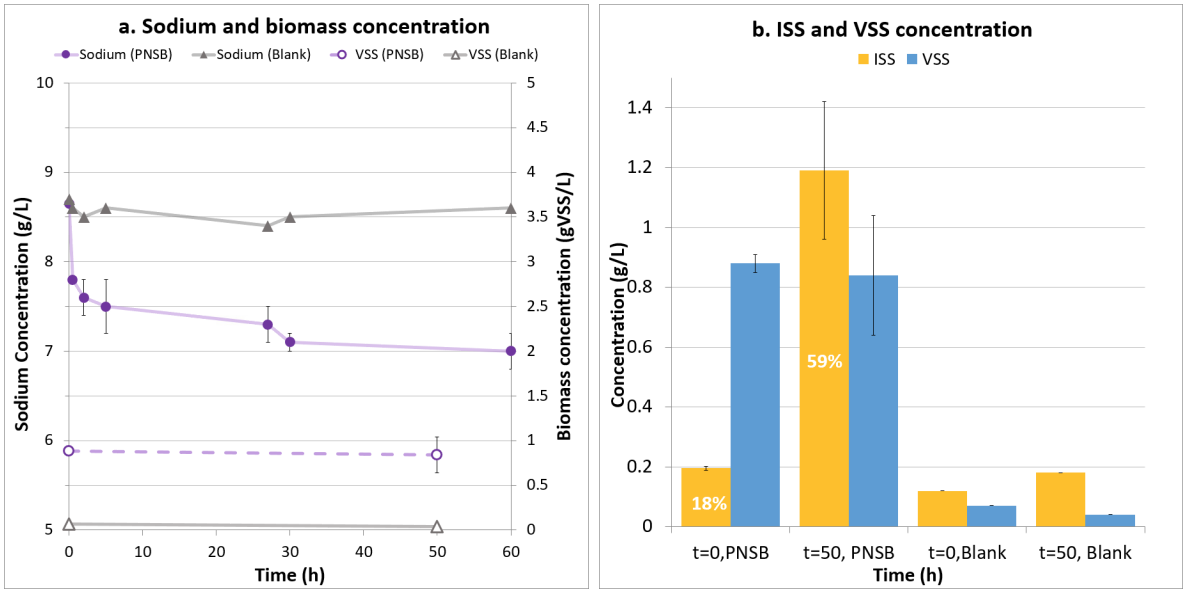

Figure 1.8: a. Sodium and biomass concentration for PNSB samples (duplicate) and blank, including error bars.
b. ISS and VSS concentration at t=0h and t=50h for PNSB and blank samples, including error bars. ISS percentage of TSS given. Due to methods error, ISS at t=0h (PNSB) calculated from VSS and earlier PNSB VSS-TSS calibration curve.

Sodium Removal Rates

Table 1.7 gives an overview of the experiments of this section in terms of sodium removal parameter k_{Na}, which is an indication of initial sodium removal speed and $R_{Na+,max}$, which gives an approximation of the maximum removable sodium percentage. The higher both of these parameters are, the more suitable a set of conditions is for microbial desalination. The underlying equation and the method fitting of curves to data of these experiments are given in Section 1.3.

Table 1.7: Comparison of sodium removal experiments in terms of k_{Na} and $Cmax$, based on curve fitting to data. More information on medium composition and manner of carrying out the experiments can be found in the Methods section 1.3. The data from the first two rows was not very well suited to this method of fitting as sodium removal was largely reversed after 48 hours, but for the sake of comparison a rudimentary fit has been made for the first 24 hours.

Biomass	**Inoculation Conc. (gVSS/L)**	**Medium**	**Initial [Na+] (g/L)**	**pH control**	**Inactive/ active**	**Growth factor [a]**	k_{Na} **(1/h^{-1})**	$R_{Na+,max}$ **(% Na$^+$ removed)**
Rps Sp.	0,01	Stevens (2017) + 24 g/L NaCl	10,8	HEPES	A	5,3	*0,06* •	*8* •
Mixed PNSB	0,01	Stevens (2017) + 24 g/L NaCl	10,8	HEPES	A	33,3†	*0,2*	*15* •
Mixed PNSB	0,05	Stevens (2017) + seawater	7,2	HEPES	A	17,28	0,19	22
Mixed PNSB	0,05	Seawater + 5 gCOD Ac	10	HEPES	A	16,8	0,22†	24
Mixed PNSB	0,05	Seawater+ 5 gCOD Ac	10	pH7	A	9,7	0,19	23
Mixed PNSB	0,02	Seawater+ 5 gCOD Ac	8,7	pH7	A	4,4	0,19	24
Mixed PNSB	0,02	Seawater+ 5 gCOD Ac	8,7	pH7	I	1,9•	0,15•	25
Mixed PNSB	0,2	Seawater+ 5 gCOD Ac	8,7	pH7	A	4,5	0,21	25
Mixed PNSB	0,2	Seawater+ 5 gCOD Ac	8,7	pH7	I	2,8	0,19	28†
Mixed PNSB	2	Seawater+ 5 gCOD Ac	8,7	pH7	A	1,5•	0,22 †	32†
Mixed PNSB	2	Seawater + 5 gCOD Ac	8,7	pH7	I	1,1•	0,27†	39†
Green Phototrophs	0,05	Seawater + nutrients	8,6	HEPES	A	22,2†	0,15 •	21
Green Phototrophs	0,05	Seawater + nutrients	8,6	pH7	A	21,1†	0,13 •	20•
Activated Sludge	0,05	Seawater+ 5 gCOD Ac	10	HEPES	A	3,0	0,15	24
Activated Sludge	0,05	Seawater + 5 gCOD Ac	11	pH7	A	6,4	0,18	22

†indicates the three highest values in terms of growth factor, k_{Na} and $R_{Na,max}$
•indicates the three lowest values for growth factor, k_{Na} and $R_{Na,max}$
[a] The growth factor indicates the growth which occurred. It is the ratio of the biomass concentration at the end of the experiment over the biomass concentration at the beginning of the experiment.

The conditions which yielded the highest final sodium removal C_{max} and the highest k_{Na} are a high inoculation concentration of 2 gVSS/L of inactive biomass, in seawater supplemented with acetate at a starting concentration of 8,7 g/L sodium. Live biomass under these conditions is hereafter the best performing. There is a considerable difference between an inoculation concentration of 0,2 gVSS/L and 2 gVSS/L, and barely a distinction between 0,02 gVSS/L and 0,2 gVSS/L both for inactive and active biomass. It must be noted that the different running times of the experiments do bias the fitting of curves.

1.5. Ion removal: integrating the results

Based on the results of the experiments described in this article, the following knowledge on ion removal has been gained:

- In the general introduction (Figure 3), three possible mechanisms for microbial desalination are discussed; uptake, binding in the EPS or precipitation. Ion exchange is eliminated as a mechanism, based upon IC results and electircal conductivity decreasing.
- Ion removal can be performed by mixed cultures of different composition, so is not limited to a certain group of organisms.
- Ion removal was seen to occur under both light and dark circumstances.
- UV inactivated biomass removes more sodium, which may hint at an active sodium export mechanism in active cells.
- There is no linear correlation between biomass concentration and sodium removal.

There are still a number of unanswered questions. Firstly, what drives the desalination? What is either the cellular advantage of collecting ions from the environment, or the involuntary process which drives it? This question is closely related to another one; by what mechanism does microbial desalination occur? If ion exchange is not the mechanism of ion removal, that leaves uptake or precipitation. A combination of both is possible as well; ions being transported over the cell membrane and precipitated within the cell.

No linear relationship between biomass concentration and sodium uptake

A curious outcome of the batch experiments, is the lack of an obvious relationship between biomass concentration and sodium removal. One would expect such a relationship in the case of cellular uptake as desalination mechanism, as presence of more cells would entail a higher uptake capacity. The lack of such a relationship is most apparent from Table 1.7; Although an inoculation concentration of 2 gVSS/L gives a significantly higher initial rate and final removal, there is no linear correlation of biomass concentration and sodium uptake and there is hardly any difference between 0,02 gVSS/L and 0,2 gVSS/L, even though there is a factor 10 difference.
A possible explanation for this is that desalination would be performed by microorganisms in order to allow the own population to grow, and therefore once a tolerable level of sodium is reached, the "motivation" to desalinate becomes less strong and the amount of ions which are removed decreases. This could be examined by examining gene expression at different times for microorganisms in a saline environment. Genes which are active at a certain point, may indicate whether this theory is correct, and which proteins are involved in ion sequestration.

Mechanism theory: compartment enclosed precipitation

This section describes a novel theory for sodium chloride sequestration by microorganisms. It is based upon the concept of bacterial microcompartments (BMCs). BMCs are intracellular compartments which make it possible to generate and maintain specific local conditions. The confined environment facilitates interactions, reactions and storage (DeLoache and Dueber, 2013). BMCs are widely spread amongst microorganisms and archae (Kerfeld et al., 2018). Encapsulin is the most characterised BMC; a virus-like protein structure that is pH and temperature stable. Encapsulins are being used to create cellular microfactories for valuable compounds (Snijder et al., 2016). BMCs are used by cells for a number of purposes, such as toxin sequestration, storage of iron compounds, as a closed environment for specific enzymatic reactions and for mineral storage (Giessen and Silver (2016); He et al. (2016)).
I propose that such a compartment may used for sodium chloride precipitation and storage. The cell would employ this mechanism in order to improve the environmental conditions for growth of it's species. Firstly, sodium and chloride diffuse into the cell. Subsequently, these compounds are directed towards a BMC. Within the BMC, the concentration of sodium and chloride ions is so high, that oversaturation is reached and solid sodium chloride or halite (the crystal form of sodium chloride) is formed. Nucleation is also important for precipitation, perhaps some compounds are present for this purpose. In solid state, the ions no longer contribute to osmotic pressure, and the environmental conditions are improved. Once a certain tolerable extracellular salt level is reached, newly grown cells do not produce BMCs and sodium removal decreases. This may explain the observed phenomenon that biomass concentration does not show a linear correlation

with sodium removal, as removal of sodium may halt once a salt level which allows growth is reached.
Exactly this mechanism has been observed in various strains of cyanobacteria by Blondeau et al. (2018). In medium with high calcium carbonate concentrations, solid calcium carbonate was precipitated in compartments, or ACC (amorphous calcium carbonate) inclusions to make the environment more liveable. The fact that such a mechanism has been previously observed, shows that it is possible to store salts in this manner on a cellular level.
Whether such compartments are formed for sodium chloride, could be examined using transmission electron microscopy (TEM), as described below.

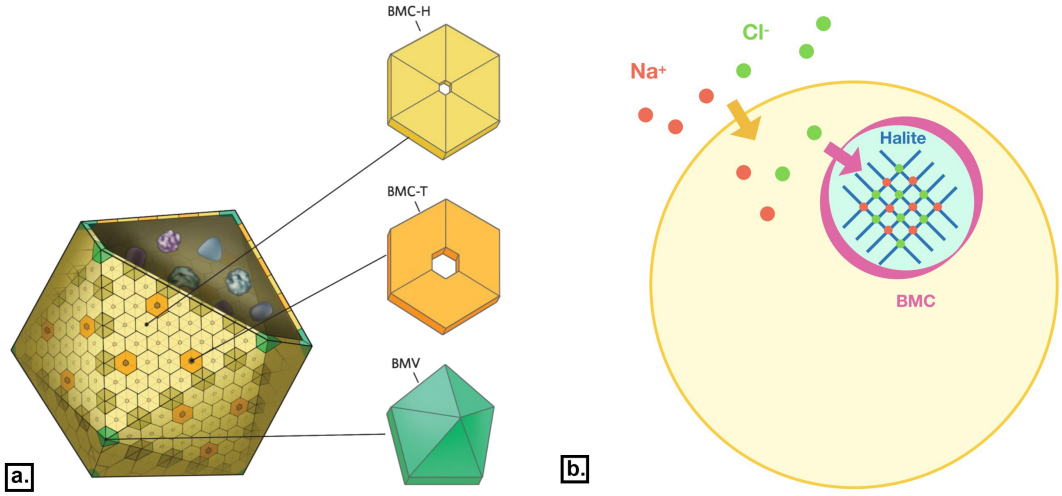

Figure 1.9: (a) The structure of encapsulin, consisting of different types of proteins forming a shell-like pocket. From: The Scientist (`https://www.the-scientist.com/infographics/infographic--bacterial-microcompartments-basics-65136`)
(b) A sodium chloride uptake mechanism, where sodium and chloride ions diffuse into the cell, are transported into the BMC where halite is formed due to a high saturation index.

TEM to verify mechanism

Transmission Electron Microscopy (TEM) was used to examine biomass from one of the batch experiments for BMC-like inclusions like in the case of Blondeau, as described above.[‖] The sample in question was a PNSB mixed culture, not UV irradiated, 2gVSS/L, cultured for 110 hours in seawater medium and thereafer frozen at -20 °C. Two TEM images taken within this research are shown in Figure 1.10c,d. For comparison, the cryo-electron microscopy of vitreous sections (CEMOVIS) images from Blondeau et al. (2018) which most clearly depict calcium carbonate inclusions are shown in Figure 1.10a,b.
It can be seen in both 1.10c,d that inclusions or bodies are present in the cell, like in the case of 1.10a,b. It may be that the inclusions which can be seen in the TEM images are used for sodium chloride storage as proposed above, or for calcium carbonate as described by Blondeau. To research what kind of compartments are present, one could attempt encapsulin isolation (for example according to the protocol of Rurup et al. (2015)) and subsequent examination of the contents, for example by taking apart the encapsulin with protease and measuring the composition of the contents by crystallography.
The inclusions as seen in the TEM images could also be something else entirely. Further analytical investigation is needed to confirm that these inclusions are BMCs. A combination of scanning transmission electron microscopy and energy dispersive X-ray spectroscopy could provide information on the composition of the inclusion.
All in all, for the theory of compartmentalised precipitation it is promising that inclusions were seen.

[‖]TEM was kindly facilitated by Duncan McMillan and performed at Nijmegen University

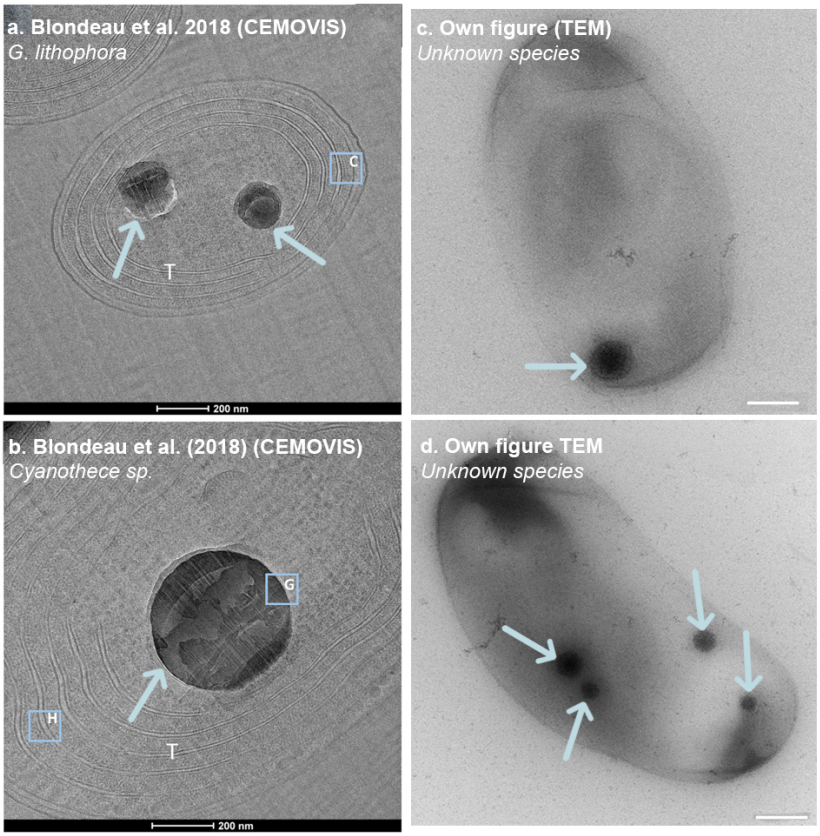

Figure 1.10: a, b. CEMOVIS image of calcium carbonate inclusions from Blondeau et al. (2018)
c, d. TEM images of seawater PNSB mixed culture, with thanks to Nijmegen University

1.6. Recommendations

The following recommendations are given for future microbial desalination research.

- In order to prove that desalination occurs by microbial methods, a mass balance of ions during the experiment must be made. This would be highly informative with regards to the mechanism of desalination. Information needed for this is the ion/precipitate content of the cell and the ion/precipitate content of the environment. Precipitate which is present in cells, at the surface of cells or in the medium could perhaps be observed by microscopy and quantified by X-ray diffraction spectroscopy and scanning transmission electron microscopy. Cellular content of ions could be determined by ash-content analysis or ion chromatography.

- For all future batch experiments, regular pH measurements would be informative. This would make it possible to include protons in the charge balance.

- Precipitation modelling, for example using PhreeqC, could be informative. An analysis could be done for precipitate formed in the medium, the exterior of the cell and the interior of the cell. The theory of compartmentalised precipitation could also be theoretically tested in this manner.

- It is currently not known whether biomass which has been used to desalinate can be used effectively again to desalinate a fresh batch of seawater, or whether they are somehow "saturated" with respect to ion removal. This may also be dependent on growth, as after a division, both new cells may have the capacity to take up sodium once more. To determine this, the broth at the end of a desalination experiment could be centrifuged in falcon tubes, and this biomass used to inoculate fresh seawater. Desalination performance of this biomass could be compared to its first performance. If desalination capacity decreases during a second run, it can be tested whether it is possible to regenerate the biomass in any way.

- Up to now, the only inoculation concentrations which were examined for desalination performance are 0,01 gVSS/L, 0,02 gVSS/L, 0,05 gVSS/L, 0,2 gVSS/L and 2 gVSS/L. An experiment could be designed where ten inoculation concentrations, for example between 0-10 gVSS/L are analysed for desalination capacity. With data from this experiment, a plot of inoculation concentration against ion removal could be made, and a curve fitted.
- To examine the location of sodium ions in or around the biomass, microscope experiments could be designed where fluorescent labeled sodium indicates the location of sodium in or around the cell. A possible problem of this, is that a fluorescent tag is rather bulky, and therefore sodium may not move across membranes in the same manner as without the tag. For such purposes, Thermofisher stocks fluorescent sodium.[**]
- Some of the experiments with PNSB cultures could also be repeated in seawater medium without acetate, as the inactive biomass is not required to grow.

1.7. Conclusion

The objective of this study was to quantify microbial desalination performance of mixed cultures under various circumstances by a series of batch experiments. The main conclusions of the work within this research line can be summarised to be:

1. Significant amounts of sodium were removed in all mixed culture experiments. The maximum sodium removal achieved was 39% in 120 hours. Different types of biomass can remove sodium and even when growth of biomass is not observed, sodium can be removed. The only experiment in which no removal was seen, was when pure *Rhodopseudomonas* was the biomass type.
2. Chloride, the prevalent anion in seawater, was also removed in significant amounts from the seawater medium by various types of biomass.
3. Light is not needed for desalination.
4. As no increase of calcium or magnesium was observed and electrical conductivity was seen to decrease, ion exchange was eliminated as a major mechanism of microbial desalination.
5. Inactive biomass removes more sodium than live biomass, which may indicate that an active sodium export process is halted in inactive cells, whilst a passive sodium import mechanism can still work.
6. There is no linear correlation between biomass concentration and ion removal. However, desalination was proven to be directly related to presence of biomass, as a blank containing just seawater showed no change in sodium concentration.

In short: Microogranisms are capable of removing sodium and chloride from seawater. Inactive biomass, inoculated at high concentration (≥ 2 gVSS/L) in seawater medium showed the highest removal of 39% and should therefore be considered for scalable desalination.

[**] https://www.thermofisher.com/nl/en/home/references/molecular-probes-the-handbook/indicators-for-na-k-cl-and-miscellan
fluorescent-na-and-k-indicators.html

2

Towards a Light Powered Biomimetic Desalination Membrane

2.1. Abstract

Biomimetic membranes offer high selectivity and permeability, and have been proposed as promising for desalination purposes. In this study, a novel concept is proposed for alight powered desalination membrane containing SyHr, a halorhodopsin protein, and a voltage gated sodium pump. Halorhodopsins are light gated chloride pumps. In order to lay the foundations for the construction of such a membrane, SyHr was produced within the solubilised membrane protein fraction at wet lab. To this end, firstly a SyHr DNA fragment including a his-tag was designed and synthesised. This fragment was restricted from a pUC-sp vector and successfully ligated into a Ptrc99a expression vector, with which E.Coli BL21 cells were transformed. Overexpression was induced using Isopropyl β-D-1-thiogalactopyranoside (IPTG), after which cells were harvested by centrifugation, passed through the cell disruptor and all membrane proteins isolated. This research lays the foundation for testing of the potential of halorhodopsins in desalination. A next study can focus on inserting the purified SyHr in a membrane to assess it's performance and stability. Although more research is needed and scalability is still a faraway station, the concept of light powered desalination with this membrane offers a novel option within the quest for desalination solutions, which could offer high selectivity, low energy cost and reduced fouling compared to seawater reverse osmosis.

2.2. Introduction

Biomimetic membrane solutions for desalination

Biomimetic membranes take inspiration from molecular systems in nature which transport solute or solvent across a membrane, which display a highly efficiency and selectivity. Using these principles and biological structures in constructed membranes offers selectivity, a sharp molecular weight cutoff and strong antifouling properties. Examples of proteins and carriers which are used in biomimetic membranes are ionophores which facilitate diffusion, S-proteins on the exterior of a membrane which bind compounds and transport proteins which shuttle materials from one side of the membrane to the other (Pressman (1976); Sleytr et al. (1999)). There are three kinds of approaches to biomimetic membrane synthesis (Shen et al., 2014):

- Biomimetic-hybrid, where biological membrane proteins or carriers are placed in artificial or natural membranes.
- Biomimetic modified, where a synthetic membrane is supplemented with functional molecules.
- Biomimetic-synthetic, wherein both channels and membrane are artificially constructed.

For the scaffold, either a lipid bilayer or a polymer based membrane is used. Lipid bilayers are a dynamic but stable barrier as seen biological cells, through which molecules can diffuse by a two-step mechanism. This diffusion is gradient driven. Synthetic lipid bilayer membranes offer excellent selectivity and permeability (Hoek and Tarabara, 2013). However, they are relatively unstable and therefore expected not to be scalable. The other option are block copolymer (BCP) membranes, which have been reported to assume a bilayer-like structure (Discher and Eisenberg, 2002). For engineering applications, these membranes are more suitable as they have good chemical and mechanical stability. Permeability for water is low and properties such as membrane thickness can be adjusted.

Case example: aquaporin membranes

Aquaporins are cilindrical proteins, ubiquitous to living cells, which transport water molecules with a high throughput and exclude ions. Kumar et al. (2007) first suggested incorporating these proteins into membranes for the purpose of desalination and proved the function of such a membrane. The permeability of such a membrane is twice as high as a RO membrane. However, scalability remains a concern due to the small scale of the proteins and the stability of the membranes (Tang et al., 2013). The robust membrane designed by Zhao et al. (2012) remained stable for weeks, which shows that there are possibilities for robust scaffolds. The largest obstacles for implementation of aquaporin membranes at this point are costs, area constraints and complex synthesis techniques (Teow and Mohammad, 2019).

The rhodopsin proteins: ion transport over the cell membrane

Rhodopsin proteins are light gated ion pumps, which are present in the cell membrane of prokaryotes and lower eukaryotes. They have a characteristic membrane-embedded seven helix structure, embedding a chromophore retinal bound in a protonated Schiff base linkage to the ϵ-amino group of a lysyl residue in the middle of the 7th helix. There are numerous groups within the opsin family which transport different types of ions, for example Bacteriorhodopsins (H^+), Channelrhodopsins (Cations, primarily Na^+) and Halorhodopsins (anions, primarily Cl^-) (Govorunova et al., 2017). Rhodopsin proteins are nothing new; as early as 1971 Bacteriorhodopsin was the first rhodopsin to be isolated, out of the purple membrane by Oesterhelt and Stoeckenius (1971). Nonetheless, research into rhopdopsins have recently experienced a surge of new interest, within the developing field of optogenetics. In optogenetics, rhodopsins play the star role as a light powered on-off switch, which controls neurons by influx of ions. Applications are both fundamental and clinical. Since the birth of this field, new characterisations of rhodopsins and analyses of their mechanisms are rapidly succeeding one another (Tan et al., 2015).

Conceptual design for a light powered desalination membrane including Halorhodopsin

Within the rhodopsin family, halorhodopsins are inward directed anion pumps, which are found in various bacteria and algae. As long as there is light (preferably 550-600 nm but they are not extremely selective), halorhodopsins have been proven to pump chloride ions inward against both concentrations and electrical gradients (Deisseroth (2010); Schobert and Lanyi (1982)). This is an excellent quality for use in desalination. If halorhodopsin could be inserted in a biomimetic membrane in combination with a voltage gated

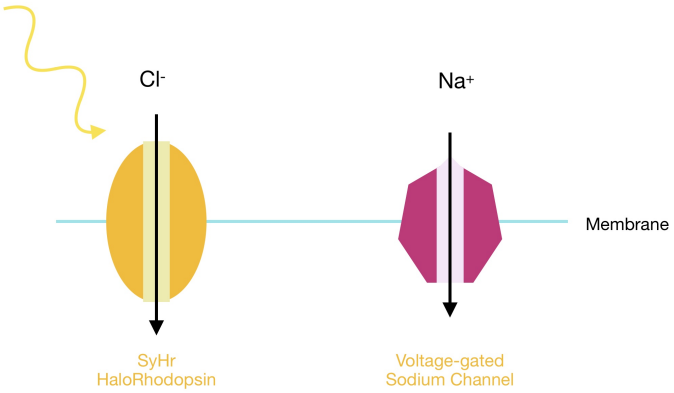

Figure 2.1: Concept for a light powered desalination membrane, where Halorhodopsin transports chloride, and a voltage gated sodium channel compensates by transporting sodium.

sodium pump, completely light-powered membrane desalination could take place. Chloride (and other anions) would be transported by halorhodopsin, after which the voltage-gated sodium pump would compensate this change in charge by transporting sodium across. The choice of voltage gated sodium channels is quite broad, there are many types which have been extensively researched structurally and mechanistically (Catterall, 2000). Finding a suitable sodium channels is therefore not expected to be a limiting factor. The concept for such a desalination membrane is shown in Figure 2.1. If such a membrane could be made and stabilised, this would be a completely new approach to desalination.

SyHr: protein of choice

In this study, it has been chosen to work with Synechocystis Halorhodopsin (SyHr). This protein was identified in 2017 by Niho et al. (2017), from a *Synechocystis* strain isolated from sulphate rich rock sediments. It is the first rhodopsin which has been observed to transport bivalent ions, as sulphate transport was measured. Sulphate transport by SyHr was demonstrated via pH measurements as protons were transported by other membrane proteins to compensate for the charge of the anions; alkalisation of the environment occurred when ions were transported. Halorhodopsins have previously been described as quite straightforward to purify (Sato et al. (2002); Hohenfeld et al. (1999)). Furthermore, it has only recently been described, which makes any discoveries more scientifically novel. The structure and ion transport mechanism of SyHr is shown in Figure 2.2. An anion enters at the exterior, is bound close to the trans-retinal and then moved across the trans-retinal to the transient binding site by a light induced configuration change. At the present time, the initial and transient binding sites are unidentified. The dissociation constant of chloride was determined to be a factor 40 stronger for chloride ions than for sulphate ions, so in a seawater environment the main transport would be of chloride ions.

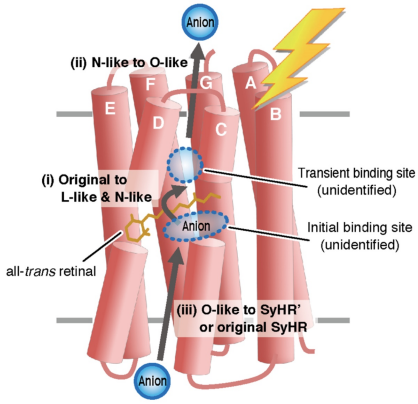

Figure 2.2: The structure and transport mechanism of SyHr. From Niho et al. (2017).

Research Objectives

The overarching research objective of this research phase is:

To develop the concept of a light driven desalination membrane which contains SyHr.

Realising such a biomimetic membrane needs extensive preperatory work. Therefore, this part of the research has been divided in two main steps: DNA work, protein extraction and purification. 2.1 shows these experiments with their respective research questions.

Table 2.1: Respective research objectives for the steps of the artificial membrane research (part 2).

Step	**Objective**
Gene preparation	Design vector including SyHr gene. Transform *E.coli* to express SyHr.
Protein expression & purification	Purify SyHr at a high enough yield to use it in following steps.

2.3. Materials & Methods

Figure 2.3 shows a visual summary of the methods employed in this research line, which are described in more detail in this section.

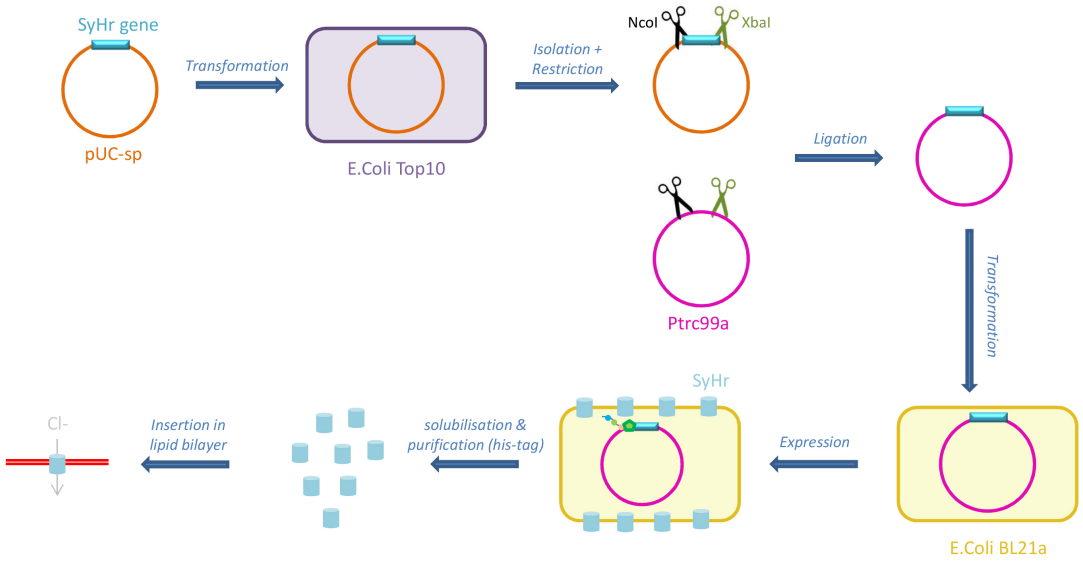

Figure 2.3: Visual summary of the methods of this research line

Gene preparation

Design of vector containing SyHr gene A design for a gene fragment was made containing the SyHr nucleotide sequence (NCBI), supplemented with NcoI and XbaI restriction sites and supplemental base pairs for successful restriction and ligation. For maximum expression, uncommon codons for E.coli were avoided according to the list provided by Novy (2001). The final gene fragment is shown fully in Appendix G. The sequence was synthesised and inserted in a pUC-sp vector containing a sequence for ampicillin resistance by Baseclear, Leiden.

Transformation of *E.Coli* Top10 with Puc-sp vector containing SyHr Chemically competent *E.coli* Top10 cells were thawed on ice (30 minutes) before the addition of plasmid DNA (5 µL, 10 ng/µL). The cells were incubated on ice (30 minutes), followed by a heat shock (60 seconds, 42°C), after which they were incubated on ice (5 minutes). The cells were allowed to warm up to room temperature before the addition of LB medium (500 µL). The cells were incubated (60 minutes, 37°C) and subsequently plated on selective LB agar plates (100µL culture, 100 µg/mL of ampicillin). The plates were then incubated overnight (37°C).

Plasmid isolation A culture was isolated from the plates by means of a pipet tip and cultivated 9 hours at 37 °C in selective LB medium. Plasmid isolation was carried out on the pellet of this culture using the Monarch Plasmid Miniprep Kit (New England Biolabs). DNA concentration was determined.

Restriction Plasmid DNA from the isolated pUC-sp vector and a Ptrc99a vector (8 µl) were mixed with CutSmart buffer (New England Biolabs) to a final volume of 20 µL and digestion was carried out using commercial NcoI and XbaI restriction enzymes (1 µL each, 37°C, 1-2 h). The reaction was terminated by addition of purple loading dye (10 µL, 6x) and the samples were analysed by gel electrophoresis (1% agarose in TAE buffer, 1x SYBR safe stain, 100 V, 400 mA, 50 min.). The corresponding bands were cut out using a scalpel and the DNA was extracted from the gel slab using the Monarch DNA gel extraction kit (New England Biolabs).

Ligation For ligation, DNA was mixed at a 1:1 ratio of insert:vector in T4 Ligase buffer to afford a final volume of 25 µL upon addition of T4 DNA ligase (1,5 µL). The mixture was incubated at room temperature (50 min), and 5 µL were directly used for transformation in four duplicates.

Transformation of *E.Coli* BL21 cells with the recombined vector Chemically competent *E.coli* BL21 cells were transformed with the Ptrc99a-SyHr vector according to the transformation method described above. To verify the vector has been correctly ligated and taken up by the cells, eight colonies from the plates were grown overnight on selective LB medium. On these cultures, plasmid isolation and restriction with NcoI and XbaI was performed as described in the previous section "restriction", but with 16 µL DNA being loaded on the agarose gel instead of 8 µL. The resulting agarose gel can confirm successful transformation.

Expression and isolation of SyHr

Expression After confirmation of successful transformation, 50 mL selective LB medium was inoculated with 1 mL of the transformed *E.Coli* BL21 colonies and grown overnight. For expression, 5 mL of this culture was then added to selective LB medium (400 mL) supplemented with 1% glucose. ITPG (400 µM) and all-trans retinal (Sigma-Aldrich, St. Louis, MO, USA) (1mM) were added when optical density reached a value of 0,6. Cultures were left for expression overnight.

Protein purification Cells were harvested by centrifugation (7000 rpm, 10 min). Cells were resuspended in 20 mL wash buffer (50mM Tris-HCl and 400 mM NaCl). Two tablets of protease and a spatula tip of DNAse were added. Cells were passed through the cell disruptor twice (35,000 psi or approximately 2,4 kbar). Cell debris was removed by centrifugation (10,000 rpm, 10 min). Supernatant was transferred to an ultracentrifugation tube and membranes were pelleted (45,000 rpm, 60 min). Pellets were resuspended in wash buffer (3 mL) and frozen for later use. Protein concentration was assessed by a BCA assay and presence of a protein with the expected length was confirmed with a polyacramide protein electrophoresis gel.

2.4. Results

Gene preparation

After initial transformation of E.coli with the pUC-sp vector and subsequent plasmid isolation, DNA concentration was found to be **46 ng/L**. The Ptrc99a vector was also isolated from a stock, the DNA concentration of this vector was found to be **26 ng/L**. After restriction, electrophoresis confirmed that the restriction enzymes had worked conform expectation, as shown in Figure 2.4a. Figure 2.4b shows the gel which was run to confirm successful transformation. It can be seen that the bands for SyHr and double restricted Ptrc99a are present in lanes 2-8, indicating successful transformation in these colonies. It was chosen to continue with colony 2.

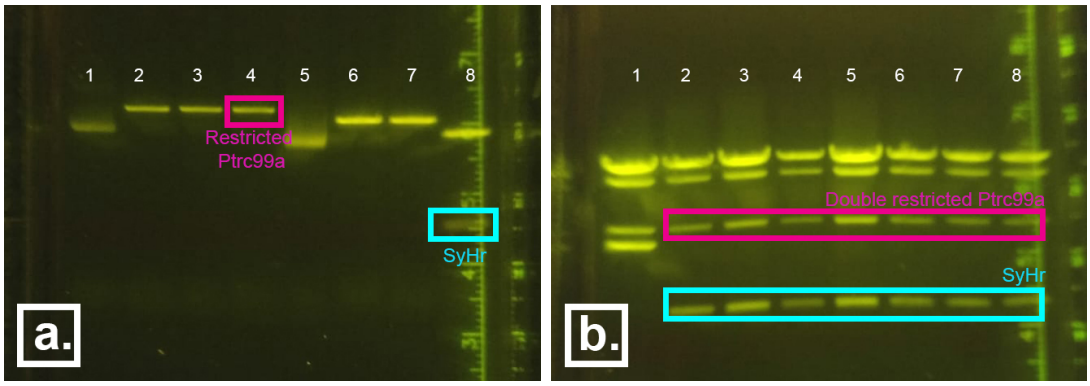

Figure 2.4: (a)Gel electrophoresis results under uV lighting. Lanes: 1.Ptrc99a unrestricted, 2.Ptrc99a + NcoI, 3.Ptrc99a + XbaI, 4.PTRC99A + NcoI + XbaI, 5.pUC-sp(SyHr) unrestricted, 6.pUC-sp (SyHr) + NcoI, 7.pUC-sp(SyHr) + XbaI, 8.pUC-sp(SyHr) + NcoI + XbaI
b)Gel electrophoresis results under UV lighting. Lanes: 1-8 all other transformation cultures grown from plates. The rows of bands respond to: unrestricted plasmid (top), single restricted plasmid (second), double restricted plasmid (third), SyHr (row 2-8, fourth)

Expression and isolation of SyHr

Succesful overexpression was confirmed by means of a polyacrimide protein gel, on which a band was visible in all fractions including SyHr. This gel is shown in Figure 2.5.

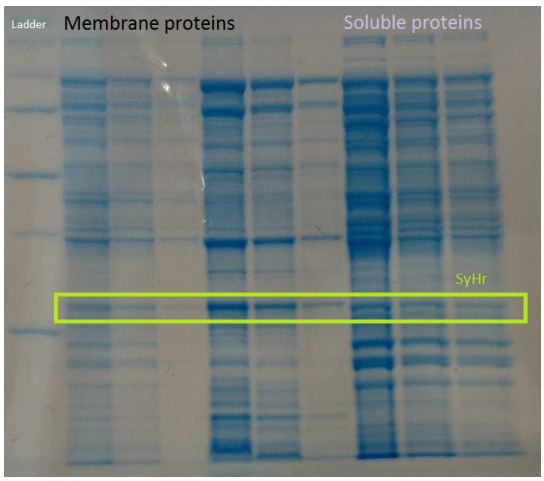

Figure 2.5: The protein gel which confirms concentrated presence of a protein with a size corresponding to SyHr.

2.5. Discussion & Recommendations

The biomimetic membrane concept described in this research offers a completely new and energy efficient way of desalination, and is therefore worth examining. Although the step of testing SyHr in an artificial membrane was not reached, obtaining the protein is already an important step to allow a next research team to complete the project. The logical next step is inserting SyHr into a membrane and evaluating its function. This could be done in membrane vesicles, which are globe shaped pockets. If SyHr and a voltage gated sodium channel are inserted into such a vesicle, one could measure ion transport by measuring ion concentrations of the solution before vesicles are added and after an incubation time. Analysis of the contents of the vesicles could be performed. Before combining with a voltage gated channel, the functioning of SyHr could be evaluated by coupling with a proton channel. When anion transport by SyHr takes place, protons are pumped to compensate the charge difference which arises due to chloride transport by SyHr, causing an easily measurable pH change. Besides vesicles, the SyHr could also be inserted into a 2D lipid bilayer or block copolymer membrane.

The most important point to be critical of in the feasibility of this membrane, is the question of scalability in isolating proteins from microorganisms. The process of obtaining pure SyHr is labour-intensive and precise, and although it could be optimised to some degree, the quality of the pure SyHr must be guaranteed. Then there is also the scalability of the membranes; a point which has been hampering the development of aquaporin membranes for several years (Teow and Mohammad, 2019). Once proper functioning of a small scale membrane is proved, testing and rapid iteration of larger membranes is advised to gain knowledge as soon as possible of the behaviour of such a membrane at a larger scale.

Besides the concept of a light powered desalination membrane, there are several other applications of this protein within the section of Biocatalysis and Organic Chemistry (BOC) at Biotechnology.

Firstly, there is interest to perform chrystallography and protein structure test on the purified SyHr as this has not yet been done and therefore contributes to fundamental knowledge. The SyHr will also be tested for integration within a titanium oxide solar energy coating due to it's ability to transport ions and thereby shift charge. There is also an idea to use the SyHr as a test protein for a new kind of scalable artificial membrane which is being developed.

2.6. Conclusion

In this paper, a new desalination concept was proposed and designed; a light powered, biomimetic desalination membrane featuring the transport protein SyHr. In order to facilitate the construction of such a membrane, the protein SyHr was obtained. To this end, the gene for SyHr was successfully inserted to a Ptrc99a vector. Subsequently, E.coli BL21 cells were showed to be transformed with this vector. Overexpression was induced, which led to abundant production of SyHr, as confirmed by a protein gel.

Although the step of placing SyHr in a membrane was not reached due to practical circumstances, the foundations have been laid to perform this last part of the experiment relatively easily. Furthermore, the SyHr can be used for many different purposes.

In short: A design was made for a biomimetic desalination membrane powered by light and methods were developed to test it.

3

A Model Approach to Assess the Feasibility of Microbial Desalination

3.1. Abstract

The topic of microbial desalination has been researched by various groups on a laboratory scale, but feasibility analyses are currently lacking. Reverse osmosis (RO) is the prevalent method of seawater desalination at this moment, but it is not yet cost effective for world wide application. In this study, two conceptual designs for full scale microbial desalination setups were proposed.

The first design entails a series of sequencing batch reactors (SBRs) for desalination using excess biomass from a wastewater treatment plant (WWTP). To evaluate feasibility of batch full scale desalination, cost comparison was made of four scenarios: seawater RO (1), three SBR steps and RO (2), seven SBR steps and RO (3) and eleven SBR steps (4). The costs per m^3 were €0,95 (1), €0,91 (2), €0,86 (3) and €0,75 (4). For agricultural or industrial use, just a series of 11 SBRs (scenario 4) could be an attractive option. For drinking water, scenario 3 is optimal as the final RO step removes enough pollutants to deliver water of (near) drinking water quality. Based on the amount of available sludge, the daily capacity for microbial desalination treatment was calculated to be 22680 m^3 seawater for Harnaschpolder WWTP (Delft, The Netherlands), so in terms of sludge this concept is feasible.

The second design entails two cross flow steps based on light powered membranes. A required membrane surface of 1,22 m^2 and a recovery of 68,4% were calculated, indicating practical feasibility of this technique. Brine was proposed to be concentrated up to 80,000 mg/L TDS. The difference in salinity between the brine and influent could be used for energy generation.

Although based on rough first estimates, the results from both models indicate that microbial desalination could be feasible at full scale. However, at present some knowledge gaps, such as whether microbial desalination is still effective at lower sodium concentrations and whether the used biomass is still fit for anaerobic digestion, need to be addressed before a full scale test is desirable.

3.2. Introduction

Current operation and efficiency of seawater desalination

Seawater desalination is becoming increasingly popular as water shortages become more pronounced and desalination technologies more efficient. Reverse Osmosis (RO) is the most popular method, accounting for over 65% of global production capacity Global Water Intelligence (2017). Energy consumption is currently equal to 3-6 kwh/m^3 in comparison to 10-15 kwh/m^3 for thermal desalination methods. It should be noted that the energy demand for thermal desalination can be lowered if solar energy or industrial heat streams are used (Voutchkov, 2018). This amounts to €0,70-€1,20/m^3 costs. Except in areas with severe water shortage, this is is not yet low enough for seawater desalination to compete with regular drinking water treatment. Although some energy is needed for pretreatment and running of the plant, the larger part of energy goes into the pressure needed in the RO unit, as shown in Figure 3.1. Energy use and costs are dependent of the salinity of the influent. This can be expressed in total dissolved solids (TDS), which gives the concentration of all ions combined, often in mg/L.

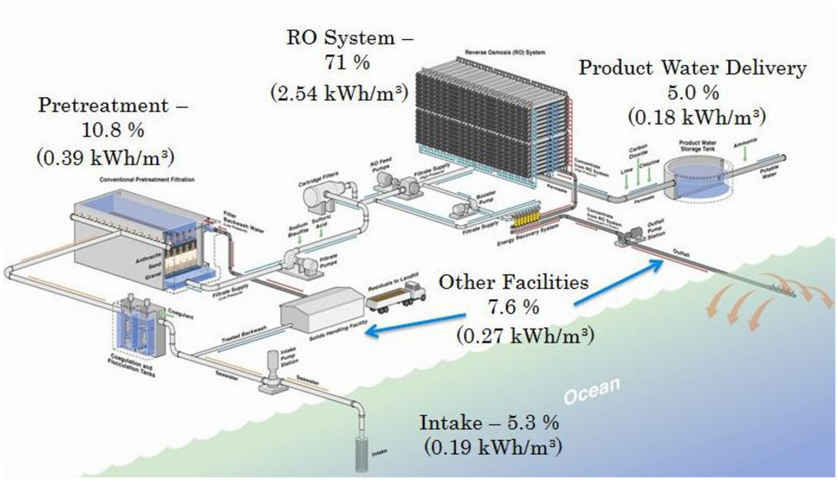

Figure 3.1: Energy consumption of a typical RO seawater desalination plant, from Voutchkov (2018)

Research gap: full scale microbial desalination

As described in the introduction of Part 1, several studies have examined microbial desalination in small scale batch or reactor experiments. Although this contributes to fundamental knowledge and the development of microbial desalination technologies, there has (to the knowledge of the author) not yet been a quantitative analysis of whether this concept could be realistic within a full scale seawater treatment line-up. Such research is relevant as it would enable a comparison to current desalination methods in terms of investment costs and energy efficiency. Also, such an analysis is valuable to assess whether microbial desalination is worth researching, or whether it is predicted to never become scalable. Amezaga et al. (2014) do offer a sketch of a full scale facility featuring their genetically modified cyanobacterial concept, but do not include a model which compares the efficiency of such a plant to current seawater desalination methods, perhaps because no results were known yet for the performance of their organisms.
In order to provide a rough analysis of microbial desalination at full scale, two conceptual designs were made. The first is based upon the batch experiments which are described in Part 1 of this thesis, the second is based upon the concept of a blight powered biodesalination membrane as described in Part 2.

Design 1: Multi step SBR system

From the research in Part 1, it can be concluded that microorganisms are capable of removing ions from seawater in batch experiments. It has been shown that various types of organisms, including activated sludge, can be used for this purpose. This is illustrated by Figure 1.5. Furthermore, the biomass does not have to be active or growing for this effect to occur (see figure 1.7). Therefore, the excess sludge which comes out of a regular water treatment or industry plant can be used as desalination sludge, without addition of a carbon source as microbial growth is not necessary. The sludge which is removed after every step could be sent to a digestor to produce biogas, for which the infrastructure is generally in place already at wastewater treatment

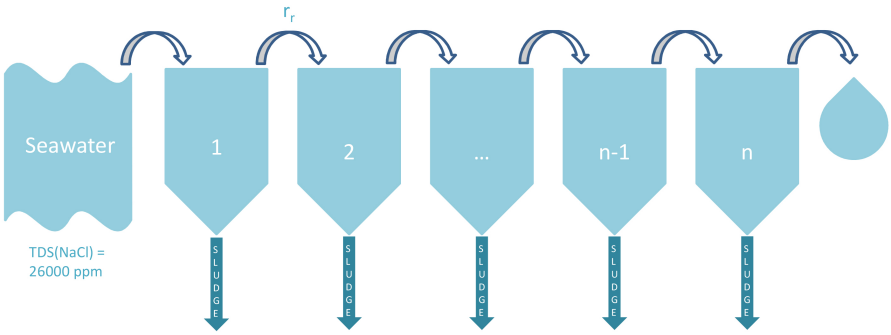

Figure 3.2: Schematic overview of multi step SBR system for seawater desalination composed of n steps

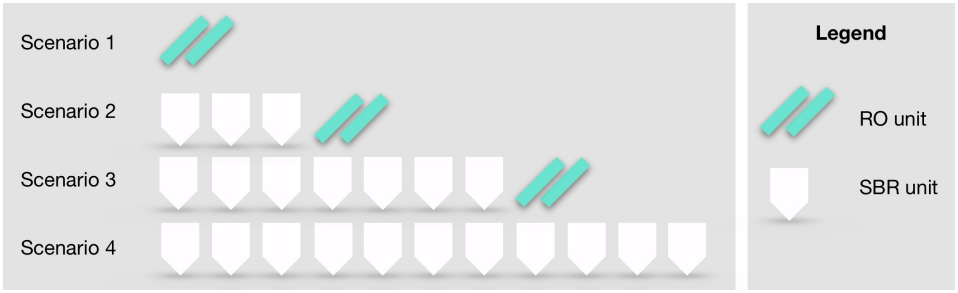

Figure 3.3: The four scenario's of model 1.

plants. This concept would work best if the installation was placed close to a wastewater treatment facility, in order to receive a constant supply of sludge without complex transport infrastructure. Whether sludge which has been used for desalination can still produce sufficient biogas in digestion, is still unsure and must be determined experimentally.
Desalination could be performed directly with any kind of waste stream biomass in a series of sequencing batch reactors (SBRs), in which the sludge can treat the seawater and decrease the ion content in several steps. This is shown in Figure 3.2. The cycle of an SBR over time consists of filling the tank with waste water (seawater in this case), reaction (removal of ions in this case), settling of the biomass, drawing of the clean water and draining of the excess sludge. As a first option, a number n of SBRs could be used which would bring the seawater to WHO acceptable standards (0-1000 TDS), without the need for any other techniques. In terms of space, this may not be efficient. Furthermore, as activated sludge is used, some kind of post-treatment will be necessary before the water can be safe for transport and consumption. It may be more efficient to combine a certain amount (<n) of biodesalination steps with RO or another kind of technology.

Model 1: Multi step SBR system

The model for this design will compares four scenario's in terms of costs, split into capital expentidures (CAPEX), operational expenditures (OPEX) and energy cost. The setup of the scenario's is shown in Figure 3.3. Firstly, in scenario 1 seawater is treated using seawater reverse osmosis, which is presently the most used desalination method. In scenario 2, the water has been treated by three desalination SBR's before RO, which is expected to decrease influent TDS by over 50%, reducing the costs of RO. In scenario 3, seven microbial desalination SBR's treat the water before reverse osmosis, further decreasing influent TDS up to brine quality. This allows for a brine water reverse osmosis setup, which saves costs. In scenario 4, seawater is treated up to WHO standards with just microbial methods.

Amount of biomass needed

Besides the cost, the amount of biomass which is needed to treat 1 m^3 will also be evaluated and compared to the average excess sludge of a WWTP. This is relevant to assess the feasibility of microbial desalination, as sludge produced by a WWTP would be the biomass for desalination. Harnaschpolder WWTP was used as an example case, as the sludge which was used in previous desalination experiments also came from this plant.

Design 2: A two-step crossflow system featuring light powered biodesalination membranes

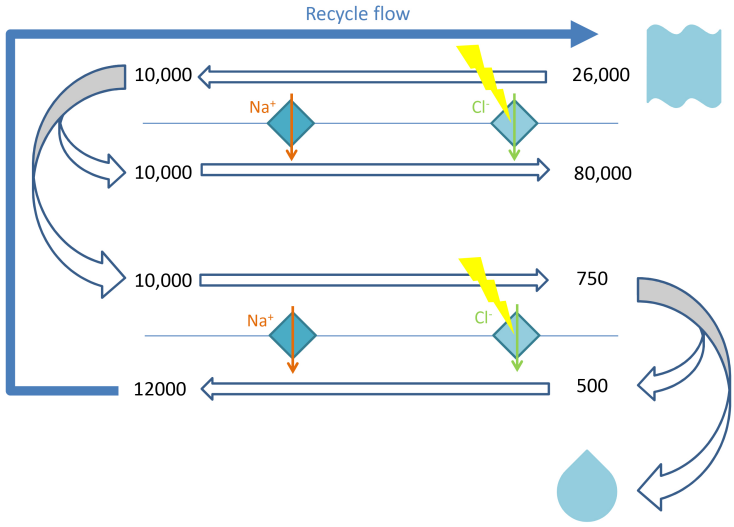

Figure 3.4: Concept of a two step cross flow biodesalination membrane powered by light. At the top right seawater enters, at the bottom right desalinated water exits. Water flows and TDS values at different phases of treatment are shown. Light is indicated by a yellow lightning bolt.

In section 2, the concept of an artificial membrane incorporating microbial membrane proteins was proposed. Anion transfer over the membrane occurs by a light powered membrane channel such as SyHr, which pumps anions even against extremely high gradients (Niho et al., 2017). Cations can be transferred in the same amounts by a voltage gated channel, which responds to a change in voltage due to anion transport. For full scale application, such a concept is interesting as it needs no pressure to separate ions from water, just a light source. Other advantages of membranes incorporating microbial transport proteins are high selectivity and reduced fouling properties. Although a lipid bilayer is only stable when constructed over a small surface, there are more scalable membranes in which biological proteins can be inserted, such as block copolymers. In the design shown in Figure 3.4, a two-step cross flow membrane setup incorporating such a membrane is shown. TDS values were chosen based upon a maximum osmotic pressure difference of a factor 3 on both sides of the membrane. A cross flow system was chosen firstly to relieve the osmotic pressure on the membrane, and secondly to further concentrate the brine, thereby decreasing the volume of the brine and increasing the efficiency of the system. After the second membrane step, the water is compliant with the WHO norm on salinity: 500-1000 mg/L TDS (World Health Organization, 2011). It is efficient to recycle the brine from the second membrane step back, as it is lower in TDS concentration than the influent seawater. The concentrated brine could even be used to generate electricity in a seawater battery, such as the one described by Kim et al. (2015). Light would need to be provided to activate the SyHr molecules, this could be realised in the form of a rollable LED sheet, which is rolled with the membrane. An example of a lighting solution which could be used is LINEARlight (Osram Technologies)*.

Model 2: Light powered membrane

It could be years before a robust and scalable membrane is found and it would be a great challenge to keep the proteins stable for long enough to successfully run such a setup. Therefore, an economical analysis is not yet of predictive value. Therefore, to offer a first evaluation of feasibility, a model was made which yields the theoretical recovery such a system could achieve and what the dimensions of the membrane would have to be to desalinate 100 m^3/h. This model can be useful to determine whether this desalination concept is worth further examining.

*https://www.osram.com/ds/ecat/Linear%20Flexible%20and%20Area%20Lighting-Digital%20Systems-/com/en/GPS01_3119470/PP_EUROPE_Europe_eCat/

Research Objectives

The objective of this study is:

To evaluate the feasibility of two scalable microbial desalination designs.

For both the designs which were described in the introduction, a model was made in order to arrive at this objective. As the two designs are of a quite different nature, there are different research sub-questions for each, as shown in Table 3.1.

Table 3.1: Research questions for the two conceptual designs

Concept	Questions
1. Multi-step SBR	How do the four scenario's compare in CAPEX, OPEX and energy costs? How much sludge is needed per m^3 seawater? What is the optimal treatment: RO, microbial desalination as a pre-step before RO or full microbial desalination?
2. Light powered membrane	What is the efficiency γ of the system? What is the minimal required area of the membrane?

3.3. Methods

Model 1: multi-step SBR

The parameters used in the model are shown in Table 3.2. These are inputs which were chosen, sourced from literature or calculated with ROSA software.
The TDS concentration after an amount n of SBR steps was calculated as follows:

$$TDS = 26000 * (1 - R_{TDS})^n \tag{3.1}$$

This equation is based upon an initial TDS value of 26000 mg/L which is representative for seawater. 25% removal per step was chosen as realistic after 4 hours based upon the results of the experiments in Section 1. This value may differ for another initial TDS starting value.
In the case of RO, the price per m^3 ($C_{RO,S1}$, $C_{RO,S2}$ and $C_{RO,S3}$)was calculated for every scenario with the influent TDS after SBR treatment using ROSA software (Reverse Osmosis System Analysis, Dow). CAPEX, OPEX and energy cost for scenario 1, 2 and 3 were calculated by multiplying the percentages $PCAP_{RO}$, POP_{RO} and PE_{RO} with the total cost for RO.
In the case of SBR's, CAPEX, OPEX and energy cost were first formulated per m^3 per SBR unit based upon literature values. For CAPEX, total SBR investment costs were converted to €/m^3 by HC and PP. Energy cost was calculated by multiplying E_{SBR} with P_E.
For every scenario, CAPEX, OPEX and energy cost were determined, based on both SBR steps and RO.

Table 3.2: Parameters for model 1: RO vs SBR system

Driver	Description	Unit	Based upon	Value
HC	Hourly water treatment capacity	m^3/h	Chosen value	100
PP	Payback period	years	Chosen value	10
R_{TDS}	TDS Removal efficiency per SBR	%	Experiments	25
P_E	Energy cost in the Netherlands	€/KWH	`https://ec.europa.eu/eurostat`	0,17
CAP_{SBR}	CAPEX per SBR	€/m^3	Yengejeh et al. (2014)	0,049
OP_{SBR}	OPEX per SBR	€/m^3	Jafarinejad (2017)	0,017
E_{SBR}	Energy consumption per SBR	KWH/m^3	Ciepliński et al. (2016)	0,012
EP_{SBR}	Energy price per SBR	€/m^3	Calculated with E_{SBR} and P_E	0,002
$PCAP_{RO}$	Percentage CAPEX of price RO	%	Fritzmann et al. (2007)	37
POP_{RO}	Percentage OPEX of price RO	%	Fritzmann et al. (2007)	19
PE_{RO}	Percentage energy of price RO	%	Fritzmann et al. (2007)	44
$C_{RO,S1}$	Costs of RO in Scenario 1	€/m^3	ROSA with TDS seawater	0,95
$C_{RO,S2}$	Costs of RO in Scenario 2	€/m^3	ROSA with TDS after 3 SBR steps	0,71
$C_{RO,S3}$	Costs of RO in Scenario 3	€/m^3	ROSA with TDS after 7 SBR steps	0,38

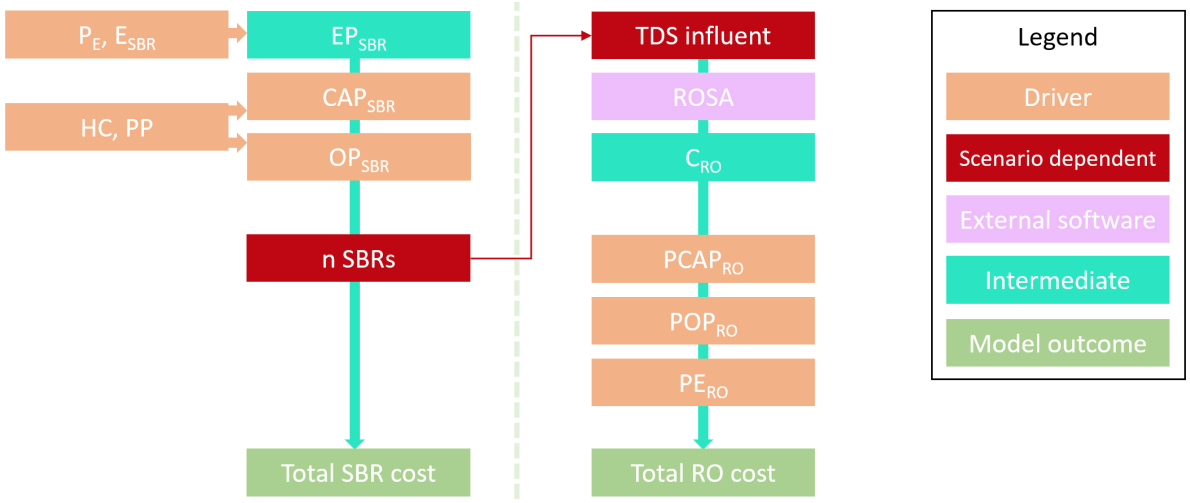

Figure 3.5: Model 1 structure

The following assumptions were made in the model:

- When TDS of inflow decreases, removal efficiency of biomass in SBR system remains the same.
- Activated sludge can directly be used for seawater desalination.
- Waste sludge can still produce biogas after being exposed to high salinities.
- The energy costs, CAPEX and OPEX are equal for all SBR steps.
- In every scenario, percentage distribution of costs within RO cost remains equal, even though salinity of influent varies.
- Costs of waste disposal are equal for all scenarios, because the SBR system makes use of the existing infrastructure of a WWTP and an RO system disposes of brine in the sea.

Amount of sludge produced

The amount of volatile suspended solids (VSS) produced at Harnaschpolder WWTP on a daily basis in excess sludge, was calculated using equation 3.2.

$$m_{sludge} = TC \cdot SP \tag{3.2}$$

Wherein:
m_{sludge}= Mass of sludge produced per day in kgVSS
TC= Treatment capacity Harnaschpolder in population equivalents, this was found to be equal to $1{,}26{\cdot}10^6$ based upon the Defluent website. †
SP= Solids production of the plant in kgVSS/day/population equivalent. Foladori et al. (2010) describes this value to vary between 0,035 and 0,085 gTSS, so 0,06 gTSS average. As a VSS:TSS ratio of 0,6 was found for Harnaschpolder sludge during calibration of the optical density to gVSS/L in article 1, 0,036 gVSS/day/population equivalent was taken.

†http://delfluent.nl/en/plant/wwtp-harnaschpolder/ratios/

Model 2: cross flow light light powered desalination membrane

The parameters of the model were defined as shown in Figure 3.6. The input parameters and their values are shown in Table 3.3. The following system of mass balance equations was used to find the values for the flow parameters Q_x. As the recycle flow Q_{cf2} influences the inflow water and the system is therefore dynamic, a simulation was run iteratively until an equilibrium was reached. The TDS input parameters were varied to obtain the best result, within the constraint that osmotic pressure difference on two sides of the membrane did not exceed a factor 3.

$$Q_{in,1} \cdot TDS1 + Q_{cf2} \cdot TDS4 = Q_{cf1} \cdot TDS3 + Q_{in2} \cdot TDS2 \tag{3.3}$$

$$Q_{in2} = Q_{in1} + Q_{cf2} - Q_{cf1} \tag{3.4}$$

Equation 3.4 can be inserted in Equation 3.3 to find Q_{cf1} and subsequently Q_{in1}, considering that during the first run, Q_{cf2} is equal to zero. Flows over the second membrane are governed by the following two equations:

$$Q_{in2} \cdot TDS2 = Q_{out} \cdot TDS5 + Q_{cf2} \cdot TDS4 \tag{3.5}$$

$$Q_{out} = Q_{in2} - Q_{cf2} \tag{3.6}$$

Which can be solved in the same manner as the first membrane.
The recovery, which is the volumetric percentage of the inflow water which can be won out of the system as treated water, can be calculated by:

$$\gamma = \frac{Q_{out}}{Q_{in1}} * 100\% \tag{3.7}$$

The required rate (r_p) of ions needing to be transferred over the membrane per hour for steps 1 and 2, can be calculated as follows:

$$r_{ions} = \frac{(TDS_{in1} - TDS2) \cdot Q_{in1}}{m_{Na/Cl}} \cdot Avo^{\ddagger} \tag{3.8}$$

From this, the required amount of proteins can be calculated according to:

$$n_p = \frac{r_{ions}}{C_{SyHr}} \tag{3.9}$$

The parameter n_p can then be inserted into Equation 3.10 to find the approximate area of the membrane.

$$A_m = \frac{n_p \cdot A_{SyHr}}{x} \tag{3.10}$$

The following assumptions were made in the model:

- A scalable, stable biomimetic membrane can be constructed.
- An osmotic pressure difference of a factor 3 can be withstood by the block copolymer membrane.
- Light supply to the membrane can be realised sufficiently.
- Values for ion transport rates reported by Niho et al. (2017) are relevant for proteins in such a membrane at these concentrations.

[‡]Values for TDS_{in1} and Q_{in1} are calculated by the iterative model, based on the recycle loop, not the initial values from the Driver table.

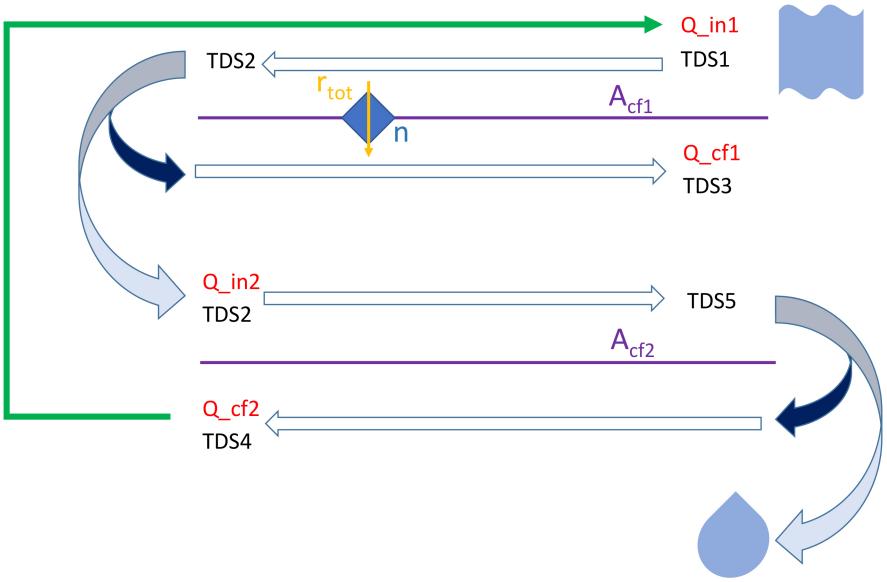

Figure 3.6: Model parameters visualised for model 2

Table 3.3: Drivers for model 2

Driver	Description	Unit	Based upon	Value
$TDS1$	Influent TDS concentration	g/m^3	Seawater TDS	26000
$TDS2$	TDS concentration after step 1	g/m^3	Chosen value	6000
$TDS3$	TDS concentration brine step 1	g/m^3	Chosen value	80000
$TDS4$	TDS concentration brine step 2	g/m^3	Chosen value	18000
$TDS5$	TDS concentration outflow	g/m^3	WHO standard	750
Q_{in1}	Flow	m^3/h	Chosen value	100
C_{SyHr}	SyHr capacity for ion transport	h^{-1}	Niho et al. (2017)	57142
A_{SyHr}	Area of one SyHr protein	m^2	Uniprot database	$8{,}1 \cdot 10^{-17}$
x	Fraction of membrane area occupied by proteins	%	Estimate by D. Mcmillan [a]	10
$m_{Na/Cl}$	Average mass of sodium and chloride	g/mol	General knowledge	29,2
Avo	Avogadro's number	#/mol	General knowledge	$6.02 \cdot 10^{23}$

[a] Assistant Professor in membrane bioinformatics at TU Delft

3.4. Results

Model 1: Multi step SBR

The costs of treatment per m^3 of seawater, split in CAPEX, OPEX and energy costs, are shown for the four scenarios in Figure 3.7. The costs per m^3 are €0,95 (1), €0,91 (2), €0,86 (3) and €0,75 (4). Incorporating SBR microbial desalination is shown to lower costs. Scenario 4 saves €0,20 per m^3; switching to this desalination method would save €1.752.000 for the whole investment period compared to RO. What must be noted, is that the effluent water from the system in scenario 4 is not of a drinking quality standard, and would need additional treatment (at additional cost) to come close to this quality. It could be used for irrigation in agriculture or for industry applications such as cooling.

For water which can be used as drinking water, scenario 3 is the best option. In comparison to scenario 1, which is the current method of treatment, the €0,09 savings per m^3 for scenario 3 would amount to €778.400 total savings over the whole investment period (assuming a treatment capacity of 100 m^3 per hour as previously defined). CAPEX amounts to the highest cost in this scenario, as investments must be done to realise both the RO and SBR systems. The SBR system accounts for 56% of costs for this scenario.

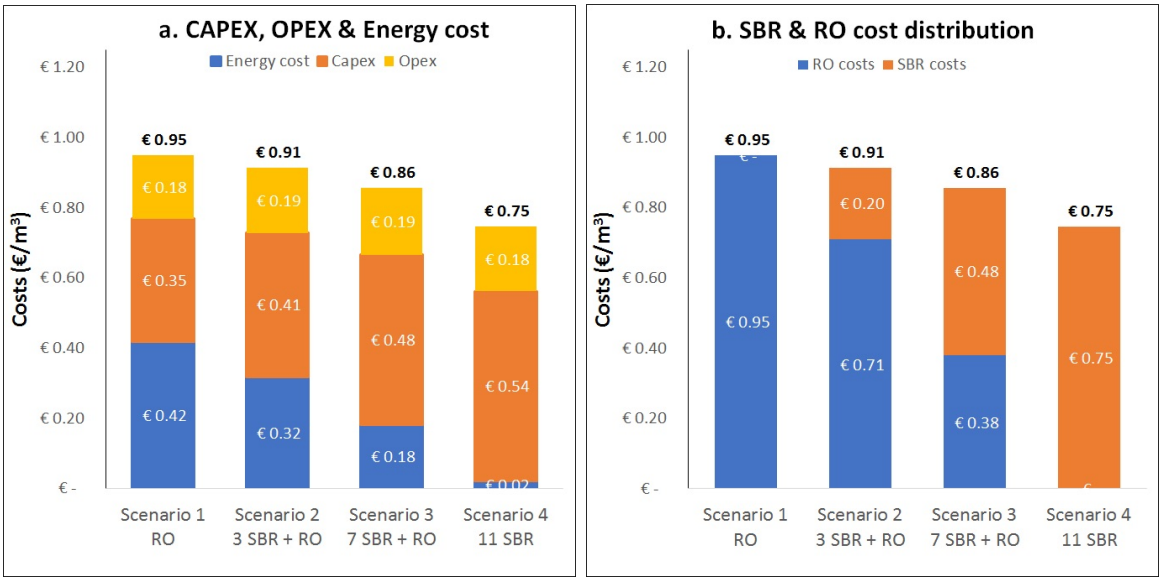

Figure 3.7: Cost comparison between the four scenarios, split in (a) CAPEX, OPEX and energy cost and (b) in RO and SBR costs

Energy consumption

Besides costs, the energy consumption of every scenario is relevant as it is directly impacts the feasibility and carbon footprint of the water treatment method. In Table 3.4, the energy consumption of every scenario is shown in household equivalents.[§] Compared to current desalination practice (scenario 1), yearly energy savings for the scenarios including microbial desalination are 145 household equivalents (scenario 2), 346 household equivalents (scenario 3) and 581 household equivalents (scenario 4). The considerable energy savings are an extra reason to consider scenario 4 as a seawater desalination system for agricultural or industrial purposes, and an advantage of scenarios 2 and 3 over 1.

Scenario	Household energy equivalent per year
1	612
2	467
3	266
4	31

Table 3.4: Energy consumption in yearly household equivalents for the four scenarios.[§]

[§]Yearly energy demand per scenario based on the aforementioned treatment capacity of 100 m^3 per hour. Average energy consumption of one household was taken to be 2990 KWH per year, as calculated by the Nibud institute for 2018: `https://www.nibud.nl/consumenten/energie-en-water/`

Amount of sludge needed

The amount of VSS in excess sludge produced daily at Harnaschpolder treatment plant was estimated to be around 45360 kgVSS/day based upon Equation 3.2.
Based upon the results of section 1, the amount of sludge needed to reduce electroconductivity of 1 m^3 seawater with 25% within a 24 hours is expected to be maximally 2 gVSS/L, so 2 kgVSS/m^3. As inoculation concentrations between 0,2 gVSS/L and 2 gVSS/L have not yet been examined, this value is a conservative estimate. Therefore, it can be calculated that for the amount of sludge produced at Harnaschpolder, 22680 m^3 of seawater can be desalinated per day. For multiple SBRs in series, this amount must be divided by the amount of SBR steps, for example in the case of scenario 4 (11 steps), 2061 m^3/day can be treated.
To conclude, in terms of available sludge at a WWTP, this design is feasible.

Model 2: Light powered crossflow membrane

The recovery γ was calculated to be 68%. As this is generally around 50% for RO, an increased efficiency and smaller brine volume would be an advantage of using such a biomimetic membrane. The membrane surface needed was calculated to be 0,62 m^2 in the case of perfect protein efficiency. If half of the proteins is expected to be active and correctly inserted (a very conservative estimate, as there are methods to do this in a near perfect manner[¶]), 1,2 m^2 would be needed.

[¶] In an unpublished paper from the Biocatalysis group at TU Delft, such a method is described where large charged groups are temporarily attached to proteins to ensure correct placement.

3.5. Discussion

Assumptions

The results from the models suggest full scale feasibility of microbial desalination concepts. However, the assumptions on which the models were built must be discussed.

Model 1 In the case of the SBR concept, equal percental removal of sions at all initial concentrations is the most vulnerable assumption. Sasaki et al. (2017) were able to remove sodium up to 94%, with a fresh culture being inoculated at 5 g/L sodium which is roughly half seawater concentration, which implies effective desalination at lower salinity. However, it was reported by Gan et al. (2016) that ion removal became less efficient at lower salinity, and such results were also seen in research line 1 of this thesis. Therefore, the assumption of equal decrease at every SBR step may be incorrect, as removal efficiency could decrease at lower salinities. A shake flask experiment where four dilutions of seawater medium are used, could provide clarity. To compensate for this uncertainty, the value of 25% removal per step which was used in the model is conservative, as removal up to 39% sodium was seen in the shake flask experiments. This higher initial uptake could partially compensate for decreased uptake at lower TDS concentrations. Whether sludge which has been used for desalination can still be used for biogas production, could also be tested in a small scale experiment. Sludge coming from a desalination experiment could be cultivated in an anaerobic shake flask or reactor to mimic an anaerobic digester, and gas formation analysed by weekly gas chromatography measurements.

Model 2 In the case of the light powered membrane concept, the most questionable assumption is the construction of a stable biomimetic membrane on a large scale. Aquaporin membranes were first proposed in 2007 by Kumar et al. (2007) after which many research groups have been working on realising this concept, but it is still not ready for scalable application due to this issue. Something which may greatly influence the scalability of this concept, is the costs. As the concept for the light powered SyHr membrane was only introduced in Research line 2, and has not even been constructed on a small scale yet, it is too early to make any predictions about costs, but for aquaporin membranes this is an important obstacle for implementation (Teow and Mohammad, 2019).

Fouling

Fouling is an important issue in membrane filtration, as it deteriorates the membrane, leading to decreased performance and a shortened lifetime. To prevent fouling, chemicals are used, which leads to extra costs and can negatively impact on the environment. The main membrane fouling issues are (Matin et al., 2011):

- Crystalline
- Organic
- Particulate and colliodal
- Microbiological

In the case of design 1, for scenario 1 (RO) the main fouling is crystalline as the feed is seawater containing many ions. In standard pretreatment, particulate matter such as sand is removed. For scenarios 2 and 3, as activated sludge has been introduced into the system, it can be expected that the reverse osmosis step is more vulnerable to organic, particulate/colloidal and microbiological fouling. A treatment step after the SBR steps would be necessary to protect the membrane. Coagulation followed by dual media filtration were found by Al-Tisan et al. (1995) to be 32-100 % effective in removing microbial contaminants from feedwater. Badruzzaman et al. (2019) describes an approach based upon the composition of the feedwater; for the expected quality of the feedwater in this case, ultrafiltration is recommended. For scenario 4, fouling is not an issue as no membrane technology is involved.
In the case of design 2, the feedwater is seawater. Therefore, crystalline fouling is expected to be the most problematic. An advantage of this design in comparison to reverse osmosis in terms of fouling, is that pressure is not applied to the water against the membrane. Ions are removed by the transporter proteins, but water stays on the feed side of the membrane and flows past. The block copolymer material of which artificial membranes including bacterial proteins are made, has even been used as a coating to prevent fouling on RO membranes due to its strong antifouling capacity (Shaffer et al., 2015). For these reasons, fouling is expected to occur less than in the case of RO.

Outlook

The results of both models indicate that it is worthwile to continue investigating the science and practical application of microbial desalination. An experiment which would contribute to understanding of a full scale system, is to replicate the SBR system with a series of upflow reactors. In the first, seawater is inoculated with 2gVSS/L of activated sludge, and from then the outflow of every reactor is led on to the next and inoculated with fresh sludge. I suggest to monitor settling time, salinity at every step, and an analysis of water quality parameters at the last step. Different residence times can be tested to find the influence of this on desalination. As for the case of the light powered membrane, it is worth examining due to the high selectivity, low energy cost and reduced fouling compared to RO. The concentrated brine flow could also be used for energy generation within a setup like proposed by Kim et al. (2015). To develop this technology, first the function of the proteins in the proposed membrane setup must be confirmed in a small scale membrane, such as a lipid bilayer vesicle. If this yields promising results, the purified protein could be inserted in a block copolymer to examine it's behaviour in a scaffold of this type. Experiments with increasing membrane sizes could be done.

3.6. Conclusion

The main messages are the following:

1. Costs for the four scenarios of model 1 were calculated to be €0,95 (1), €0,91 (2), €0,86 (3) and €0,75 (4). Therefore, it can be concluded that incorporating a microbial desalination SBR system is advantageous in terms of costs, saving up to €1.752.000 over the investment period of 10 years. It was also found that the scenarios including microbial desalination saved 145 (2), 346 (3) and 581 (4) household equivalents of energy in comparison to seawater RO (1).

2. Using just a series of SBRs is the most financially attractive desalination option, however the water quality is too poor for drinking water use. Therefore, a system of seven SBRs followed by RO is the best option for drinking water.

3. With the daily excess sludge production of Harnaschpolder WWTP, a desalination capacity of 22680 m^3 was calculated. This number should be corrected for the amount of SBRs used.

4. For model 2, a recovery of 68 % was predicted, which is more efficient than RO (generally about 50%). The membrane surface which was needed was calculated to be <1,2 m^2. Both these outcomes indicate practical feasibility of a full scale installation. It is too early to evaluate technical feasibility.

5. From the models implemented in this study, both conceptual designs for microbial desalination seem feasible. It must, however, be considered that at this point many assumptions had to be made as the designs are still conceptual.

In short: Two models for microbial desalination designs showed promising outcomes for feasibility; either using SBRs in series or a biomimetic membrane. However, many assumptions need to be validated by research.

General Conclusion & Outlook

Summary of conclusions from the three articles

This thesis deals with the concept of microbial desalination. Three research lines were executed. This section summarises the main conclusions.

1. **Determining the Optimal Conditions for Microbial Desalination through Batch Experiments** The results of this research line show that various mixed cultures are capable or removing sodium and chloride from artificial seawater, or Stevens medium supplemented with sodium chloride. This desalination also occured when no growth of biomass was observed. Light is not required for desalination. Ion chromatography and electroconductivity results indicated that ion exchange was not an important desalination mechanism. The most successful removal which was observed (39% sodium), occured under the conditions of inactive biomass at 2 gVSS/L inoculation concentration.

2. **A Light Powered Bio-Inspired Desalination Membrane** A new desalination concept was proposed and designed; a light powered, biomimetic desalination membrane featuring the transport protein SyHr. In order to facilitate the construction of such a membrane, the protein SyHr was obtained. To this end, the gene for SyHr was successfully inserted to a Ptrc99a vector. Subsequently, E.coli BL21 cells were showed to be transformed with this vector. Overexpression was induced, which led to abundant production of SyHr, as confirmed by a protein gel.
   Although the step of placing SyHr in a membrane was not reached due to practical circumstances, the foundations have been laid to perform this last part of the experiment relatively easily. Furthermore, the SyHr can be used for many different purposes.

3. **A Model Approach for Assessing the Feasibility of Microbial Desalination** To evaluate feasibility of batch full scale desalination, cost comparison was made of four scenarios: seawater RO (1), three SBR steps and RO (2), seven SBR steps and RO (3) and eleven SBR steps (4). Costs for the four scenarios per m^3 were calculated to be €0,95 (1), €0,91 (2), €0,86 (3) and €0,75 (4). Therefore, it can be concluded that incorporating a microbial desalination SBR system is financially attractive. A series of 11 SBRs (4) is the most financially attractive desalination option for industry or agriculture. A system of seven SBRs followed by RO (3) is the best option for drinking water. With the daily excess sludge production of Harnaschpolder WWTP, a desalination capacity of 22680 m^3 was calculated. For the second design, a biomimetic membrane, a recovery of 68% was predicted. This is more efficient than RO. The membrane surface which was needed to treat 100 m^3/h was calculated to be <1,22 m^2.
   Based upon the models implemented in this study, both a series of SBRs and a light powered membrane were calculated to be feasible. It must, however, be considered that at this point many assumptions had to be made as the designs are still conceptual. This study, nonetheless, paves the way for new science and technology within microbial desalination.

Overall Conclusion

The research question for this combined work was formulated as:

> **"Is there potential in using biological principles to desalinate seawater?"**

As summarised above, it was demonstrated that microorganisms are capable of removing sodium from seawater medium up to 39%. Chloride was shown to be removed alongide with sodium, various mixed cultures were found capable of desalination and dark conditions do not limit performance. All these results are positive for practical application. Furthermore, EPS exchange was eliminated as a desalination mechanism and a new mechanism for sodium chloride storage was proposed. In the second article, the wide potential of microbial methods in desalination was demonstrated in the design for a light powered membrane. Finally, both this membrane and full scale desalination in batches were found to be feasible in models. Therefore, based on the results from all research lines it can be concluded that there is potential in microbial desalination, and this topic is worth examining in the future.

Impact

This section describes the substantial contribution of this work to current knowledge microbial desalination.

Firstly, the capacity of mixed cultures to desalinate was proven, in active and inactive state. As mixed cultures had not previously been used in these experiments but do have practical and financial advantages in comparison to pure cultures, this is relevant information. It was shown that inoculum from a mixed PNSB reactor, activated sludge and a green phototroph culture were capable of removing sodium from seawater.

Secondly, the removal of chloride was shown by ion chromatography. As previous studies measured only sodium concentration to indicate desalination, this finding confirms the assumption that chloride decreases with sodium. Furthermore, the influence of pH control was found to be negligible, which indicates that pH and presence of protons are of minor influence.

Previous research has shown capacity of various organisms to desalinate, but without delving into the mechanism of why and how (with the exception of Amezaga et al. (2014) who describe a theoretical mechanism for genetically modified cells). Based upon this work, ion exchange can now be eliminated as a possible mechanism of desalination.

Furthermore, a driver and mechanism were proposed based on results. The driver of microorganisms to take up salt would be to lower the sodium concentration sufficiently for the population to grow. The mechanism would be via local oversaturation of sodium and chloride and subsequent precipitation of sodium chloride in a bacterial microcompartment. The fact that this behaviour was previously seen in cyanobacteria storing calcium carbonate shows that it is feasible (Blondeau et al., 2018). In TEM, inclusions were seen in cells which had been cultivated in seawater, possibly confirming this theory. Although the time frame of the thesis did not allow to prove this mechanism, it offers an explanation for microbial desalination and it is a starting point for future research.

A new technology was proposed wherein microbial transport proteins are integrated into a membrane for light powered desalination. Considering that this concept is completely new, it contributes to creative problem solving within desalination science and fosters bio-based innovation. As the gene design and expression of SyHr (Synechocystis Halorhodopsin, a light powered anion pump) have already been performed within this research, integration into an artificial membrane to test desalination capacity could be realised fast in future research projects.

Finally, this thesis offers a first quantitative evaluation of feasibility of microbial desalination for full scale water treatment. Where previous work does not yet consider full scale installations, the third research line shows that implementation of microbial desalination by a series of SBRs, with ot withour RO, reduces the cost of seawater desalination and that the amount of sludge which is needed can realistically be supplied by a WWTP. The second model shows that the design for a light powered desalination membrane is theoretically feasible, as surface area (1,2 m^2) and recovery (68%) are comparable to (and even significantly better than) RO (respectively around 50% recovery and 10+ m^2 area needed).

Outlook

This thesis makes a strong case for microbial desalination. Working on this topic has led me to believe that this approach is both promising for practical application, as highly interesting in terms of fundamental knowledge concerning ion sequestration by microorganisms. It is now for science and the industry to recognise the scientific and societal value of this topic and further develop it. After all, sodium concentration decreases up to 39% were seen; this presents opportunities for desalination via a method which has not yet been developed. Before proceeding to finding applications, the scientific community must be convinced. Therefore, the most important next research step is to prove the removal further: by making a mass balance of sodium and chloride as described in section 1.5, and to analyse where the ions are present at the end of the experiment. Proving the desalination mechanism is also important, not in the least because it will offer opportunities for optimisation of desalination performance. Once the mechanism is clear and the ion balance has been closed, one or multiple industry parties could be involved in some kind of pilot scale installation. This will provide valuable information of the long term performance of such a system, and the properties of the sludge which comes out of it. If successful, designs for a larger scale can be made.

In short, this innovative work has advanced knowledge in several directions and paved the way for the further development of microbial desalination

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A

Presentations and documents

This appendix contains an overview of presentations given about this thesis topic and documents which were made for internal and external parties. As this project was started from scratch, a considerable amount of time was spent on project management and reaching out to other parties in the first phase. This appendix quantifies this output.

Presentations

Table A.1 gives an overview of presentations given about this thesis topic in addition to kickoff, midterm and green light presentations.

Table A.1: Presentations given about microbial desalination

Date	**Audience**	**Type**
May 6th 2018	Dopper Changemaker Challenge	Concept Pitch
November 5th 2018	MB5.0 KNVM Meeting	Pitch and poster presentation
November 6th 2018	EBT Seminar	Introduction presentation
May 2nd 2019	Sanitary Engineering Colloquium	Results presentation
May 3rd 2019	EBT Seminar	Results presentation

Documents

Table A.2 gives an overview of documents made during this this thesis for various purposes. These documents can be found on the shared Google drive or requested from the author.

Table A.2: Documents made within this project

Date	**For whom**	**Contents**
August 2018	David Weissbrodt, Merle de Kreuk (TUD)	Project proposal, first literature survey and planning
September 2018	Baoxia Mi, Shaofan Li (UC Berkeley)	Project outline and proposal for joint work
November 2018	Anna Amtmann (Glasgow)	Project outline and enquiry after continuation of her work
December 2018	Duncan Mcmillan (TUD)	Project outline and proposal for joint work
January 2019	Duncan Mcmillan (TUD)	Research document on opsin proteins
March 2019	Stefan Marsden, Albert Hérnandez (TUD)	Light Powered Desalination Membrane: a Summary
March 2019	Damian Palin (Cornell University)	Summary and Precipitation hypothesis

B

Visual appearance of shake flasks

Day	Mixed PNSB Saline	*Rhodopseudomonas* Saline	Mixed PNSB Saline	*Rhodopseudomonas* Control
0				
1				
3				
7				

Figure B.1: Visual appearance of *Rhodopseudomonas* vs mixed PNSB culture shake flasks over time.

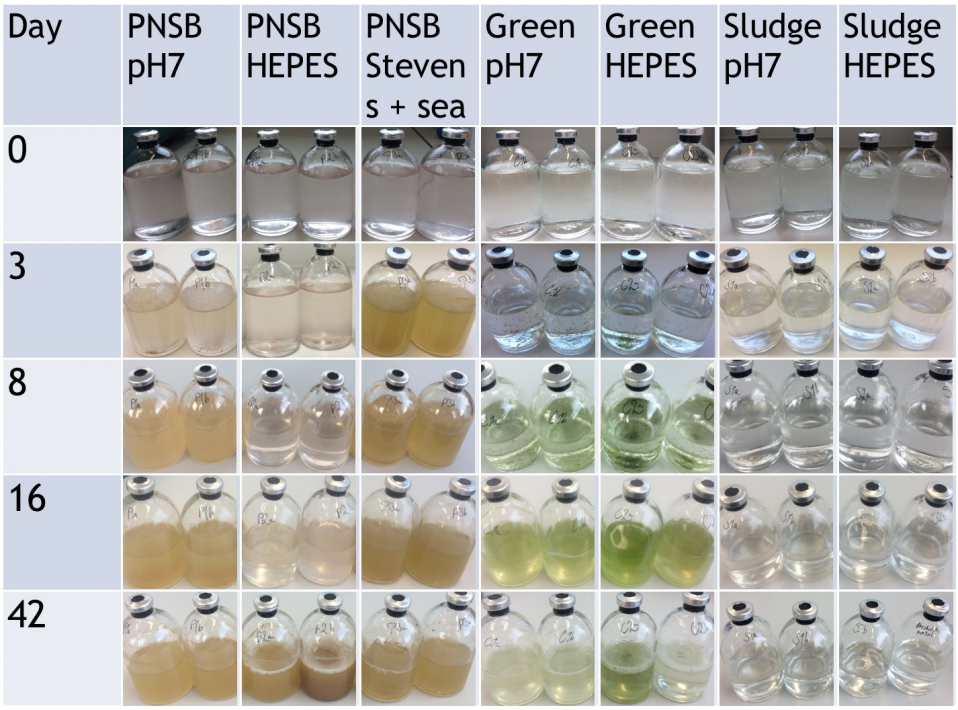


Figure B.2: Visual appearance of biomass types and pH control experiment over time.

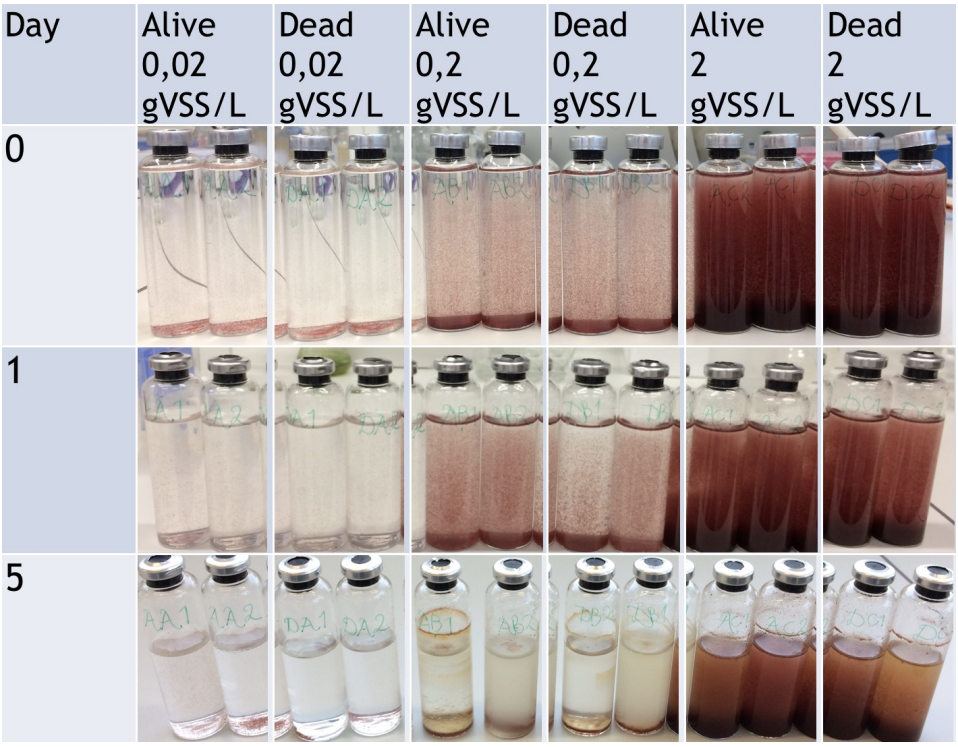


Figure B.3: Visual appearance of dead-alive and inoculation concentration experiment over time

C

Experiment: Microbial community composition at various NaCl concentrations

Research Question

The research question for this experiment was formulated to be:

> How does the microbial community of a PNSB enriched mixed culture change over time at different salinities and does desalination occur?

Materials and Methods

A shake flask experiment was peformed a PNSB mixed culture from a PNSB enriched reactor was cultivated under various salt concentrations. The basic medium composition was the same as the 0,2 g/L sodium variant used in Experiment 1, as shown in Table 1.4. This medium was used in all shake flasks, but supplemented with various concentrations of sodium chloride as shown in Table C.1. Inoculum was taken from a continuous reactor containing a mixed PNSB culture which was enriched by Stevens (2017) and Ligtenberg (2017) from an activated sludge sample from Harnaschpolder WWTP, The Netherlands. In the cases of the bottles which have sodium chloride concentrations from 0-12 g/L, inoculum was taken directly from the reactor. In the case of 24 g/L sodium chloride, in one sample fresh inoculum from the reactor was used, and in another an acclimatised inoculum which came from the same reactor but has already been cultivated at 24 g/L NaCl for 14 days.

Table C.1: Composition of the bottles for Experiment 1B

Inoculum	**g/L Additional NaCl**
1 mL mixed culture (reactor)	0 g/L
1 mL mixed culture (reactor)	6 g/L
1 mL mixed culture (reactor)	12 g/L
1 mL mixed culture (reactor)	24 g/L
1 mL mixed culture (acclimatised)	24 g/L

100 mL shake flasks were filled with medium as described before, inoculated, then capped and flushed with argon for five minutes. Flasks were cultured for 28 days at 150 rpm shaker speed. The setup was illuminated using Gamma Breedstraler lamps of 120 W, filtered for infrared light (>700 nm). The experiment was performed in duplicate. Biomass concentration was determines spectrophotometrically four times, at 0, 10, 17 and 28 days. At 0 and 28 days sodium concentration was determined using the Laqua Twin Sodium Meter. A wavelength scan from 400-1000 nm was performed on the initial inoculum and the samples from the final day.

Results

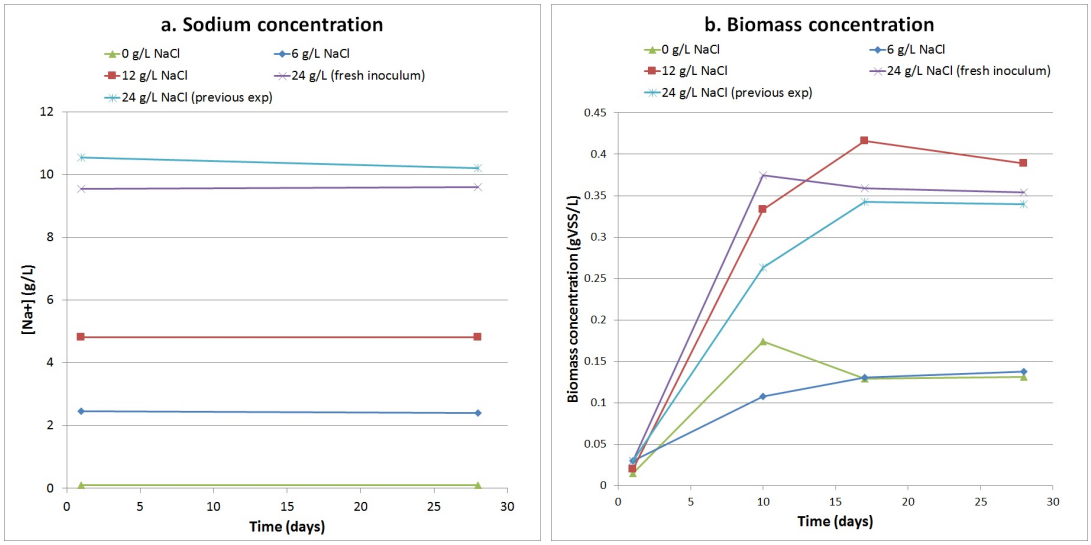


Figure C.1: (a) Sodium concentration and (b) Biomass concentration as measured in the various medium salinities of experiment 2, based upon single measurements.

In Figure C.1, the biomass and sodium concentrations are shown for the duration of the experiment. Notably, the fastest growth and highest final biomass concentrations were seen at 12 g/L and 24 g/L sodium chloride. This corresponds with the observation in experiment 1, where the mixed culture grew better in the 24 g/L sodium chloride medium than at lower salinity. As shown in Figure C.1b, the sodium concentrations at the beginning and ending of the experiment do not show pronounced change.
In Figure C.2, the community composition of the various samples of this experiment can be seen. It can be concluded that saline medium enriches for *Rhodobacter*, and that *Rhodopseudomonas* and *Thiobaca* become less prevalent as salinity increases. as also seen in Experiment 1A (Figure 1.3). If a culture is used which is acclimatised, the enrichment for *Rhodobacter* is more pronounced, as the culture has been enriching in saline medium for a longer period of time.

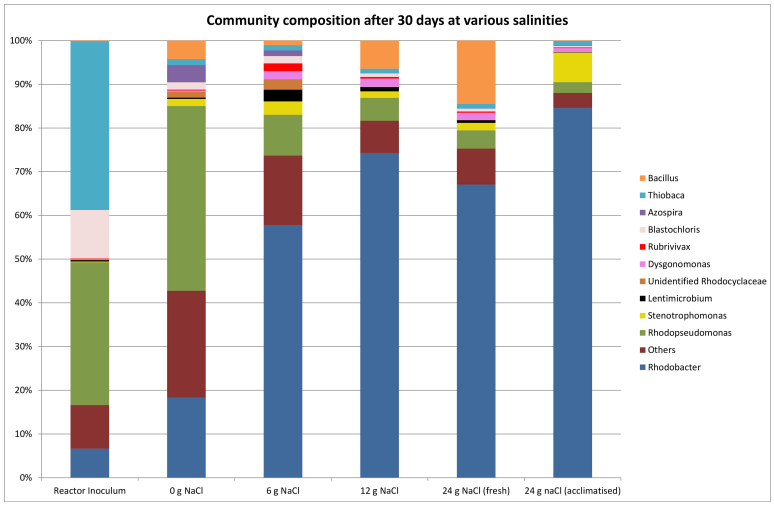


Figure C.2: Community composition at various sodium chloride concentrations, reactor inoculum at t=0 hours and samples at t=30 days (end of experiment)

Finally, Figure C.3 shows the visual appearance of the bottles during the experiment. It can be seen that an orange-yellow colour corresponds to high amount of *Rhodobacter*, although increased salinity appears to lend a more pale, yellow colour to bottles which have similar proportions of *Rhodobacter*.

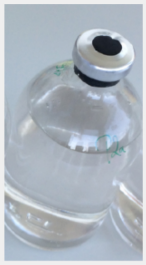
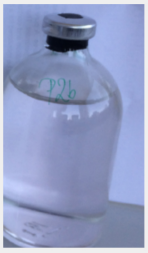
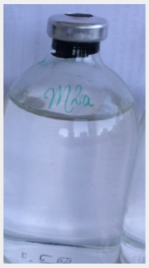
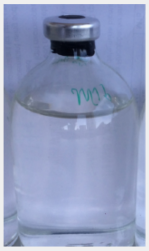
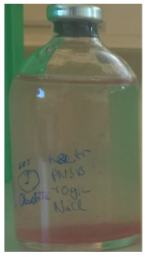
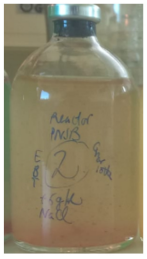
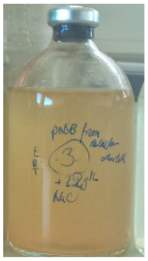
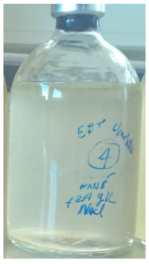
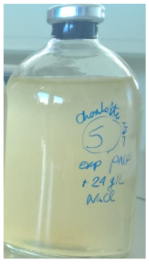
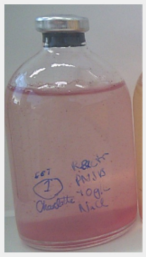
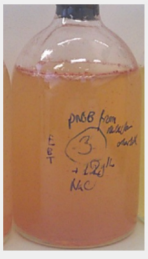
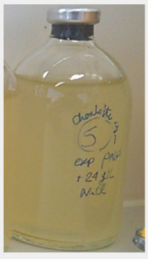
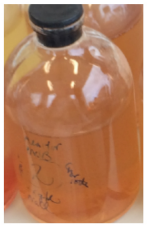
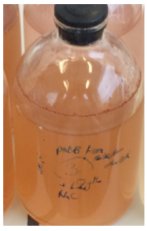
Day	0 g NaCl	6 g NaCl	12 g NaCl	24 g NaCl reactor	24 g NaCl prev exp
0					
7					
14					
30					

Figure C.3: Visual appearance of shake flasks during the experiment.

D

Experiment: Enrichment of green phototrophs from pond water

Research Objective

The research question of this experiment was formulated to be:

> To enrich for green phototrophs in pond water, in order to assess the desalination capacity of algae and cyanobacteria.

Materials and methods

A sample of pond water was taken from the rectangular pond next to the car park behind Applied Sciences, TU Delft, Delft, the Netherlands. 5 mL of this water was cultivated in a 100 mL erlenmeyer with 45mL BG11 medium, of which the composition is shown in Table D.1. For five days the culture was stirred under continuous illumination. Illumination was provided by Gamma Breedstraler lamps of 120 W. Cultivation was aerobic (cotton wool stopper) in order for sufficient carbon dioxide to be available as a carbon and energy source. The work of Mooij (2016) was used to develop these methods.

Table D.1: BG11 Medium composition

Component	**Concentration per L**
$NaNO_3$	17,6 mM
K_2HPO_4	0,022 mM
Na_2CO_3	0,18 mM
$MgSO_4 * 7H_2O$	0,03 mM
$Citricacid * H2O$	0,03 mM
$CaCl_2 * 2H_20$	0,2 mM
Ammonium ferric citrate	0,02 mM
$Na_2EDTA * 2H_2O$	0,002 mM
Trace Element Solution	1 mL

Results

After five days of cultivation, the culture had a bright green colour, indicating the presence of green phototrophs and thereby success of the enrichment. The colour evolution of the culture over time is shown in Figure D.1.

Location of glycerol stocks

In case anybody around Applied Sciences needs a green phototroph culture in the future, glycerol stocks were made of both my own enrichment and the co-culture sent by Wageningen University (described in Methods

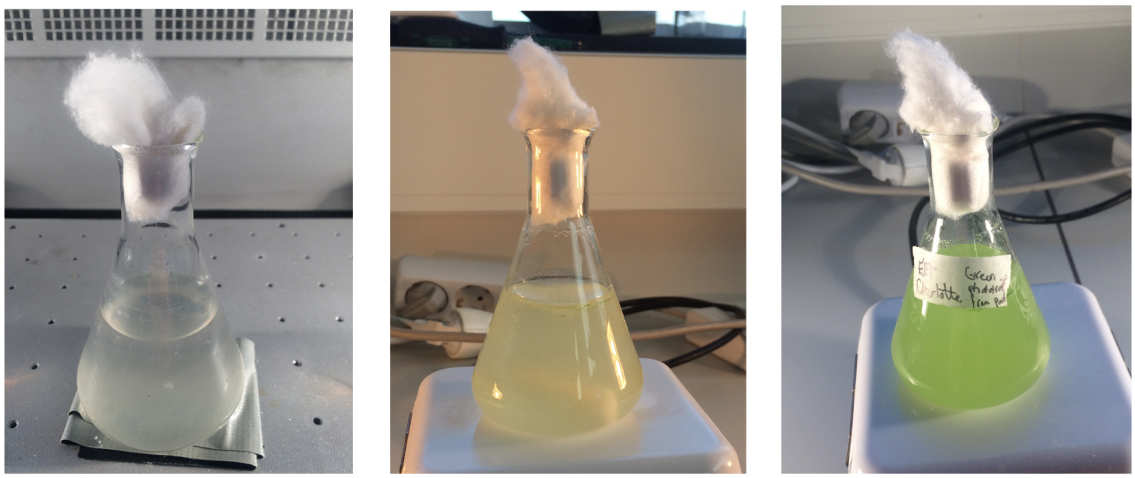


Figure D.1: Photographs of the enrichment culture on day 0, day 1 and day 5, from left to right. The evolution of the colour to bright green indicates presence of algae and cyanobacteria.

Article 1). These can be found in the -80 °C freezer in room B2.460 in drawer 36B.

E

Supplemental Information Biomass types experiment

Acetate consumption

HPLC measurements to determine acetate consumption were performed on the supernatant of a selection of samples from the PNSB and sludge cultures of the biomass types and pH control experiment, of which the results are shown in Figure E.1. For green phototrophs, acetate consumption is redundant, as no organic carbon source was added to the phototroph seawater medium in order to select for carbon fixating species.

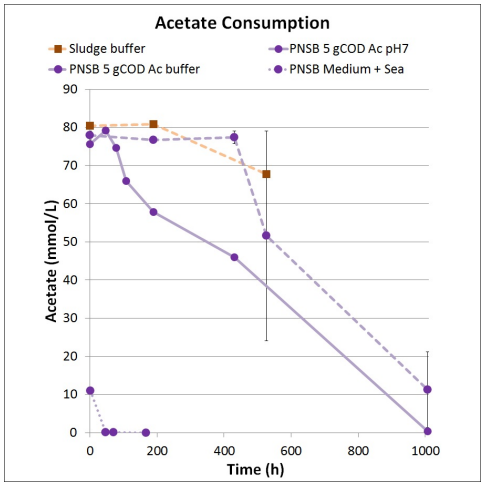


Figure E.1: Acetate consumption based upon HPLC measurements, for all three PNSB cultures and the buffer sludge culture.

Results up to 1007 hours

In article 1, sodium values for the biomass types and pH control experiment are given up to 550 hours. One measurement of optical density and sodium concentration was also done at 1007 hours. This was not shown in the article as the relevant sodium decrease at the beginning of the experiment becomes more compressed. Figure E.2 shows the sodium and biomass concentration curves over time up to 1007 hours.

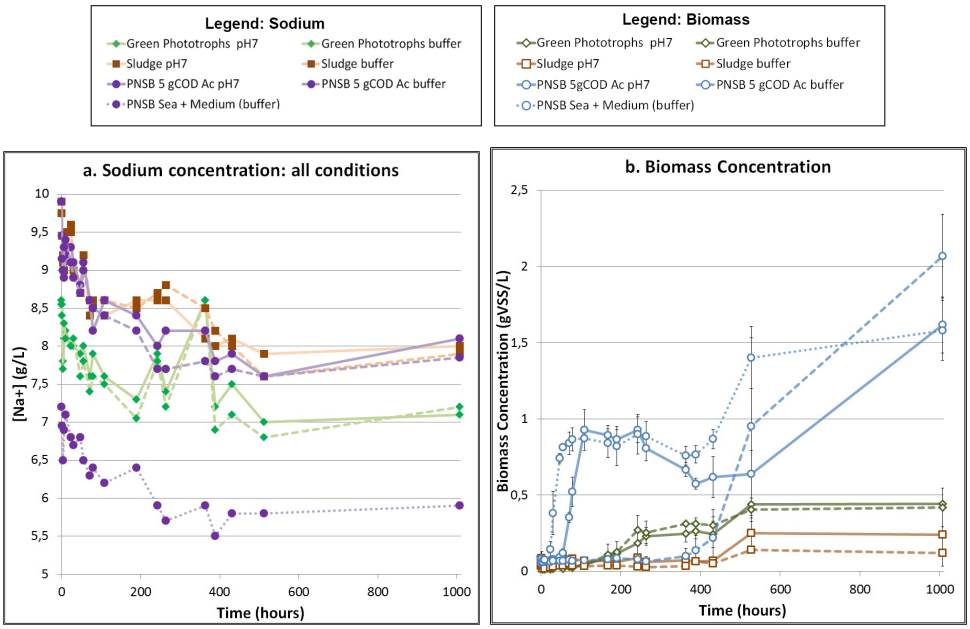


Figure E.2: a. Sodium concentration in experiment 3 up to 1007 hours
b. Biomass concentration in experiment 3 up to 1007 hours

Community composition

The community composition of all PNSB cultures and the pH7 sludge culture are shown in Figure E.3

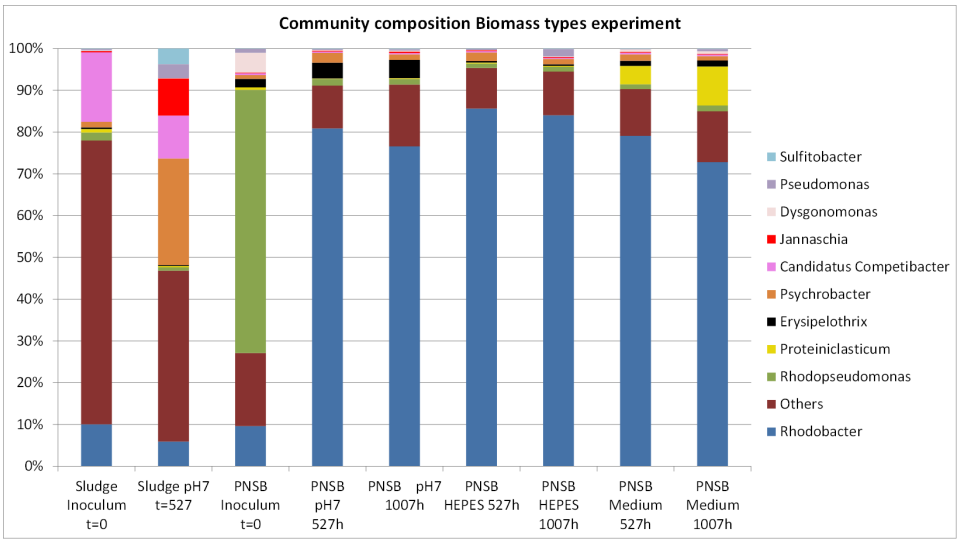


Figure E.3: Community composition of various cultures during the biomass types experiment, at 0, 527 and 1007 hours.

F

Supplemental information active/inactive experiment

Community composition of active and inactive inoculum

Figure F.1 shows community composition of the active and UV-irradiated inoculum.

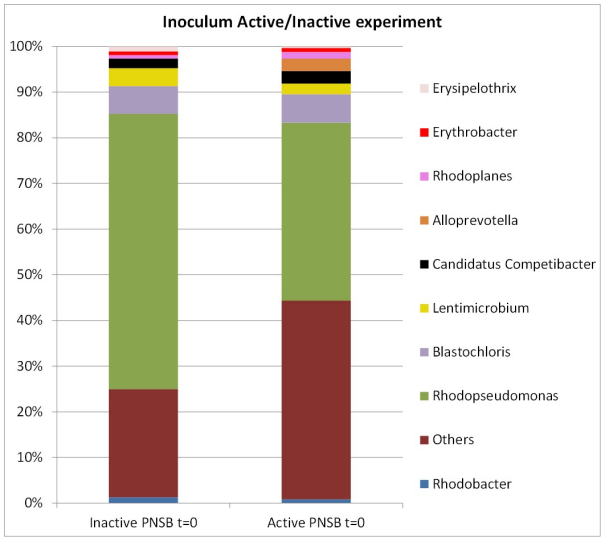


Figure F.1: Community composition of active and UV inactivated inoculum

G

SyHr DNA fragment

Figure E.1 shows the SyHr DNA fragment which was designed and synthesised within a pUC SP vector.

Figure G.1: Complete sequence of SyHr DNA which was designed to be inserted in a pUC SP vector.

Location of materials

A selection of materials from this research was kept for future use:

- Glycerol stocks of the co-culture from Wageningen
- Glycerol stocks of green phototroph enrichment
- Pellets of selected experiments
- *E.coli* BL21 transformed to express SyHr
- Trans-retinal for SyHr expression
- Membrane protein fraction after SyHr overexpression

The first three can be found in the -80 °C freezer in room B2.460 in drawer 36B, in a box labeled "Charlotte Meerstadt, Microbial Desalination".
The final three, related to protein purification, have been placed under care of Duncan McMillan.

Anyone wishing to use the Laqua Twin Sodium Meter can contact Marta Cerruti.