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Effect of urine dilution on the biofilm formation in a MBMBR setup

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TABLE OF CONTENTS

USED ABBREVIATIONS

AOB	Ammonium Oxidizing Bacteria
AOO	Ammonium Oxidizing Organism
DO	Dissolved Oxygen
EC	Electrical Conductivity
ESA	European Space Agency
HRT	Hydraulic Retention Time
ISS	International Space Station
MBR	Membrane Bioreactor
MBMBR	Membrane Moving Bed Biofilm Reactor
MBBR	Moving Bed Bioreactor
NASA	National Aeronautics and Space Administration
NOB	Nitrite Oxidizing Bacteria
PBBR	Packed Bed Biofilm Reactor
PVA	Polyvinyl Alcohol
SRT	Sludge Retention Time
SVI	Sludge Volume Index
RO	Reverse Osmosis
TSS	Total Suspended Solids
VCD	Vapor Compression Distillation
VSS	Volatile Suspended Solids
WTUB	Water Treatment Unit Breadboard

LIST OF FIGURES

LIST OF TABLES

ABSTRACT

Water accounts for 90% of the life support consumables and is pivotal for long-term Space mission. Currently physicochemical water recycling systems are being used to recover water in Space. A new approach to recover water is the combination of biological and physicochemical processes. It is believed that the integration of these two may offer an increase in water recycling efficiencies. Urine is an important water source to be treated. This component contains relatively high concentrations of inorganic salts such as sodium chloride (8 g L^{-1}), potassium chloride (1.6 g L^{-1}), potassium sulfate (2.6 g L^{-1}), magnesium sulfate (0.8 g L^{-1}), magnesium carbonate (0.1 g L^{-1}), potassium bicarbonate (0.7 g L^{-1}) and potassium phosphate (0.2 g L^{-1}). A nitrification bioreactor is essential in a wastewater treatment system for Space applications to convert urea to nitrate. In Space, microgravity conditions are present and thus it is not possible to provide bubble aeration to suspended sludge in the bioreactor. The only way to provide oxygen to the microorganisms is by diffusion. That is why an attached growth (= biofilm) system is required for Space applications. In literature, adaptation of an established nitrification biofilm to high salinity has been described, yet for practical implementations it would be more appealing if a salt-adapted or -tolerant nitrifying sludge in suspension could be shifted towards biofilm growth mode. However, from literature it is still not known how salinity affects the biofilm formation in a bioreactor treating urine.

The overall purpose of this study was to investigate the effect of salinity on nitrification activity and biofilm formation in a Moving Bed Membrane Bioreactor (MBMBR). This research is divided in different goals: (i) identify a suitable sludge for the inoculation of the bioreactors, (ii) study the effect of salinity on biofilm formation (stage 1 MBMBR), (iii) investigate the effect of wash-out as selective pressure for biofilm formation (stage 2 MBMBR) and (iv) assess the effect of an altered divalent/monovalent cation ratio of biofilm formation (stage 3 MBMBR).

It is crucial that a right inoculum is chosen when operating a nitrification bioreactor at high salinity. It should be rich in Ammonium Oxidizing Organisms (AOO; bacteria and/or archaea) and Nitrite Oxidizing Bacteria (NOB) and maintain activity under a high salinity. In this part of the research batch experiments were performed for different sludge types, either commercialized for aquaculture applications or derived from manure, wastewater and landfill leachate treatment facilities. AOO and NOB activity were analyzed separately at the salinity of nitrified urine (45 mS cm^{-1}) as well as at the salinity the sludge was used to grow at. These experiments revealed that the aquaculture inoculum had the highest activity for both AOO and NOB at original salt conditions and under nitrified urine conditions. The inhibition of AOO and NOB was 0% and 18%, respectively. The results of the inoculum screening provide some support for the fact that AOO are more sensitive to salt stress than NOB. The aquaculture inoculum was chosen for the inoculation of the bioreactors.

Stage 1 of the bioreactor experiments deals with salinity. Microorganisms in suspension have to cope with salt stress. This is costly from a bioenergetic point of view. It is possible that microorganisms will prefer attached growth to suspended growth to be protected from salt stress. The goal of stage 1 was to study the effect of salinity as a driver for biofilm formation. Two bioreactors were operated for 30 days at different salinities: 15 mS cm^{-1} and 45 mS cm^{-1}. Sodium and chloride were chosen as ion source. These two ions are most abundant in urine. Polyvinyl Alcohol (PVA) beads were used as carrier material in both bioreactors. No sludge was taken out of the MBMBRs during operation. Micro-scale activity tests and Lowry protein determination were performed weekly, both for the attached and suspended sludge, in order to quantify biomass-specific conversion rates. The suspended sludge did not suffer from high salinity according to the results obtained in the activity tests. No

biomass developed on the beads however. The microorganisms in suspension were seemingly not sufficiently stressed to form rapidly biofilm.

The aim of stage 2 was to investigate the effect of mixed liquor wash-out as a selective pressure for biofilm formation in both bioreactors. Literature has proven that a low bulk Sludge Retention Time (SRT) is critical for biofilm formation. Microorganisms in suspension get washed out and microorganisms growing on the carriers are retained. The salinity of the reactors was identical as in stage 1, only the floccular SRT was decreased to 8 days (no sludge was taken out in stage 1). 1.14 L d^{-1} effluent was taken out by membrane permeation (solid/liquid separation) and 1.14 L d^{-1} direct from the bioreactors. The reactors were operated for 35 days. During reactor operation, micro scale batch activity tests (250µL), macro scale activity test (50mL) and Lowry extraction were performed for the PVA beads and for the suspensions. No AOO and NOB activity was present on the beads. Furthermore, no proteins and hence no biomass could be detected on the carriers. The results of the micro scale batch experiments showed that the activity of the suspended sludge did not decrease by salt stress. This was in correspondence with the results of the inoculum screening and the experiments in stage 1. From these results it can be concluded that the aquaculture inoculum sludge was tolerant to salt stress. Possible reasons for lack of a biofilm could be due to: the detection limit of the Lowry extraction, length of the experiments, shear effects, absence of COD in the influent and inoculum selection.

Divalent cations such as calcium bridge negatively charged functional groups on extracellular polymeric substances and bacterial surfaces. This connection enhances the strength of the biofilm according to the divalent cation bridging theory. An increase in monovalent ion concentration will then supposedly result in deterioration of the biofilm structure and increased detachment of the biofilm. In stage 3, the importance of a high divalent/monovalent cation ratio was studied. Both MBMBRs were operated for 15 days at an identical salinity of 15 mS cm^{-1} and a SRT of 8 day. One reactor was running on a divalent/monovalent cation ratio of 0.002 mg Ca^{2+} mg^{-1} Na^{+}, while the other one received 1.93 mg Ca^{2+} mg^{-1} Na^{+}. Macro scale batch activity test (50mL) and Lowry protein determination were performed during operation. The results indicated that there was no AOO and NOB activity on the carrier. Proteins were detected in the reactor running on a low divalent/monovalent cation ratio, in contrast to the hypothesis. Visually, biofilm was observed in both bioreactors. The results indicate that a low SRT is of great importance for biofilm formation.

Overall, this research shows that biofilm formation in a floccular system used to or tolerant to high salinity is challenging. A key aspect for biofilm formation is the SRT of the floccular sludge, in function of the process temperature. More research is still needed to study the effect of salinity on biofilm formation and to explore the effect of a high divalent/monovalent cation ratio. These findings will not only allow to further implement urine nitrification to Space treatment conditions, but will also open up terrestrial applications for start-up and robustness of industrial biofilm treatment systems for wastewaters with high and/or varying salinity.

SAMENVATTING

Het effect van zout op de biofilm vorming in een "Moving bed mebrane bioreactor" (M*BMBR).*

Water is goed voor 90% van het verbruik in een levensondersteunend systeem en is vitaal voor lange termijn ruimtemissies. Momenteel worden er in de ruimte fysische systemen gebruikt om water te herwinnen. Een nieuwe werkwijze om water te recupereren in de ruimte, is gebaseerd op de combinatie van biologische en fysisch-chemische processen. Er wordt verondersteld dat een integratie van beide systemen kan leiden tot een verhoging van de water herwinningefficiëntie. Urine is een belangrijke bron van water die dient behandeld te worden. Deze component bevat relatieve hoge concentraties aan anorganische zouten zoals natriumchloride (8 g L^{-1}), kaliumchloride (1,6 g L^{-1}), kaliumsulfaat (2,6 g L^{-1}), magnesiumsulfaat (0,8 g L^{-1}), magnesiumcarbonaat (0,1 g L^{-1}), kaliumbicarbonaat (0,7 g L^{-1}) en kaliumfosfaat (0,2 g L^{-1}). Een nitrificatie bioreactor waarin ureum wordt omgezet in nitraat is essentieel in een waterzuiveringsysteem voor ruimtetoepassingen. In de ruimte zijn er microgravitationele condities aanwezig. Daardoor is het niet mogelijk om bubbelbeluchting te voorzien aan gesuspendeerd slib in een bioreactor. De enige manier om zuurstof te transfereren aan de micro-organismen is doormiddel van diffusie. Omwille van die reden is het cruciaal dat er gehechte groei (= biofilm) aanwezig is in een bioreactor voor ruimtetoepassingen. In de literatuur is de aanpassing van een nitrificatie biofilm naar hoog zoutgehalte reeds beschreven. Omwille van de praktische implementatie, zou het aantrekkelijker zijn om te starten met nitrificerend slib dat gewoon of tolerant is aan zout. Daarna kan biofilm groei gestimuleerd worden. Echter, uit de literatuur is het nog niet bekend hoe zout invloed heeft op de biofilm vorming in een bioreactor die urine behandelt.

Het globale doel van deze thesis, was het bestuderen van het effect van zout op de nitrificatie activiteit en de biofilm vorming in een MBMBR. Het onderzoek is onderverdeeld in verschillende doelen: (i) het identificeren van een geschikt slib voor inoculatie van de bioreactoren, (ii) het onderzoeken van het effect van het zoutgehalte op biofilm vorming (fase 1 MBMBR), (iii) het bestuderen van het effect van uitspoeling als een selectietechniek voor de biofilm vorming (fase 2 MBMBR) en (iv) het bestuderen van het effect van een gewijzigde tweewaardige/eenwaardige kationen verhouding (fase 3 MBMBR).

Het is cruciaal dat een goed inoculum gekozen wordt voor een nitrificatie bioreactor die geopereerd wordt op hoog zoutgehalte. Het inoculum moet rijk zijn aan Ammonium Oxiderende Organismen (AOO: bacteriën en/of archaea) en Nitriet Oxiderende Bacteriën (NOB). Bovendien moet het zijn activiteit kunnen behouden onder hoog zoutgehalte. In dit deel van het onderzoek werden er batch experimenten uitgevoerd voor verschillende soorten slib. Het slib was afkomstig van commercialiseerde bronnen voor aquacultuur toepassingen, mestverwerking, afvalwaterzuivering en stortplaatsen. De AOO en NOB activiteit werden afzonderlijk geanalyseerd bij het zoutgehalte van genitrificeerd urine (45 mS cm^{-1}) en het zoutgehalte waarbij het slib was aangepast. Deze experimenten toonden aan dat het aquacultuur inoculum de hoogste activiteit had voor zowel AOO en NOB bij beide zout condities (genitrificeerde urine en het zoutgehalte waarbij het slib was aangepast). De AOO en NOB van dit slib werden respectievelijk 0% en 18% geïnhibeerd. De resultaten van dit deel van de experimenten tonen aan dat AOO gevoeliger zijn voor zoutstress dan NOB. Het aquacultuur inoculum werd uiteindelijk gekozen voor de inoculatie van de bioreactoren.

In fase 1 van de bioreactor experimenten staat zoutgehalte centraal. Micro-organismen in suspensie hebben te maken met zoutstress en dit is kostelijk vanuit een bio-energetisch oogpunt. Daarom is het mogelijk dat micro-organismen in suspensie gehechte groei verkiezen. Door groei in biofilms op dragermateriaal, zou het kunnen dat de micro-organismen beschermd zijn tegen zoutstress. In fase 1, was het de bedoeling om het effect van zoutgehalte te onderzoeken als drijvende kracht voor biofilm vorming. Twee bioreactoren werden geopereerd voor 30 dagen bij verschillende zoutgehaltes: 15 mS cm^{-1} en 45 mS cm^{-1}. Polyvinylalcohol (PVA) bolletjes werd

gebruikt als dragermateriaal voor beide bioreactoren. Er werd geen slib verwijderd uit de MBMBRs gedurende deze experimenten. Microschaal activiteitstesten (250µL) en Lowry eiwit analyses werden wekelijks uitgevoerd voor het gehechte (biofilm) en gesuspendeerde slib om de biomassa specifieke conversie snelheid te bepalen. Volgens de verkregen resultaten van de microschaal activiteitstesten, werd het slib niet benadeeld door een hoog zoutgehalte. Weliswaar was er nog geen biofilm vorming op de PVA bolletjes. De micro-organismen in suspensie ondervonden schijnbaar niet voldoende druk om snel biofilm vorming te vertonen.

In fase 2 werd het effect van uitspoeling onderzocht als selectiemiddel voor biofilm vorming. De literatuur heeft reeds aangetoond dat een lage bulk Slib Retentie Tijd (SRT) noodzakelijk is voor de vorming van biofilm. Micro-organismen in suspensie worden als het ware uitgewassen en micro-organismen gehecht aan een drager (PVA bolletjes) worden vastgehouden in de reactor. De reactoren werden geopereerd op een zoutgehalte identiek als in fase 1. Enkel de bulk SRT werd verlaagd tot 8 dagen (in fase 1 werd er geen slib uit de reactor gehaald). 1,14 L d^{-1} effluent werd verwijderd door het membraan (vast/vloeistofscheiding) en 1,14 L d^{-1} gesuspendeerd slib werd rechtstreeks uit de reactoren ontrokken. De reactoren werden bediend gedurende 35 dagen. Tijdens deze reactor experimenten werden er microschaal batch activiteitstesten (250 µl), macroschaal activiteitstesten (50 ml) en Lowry extractie uitgevoerd voor de PVA bolletjes en voor het gesuspendeerd slib apart. Er was geen AOO en NOB activiteit aanwezig op het PVA dragermateriaal en er werden ook geen eiwitten gedetecteerd op de PVA bolletjes. De resultaten van de microschaal batch experimenten toonden aan dat de activiteit van het gesuspendeerde slib niet afnam door zoutstress. Dit was in overeenstemming met de resultaten van de inoculum "screening" en de experimenten in fase 1. Uit deze resultaten kan worden geconcludeerd dat het aquacultuur inoculum tolerant was tegen zoutstress. Mogelijke redenen voor het ontbreken van een biofilm kan te wijten zijn aan: de detectielimiet van de Lowry bepaling, de lengte van de experimenten, shear effecten, afwezigheid van COD in het influent en inoculum selectie.

Tweewaardige kationen zoals calcium binden aan negatief geladen functionele groepen van extracellulaire polymere stoffen en bacteriële oppervlakken. Deze connecties verhogen de sterkte van de biofilm volgens de "divalent cation bridging theory". Een verhoging van de eenwaardige ionen concentratie daarentegen, zal dan vermeend leiden tot een desintegratie van de biofilm structuur en een verhoogd loskomen van de biofilm. In fase 3 werd het belang van een hoge tweewaardige/eenwaardige kationen ratio onderzocht. Beide MBMBRs waren in gebruik gedurende 15 dagen bij een gelijkaardig zoutgehalte (15 mS cm^{-1}) en een SRT van 8 dagen. Een reactor werd operationeel gemaakt op een tweewaardige/eenwaardige kationen verhouding van 0,002 mg Ca^{2+} mg^{-1} Na^{+} en een andere bioreactor op een tweewaardige/eenwaardige kationen verhouding van 1,93 mg Ca^{2+} mg^{-1} Na^{+}. Macroschaal batch activiteitstesten (50 ml) en Lowry eiwit bepaling werden uitgevoerd tijdens de reactor experimenten. De resultaten tonen aan dat er geen AOO en NOB activiteit aanwezig was op het PVA dragermateriaal. Eiwitten werden gedetecteerd in de reactor die werkte op een lage tweewaardige/eenwaardige kationen verhouding. Visueel werd er biofilm waargenomen in beide bioreactoren. De resultaten geven aan dat een lage SRT van groot belang is voor biofilm vorming.

Dit onderzoek toont aan dat biofilm vorming in een floculerend systeem, gewoon aan of tolerant voor hoog zoutgehalte, uitdagend is. Een belangrijk aspect van biofilm vorming is de bulk SRT als functie van de temperatuur. Er is meer onderzoek nodig om de invloed van het zoutgehalte op de biofilm vorming te bestuderen en om het effect van een hoge tweewaardige/eenwaardige kationen verhouding te onderzoeken. Deze bevindingen zullen niet alleen toepasbaar zijn voor urine nitrificatie in de ruimte, maar ze zullen ook belangrijk zijn voor aardse toepassingen. Ondermeer in industriële biofilm zuiveringssystemen voor afvalwater met een hoge en/of wisselende zoutgehalte.

PART 1: LITERATURE STUDY

1. INTRODUCTION

Mankind has been investigating Space from the early beginnings of the history. From the Greek philosophers to the Soviet scientists all had the same curiosity…discover the secrets of the universe.

The main problem in Space exploration is shifting from short-term Space missions to long-term missions. For short-term mission all consumables such as food, water and oxygen can be provided at launch. The waste streams generated can return to Earth. This system is reported as an open-loop life support system (Golub & Wydeven, 1992). However for long-term mission supply becomes difficult. To be independent of supply from Earth, a closed-life support system needs to be present. A closed-life support system compromises several functions: atmosphere regeneration, waste treatment, water recycling and generation of food accounts (Gòdia et al., 2004). Water recycling is of the upmost importance in Space. It accounts for 90% of the life support consumables (considering the fact that no recycling system is present) (Tamponnet et al., 1999). Currently physicochemical water recycling systems are already being used. For example on the International Space Station (ISS) a water recovery and management systems provides potable water, water for the electrolysis unit for oxygen production, urinal flush water and hygiene water. The water recovery system consists of different units (see section 2.3). The main part of the urine processor assembly (processes pre-treaded urine) is based on Vapor Compression Distillation (VCD) (Carter et al., 2013). According to Udert & Wächter (2012) this water recovery step uses more energy than Reverse Osmosis (RO), electrodialysis and a Membrane Bioreactor (MBR). The urine processor assembly was designed to recover 85% of the water coming from pre-treated urine. Due to technical complications (see section 2), it is currently running at a recovery of 70% (Carter, 2010).

A new approach for water recycling is the combination of biological and physicochemical processes. It is believed that the integration of biological systems with physiochemical systems may offer an increase in water recycling efficiencies (Kubista, 2012). The equivalent system mass is a method to evaluate trade study options for life support programs. It can be used to identify which of several options meets all requirements i.e. lowest launch cost related to the mass volume power, cooling and crewtime needs (Levri et al., 2003). Anderson (2004) found that an integrated system has a lower equivalent mass system than the present water recovery technologies used on the ISS (see subsection 2.4). A novel system that is being developed by the National Aeronautics and Space Administration (NASA) is the alternative water processor. This project aims to develop a water recovery system based on biological and physical processes. The basis for the alternative water processor is a biological water processor and a forward osmosis secondary treatment unit. The former mineralizes organics such as carbon and nitrogen. The latter removes solids and inorganic ions. It is expected that the system will recycle 95% of the water. In the future, the alternative water processor could replace water recycling systems that are present on the ISS (Barta et al., 2014).

This thesis contributes to the Water Treatment Unit Breadboard (WTUB) project sponsored by the Belgian Science Policy through the European Space Agency (ESA). The WTUB project aims to recycle urine, condensate and grey water by combining biological and physicochemical wastewater treatment technologies. A water recovery of 90% is a main goal. The objective of the installation is to produce hygiene water that complies with the ESA hygiene water standard. The whole process consists of a

crystallization unit, a nitrification reactor with ultrafiltration, an electrodialysis step, nanofiltration step and a RO step (see section 2.4).

The main nitrogen component of urine is urea (see section 2.1). This component will be further hydrolyzed into ammonia and carbamate by bacteria producing the enzyme urease. After the hydrolysis the pH rises to around 9 due to the release of ammonia (**Table 3**). The augmentation in pH results in more volatilization of ammonia (toxic). Volatilization of ammonia can be prevented by stabilizing the ammonia to nitrate by nitrification (Maurer et al., 2006). In the WTUB applying a high pH will stabilize urine (no hydrolyzation). Afterwards hydrolyzation and nitrification will take place in the bioreactor. Nitrification is also important from a process-orientated point of view. The RO membrane and ultrafiltration membrane are more permeable for ammonia than for nitrate. This results in low retention of ammonia. In order to comply with the ESA hygiene water standard (**Table 5**), the nitrification step needs to convert ammonia into nitrate. Then in a final RO step nitrate can be removed in a satisfactory way (Clauwaert et al., 2014).

Nitrification is a technology that is already in use for wastewater treatment technologies on Earth. Integrating this technique in a Space water recycling system requires an understanding of the physical conditions in Space. One important feature of Space is the fact that microgravity conditions are present. When supplying oxygen under microgravity conditions, the generated bubbles will not rise to the surface due to a lack of convection driven by density changes (Benoit & Klaus, 2007). And thus oxygen will need to be supplied by diffusion.

Different reactors exist to perform urine nitrification (**Table 7** and **Table 8**). However in the WTUB project an attached growth system will be used. Attached growth is necessary because in Space microgravity conditions are present. And thus it is not possible to provide bubble aeration to suspended sludge. The biomass will be held on carrier material in the reactor. However it is not know whether the biomass will grow completely on the carrier. To prevent transfer to the subsequent compartment, a combination of membrane and a carrier will be used in the WTUB. If no membrane or carrier would be used during the WTUB, the Hydraulic Retention Time (HRT) and the Sludge Retention Time (SRT) of the bioreactor will be identical (Rabaey, 2014). This could result in wash-out of the nitrifying microorganisms, because nitrifying microorganisms have a low maximum growth yield (five times lower than heterotrophic bacteria) (Lee et al., 2006; Blackburne et al., 2007).

The bioreactor that will be operated during this thesis will be referred to as a Membrane Bed Biofilm Reactor (MBMBR). The effect of urine dilution on the biofilm formation on the carriers will be investigated throughout this research. Urine contains a lot of ions and many other compounds (**Table 1**). It is not clear what the effect of salinity is on biofilm formation. Salt stress can be a driver for biofilm formation, but there is a possibility that a low SRT (wash-out) is essential for biofilm growth. A last aspect that will be investigated is the effect of a high divalent/monovalent cation ratio for biofilm formation. This effects the biofilm structure according to the divalent cation bridging theory (Sobeck & Higgins, 2002). The carrier material will be made of polyvinyl Alcohol (PVA) beads (see section 3.1).

2. WATER RECYCLING IN SPACE

2.1. THE COMPOSITION OF URINE

In the WTUB urine, condensate and grey water will be collected and treated to hygiene water. Urine is generally considered a waste stream however it can serve as a source of water and nutrients (Maurer et al., 2006). Especially for long-term Space missions reusing urine is of great importance. In this section, the chemical composition before and after complete urine hydrolysis will be discussed. Furthermore the effect of Space conditions on the urinary excretion will be presented.

Putnam (1971) lists 68 components in urine that have an individual maximum concentration of more than 10 mg L^{-1}. It concerns electrolytes, vitamins, hormones, nitrogenous compounds, organic acids and miscellaneous organic acids. In **Table 1** twelve chemical components are selected.

Table 1: Selection of 12 chemical components in human urine adapted from Putnam (1971).

Component	Range	
	Min (mg L^{-1})	Max (mg L^{-1})
Urea	9300	23300
Chloride	1870	8400
Sodium	1170	4390
Potassium	750	2610
Creatinine	670	2150
Sulfur, Inorganic	163	1800
Hippuric Acid	50	1670
Phosphorus Total	470	1070
Citric Acid	90	930
Glucuronic Acid	70	880
Ammonia	200	730
Uric Acid	40	670

There is a difference between fresh and stored urine. When no preliminary measures are taken, urine hydrolysis occurs due to microbial ureolysis by microorganisms present in collection systems. Urea the main component of urine will be hydrolyzed into ammonia and carbamate by bacteria producing the enzyme urease. This enzyme, which is also called urea amidohydrolase, catalyzes the hydrolysis of urea (Mobley & Hausinger, 1989). It is found in nature in some eucaryotics (e.g. jack bean) and some microorganisms. Some Ammonia Oxidizing Bacteria (AOB) have urease activity (**Table 2**).

Table 2: Urease activity of AOB species adapted from(Koops et al. 2006).

Species	Urease activity
Nitrosomonas europaea	-
Nitrosomonas eutropha	-
Nitrosomonas communis	-
Nitrosomonas mobilis	-
Nitrosomonas communis	-
Nitrosococcus halophilus	-
Nitrosomonas nitrosa	+
Nitrosomonas urease	+
Nitrosomonas oligotropha	+
Nitrosomonas marina	+
Nitrosomonas aestuarii	+
Nitrosomonas cryotolerans	+
Nitrosolobus multiformis	+/-
Nitrosovibrio tenuis	+/-
Nitrosospira briensis	+/-
Nitrosococcus halophilus	+/-

Symbol -, not present, +, present, +/-, present in some strains

After the bioprocess, the molecule carbamate will decompose spontaneously to one molecule carbonic acid and ammonia. The carbonic acid proton will dissociate and ammonia will equilibrate with water at physiological pH. This results in an increase of pH (Mobley & Hausinger, 1989). The hydrolyzation reaction of urea to ammonium can be written as: (Udert et al., 2003)

$CO(NH_2)_2 + 2H_2O + H^+ \rightarrow 2NH_4^+ + HCO_3^-$ (Equation 1)

Chen (2009) compares the composition of collected urine and urine after complete hydrolysis in literature. The data of this study are shown in **Table 3**.

Table 3: Composition of urine before and after complete hydrolysis adapted from (Chen, 2009).

	Fresh urine			Hydrolyzed		
Parameter	(Geigy, 1977)	(Udert, 2002)	(Chen, 2009)	(Kirchmann & Pettersson, 1995)	(Udert, 2002)	(Chen, 2009)
pH	6.2	6.2	6.4	9.0	9.0	9.2
Total nitrogen (mg N L^{-1})	8830	9200	5980	7909	9200	5900
Total kjeldahl nitrogen (mg N L^{-1})	-	-	-	-	-	5600
Total ammonia nitrogen (mg N L^{-1})	463	480	374	7572	8100	5380
Chemical oxygen demand (mg O_2 L^{-1})	-	10000	6700	-	10000	6700
Potassium (mg L^{-1})	2737	2200	1200	3485	2200	1200
Sodium (mg L^{-1})	3450	2600	3067	2842	2600	3067
Calcium (mg L^{-1})	233	190	134	40.4	0	13.4
Magnesium (mg L^{-1})	119	100	55	4.5	0	1.01
Chloride (mg L^{-1})	4970	3800	5077	6772	3800	5077
Total phosphor (mg L^{-1})	800-2000	740	735	606	540	550

Table 3 shows that hydrolysis changes the composition of urine. There is a notable difference in pH, total ammonia nitrogen, calcium, total phosphor and magnesium concentration. First, the rise in pH and in total ammonia nitrogen concentration is due to hydrolysis of urea. The difference in calcium, magnesium and total phosphor is because of precipitation of minerals. These precipitates consist of three main crystalline compounds: struvite ($MgNH_4P0_4.6H_20$), hydroxyapatite ($Ca_5(P0_4)_3(OH)$) and calcite ($CaCO_3$). The origin of the formation of the different minerals is due to microbial ureolysis. As stated before, this process gives rise to pH

and releases ammonia and carbonate. This change induces formation of hydroxyapatite and precipitation of struvite and calcite (Udert, 2002).

Space conditions can alter the composition of human urine. An important physiological change is bone loss. This results in higher urinary calcium excretion. Two types of studies exist that characterize bone loss: bed rest and Space flight mission. It is reported that ground-based studies are a viable alternative to assess Space flight induced bone loss. However these studies have a smaller magnitude than their analog (Smith et al., 2012).-

The biochemistry of the bone alters rapidly after humans enter the new environment (i.e. bed rest or Space flight). This is assumed to be associated with a loss of bone and a corresponding release of calcium. Calcium on its account will suppress the parathyroid hormone. The suppression of this hormone will decrease the 1,25-dihydroxyvitamin D concentration and also the calcium absorption (Smith et al., 2012). These biochemical changes increase urinary calcium excretion. Calcium excretion will result in a greater risk of renal stone formation. This disease is not desired for crewmembers (Smith et al., 2012). Renal stones consist of a variety of components: calcium oxalate, calcium phosphate, uric acid, cystine and struvite (Whitson et al., 1997).

Urinary calcium became a problem on the ISS not because of medical reason but due to the technical operation of the urine processor assembly (see section 2.3). During deployment, failure of the system occurred in the distillation assembly. The problem of the failure was located in the evaporator. It contained a large amount of solids. These solids cause blockage of the brine pilot tube (removes brine from distillation assembly) and resulted in a flooding of the distillation assembly (Carter, 2010). Solid precipitates in the distillation assembly evaporator are shown in **Figure 1**.

Figure 1: Precipitation in the Distillation Assembly Evaporator (Carter, 2010).

After analyzing the solids it was clear that they mainly comprised calcium sulfate. The presence of sulfate is due to the use of sulfuric acid in the pretreatment of urine. Sulfuric acid is a preservative added to urine to lower the pH as to prevent the formation of ammonia gas (Smith et al., 2012; Carter, 2010). Calcium results from bone loss. The urine processor assembly is tested on Earth and had no problem in recovering 85% of the water from pretreated urine. However because of higher urinary calcium excretion, the calcium concentration was higher than on Earth. The result is that calcium sulfate exceeds its solubility limit and precipitates. A way to avoid precipitation is by using another mineral acid. Muirhead & Carrier (2012) recommend phosphoric acid as a safe substitute for sulfuric acid. The upper limit of calcium concentration (i.e. the concentration were precipitation occurs) is 283 mg L^{-1} at a recovery of 85% water from the pretreated urine (Carter, 2010). Because of the failure it was concluded that an 85% recovery of water from pretreated urine is not feasible. Currently the urine processor assembly is running at a recovery of 70% (Carter, 2010).

2.2. WATER STREAMS

In the WTUB project three different wastewater streams will be collected: grey water, urine and condensate. These streams will be further processed into hygiene water that complies with the ESA hygiene water standard (**Table 5**). The term grey water is defined in literature as wastewater produced from households that comprises water originating from shower, handbasins baths, washing machines, dishwashers, laundries and kitchen sink (Eriksson, 2002).

Humans require water for different handlings throughout the day. Also in Space water is necessary. **Table 4** gives an overview of: the different water requirements in Space, the liquid waste streams generated in Space and the average household use per capita per day. The total amount of water needed in Space on a mass base is 29.29 kg per person per day. From the data it is clear that laundry and personal hygiene have a great impact.

In the WTUB project three streams will be collected and processed. The first one (i.e. grey water) comprises of three different liquid streams: dishwashing, water for personal hygiene and laundry. They also have the greatest burden on a mass base. The total amount of grey water generated per person per day is 25.23 kg. The condensates (i.e. metabolic perspiration and respiration) account for 2.28 kg of the total mass. Finally urine comprises only 1.50 kg. Thus a total amount of 29.01 kg needs to be collected and further treated.

In **Table 4** an overview is given of the different water use per capita per day expressed in kg (considering that 1 litre = 1 kg). Toilet use has a great burden on water use in a household. Also personal hygiene, laundry and especially outdoor use are important consumers. The data are from a study conducted in the United States. It is not representative for world water use. The total average household consumption in the United States is approximately 22 greater than the water requirements for Space.

Table 4: Water in Space and on Earth.

Water for	**Requirements per person per day in Space (kg)** (Tamponnet et al., 1999)	**Liquid waste streams per person per day in Space (kg)** (Tamponnet et al., 1999)	**Requirements per capita per day (kg)** (Mayer & DeOreo, 1999)
Food re-hydration/ preparation	1.94	-	-
Drinking	1.62	-	-
Dishwashing	5.46	5.46	3.80
Personal hygiene	7.28	7.27	48.00
Laundry	12.50	12.50	56.8
Toilet	0.50	0.50	70.0
Metabolic perspiration and respiration	-	2.28	-
Urine	-	1.50	-
Faeces	-		-
Total outdoor	-	0.09	381.60
Other	-	-	89.80
Sub-total	**29.29**	**29.6**	**650.00**

The product stream that will be produced is hygiene water. The composition of this water has to comply with ESA standards (**Table 5**).

Table 5: ESA hygiene and drinking water standards (Tamponnet et al., 1999).

Parameter	Hygiene water	Drinking water
pH	5-8.5	6.5-8.5
Conductivity (mS cm^{-1})	3	0.75
Turbidity (NTU)	10	2.5
TOC (ppm)	10	0.5
F^- (ppm)	10	1
Cl (ppm)	1000	200
NO_2^- (ppm)	50	25
PO_4^{2-} (ppm)	50	5
SO_4^{2-} (ppm)	TBD	250
$Na^=$ (ppm)	750	150
K^+ (ppm)	120	12
NH_4^+ (ppm)	0.5	0.5

TBD=To be determined

When considering a long-term Space mission of three years, the water requirements are 32072.55 kg per person (Data in **Table 4** as basis for the requirements for one day). And thus recycling water is important. Different technologies nowadays exist that process water and remove salts. These will be discussed in section 2.3.

2.3. PRESENT TECHNOLOGIES

The most diluted stream that will be collected in the WTUB project is urine. It contains a lot of ions and other components (**Table 1**). In this section vapor compression distillation (VCD), RO and electrodialyis will be discussed. The latter two will be used in the WTUB project. A number of other technologies exist to recover water: thermoelectric integrated membrane evaporation system, vapor phase catalytic ammonia removal, lyophilization, freeze thaw and air evaporation systems (Wydeven, 1988; Udert & Wächter, 2012; Maurer et al., 2006). However these other technologies will not be discussed. The main focus of this literature study is the technologies present onboard of the ISS and the technologies used for the WTUB.

Figure 2 is a scheme of the water recovery and management system on the ISS. This system is part of the United States segment and was delivered on November 14, 2008. This system ensures the production of potable water, urinal flush water, and water for oxygen generation. The different waste streams that are collected by the system are: urine, condensate and sabatier product water. The subsystem that is responsible for processing these waste streams is the water recovery system. The water recovery system is further subdivided in two systems i.e the urine processor assembly (including the VCD) and the water processor assembly. The latter takes care of the distillate, condensate and water from the sabatier process (Carter et al., 2013).

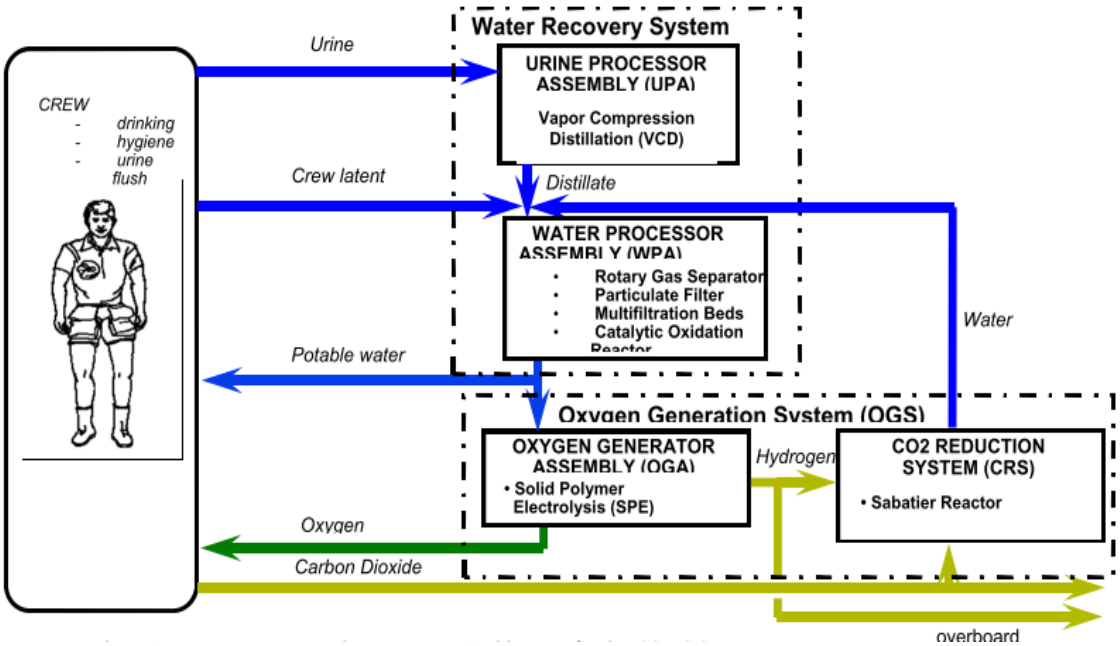


Figure 2: Water recovery and management system for the ISS US segment adapted from Carter et al. (2013).

Urine is collected in the waste and hygiene compartment. Then it is further treated with flush water and chemicals such as chromium trioxide and sulfuric acid. These chemicals have two main functions: control of microbial growth and control of ammonia formation (Carter et al., 2013). Sulphuric acid suppresses microbial hydrolysis of urea by keeping the pH low (Maurer et al., 2006).

The urine processor assembly is a complex system that consists of different parts. In **Figure 3**, a general overview of this system is shown. The wastewater tank stores pretreated urine. When a certain amount of pretreated urine is collected, the urine processor assembly starts working. The fluid is pumped between the distillation assembly and the recycle filter, and this has three objectives.

1. Pumping pretreated urine from the tank to the distillation assembly
2. Recycling concentrated waste (brine) from the distillation assembly to the recycle filters and back again.
3. Finally pumping distillate of the urine processor assembly to the water processor assembly

The most important part of the urine processor assembly is the distillation assembly (on the left in **Figure 3**). It makes use of VCD technology. Basically pretreated urine is evaporated at low pressure. Then the water vapor is compressed and afterwards condensed on the opposite side of the evaporator surface (Carter et al., 2013). Condensing of the vapor is an advantage of the VCD process, because the latent heat of condensation is recovered from the water. The evaporator and the condenser are side by side, separated by a heat exchange surface and latent heat is recovered to heat up the wastewater. The VCD process requires energy for compression of the vapor and energy to overcome thermal and mechanical losses. The overall process is able to recover 96% (i.e. ideal conditions, because of precipitation only 70% recovery see section 2.1) water from urine. It concentrates the urine up to 50% solids (Wydeven, 1988). To induce phase-separation in microgravity, the evaporator, condenser and product water collector are rotated. This provides sufficient centrifugal force for separation. The primary energy demand for the VCD process is 336 W h L^{-1}_{urine} (Udert & Wächter, 2012).

After the urine processor assembly, the produced distillate is mixed with condensate and sabatier water and further processed in the water processor assembly.

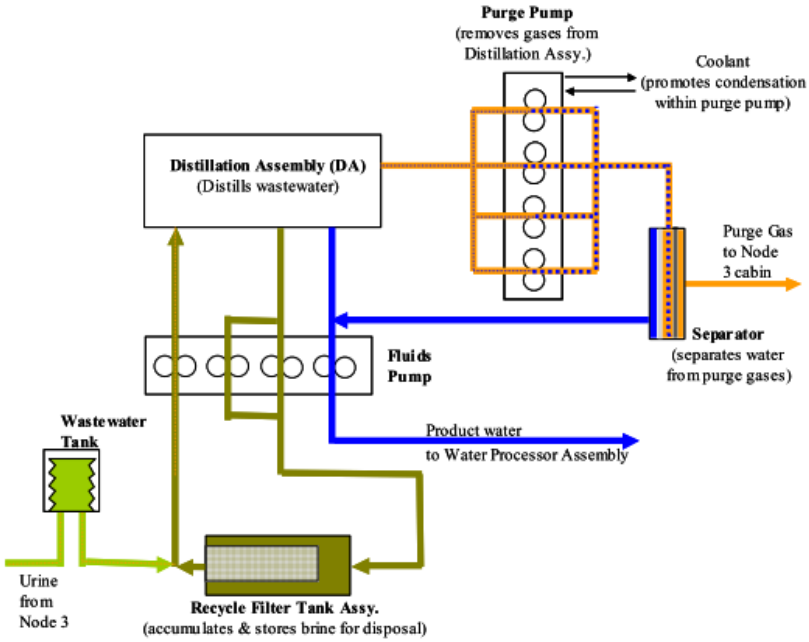


Figure 3: Overview of the Urine processor assembly (Carter et al., 2013).

Figure 4 illustrates the principle of RO. A difference in osmotic pressure results in an osmotic flow from the permeate to the feed. This is called osmosis. To produce clean water from a solution containing salts, a pressure needs to be applied greater than the osmotic pressure. RO makes use of a semi-permeable membrane. This membrane enables water to pass and retains small molecules such as ions (Verliefde, 2014). The energy consumption of RO is 30.4 W h L^{-1}_{urine} (Udert & Wächter, 2012).

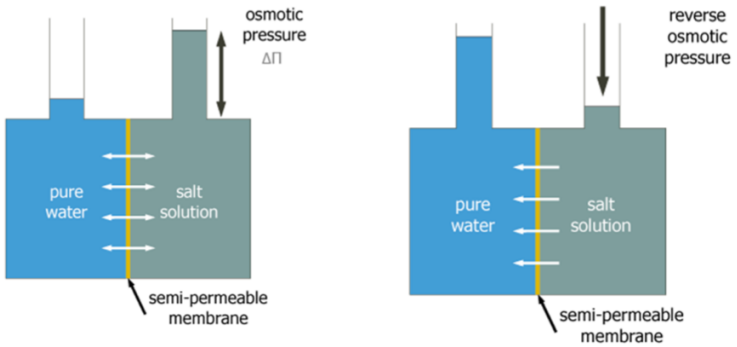


Figure 4: An illustration of osmotic pressure and reverse osmotic pressure (Verliefde, 2014).

Electrodialysis (**Figure 5**) will be used in the WTUB project. In this process anionic and cationic membranes are arranged in an alternating pattern between the anode and the cathode. A cell pair is the combination of an anion and a cation membrane. A saline solution is pumped through these cells and at the same time an electrical potential is maintained across the electrodes. Cations (positive) will migrate towards the cathode and anions (negative) migrate to the anode. Cations can migrate through the negatively charged cation exchange membrane. However the positively charged anion membrane retains them. Anions on there account can migrate through the positively charged anion membrane. These ions are retained at the cation exchange membrane. The result of electrodialysis is that one of the cell pairs get depleted of ions and the other cell gets enriched with ions (Baker, 2004). The energy consumption of electrodialysis is 97.6 W h L^{-1}_{urine} (Udert & Wächter, 2012).

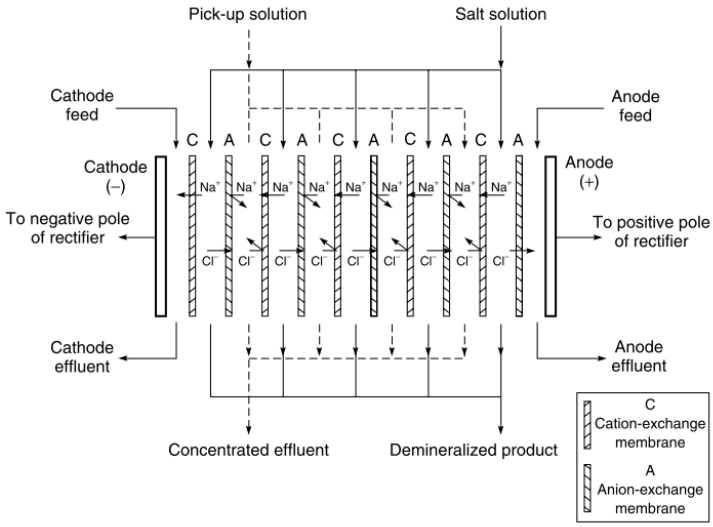


Figure 5: An illustration of electrodialysis (Baker, 2004).

Table 6 gives an overview of the primary energy demands per liter urine.

Table 6: A summary of water recovery technologies and their primary energy demands.

Technology	**Primary energy demands (W h L^{-1}_{urine})** (Udert & Wächter, 2012)
VCD	336.0
RO	30.4
Electrodialysis	97.6
MBR	129.6

2.4. FUTURE TECHNOLOGIES FOR LONG-TERM MISSIONS

In subsection 2.3 the water recovery system of the ISS was described. It is based on different units and processes to obtain high quality water for potable use. A disadvantage of this treatment system is the production of toxic brine that cannot be recovered. This is not desired for closed-loop life support systems in Space. Hence this is one reason why other water treatment architectures need to be developed. A manner to overcome this hurdle is by converting the urea by means of ureolysis and subsequently nitrification/denitrification. Thus no chromium trioxide is added for stabilizing the urine (Lunn, 2012).

Another motive to justify the need for alternative water treatment systems is based on energy requirements. As mentioned in subsection 2.3 the urine processor assembly uses VCD. This technology has a primary energy demand of 336 W h L^{-1}_{urine}

Table 6). This is higher than the primary energy demands of RO and electrodialysis. Also a biological treatment system such as a MBR has a primary energy demand of 129.6 W h L^{-1}_{urine} (Udert & Wächter, 2012).

An import difficulty in Space applications is the mass and the associated costs. The equivalent system mass is used to quantify the launch costs of the life support system and its supporting systems such as power generation, cooling, crew time (Levri et al., 2003). In the trade study of Anderson (2004), the equivalent system mass of the water recovery technologies on the ISS and from an integrated system that combines biological

and physiochemical processes were investigated. The water recovery system of the ISS (see section 2.3) performed better for short missions (i.e. 30-90 days). However for long duration missions (i.e. 360-500 days) the integrated system outperformed the water recovery system. The equivalent system mass for mission of 500 days was around 4200 kg for the integrated system and around 4800 kg for the water recovery system of the ISS.

Nowadays NASA and ESA are doing research on alternative water recycling systems. A number of approaches exist based on the scope of the mission. When the main object of urine treatment is stabilizing the urea, a combined nitrification and denitrification reactor can be used. The produced nitrogen can be used as pressurizing gas (Lunn, 2012). The NASA is developing the alternative water processor. This project aims to develop a novel water recovery system for long duration missions. The main parts of this technology are two systems: a biological water processor and a forward osmosis secondary treatment system. The biological water processor consists of four membrane aerated biological reactors. The bacteria in the reactor metabolize organics located in the wastewater to carbon dioxide. Also nitrification and denitrification takes place in the reactors. The biological water processor is the first process step. After biological treatment, the forward osmosis secondary treatment system removes dissolved solids that are still present in the effluent of the biological water processor. This system consists of a forward osmosis membrane module and a RO system (Pickering et al., 2013). Forward osmosis uses the osmotic pressure difference across a semi-permeable membrane as the driving force for transport of water through the membrane. This technology results in concentration of a feed stream and dilution of a concentrated stream (Cath et al., 2006). This concentrated stream is the driving force in forward osmosis. To conclude, the alternative water processor will treat urine, condensate, hygiene water and laundry water. The biological water processor is intended to convert 90% of the total organic carbon to carbon dioxide. The overall process is expected to recover 95% of the initial wastewater volume. The remaining 5% will be concentrated in a brine stream (Pickering et al., 2013).

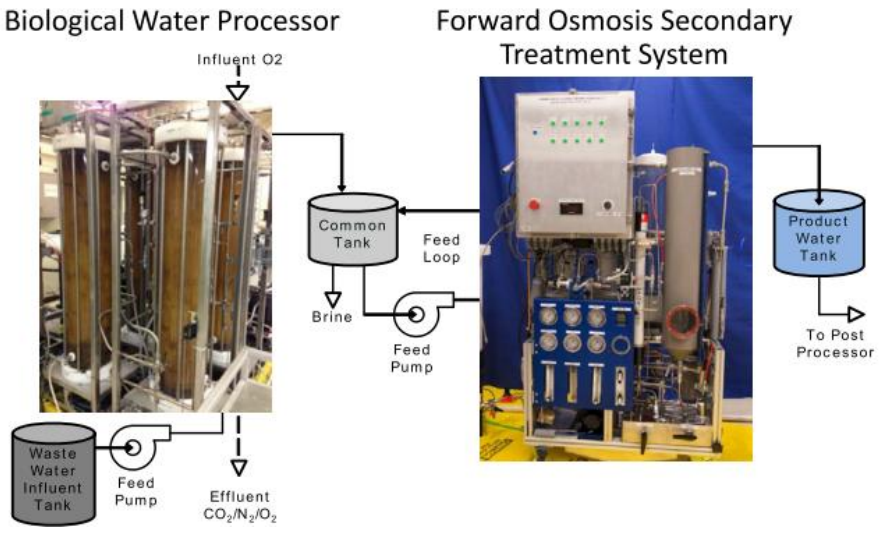


Figure 6: An illustration of the alternative water processor (Barta et al., 2014).

The forword osmosis secondary treatment system consist of a forward osmosis membrane module and a RO system.

ESA is also exploring novel technologies. Basically nitrification is combined with different physical technologies. The aim of this project is to design and development a WTUB for one person. A schematic overview of the WTUB is given in **Figure 7**. It is a combination of three units: urine treatment unit, grey water unit and electrodialysis unit. In the ESA project, the focus for the nitrogen lies on stabilizing urea by ureolysis and nitrification. Nitrogen losses (N_2O or N_2 through denitrification) should be minimal, since nitrogen gas is considered as a loss in a closed loop life support system (Demey et al., 2013).

As stated before, three different streams will be collected. Urine (containing flush water), grey water and condensate will be processed in the urine treatment unit. The first step in the urine treatment unit is crystallization. This step is of great importance to avoid scaling in the different units of the WTUB (no anti-scaling agent used). Thereafter follows a bioreactor that stabilizes urea to nitrate. After nitrification there is still an electrodialysis process unit step. The processed stream of the urine treatment unit is mixed with grey water to be further processed in the grey water treatment unit. This processing unit contains nanofiltration and RO. The WTUB project aims to recycle 90% of the water. Note that the bioreactor is the main focus of this thesis research (Demey et al., 2013).

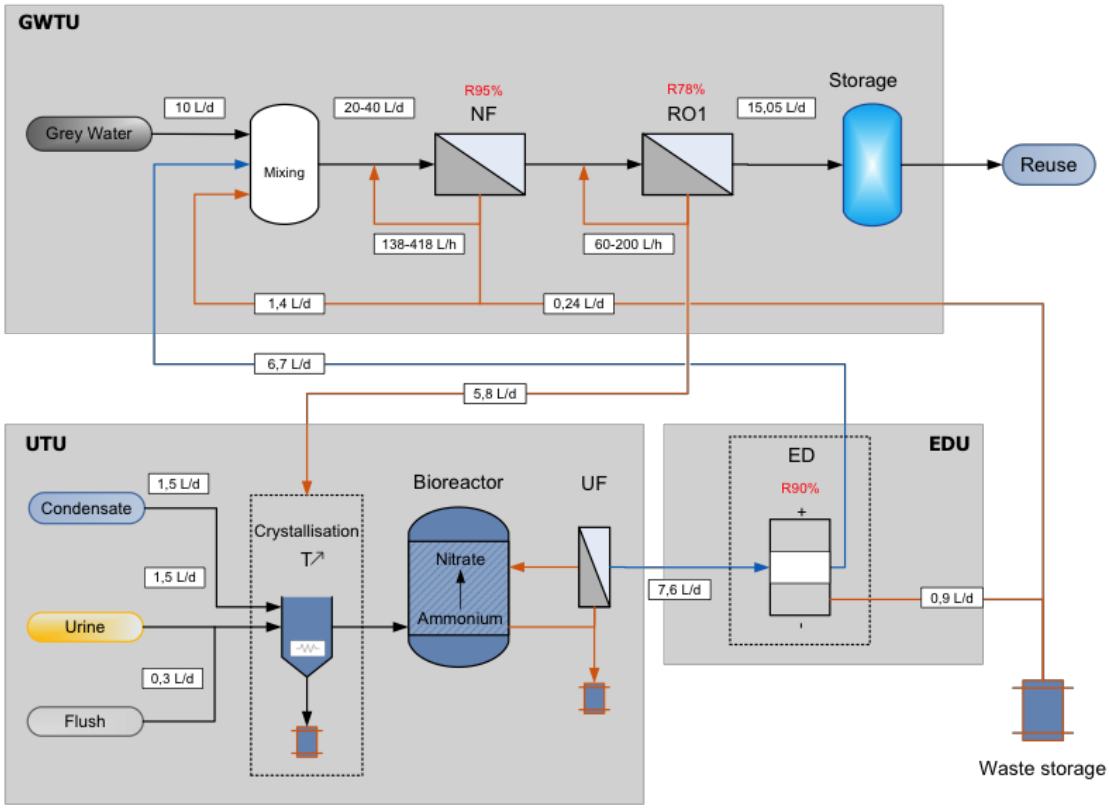


Figure 7: Schematically overview of the WTUB (Demey et al., 2013).

3. NITRIFICATION

In this chapter the focus is on aerobic nitrification. Below an overview is given of the oxidation of ammonium to nitrate. Furthermore the corresponding oxidation state is shown. Ammonium originates from the hydrolysis of urea (see section 2.1). The combination of both nitritation and nitratation is addressed as nitrification. The oxidation of ammonia to nitrite is done by autotrophic Ammonium Oxidizing Bacteria (AOB). The conversion to nitrate is performed by autotrophic Nitrite Oxidizing Bacteria (NOB) (Bock & Wagner, 2006). The oxidation of

ammonia or nitrite by AOB or NOB, is important to generate energy for growth. These bacteria are chemical autotrophic microorganism and they obtain their carbon from dissolved carbon dioxide (Chen, 2009).

$$Urea \xrightarrow{Urease} \underset{-3}{NH_4^+} \xrightarrow{AOB} \underset{-1}{NH_2OH} \xrightarrow{AOB} \underset{+3}{NO_2^-} \xrightarrow{NOB} \underset{+5}{NO_3^-}$$

First ammonia is converted to nitrite by two successive steps (Equation 3 and 4). This process is referred to as nitritation. As reported by Suzuki & Dular (1974) ammonia is the substrate rather than ammonium. These two molecules are in equilibrium and the conversion is temperature and pH dependent. This equilibrium reaction can be written as follows:

$NH_4^+ + OH^- \leftrightarrow NH_3 + H_2O$ (Equation 2) (Chen, 2009)

After oxidation of ammonia, nitrite is oxidized to nitrate. This conversion is called nitratation. As stated before nitritation is a two-step oxidation reaction. Ammonia, the actual substrate (Suzuki & Dular, 1974), is first oxidized to hydroxylamine. The enzyme responsible for this conversion is ammonia monooxygenase. After this reaction hydroxylamine is oxidized to nitrite by the enzyme hydroxylamine oxidoreductase. All these reactions can be written as followed:

$NH_3 + O_2 + 2H^+ + 2e^- \rightarrow NH_2OH + H_2O$ (Equation 3)

$NH_2OH + H_2O \rightarrow NO_2^- + 5H^+ + 4e^-$ (Equation 4)

$0.5O_2 + 2H^+ + 2e^- \rightarrow H_2O$ (Equation 5)

Net reaction: $NH_3 + 1.5O_2 \rightarrow NO_2^- + H^+ + H_2O$ (Equation 6) ($\Delta G^{\circ\prime}$ = -235 kJ mol^{-1} ammonium) (Bock & Wagner, 2006)

According to Koops & Pommerening, (2001) two different phylogentical groups of AOB exist and 25 cultured species are present. The first group belongs to the γ subclass of the Proteobacteria. *Nitrosococcus* is the only genus located in this group. A second group can be found in the β subclass of the Proteobacteria. Two clusters are present: *Nitrosospira* and *Nitrosomonas*. Not only bacteria have nitrification activity but also aerobic ammonium-oxidizing archaea such as *Nitrosopumilus maritimus* and *Nitrososphaera viennensis* are reported to have this functionality (Martens-Habbena et al., 2009).

Nitratation occurs by the NOB microorganisms. The reaction is catalyzed by the enzyme nitrite oxidoreductase. The change in Gibbs free energy is 54 kJ mol^{-1} nitrite. The overall reaction can be summarized as:

$NO_2^- + 0.5O_2 \rightarrow NO_3^-$ (Equation 7) (Bock & Wagner, 2006)

The NOB have four different groups and 8 cultured species exist. The group that belongs to the α subclass of the Proteobacteria is the major group. Nitrobacter only represents this group. There are also two marine species: *Nitrococcus mobilis* and *Nitrospina gracilis*. These are respectively assigned to γ subclass and δ subclass of the Proteobacteria. A last group described belongs to the genus *Nitrospira* and are represented by two species: *Nitrospira marina* and *Nitrospira moscoviensis*. These two species are a distinct phylum close to the δ subclass of the Proteobacteria (Bock & Wagner, 2006). **Figure 8** represents the phylogenetic tree of AOB (red) and NOB (green).

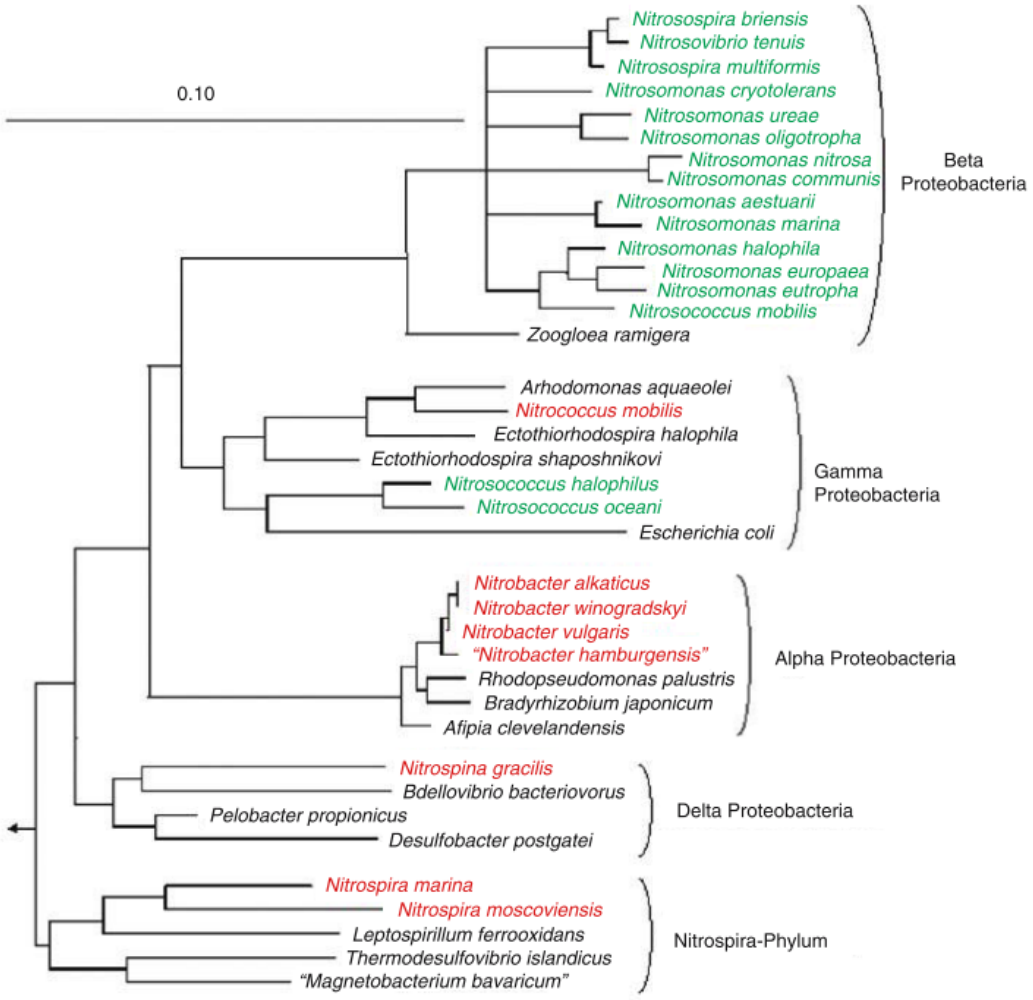


Figure 8: 16S rRNA-based tree reflecting the phylogenetic relationship of AOB and NOB (Bock & Wagner, 2006).

The overall nitrification reaction can be summarized as:

$NH_4^+ + 2O_2 \rightarrow NO_3^- + H_2O + 2H^+$ (Equation 8) (Chen, 2009)

When assuming that the empirical formula of a bacterial cell corresponds to $C_5H_7O_2N$, oxidation and synthesis of biomass can be expressed as (Wiesmann & Libra, 1999) :

$NH_4^+ + 1.98HCO_3^- + 1.87O_2 \rightarrow 0.98NO_3^- + 1.88H^+ + 1.88HCO_3^- + 1.041H_2O + 0.021\ C_5H_7O_2N$ (Equation 9)

Respecting the stoichiometry of reaction (Equation 9), 10 mg NH_4^+-N generates 1.7 mg biomass. Oxygen consumption is also important. For nitrification of 1 mg NH_4^+-N to nitrate, 4.27 mg O_2 is consumed (Chen, 2009). This value does not take the consumption of oxygen by heterotrophic organisms in to account. Urine also contains organic components (**Table 3**). Thus heterotrophs compete with oxygen with the nitrifying bacteria (Chen, 2009).

The cell yield for *Nitrobacter* (NOB) and *Nitrosomonas* (AOB) is 0.072 gVSS gN^{-1} and 0.014 gVSS gN^{-1} respectively (Blackburne et al., 2007).

3.1. NITRIFICATION AND BIOREACTORS

There are a number of reactor designs examined in literature for the nitrification of urine (**Table 1** and **Table 7**). Two main configurations exist: attached growth and suspended growth.

Table 7: Schematic overview of reactor configurations for attached growth adapted from (Clauwaert, 2014).

Reference	(Gòdia et al., 2002)	(Feng et al., 2008)	(Udert et al., 2003)	(Udert & Wächter, 2012)
Reactor technology			MBBR	MBR
Carrier material	Polystyrene beads	Ceramic rings	Plastic rings	Silicone tubes
Dilution % artifical urine	N/A	10	22-121*//	45
N oxidation rate ($g\ N\ L^{-1}\ d^{-1}$)	1.35	0.05	0.38	0.18
pH	8.1	6.5-7.0	6.4	6.3
T (°C)	28	27	25	23
DO ($mg\ O_2\ L^{-1}$)	1-8	0.3	3.0-5.2	4.0**
Inoculum	Undefined	Undefined	Undefined	Undefined
Effluent	100% NO_3^-	100% NO_3^-	50% NO_3^- 50% NH_4^+	50% NO_3^- 50% NH_4^+

*Artificially concentrated through NH_4HCO_3 addition

**Through additional bubble aeration in the bulk liquid

Table 8: Schematic overview of reactor configurations for suspended growth adapted from (Clauwaert, 2014).

Reference	(Chen, 2009)	(Sun et al., 2012)	(Udert et al., 2003)	(Udert et al., 2003)	(Sun et al., 2012)
Reactor technology	SBR	SBR	SBR	CSTR	MBR
Dilution % artifical urine	N/A	15	N/A	N/A	19
Dilution % real human urine	10-29	28-47	13-42	137	28
N oxidation rate ($g\ N\ L^{-1}\ d^{-1}$)	1.95	0.4-0.75	0.3-1.3	0.8	0.25
pH	7.6	6.4-6.5	6.0-8.8**	6.9	6.3
T (°C)	25	25-35	24.5	30	35
DO ($mg\ O_2\ L^{-1}$)	1.5	2	2.0-4.5	2.0-4.5	3
Inoculum	Undefined	Undefined	Undefined	Undefined	Undefined
Effluent	100% NO_3^-	50% NO_3^- 50% NH_4^+	50% NO_3^- 50% NH_4^+	50% NO_3^- 50% NH_4^+	50% NO_3^- 50% NH_4^+

SBR=Sequencing bioreactor

CSTR=Continuous stirred tank reactor

*With some higher outliers (7.6-8.9)***Submerged hollow-fiber membranes

**Intended outcome, for partial nitritation/anammox or chemical nitrite oxidation experiments

The reactor configuration that will be used during this research is a MBMBR. This is a reactor that combines MBBR and MBR technology (Leiknes & Ødegaard, 2007). Ivanovic (2011) has performed research on MBMBRs and defines a MBMBR technology as "*a wastewater treatment system that combines biological wastewater treatment with a biofilm process and with membrane separation for clarification and purification of biologically treated wastewater".*

An MBBR setup makes use of free-floating carrier for the growth and fixation of activated sludge. Thus biofilm formation is a necessity (Skerjanec et al., 2009). This reactor configuration can be used for nitrification of urine (**Table 7**) and mixing of the reactor occurs by aeration or by mechanical mixing (Zimmerman et al., 2005). An overview of a MBBR system is shown in **Figure 9**.

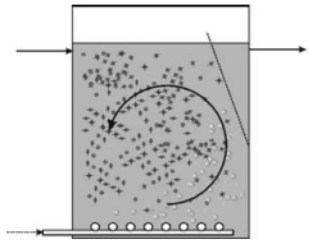


Figure 9: A simplified overview of a MBBR (Leiknes & Ødegaard, 2007).

A number of carrier materials can be used in a MBBR (**Table 7**). In the WTUB project PVA beads will be used. According to Skerjanec et al. (2009) these carriers have the similar nitrification efficiencies as the K1 carrier of the Kaldnes company (Norway). In this study the nitrification rate of both carriers was around 1 g NH_4^+-N $L^{-1}d^{-1}$.

The spherical PVA beads are 4 mm in diameter, hydrophilic in nature and have a volumetric density of 670 g L^{-1} (Szentqyörgyi et al., 2010). Moreover, they have a porous structure containing a continuum of passages of 10 to 20 µm in diameter. This structure allows microorganisms to penetrate and colonize the material (Rouse et al., 2005). A typical volumetric packing ratio of the beads is between 5% and 15% (in the thesis a volumetric packing ratio of 10% is chosen). This is much lower that the Kaldnes K1 carrier (volumetric packaging ratio 30%-70%). The carrier is also lighter than water (specific gravity 1.025) (Levstek et al., 2010).

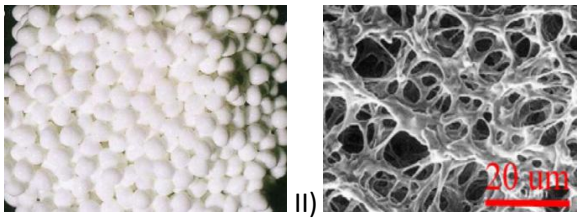


Figure 10: I) PVA beads II) microscopic structure of a PVA carrier (Levstek et al., 2010).

The configuration of a MBR is different than for a MBBR. In a MBR, the biomass is suspended and not attached to a carrier. A key difference between these two configurations is the use of membrane technology for the separation of solids and liquids. Typical membranes for MBR configurations are in the micro and ultra-filtration range. A MBR exists in two modes: cross flow or submerged (Rabaey, 2014). In this research a submerged configuration will be used because of the expertise, the availability of the technology and knowhow of a submerged membrane configuration in the laboratory of microbial ecology and technology (Muys, 2014). Note that in the WTUB project, a crossflow system will be used.

In this thesis study two MBMBR will be operated at different salinities. It is still not know whether the microorganisms will prefer the carriers over the suspension. Moreover the nitrification can be performed by the attached growth biomass and the suspended biomass. Reboleiro-Rivas et al. (2014) found that the abundance and dynamics of denitrifiers, AOB and NOB was the same for both suspended biomass and the attached biomass in a MBMBR. Additionally in this article it is concluded that suspended and attached biomass could play an equal role in the overall nitrification process of their MBMBR. However the influence of salinity was not examined in this article. In the contrary Onnis-Hayden et al. (2011) found that the nitrifying population was more abundant on the carrier than in suspension. The reactor setup was an intergraded fixed film activated sludge configuration. No membrane was present.

3.2. REACTOR CONDITIONS

Nitrification is a biological process and thus when conducting nitrification in a reactor, the conditions need to be optimal for the nitrifying microorganisms. Several parameters are known that influence nitrification. Some of these factors will be discussed below.

3.2.1. TEMPERATURE

According to Chen (2009) temperature is a critical factor effecting nitrification. However it is not that straightforward to determine the influence of temperature on nitrification. Van Hulle et al. (2010) mentions two opposite effects of an increase in temperature. The organisms will have a higher activity according to the Arrhenius principle. However this increase will be maintained until a certain temperature. Where after a decrease in activity will follow. The second effect is an increased ammonia inhibition due to a rise in temperature that results in a higher ammonia/ammonium ratio (see subsection 3.2).

The optimal temperature range lies between 28°C and 32°C. Furthermore the specific growth rate of nitrifying microorganisms will decrease half for every 6°C decrease in temperature (Chen, 2009). This can be different for pure cultures. Grunditz & Gunnel (2001) have conducted experiments on pure cultures. In this research, they found that the optimum temperature for *Nitrosomonas*, an AOB organisms, was 35°C and for *Nitrobacter* 38°C.

Courtens et al. (2014) tested the effect of salinity on the thermotolerance of mesophilic nitrifying sludge (34°C). This study demonstrated that salt amendment is a tool for a more efficient temperature transition for mesophilic sludge. 5 g NaCl L^{-1} increased the specific activity (mg N g^{-1} VSS d^{-1}) of AOB by 20-21% at 40 and 45°C. The specific activity of NOB remained unchanged at 40 °C, yet decreased by 83% at 45°C.

3.2.2. OXYGEN

It is important that during reactor operations sufficient oxygen is supplied. This because nitrification requires oxygen (see reaction 10) The optimal dissolved oxygen (DO) concentration lies between 2.0 and 3.0 mg O_2 L^{-1} (Chen, 2009).

When oxygen is limited due to low DO, the activity of AOB and NOB are influenced differently. Hanaki et al. (1990) performed a lab-scale experiment with a DO level of 0.5 mg O_2 L^{-1}. The results indicated that AOB activity was not influenced, yet NOB activity was inhibited. These conditions let to an accumulation of nitrite. This effect may be due to the fact that more energy is released per amount of oxygen consumed by AOB than NOB (156 kJ mol^{-1} oxygen and 108 kJ mol^{-1} oxygen respectively) (Van Hulle et al., 2010).

3.2.3. PH

Starting from urea, ureolysis will increase the pH (see section 2.1). However when nitrification initiates, the pH drops again (Equation 9). The effect of nitrification (2 mol H^+/mol N produced) is stronger than the hydrolysis of urea (0.5 mol H^+/mol N consumed). The pH is important because it influences the ammonia/ammonium and the nitrous acid/nitrite equilibria (see 3.2.4). The AOB organisms prefer slightly alkaline conditions because ammonia is the substrate. However at a certain suboptimal pH ammonia and nitrous acid can be come inhibitory on both AOB and NOB. This will be discussed in 3.2.4 (Van Hulle et al., 2010). When the pH is below 7, carbon limitation might occur because of carbon dioxide stripping. This will result in a decrease of the nitrification rate (Wett & Rauch, 2003). In the research of Udert (2002), an unknown effect related to the pH was the inhibition of ammonia oxidizers at a pH below 6.

On optimal pH for nitrification according to Chen (2009) is between 7.2 and 8.

3.2.4. FREE AMMONIA AND FREE NITROUS ACID

Free ammonia and free nitrous acid tend to inhibit nitrification, however AOB and NOB are affected differently. The concentration of ammonia and total nitrous acid can be determined from a total ammonia and total nitrite analysis. The calculation can be derived from equations 10 and (11). The total ammonia and total nitrous acid is the summation of unionized and ionized compounds (Anthonisen et al., 1976).

$$\frac{Total\ NH_3}{(NH_3 + NH_4^+)} = \frac{10^{pH}}{e^{\frac{6344}{273+T}} + 10^{pH}} \quad \text{(Equation10)}$$

$$\frac{Total\ HNO_2}{(HNO_2 + NO_2^-)} = \frac{10^{-pH}}{e^{\frac{-2300}{273+T}} + 10^{-pH}} \quad \text{(Equation11)}$$

The ammonia and nitrous acid concentration are dependent of temperature and pH. When the pH increases, the ammonia concentration increase, whereas the nitrous acid concentration decreases (Van Hulle et al., 2010). When looking at nitrification inhibition, ammonia will be the inhibitor at high pH (values above 8) and nitrous acid at low pH (values below 7.5) (Van Hulle et al., 2010).

Concentrations of ammonia inhibition are different for AOB and NOB. Values lay respectively around 8-120 mg N L^{-1} and 0.2-2.8 mg N L^{-1}. For nitrous acid inhibition values lay between 0.08-0.82 mg N L^{-1} and 0.06-0.83 mg N L^{-1} for respectively AOB and NOB respectively (Anthonisen et al., 1976).

3.2.5. SALINITY-CONDUCTIVITY-OSMOTIC PRESSURE

In wastewater treatment plants, salt can be a stress factor. Especially for certain industries such as cheese manufacturing, seafood processing, the production of chemicals and pharmaceuticals, oil and gas recovery. They produce a lot of inorganic salt, ending up in their wastewater (Moussa et al., 2006). The WTUB also deals with high salt concentrations coming from urine (**Table 1**). Inorganic salts present in urine are sodium chloride (8 g L^{-1}), potassium chloride (1.6 g L^{-1}), potassium sulfate (2.6 g L^{-1}), magnesium sulfate (0.8 g L^{-1}), magnesium carbonate (0.1 g L^{-1}), potassium bicarbonate (0.7 g L^{-1}) and potassium phosphate (0.2 g L^{-1}) (Putnam, 1971).

In literature it can be found that certain AOB and NOB are halotolerant, moderately halophilic or obligately halophilic (Koops & Pommerening, 2001). An overview of these species is given in **Table 9**.

Table 9: Halotolerant or halophilc character of AOB and NOB species (Koops & Pommerening, 2001).

Ecophysiological parameter	Species
AOB	
Halotolerant or moderately halophilic	*Nitrosomonas europaea*
	Nitrosomonas eutropha
	Nitrosomonas halophila
	Nitrosococcus mobilis
Obligately halophilic	*Nitrosomonas marina*
	Nitrosomonas sp. III
	Nitrosomonas aestuarii
	Nitrosomonas cryotolerans
	Nitrosococcus oceani
	Nitrosococcus halophilus
NOB	
Halotolerant or moderately halophilic	*Nitrobacter alkalicus*
Obligately halophilic	*Nitrococcus mobilis*
	Nitrospina gracilis Nitrospira marina

There are two strategies for microorganisms to cope with a salty environment. The first strategy is the ”salt in strategy”. Cells will maintain high intracellular salt concentration. In this strategy, the intracellular system need to be adapted to the presence of high salt concentrations. A second strategy is the “compatible solute strategy”. Solutes such as glycerol, ectoine, sucrose, trehalose etc. will be synthesized by the cell or taken up from the medium. Both strategies are costly from a bioenergetic point of view. The strategy implemented depends on the microorganism (Oren., 1999). In literature no information could be found concerning the adaptation strategy of nitrifying microorganisms to salt.

For nitrifying microorganisms, it is costly to thrive at high saline environments. Their electron donors (ammonia and nitrite) are relatively oxidized and thus little energy can be gained from electron transport to oxygen. Moreover most of the energy generated needs to be used to make uphill electron transport to produce NADPH (reducing power necessary for autotrophic carbon dioxide fixation in the Calvin cycle) (Oren., 1999). Moussa et al. (2006) examined the effect of salinity on the activity, the composition of nitrifying microorganisms and floc characteristics of nitrifying sludge. In this study adapted and non-adapted cultures were tested. Before the experiments the reactor of the adapted culture was continuously operated at 10 g NaCl-Cl L^{-1}. During the salt experiments, the salt concentration was gradually increased from 5 to 40 g NaCl-Cl L^{-1}. The results indicate that the activities of AOB and NOB dropped respectively 36% and 11%. Hence it was concluded that AOB were more sensitive towards salt stress. No difference in activity could be observed between the adapted and the non-adapted cultures. When increasing concentrations up to 40 g NaCl-Cl L^{-1}, the inhibition reached 95% for both AOB and NOB. Dinçer & Kargi (2010) concluded that NOB are more effected by salt because nitrite accumulates. However Moussa et al. (2006) discusses that his is not a direct effect of salt stress, but due to other factors such as oxygen limitation, phosphorous limitation and maybe the presence of toxicants. Oxygen limitation is due to the fact that salts influences the maximum solubility of oxygen. The solubility of a gas in an aqueous solution decreases by the addition of electrolytes. This phenomenon is known as salting out (Bank et al., 1967).

Urine contains not only sodium ions but also other cations such as potassium, magnesium and calcium (**Table 3**). In biology extensive research has been done concerning the effects of cations on the physiological processes of various organisms. There are three basic effects: toxicity, antagonism and stimulation. The first phenomenon (i.e. toxicity) is present for all cations. At certain concentrations, cations become toxic. Moreover for different

cations the toxicity increases with the valence and the atomic weight of the ion (Kugelman & McCarty, 1965). However when different cations are present in a medium the toxicity varies. Antagonism is the ability of one cation to decrease the toxicity of another cation. Stimulation is the effect that a cation will stimulate the toxic effect of another cation This occurs at concentrations below toxicity (Kugelman & McCarty, 1965). In **Table 10** a general overview of different toxic cations is given with their antagonist and stimulator.

Table 10: An overview of toxic cations and their corresponding antagonist and stimulator adapted from (Kugelman & McCarty, 1965).

Toxic Cation	Na	NH_4	K	Ca	Mg
Antagonist	K	Na	Na,K,Mg,NH_4	Na,K	Na,K
Stimulator	NH_4,Ca,Mg	K,Ca,Mg	-	NH_4,Mg	Ca

In literature different methods exist to express salinity. Some examples are the amounts of inorganic salts expressed in g L^{-1}, using conductivity measurements or by osmotic pressure. In the article of (Moussa et al., 2006), the salt levels are expressed in g NaCl-Cl L^{-1}.

The conductivity is defined as the reciprocal of the resistivity normalized to a 1 cm cube of a liquid at a specified temperature. The units are Siemens cm^{-1} (S cm^{-1}) (Miller et al., 1988). Another way to express the salt content of a solution is by the osmotic pressure. The osmotic pressure is the pressure generated by the osmotic flow of water through a semi-permeable membrane. The water flows from an aqueous compartment containing low solute concentration an aqueous compartment with higher concentration. Movements of water across a semi-permeable mebrane are thus driven by difference in osmotic pressure. For a cell three situations exist: the cell is surrounded by an isotonic, a hypertonic or a hypotonic solution. In the first situation there is no difference in osmotic pressure and thus the cell neither gains nor loses water. For the last two conditions an osmotic pressure difference exist, so water will flow through the membrane. In the case of a hypertonic solution, the cell will shrink and in the case of the hypotonic solution, the cell will swell (David & Michael, 2008).

Table 11 gives a calculated overview of the osmotic pressure and electrical conductivity (EC) for different forms of urine (values as used in WTUB appendix). To calculate the osmotic pressure, the van't Hoff equation can be used (Equation 12) where T is the absolute temperature, R is the gas constant and ic is the osmolarity of the solution. When a solution contains several solutes, the sum of the contributing solutes needs to be taken (David & Michael, 2008).

$\Pi = icRT$ (Equation 12)

Table 11: Calculated osmotic pressure and electrical conductivity of different forms of urine (Clauwaert et al., 2014).

Parameter	EC (mS cm^{-1})	Osmotic pressure (bar)
Fresh urine	27.2	10.8
Nitrified urine	82.9	34.5
Hydrolysed urine	86.2	35.8

3.3. NITRIFICATION AND BIOFILMS

A biofilm can be defined as a complex structure of cellular products ands cells attached to a solid or substratum. There are mainly two types of biofilm reactors: those on a fixed substratum and biofilms on suspended particles (Tijhuis, 1994).

The formation of a biofilm is characterized by four distinct stages. In the first stage cells attach to the surface. Then a slime-like matrix containing extracellular polymeric substances is produced i.e. irreversible attachment. These substances are import for the support of the biofilm. They are made up of polysaccharides, nucleic acids and proteins. The following stage is called maturation. In this stage the formation of a complex architecture of channels and pores are formed. Detachment is the final stage (Stoodley et al., 2002). In this stage loss of parts of the biofilm occurs into the bulk liquid (Tijhuis, 1994).

For nitrifying microorganisms it can be beneficial to immobilize them because of their low growth rate and growth yield (Tijhuis, 1994). Biofilms favor the development of slow growing organisms such as autotrophs and reduce their wash-out from the reactor (Lee et al., 2006). However immobilization can impose problems for oxygen transport. Oxygen can penetrate up to 100 -200 µm. That is why the thickness of a biofilm has to lie between 100-200 µm (Tijhuis et al., 1994).

It is interesting to have an understanding about the specific nitrifying microorganisms present in a biofilm. Okabe et al. (1999) investigated the special organization of AOB and NOB in wastewater biofilms and in autotrophic nitrifying biofilms. This study revealed that the genus *Nitrosomonas* was the dominant AOB for both types of biofilms. No bacteria from the genus *Nitrobacter* were detected. For the NOB *Nitrospira*-like bacteria were dominant.

At present, no information about the effect of salinity on a nitrifying biofilm could be found. In activated sludge processes there is already some research about the effect of divalent and monovalent cations on settling characteristics. Sobeck & Higgins (2002) observed that divalent cations such as calcium and magnesium improve floc strength. The addition of sodium, a monovalent cation, resulted in deterioration of the flocs. The divalent cations are being leached by sodium (Bin Ismail, 2013). An explanation for this phenomenon can be found in the divalent cation bridging theory. This theory states that divalent cations bind negatively charged functional groups on extracellular polymeric substances and bacterial surfaces. This bridging promotes bioflocullation (Sobeck & Higgins, 2002). **Figure 11** illustrates this divalent cation bridging theory: On the left divalent cations and on the right monvalent cations competing with divalent cations on the sites of the extracellular polymeric substances.

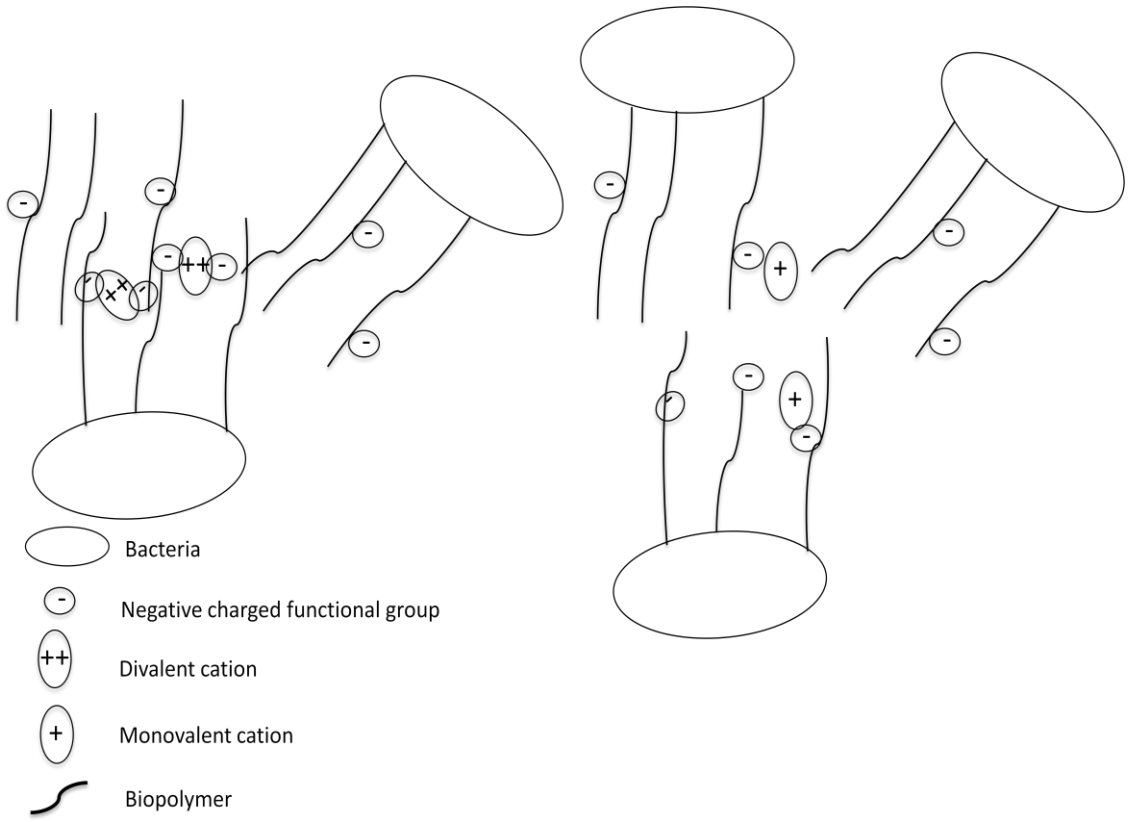


Figure 11: Divalent cation bridging theory.

Sobeck & Higgins (2002) also discusses the double layer theory, a classical colloidal theory to explain the mechanisms of biofloculation. Basically this implies that addition of cations such as sodium or calcium improves biofloculation. In the study of Cousin & Ganczarczyk (1998), addition of sodium increased the floc porosity and floc size. Sobeck & Higgins (2002) reports that studies supporting the double layer theory have been conducted by short duration batch tests. While research supporting the divalent cation bridging theory have used continuous flow reactors. Cation exchange can only occur at readily available sites within the floc during batch addition. And thus the full extent of change of floc properties and floc structure may not be observed during short duration batch studies. According to Higgins & Novak (1997) a divalent/monavalent ratio higher than 0.43 (mg mg^{-1}) improves floc properties and settling.

The effect of increased biofilm strength by a higher calcium concentration (divalent cation) was observed by Ahimou et al. (2007), Huang & Pinder (1995) and Körstgens et al. (2001). According to Eldyasti et al. (2013), the detachment rate of a biofilm decreases at high calcium concentration. These studies do not discuss the importance of the divalent/monovalent cation ratio. Furthermore no studies have been found in literature concerning the effect of calcium concentration on the strength of nitrifying biofilms.

When considering the information stated above, high salinity levels (i.e. high sodium concentration), can have an effect on the biofilm of a nitrifying population. It is interesting to examine the effect of a high divalent/monovalent ratio on the biofilm formation of a carrier.

4. RESEARCH OBJECTIVES

In this thesis research, the effect of increasing salt concentration (decreasing urine dilution) on the biofilm formation in a MBMBR will be investigated. It is not known what the effect will be of high salinity on the biofilm formation. Nitrified urine can exert high stress on microorganisms in the form of salinity (**Table 11**).

As mentioned in the literature study (see section 3.3), high salinity levels may reduce the strength of the biofilm by leaching of caclium Moreover salinity has consequences from a bioenergetic point of view. The microorganism can change the intracellular ion concentration or synthesize compatible solutes. This is costly for the overall metabolism (see 3.2.5). Thus how will the activity of both AOB and NOB be affected by increasing salt concentrations? Is it possible that salinity is a driver for biofilm formation? Moreover the combination of membrane filtration and PVA beads eliminates the stress of wash-out. And thus maybe salinity alone will not cause a competitive advantage of biofilm growth. This could result in a hybrid situation where the suspended biomass and attached biomass play an equal role in the nitrification process (Reboleiro-Rivas et al., 2014).

This thesis research is divided in two parts.

Part 1: When using a nitrification reactor it is of great importance that the right inoculum is selected. It should have a high activity when fed with urine. Therefore inoculum has to be used that maintains high activity under high salinity. As mentioned in section 2.1, urine contains a lot of salts. Salinity influences the nitrification (see 3.2.5). When operation a reactor it is important to have sludge that is able to operate at stress conditions exerted by urine. That is why a screening of different sludge types at two salinity levels will be done (i.e. normal conditions at which the sludge is adapted to and nitrified urine salt conditions). This gives a general overview of the adapting performance of the sludge to high salinity levels. Furthermore the results may indicate which functional groups (AOB/NOB) are more sensitive toward salts. An inoculum will be selected for Part 2 based on these results.

Bioreactors will be operated in the second part of the research. The bioreactor that will be studied is a MBMBR. This research part consists out of three stages.

Stage1: In this stage the selected sludge of stage 1 will be used in a MBMBR. Basically two MBMBRs will be operated at increasing salinity levels (decreased urine dilution). During this stage no sludge will be removed from the reactors (SRT infinite). Throughout this phase the effect of salinity on the biofilm formation will be investigated. Does salt stress select for biofilm formation? How will the biomass be divided over the biofilm and suspension? Is biofilm formation an issue at increasing salinity? Will this be in correspondence with the results obtained in stage 1?

Stage 2: At this stage the effect of decreasing SRT will be investigated to induce a selective pressure (wash-out). How will a decreasing SRT effect the biofilm formation at low salinity and high salinity MBMBR?

Stage 3: The divalent/monovalent cation ratio of one bioreactor will be changed in this stage of the experiments. The divalent cation bridging theory will be evaluated for the bioreactors.

PART 2: MATERIALS AND METHODS

1. BATCH EXPERIMENTS

1.1. EFFECT OF SALINITY ON THE ACTIVITY OF AOO AND NOB FOR DIFFERENT TYPES OF SLUDGE

The experiments were organized as follows: collection and storage of the sludge, activation of the sludge, washing and activity test. Freshly collected activated sludge (0.2 - 10 L) was stored at 4°C prior to analysis. An overview of the original salt conditions at which the sludge types were adapted to before the experiments is presented in **Table 12**. All data are ranked in order of decreasing salinity. The specific N-load is normalized to 20°C by the Arrhenius equation (see section subsection 5.6 PART 2).

Table 12: Overview of the tested sludge types.

Company location	Type of wastewater	Process configuration	pH	T	Influent concentration	N-Load	Specific N-load	Salinity	Normalized N-load 20°C**
				°C	mg NH_4^+-N L^{-1}	mg NH_4^+-N $L^{-1}d^{-1}$	mg NH_4^+-N g^{-1} VSS d^{-1}	mS cm^{-1}	mg NH_4^+-N g^{-1} VSS d^{-1}
Ugent shrimp tank-Ghent	Aquaculture	Fixed bed	8.2	28	<0.1	n.d.	n.d.	90	n.d.
DOW - Terneuzen	Industrial wastewater	Carrousel	7.2-7.5	23-28	15	n.d.	6	70	7
Sealife - Blankenberge (Octopus)	Aquaculture	MBBR	8.2	15	0.1	n.d.	n.d.	46	n.d.
Ugent Labscale reactor - Ghent	Autothropic medium	SBR	7.8	26	Variable	250	133	37	158
Decostere - Kortemark	Thin fraction pig manure	n.d.	7.7	n.d.	n.d.	n.d.	n.d.	25	n.d.
DANIS NV - Izegem	Pig manure	n.d.	7.7	26-27	4500	162	41	25	49
Avecom – Wondelgem	Aquaculture inoculum	Fed batch	7.0	24	n.d.	n.d.	230-350	23	273-415
Hooge Maey - Antwerp	Wastewater landfill	Circuit reactor	n.d.	25-30	400	n.d.	n.d.	9	n.d.
Imog – Moen	Landfill leachate	Batchreactor	7.0-8.5	20-30	330	69	17	9	20
RWZI dokhaven - Nieuwveer	Municipal wastewater	AB-process	7.6	14	17	125*	37	2	44
Inagro - Rumbeke	Aquaculture	MBBR (Polyethyleen beads)	8.5	22	0.1	7.2	36	1	43
SeaLIfe – Blankberge (Piranha)	Aquaculture	Packed Bed Reactor	6.0-7.0	20	<0.1	n.d.	n.d.	1	n.d.

Data provided in collaboration with companies. EC and pH measured in laboratory. The normalized N-loads are obtained by the Arrhenius equation.

n.d.= no data

SBR= Sequencing batch reactor

After storage (2 - 5 days), the sludge was activated by spiking with ammonium chloride (0.05 g N L^{-1}) or with sodium nitrite (0.05 g N L^{-1}). 1 L sludge was divided over two Erlenmeyers (2 L working volume). The Erlenmeyers were placed on a shaker (Innova 2300, eppendorf, 111 rpm) in a temperature controlled room of 20 °C. Strip test for ammonium and nitrite concentrations (section 3.1) were taken at regular time intervals to have a rapid indication about the volumetric rate of the nitrifying sludge.

Ammonium Oxidizing Organisms (AOO) and NOB activity were analyzed separately at the salinity at which the sludge was adapted to, as well as the salinity of nitrified urine (45 mS cm^{-1}) (Muys, 2014). Before the batch test, the sludge was washed with buffer solutions that had the same conductivity to which it was adapted. The buffer had the following composition: 8.316 g L^{-1} potassium dihydrogen phosphate, 14.516 g L^{-1} dipotassium hydrogen phosphate, 2.222 g L^{-1} sodium bicarbonate. The pH was corrected to 7 with hydrogen chloride (0.02 M, Chem Lab). The sludge was divided over tubes (250 mL) and centrifuged at 3000 rpm for 10 minutes. The supernatant was then removed and buffer was added as washing solution (1/2 of original volume). Buffer and settled sludge was vortexed (Thermo Scientific, RC 6+ Centrifuge) until the pellet was re-suspended. The sludge was again centrifuged at 3000 rpm for 10 min and the supernatant was removed. Buffer was added and the pellet was vortexed again. The sludge was than collected in an Erlenmeyer (working volume 2 L) and homogenized by shaking manually. Four liter of buffer was prepared for every activity test.

The EC of the buffer was 11 mS cm^{-1}. Some sludge types were adapted to salinities lower than 11 mS cm^{-1}. And thus the buffer was diluted with demi-water for some sludge types (RWZI dokhaven – Nieuwveer, Sealife – Bankenberge (Piranha) and Inagro – Rumbeke) until an EC of 2 mS cm^{-1}. For the actual batch test, the sludge was divided over two Erlenmeyers. Sodium chloride was added to one Erlenmeyer to raise the EC until 45 mS cm^{-1}. Batch tests were performed in triplicate, in Erlenmeyers with a volume of 250 mL. 50 mL sludge was added to the Erlenmeyer and diluted with 45 mL buffer. 5 mL of an ammonium chloride or sodium nitrite solution was added to the Erlenmeyers (1 g N L^{-1}). The working volume of the Erlenmeyers was 100 mL. These Erlenmeyers were put in a box on a shaker (Innova 2300, eppendorf, 111 rpm). An indication of the volumetric rate could be obtained from activation of the sludge by measuring the ammonium and/or nitrite concentration with strip tests. The initial ammonium or nitrite concentrations varied according to the activity of the sludge. Highly active sludge types were spiked to a final concentration of 0.05 g N L^{-1} ammonium (for ammonia oxidizing activity) or nitrite (for nitrite oxidizing activity) and less active sludge types (such as the sludge from Sealife, Inagro and Ugent shrimps tank) were spiked to a final 0.02 – 0.015 g N L^{-1}. 3 mL of sample was taken every 1 to 12 hours. The sample was first filtered with a 0.45 µm pore size filter (Chromafil^{R} Xtra) and stored in the fridge at 4°C for 1 to 30 days. During the batch tests DO and pH was measured in the Erlenmeyers. DO was measured every 4 hours and the pH was measured at the beginning and at the end of the experiments. Twelve Erlenmeyers were needed to test the nitrification activity of one type of sludge.

An overview of the experimental setup is shown in **Figure 12**. The Erlenmeyers were protected from light during the experiments. NOB were not suppressed during the AOO experiments. Only ammonium oxidation was analyzed.

$Triplicate_1$	AOO_{SSA}	NOB_{SSA}	AOO_{NC}	NOB_{NC}
$Triplicate_2$	AOO_{SSA}	NOB_{SSA}	AOO_{NC}	NOB_{NC}
$Triplicate_3$	AOO_{SSA}	NOB_{SSA}	AOO_{NC}	NOB_{NC}

Figure 12: Experimental setup for the batch experiments.

SSA=Salinity

NC=Nitrified urine conditions (high salinity conditions 45 mS cm^{-1})

1.2. SALINITY PROFILE ABIL AVECOM

The activity of the AOO and NOB of the sludge from the company Avecom (Wondelgem, 1-12-14) was tested at four different salinities. The EC and the corresponding sodium chloride concentration during the experiment are shown in **Table 13**. The same procedure was followed as for the batch experiments (see section 1.1 PART 2). All experiments were performed in triplicate on a shaker (Innova 2300, eppendorf, 111 rpm) at 20°C.

Table 13: EC and sodium chloride concentration during experiment.

EC	Sodium chloride added (g L^{-1})
3	0.0
23	5.6
34	9.4
48	11.3

2. MBMBR EXPERIMENTS

This part of the research was divided over three stages. In stage 1, the effect of increasing salinity on the biofilm formation was studied. In stage 2, the floccular SRT of both reactors was decreased. In the final stage, the effect of the divalent and monovalent cation ratio was investigated.

2.1. EXPERIMENTAL SETUP OF MBMBRS

During stage 1 and stage 2, MBMBR reactors were operated at two different salinities. The first reactor was operated at a salinity of 15 mS cm^{-1} and the second MBMBR was operated at nitrified urine conditions i.e. a salinity of 45 mS cm^{-1} (EC profile fluctuated during experiments see **Figure 22** and **Figure 35**). Both reactors had a dimension of 50X20X10 cm. The working volume was 9.0 L. Four pumps were used for the experimental setup: two for the influent (Prominents 0.4 and Prominent 2.4) and two for the effluent (Watson Marlow 603 S and Watson Marlow 313 S). The membrane (KUBOTA Membrane Europe LTD) used for the MBMBRs had a pore diameter of 0.4 µm, a surface area of 0.8 m^2 and a capacity of 1-1.5 m^3 d^{-1}. PVA beads were used (KURARAY AQUA Co) as carrier material. The beads had a wet weight of 27.8±2.8 mg and a dry weight of 4.3±0.1 mg (Lindeboom et al., 2014). The beads consist 84% of water. Properties of these beads are discussed in the literature study (see subsection 3.1 PART 1). In order to provide sufficient circulation of the PVA beads, two vacuum pumps (VMR Mini diaphragm vacuum gas pump) were used per bioreactor. These pumps had a double functionality: provide circulation and aeration. The flow rate of these aeration pumps was 5 L min^{-1}. During stage 1, no pH controller was used. The pH was manually controlled by addition of bicarbonate in the influent. The bicarbonate factor of the influent varied between 6 to 12 g $NaHCO_3$ $g^{-1}N$. The inoculum provided by Avecom, was initially attached to calcium carbonate as described in Grommen et al. (2002) and Rombaut et al. (2003). In order to flush out the calcium carbonate, a bicarbonate factor of 6 g $NaHCO_3$ g N^{-1} was initially used. Produced protons from nitrification push the carbonate equilibrium to the formation of CO_2. When the pH started dropping in the MBMBRs, the bicarbonate factor was raised to 12 g $NaHCO_3$ g N^{-1}. In the second stage of the experiments, a pH controller (Prominent dulcometer; prominent dulcotest pH electrode PHEP-112) was used. The probe was introduced from above the reactor through a glass tube to protect. Only base (sodium hydroxide solution 1 M) was added to both bioreactors because nitrification produces protons (see section 3 PART 1) and pH decreases as a result. Daily samples were taken of the MBMBR at the end of every cycle (see subsection 2.2 PART 2). These samples were analyzed for nitrate, nitrite (ion chromatography) and ammonium

(high throughput) (see subsection 3.2 and 3.3). EC, DO and pH were measured on a daily basis. An illustration of the experimental setup is shown in **Figure 13** and in **Figure 14**. The setup was the same for both bioreactors.

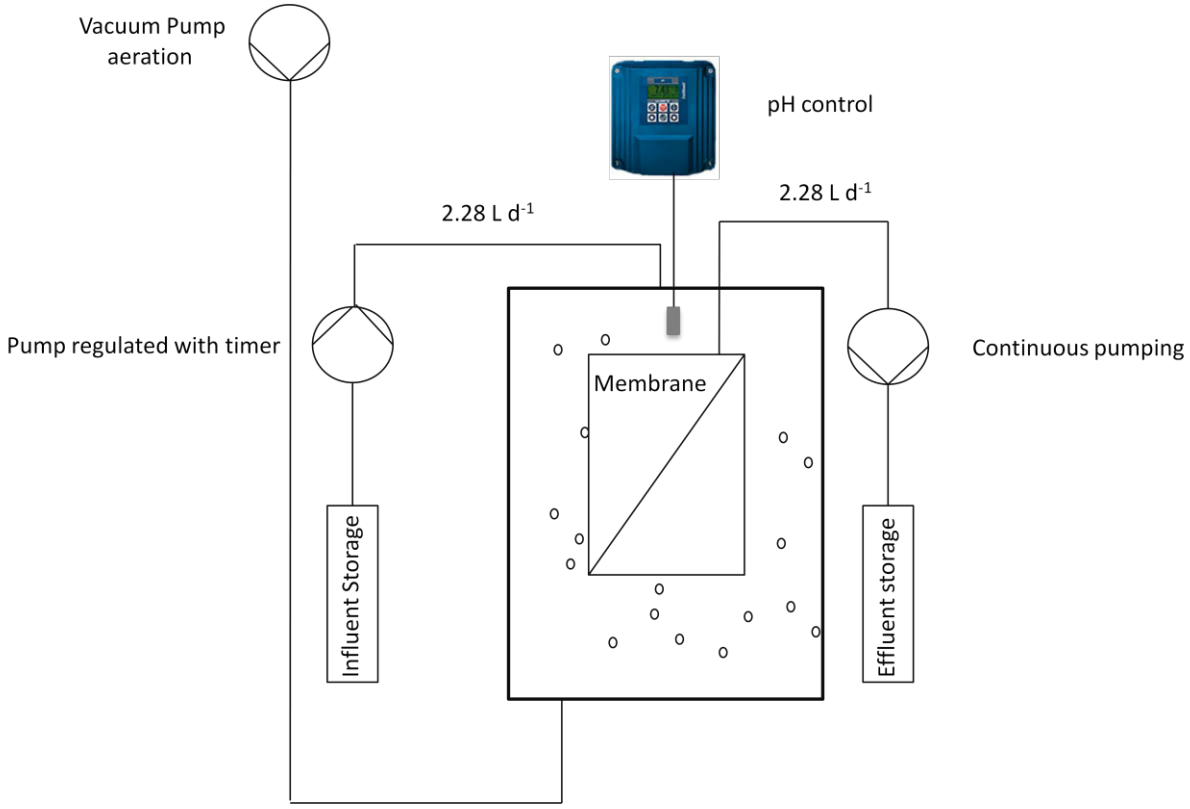


Figure 13: MBMBR setup.

The setup of **Figure 13** was the same for both reactors. The circular shaped objects in the reactor represent PVA beads.

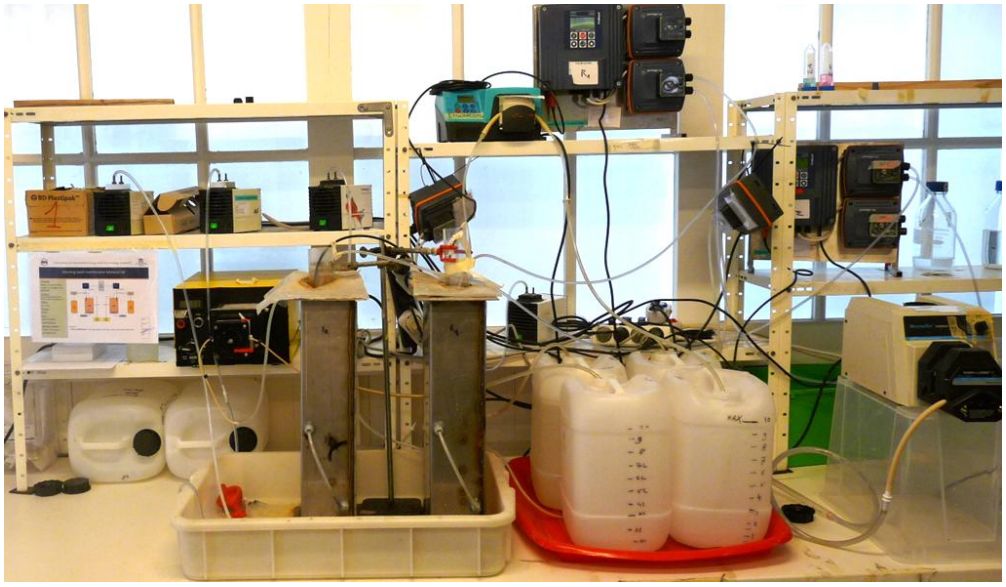


Figure 14: Picture of test setup.

2.2. REACTOR OPERATIONS

The influent flow rate of MBMBR1 and MBMBR2 was kept the same during the operation of the experiments: 2.28 L d^{-1}. Dosage was not continuous during the reactor operation. Six dosage cycles were chosen throughout the day to spread the nitrogen load equally in time. Pumping started every four hours. There was a dosage cycle for 15 minutes or 1.5 hours for the high salinity and the low salinity reactor respectively. The cycle comprised pumping of influent and subsequent nitrification in the reactor without pumping. At the end of the cycle, samples were taken for analysis. Effluent pumps were operated continuously. The volumetric nitrogen-loading rate was kept constant for both reactors. The only variable factor between the bioreactors was the sodium chloride content of the influent (**Table 15**).

An overview of the operating parameters for the bioreactors is shown in **Table 14**. During stage 2, the floccular SRT was manually reduced by taken out 1.14 L reactor broth extra a day with a second effluent pump (8 minutes, 8.5 L h^{-1}) (Masterflex 77200-50) used for both bioreactors. This was done every morning at one time point, not continuously. The broth was collected with a volumetric flask of 2 L to determine exactly how much liquor was removed. A tube was connected with the pump. A sieve was place on the opening of the tubing, to prevent removal of PVA beads. The effluent pumps connected with the membranes, took out another 1.14 L a day continuously.

Table 14: An overview of the operation parameters throughout the reactor experiments.

Operating parameters	**MBMBR1** (stage1)	**MBMBR2** (stage1)	**MBMBR1** (stage2)	**MBMBR2** (stage2)	**MBMBR1** (stage3)	**MBMBR2** (stage3)
EC (mS cm^{-1})	7*/15**	7*/45**	15	45	15	20*/15**
Gas flow (L min^{-1})	5	5	5	5	5	5
pH	6.5-7.5	6.5-7.5	6.5-7.5	6.5-7.5	6.5-7.5	6.5-7.5
Nitrogen loading rate (mg N L^{-1} d^{-1})	112*/313**	112*/313**	313*/50**	313*/50**	50	50
Working volume (L)	9	9	9	9	9	9
HRT (d)	4	4	4	4	4	4
SRT (d)	∞	∞	∞*/8*	∞/8*	8	8
Temperature	22-24	22-24	22-24	22-24	22-24	22-24

*Start-up

**Target values

The influent composition of MBMBR2 was changed in stage 3 of the experiments. During this part of the research, the effect of a higher divalent/monavelent cation ratio was studied. The divalent/monavelent cation ratio was 1.93 Ca^{2+}g Na g^{-1} in MBMBR2 0.002 Ca^{2+}g Na g^{-1} in MBMBR1.

Table 15: Influent composition throughout the reactor experiments.

Operating parameters	**MBMBR1** (stage1)	**MBMBR2** (stage1)	**MBMBR1** (stage2)	**MBMBR2** (stage2)	**MBMBR1** (stage3)	**MBMBR2** (stage3)
Sodium chloride (g L^{-1})	4.5	18	4.5	18	6	2
$CaCl_2$ (g L^{-1})	-	-	-	-	-	4.4
pH	8	8	8	8	-	8
EC (mS cm^{-1})	15	45	15	45	15	15
NH_4HCO_3 (mg N L^{-1})	450*/1253**	450*/1253**	1253*/183**	1253*/183**	183	183
Bicarbonate factor (g$NaHCO_3$ gN^{-1})	6*/12**	6*/12**	12	12	-	-
Trace element A (mL L^{-1})	0.1	0.1	0.1	0.1	0.1	0.1
Trace element B (mL L^{-1})	0.1	0.1	0.1	0.1	0.1	0.1
Temperature	22-24	22-24	22-24	22-24	22-24	22-24

**Start-up

**Target values

An overview of the chemical composition of trace element A and B is presented in **Table 16** and **Table 17**.

Table 16: Composition trace element A.

Chemical	**Concentration (g L^{-1})**
$FeSO_4.7H_2O$	9
Na_2EDTA	6

Table 17: Composition trace element B.

Chemical	Concentration ($g\ L^{-1}$)
$Na_2EDTA.2H_2O$	11.777
$ZnSO_4.7H_2O$	0.338
$CaCl_2.6H_2O$	0.188
$MnCl_2.4H_2O$	0.494
$CuSO_4.5H_2O$	0.196
$Na_2MoO_4.2H_2O$	0.173
$NiCl_2.6H_2O$	0.149
$Na_2SeO_4.10H_2O$	0.165
H_3BO_3	0.011
$NaWO_4.2H_2O$	0.039

2.3. INOCULUM REACTORS

The sludge utilized to inoculate the MBMBR was from the company Avecom (Wondelgem, 17-1-15). The sludge was first activated with the addition of ammonium chloride for two weeks prior to inoculation. The inoculation conditions for both bioreactors are shown in **Table 18**.

Table 18: Inoculum MBMR1 and MBMBR2.

MBMBR	TSS (g/L)	VSS (g/L)	EC ($mS\ cm^{-1}$
1	14.79±0.78	2.70±0.08	7
2	12.95±0.60	2.23±0.09	7

The PVA beads were introduced in the reactor after two days of steady operation of the MBMBR. A volume of 800 mL beads was inserted in every bioreactor. The packing ratio was 9%.

1.14 L sludge was taken out daily to reduce the bulk SRT. The sludge was then stored at 4°C. The harvested sludge of the 15 $mS\ cm^{-1}$ reactor was used for the inoculation of stage 3 of the MBMBR experiments. The sludge was stored for 2 weeks prior to inoculation. This stored sludge was first mixed with the broth of the 15 $mS\ cm^{-1}$ reactor.

2.4. MICRO SCALE BATCH EXPERIMENTS

Activity tests were setup to determine the AOO and NOB activity of suspension and of the beads. A 96 well plate (well volume 360 µL) was used for the inoculation of the wells. An overview of the well plate of the batch experiments is illustrated in **Figure 15** and **Figure 16**.

<>	1	2	3	4	5	6	7	8	9	10	11	12
A	NOB **SS+B** 45mS cm^{-1}				NOB **SS** 15mS cm^{-1}	NOB **B** 15mS cm^{-1}	NOB **SS** 45mS cm^{-1}	NOB **B** 45mS cm^{-1}	NOB **SS** 15mS cm^{-1}	NOB **B** 15mS cm^{-1}	NOB **SS** 45mS cm^{-1}	NOB **B** 45mS cm^{-1}
B	NOB **SS+B** 15mS cm^{-1}											
C	AOO **SS+B** 45mS cm^{-1}											
D	AOO **SS+B** 15mS cm^{-1}											
E	NOB **SS+B** 45mS cm^{-1}				AOO **SS** 15mS cm^{-1}	AOO **B** 15mS cm^{-1}	AOO **SS** 45mS cm^{-1}	AOO **B** 45mS cm^{-1}	AOO **SS** 15mS cm^{-1}	AOO **B** 15mS cm^{-1}	AOO **SS** 45mS cm^{-1}	AOO **B** 45mS cm^{-1}
F	NOB **SS+B** 15mS cm^{-1}											
G	AOO **SS+B** 45mS cm^{-1}											
H	AOO **SS+B** 15mS cm^{-1}											

Figure 15: Schematic overview of a activity test for MBMBR1 and MBMBR2.

Red boxes= MBMBR1

White boxes= MBMBR2

Blue boxes= positives MBMBR1

Brown boxes: positives MBMBR2

SS= Suspension of reactor

B= Bead reactor

Positive controls were used for the results.

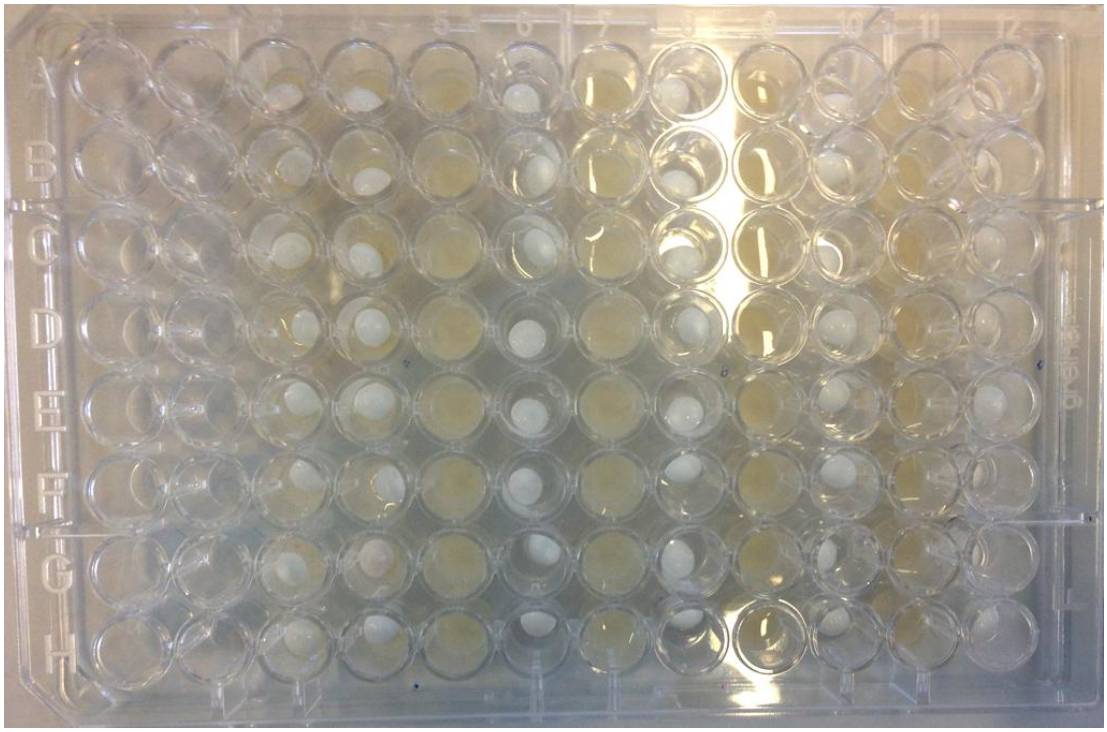


Figure 16: Picture inoculation plate.

Positive controls were performed in duplicate for this test. Other experiments were done in triplicate.

The batch tests were conducted three times for stage 1 (day 20, 27 and 34 of reactor operation) and two times for stage 2 (day 41 and 48 of reactor operation). Suspension and the beads of the reactor were tested together (positive control) and separately. The experiments were performed in triplicate for the positive controls and the other activity tests were performed in quadruplicate. Beads and suspension of both bioreactors were

tested at two salinities (15 mS cm^{-1} and 45 mS cm^{-1}). Before the test, the suspension was centrifuged (Eppendorf centrifuge 5430, 3000 rpm 10 minutes). The supernatant was replaced by a salty solution (sodium chloride added to demi-water until a EC of 15 mS cm^{-1} and 45 mS cm^{-1} depending on the test). The beads were manually removed out of the bioreactor. These carriers were then carefully washed with milli-Q water to removed suspended solids. 60 µl of suspension (test suspension) or one bead (test bead) was introduced in a well. No more than one bead could be added to the well. An extra volume of 20 µL of solution (EC 15 mS cm^{-1} or 45 mS cm^{-1} depending on the test) was added to the wells filled with beads. All wells were spiked with 150 µL (80 mg N L^{-1}) of ammonium chloride or sodium nitrite, depending on the test. The final volume of the wells was 210 µL with a final nitrogen concentration of 50 mg N L^{-1}. As positive control, a bead and 60 µL of suspension from the bioreactor were combined. These positives were also tested for AOO and NOB activity at two different salinities. The active volume of the positives was 250 µL.

2.5. MACRO SCALE BATCH EXPERIMENTS

Macro scale experiments were executed for both reactors. These test were setup to have an indication about the activity of AOO and NOB on the beads. Furthermore it was also important to verify the results of the micro batch experiments on a macro level. The tests were done in Erlenmeyers with a working volume of 100 mL. The active volume was 50 mL. All test were performed at the salinity of the corresponding reactor (15 mS cm^{-1} or 45 mS cm^{-1}). Suspension and beads were tested separately. Before an influent cycle, 75 mL of sludge was taken out of the reactors and 15 mL beads. The beads were washed with filtered effluent. 75 mL of suspension was divided over three Erlenmeyers. For the activity test of the beads, 75 mL of effluent was used. This effluent was first filtered with a 45 µm pore size filter (Chromafil^{R} Xtra) and divided over three Erlenmeyers. 5 mL beads were added to every Erlenmeyer. The volume packing ratio during the batch experiment was 10%. AOO and NOB activity was tested in one Erlenmeyer by the addition of 5 mL of ammonium chloride and 5 mL of sodium nitrite. The initial concentration of ammonium and nitrite was 30 mg N L^{-1}. 12 Erlenmeyers were used in total for both reactors. The Erlenmeyers were put on a shaker (Innova 2300, eppendorf, 111 rpm) in the dark, for the actual test.

2.6. MICROSCOPY

Samples of the two bioreactors were analyzed with light microscopy and fluorescent microscopy on a magnification of 10X, 40 X and 100X (Zeiss microscope, Axioskop 2 plus).

3. ANALYTIC METHODS

3.1. TEST STRIPS

Ammonium, nitrite and nitrate test strips (MQuant^{TM}) were used to have a rapid indication of the concentration during the batch experiments. The ammonium, nitrite and nitrate strips had a range of 0-310 NH_4^+-N L^{-1}, 0.6-24 NO2 - N L^{-1} and 2.3-113 mg NO3 - N L^{-1} respectively.

3.2. NITRITE AND AMMONIUM CONCENTRATION

The ammonium concentration was analyzed spectrophotometrically according to the Berthelot reaction (Bucur et al., 2006). The absorbance was measured at 690 nm instead of 700 nm with a Tecan infinite plate reader (see **Figure 17**).

Figure 17: Tecan plate reader (Tecan, 2015).

The nitrite concentration was also measured with the plate reader. It was determined spectrophotometrically by the Montgomerey reaction (Montgomerey & Dymock, 1961). High throughput analyses were done in duplicate or in triplicate on 96 well plates. 2 µL samples was taken and the total volume of the well was 235 µL and 290 µL for the ammonium and nitrite determination respectively.

3.3. ION CHROMATOGRAPHY

Nitrite and nitrate were determined with a Compact Ion Chromatograp (Metrohm, Switzerland) equipped with a conductivity detector. 1 mL samples was diluted with milli-Q water for analysis.

3.4. TSS AND VSS

Total suspended solids (TSS) and volatile suspended solids (VSS) were determined according to Standard Methods 2540D and E (APHA, 1997). These test were performed in triplicate. A volume of 5 mL for every triplicate was taken for analysis.

3.5. PH

As stated before the pH during the experiments was 6.5 ±7.5. The pH was measured with a digital pH meter (744 pH meter, electrode 6.0228.020 Primatrode, Metrohm). Measurement for the bioreactor was only necessary in stage 1. For stage 2, pH controllers were installed.

3.6. ELECTRICAL CONDUCTIVITY

The EC was measured with a temperature controlled (20 °C) multi analyzer (C833 multi channel analyzer, CONSORT) and an EC electrode (6.0912.110 conductivity measuring cell c= 0.8 cm^{-1}).

3.7. DISSOLVED OXYGEN

The DO was measured with a portable DO meter (HQ40d, HACH).

3.8. PROTEIN CONCENTRATION

Protein concentration was determined with the Lowry method (1951). Bovine serum albumin was used as a standard. This was done for suspension and beads. The beads were cut in little peaces with a scalpel in order to improve the extraction. For every Lowry determination, 1 mL of suspension was used and 3 to 4 beads. All tests were performed in triplicate.

3.9. SLUDGE VOLUME INDEX

The Sludge Volume Index (SVI) is the volume of sludge occupied by 1 gram of suspension after 30 minutes of settling (Rabaey, 2014). This parameter was determined weekly for stage 1 and 2 (day 6, 13, 20, 27, 34, 41 and 48 of reactor operation) and for stage 3 (day 0, 2, 7 12 of reactor operation). A volume of 200 mL was taken from the reactors. The suspension was introduced in a cone shaped graduated cylinder. The volume of settled sludge was recorded after 5 minutes and after 30 minutes. The following formula was used to calculate the SVI:

$$SVI(mL\ g^{-1}) = \frac{Settled\ Volume(mL)}{TSS(g)} \quad \text{(Equation 13)}$$

4. STATISTICS

Analysis of covariance was used to compare the significance of salinity effect. A significance level of 0.05 was chosen. Linearity of the regression, normality of the residuals and homogeneity of error variances was verified. Normality of the residuals was statistical analyzed with a Shapiro-Wilk normality test. The software package R (version 2.13.2) for Mac OS X was employed for the statistical analyses (R Core Team, 2013). An overview of the script is shown in the Appendix. Wilcoxon signed-rank test, a non – parametric statistical hypothesis test, was used if the hypothesis of normality was rejected.

5. CALCULATIONS

5.1. VOLUMETRIC RATE

High throughput ammonium and nitrite analysis was performed to determine the volumetric conversion rate of AOO and NOB at the different salinities. **Figure 18** is an example of the analyzed results of a batch experiment. A slope can be calculated by linear regression. This slope represents the volumetric rate expressed in mg N L^{-1} h^{-1}. The specific rate could be calculated by dividing the volumetric rate by the VSS concentration. All batch tests were performed in triplicate (three Erlenmeyers). Other figures for the determination of the volumetric conversion rate are shown in the Appendix.

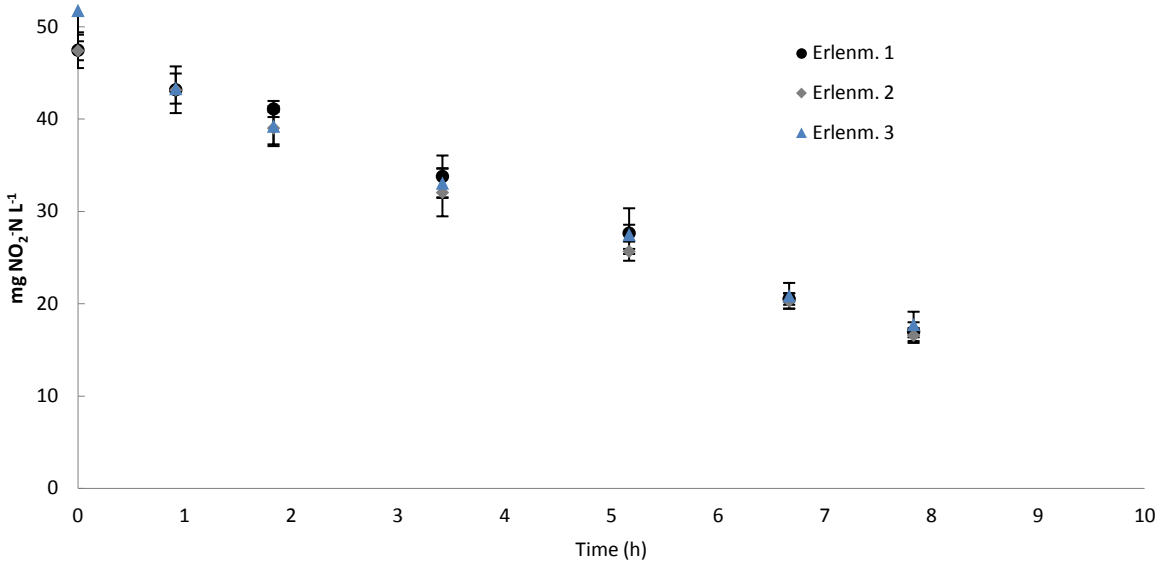


Figure 18: Result batch experiments.

The NOB data originates from the sludge OLAND labscale reactor- Ghent. The EC during this experiment was 46.6 mS cm^{-1}.

5.2. INHIBITION PERCENTAGE

An overview of the calculated specific rates determined from the batch experiments are shown in **Table 19**.The corresponding difference in EC between the salinity (expressed in mS cm^{-1}) of nitrified urine conditions (EC 45 mS cm^{-1}) and the salinity at which the sludge was adapted to (ΔEC) (salt shock) is also shown. The inhibition percentage for AOO and NOB was calculated as follows:

$$Inhibition\ \% = 1 - \frac{Activity_{SSA}}{Activity_{NC}} \quad \text{(Equation 14)}$$

SSA= Salinity at which the sludge is adapted to
NC= Nitrified urine conditions

5.3. INDICATION FACTOR

The indication factor can be calculated by the following formula:

$$Indicator\ factor\ (\%) = \frac{\mathrm{Re}\,lative\ rate_{SSA} - \mathrm{Re}\,lative\ rate_{NC}}{\mathrm{Re}\,lative\ rate_{SSA}} \text{ (Equation 15)}$$

SSA= Salinity at which the sludge is adapted to
NC= Nitrified urine conditions

5.4. AMMONIUM REMOVAL AND NITRIFICATION EFFICIENCY

The ammonium removal efficiency and the nitrification efficiency was calculated as follows:

$$NH_4^+\ removed\ (\%) = \frac{NH_4^+ Influent - NH_4^+ Effluent}{NH_4^+ Influent} \text{ (Equation 16)}$$

$$Nitrification\ efficiency\ (\%) = \frac{NO_3^- Effluent - NO_3^- Influent}{NH_4^+ Influent - NH_4^+ Effluent} \text{ (Equation 17)}$$

5.5. SRT

The SRT was calculated as follows:

$$SRT = \frac{V\ .\ X}{(V\ .\ \frac{\Delta X}{\Delta t} - Qw.\ X)} \text{ (Equation 18) (Rabaey, 2014)}$$

Qw= Waste sludge = 1.14 L d^{-1}
X= Biomass concentration (gVSS L^{-1})
V= Volume reactor (L)
t= time (d)

5.6. ARRHENIUS EQUATION

$$N - Load_{20°C} = N - Load_T \vartheta^{(20°C - T)} \text{ (Equation 19)}$$

T= temperature of operation at company reactor expressed in °C
ϑ= 1.23 = Temperature sensitivity for nitrification (Henze, 2008)

PART 3: RESULTS

1. BATCH EXPERIMENTS

1.1. EFFECT OF SALINITY ON THE ACTIVITY OF AOO AND NOB FOR DIFFERENT SLUDGE TYPES

The goal of this research stage was to assess the effect of salinity on the activity of AOO and NOB for different sludge types. With the results obtained in this part of the research, a selection could be made of a suitable nitrifying sludge that has a low inhibition towards salinity for both AOO and NOB activity and performs well at the desired reactor conditions i.e. temperature around 20°C and pH 7. Twelve sludge types were tested originating from wastewater treatment, manure treatment, landfill leaching, marine aquaculture and seawater aquaculture adapted to different salinities (**Table 19**). All data presented here are co-data obtained together with Coessens (2015).

Table 19: Results batch experiments AOO and NOB.

Company-location	SSA	NC	ΔEC	AOO SSA activity	AOO NC activity	NOB SSA activity	NOB NC activity	AOO inhibition	NOB inhibition	Biomass concentration
	mS cm^{-1}	mS cm^{-1}	mS cm^{-1}	mgN g^{-1}VSS L^{-1}	mgN g^{-1}VSS L^{-1}	mgN g^{-1}VSS L^{-1}	mgN g^{-1}VSS L^{-1}	%	%	gVSS L^{-1}
Inagro – Rumbeke •	3	46	43	n.a.o.	n.a.o.	n.a.o.	n.a.o.	-	-	0.20±0.01
RWZI dokhaven - Nieuwveer •	3	44	41	59±2	n.a.o.	86±11	2.9±0.3*	100%	97%	1.73±0.12
Hooge Maey – Antwerp •	3	43	40	4±1	n.a.o.	18±2	15±2	100%	0%	0.82±0.03
Ugent shrimp tank-Ghent •	87	47	40	n.a.o.	n.a.o.	n.a.o.	n.a.o.	-	-	0.72±0.10
SeaLIfe – Blankberge (Piranha) •	3	42	39	19±2	n.a.o.	15±4	n.a.o.	100%	100%	0.13±0.05
Imog – Moen •	7	45	38	101±10	30±2*	131±20	56±3*	70%	57%	3.44±0.06
Decostere – Kortemark •	17	45	28	10±1	7.3±0.5*	6±1	10±1*	30%	-55%	0.40±0.02
DANIS NV – Izegem •	18	45	27	26±6	6±3*	8±3	7±4	77%	0%	0.41±0.01; 0.39±0.03**
Avecom – Wondelgem	23	48	25	157±27	149±20	555±18	454±20*	0%	18%	0.66±0.02
DOW – Terneuzen •	62	44	18	n.a.o.	n.a.o.	4.3±0.3	5.3±0.3*	-	-23%	0.62±0.03
OLAND Labscale reactor - Ghent	37	47	10	124±11	114±6	n.a.o.	n.a.o.	0%	-	0.65 ±0.02
Sealife - Blankenberge (Octopus) •	46	46	0	n.a.o.	n.a.o.	n.a.o.	n.a.o.	-	-	0.05±0.00

*Significant difference between activities

**Biomass concentrations for AOO and NOB tests were different

** The actual salinity at which the sludge was adapted to was 23 mS cm^{-1}. However it is distributed at 3.44 mS cm^{-1}. The experiments were performed at 23 mS cm^{-1}.

n.a.o. = no activity observed

Results in **Table 19** are ranked from high to low salt shock (ΔEC). AOO and NOB were tested at two salinities: salinity at which the sludge was adapted to (SSA) and nitrified urine conditions (NC). Analysis of covariance was used as statistical method to compare the salinities of a specific sludge type for two salinities. A significance level of 5% was used. The actual EC values during the test are shown in the table. An inhibition percentage of 0% was chosen if the activities were not significant different ($p > 0.05$). Biomass concentrations during the test are shown in the last column. Inagro, Sealife and Ugent shrimp tank were spiked with a lower concentration than the other sludge types. Markings indicate the origin of the sludge: • aquaculture, • wastewater treatment • Landfill and • manure treatment. Two sludge types are not marked: Avecom (aquaculture inoculum) and OLAND labscale reactor.

As shown in Table 19, not all sludge types had AOO or NOB activity at the different tested salinities. During the high throughput analyses, the volumetric rate was sometimes smaller than 1 mg N L^{-1} d^{-1}. Slightly negative volumetric rates were also observed. The standard error of the high throughput method is higher than 1 mg N L^{-1} d^{-1}. That is why no activity could be determined for these experiments. The highest AOO activity and NOB activity for both salinities was from the ABIL nitrifying sludge from Avecom. The least inhibited sludge for AOO was the sludge from OLAND labscale reactor and the sludge from Avecom – Wondelgem. For NOB no inhibition occurred for DANIS NV – Izegem and for Hooge Maey - Antwerp. Negative inhibitions and thus increased activity observed at nitrified conditions were measured for DOW – Terneuzen and Decostere – Kortemark. Only the NOB inhibition column had negative values.

Table 20 is a representation of the data from the experiments and data provided by the companies. All batch experiments were performed at 20°C. Company data needed to be adjusted because growth rates and substrate uptake rates are influenced by temperature according to the Arrhenius equation (see subsection 5.6). All data were recalculated based on the experimental temperature of 20 °C.

Table 20: AOO activity results from batch experiments and company data.

Company location	AOO SSA activity (Experiments)	Specific N-load (Company)	Corrected Specific N-load (Company)	Temperature
	mg NH_4^+-N g^{-1}VSS d^{-1}	mg NH_4^+-N g^{-1}VSS d^{-1}	mg NH_4^+-N g^{-1}VSS d^{-1}	°C
Ugent shrimp tank-Ghent •	n.a.o.	n.d.	n.d.	28
DOW – Terneuzen •	n.a.o.	6	7	23-28
Sealife - Blankenberge (Octopus) •	n.a.o.	n.d.	n.d.	15
OLAND Labscale reactor - Ghent	124±11	133	158	26
Decostere – Kortemark •	11±1	n.d.	n.d.	n.d.
DANIS NV – Izegem •	26±6	41	49	26-27
Avecom – Wondelgem	70±4	230-350	273-415	24
Hooge Maey – Antwerp •	4±1	n.d.	n.d.	25-30
Imog – Moen •	101±10	17	20	20-30
RWZI dokhaven - Nieuwveer •	59±2	37	44	14
Inagro – Rumbeke •	n.a.o.	36	43	22
SeaLIfe – Blankberge (Piranha) •	19±2	n.d.	n.d.	20

n.a.o.= no activity observed

n.d.: no data

SSA= Salinity at which the sludge was adapted to

The companies only provided N-loads. To compare the data, the specific N-loads are regarded as maximum activity of AOO. N-loads are correct with the Arrhenius equation. Markings indicate the origin of the sludge: • aquaculture, • wastewater treatment • Landfill and • manure treatment. Two sludge types are not marked: Avecom (aquaculture inoculum) and OLAND labscale reactor (partial nitrification denitrification).

No activity could be observed for AOO and NOB from the sludge Ugent shrimps tank-Ghent (**Table 19**). This sludge was used to a pH of 8.2 and a temperature of 28°C whereas during the experiment the pH was 7.0 en the temperature 20°C (all company data is shown in **Table 12**). Absence of activity was also observed for Sealife-Blankenberge (Octopus) sludge and the Inagro-Rumbeke sludge (**Table 19**). The former (Octopus) also encountered a pH (ΔpH = 1.2) and temperature (ΔT = 5°C) difference relatively to the original conditions. Both sludge types had initially a biofilm structure. The biomass concentration of Inagro-Rumbeke was relatively low (0,05 ± 0,00 gVSS L^{-1}) during the batch experiments. Inagro-Rumbeke sludge was adapted to high pH values (ΔpH = 1.5) and the temperature difference between original conditions and experimental conditions was 2°C.

The wastewater treatment sludge coming from DOW-Terneuzen did not show any AOO activity, yet NOB activity was present for the two tested salinities (**Table 19**). The data provided by DOW indicate that the sludge had AOO activity (7 mg $N\text{-}NH_4^+$ g^{-1}VSS d^{-1}) prior to sampling. The experiments for this sludge were repeated two times. However there was no AOO activity observed for both experiments at the conductivity the sludge was used to. This sludge was spiked with 60 mg N L^{-1} during the experiment. The biomass concentration was 0.62 ± 0.03 g VSS L^{-1}. Hence, the specific N-load was 98 mg $N\text{-}NH_4^+$ g^{-1}VSS d^{-1}. This is much higher than the specific N-load (company data) at which the sludge was adapted to.

No NOB activity could be observed for the OLAND labscale reactor - Ghent. This is a logic observation because the labscale reactor was operated at partial nitrification combined with anaerobic ammonium oxidation, where nitrite oxidation is not desired. This sludge was gradually adapted to an increased salinity (0-60 mS cm^{-1}) during the operations in the OLAND labscale reactor. No AOO inhibition was observed at a high conductivity (45 mS cm^{-1}). The sludge encountered a salt shock of 9.2 mS cm^{-1}. The specific N-load (company data) is higher than the AOO activity during the batch experiments (**Table 20**).

AOO activity of Decostere - Kortemark sludge was inhibited by 30%, yet the NOB activity increased at 45 mS cm^{-1}. The DOW-Terneuzen sludge also had an increased NOB activity at a high salinity (45 mS cm^{-1}). A negative inhibition percentage (thus an increased activity at high salinity conditions) was not detected in any of the batch sludge tests for AOO. Decostere – Kortemark sludge and Danis NV was sludge originating from a pig manure treatment. The Danis sludge experienced a salt shock (increase of 27.40 mS cm^{-1}). The AOO activity rate was 77% inhibited while the NOB were not inhibited. N-load provided from the company data shows that the AOO activity during the test was lower.

The activity during the batch test with RWZI Dokhaven sludge was higher than the activity data obtained from the company (**Table 20**). The temperature during the batch experiment (20°C) was 6°C higher than the temperature the sludge was adapted to. Moreover, the pH was higher (ΔpH = 0.6) during the batch experiments. This difference in pH and temperature did not affect the sludge activity. The sludge RWZI dokhaven – Nieuwveer was strongly inhibited by in an increase in salinity (ΔEC = 41 mS cm^{-1}) for both AOO and NOB: 100% and NOB 97% respectively. The salt shock could also have affected the sludge coming from SeaLIfe – Blankberge (Piranha). The salt shock for this sludge type was (ΔEC = 39 mS cm^{-1}) as was the shock endured by RWZI dokhaven-Nieuwveer.

For the Hooge Maey – Antwerp sludge originating from a landfill leachate treatment plant, the AOO activity was 100% inhibited at high salinity conditions. The NOB activity did not change. The sludge experienced a temperature difference (ΔT = 5-10°C) during the experiments.

The activity of Imog – Moen sludge was five times higher than the activity provided by the company (**Table 20**). However the activity at 45 mS cm^{-1} was strongly inhibited. A 70% inhibition occurred for the AOO and 57% for the NOB. The salt shock was a 36.16 mS cm^{-1}.

The Avecom sludge experienced a salt shock of 25 mS cm^{-1} (ΔEC), yet the AOO activity was not inhibited and the NOB activity was inhibited by 18%. Of all the different sludges tested, the Avecom sludge had the highest NOB activity and AOO activity for both high salinity conditions and original salt conditions.

A Graphical representation of AOO and NOB activity for the two EC conditions is illustrated in **Figure 19** (AOO) and **Figure 20** (NOB).

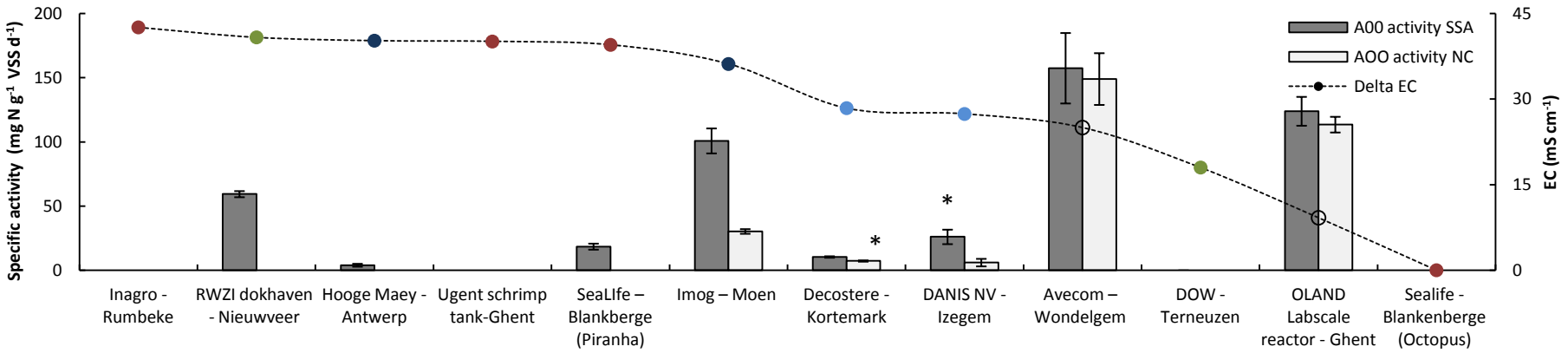


Figure 19: Specific activities for AOO at two salinities.

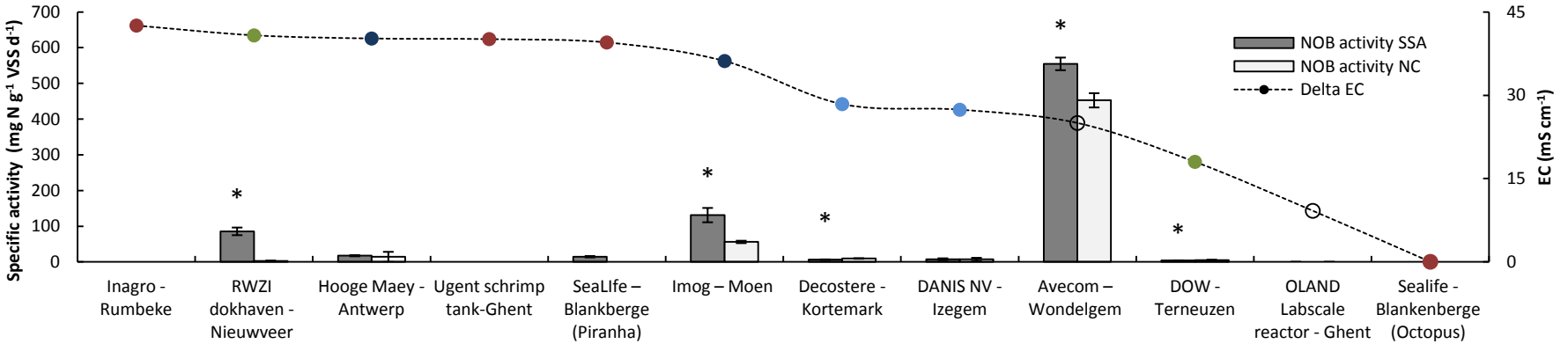


Figure 20: Specific activities for NOB at two salinities.

An overview of the specific rates (AOO and NOB) tested at two salinities: salinity at which the sludge was adapted to (SSA) and nitrified urine conditions (NC). There was no significant difference between the SSA and NC activities for Hooge Maey- Antwerpen and Danis NV-Izegem. Significance is marked with *. OLAND labscale reactor was operated at partial nitrification denitrification. Markings indicate the origin of the sludge: • aquaculture, • wastewater treatment • Landfill and • manure treatment.

Table 21 represents the relative rates calculated for the two salinity experiments for all different tested sludge types. The formula for the indication factor can be found in subsection 5.3 PART 2.

Table 21: Relative rates calculated for two salinities and an indicator factor.

Company-location	Relative rate SSA(NOB AOO^{-1})	Relative rate NC (NOB AOO^{-1})	Indication factor (%)
Inagro – Rumbeke •	***-	***-	-
RWZI dokhaven - Nieuwveer •	1.4	**_	-
Hooge Maey – Antwerp •	4.3	*-	-
Ugent shrimp tank-Ghent •	***-	*-	-
SeaLIfe – Blankberge (Piranha) •	***-	***-	-
Imog – Moen •	1.3	1.8	-42%
Decostere – Kortemark •	0.6	1.3	-121%
DANIS NV – Izegem •	0.3	1.2	-300%
Avecom – Wondelgem	3.5	3.0	14%
DOW – Terneuzen •	***-	*-	-
OLAND Labscale reactor - Ghent	0.0	**-	-
Sealife - Blankenberge (Octopus) •	***-	***-	-

Relative rates could not be determined for: *Only NOB activity observed; ** only AOO activity observed and *** no AOO or NOB activity.

Markings in **Table 21** indicate the origin of the sludge: • aquaculture, • wastewater treatment • Landfill and • manure treatment.

The relative rate was greater than one for most sludge types. For these sludge types, the NOB activity was higher than the AOO activity. This was not the case for DANIS NV – Izegem, Decostere – Kortemark and SeaLIfe – Blankberge (Piranha).

An indicator factor could be calculated for Avecom-Wondelgem, Decostere-Kortemark and DANIS NV-Izegem. This indicator points out whether AOO or NOB performed better at high salt conditions during the batch experiments. A negative values of the indicator factor implies that the AOO activity decreased more than the NOB activity, the NOB activity increased more than the AOO activity, the NOB activity did not change and the AOO activity decreased or the NOB activity increased and the AOO activity did not change.

The relative rate was greater than one for most sludge types. For these sludge types, the NOB activity was higher than the AOO activity. This was not the case for DANIS NV – Izegem, Decostere – Kortemark and SeaLIfe – Blankberge (Piranha).

1.2. SALINITY PROFILE ABIL AVECOM

During these experiments, the nitrification activity of the sludge from the company Avecom – Wondelgem was tested at four different salinities. These experiments were setup to have an indication about the effect of salinity on the nitrification activity of the sludge. An overview of the AOO and NOB inhibition percentages are shown in **Table 22**. Resuls of 23 and 48 mS cm^{-1} tests were also used for the sludge screening.

Table 22: Results salinity profile ABIL Avecom.

EC	Sodium chloride	ΔEC	AOO inhibition	NOB inhibition	Relative rate	Indicator factor
mS cm^{-1}	(g L^{-1})	mS cm^{-1}	%	%	NOB AOO^{-1}	%
3	0	20	44	0	6.2	-43%
23	5.6	0	-	-	3.5	-
34	9.4	11	0	0	4.1	-10%
48	11.3	25	0	18	3.0	51%

* A negative values of the indicator factor indicate that the AOO activity decreased more than the NOB activity, the NOB activity increased more than the AOO activity, the NOB activity did not change and the AOO activity decreased or the NOB activity increased and the AOO activity did not change.

Data in **Table 22** are ranked from high to low salinity. The sludge was adapted to an EC of 23 mS cm^{-1}. The inhibition percentage for AOO and NOB, relative rates and indicator factor was also determined. The inhibition percentage and the indicator factor were weighed with the results of 23 mS cm^{-1}. All test were performed on the same day. Results of 23 and 48 mS cm^{-1} were also used for batch screening.

AOO and NOB activities are graphically illustrated in **Figure 21**.

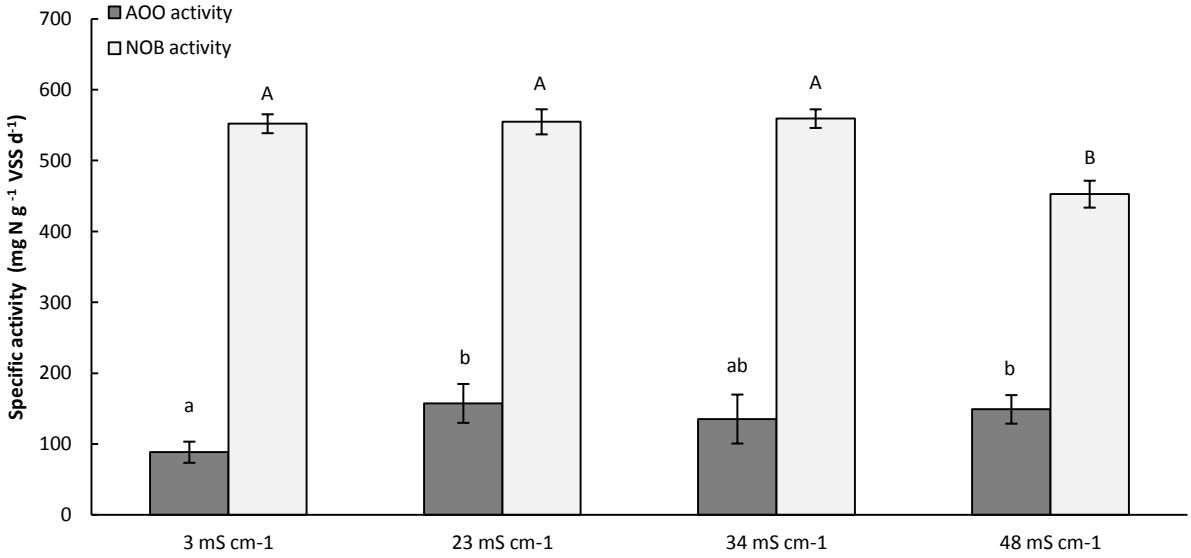


Figure 21: An overview of the AOO and NOB activity for Avecom sludge.

Activity for AOO and NOB at four salinities presented in **Figure 21**. Error bars show standard error. Significance comparison is shown separately for AOO (minuscule letters) and NOB (capital letters). Two activities were not significant different if the letter is the same (e.g. a at 3 mS cm^{-1} and ab at 34 mS cm^{-1} or ab at 34 mS cm^{-1} and b at 48 mS cm^{-1}) ($p>0.05$). Two activities were significant different, if the letters are different (e.g. A at 3 mS cm^{-1} and B at 48 mS cm^{-1} or a at 3 mS cm^{-1} and b at 23 mS cm^{-1}).

The AOO activities were smaller than the NOB activities for all salinities. The sludge was adapted to a salinity of 23 mS cm^{-1}. However it was delivered at an EC of 3 mS cm^{-1}.

2. MBMBR

2.1. REACTOR OPERATION STAGE 1 AND 2

During stage 1 and 2 of the experiments, the MBMBRs were operated at two electrical conductivities: 15 mS cm^{-1} and 45 mS cm^{-1}. In stage 1, the effluent was drown from the membrane (2.28 L d^{-1}) and in the second stage the bulk SRT of the reactor was decreased to 8 days by taking half of the effluent (1.14 L d^{-1}) by membrane permeation and the other half direct from the reactor (1.14 L d^{-1}). Stage 1 was operated for 30 days and stage 2 for 35 days. No biomass could be detected on the beads with the Lowry protein determination method for stage 1 and stage 2.

The EC profile during reactor operation is shown in **Figure 22**. The red line marks the separation between stage 1 and stage 2 of the experiments.

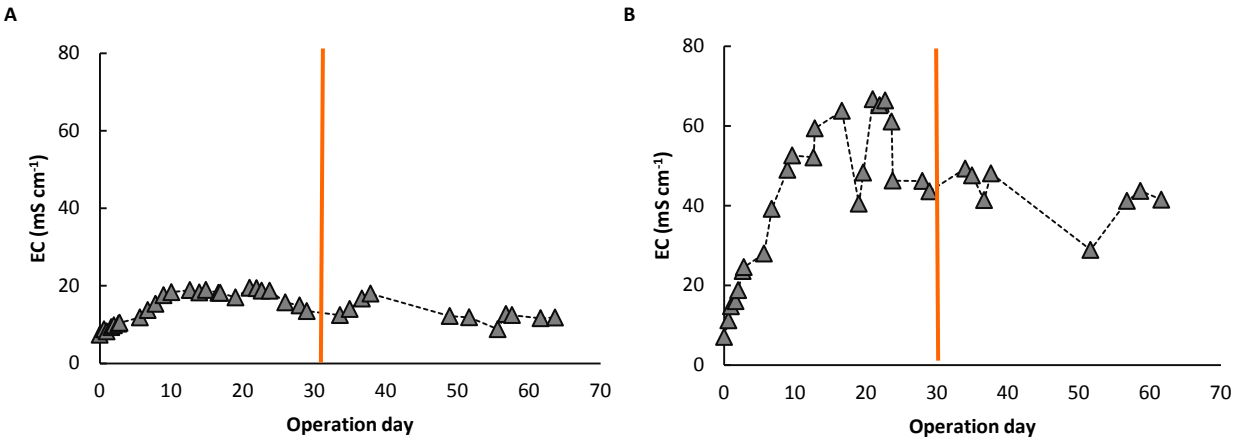


Figure 22: Electrical conductivity of low (A) and high (B) salinity reactor.

The initial EC of the reactors was 7.34 mS cm^{-1}. The target EC (15 and 45 mS cm^{-1}) for both reactors was achieved after 10 and 12 days respectively. This was higher than two times the HRT (8 days). The EC values of both bioreactors fluctuated during operation and the reactors were not operated at the target EC.

Sodium hydrogen carbonate and ammonium hydrogen carbonate was added as pH-buffering agent to cope with protons produced from nitrification. During operation carbon dioxide was stripped due to acidification and aeration (van Hulle et al., 2010). This lowered the EC of the reactor effluent relatively to the influent. That was why the EC was overcompensated by adding sodium chloride to anticipate this EC drop. This was done after 10 days of operation and can be observed in **Figure 22**. The EC of the effluent during day 10 and day 25 of operation was higher than the target values of 15 mS cm^{-1} and 45 mS cm^{-1}. As seen in **Figure 22**, too much sodium chloride was added.

pH controllers were installed after 30 days of operation. And thus no sodium hydrogen carbonate addition was necessary from that day on. Nota that ammonium hydrogen carbonate was still used as nitrogen source for the influent.

The EC suddenly drops at day 56 of operation for stage 1 and stage 2 (see **Figure 22**). Tap water was added to dilute the high ammonium concentrations in both bioreactors. That is why the EC decreased abruptly.

The pH profile during reactor operation is presented in **Figure 23** and **Figure 24** for the high and low salinity reactor respectively. The red line marks the separation between stage 1 and stage 2 of the experiments.

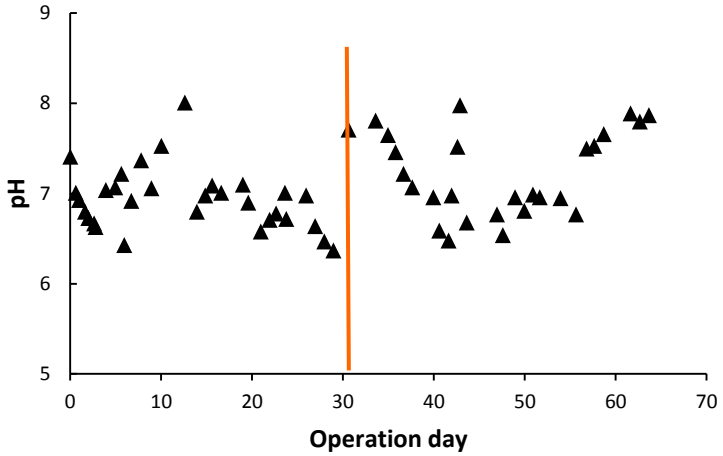


Figure 23: pH profile stage 1 and stage 2 of the low salinity reactor.

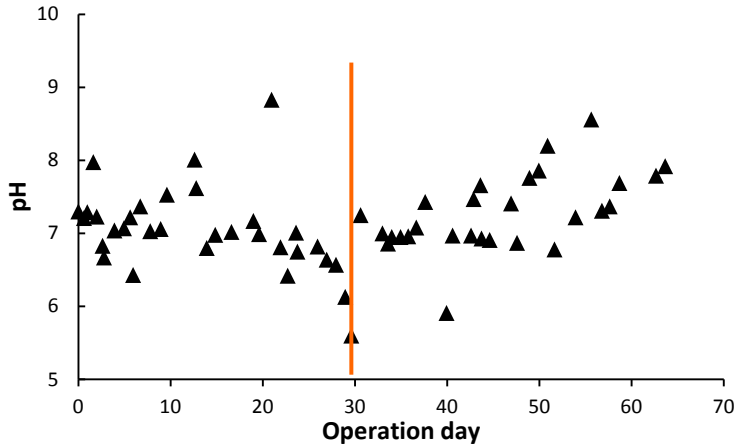


Figure 24: pH profile stage 1 and stage 2 of the high salinity reactor.

The pH fluctuated during the reactor operation. In stage 1 of the experiments, no pH controllers were installed. pH controllers were placed in stage 2. No acid was dosed to the reactor only base.

The calculated SRT for stage 2 of the reactor experiments is presented in **Figure 25**.

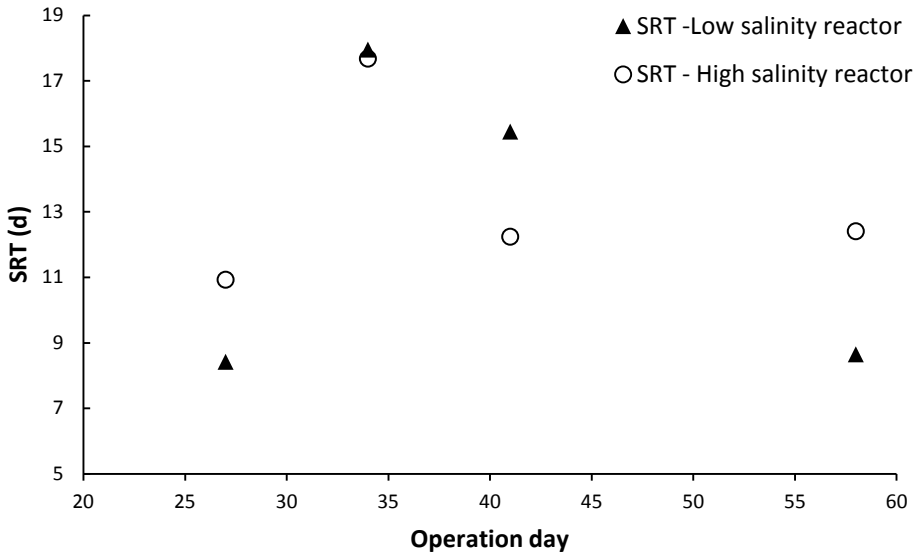


Figure 25: SRT for both reactors throughout stage 2.

The exact SRT values are calculated with the formula in subsection 5.5 PART 2.

An overview of the VSS and TSS ratio is shown in **Figure 26**. The red line marks the separation between stage 1 and stage 2 of the experiments.

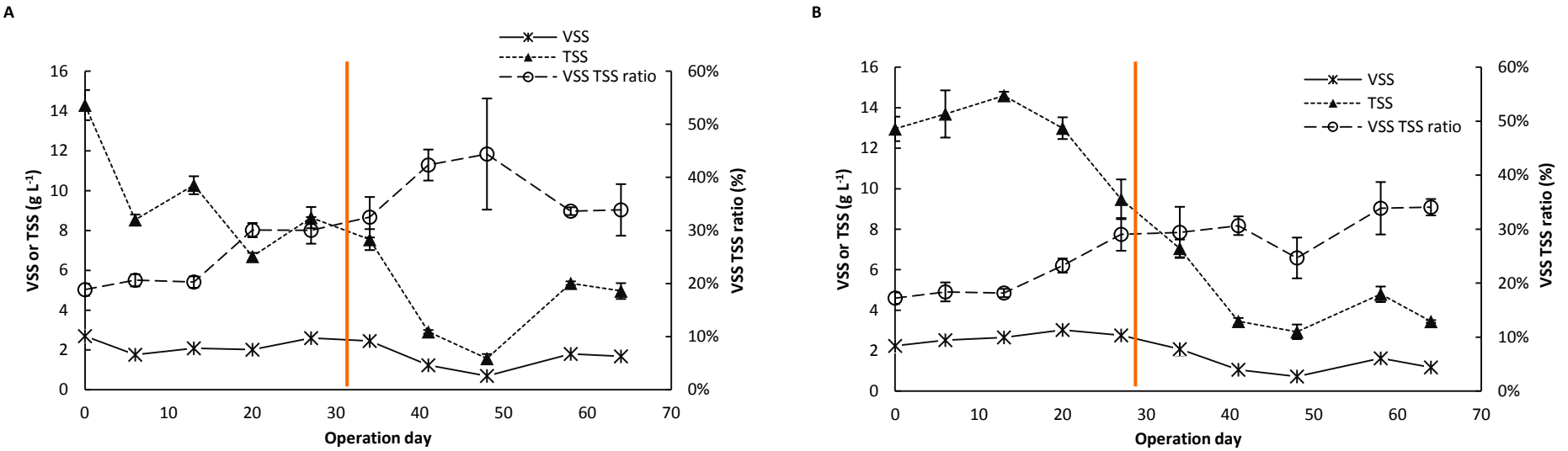


Figure 26: Volatile and suspended solids of MBMBRs for the low salinity reactor (A) and for the high salinity reactor (B).

The initial VSS concentration was 2.70 ± 0.08 g L^{-1} and 2.23 ± 0.09 g L^{-1} for the low salinity reactor and the high salinity reactor respectively. VSS over TSS ratio for both MBMBRs increased over time. This ratio was 44 % and 25 % for the 15 mS cm^{-1} and 45 mS cm^{-1} reactors respectively after 50 days of operation while the VSS over TSS ratio was initially 19% and 17% for the low and high salinity reactor respectively. The initial low VSS TSS ratio was due to calcium carbonate present in the inoculum (Grommen et al., 2002; Rombaut et al., 2003). During inoculation, the sludge had a white color. The color of the sludge changed over time. After a few weeks, a brownish color was observed. The low salinity reactor (15 mS cm^{-1}) had a TSS concentration of 14.30 ± 0.76 g L^{-1} at the beginning of the reactor operation. The initial TSS concentration of the high salinity reactor was 12.96 ± 0.60 g L^{-1}. After 20 days of operation the TSS concentration of the low salinity reactor was 6.70 ± 0.18 g L^{-1} and 12.99 ± 0.53 g L^{-1} for the high salinity reactor.

During the first 30 days of operation (stage 1), the bulk SRT was kept at infinite (HRT 4 days). No sludge was taken out of the reactor. The VSS concentration decreased during this period for the low salinity reactor compared to the initial condition. On the other hand, the VSS concentration of the high salinity reactor increased during this period. After 30 days of operation, the SRT was decreased to 8 days to favor microorganisms forming biofilms on the PVA beads. In this period, the VSS concentration and TSS concentration decreased in both bioreactors. In the low salinity reactor, the VSS and TSS concentration decreased by 28% and 53% respectively. In the high salinity reactor, the VSS and TSS concentration decreased by 35% and 58% respectively.

The last two data points in **Figure 26** are data from a new experiment. The suspension of both bioreactors was removed after the reactors crashed (high ammonium and no nitrogen removal anymore) on operation day 56. MBMBRs were re-inoculated with harvested sludge.

An overview of SVI-5 and SVI-30 is presented in **Figure 27**.

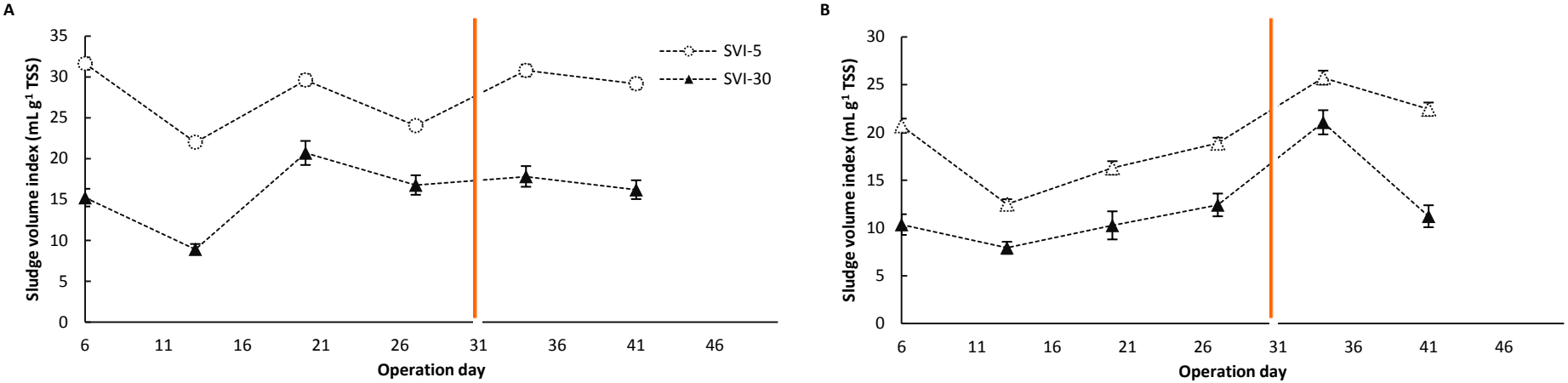
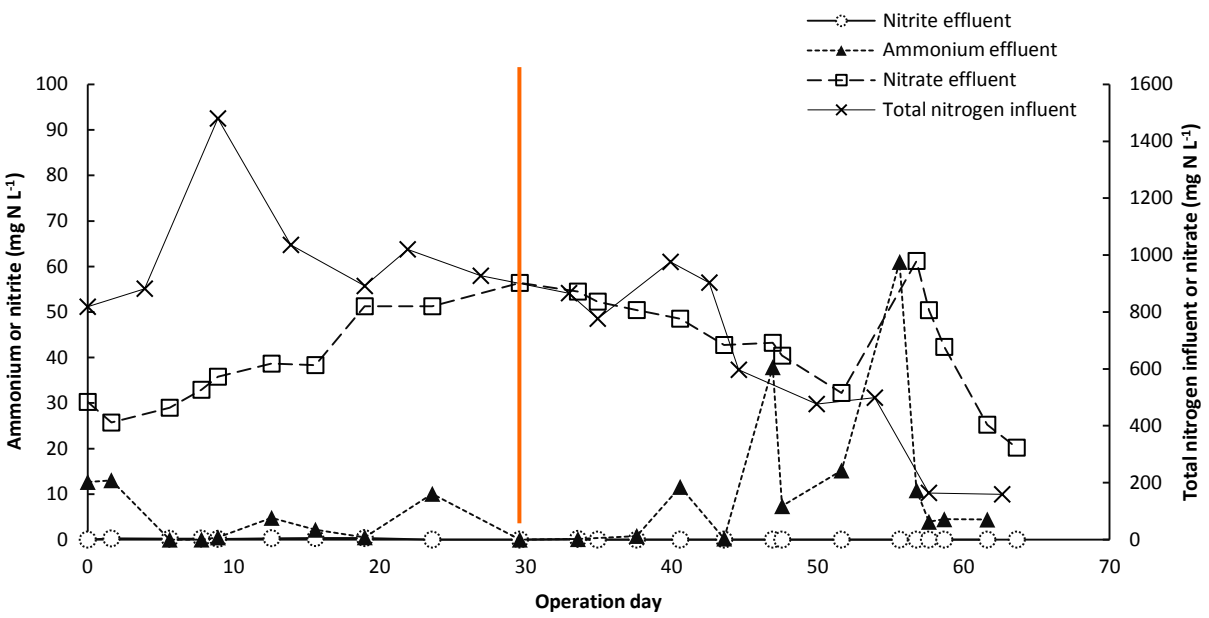


Figure 27: Sludge volume index 5 and 30 for low (A) and high (B) salinity reactors.

The SVI was first determined after 6 days of operation. The SVI of both reactors followed the TSS concentration pattern between 6 and 30 days of operation. SVI data of the low salinity bioreactor were always higher than the data of high salinity reactor except for operation day 34.

Figure 28 gives an overview of the nitrogen species in the effluent and the total nitrogen of the influent for both bioreactors.

A



B

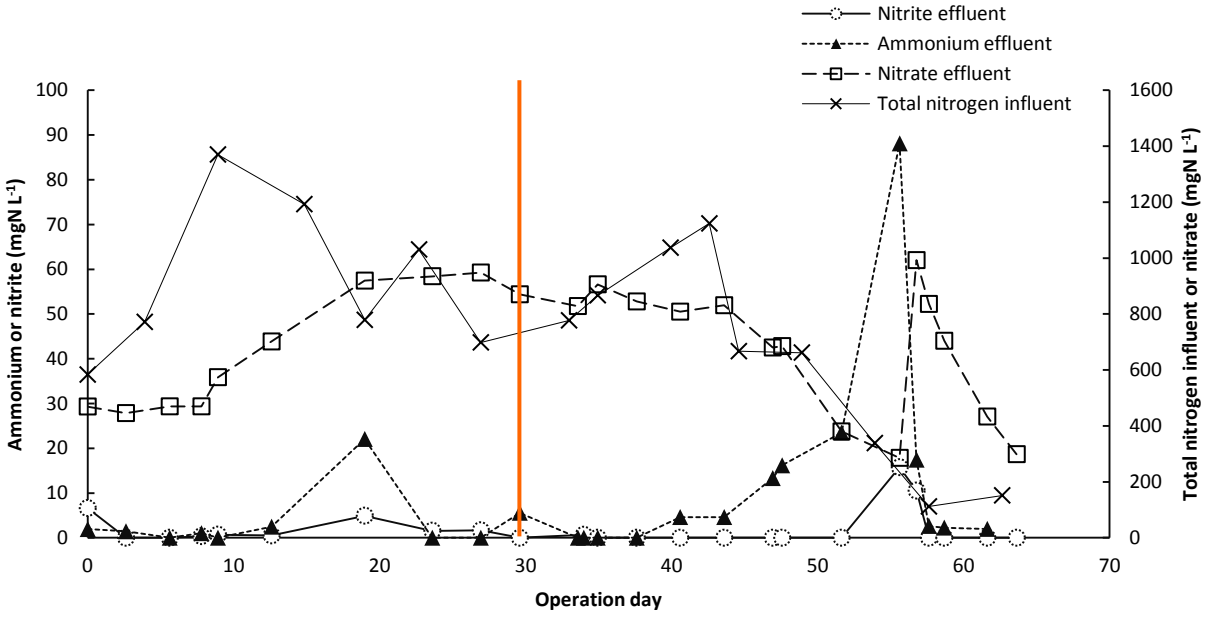


Figure 28: Nitrogen species in effluent and total nitrogen concentration in influent for Low salinity reactor (A) and high salinity reactor (B).

Ammonium and nitrite concentrations are expressed in the left axis and nitrate and the total nitrogen are expressed in the right axis. The red line marks the separation between stage 1 and stage 2 of the experiments. Nitrite in the effluent was always low for both MBMBRs.

Only after 58 days of operation, the nitrite concentration of the high salinity reactor increased to 17 mg N L^{-1}. The initial nitrate concentration was 483 mg N L^{-1} for the low salinity reactor and 469 mg N L^{-1} for the high salinity reactor. The sludge was activated with ammonium chloride for two weeks prior to inoculation. The

inoculum wasn't washed before the experiments. The total nitrogen loading was steadily increased for both reactors. The nitrogen load was decreased to avoid toxicity for stage 2 (SRT 8 days).

The ammonium concentration for the low salinity reactor showed four peaks after 24, 41, 47 and 56 days of operation. The ammonium concentrations during this period were: 10, 12, 38 and 61 mg N L^{-1} respectively. The first peak was due to a high dosage of sodium hydrogen carbonate and a corresponding rise in pH to 8. Sodium hydrogen carbonate had to be dosed to the influent because there were no pH controllers present for the first 30 days of operation. The second peak had an ammonium concentration of 10 mg N L^{-1}. This rise was the result of a broken timer that controlled the influent dosage. The last three peaks were the consequence of a high nitrogen load. The nitrogen load was not decreased properly when the floccular SRT was decreased. Thus due to a decrease in SRT, the VSS concentration decreased. However the specific nitrogen load increased. The high salinity MBMBR had a peak in ammonium and nitrite concentration on day 19 of reactor operation. This measurement was taken during dosage of the influent. All measurements were taken the morning right before the influent pumps started. The ammonium and nitrite concentration were 22 and 4 mg N L^{-1} respectively. There was also a peak in ammonium concentration on day 56 of operation. The ammonium concentration was 88 mg N L^{-1}. The nitrogen load was not decreased properly as explained before.

Both reactors crashed (high ammonium concentration and no nitrogen removal) after 56 days of operation. The ammonium concentration after 56 days was 88 mg N L^{-1}. Reactors were re-inoculated with harvested sludge.

The ammonium removal efficiencies and nitrification efficiencies are presented in **Figure 29**.

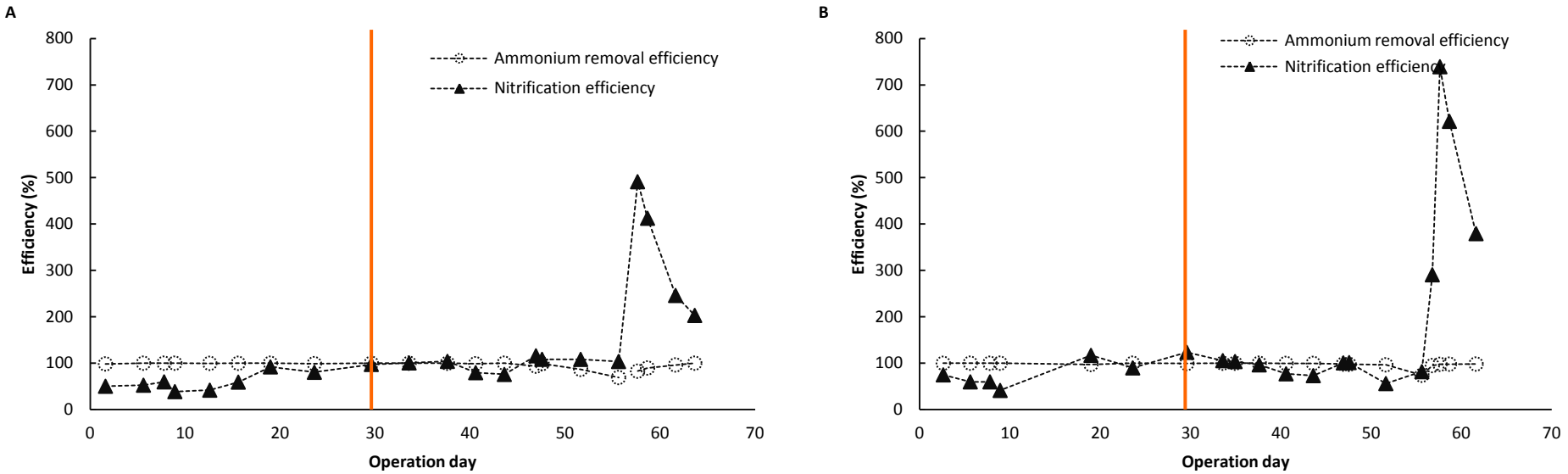


Figure 29: Ammonium removal efficiency and nitrification efficiency Low salinity reactor (A) and high salinity reactor (B).

The nitrification efficiency was lower than the ammonium removal efficiency for the first 20 days of operation. There was no build up of nitrite and the biomass concentration of the low salinity reactor even decreased. The nitrification efficiencies were higher than 100% for both bioreactors after 56 days of operation. The loading rate was decreased on that day. There were still no steady state conditions in both bioreactors. The ammonium concentration of the influent was low in comparison with the nitrate concentration of the effluent. The ammonium removal efficiency characterizes the removal of ammonium. The nitrification efficiency characterizes the conversion of ammonium to nitrate (see subsection 5.4).

Free ammonium and free nitrous acid is presented in **Figure 30**.

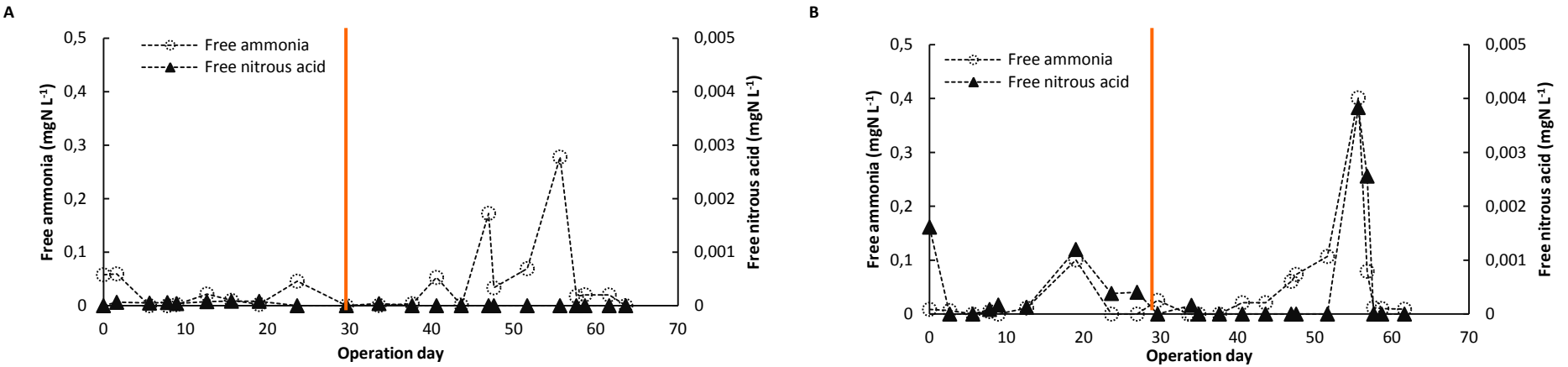
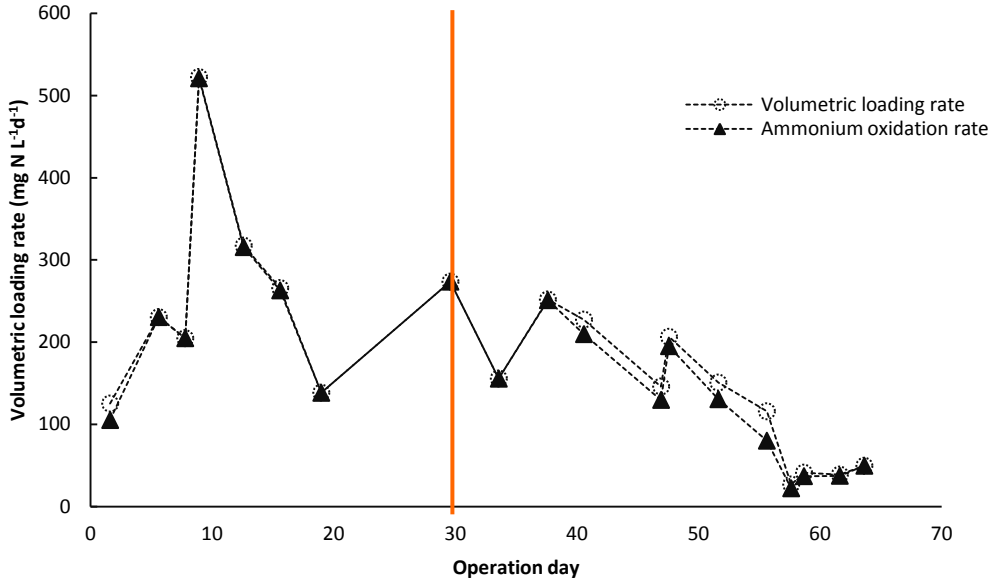


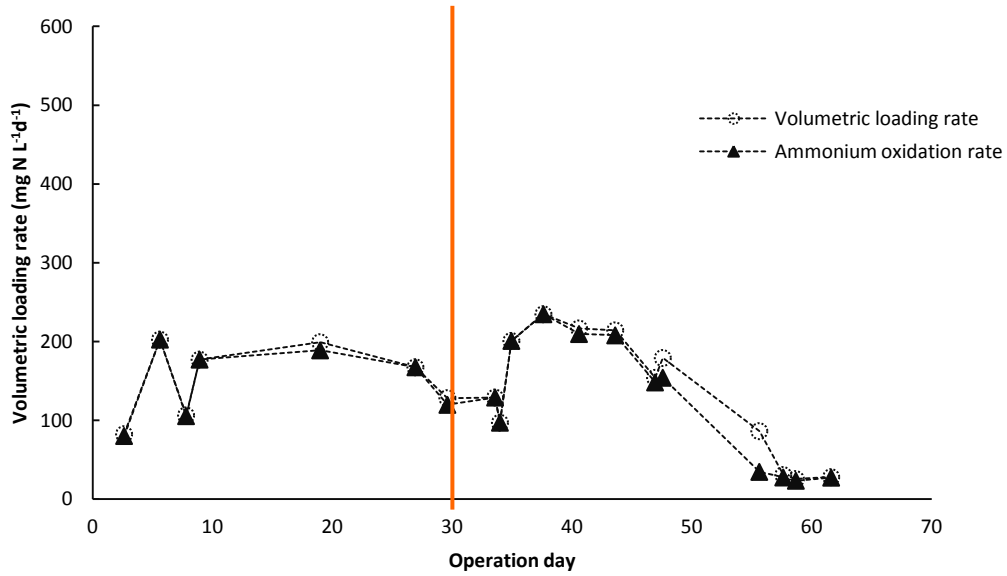
Figure 30: Free ammonium and free nitrous acid for the low salinity reactor (A) and high salinity reactor (B).

Free ammonium became inhibitory for NOB in both bioreactors at day 56 (see **Figure 30**). The free ammonium concentrations were respectively 0.28 and 0.40 mg N L^{-1} at that moment. There were no other inhibitory effects for AOB or NOB during reactor operation. Toxicity limits for AOB and NOB are discussed in the literature study (see 3.2.4 PART 1).

An overview of the volumetric loading rate and ammonium oxidation rate is shown in **Figure 31**.

A





B

Figure 31: Volumetric loading rate and ammonium oxidation rate for the Low salinity reactor (A) and high salinity reactor (B).

The volumetric loading rate was higher than the ammonium oxidation rate for both reactors after 56 days of reactor operation.

Microscopy has been performed for both reactors. An illustration of live/dead staining fom the low salinity reactor is shown in **Figure 32** (magnification 10X). Two light microscopy images are shown in **Figure 33** and **Figure 34**.

A B

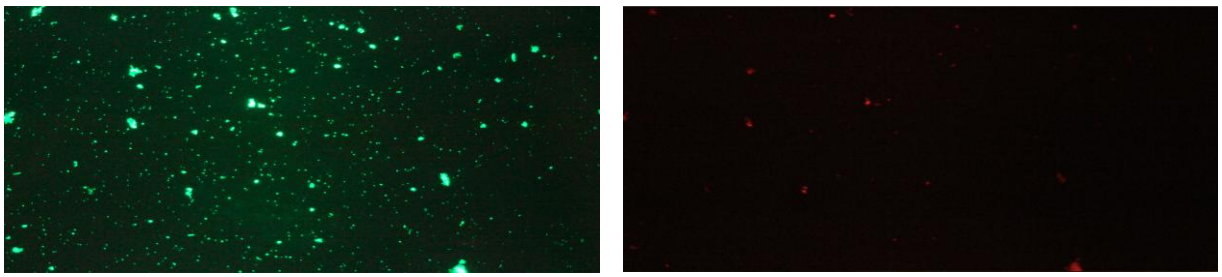


Figure 32: Microscopy live (A) dead (B) staining.

The big shaped clustering objects were not moving underneath the microscope. This could be a salt crystal originating from the influent. The smallest dots were moving around.

Two light microscopy images are shown in **Figure 33** and **Figure 34**.

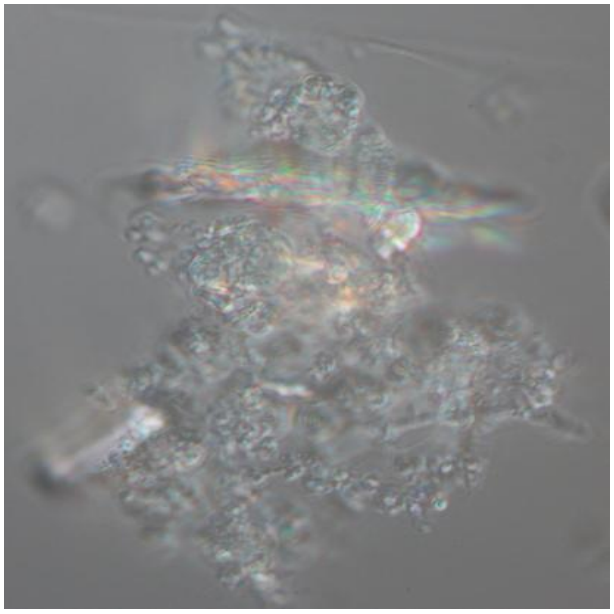


Figure 33: Light microscopy.

Figure 33 could be a salt crystal. The magnification is 100X. The sample was taken from the low salinity reactor.

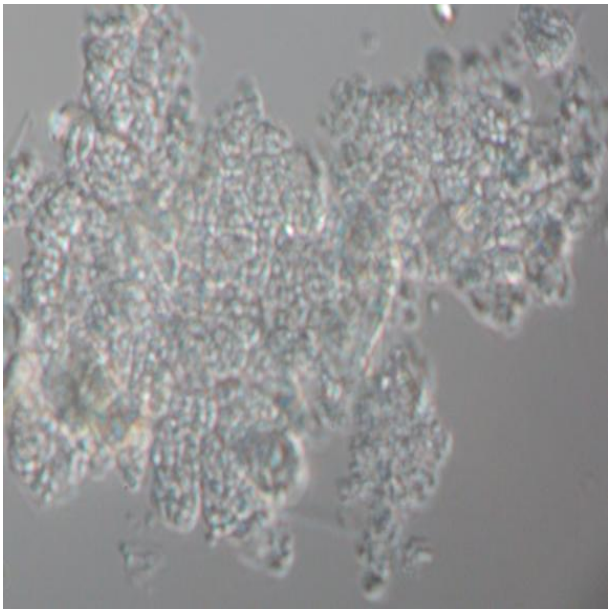


Figure 34: Light microscopy.

The **Figure 33** can be a salt crystal. Microorganisms look attached to this object. **Figure 34** shows a cluster of microorganisms. No morphological difference could be observed between both reactors.

2.2. MICRO SCALE BATCH EXPERIMENTS

These experiments were executed in 96 well plates. The main objective was to assess the AOO and NOB activity of the suspension and the beads. AOO and NOB activity was tested separately during the tests. Beads and suspension of both high and low salinity reactor (15 mS cm^{-1} and 45 mS cm^{-1}) were always tested at two salinities: 15 mS cm^{-1} and 45 mS cm^{-1}. This was done to have an indication of the adaption performance to salinity over time. The activities of the suspensions of the two operated reactors are shown in **Table 23**.

Table 23: Results of micro scale batch experiments for suspension.

Operation (d)	AOO activity (mg N g^{-1} VSS d^{-1})		NOB activity (mg N g^{-1} VSS d^{-1})		AOO activity (mg N g^{-1} VSSd^{-1})		NOB activity (mg N g^{-1} VSS d^{-1})	
	A	B	A	B	A	B	A	B
20	81±5	71±6	361±22	303±28	75±5	79±6	240±14	216±14
27	95±12	76±4	318±7	305±7	80±28	77±21	195±21	285±18*
34	59±2	59±3	339±45	310±42	69±9	64±7	281±11	293±30
41	38±10	34±7	311±21	207±14*	32±10	34±7	165±5	179±11
48	65±13	52±7	29±95	542±69*	49±12	35±7	439±43	517±44

*= Significant difference

A= 15 mS cm^{-1}

B= 45 mS cm^{-1}

The third and second column of **Table 23** are the AOO and NOB results of the reactor operating at 15 mS cm^{-1} and the last two columns are the AOO and NOB results of the 45 mS cm^{-1} reactor. Operation was the period at which the test was executed relative to the time of reactor operation. The reactors were operated at infinite bulk SRT during experiments of day 20, 27 and 34. For the last two experiments, the reactors were operated at 8 days SRT. The results of A and B were statistically analyzed with analysis of covariance. A significant level of 0.05 was chosen.

The results indicated that there was no significant difference between the two salinities for the AOO activity of both reactors. For the NOB, no significant difference in activity could be observed for the high salinity reactor on operation days 20, 27 and 34. The NOB activity on operation day 41 was significantly different for salinity A than for salinity B. This altered at operation day 48. The results for the NOB of the high salinity reactor showed that there was no significant difference in activity for salinity A or B except for operation day 27. The NOB activity was significant higher for salinity B (45 mS cm^{-1}) than for salinity A (15 mS cm^{-1}).

The carriers of both MBMBRs were also tested for AOO and NOB activity on two salinities. The standard deviation of the triplicates of the ammonium and nitrite standards was higher than 10 mg N L^{-1}. The decrease in concentration of the wells of the beads was smaller than 10 mg N L^{-1} in a time span of 28 hours. Increased concentration during the experiments was also observed. That was why only a few results were obtained for the test. These results are shown in **Table 24**. The Lowry protein determination method indicated that no biomass was present on the beads during the activity tests. A visual inspection (dissection of the beads) indicated that no biomass was present.

Table 24: Results of micro scale batch experiments for PVA carriers.

Operation (d)	AOO activity (mg N L^{-1} d^{-1})		NOB activity (mg N L^{-1} d^{-1})		AOO activity (mg N L^{-1} d^{-1})		NOB activity (mg N L^{-1} d^{-1})	
	A	B	A	B	A	B	A	B
20	n.a.c.d	n.a.c.d	n.a.c.d	n.a.c.d	n.a.c.d	n.a.c.d	n.a.c.d	n.a.c.d
27	n.a.c.d	n.a.c.d	n.a.c.d	n.a.c.d	0.35±0.08	n.a.c.d	0.15±0.05	0.15±0.3
34	0.25±0.06	0.15±0.06	n.a.c.d	n.a.c.d	0.15±0.08	n.a.c.d	n.a.c.d	n.a.c.d
41	0.25±0.04	n.a.c.d	n.a.c.d	n.a.c.d	0.19±0.04	n.a.c.d	n.a.c.d	n.a.c.d
48	n.a.c.d	n.a.c.d	n.a.c.d	n.a.c.d	n.a.c.d	n.a.c.d	n.a.c.d	n.a.c.d

n.a.c.d.= no activity could be determined

A= 15 mS cm^{-1}

B= 45 mS cm^{-1}

All activities for five micro scale experiments. The third and second column are the AOO and NOB results of the reactor operating at 15 mS cm^{-1} and the last two columns are the AOO and NOB results of the 45 mS cm^{-1} reactor. The reactors were operated at infinite floccular SRT during the test of operation day 20, 27 and 34. For the last two experiments, the reactors were operated at 8 days SRT. The results of A and B were statistically analyzed with analysis of covariance. A significant level of 0.05 was chosen. All activities are expressed per liter of reactor volume.

The 96 well plate of the micro batch experiments is presented in **Figure 15**. The results shown in **Table 23** are the data of the positive tests (bead + suspension).

2.3. REACTOR OPERATION STAGE 3

During these experiments the reactors were re-inoculated with sludge harvested from the previous experiments (stage 2). In this stage of the research the importance of a high divalent/monovalent cation ratio was tested. The divalent/monovalent cation ratio of one reactor was raised to 1.93 g Ca^{2+} g^{-1} Na^{+}. The MBMBRs were operated for 15 days. The HRT was 4 days. As in stage 2, sludge was taken out of the reactor (1.14 L d^{-1}). Membranes were also used to take out liquid (1.14 L d^{-1}). An overview of the EC profile for both reactors is presented in **Figure 35**.

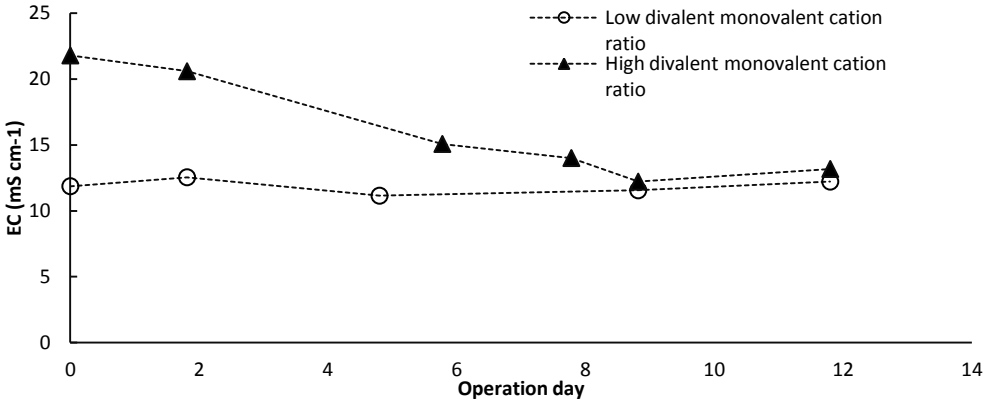


Figure 35: EC profile during reactor operation.

The reactor running on a low divalent/monovalent cation ratio had an initial EC of 11.87 mS cm^{-1}. The EC of the reactor with a high divalent/monovalent cation ratio was initially increased to 21.8 mS cm^{-1} by the addition of calcium chloride at the start of the reactor experiments (calcium was also dosed by the influent (see subsection 2.2 PART 2). After two HRTs, the EC of the MBMBR operating on a high divalent/monovalent cation ratio was stabilized to 11.56 mS cm^{-1}.

The pH profile during operation is presented in **Figure 36** and in **Figure 37**.

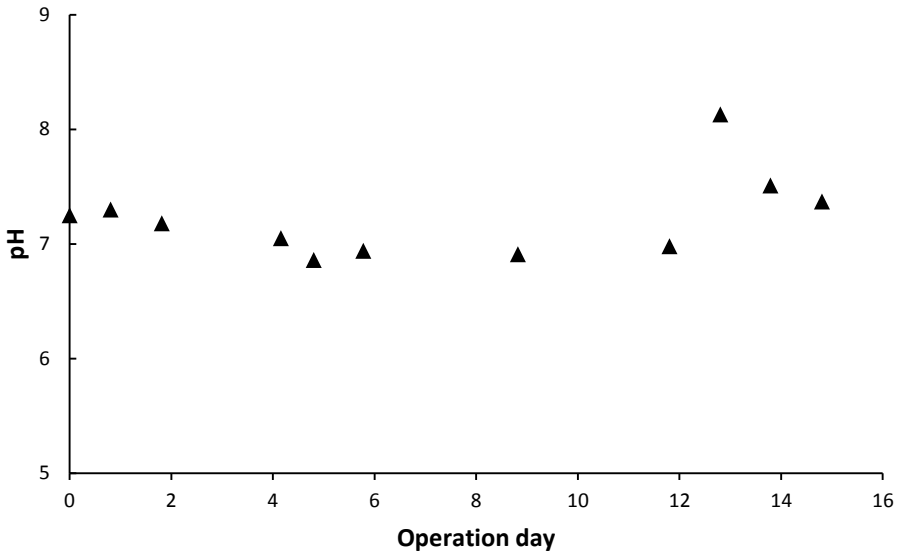


Figure 36: pH profile stage 3 reactor running on a low divalent/monovalent cation ratio.

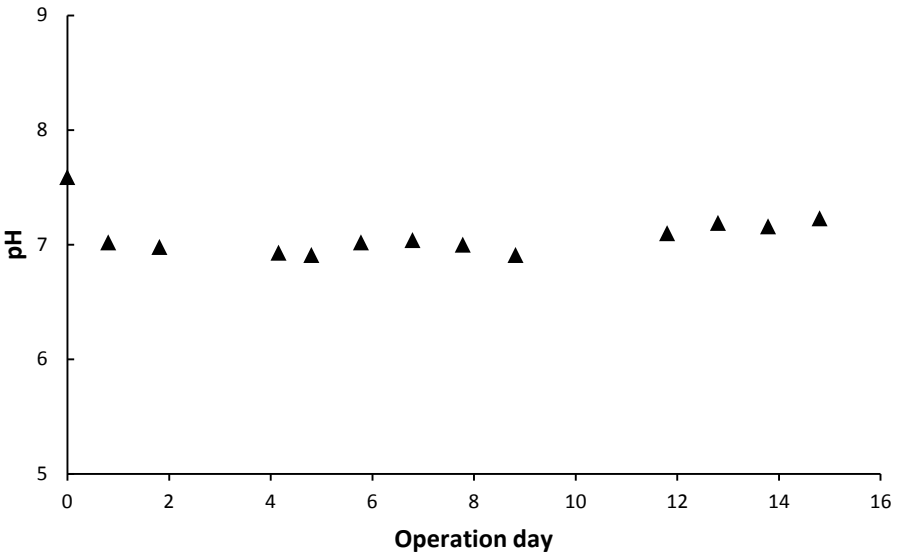
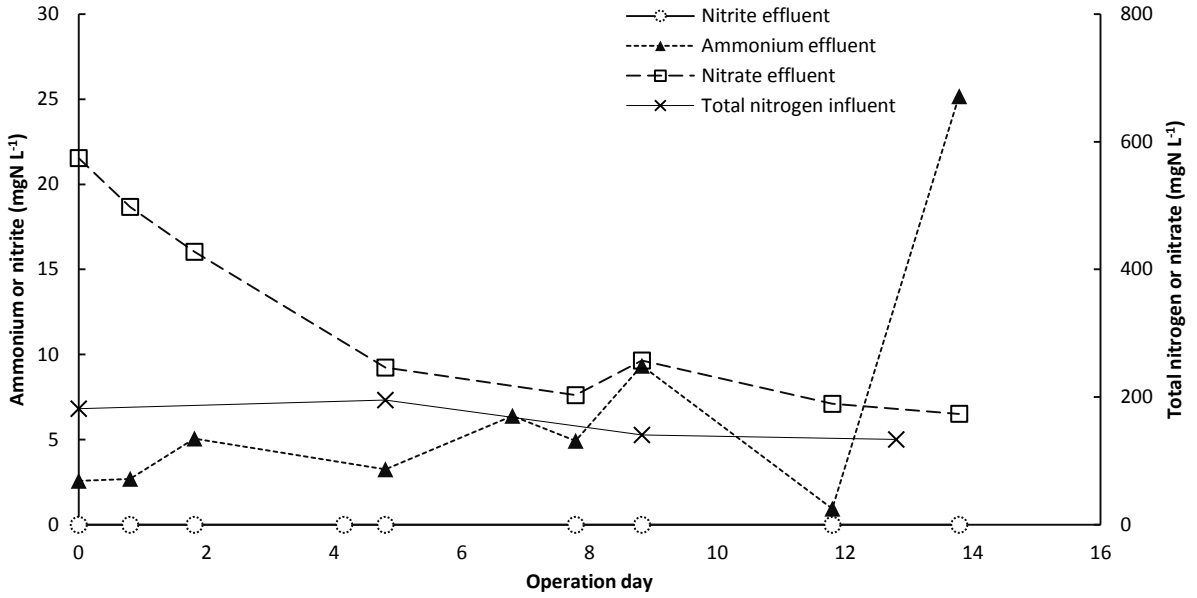


Figure 37: pH profile stage 3 reactor running on a high divalent/monovalent cation ratio.

An overview of the nitrogen species in the effluent en the total nitrogen of the influent is shown in **Figure 38**.

A



B

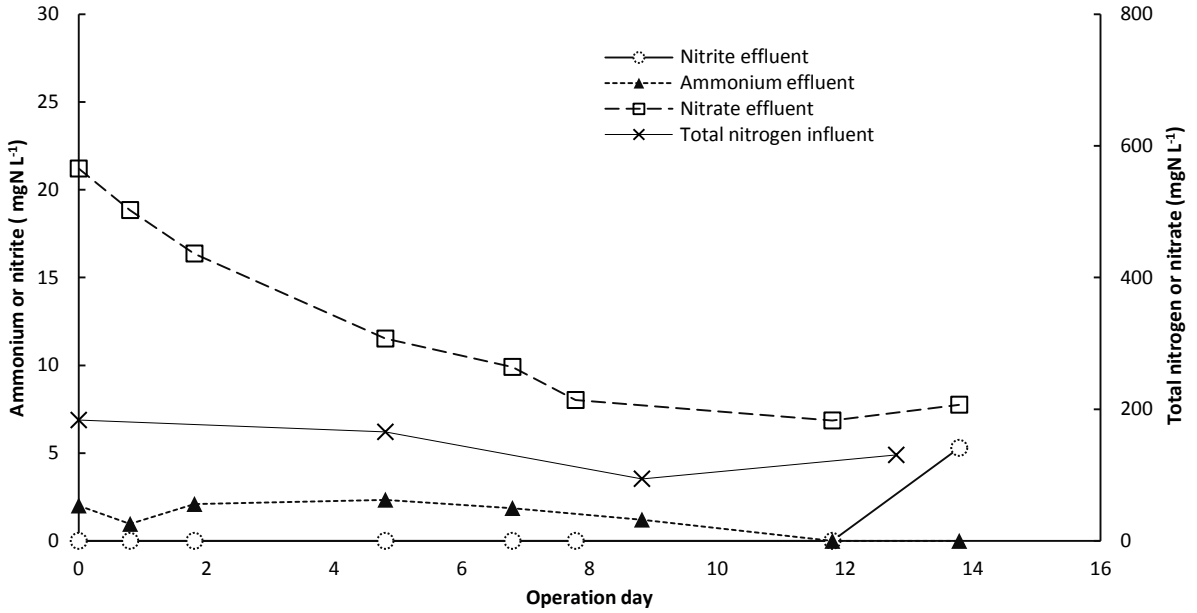


Figure 38: Nitrogen species in effluent and total nitrogen concentration in influent for reactor running on low divalent/monovalent cation ratio (A) and reactor running on high divalent/monovalent cation ratio (B).

The ammonium concentration was 25 mg N L^{-1} on day 15 in the reactor running on a low divalent/monovalent cation ratio. The reactor running on a high divalent/monovalent cation ratio had a high nitrite concentration (5 mg N L^{-1}) on the same day. The nitrate concentration was initially high (564-575 mg N L^{-1}). The sludge was not washed before inoculation. Total nitrogen concentration and nitrate concentrations converge for both reactors. There was still no steady state after two HRTs (8 days). The nitrite and ammonium concentrations in both MBMBRs were higher than zero during reactor operation. Only on day 12, the ammonium concentration decreased to zero in both bioreactors.

The VSS and TSS concentration is presented in **Figure 39**. SVI data are shown in **Figure 40**.

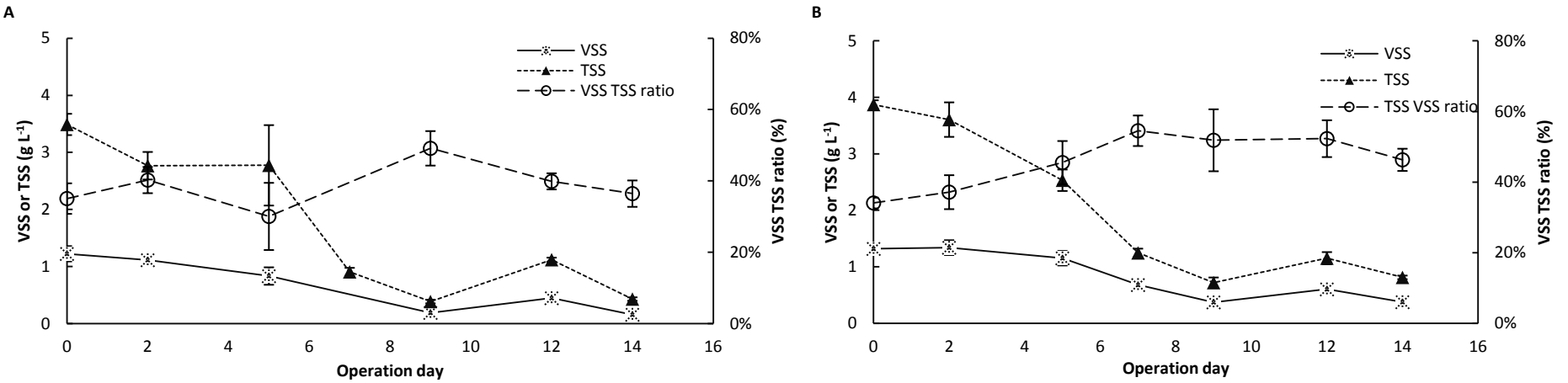


Figure 39: Volatile and suspended solids of MBMBRsfor reactor running on low divalent/monovalent cation ratio (A) and reactor running on high divalent/monovalent cation ratio (B).

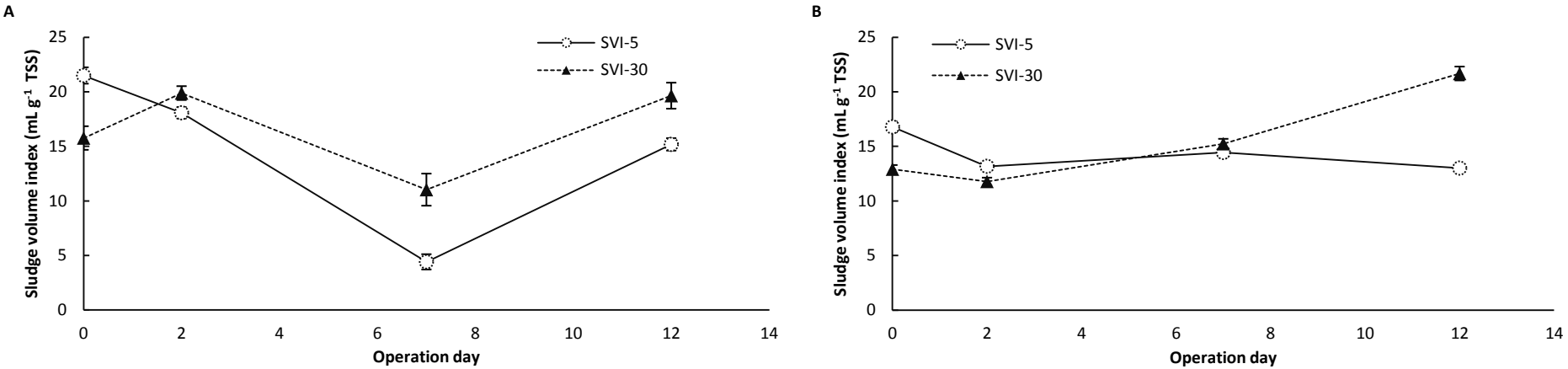


Figure 40: Sludge volume index 5 and 30 for reactor running on low divalent/monovalent cation ratio (A) and reactor running on high divalent/monovalent cation ratio (B).

The VSS and TSS concentration (see **Figure 39**) of both bioreactors increased. The VSS TSS ratio of the reactor operation on a low divalent/monovalent cation did not change. The VSS TSS concentration of the reactor running on high divalent/monovalent cation ratio increased over de operation period from 36% to 46%.

The ammonium removal efficiency during the experiments is presented in **Figure 41**. Free ammonium and free nitrous acid is shown in **Figure 42**.

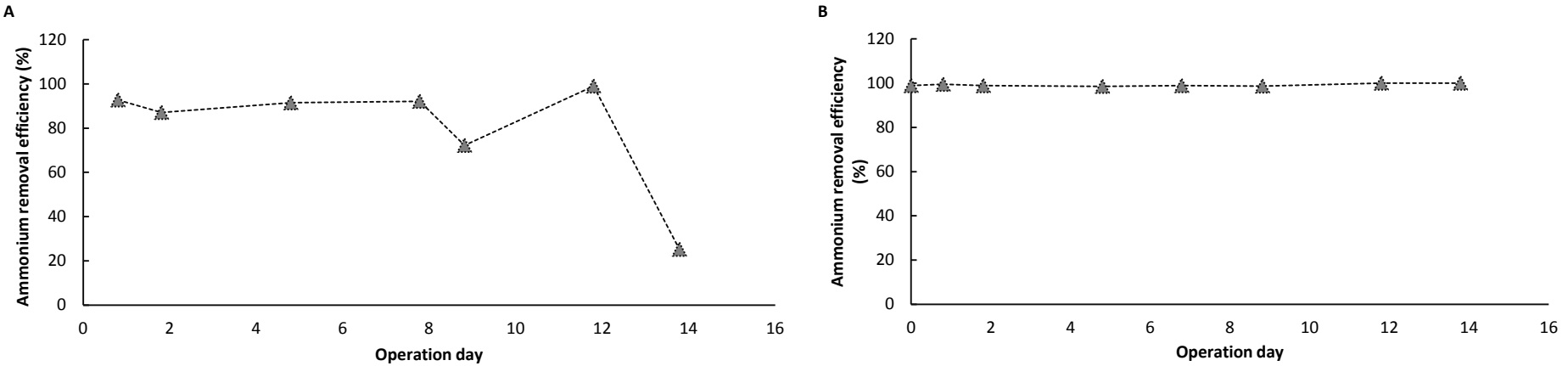


Figure 41: Ammonium removal efficiency and nitrification efficiency for reactor running on low divalent/monovalent cation ratio (A) and reactor running on high divalent/monovalent cation ratio (B).

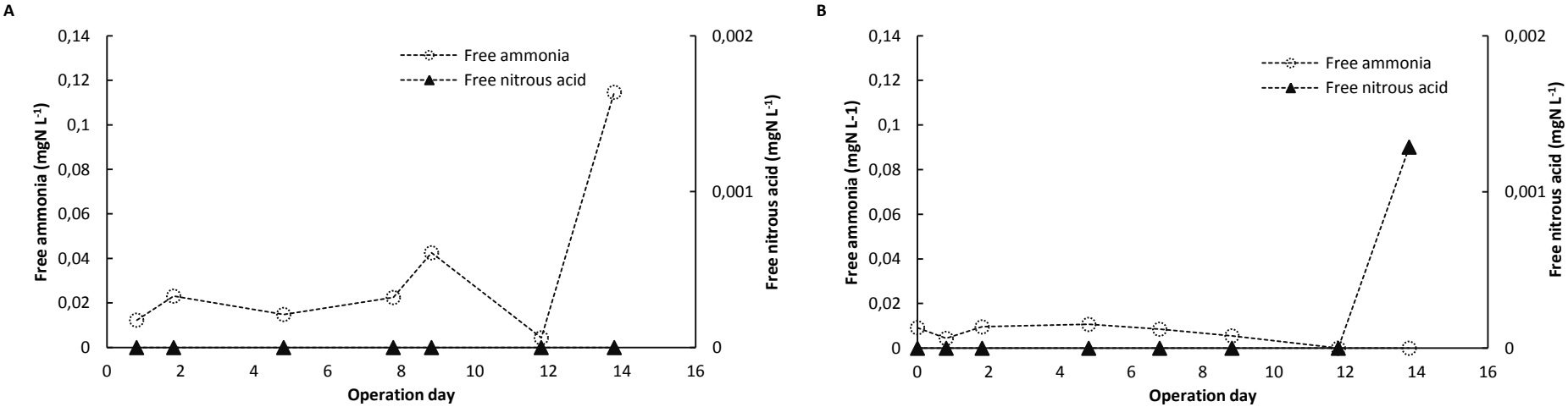


Figure 42: Free ammonium and free nitrous acid for reactor running on low divalent/monovalent cation ratio (A) and reactor running on high divalent/monovalent cation ratio (B)..

The ammonium removal efficiency of reactor running at a low divalent/monovalent cation ratio showed a decrease at day 9 (72%) and day 14 (25%). The bioreactor running at a high divalent/monovalent cation ratio had a stable ammonium removal efficiency.

Free Ammonia became inhibitory for NOB (see **Figure 42**) in the MBMBR running on a low divalent/monovalent cation ratio (toxicity AOB and NOB see 3.2.4 PART 1).

The volumetric loading rates and ammonium oxidation rates of both MBMBRs are presented in **Figure 43**.

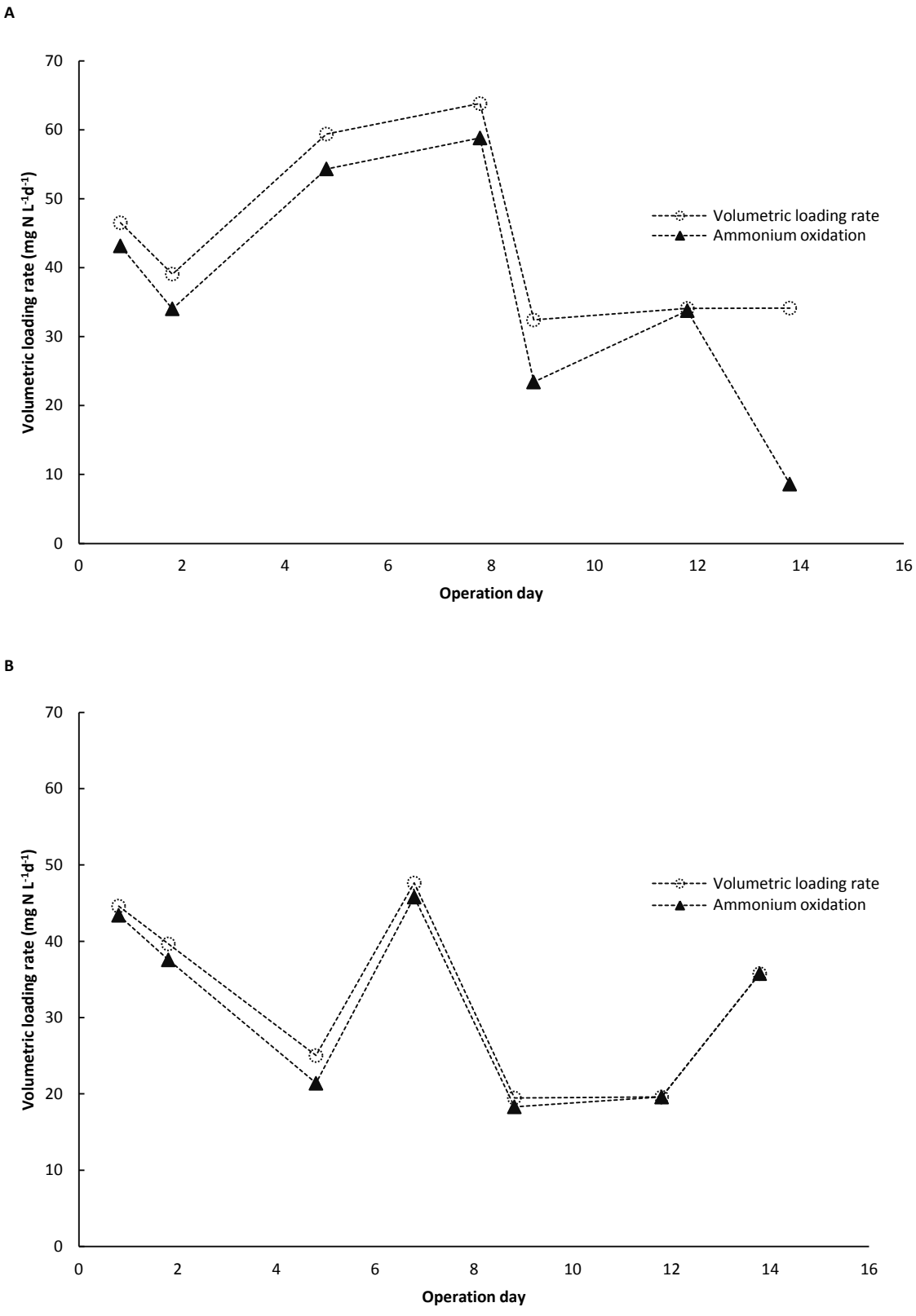


Figure 43: Volumetric loading rate and ammonium oxidation rate for reactor running on low divalent/monovalent cation ratio (A) and reactor running on high divalent/monovalent cation ratio (B).

2.4. MACRO SCALE BATCH EXPERIMENTS

Macro scale batch experiments were performed. No activity could be determined for the beads of both bioreactors in a larger volume (50 mL).

2.5. PROTEIN DETERMINATION

Proteins were analyzed with the Lowry protein determination method. No proteins could be determined on the beads in stage 1 and stage 2 of the reactor experiments. In stage 3, Proteins were observed after 14 days of operation (altered divalent/monovalent cation ratio). The extraction liquid (1 M NaOH) was reduced from 1 mL to 0.5 mL in order to reach the detection limit of the analysis. The Lowry protein analysis showed proteins (on the beads) in the MBMBR running on a low divalent/monovalent cation ratio. No proteins (on the beads) could be determined for the reactor running on a high divalent/monovalent cation ratio.

The results of the Lowry protein analysis is shown in **Table 25** for day 14 of the reactor operation of stage 3.

Table 25: Results Lowry protein extraction and volatile suspended solids.

Location	Protein (µg BSA mL^{-1})	Biomass (gVSS mL^{-1})	Distribution (gVSS g^{-1}Total VSS)
Suspension	6±2	$(0.16\pm0.1)10^{-3}$	60%
Beads	1.9±0.7	$(0.5\pm0.2)10^{-4}$	40%

Data for the beads are expressed per bead and not per mL.

The detection limit of the Lowry method is 10 µg mL^{-1}. This is higher than the results of the protein analysis (Waterborg, 2009).

A visual inspection, showed biofilm growth in both bioreactors. A picture of biofilm growth on the carrier is shown in **Figure 44**.

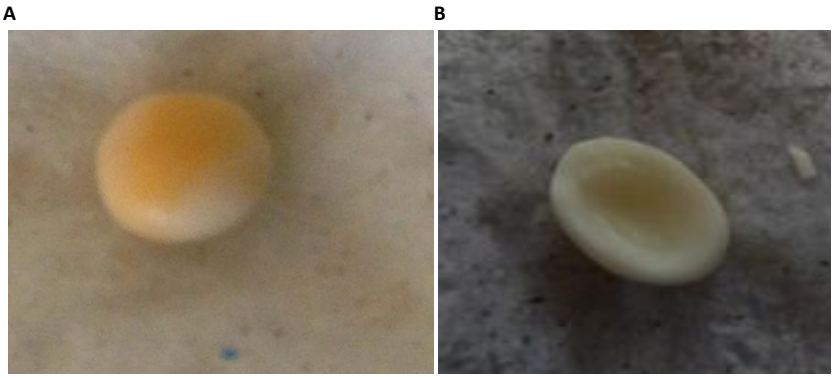


Figure 44: Pictures of beads intact (A) and cut in half (B).

PART 4: DISCUSSION

1. BATCH EXPERIMENTS

1.1. EFFECT OF SALINITY ON THE ACTIVITY OF AOO AND NOB FOR DIFFERENT TYPES OF SLUDGE

This study investigated the effect of salinity on the nitrification activity of AOO and NOB. Different activated sludge types were studied adapted to a variety of salinities.

From the batch experiments (**Table 19** and company data **Table 12**), it can be seen that different factors could have affected the activity of the nitrifiers during the experiments such as pH difference, low biomass concentration, temperature difference, loss of biofilm structure, composition of the original medium and continuous dosing versus spike dosage.

An interesting parameter is salt shock (ΔEC). This parameter is the difference in EC between original salt conditions and the EC of nitrified urine conditions (45 mS cm^{-1}). Intuitively, it could be expected that a higher salt shock would results in a higher inhibition percentage. **Figure 45** is an illustration of the inhibition percentage as function of the salt shock.

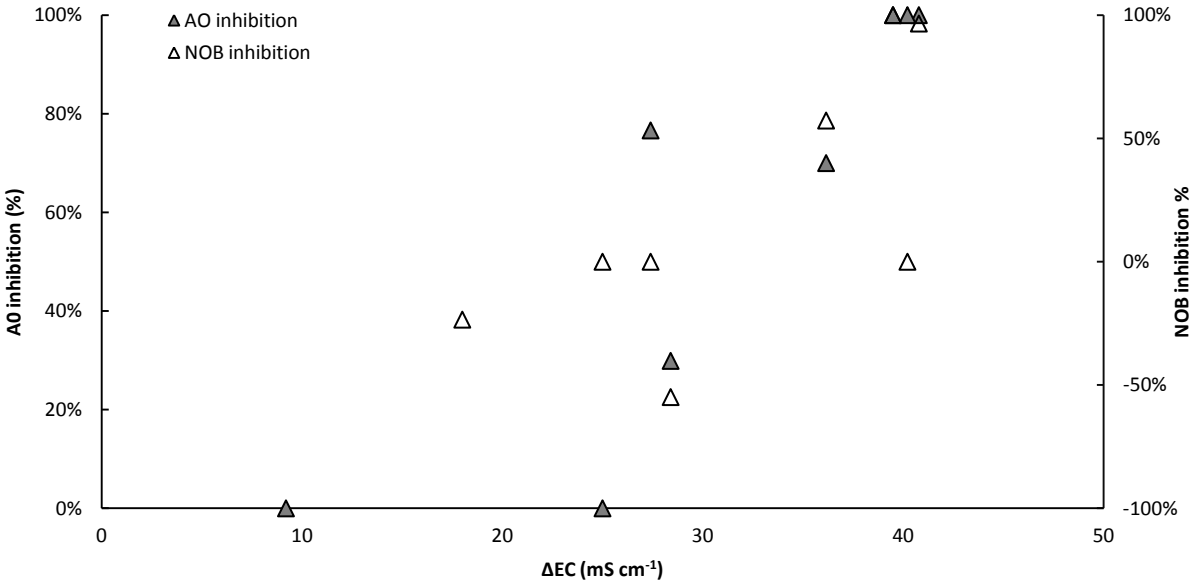


Figure 45: An overview of the inhibition percentage for all tested sludge types for AOO and NOB.

From **Figure 45**, it can be concluded that salt shock (ΔEC) and inhibition percentage were not linear during the experiments. Some sludge types performed remarkably well even if the salt shock was high such as Decostere – Kortemark, Danis NV – Izegem sludge and Avecom – Wondelgem sludge. An interesting observation from the batch tests are the two negative inhibition percentages observed for the NOB from Decostere –Kortemark sludge and Dow – Terneuzen sludge (difference in activity was statistically analyzed with analysis of covariances). Microorganisms can cope with a salty environment by using two distinct strategies (i.e. "salt in strategy" and "compatible solute strategy" see 3.2.5 PART 1). Both strategies require energy. When the salinity of the environment is increased, microorganisms need more energy to endure the salty environment. An increase in activity at higher salinity is not straightforward. In literature no similar results could be found.

Remarkably, the sludge from Avecom had the highest AOO and NOB activity for original salt conditions and nitrified urine conditions. It endured a salt shock of 25 mS cm^{-1}. This sludge is referred to as ammonia binding inoculum liquid (Grommen et al., 2002; Rombaut et al., 2003). It is typically fed with ammonium chloride (117 mg TAN L^{-1} d^{-1}) and sodium nitrite (58 mg N L^{-1} d^{-1}) (Grommen et al., 2002). The feed also contains calcium carbonate. This chemical has three functions: carrier matrix, a pH-buffer and a carbon source for the autotrophic microorganisms. It is possible that due to the calcium carbonate, the sludge had high activity in our experiments. The calcium carbonate carrier may play an import role for protection of the sludge to salinity. However in the article of Grommen et al. (2002) the specific function of the carrier (except for pH-buffer, matrix or carbon source) is not specified.

A relative NOB AOO^{-1} activity from the batch experiments could be determined (**Table 21**). It can be concluded from the batch tests that the AOO activities were in most tests lower than the NOB activities. This was not the case for Danis NV-Izegem sludge and Decostere-Kortemark sludge for the tests of the original salt conditions. The NOB AOO^{-1} ratio was lower than one (NOB activity < AOO activity) for these two sludges. However at high salinity conditions (45 mS cm^{-1}), the relative activity was higher than one (NOB activity > AOO activity) for all sludge types. These findings are in line with literature. The oxidation reaction of ammonium to nitrite generates more energy than the oxidation of nitrite to nitrate. As a result, the NOB need to convert more substrate than the AOO in order to supply an equal amount of energy (Rabaey, 2015). It is not clear why AOO activity is higher for both pig manure sludge types. This could be due to the conditions where the sludge was adapted to. It could be possible that AOO growth was stimulated by ammonium chloride addition. The company did not provide any other information than stated in **Table 12**. Note that the relative abundance of AOO and NOB was not determined in the biomass. Thus it is possible that a higher activity of NOB relative to AOO, was due to a higher abundance of NOB in de biomass.

An interesting value that can be derived from the relative rates is the indicator factor. This factor characterizes the change in relative activity at higher salinity. And thus it gives an indication which type of organisms (AOO or NOB) are more inhibited by salinity. A negative percentage indicated that (i) AOO decreased more than the NOB activity, (ii) the AOO activity decreased and the NOB activity did not change, (iii) the NOB activity rate increased more than the AOO activity or (iv) the NOB activity increased and the AOO activity did not change. This percentage could not be determined for every sludge type because AOO or NOB activity was not always observed during the batch experiments (**Table 21**).

During these batch tests, AOO were more sensitive to salt stress than NOB. This is supported by (i) the indicator factor: three sludge types showed a negative indicator factor and only one had a positive value. (ii) The hypothesis that NOB were more sensitive to salt stress, is also noticeable from the inhibition percentages of AOO and NOB. For the AOO, four distinct sludge types were 100% inhibited at high salinity conditions. In the contrary only one sludge type was 100% inhibited at high salinity conditions for NOB (SeaLIfe – Blankberge (Piranha)). (iii) Furthermore, some sludge types had negative NOB inhibition percentages. This indicates that the activity of the NOB increased at high salinity conditions (45 mS cm^{-1}). (iv) A last observation that supports the hypothesis that AOO were more sensitive to salinity than NOB, is the fact that the NOB inhibition percentages were smaller than the corresponding AOO inhibition percentages.

In these experiments, AOO and NOB activity was examined at the salinity at which the sludge was adapted to and at high salinity conditions (nitrified urine conditions). For the high salinity batch experiments, 20 NaCl g L^{-1} was added directly to the Erlenmeyer. The sludge had to cope with an immediate salt shock. From a bioenergetic point of view, a salty environment requires energy (salt in strategy and compatible solute strategy see 3.2.5). It could be expected that inhibition would be greater when the salt shock was higher. However from these results, it is clear that this was not the case for all tested sludge types. Some sludge types were inhibited by salinity, for other sludge no activity was observed at high salinity conditions. The NOB activity also increased

relatively to the AOO activity at high salinity conditions for Imog, Decostere and Danis NV (**Table 21**). From the results it can be concluded that AOO were more sensitive towards salt stress. Literature is inconclusive about this matter. **Table 26** list four studies with there outcome regarding salt sensitivity and nitrifiers. Note that the studies from Bassin et al. (2011), Dincer & Kargi (1999) and Moussa et al. (2006) were performed in a bioreactor and not in batch experiments. Only the study of Hunik et al. (1992) and Hunik et al. (1993) were performed in batch. In the articles listed in **Table 26**, the salinity was gradual increased. The salt shock applied to the bioreactors are listed in **Table 26**.

Two of the studies listed in **Table 26**, indicating that NOB were more sensitive to salinity, concluded this from the accumulation of nitrite. Moussa et al. (2006) mentions that nitrite accumulation is not a direct cause of salt stress but due to secondary factors such as oxygen limitation, phosphorous limitation and/or the presence of toxicants. Salt has an effect on the solubility of oxygen (Bank et al., 1967). This can lead to limited oxygen availability for nitrifiers. Note that the DO was always higher than 2 mg O_2 L^{-1} during the batch experiments. All Erlenmeyers were put on a shaker. In the studies listed in **Table 26** only one sludge types was tested. The salt level was also not equal.

Table 26: A comparison with literature.

Study	Group most sensitive to salt	Salt NaCl (g L^{-1})	Salt shock (g L^{-1})	Salt shock (mS cm^{-1})	Type of reactor	Type of nitrifiers
(Moussa et al., 2006)	AOO	25 and 101	5	10	Domestic WWTP	WWTP inoculum
(Hunik et al., 1993) and (Hunik et al., 1992)	AOO	29	-		Chemostat	*Nitrobacter agilis* and *Nitrosomonas europaea*
(Dincer & Kargi, 1999)	NOB	60	10	20	Aerated reactor	Labscale shaker inoculum
(Bassin et al., 2011)	NOB	33	11	22	Aerobic granular sludge reactor	WWTP inoculum

Salt shock in EC units were calculated using the internal document of Clauwaert et al. 2014.

1.2. SALINITY PROFILE ABIL AVECOM

These experiments were setup to have an indication about the salinity profile of the sludge coming from the company Avecom-Wondelgem. This sludge was also used for the reactor experiments. It can be concluded from the results that the Avecom sludge is able to withstand a variety of salinities for both AOO and NOB (**Table 22**).

An important remark is that the sludge coming from Avecom is distributed on an EC of 3 mS cm^{-1}. However the salinity at which the sludge was being cultivated, is 23 mS cm^{-1}. It is not known how this has affected the experiments.

2. BIOREACTOR OPERATION

A bioreactor was operated in a second part of the research. As mentioned in the literature study (see section 1 PART 1), it was intended to operate a bioreactor that combined membrane permeation and biofilm growth (PVA carriers). The MBMBR was operated for 80 days. The objective of the reactor experiments was to study the effect of urine dilution (decreasing salinity) on the biofilm formation. Three different hypotheses were tested during operation: the effect of salinity on the biofilm formation (stage 1), the influence of wash-out (stage 2) and an altered divalent/monovalent cation ratio (stage 3).

The choice of reactor (membrane permeation and attached growth) was a result of the requirements for a biological treatment step in a wastewater treatment system for space application, in the context of the WTUB project. In a space environment, reduced gravity (microgravity) doesn't allow bubble aeration from bottom to top in a bioreactor. As the gravity driven convection through differences in densities can become reduced by up to six orders of magnitude, transfer of oxygen mainly relies on diffusion (Benoit & Klaus, 2007). Growing the microorganisms in a biofilm based bioreactor might allow for a convenient continuous replenishment of oxygen rich liquid to the microorganisms, without a rapid clogging of a diffusion based aeration system (e.g. diffusion based membrane aeration with hollow-fiber membranes). This is the rationale for investigating biofilm formation on a carrier material, although bubble aeration was still allowed for the time being in this WTUB project. After providing a proof of principle with bubble aeration, future developments will have to focus on diffusion based aeration systems.

Other solutions might be feasible as well, like a hollow-fiber based biofilm reactor (Lunn, 2005). It has been observed that non-motile microorganisms have a higher growth rate in reduced gravity conditions due to a better homogenization in the liquid, which enables a better access to the bulk solution (Benoit & Klaus, 2007). However, in the context of the WTUB project conducted for ESA, growing the biomass in a biofilm was a requirement. An additional advantage of a biofilm based system might be a higher robustness towards processes disturbances (pH, ammonia, nitrite) (Rabaey, 2014).

A membrane was essential for space application. Membrane filtration reduces the risk of pathogen breakthrough to other parts of the treatment system (see subsection 2.3 PART 1 WTUB). The membrane was located inside the bioreactor (submerged configuration). In the WTUB hardware, the membrane will be located outside the bioreactor (i.e. cross flow operation). The performance of a carrier is dependent on the available surface area for growth. That was an important reason for the choice of the PVA carrier (specific surface area 2500 $m^2 m^{-3}$) (Levstek et al., 2010).

The main objective of this thesis research was to study the effect of salinity on the biofilm formation on PVA carrier material. There are two research methods to assess this question. (i) In one framework salt can be applied first (e.g. bioreactor at two different salinities) and this kind of study mainly focuses on the formation of biofilm at different salinities on the carrier. (ii) In another framework, growing a biofilm first without the microorganisms being exposed to salinity stress and then later, the salinity could be increased. In literature, adaptation of a nitrification biofilm to high salinity has been described by (Windey et al., 2005). For practical implementations it would be more interesting if a salt-adapted or -tolerant nitrifying sludge in suspension could be shifted towards biofilm growth mode. For transport of a biofilm more volume is needed than for dense floccular sludge (e.g. transport to Space). Furthermore storage and transport may lead to decay of the biofilm structure and thus suspended growth may dominate again. Therefore, in this thesis research the first framework was chosen and, virgin carriers were used to investigate the biofilm growth directly in a saline environment.

2.1. SALINITY

As seen on **Figure 22**, the EC showed a clear fluctuating pattern during reactor operation. There was no stable operation on the target EC of 15 mS cm^{-1} or at 45 mS cm^{-1}. This was due to a constant change in influent composition. For example when the bulk SRT was set at 8 days, the nitrogen load was decreased. A decrease in ammonium in the influent would reduce the EC. That was why extra sodium chloride had to be added to compensate the expected EC drop. This was not done perfectly as seen in the graphs on **Figure 22**. As discussed before, the EC of the influent does not relate one on one to the EC of the effluent.

In these experiments, salinity was expressed as EC. In literature, articles concerning nitrification and salinity are expressed in g NaCl L^{-1}, % NaCl and g NaCl-Na^{+} L^{-1} (Dincer and Kargi, 1999; Jonassen, 2013; Hunik et al., 1993; Hunik et al., 1992; Moussa et al., 2006). This is a simple method and it is also a straightforward way to compare studies. However it does not take the increase in salinity in account due to the presence of other ions such as nitrate, ammonium, nitrite, phosphate, etc (e.g. buffer). In the contrary the EC is a summary of the salinity caused by all ions in suspension. As such, this parameter is easy to use for experiments.

A disadvantage of the use of EC for salinity is that ion species are not specified. Two suspensions can have the same EC, but have a different ion combination. The toxic character of an ammonium solution with a same EC as a solution with only sodium as cation, will have another toxicity effect on the microorganisms. Thus the use of EC is interesting from a salinity point of view. However it does not specify the cation present. And this is important from a toxicity perspective.

2.2. AMMONIUM REMOVAL EFFICIENCY AND NITRIFICATION EFFICIENCY

Ammonium removal efficiency and nitrification efficiency was also determined (see **Figure 29** and **Figure 41**) according to the formulas in section 5 PART 2. In **Figure 29**, the nitrification efficiency is higher than 100% (day 57-63 both bioreactors). This was due to the calculation method of the nitrification efficiency (NO_3eff-NO_3inf/NH_4inf-NH_4eff). The ammonium concentration and the nitrate concentration in the effluent increased on day 56 of reactor operations (stage 2). This resulted in a nitrification efficiency higher than 100%.

The nitrification efficiency was not calculated for stage 3 of the reactor experiments. Because the sludge was not washed prior to inoculation and thus the nitrate concentration was high (564-575 mg N L^{-1}) relative to the ammonium influent concentration (100 mg N L^{-1}). This would have resulted in a nitrification efficiency higher than 100%.

2.3. MICRO AND MACRO SCALE BATCH EXPERIMENTS

The micro batch experiments were performed in 96 well plates. The goal of these experiments was to determine the activity on the carriers and investigate if there was a shift in activity for the beads and suspension at higher or lower EC for both bioreactors.

The result for the carrier activity tests indicated no AOO or NOB activity for both bioreactors (**Table 24**). Only for some experiments, activity was observed. However the activity measured was low, and the corresponding standard deviation was high (relatively to the activity). One bead was used per well for every test. There is some variation between the volumes of the beads (Lindeboom et al., 2014). This could have changed the initial conditions of the test. Because of the variation in bead volume and the poor data, macro scale batch experiments were performed in Erlenmeyers of 100 mL to verify the results. The results of the macro scale experiments (50 mL) indicated that there was no AOO and NOB activity on the beads.

Activity could be determined for the suspension (**Table 23**) through the 96 well plate experiments. The objective of these experiments was to investigate if the nitrifiers were adapted to the salinity conditions of the bioreactor. That was why the experiments were always performed at two electrical conductivities. These results show that the microorganisms were resilient to salt stress. Furthermore the activity of both bioreactors did not shift to higher or lower EC values. This was statistically validated by analysis of covariances.

2.4. PROTEIN DETERMINATION WITH LOWRY

The determination of proteins was performed with the Lowry extraction method. The objective of these tests was to quantify the biomass present on the beads. It can be seen from the results of the Lowry method (**Table 25**) that proteins could be determined only in stage 3 of the reactor experiments. The results indicated that proteins on the beads were only present in the reactor running on a low divalent/monovalent cation ratio. This was the low salinity reactor during stage 1 and stage 2 of the experiments. Proteins could probably not be determined in stage 1 and stage 2 because of the detection limit of the Lowry method. The detection limit of the Lowry method is 10 µg mL^{-1} (Waterborg, 2009). The extraction medium was halved and thus proteins were up concentrated for the last Lowry protein determination. This is an important reason why proteins could be determined. Note that the results were still lower than the detection limit of the Lowry method so the actual protein concentration could not be measured precisely.

From the divalent cation bridging theory, it was expected that the reactor running on a high divalent/monovalent cation ratio would have biofilm growth on the beads. This was not the case only in the reactor running on a low divalent/monovalent cation ratio proteins were determined with the Lowry extraction method. The lack of proteins in the reactor running on a high divalent/monovalent cation ratio could be due to interference of calcium with the extraction method (Morrissey, 1989).

Table 25 shows the results of the protein analysis on day 14 of the reactor operation experiments (stage 3). The results indicate that 60% of the VSS is located in suspension and 40% on the beads. The biomass distribution would be much higher for the suspension but the floccular SRT was set at 8 days. The VSS concentration in suspension was low as a result.

Proteins were characterized with the Lowry extraction method (Lowry, 1951). In literature other colorimetrical methods exist such as Smith et al. (1985) and Bradford (1976). An interesting technique is the use of in fluorescent in situ hybridization. Okabe et al. (1999) has analyzed nitrifying biofilm with in situ hybridization techniques. The special organization of AOB and NOB could be determined.

Colorimetrical techniques and in situ hybridization are both indirect methods to characterize the biofilm. Colorimetrical methods are better to quantify the growth of biofilm over time. When the biofilm is fully integrated in the structure of the carrier, in situ hybridization could be used.

3. GENERAL DISCUSSION

The topic of this thesis is the effect of urine dilution on the biofilm formation in a MBMBR. Urine, condensate and gray water will be treated to hygienic water in the WTUB (see subsection 2.4 PART 1). In this project, hydrolyzation and nitrification will take place in the same bioreactor (see **Figure 7**). Urine contains a number of different ions including calcium, chloride, potassium, sodium etc. The most common cation and anion in urine are respectively sodium and chloride (**Table 3**).

The influent composition during bioreactor operation contained ammonium as nitrogen source and sodium and chloride as ions. Other ions were not introduced in the influent. Moreover, urine contains organic compound such as creatine and citric acid (**Table 1**). However no carbon source was inserted in the influent (**Table 15**).

This research does not study directly the effect of urine dilution, but examines the salinity exerted by urine. That was why no carbon was added and only sodium and chloride as ions. Since they are the most abundant ion present in urine. Other cations such as calcium and potassium were not added.

3.1. INOCULUM SELECTION

The sludge selected for inoculation was ABIL from the company Avecom. The batch screening indicated that this sludge was most resilient to salinity (**Table 19**). It had the highest AOO and NOB activity for both original salt and nitrified urine conditions. The AOO were not inhibited at high salinity conditions and the NOB were only inhibited for 18% (see section 1 PART 3). The high elasticity to salt stress could also be observed in the salinity profile experiments (**Figure 21**). Note that the result for 23 mS cm^{-1} and 45 mS cm^{-1} were also used for the batch screening.

The sludge was chosen for inoculation because of the high nitrifying performance under salt stress. The resilience to salt stress was also observed during reactor operation. The micro batch experiments (96 well plates) indicated that there was no shift in optimum activity to higher or lower EC values for both bioreactors (see subsection 2.2 PART 3). Thus the batch experiments, the salinity profile experiments and the micro scale batch test conclude that the ABIL inoculum is resilient to salinity for both AOO and NOB.

Sludge selection could have had a major influence on the result of the reactor experiments, especially for stage 1 of the reactor operation experiments. During this stage, the reactors were running on two different electrical conductivities. No sludge was taken out of the reactor (except 15 mL 7 d^{-1} for analysis of TSS VSS). In these experiments, salinity can be seen as a selection technique for biofilm growth on the PVA carrier. Microorganisms in suspension have to cope with salinity and a saline environment is costly from a bioenergetic point of view (Oren., 1999). That was why the proposed hypothesis was: microorganisms in the high salinity reactor would prefer the carrier over the suspension because biofilm growth could result in an extra protection to salinity for the microorganisms. However this was not observed during the reactor experiments. This could be due to a number of reasons: (i) Avecom sludge is resilient to salt stress. It does not feel any pressure to migrate to the carrier. (ii) Salinity has no influence on biofilm formation. (iii) No organics were added to the influent and thus heterotrophic growth was not stimulated in the bioreactors. (iv) Shear effects could have resulted in a higher detachment rate than biofilm growth.

The resilience to salt stress of the Avecom sludge, was already proven in the batch screening, the salinity profile experiments and the micro scale batch tests (see subsection 1.1 and 1.2 and 2.2 PART 3). This could be

an important reason for lack in biofilm formation. Yet it is also possible that biofilm growth do not protect the microorganisms to salt stress. And thus it is not a selective advantage for the microorganisms.

The fact that salinity has no influence on biofilm formation is the contrary of the hypothesis. This could be the main reason of lack in biofilm in the first stage of the reactor experiments. Thus salinity does not pose a selective pressure on microorganisms. In the experiment of stage 1, it took 10 and 12 days respectively for the low and high MBMBR to reach the target EC. The experiment lasted 30 days. Thus the EC of both reactors was actually 20 days at the target EC (**Figure 22**). This could have been too short to see biofilm formation. After 30 days, the bulk SRT was decreased.

In the study of Udert & Wächter (2012) a membrane aerated biofilm reactor was used and biofilm growth was observed. The carrier material was made of silicone tubing and the reactor was fed with 45% diluted real human urine (**Table 7**). In the article it was reported that a biofilm structure was present and no problems were reported concerning a lack in biofilm by salinity effects (salinity caused by human urine). Note that the SRT of suspension and the time for biofilm formation was not specified in Udert & Wächter (2012). An important difference with this thesis research, is that the influent contained an average of 4.5 g L^{-1} dissolved COD. In the study of Udert et al. (2003) a MBBR with plastic rings as carriers were used fed with real human urine (dilution 22-131%). The carriers were already inoculated from a previous experiment. In the article there is nothing mentioned concerning loss in biofilm structure due to salinity. The HRT during operation was 4.8 days.

From the experiments in this thesis, it cannot be concluded that salinity effected the biofilm formation. The only hard evidence is that the sludge was maybe too resilient to salinity. To prove the hypothesis of salinity as a selection pressure, it was maybe interesting to choose an inoculum that has high activity at the original salt conditions, yet inhibited at nitrified urine conditions for both AOO and NOB. The sludge cannot be 100% inhibited at high saline conditions. Otherwise toxicity problems can occur and the experiment will fail. An interesting sludge that has these requirements is the sludge coming from the company Imog (**Figure 19** and **Figure 20**). This sludge had a high AOO activity and was 70% inhibited at high salinity conditions. The NOB activity was the second highest after the sludge from Avecom. The NOB activity was also inhibited for 57% at high salinity conditions. This sludge is very appealing to examine the hypothesis of salinity for future research.

It could also be interesting to select a sludge that was already used to biofilm growth such as sludge from: Inagro, Ugent shrimps tank, Sealife (Piranha) and OLAND lab scale reactor. However the sludge from Ugent schrimpt tank and Sealife had no activity. The sludge from Sealife was 100% inhibited at high salinity conditions. The OLAND sludge is interesting. It had high AOO activity, yet no NOB activity. That is why a mixture of OLAND and Imog might compensate this.

3.2. BIOFILM FORMATION

In space, bioprocesses encounter the effects of reduced gravity conditions (microgravity). Transfer of oxygen towards the microorganisms is expected to occur mainly by diffusion. During this research, the main goal was to have biofilm growth on the porous PVA carrier. As discussed in section 2 (PART 4) two methods exist to tackle the research question: (i) investigate the biofilm formation at two different salinities or (ii) start with a biofilm. In this research the first option was chosen.

The results of micro and macro batch experiments indicated that no (not reliable) AOO and NOB activity was present on the beads. For the Lowry protein extraction method, only proteins could be determined in the reactor running on a low divalent/monovalent cation ratio (stage 3 reactor experiments). Calcium interferes with the Lowry method (Morrissey, 1989). This could explain the lack of proteins on the beads from the reactor

running on a high divalent/monovalent cation ratio. A visual observation indicated that biofilm growth was present in both bioreactors.

An important observation in both bioreactors is that biofilm growth is very heterogeneous. Some carriers show biofilm growth, but others appear untouched. Also the biofilm is not spread out over the whole carrier. Very concentrated growth patterns can be observed in both bioreactors (see **Figure 44**).

The hypothesis that salinity influences the biofilm formation could not be concluded or rejected (see subsection 3.1 PART 4). However the effect of shear may well be important for biofilm formation. During operation, some PVA carriers were stuck between openings of the membrane and the wall of the bioreactor. There were also some carriers on the bottom that were not circulating. As a result some carrier did not encounter shear by collision with the wall and other carriers. It could be possible that these beads were the anchoring place for biofilm growth. Heijnen et al. (1992) mentions three distinct stages in a biofilm formation process: (i) Outgrowth of single cells to micro-colonies, (ii) the outgrowth of micro-colonies to small biofilms and (iii) the outgrowth of biofilms. Heijnen et al. (1992) discusses that the first stage is influenced by the carrier surface roughness. The article describes that rough carriers give the best biofilm development. Initially the biofilm is formed in the surface cavities of the particles. The micro-colonies are protected from shear effects. Protection could have occurred on the bottom and between the wall en the membrane of the MBMBRs. As such micro-colonies were formed. The second stage in the biofilm formation process (outgrowth to small biofilms) is negatively influenced by the concentration of carrier material. In the research, a volumetric packing ratio of 9% was deployed during reactor operation. Particle-particle collision was observed in the bioreactor. This is an effect of a high concentration of carrier material and leads to detachment of biofilm. This could have been an important reason for lack/slow biofilm formation on the beads. Thus basically, biofilm growth observed in the bioreactors in stage 3 (**Table 25**), could be due the location of the carriers.

Heijnen et al. (1992) discusses the importance of carrier characteristics and SRT of the suspension. As carrier material, PVA beads were chosen. These beads have a smooth surface and are porous. Moreover they have a large specific surface area (2500 $m^2\ m^{-3}$) (Levstek et al., 2010). The porous structure is supposed to enable growth inside. The SRT of the suspension is also important for biofilm formation. If the SRT is too high, biomass is washed out slower and the microorganisms in suspension have the ability to consume more substrate as compared to lower retention times. In stage 2 of the reactor experiments, the SRT was decreased to 8 days (sludge was taken out directly by bypassing the membrane filtration of the MBMBR). The maximum growth rate for *Nitrosomonas and Nitrobacter* is respectively 0,54 d^{-1} and 0,67 d^{-1} (Blackburne et al., 2007). Thus the corresponding minimal doubling time for *Nitrosomonas* and *Nitrobacter* is 1,85 d and 1,49 d respectively (μ_{max}= doubling $time^{-1}$). The minimum doubling times of *Nitrosomonas* AOB and *Nitrobacter* a NOB was still smaller than the SRT.

The importance of a low bulk SRT is observed by Heijnen et al. (1992), but also by (Prat, 2015). In the thesis of Prat (2015), one sequencing batch reactors and a MBBR was operated for the development of thermophilic nitrification. PVA carriers were used in one reactor. The carrier material was the same as used in this thesis. An interesting finding of Prat (2015) was that SRT of the suspension needed to be decreased in order to have biofilm formation. The study of Prat (2015) is difficult to compare because of the high temperature conditions (38-42 °C) and low salinity (0.8- 4.2 mS cm^{-1}) conditions. The biofilm of Prat (2015) was equally covered over the whole carrier. However dissection of the biofilm showed no growth inside the carrier. Growth inside the carrier was observed in the MBMBRs of this research (see **Figure 44**).

Note that the SRT of suspension was decrease throughout stage 2 and 3 of the reactor experiments. No carriers were taken out of the bioreactor (only for the activity tests and the Lowry extraction). According to Henze et al.

(2002) a bulk SRT of 5 days is necessary for nitrification at 20°C. More sludge decay was expected by salt stress. That was why the floccular SRT was set at 8 days.

During this research, it was observed that biofilm grew on silicone material. This silicone material was placed between glass and the iron wall of the bioreactor. This was done to prevent water leaking out of the reactor.

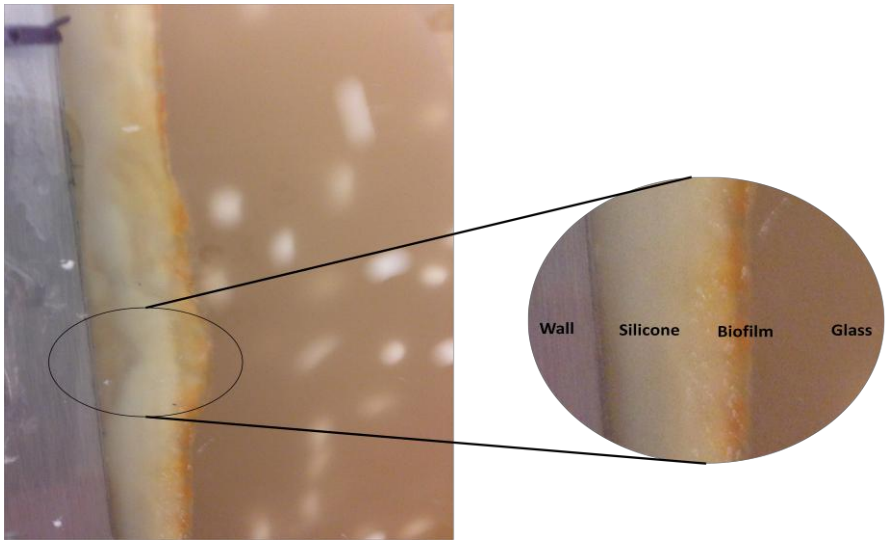


Figure 46: Picture biofilm growth on silicone material.

Silicones are often used as support material for tests with biofilm growth in membrane aerated biofilm reactors (Kubista, 2012; Pickering et al., 2013; Udert & Wächter, 2012). Oxygen is four to five times more soluble in silicone than in water (Casey et al., 1999).

3.3. DIVALENT CATION BRIDGING THEORY

The divalent cation bridging theory states that divalent cations such as calcium and magnesium bind negatively charged functional groups with the extracellular polymeric substances and this promotes biofloculation. Monovalent cations replace the divalent cations and induce deterioration of the floc (see subsection 3.3 PART1) (Sobeck & Higgins, 2002).

The divalent/monovalent cation ratio was altered for one bioreactor in stage 3 of the reactor experiments. The hypothesis was that a high divalent/monovalent cation ratio would increase the strength of the biofilm as with the floc structure (Bin Ismail, 2013; Higgins & Novak, 1997 Sobeck & Higgins, 2002). Increased biofilm strength by a higher calcium concentration, was observed by Ahimou et al. (2007; Huang & Pinder (1995); Körstgens et al. (2001). In these articles the importance of a divalent/monovalent cation ratio is not discussed. Eldyasti et al. (2013) proved that the detachment rate of a biofilm decreased at higher calcium concentrations.

During reactor operation biofilm growth was detected with the Lowry extraction method in the reactor running on a low divalent/monovalent cation ratio. Visual inspection indicated that biofilm was present in both bioreactor. The lack of detection by the Lowry method could be due to (i) calcium interference (Morrissey, 1989) and (ii) the of length of the experiment. Stage 3 of the reactor experiments, had run for only 15 days. That is why it is not possible to make any conclusion regarding biofilm growth. The SRT in stage 3 was also 8 days.

4. CONCLUSION

The sludge bred for aquaculture applications was highly resilient to salt stress. This sludge did not show any difficulty to perform complete nitrification at high rates at electrical conductivities values between 3 mS cm^{-1} and 45 mS cm^{-1}. This sludge is highly interesting for treatment of wastewater fluctuating in conductivity. The batch experiments also show that AOO are more sensitive to salt stress than NOB.

The reactor experiments consisted of three stages. In every reactor stage, a hypothesis was tested.

In stage 1, the effect of the level of salinity (15 mS cm^{-1} and 45 mS cm^{-1}) on the biofilm formation was tested; conclusion: according to the results, no differential effect on biofilm formation could be observed.

In stage 2, the focus was on imposing a selective pressure by setting the floccular SRT at 8 days (in stage 1 no sludge was taken out of the MBMBRs) ; conclusion: no biofilm growth was detected during this stage.

In stage 3, the divalent/monovalent cation ratio of one bioreactor was modified to 1.93 mg Ca^{2+} mg^{-1} Na^{+} the other MBMBR was operated at a ratio of 0.002 mg Ca^{2+} mg^{-1}; conclusion: the divalent/monovalent ratio had no effect on the biofilm formation. The SRT was kept at 8 days during stage 3 of the reactor experiments. Biofilm growth was detected in both bioreactors. The reactor experiments indicate that a decrease of SRT of the mixed liquor was essential for biofilm formation.

5. FURTHER RESEARCH

5.1. ADDITIONAL EXPERIMENTS

(i) The Lowry method indicated that biomass was present in the reactor running on a low divalent/monovalent cation ratio. Visually, biofilm growth could be observed in both bioreactors. The micro and macro scale batch experiments (see subsection 2.2 and 2.4) showed that no AOO and NOB activity was present on the carrier. An additional batch experiment to verify if the biofilm consists out of heterotrophs is important. This is possible by e.g. performing an acetate activity tests or by analyzing the microbial community on the beads. However there was still not enough biomass present.

(ii) It would be interesting to reevaluate stage 1 of the experiments. As discussed, this experiment only ran for 20 days. When redoing the experiments, it is important to operate the reactors for a longer period (e.g. 100 days). In addition a combination of OLAND labscale sludge can be used for inoculation, in combination with another inoculum. The sludge from OLAND labscale reactor is already in biofilm growth mode. However this sludge has no NOB activity. That is why the sludge type Imog could be mixed with OLAND labscale sludge. Imog has AOO and NOB activity (**Table 19**). Furthermore this sludge is inhibited for 70% and 57% for AOO and NOB respectively at high salinity conditions (45 mS cm^{-1}). A combination of these sludge types is interesting to test the hypothesis of salinity because: *(i)* The combined sludge has the potential to form biofilm and *(ii)* the sludge is inhibited at high salinity conditions. Thus it is expected that microorganisms in suspension will migrate to the carrier to grow on biofilm in order to be protected to the salt stress. Also the effect of shear needs to be studied by lowering the volumetric packing ratio of the carrier.

(iii) In the reactor experiments, ammonium was used as nitrogen source for the influent. Sodium and chloride were used as ions. For further research, synthetic urine and real urine can be employed. As in the WTUB, hydrolysis and nitrification will take place in the same bioreactor. Furthermore, it is also interesting to test the effect of organics to stimulate heterotrophic growth.

5.2. BIOREACTORS IN SPACE

This thesis was in line with the WTUB project sponsored by the ESA. A biofilm needs to present because oxygen can only be provided by diffusion. For Space applications, it is not feasible that the wastewater treatment system is not up and running from the start. Thus a simple system needs to be developed for direct employment of the bioreactor in space. For example immobilization can be interesting for space applications as performed by Hsieh et al. (2002). PVA, water and alginic acid sodium salt were first mixed, cooled and than a concentrated nitrifying culture was added.

The MBMBR setup is studied during this thesis. This may be interesting for Space applications. However it is not known if these PVA beads will topple the oxygen transfer limitations (diffusion). Most biological system that are studied for Space application are based on hollow-fiber membrane bioreactors (Kubista, 2012). They use the hollow-fiber structure as support for biofilm formation. Moreover the fiber is pressurized and oxygen flows from inside the fiber material to the reactor medium. Thus penetrating the biofilm from the inside. In a later stage it could be necessary that the WTUB shift to hollow-fiber membrane technology.

The findings of this research are not only interesting for nitrifying Space treatment technologies, but will also open up terrestrial applications such as in fish canning industries. The wastewater in these industries can contain high salt levels and can cause failure of biological processes (Figueroa et al., 2008).

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APPENDIX

I. VAPOR COMPRESSION DISTILLATION

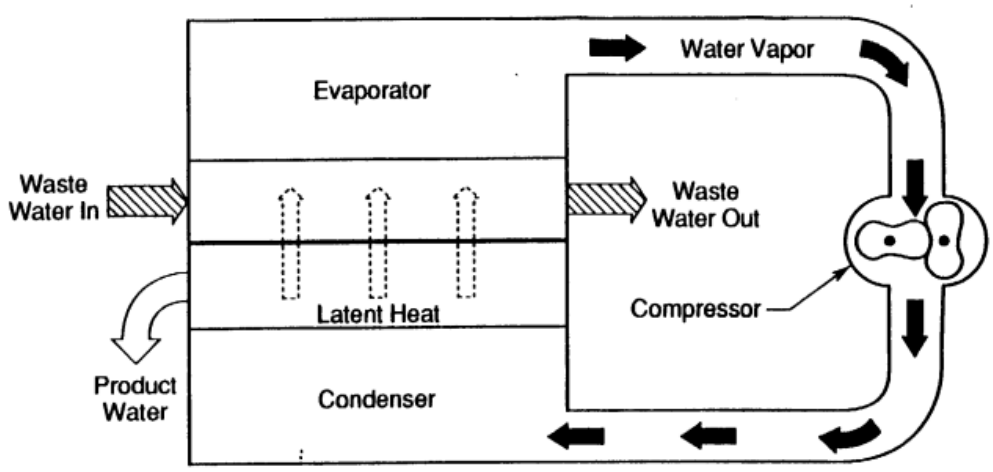

Figure 47: An illustration of vapor compression distillation (Wydeven, 1988).

II. WTUB VALUES FRESH, HYDROLYZED AND NITRIFIED URINE

		Fresh	Hydrolyzed	Nitrified
Cations		**mg L^{-1}**	**mg L^{-1}**	**mg L^{-1}**
Aluminum	Al^{3+}	0	0	0
Ammonium	NH^{4+}	480	9200	0
Barium	Ba^{2+}	0	0	0
Calcium	Ca^{2+}	190	190	190
Copper	Cu^{2+}			
Hydrogen	H^{+}			
Ferrous ion	Fe^{2+}			
Ferric ion	Fe^{3+}			
Magnesium	Mg^{2+}	100	100	100
Manganese	Mn^{2+}			
Potassium	K^{+}	2200	2200	2200
Sodium	Na^{+}	2600	2600	13766
Strontium	Sr^{2+}			
Anions		**mg L^{-1}**	**mg L^{-1}**	**mg L^{-1}**
Bicarbonate	HCO3-	724.39	26790	0
Carbonate	CO_3^{2-}		1720	0
Chloride	Cl^{-}	3800	3800	3800
Fluoride	F^{-}			
Iodine	I^{-}			
Hydroxide	OH^{-}		0.17	0.17
Nitrate	NO_3^{-}	1	0	31000
Phosphate (dibasic)	PO_4^{3}-			
Phosphate (tribasic)	HPO_4^{2-}	1200	2400	2400
Phosphate (monobasic)	$H2PO_4$-	1200		
Sulfate	SO_4^{2}-	748	748	748
Bisulfate	HSO_4^{-}			
Bisulfite	SO_3^{2-}			
Organic acids	OA-	2500		
Sulfide	S^{2-}			
Alkalinity-P	-	-	-	-
Alkalinity-M	-	-	-	-
Neutral		**mg L^{-1}**	**mg L^{-1}**	**mg L^{-1}**
Carbon dioxide	CO_2	0	90.82	90.82
Silica	SiO_2	0	20	20

Table 27: Values WTUB project.

III. R SCRIPT ANALYSIS OF COVARIANCES

Script used for data analysis:

```
table=read.table('stat.txt',header=T, dec=",", fill=T)
modelabilAO = lm(table$conc_abil_a ~ table$tijd_abil_a+table$sal_abil_a+table$tijd_abil_a*table$sal_abil_a)
summary(modelabilAO)
shapiro.test(modelabilAO$residuals)
plot(table$tijd_abil_a, table$conc_abil_a)
plot(model$residuals_abil_a)
qqnorm(model$residuals_abil_a)
qqline(model$residuals_abil_a)
```

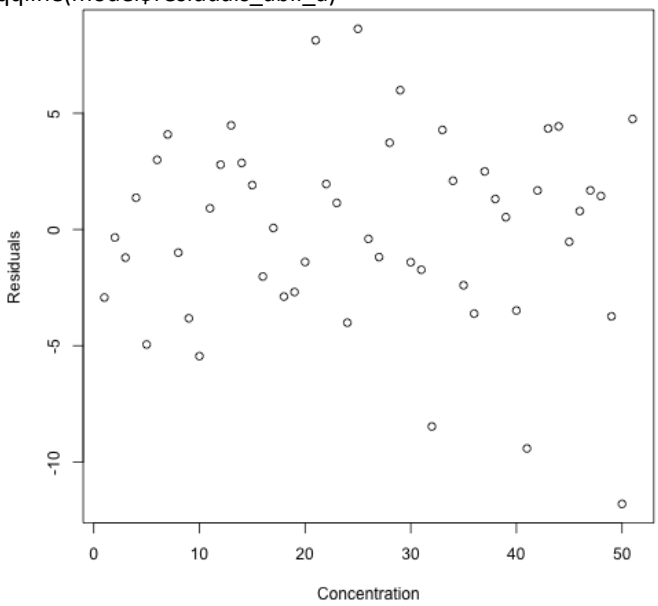

Figure 48: Plot of residuals.

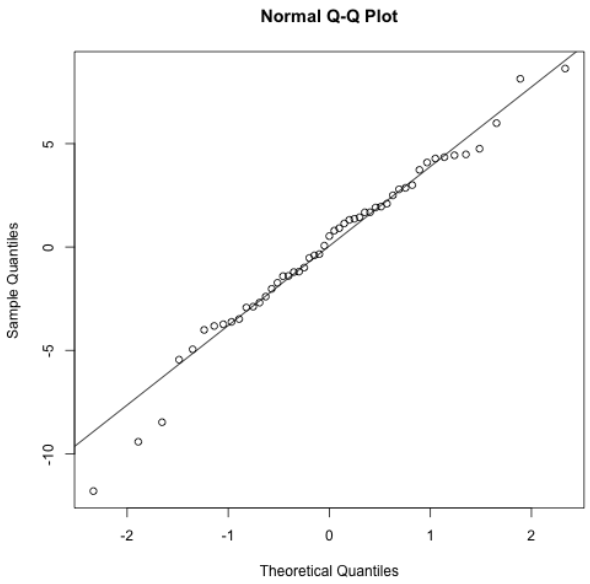

Figure 49: Normal probability plot.

IV. RAW RESULTS BATCH EXPERIMENTSRESULTS OF THE AOO

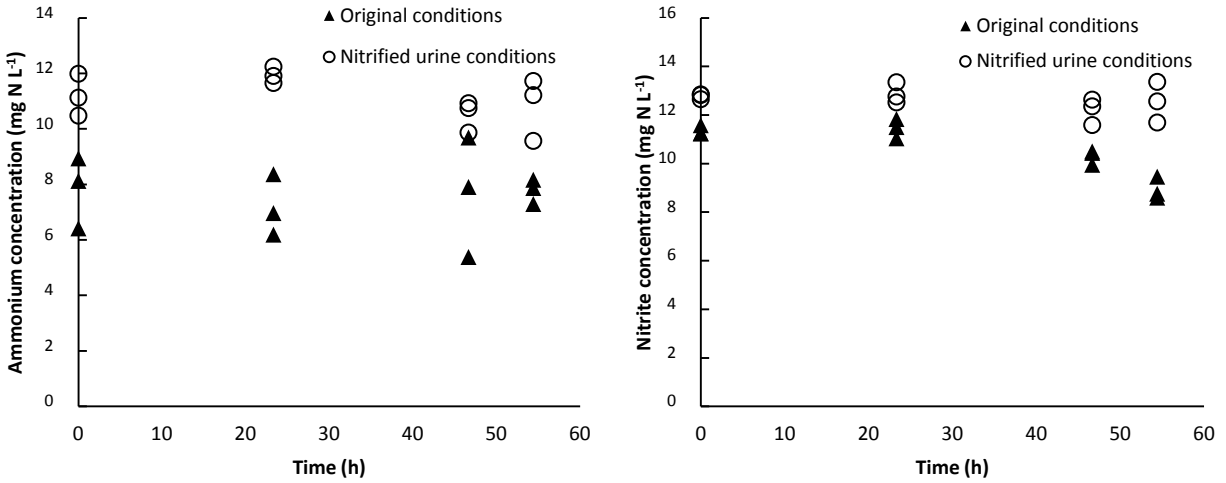

Figure 50: Results Inagro – Rumbeke for AOO (left) and NOB screening (right).

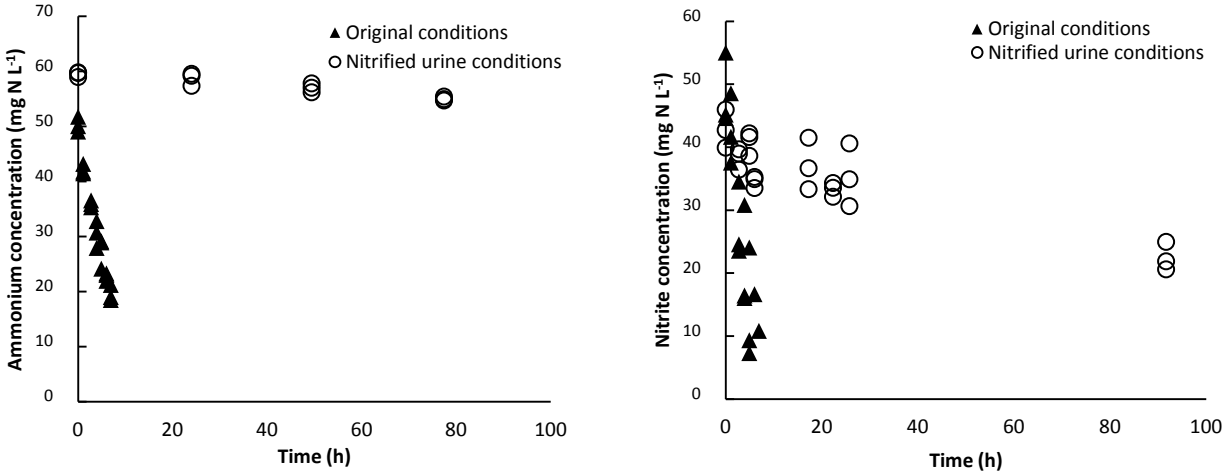

Figure 51: Results RWZI dokhaven – Nieuwveer for AOO (left) and NOB screening (right).

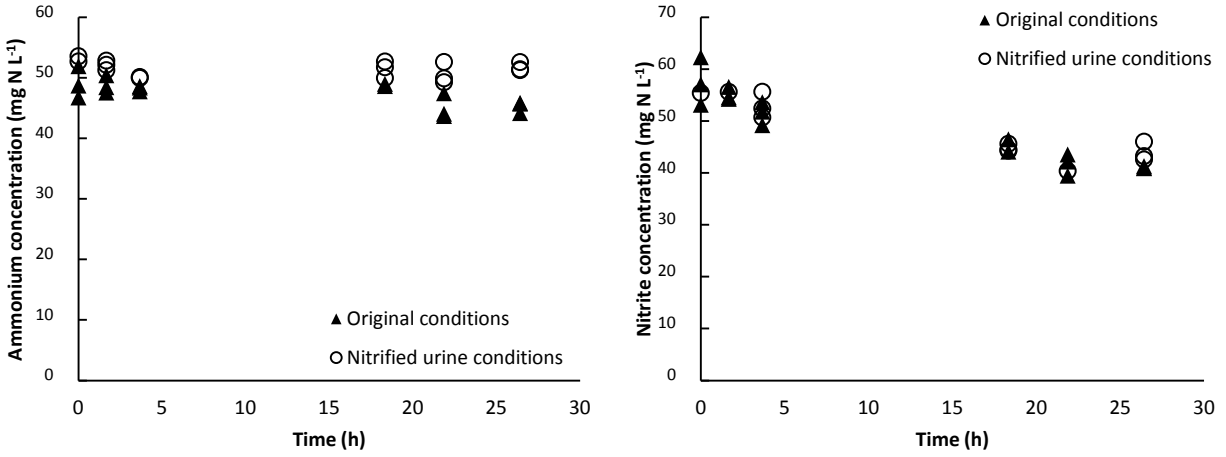

Figure 52: Results Hooge Maey – Antwerp for AOO (left) and NOB screening (right).

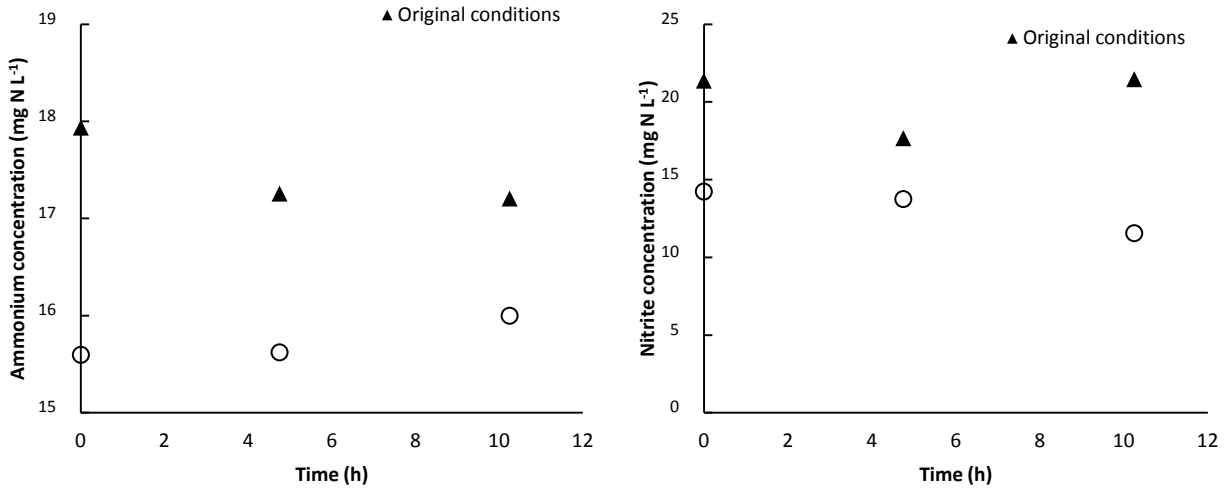

Figure 53: Results Ugent shrimps tank – Ghent for AOO (left) and NOB screening (right).

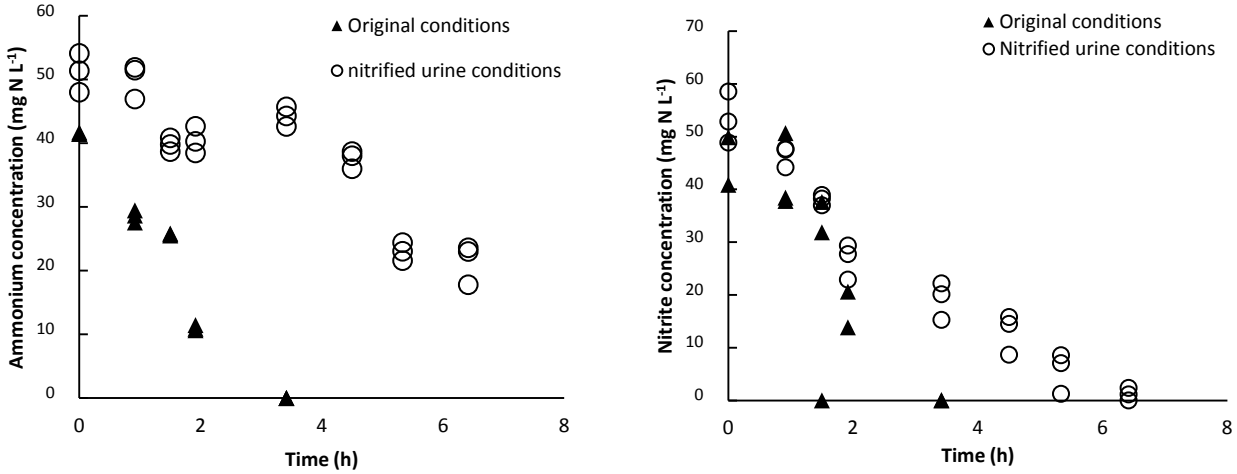

Figure 54: Results Imog – Moen for AOO (left) and NOB screening (right).

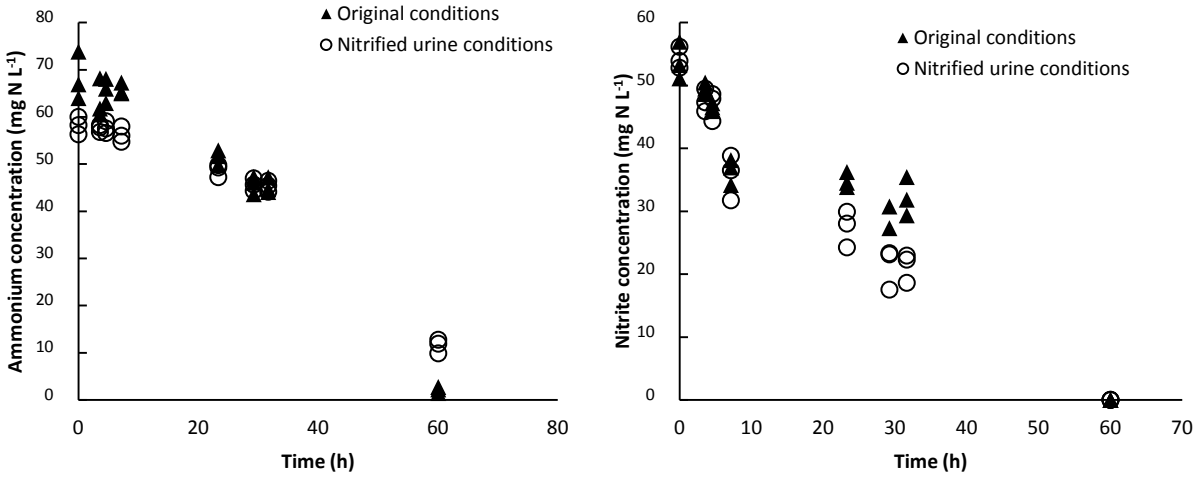

Figure 55: Results Decostere – Kortemark Results AOO screening (left) and NOB screening (right).

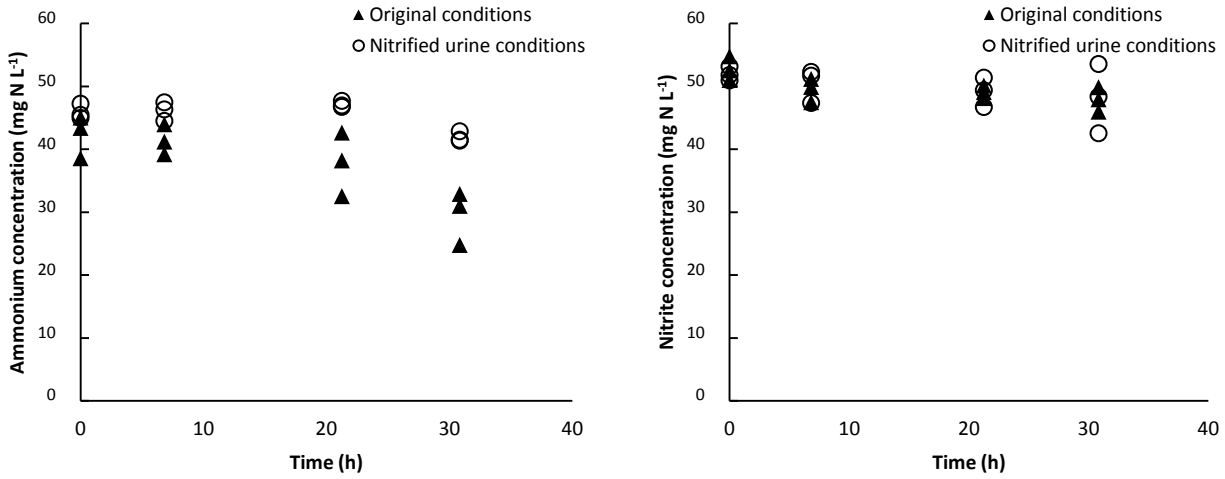

Figure 56: Results Danis NV – Izegem for AOO (left) and NOB screening (right).

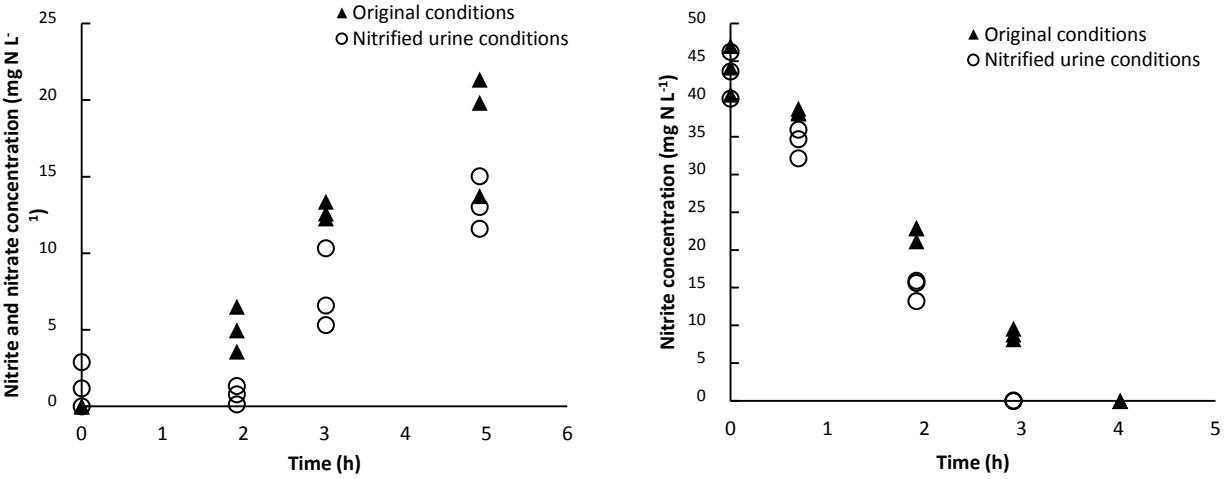

Figure 57: Results Avecom – Wondelgem for AOO (left) and NOB screening (right).

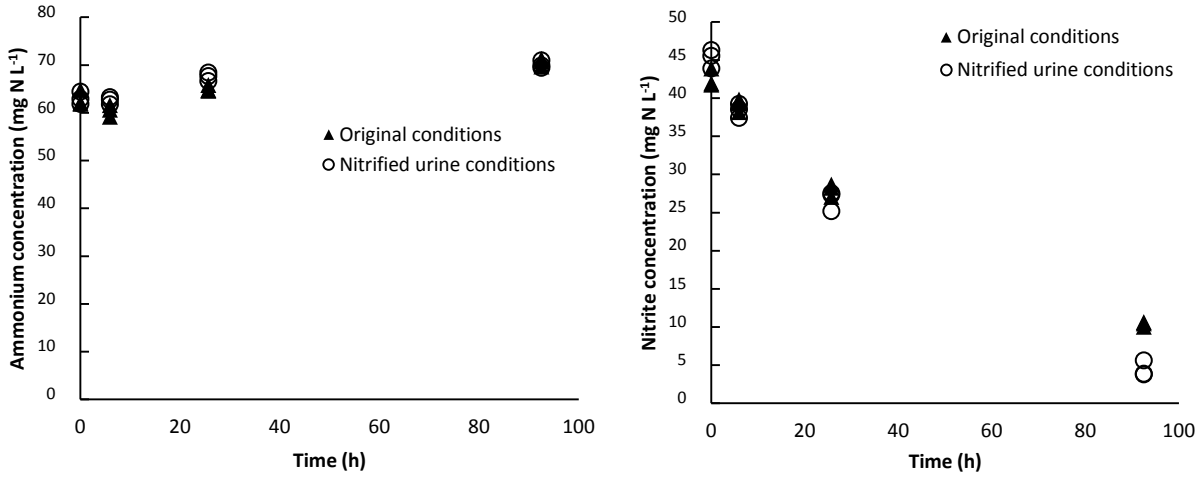

Figure 58: Results DOW – Terneuzen for AOO (left) and NOB screening (right).

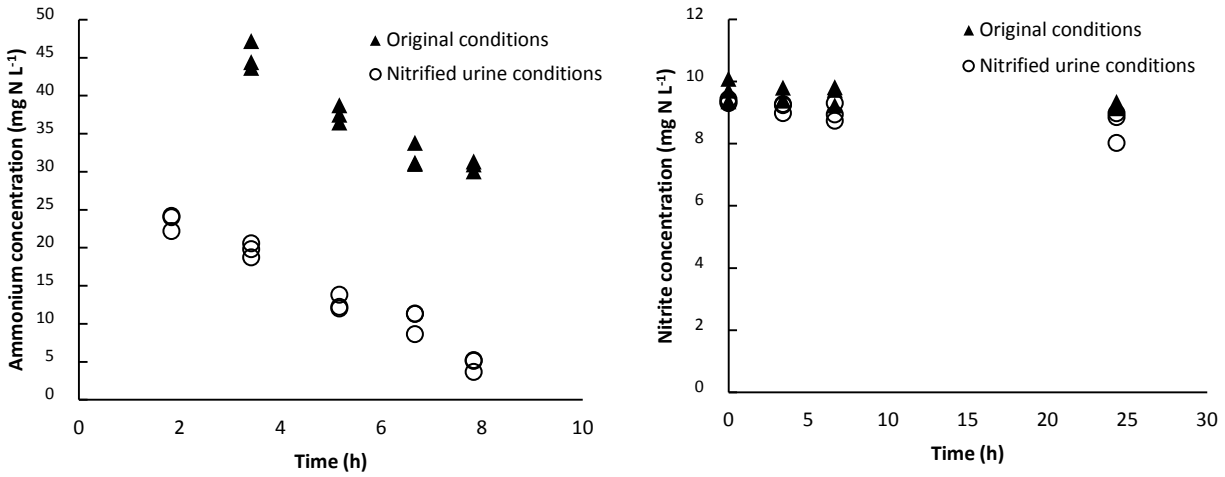

Figure 59: Results OLAND Labscale reactor – Ghent for AOO (left) and NOB screening (right).

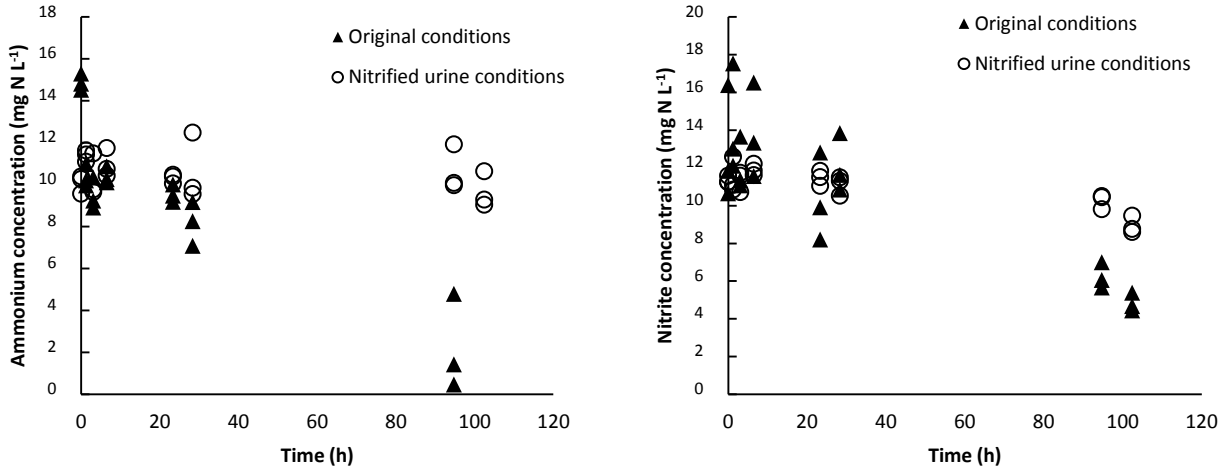

Figure 60: Results SeaLife – Ghent (Piranha) for AOO (left) and NOB screening (right).

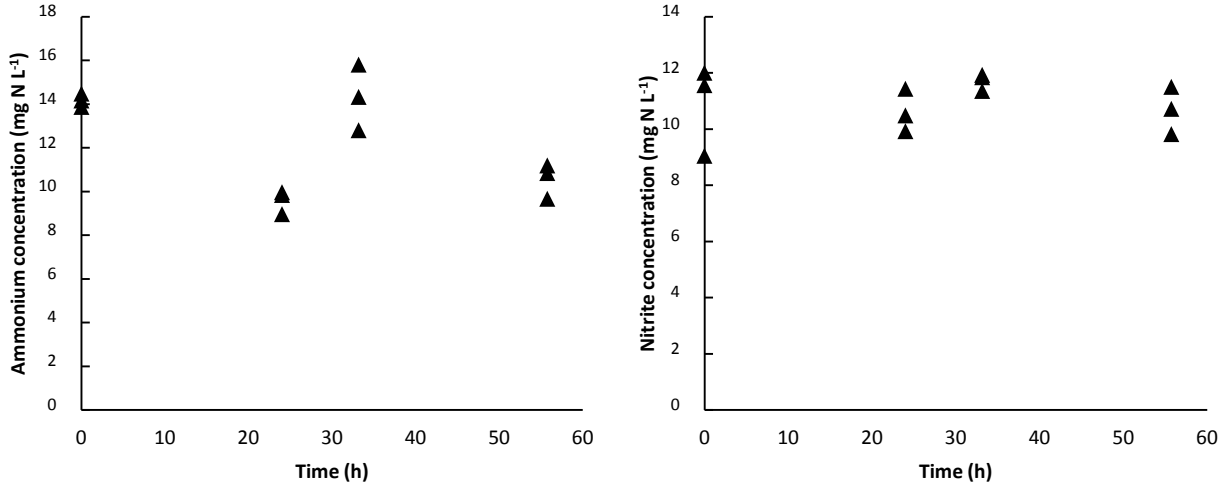

Figure 61: Results SeaLife – Ghent (Octopus) for AOO (left) and NOB screening (right).