UNIVERSITEIT GENT

Faculteit Bio-ingenieurswetenschappen

Academiejaar 2014 – 2015

Start-up of a nitrification reactor for wastewaters high in nitrogen and salinity: urine as a model

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Masterproef voorgedragen tot het behalen van de graad van
Master in de bio-ingenieurswetenschappen: Milieutechnologie

ACKNOWLEDGEMENTS

"If I have seen further it is by standing on the shoulders of Giants."
— Isaac Newton (1676)

Dear reader,

A year filled to the brink with deadly deadlines and clustering clusters has inexplicably changed something inside me. LabMET has been a wonderful environment to "do science" and maybe a place where I grew a bit more mature, though some would question this. But I know I enjoyed!

First of all, I would like to thank both my promotors, prof. dr. ir. Siegfried E. Vlaeminck and dr. ir. Peter Clauwaert. Siegfried, thank you for your guidance throughout the year concerning experimental design, your enthusiasm for research and your seemingly endless patience while you were awaiting my literature study. Peter, your endless knowledge about BLSS related stuff was a real help to put things in the right perspective. I would also like to thank you for proofreading my thesis with the greatest attention for detail.

The regular student-N-clusters and ESA clusters were an annoyance at first, but the fast follow-up on issues proved to be indispensible for the work I was performing and to improve my presentation skills.

Joeri, throughout the year you have always found a way to (re)motivate me. At times it was a tough cookie, but in the end everything was worth it. Thank you for operating my experiments while I wasn't around. I couldn't have wished for a better tutor. And you will still get a homemade Dr. Oetker cake from me!

All labmetians have contributed to the amazing experience, fun talks and valuable help in and out the lab. I want to especially express my gratitude towards Way, Xu, Marta, Cristina, Naya, Anthonin, Christine, Marlies, Dries, Chiara, Ralph, José, Oliver, and many more… for an unforgettable year!

To all students and internships at LabMET during 2014-2015: this was by far the hardest year in terms of workload, but in those hard moments you were always there (…like, for real). For everything related and unrelated to the lab, I wish to thank Koen, Veerle, Dorine, Esther, Nele, Abbas, Matthias, Mathias, Delphine, Pieter, Hildebrand, Domien, , Charlotte, …

To my team mates and buddies at the badminton. I didn't attend the training sessions on Tuesday and Thursday as much as I would have wanted (that's an understatement), but I enjoyed every moment I of the competition and the very rare training sessions which I did attend. Playing a competition match together or just some friendly sparring gave me a lot of mental energy to finish this work.

I would also like to thank collectively, everyone who taught me anything, and especially Mams and my high school teachers who sparked my interest in science and motivated me to pursue a degree in the field I know deeply love.

And last but certainly not least: my family for supporting me in more ways than I can express or imagine.

Wout Coessens – June 2015

ABSTRACT

Sustainability requires a paradigm shift in modern wastewater treatment systems toward higher resource efficiency. Source separated urine is an ideal target stream to implement resource recovery of nutrients, given its high nitrogen (N) concentration, however combined with a high salinity. Urea hydrolysis readily occurs in storage tanks yielding a pH increase. This triggers precipitation of salts (struvite, hydroxyapatite) and volatilization of ammonia (NH_3), leading to nutrient losses from the solution. In case of precipitating salts, scaling can obstruct pipelines and other equipment. In case of volatilization, additional emissions are posed upon the environment. An attractive solution is to stabilize urine by converting all its nitrogen to nitrate (NO_3^-) through nitrification. The rapid startup of urine nitrification reactors is indispensable for a bioregenerative life support system (BLSS) in space exploration, but challenging due to the slow growth of nitrifying microorganisms and the necessity of biomass adaptation to the high salinity. By rapid startup, costs and duration of the startup could be reduced. The startup period could be reduced by selection of an optimal inoculum. Subsequently, cultivation of microalgae, such as the cyanobacterium *Arthrospira platensis,* might present an appealing option to upcycle the nitrogen from the stabilized urine to organic nitrogen, available for reuse. *A*. *platensis* is a rich source of proteins, vitamins and amino acids. It is currently used as a supplement in aquaculture feed, fodder and it has been used for human consumption for centuries. The potential of *A*. *platensis* to grow on minimally diluted nitrified urine has not yet been explored.

A preliminary screening of twelve (12) nitrifying consortia was performed to determine the nitrification potential under high salt conditions and nitrogen loading rates. A commercially produced aquaculture inoculum (ABIL) was found to be the best performing nitrifying consortium due to high nitrogen oxidation rates (143 mg NH_4^+-N (g VSS)$^{-1}$ d^{-1} and 555 mg NO_2^--N (g VSS)$^{-1}$ d^{-1}) and low inhibition by a salt shock of 45 mS cm^{-1}.

With the optimal inoculum ABIL, a membrane bioreactor (MBR) was inoculated at 1.32 g VSS L^{-1}. Startup of urine nitrification in the MBR was the fastest so far achieved on undiluted urine (12 days). During the operation, a maximum nitrogen loading rate of 500 mg N L^{-1} d^{-1} was achieved with synthetic urine. Due to failure of the pH controller on day 68 and power shutdown on day 69, the adapting biomass was severely shocked and the nitrogen loading rate had to be reduced. Recovery of nitrification performance was achieved up to 250 mg N L^{-1} d^{-1}. The synthetic influent was substituted by real human urine on day 118. A loading rate could be maintained at 250 mg N L^{-1} d^{-1}. This is the first urine nitrification reactor system fed with real urine without nitrite accumulation.

For nutrient recovery and upgrading, the feasibility of *A. platensis* cultivation was investigated on different N sources (nitrate, ammonium and urea) at different concentrations and salinities by modification of standard Zarrouk medium. The most optimal nitrogen source was nitrate, at a nitrogen concentration of 1 g N L^{-1}. Higher concentrations of nitrate had an inhibitory effect on the growth of *A*. *platensis*. The effect of salinity on growth was limited. In a second experiment, growth feasibility on non-hydrolyzed, hydrolyzed and nitrified urine was assessed. The latter was the most optimal medium at a concentration of 1 g N L^{-1}. A (semi) continuous microalgae cultivation system fed with undiluted urine may be feasible when operated correctly. Urine nitrification treatment and subsequent valorization (single cell protein – SCP, agricultural fertilizers, oxygen production in bioregenerative life support systems – BLSS) shows high potential for increasing the efficiency of the nutrient cycle, lowering environmental impact as well as pressure on natural resources.

Samenvatting

Duurzaamheid vereist een verschuiving van hedendaagse waterzuiveringstechnieken (nutriënten verwijdering) naar nutriënten herwinning. Brongescheiden urine is een ideale afvalstroom voor nutriëntenherwinning, gegeven de hoge stikstofconcentratie. Een moeilijkheid voor de applicatie van brongescheiden urine is de hoge saliniteit. De hydrolyse van ureum aanwezig in urine gebeurt vlot in opslagcontainers en resulteert in een stijging van de pH. Dit induceert de precipitatie van zouten (struviet, hydroxyapathiet) en vervluchtiging van ammoniak(NH_3). Dit zorgt ervoor dat nutriënten uit de vloeistof verloren gaan. Precipiterende zouten kunnen verstoppingen veroorzaken. Ammoniakvervluchtiging zorgt voor extra emissie in het milieu. Een mogelijke oplossing voor deze problemen is om de urine te stabiliseren door organische stikstof en ammonium om te zetten naar nitraat (NO_3^-) door middel van nitrificatie. De snelle opstart van nitrificatiereactors voor urine is van groot belang voor bioregenerative life support systems (BLSS) in de ruimtevaart. Dit wordt bemoeilijkt door de trage groei van nitrificerende micro-organismen en de noodzaak om de biomassa te adapteren aan het hoge zoutgehalte. De kosten en de tijd nodig voor op het opstarten van een dergelijke reactor worden op deze manier gereduceerd. De opstartperiode kan verkort worden door het selecteren van een optimaal inoculum. Vervolgens kan de nitraat stikstof van de gestabiliseerde urine worden omgezet in organische vorm, beschikbaar voor nutriënten hergebruik, door de cultivatie van micro-algen zoals de cyanobacterie *Arthrospira platensis. A. platensis* is een rijke bron aan proteïnen, vitaminen en aminozuren. Hedendaags wordt het gebruikt als supplement in de aquacultuur en in veevoeder. Deze cyanobacterie wordt ook reeds lang gebruikt voor menselijke consumptie. Er werd nog niet onderzocht of *A. platensis* kan gecultiveerd worden op minimaal verdunde genitrificeerde urine.

Een verkennende studie werd uitgevoerd met twaalf (12) nitrificerende consortiums om de nitrificatiecapaciteit te bepalen onder hoge saliniteit en hoge stikstofbelasting. Een commercieel geproduceerd inoculum voor aquacultuur (ABIL) werd als best presterend consortium geselecteerd. Dit consortium heeft een hoge tolerantie voor zoutschokken naar 45 mS cm^{-1} en hoge specifieke oxidatiesnelheden van ammoniak en nitriet (143 mg NH_4^+-N (g VSS)$^{-1}$ d^{-1} en 555 mg NO_2^--N (g VSS)$^{-1}$ d^{-1}).

Een membraanbioreactor (MBR) werd geïnoculeerd met ABIL bij een VSS-concentratie van 1.32 g VSS L^{-1}. De opstart van de MBR werd vervolledigd na slechts 12 dagen, het snelste tot op heden voor een nitrificatie reactor voor onverdunde urine. Gedurende de werking van de MBR werd een maximale stikstofbelasting van 500 mg N L^{-1} d^{-1} gehaald met synthetische urine. Wegens het falen van de pH-controller op dag 68 van operatie en het stilleggen van de elektriciteit in het gebouw op dag 69 werd de biomassa hevig geschokt. De stikstofbelasting moest worden verlaagd om de stabiele operatie van de reactor te kunnen blijven garanderen. Het herstel van nitrificatie was beperkt tot een stikstofbelasting van 250 mg N L^{-1} d^{-1}. Op dag 118 werd het synthetische influent vervangen door gecollecteerde menselijke urine. De stikstofbelasting kon worden behouden op 250 mg N L^{-1} d^{-1}. Het is de eerste keer dat een nitrificerend systeem voor menselijke urine geen nitrietaccumulatie vertoont.

Voor de nutriëntenherwinning werd de cultivatie van *A. platensis* onderzocht met verschillende stikstofbronnen (nitraat, ammonium en ureum), elk bij verschillende concentraties en verschillende zoutgehaltes.De verschillende media werden bereid op basis van het standaard Zarrouk medium, met aangepaste stikstofbron, concentratie en/of zoutgehalte. Nitraat bij een stikstofconcentratie van

1 g N L^{-1} werd het beste benut. Hogere concentraties zorgden ervoor dat groei werd geïnhibeerd. Het effect van een verhoogde saliniteit was gering. In een tweede experiment werd de groei op een urinematrix onderzocht. Verse (niet-gehydrolyseerde), gehydrolyseerde en genitrificeerde urine werden als medium gebruikt. Deze laatste was het meest optimale medium, tevens bij een concentratie van 1 g N L^{-1} zoals bevestigd door de eerste test. Een (semi) continu systeem voor de cultivatie van algen op genitrificeerde urine lijkt mogelijk mits adequate systeemcontrole.

De behandeling van urine door nitrificatie en opeenvolgende valorisatie (microbieel eiwit, meststoffen of zuurstofproductie in BLSS systemen) heeft het potentieel om de efficiëntie van de nutriëntencyclus te verhogen alsook om de impact op het milieu en natuurlijke grondstoffen te verminderen.

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ABBREVIATIONS

AOA	Ammonia Oxidizing Archaea
AOB	Ammonia Oxidizing Bacteria
AOM	Ammonia Oxidizing Microorganisms
BLSS	Bioregenerative Life Support System
COD	Chemical Oxygen Demand
DO	Dissolved Oxygen
EC	Electrical Conductivity
FA	Free Ammonia
FNA	Free Nitrous Acid
HRT	Hydraulic Retention Time
MBR	Membrane Bioreactor
N	Nitrogen
NOB	Nitrite Oxidizing Bacteria
SBR	Sequencing Batch Reactor
sNLR	specific Nitrogen Loading Rate
TKN	Total Kjeldahl Nitrogen
TSS	Total Suspended Solids
vNLR	volumetric Nitrogen Loading Rate
VSS	Volatile Suspended Solids
WWTP	Wastewater Treatment Plant

PART 1: Literature study

1 Nutrient recovery

In the light of rising consumption levels of natural resources on a global scale, technologies related to the recovery of rare earth materials, nutrients and energy need to be further explored and optimized. In this study, the focus is on nitrogen recovery from urine.

1.1 Nitrogen recovery on earth

Nitrogen (N) is an essential element to life, as it is a constituent of many biomolecules such as amino acids, proteins, and DNA. Even though nitrogen is abundant in the atmosphere, it is sequestered in the form of inert nitrogen gas (N_2), which makes up approximately 78% of the atmosphere. In order to grow, living organisms need a more reactive nitrogen compound which can be metabolized.

Nitrogen fixation, or the production of reactive nitrogen from nitrogen gas, is achieved by various processes (biological fixation, anthropogenic fixation, lightning events, etc.). Biological nitrogen fixation is accomplished by prokaryotes. Several specialized bacteria and archaea are capable of nitrogen fixation. These organisms are called diazotrophs. *Rhizobia* bacteria in the root nodules of *Leguminosae* plants, as well as free-living bacteria such as *Cyanobacteria* act as biological operators for nitrogen fixation (Vlaeminck 2009). Although biological nitrogen fixation is thus possible, the fixation rates may be too low to have industrial relevance. Anthropogenic fixation of nitrogen is therefore achieved via the Haber Bosch process (equation (1)), which is the most widely used production pathway for N fertilizers.

$$\mathbf{3H_2 + N_2 \rightarrow 2NH_3} \qquad \mathbf{\Delta H_f^0 = -92.4 \frac{kJ}{mol\ NH_3}} \qquad \textbf{(1)}$$

The process is an exothermic reaction of hydrogen gas (H_2) with N_2, resulting in formation of ammonia (NH_3). The reaction proceeds under high pressure and temperature (300 bar and 500 °C) to overcome the triple covalent bond of dinitrogen (941 kJ (mol N_2)$^{-1}$) and therefore results in a high energy demand. Current state of the art technologies require 37 MJ kg^{-1} NH_3-N (Maurer et al. 2003). Due to the high demand for fertilizers, combined with high energy demand per kilogram ammonia produced, the Haber Bosch industry consumes 2% of the global energy demand and accounts for 1.6% of the total anthropological CO_2 emissions (Liu 2014) since the hydrogen is generally produced from fossil fuels (e.g. methane reforming). In Flanders alone, 0.533 Tg N is annually fixed via this energy intensive industry (Coppens et al. 2013). Nitrogen fixation also occurs in the atmosphere during lightning events, although this production rate is rather modest (globally 2.4 Tg N y^{-1}; Galloway et al. 2004) in comparison with anthropogenic nitrogen fixation in the Haber Bosch process. Today, an estimated 132 Tg N are produced annually by the Haber-Bosch process, mainly for use in fertilizers as a nitrogen source, but also for use in explosives and the chemical industry (Tanabe & Nishibayashi 2013).

It is clear that to sustain a growing population, a sustainable alternative, in terms of eco-effectiveness as well as an eco-efficiency, to the fossil fuel driven ammonia production is required. The ammonia production through the Haber Bosch process can become eco-effective by using hydrogen that is generated from renewable energy sources (e.g. electrolysis driven by wind power),

however this is no guarantee that the process is eco-efficient (e.g. in terms of energy efficiency) as well. The recovery of nitrogen from waste streams can be both eco-effective as well as eco-efficient if e.g. renewable energy is used to recover the nitrogen in a more energy efficient way than the Haber Bosch process.

High concentrations of reactive nitrogen are already present in many waste streams (human urine, thin manure digestate, industrial wastewater, etc.) and can be recovered physicochemically. In domestic wastewater, 80% of the nitrogen originates from urine (Jin et al. 2013). Despite this, the volume contributed by urine is less than 1%. Present wastewater treatment installations aim to remove nitrogen from domestic wastewater, rather than recovery. This way, reactive nitrogen is prevented to access local water bodies which are vulnerable to eutrophication and degradation of biodiversity. Processes presently used for removal of nitrogen are nitrification/denitrification, nitritation/denitritation and Anammox (Vlaeminck et al. 2012). However, in the current view of sustainability, focus is shifting from nitrogen removal to nitrogen recovery. For nitrogen recovery to become economically viable, concentrated waste streams are targeted to optimize the nitrogen recovery yield and reduce costs. As previously mentioned, thin manure digestate, industrial wastewater and human urine (source separated) are potential candidates for sustainable nitrogen recovery.

1.2 MELiSSA

Crew members on long duration space missions are in need of supplies. Food, water, oxygen, as well as tools and spare parts are among the necessities of life in space. All of these are currently delivered to the crew by an extremely expensive delivery system, with an estimated cost of U.S. $30,000 per kg payload sent to the International Space Station (ISS) (Berkovich et al. 2004). There are efforts by SpaceX to design and test rockets which can return after a mission and thus make spaceflight more renewable (SpaceX 2013). There's still a problem when it comes to manned missions to other planetary bodies (for example Mars). Frequent supplying to Mars is not desirable, because Mars is between 54.6 million and 401 million kilometers from earth (depending on its relative position to earth) and the journey time is estimated to last 210 days (Menezes et al. 2015). This is were closed ecological systems (CES) come into the picture. Closed ecological systems are artificial systems which resembles an ecosystem, only that they are man-made (Nelson et al. 2003). CES are used to investigate relations of components of ecosystems. They can also be used for investigating life support system for space exploration and habitation. Examples of such systems are NASA's CELSS (Controlled Ecological Life Support System), University of Arizona's Biosphere2 or the Russian Bios-3 in Krasnoyarsk.

The European Space Agency (ESA) has been developing its own life support system. A complete gas, liquid and solid waste recovery system, named the Micro Ecological Life Support System Alternative (MELiSSA). It aims to regenerate oxygen, potable water and food (higher plants, as well as edible phototrophic bacteria) from carbon dioxide, urine and feces. Figure 1 represents a schematic overview of the MELiSSA concept. It consists of 5 compartments: an anaerobic compartment, a photoheterotrophic compartment, a nitrifying reactor, a photoautotrophic compartment and a crew compartment.

The anaerobic compartment digests the inedible crop residues, kitchen waste and faeces to produce volatile fatty acids (VFA) and carbon dioxide. Complex molecules are hydrolyzed and converted to CO_2, VFA and ammonia by acidogenesis. The reactor is operated slightly acidic pH (~5,3) and at high

temperature (55°C), preventing methanogenesis, which would exclude valuable carbon from the closed system in the form of CH_4 (Hendrickx et al. 2006).The second compartment aims to remove VFA through the conversion into edible biomass and CO_2 by photoheterotrophic bacteria (*Rhodospirillum rubrus*) (Hendrickx et al. 2006; Vrati 1984).

Following the conversion of VFA, ammonia remains in the waste stream. Ammonia is a volatile substance and toxic in the gas phase. Ammonia release into the crew compartment, may cause serious problems due to odor nuisance and toxicity. In order to retain all nitrogen in liquid form for the phototrophic growth of biomass, ammonia is converted to nitrate via the nitrification pathway. Plants and cyanobacteria can assimilate nitrogen via ammonium and nitrate. However, nitrate is the preferred substance in space applications because it is neither volatile nor toxic to humans, while ammonia is both volatile and toxic to humans. Furthermore, it was shown that ammonia can be toxic to the roots of tomato plants at concentrations of 3.0 mmol L^{-1} (Ti-da et al. 2008). Ammonia oxidizers and nitrite oxidizers work together to form nitrate in the third compartment. During this process, oxygen is consumed.

In the fourth compartment, higher plants and cyanobacteria are grown which are used as food resource (Gòdia et al. 2004). Carbon dioxide is consumed and oxygen produced. Nutrients such as N, P and K are extracted from the stream and assimilated by plants. Also potable water is recovered from evapotranspiration of higher plants.

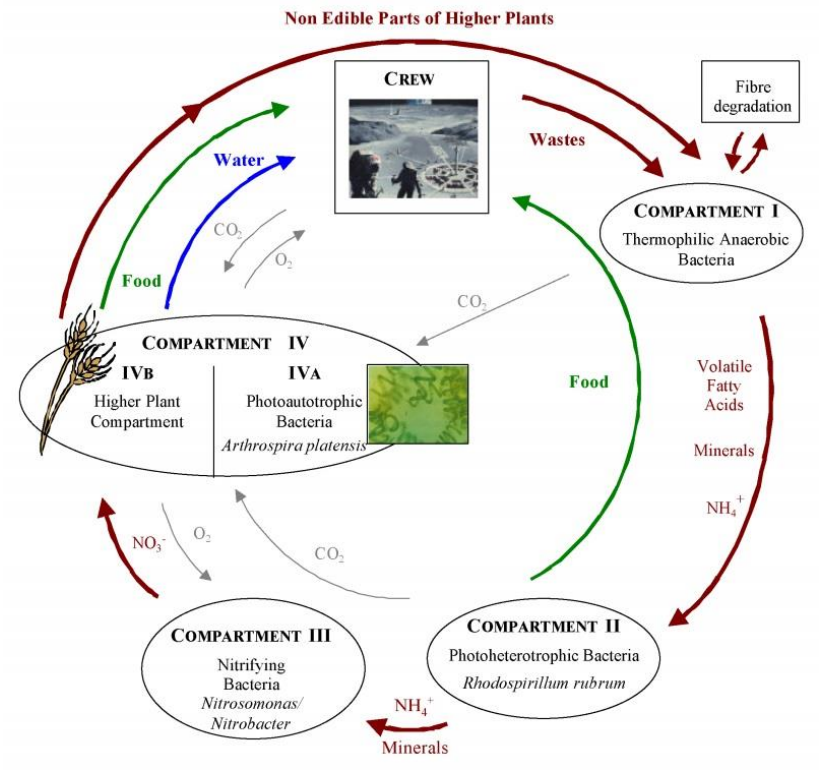

Figure 1: Scheme of the MELiSSA loop (Micro-Ecological Life Support System Alternative). Figure from Hendrickx et al. 2006

In this study, the focus is on the nitrification of ammonia to nitrate. Hence the nitrification compartment (C III) is of special interest. Volatilization of components in the closed ecological system are mostly not desired in space, for practical and operational reasons. Reaction rates become

optimal when only one phase (liquid) occurs which is easier to mix than two different states (for example liquid and gaseous).

When dosing urine to the anaerobic compartment, methanogenesis is inhibited by high concentrations of ammonia. This prevents production of the undesired methane. However, not only methanogenesis is repressed, also the fermentation experiences toxic effects by the high ammonia concentrations when urine hydrolyzes. For that reason it is better to dose the urine directly to the nitrification compartment instead of the anaerobic compartment.

2 Urine

Human urine is conventionally considered a waste product, but due to the high nutrient concentrations, it is a very interesting stream for nutrient recovery for both terrestrial as space applications.

2.1 Composition

Maurer et al. (2006) reports on the composition of urine from different urine collection systems (Table 1). Urine is characterized by high concentrations of nitrogen (~ 9 g N L^{-1}), which is predominantly present as urea (85% of total N) in fresh urine (Udert et al. 2006). Other nitrogen containing molecules include amino acids, creatine, creatinine, but these are only present in a far lower extent. Also high concentrations of phosphate-phosphorous (0.8 to 2.0 g $P.L^{-1}$) and high salt concentrations define this waste stream (Maurer et al. 2006).

Urine composition nevertheless varies not only per person, but also over time due to variations in diet and daily activities. Also processing of urine may change composition: e.g. non-sterile storage tanks or transport piping, addition of Ca/Mg when diluted with tapwater.

Table 1: Composition of undiluted fresh human urine and undiluted human urine collected from a workplace collection system (fully hydrolyzed) – Table from Maurer et al. (2006)

Parameter	Unit	Fresh urine	Workplace
pH	(-)	6,2	9,1
N_{tot}	mg N L^{-1}	8830	9200
TAN	mg N L^{-1}	463	8100
P_{tot}	mg P L^{-1}	800-2000	540
Na	mg Na L^{-1}	3450	2600
Cl	mg Cl L^{-1}	4970	3800
Ca	mg Ca L^{-1}	233	0
Mg	mg Mg L^{-1}	119	0

2.2 Storage

Storage of urine is difficult because of the microbial hydrolysis of urea. Ureolysis results in ammonium, ammonia and bicarbonate according to equation (2). Due to the production of ammonia, the pH will increase.

$$\mathbf{CO(NH_2)_2 + 2H_2O \xrightarrow{urease} NH_3 + NH_4^+ + HCO_3^-} \quad \textbf{(2)}$$

During storage and in presence of urease, hydrolysis proceeds and raises the pH of the storage solution, as can be clearly seen when comparing urine from a storage or collection tank with fresh urine (Table 1). The rise of pH triggers a shift of ionic equilibria. The results of hydrolysis during storage and subsequent precipitation of phosphate minerals are clear from Table 1: total ammonia nitrogen concentration (TAN), has increased due to urease activity while total nitrogen remains in the same range. In tandem, pH rises from slightly acidic (pH 6.2) up to pH 9.1. Total phosphate concentrations are much lower (depending on the sample) while calcium and magnesium, being common solutes integrating in phosphate minerals, have been totally depleted from the solution. The latter is explained by the precipitation of struvite, hydroxyapatite, octacalcium phosphate, etc. at high pH (Udert, Larsen & Gujer 2003). In diluted urine, calcite ($CaCO_3$) is a common precipitate, since tap water usually contains a lot of calcium. This can result in the clogging of pipes, causing the need for regular maintenance of the infrastructures.

It is clear that precipitation of phosphate minerals is undesirable due to clogging and nitrogen loss. However, urease-producing bacteria are considered omnipresent (Mobley & Hausinger 1989), complicating storage of urine and compromising full nitrogen recovery. Two strategies can be adopted to prevent nitrogen loss from source separated urine during storage. Addition of 60meq sulfuric acid or acetic acid per liter of urine is sufficient to acidify the solution and stop hydrolysis (Hellström et al. 1999). The equilibrium between ammonia (NH_3) and ammonium (NH_4^+) shifts to the latter at low pH, as the pKa for the equilibrium NH_3/NH_4^+ is 9.3. As a result, total ammonia nitrogen already present in fresh urine will be contained and will not volatilize, as ammonium is very soluble and ammonia is rather volatile. At the same time, phosphate equilibria are shifting towards phosphoric acid, reducing the concentration of phosphate ions in the solution, which in turn reduces the precipitation potential of phosphate minerals (Udert, Larsen & Gujer 2003).

The second strategy to inhibit urease activity, is to add base to inhibit bacterial ureolytic activity. There are a few points of attention to this method. To begin with, raising the pH induces precipitation, which means precipitation should occur in a controlled way to avoid clogging issues. The removal of bivalent cations (Ca^{2+}, Mg^{2+}, …) during this precipitation reduces the risk of scaling when using membrane based technologies downstream in the urine treatment train. Secondly, the NH_3/NH_4^+ equilibrium is shifting towards ammonia, which means, as soon as hydrolysis starts, pH will still be rather high, meaning the formed ammonia could volatilize easily, another cause of nitrogen loss, reducing the recovery efficiency once more. On the other hand, if the ureolysis occurs in a nitrification reactor, where ammonia oxidation increases the proton concentration, the urea hydrolysis occurs at a neutral pH and the base dosed for storing reduces the need to dose base in the nitrification reactor significantly.

3 Nitrification

3.1 General principles

Nitrification is the biological oxidation of ammonia (NH_3) to nitrate (NO_3^-). It is an important part of the nitrogen cycle, as it converts potentially toxic ammonia, while the nitrate produced is used by higher plants as substrate for growth. Nitrification is a two-step process. First, ammonia is oxidized to nitrite and subsequently nitrite is oxidized to nitrate. Two groups of bacteria are responsible for these processes.

The first group are the chemolithoautotrophic ammonia oxidizing bacteria (AOB) typically members of *Nitrosomonas* and *Nitrosococcus* genera. The oxidation is facilitated by (bacterial) ammonia monooxygenase (AMO), enzymes present in AOB. This first step of the nitrification process is called nitritation and the complete reaction is given in equation (3) (Wiesmann & Libra 1999).

$$\mathbf{NH_4^+ + 1.98\ HCO_3^- + 1.38\ O_2 \rightarrow 0.0182\ C_5H_7O_2N + 0.98\ NO_2^- + 1.04\ H_2O + 1.89\ H_2CO_3} \quad \textbf{(3)}$$

Cell biomass elemental composition is assumed to be $C_5H_7O_2N$. The nitritation reaction results in a pH decrease because more protons are generated than consumed in the process of nitrite formation (Kowalchuk & Stephen 2001).

$$\mathbf{NH_3 + O_2 + 2\ H^+ + 2\ e^-} \xrightarrow{\textbf{AMO}} \mathbf{NH_2OH + H_2O}$$

$$\mathbf{NH_2OH + H_2O} \xrightarrow{\textbf{HAO}} \mathbf{NO_2^- + 5\ H^+ + 4\ e^-}$$

$$\mathbf{0.5\ O_2 + 2\ H^+ + 2\ e^-} \xrightarrow{\textbf{terminal oxidase}} \mathbf{H_2O}$$

$$\mathbf{NH_3 + 1.5\ O_2} \xrightarrow{\textbf{AMO}} \mathbf{NO_2^- + H^+ + H_2O}$$

Besides bacteria, also chemolithoautotrophic members of the archaeal domain, are capable to oxidize ammonia. These ammonia oxidizing archaea (AOA) use a similar enzyme to the bacterial AMO's (Könneke et al. 2005; Nicol & Schleper 2006; J. You et al. 2009). Recent studies (Brown et al. 2013) have found a higher abundance of these AOA's in comparison to AOB in aquaculture systems. In general, ammonia oxidizing microorganisms (AOM) are either members of AOB or members of AOA.

The second group of bacteria consists of nitrite oxidizing bacteria (NOB). These obligate aerobic bacteria use nitrite as electron donor, oxidizing it to nitrate with oxygen as electron acceptor. This step of the nitrification process is called nitratation and is given by equation (4) (Wiesmann & Libra 1999). Typical NOB species are *Nitrospira* and *Nitrobacter*.

$$\mathbf{NO_2^- + 0.02\ H_2CO_3 + 0.48\ O_2 + 0.005\ NH_4^+ + 0.005\ HCO_3^-} \rightarrow \mathbf{0.005\ C_5H_7O_2N + NO_3^- + 0.015\ H_2O} \quad \textbf{(4)}$$

3.1.1 Nitrification kinetics: K- and r-strategists

In respect to substrate uptake kinetics, two types of (micro)organisms are distinguished. Microorganisms classified as r-strategists have a low substrate affinity and a high maximum uptake rate. These r-strategists are opportunistic and outcompete other species in the presence of high substrate concentrations. Applying Monod-kinetics (equation (5)), a high half saturation constant (K_s) and high maximum growth rate (μ_{max}) are typical features of r-strategists. Organisms applying this strategy are usually found abundantly in concentrated waste streams, such as manure digestate and many industrial waste streams.

$$\mu = \mu_{max} \cdot \frac{S}{K_s + S} \quad \textbf{(5)}$$

Another strategy is the K-strategy. It involves a high substrate affinity (low K_s), enabling it to utilize very low concentrations of substrate more efficiently than competitor species, as depicted in Figure 2. K-strategists are dominant species in systems with low concentrations of substrate, such as aquaria or pond systems. As Brown et al. (2013) found AOA being more abundant in aquaculture systems, this suggests AOA are mostly K-strategists.

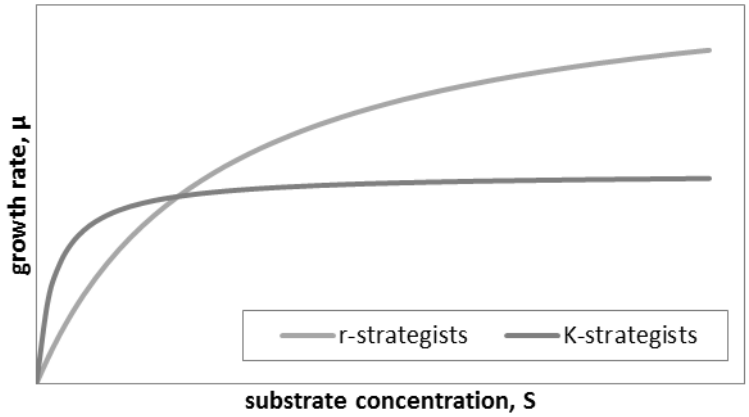

Figure 2: Effect of substrate concentrations (S) on growth rate (μ) of r-strategists and K-strategists based on the Monod equation

Examples of nitrite oxidizing r-strategists are members of the *Nitrobacter*-genus, with typically high specific substrate oxidation rates of around 93.8 mg NO2--N g^{-1} NOB h^{-1} (Kim & Kim 2006). In contrast, members of the genus *Nitrospira* are clear K-strategists, having very low substrate oxidation rates around 10.5 mg NO2--N g^{-1} NOB h^{-1} (Kim & Kim 2006). Ammonia oxidizing *Nitrosomonas* and *Nitrosospira* respectively have a high (up to 4.2 mg NH_3-N.L^{-1}) and low (0.56 mg NH_3-N.L^{-1}) half saturation constant (Suzuki & Dular 1974; Schramm et al. 1999).

An overview of literature K_s values is given in Table 2.

Table 2: K_s values of several genera involved in nitrification.

Type/Genus	K_s (mg N/L)	References
Ammonia oxidizers		
Nitrosomonas	2.8 – 4.2	Suzuki & Dular (1974)
	14	Belser & Schmidt (1980)
Nitrosospira	0.56	Schramm et al. (1999)
Nitrite Oxidizers		
Nitrobacter	1.9	Blackburne et al. (2007)
Nitrospira	0.14	Schramm et al. (1999)

3.2 Process parameters

The performance of the nitrification process is dependent on various parameters. A summary of the optimal range for each parameter discussed is given in Table 4. It should be noted that salinity is influencing the nitrification process, but is not listed among the discussed process parameters. In section 4.1 the effects of salinity are discussed in greater detail.

3.2.1 Inhibition by free ammonia (FA) and free nitrous acid (FNA)

Ammonia oxidizing microorganisms (AOM) use the unionized free ammonia (FA), as substrate, rather than the ionized ammonium (Suzuki & Dular 1974). In a similar way, NOB prefer to consume unionized free nitrous acid (FA), HNO_2, instead of the ionized nitrite, NO_2^-. However, both compounds are toxic to both AOM and NOB. Anthonisen et al. (1976).

Table 3: Nitrification inhibition of AOB and NOB by the substances Free Ammonia (FA) and Free Nitrous acid (FNA), as reported by Anthonisen et al. (1976)

Component	AOB	NOB
FA (mg $N.L^{-1}$)	8 – 120	0.08 – 0.82
FNA (mg $N.L^{-1}$)	0.2 – 2.8	0.06 – 0.83

Concentrations of both FA and FNA are in equilibrium with their ionized forms ammonium and nitrous acid (equations (6) and (7)).

$$\mathbf{NH_3 + H^+ \leftrightarrow NH_4^+} \qquad \boldsymbol{pK_a = 9.25} \qquad \textbf{(6)}$$

$$\mathbf{NO_2^- + H^+ \leftrightarrow HNO_2} \qquad \boldsymbol{pK_a = 3.40} \qquad \textbf{(7)}$$

The present concentrations of NH_3 and HNO_2 are dependent on pH and temperature as well as total ammonia nitrogen (TAN) and total nitrite nitrogen (TNO_2) respectively. The relationships are given in equations (6) and (7). Note that the pK_a-value of both equations is true at a temperature of 25°C. Temperature dependence of the acid dissociation constant (K_a) is given by the Van 't Hoff equation (8). At higher pH, toxic NH_3 becomes the predominant form in the system, while at lower pH, toxic HNO_2 takes over from NO_2^-. Because both unionized forms are substrate as well as inhibitor, pH influences activity of the nitrifiers (AOM and NOB) in different ways as will be discussed in section 3.2.2.

$$\frac{\mathbf{d}\left(\mathbf{ln}(\mathbf{K_a})\right)}{\mathbf{d}(\mathbf{T})} = \frac{\mathbf{\Delta H^0}}{\mathbf{RT^2}} \qquad \textbf{(8)}$$

Free nitrous acid (FNA) is another toxic molecule to nitrifiers. In systems with pH as low as 7.5, FNA is formed when nitrite accumulates. Nitrite buildup can occur at low DO concentrations as well as in systems in which AOM have higher oxidation rates than NOB. FA is the prominent inhibitory factor at pH above 8 and FNA is the prominent inhibitor at pH below 7.5 (Van Hulle et al. 2010).

3.2.2 pH

The influence of pH on the ratios NH_3/NH_4^+ and NO_2^-/HNO_2 is explained in 3.2.1.Another issue at low pH is the CO_2 stripping as showed in equation (9).

$$\mathbf{HCO_{3,aq}^- + H^+ \leftrightarrow H_2CO_{3,aq} \leftrightarrow H_2O + CO_{2,gas}} \qquad \textbf{(9)}$$

Inorganic carbon is the main carbon source for AOM and NOB. Bicarbonate (HCO_3^-) is the main source of carbon. When pH decreases, the equilibrium in equation (9) shifts to less soluble carbon dioxide (CO_2). As a result, carbon escapes to the atmosphere and the nitrifiers are depleted of their

preferred carbon source. NOB have been reported to grow mixotrophically (Degrange et al. 1997). In mixed populations however, heterotrophs utilize most organic substrate and mixotrophic growth of NOB is limited. Hence, growth and nitrification become restricted when bicarbonate is depleted or volatilized.

Bicarbonate is the main buffer present in regular wastewaters, consuming protons while preventing a pH-drop at the cost of carbon losses from the wastewater.

Due to the equilibria of NH_3/NH_4^+, NO_2^-/HNO_2 and HCO_3^-/ H_2CO_3 combined, the optimal pH is around 8, as can be seen in Figure 3 (a) and (b) (Grunditz & Dalhammar 2001).

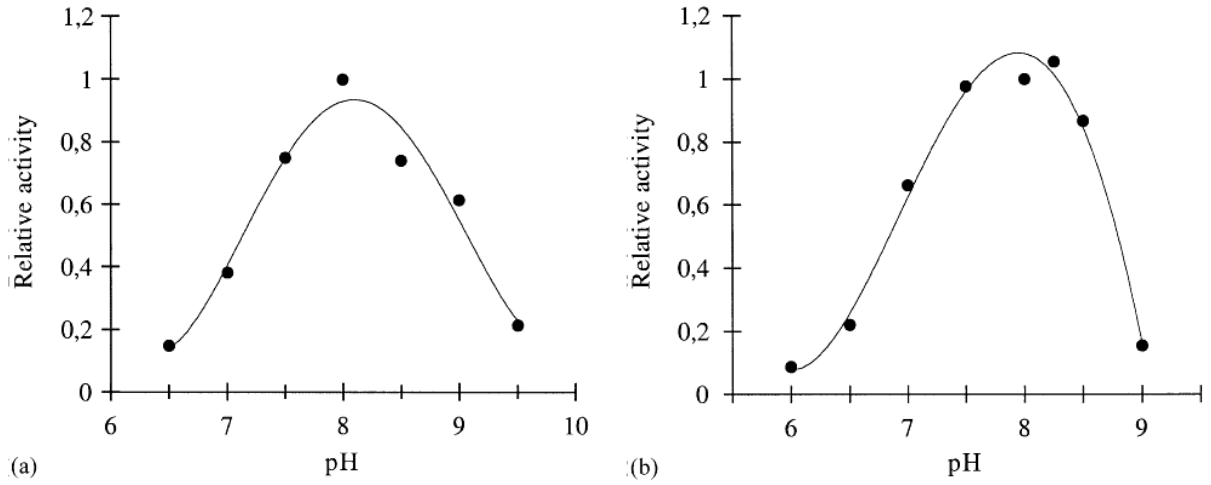

Figure 3: Relative ammonia (a) and nitrite (b) oxidation activity of respectively *Nitrosomonas* and *Nitrobacter* related to pH. Figure from Grunditz & Dalhammar 2001

3.2.3 Oxygen

In equations (3) and (4), oxygen is consumed and respectively ammonia and nitrite are oxidized. In systems with limited dissolved oxygen (DO), competition for DO occurs between AOM, NOB and heterotrophic microorganisms. Hunik et al. (1994) found half saturation constants for ammonia oxidizer *Nitrosomonas europaea* and nitrite oxidizer *Nitrobacter agilis* to be 0.16 mg O_2 L^{-1} and 0.54 mg O_2 L^{-1} respectively. Ammonia oxidizers (represented by *N. europaea* in the study of Hunik and colleagues) are able to thrive at lower oxygen concentrations compared to NOB (represented by *N. agilis* in the study of Hunik and colleagues). Oxygen half saturation constants for AOB and NOB of respectively 0.2-0.4 mg O_2 L^{-1} and 1.2-1.5 mg O_2 L^{-1} are reported by Peng & Zhu (2006). With oxygen levels above 2 mg O_2 L^{-1}, the nitrification process proceeds at rates higher than 80% of the maximum process rate (Figure 4).

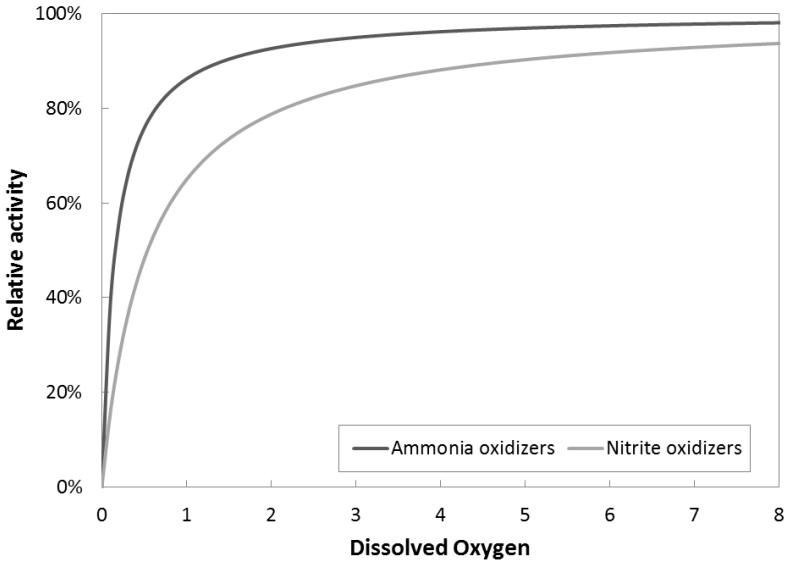

Figure 4: Relative growth rate of ammonia oxidizing microorganisms and nitrite oxidizing bacteria in relation with the DO concentration. Data based on Hunik et al. (1994)

The difference in half saturation constants between AOM and NOB implies an important effect in systems with limited DO. At decreasing DO levels, NOB activity will decline while AOM retain the ability to oxidize ammonia. As a result, nitrite accumulation occurs, which can result in an increase of toxic FNA if pH drops below 7.5.

From equation (3), it follows that oxygen demand (OD) for AOM is 3.15 mg O_2 mg^{-1} NH_4^+-$N_{consumed}$. Applying the same reasoning (equation (4)), OD for NOB is 1.12 mg O_2 mg^{-1} $NO_2^-N_{consumed}$. Note that consumed nitrogen is calculated as nitrogen oxidized and nitrogen accumulated in biomass. Oxygen demand for complete nitrification is 4.25 mg O_2 mg^{-1} $N_{consumed}$. This is slightly less than the cumulative (specific) OD of AOM and NOB because AOM biomass accumulates 2% of nitrogen consumed, so NOB can only consume 98% of total removed nitrogen and thus less oxygen is required during nitratation.

3.2.4 Temperature

Besides the effect of temperature on FA and FNA (3.2.1), temperature has a well-known effect to increase microbial activity until the optimum temperature is reached. A further increase in temperature results most often in a sharp drop of activity (Figure 5, Grunditz & Dalhammar (2001)). Another effect of elevated temperature is an increased decay rate, because cells grow and reproduce more rapidly, but also die earlier.

Grunditz & Dalhammar (2001) studied inhibition of ammonia and nitrite oxidation in relation to the reactor temperature. Tests were performed using pure *Nitrobacter* and *Nitrosomonas* strains isolated from activated sludge of a municipal WWTP. The authors found that *Nitrobacter* reached an optimum at 38°C (Figure 5b), while *Nitrosomonas* reached an optimum at 35°C (Figure 5a). Oxidation activity at temperatures beyond 40°C (*Nitrosomonas*) and 45°C (*Nitrobacter*) dropped sharply, indicating enzymatic inhibition. The authors also found oxidation rates became very slow at temperatures lower than 20°C.

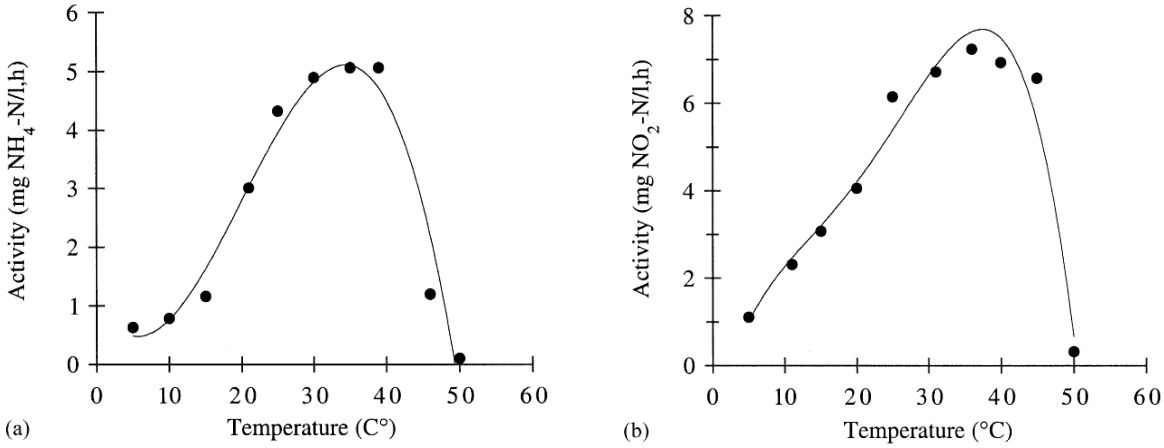

Figure 5: Relative ammonia (a) and nitrite (b) oxidation activity of respectively *Nitrosomonas* and *Nitrobacter* related to temperature. Figure from Grunditz & Dalhammar 2001

Mesophilic nitrification (Courtens et al. 2014) can be applied in industry, where cooling costs are high for treating high temperature wastewaters. Mesophilic nitrifying sludge was adapted to 42.5°C and 47.5°C, respectively for nitritation and nitratation.

3.2.5 Light

Light exposure inhibits both AOM and NOB due to oxidation of cytochrome c (Van Hulle et al. 2010). Light wavelengths of 410-415nm appeared to be causal for light inhibition (Alleman et al. 1987). Alleman et al. (1987) also investigated the combined effect of anoxic conditions and light exposure. Enriched *Nitrosomonas* cultures were found to be inhibited (reversibly) by ambient light contact during as little as 10 minutes in the presence of oxygen. Under anoxic conditions, cells were protected from inhibition. This is probably because cytochrome c was not utilized by the microorganisms during light exposure under anoxic conditions.

Merbt et al. (2012) compared photo inhibition of ammonia oxidation between AOB (*Nitrosomonas europaea* and *Nitrosospira multiformis*) and AOA (*Nitrosopulimus maritimus* and *Nitrosotalea devanaterra*). The authors found all strains were inhibited by continuous illumination of the greatest tested intensities (500 µE m^{-2} s^{-1}), but continuous illumination at lower intensities (60-15 µE m^{-2} s^{-1}) revealed archaeal strains were more sensitive than bacterial strains. Furthermore, AOA showed no signs of recovery during subsequent dark phases, whereas AOB strains recovered partial or fully. Specific growth rate of *N. europaea* showed no decrease after recovery phases, while specific growth rate of *N. multiformis* after dark phases were reduced only by 14% compared to 41% under continuous illumination.

The optimal range of physicochemical parameters on nitrifications is summarized in Table 4.

Table 4: Optimal ranges of several parameters for nitrification.

Parameter	Optimal range	Unit	Source
Temperature	25-40	°C	Grunditz & Dalhammar (2001)
Oxygen	>2	mg O_2 L^{-1}	Hunik et al. (1994)
NH_3	<8* and <0.08**	mg NH_3-N L^{-1}	Anthonisen et al. (1976)
HNO_2	<0.2* and <0.06**	mg NO_2-N L^{-1}	Anthonisen et al. (1976)
pH	7.5-8	-	Van Hulle et al. (2010)

*Ammonia oxidizers
**Nitrite oxidizing bacteria

4 Nitrification at high salinity

Many waste streams, including urine, contain a considerable amount of inorganic salts. The increased osmotic pressure causes stress to micro-organisms which are not adapted to growing in a solution with high salt concentration. It is important to understand how bacterial communities respond to changes in salinity.

4.1 Salt inhibition

In optimal conditions, the cell osmotic pressure is slightly higher than the environmental osmotic pressure. This is considered a driving force for cell growth and division (Welsh 2000). In saline environments, non-adapted microorganisms have hypotonic cell turgor. Water thereby leaves the cell and the resulting drought stress will affect the cell's activities. Stressed cells therefore use more energy to balance the cell osmotic pressure to the bulk solution in comparison to unstressed cells.

Panswad & Anan (1999) shocked an aerobic non-adapted activated sludge from 0 to 30 g $NaCl.L^{-1}$. An increased specific oxygen uptake rate (SOUR) was observed, combined with decreased specific ammonia uptake rate (SAUR), from 5.40 to 9.72 mg $O_2.g^{-1}$ $MLSS.h^{-1}$ and from 4.76 to 2.14 mg NH_3-$N.g^{-1}$ $MLSS.h^{-1}$, respectively. This is a decrease of SAUR by 55% and a SOUR increase by 80%. It is postulated the increase of SOUR is due to an increased amount of energy needed to preserve the moisture balance, while less substrate is oxidized.

Li et al. (2009) observed a decrease of SOUR and specific nitrification rate (SNR) when raising the salt level to 30 g NaCl L^{-1}. SOUR and SNR at 0 g NaCl L^{-1} was 19.36 mg O_2 L^{-1} and 1.99 mg NH_4^+-N (g VSS)$^{-1}$ h^{-1} respectively. When salinity was increased to 30 g NaCl L^{-1}, SOUR and SNR decreased by 69.9% and 86.4% respectively.

4.2 Microbial adaptation to high salinity

Although activity inhibition can occur because of salt stress, microorganisms have also developed strategies to adapt to a change in salinity. According to Oren (1999), two strategies are adopted by microorganisms to avoid hypotonic cell turgor. The first possible strategy consists of maintaining a high intracellular salt concentration. This strategy is observed in an archaeal group (Order of *Halobacteriales*) and a bacterial group (Order of *Halanaerobiales*). These microorganisms are adapted to salinity by controlling the cell's cytoplasm ionic composition. Usually, the cytoplasmatic ionic composition differs from the environmental ionic composition (mostly NaCl). Instead, in the cytoplasm elevated concentrations of inorganic osmotic solutes (such as KCl) are found. No organic solutes have been reported in representatives of these groups.

However, the known nitrifiers are no members of *Halobacteriales* or *Halanaerobiales* and will therefore apply a different strategy, which is applied by most other micro-organisms The osmotic pressure is balanced to the environmental osmotic pressure with (small) organic compatible solutes in the cytoplasm. This strategy does not require specifically adapted intracellular systems. The organic solutes are synthesized by the cell, or when available, taken from the environment. Examples of such solutes are sugars and derivatives, amino acids and derivatives, etc. (Oren 1999).

On a side note, many of these compatible solutes can act as either osmoprotectant and thermoprotectant (Caldas et al. 1999; Courtens et al. 2014). Welsh (2000) states compatible solutes can have many other useful applications in cells. They can act as intracellular reserves of nutrients during starvation periods, induce thermotolerance (trehalose), freeze tolerance (sugars, glycerol, glycine), and desiccation tolerance (trehalose, sucrose, ectoine, hydroxyectoine).

Adaptation strategies for nitrification

Many studies (Bassin et al. 2011; Bassin et al. 2012; Moussa, Sumanasekera, et al. 2006; Pronk et al. 2014) focus on adaptation of well-characterized freshwater nitrifying microbial communities to elevated salinity of wastewaters. Inocula were obtained from WWTP in most cases. The best results are obtained when the salinity is gradually increased. Shock loads rarely give satisfactory results compared to gradual loading increase, as will be discussed below.

Cui et al. (2013) collected estuarine samples to cultivate nitrifiers on hypersaline wastewater (28-32 g NaCl L^{-1}). The specific ammonia oxidation rate of the resulting sludge showed an optimum at around 40 g NaCl L^{-1} (Figure 6). Uygur (2006) tested ammonia oxidation activity by a mixed freshwater nitrifying consortia (*Nitrosomonas*, *Nitrobacter* as well as a heterotrophic mixed culture) at salt concentrations between 0 and 60 g NaCl L^{-1} as well as ammonia oxidation by acclimated nitrifying mixed culture (freshwater mixed culture with added *Halobacter* in order to alleviate the adverse effect of salt). No optimum was observed, as the ammonia oxidation rates for both consortia decreased with increasing salinity (Figure 6). The observed difference between Uygur's and Cui et al.'s studies is to be expected, as Cui et al. used marine species while Uygur mainly used freshwater species. From Figure 6, it is clear that different kinds of bacteria react differently to salt stress. When comparing to the non-adapted freshwater community in Uygur's study, the salt acclimated consortium shows an oxidation rate curve shifted to higher salinities. The optimal oxidation rates are achieved when no salt is present. However, for the ammonia oxidation rate of the halophilic nitrifying community of Cui et al., a clear optimum is distinguished at around 30 to 40 g NaCl L^{-1}. The nitrite oxidation rate of the halophilic community in Cui's study shows an optimal range between 20 and 40 g NaCl L^{-1}. This is caused by continuous competition in the estuary sediments. Salt adapted microorganisms are at an advantage in saline environments, and will eventually dominate due to higher growth rates compared to competitive microorganisms.

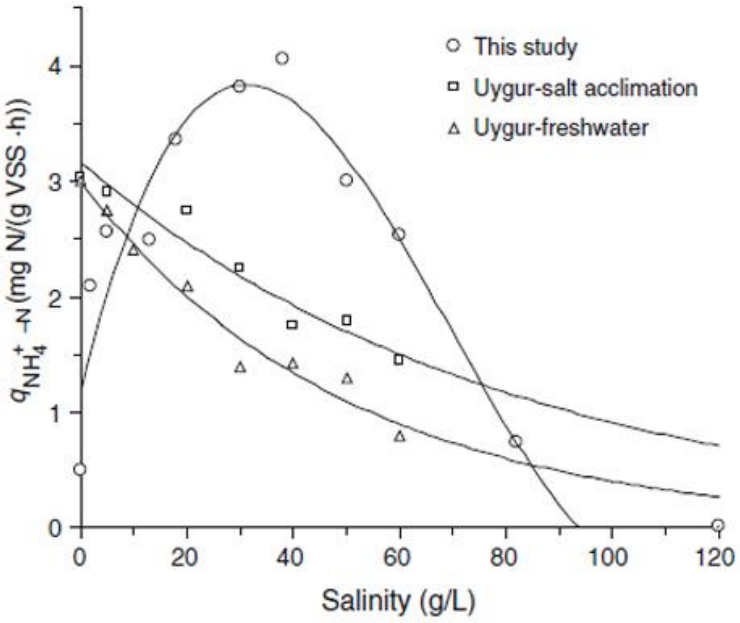

Figure 6: Comparison of specific ammonium oxidation rates of nitrifying sludge sampled from estuarine sediments (Cui et al. 2013), enriched freshwater nitrifying sludge with *Halobacter* and non-enriched freshwater nitrifying sludge without *Halobacter* (Uygur 2006) at different salt concentrations. Figure from Cui et al. 2013.

Bassin et al. (2012) reports on the effects of different salt adaptation strategies on nitrifying sludge in a sequencing batch reactor (SBR). Gradually elevated salt levels (up to 20 g NaCl L^{-1}) affected the microbial community less than was the case for shock wise elevated salt levels. By gradual adaptation, a stable community with 90% similarity to the inoculum was obtained. Shock wise adaptation to 10 and consecutively 20 g NaCl.L^{-1} over ten weeks only showed 50% similarity to the inoculum. Most of the tested members of the *Nitrosomonas* group were no longer detected by DGGE analysis when the salt level was increased shock wise from 0 to 10 g NaCl L^{-1}. They remained undetected by DGGE upon increasing the level to 20 g NaCl L^{-1}. However, FISH analysis showed they were still present, but under the detection limit of DGGE analysis. This change of community was not observed in the SBR with gradually increased salt concentrations. The authors suggest that the two step (shock wise) adaptation was more severe to AOB than the four step (gradual) adaptation. Additionally, the authors only detected *Nitrospira moscoviensis* during the later stages of the shock wise adaptation process, indicating the affinity of this strain to salinity. Nitrifying activity of the gradually adapted biomass was reduced by 50% (from 14 to 7 mg N.(g VSS)$^{-1}$.h), while the activity of the shock wise adapted biomass decreased by 60%. These findings suggest slow adaptation methods for freshwater microbial communities are advisable, because shock wise adaptation to salinity decreased ammonia oxidation rates more than gradual adaptation (Figure 7). Settling of the sludge increased with salinity. The authors suggest this could be due to the increased water density, which potentially induces washing out of poor settling sludge flocs.

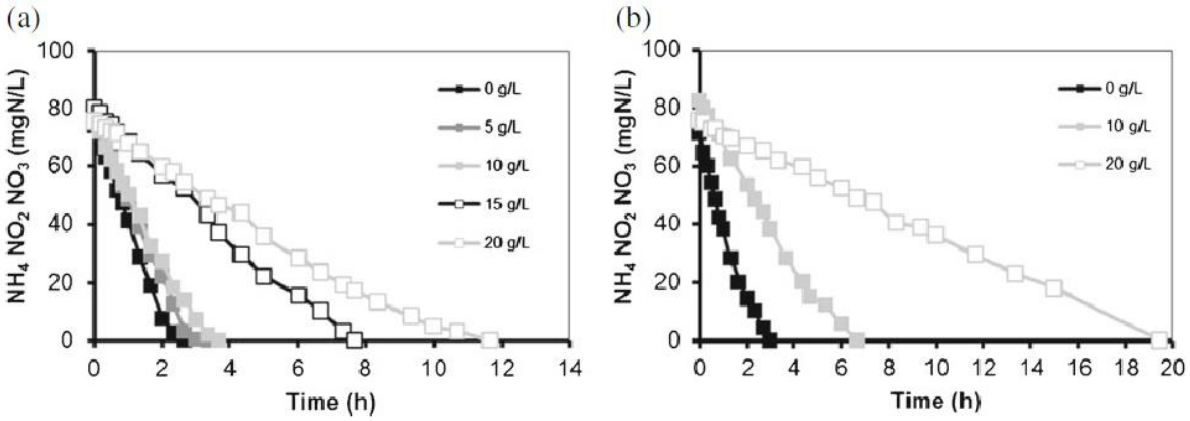

Figure 7: Ammonium profiles in batch activity experiment for SBR with (a) gradual adaptation to salinity and (b) shockwise adaptation to salinity, during different operational phases. Figure from Bassin et al. 2012.

Campos et al. (2002) studied the effect of increased salt concentration and increasing ammonia loading rate on nitrifiers in an activated sludge unit over 144 days. Ammonia loading rate was increased from day 46 (1 g NH_4^+-N.L^{-1}.d^{-1}) until day 70 (4 g NH_4^+-N.L^{-1}.d^{-1}). After 138 days, the authors observed a sharply reduced nitrification activity, which was when salt concentration in the reactor reached 525 mM (i.e. influent was a solution containing different salts: 13.7 g NaCl.L^{-1}, 19.9 g $NaNO_3$.L^{-1} and 8.3 g Na_2SO_4.L^{-1}). Up to 525 mM salt concentrations, the reactor was able to oxidize ammonia with an efficiency of 99.5% at loading rates of 4 g NH_4^+-N.L^{-1}.d^{-1} while the biomass concentration increased from 3.4 to 20 g VSS.L^{-1}. Table 5 gives an overview of the different nitrification process adaptations.

Table 5: Comparison of nitrification reactors at high salinity

	Unit	Bassin et al. (2011)		Bassin et al. (2012)		Campos et al. (2002)	Cui et al. (2013)	Moussa et al. (2006)
Reactor technology	-	MBBR	SBR	SBR	SBR	Act. sludge unit + settler	SBR	SBR
Inoculum	-	-[a]	-[a]	Act. sludge	Act. sludge	-	Halophilic	Enriched culture
Influent type	-	Industrial saline WW	Industrial saline WW	Synthetic	Synthetic	-	Salinized sewage	Synthetic
Salinity	**g NaCl L^{-1}**	13[b]	13[b]	0-5-10-15-20	0-10-20	525 mM[c]	28-32	0-40
pH	-	6.5-8.0	6.5-8.0	6.8-7.5	6.8-7.5	7.8	7.2-7.7	7.5
Temperature	**°C**	22	22	24	24	20	24	30°C
Dissolved oxygen	**mg O_2 L^{-1}**	>3	>3	>6	>6	>2	>3.5	-
$C_{influent}$	**mg NH_4 L^{-1}**	40-50	40-50	85	85	-	32-85	-
Nitrification efficiency	**%**	70%	>80%	50%	40%	±100%	±100%	-
Loading rate	**mg N L^{-1} d^{-1}**	20-25	20-25	50-115	30-100	4000	16-90	-
Inhibition	**%**	-	-	50%	60%	30%	-	60%

[a] not specified

[b] maximum

[c] salt solution containing 13.7 g $NaCl.L^{-1}$, 19.9 $NaNO_3.L^{-1}$ and 8.3 g $Na_2SO_4.L^{-1}$

4.3 Urine nitrification

Urease in (stored) urine hydrolyzes with formation of ammonia and carbon dioxide, causing an increase of the pH (equation (2)). Together with the pH rise, a shift of the chemical equilibrium between ammonia and ammonium towards ammonia is observed (equation (6)). With a large portion of nitrogen in ammonia form, nitrogen loss by volatilization poses a problem to nitrogen recovery from the source separated urine. As a way to stabilize this ammonia nitrogen, several authors (Udert, Fux, et al. 2003; Udert & Wächter 2012; Sun et al. 2012; Feng et al. 2008) have investigated nitrification of human urine.

Udert, Fux, et al. (2003) investigated urine nitrification in a moving bed biofilm reactor (MBBR) aiming to produce an ammonium nitrate solution, which is already the main fertilizer in Europe (UNIDO 1998). Urine containing 7.1 g TAN-N.L^{-1} at pH 9.2 was used as influent. No pH control was used, except in cases of aeration failure. Temperature was kept stable at 25.3°C. Nitrification efficiency was about 50% from day 23 until the end of the experiment (day 79). Nitrification ceased at 50%, due to low pH (pH < 6). Less than 1% of the effluent nitrogen was nitrite, indicating nitrite oxidation was not rate limiting. Maximum nitrification rate was 380 mg N L^{-1} d^{-1}.

Sun et al. (2012) achieved aerobic nitrifying granules in a sequencing batch reactor (SBR) setup, starting from seed aerobic granules cultivated during a previous study. The obtained nitrifying granules had very good settling characteristics (60 m h^{-1}) as well as a volumetric loading rate around 194 mg NH_4^+-N g SS^{-1}.d^{-1}. pH was controlled at 7.5 and temperature at 25°C. Due to the high total ammonia concentration (250 mg NH_4^+-N L^{-1}, corresponding to 7.2 mg FA.L^{-1}), nitrite buildup occurred from day 30 onwards. However, ammonia oxidation reached 90% on day 75 of operation, successfully stabilizing urine into nitrite with aerobic nitrifying granules.

Feng et al. (2008) investigated the potential of nitrified human urine as culture medium for *A. platensis*. Urine nitrification was performed in a packed bed bioreactor at 27°C. When pH was uncontrolled, nitrification performance was only 50%. The authors suggest this is due to the NH_3/NH_4^+ equilibrium shifting towards ammonium, resulting in an ammonia shortage. When adjusting pH daily to pH 8, a nitrification efficiency of 95% was achieved. In this study, a 10 fold dilution of human urine was used as influent.

Udert & Wächter (2012) operated a membrane aerated biofilm reactor for over 12 months steadily, aiming to achieve complete nutrient recovery in a dry end product. As a first step, source separated urine was nitrified, controlling the pH via influent between 6.2 and 7.0, depending on setpoint. Influent ammonia concentration was 2.39 g NH_4-N L^{-1} and pH was around 8.7. Ammonia concentrations in the influent were considerably lower than common concentrations in fresh urine due to ammonia volatilization. An ammonia nitrate solution was produced with a ratio of ammonia to nitrate between 0.87 and 1.15 g N (g N)$^{-1}$). Maximum volumetric loading rate achieved is 180 mg N L^{-1} d^{-1}. Substantial nitrogen loss was recorded at high pH setpoints. At pH 6.7, a nitrogen loss of 24% was observed. Ammonia volatilization in this pH range likely did not induce the nitrogen loss. A thick biofilm was established, due to low shear stress by aeration. This thick biofilm, combined with the high COD over N ratio which was caused by ammonia volatilization during storage, the nitrogen loss was likely induced by heterotrophic denitrification in anoxic zones where oxygen diffusion did not reach the base of the biofilm.

5 Microalgae

Microalgae are a mixed group of photosynthetic eukaryotes and prokaryotes. Cyanobacteria are not strictly considered algae, but are regularly mentioned being algae for convenience matters.

5.1 Spirulina

A. platensis (Cyanobacteria) is a well-known representative of prokaryotic microalgae and is also known as Spirulina. The filamentous photosynthetic prokaryote occurs naturally in alkaline lakes (pH 9-11) and has been used for centuries as a food source or as supplementing food by tribes living near the Chad Lake in Africa as well as in Mexico (Usharani et al. 2012). It is considered a rich source of microbial protein, or single cell protein (SCP), because of the distinctive high protein content. Other than proteins, it contains minerals, vitamins, fatty acids and amino acids.

Applications for Spirulina are plentiful, but the cyanobacteria is mostly used in the food or fodder sector The dried cultivation product is used as a protein supplement in shrimp, fish and chicken feed as well as food (Usharani et al. 2012). The industrial potential was investigated (Ciferri & Tiboni 1985) and it was found that Spirulina is a good source of chemical and other basic commodities such as enzymes, lubricants, vitamins and pigments, among others. The pharmaceutic and food industry and even the cosmetic industry have uses for Spirulina-based products.

5.2 Microalgae cultivation

Spirulina is one of the most commonly cultivated microalgae because it is very rich in protein, contains minerals, vitamins, essential fatty acids and amino acids (Usharani et al. 2012). Fresh water and wastewater are both used to cultivate the microalgae but wastewater is utilized for fodder grade SCP rather than SCP for human consumption. For the purpose of human nutrition, fresh water is used.

Two common cultivation systems exist (Muñoz & Guieysse 2006): the open railway pond is the cheapest configuration to construct and operate, but is not as efficient as the closed system (flat plate or tubular photobioreactors). Due to the limited settling characteristics of the microalgae, harvesting of the biomass remains a challenging and costly affair, accounting for 20-30% of the total operation costs.

Muñoz & Guieysse (2006) list the major environmental parameters affecting the cultivation process: light supply, temperature, pH, dissolved oxygen (DO) concentration and reactor infection with predators (such as parasitical fungi). The major configuration parameters are HRT, mixing and the surface over volume ratio (S/V).

Rodrigues et al. (2010) investigated the use of simultaneous ammonia (NH_4Cl) and nitrate (KNO_3) as nitrogen source in order to reduce the cost of conventionally used culture media (only KNO_3). The authors found that a 30% reduced cost can be achieved by replacing 41% of the nitrate nitrogen source with ammonium chloride.

5.3 Urine as a growth medium for microalgae

Several authors have studied the growth of microalgae on (diluted) urine.

Yang et al. (2008) used Spirulina to treat urine in a batch setup, aiming on development of a bioregenerative life support system for space applications. Problems arose with NaCl concentrations in the urine.

Feng et al. (2007) showed that nitrate is the preferred nitrogen source of Spirulina, indicating nitrified urine is a feasible growth medium for microalgae cultivation. Feng et al. (2008) continued to investigate nitrification of human urine with and without pH control. Only 50% NH_4 could be converted to nitrate without pH control, while 95% nitrification efficiency was achieved when daily adjusting the pH to 8.0.

Chang et al. (2013) investigated the coupling of a biomass production with wastewater treatment. The wastewater was 120 times diluted real human urine. Nutrient removal efficiencies of 97%, 96.5% and 85-98% were achieved for respectively NH_4, total P and urea.

Tuantet et al. (2014) showed that *Chlorella sorokiniana* can grow on concentrated urine up to 1.4 g NH_4-N L^{-1}, but optimal growth was observed on 20 times diluted urine. The author also showed the necessity to add trace elements to human urine for optimal microalgae growth.

6 Research objectives

Source separated urine is an interesting waste stream for stabilization and recovery of nitrogen. However, nitrifying consortia need to adapt to high nitrogen loading rates as well as high salinity of the source separated urine.

Therefore, a first objective of this study is to select an optimal nitrifying inoculum for the startup of a nitrification reactor. Time is an important aspect because nitrifying microorganisms have low growth yields and startup of such a system is rather slow. to stabilize nitrogen present in urine.

A second objective is to evaluate adaptation period of the selected consortium in a continuous setup. For this, a membrane bioreactor (8L) will be utilized, and the startup will be investigated using synthetic urine as a waste stream. This is to maintain a constant composition of the influent, eliminating factors other than influent dilution and nitrogen loading rate. Startup will be considered as successful when the MBR is running stable at the highest N loading (500 mg N L^{-1} d^{-1}) for 2 hydraulic retention times (approximately 16 days).

Once startup has been accomplished, influent will be changed by batches of real human urine. This will be collected and freshly stored in the freezer. Several batches of urine with different electrical conductivities and nitrogen concentrations will be prepared and fed to the MBR to simulate real life conditions.

As a final objective, the nitrified effluent will be used as a source for nitrogen valorization. Growth rates and protein composition of *A. platensis* will be investigated under different salinities, nitrogen concentrations and feed type (ammonium vs. nitrate) to test the affinity on food sources.

PART 2: Materials and Methods

1 Screening nitrifying consortia

1.1 Nitrifying consortia

Twelve nitrifying consortia were tested for nitrification activity to select an optimal inoculum under the projected operation conditions (pH 7, 20°C, 45 $mS.cm^{-1}$) for the urine nitrification reactor. Nitrifying consortia were selected from a wide spectrum of wastewater types on a wide range of electrical conductivities and nitrogen loading rates.

Manure treatment from piggeries (Danis, DeCoster), biological filtration of freshwater (Inagro) and saltwater (Shrimp) aquaculture were sampled to collect nitrifying active sludge. An industrial wastewater treatment consortium (DOW) and a synthetic nitrifying filter inoculum (ABIL) were obtained respectively from DOW in Terneuzen and from Avecom in Wondelgem. An experimental OLAND (Oxygen Limited Autotrophic Nitrification Denitrification) setup at Ghent University provided a consortium (OLAND). Treatments of percolate at landfill sites in Moen (Imog) and Antwerp (Hoge maey) provided Imog and HANDS respectively. The Sealife center in Blankenbergen cooperated in collection of two aquarium filter materials. The first was collected from a saline aquarium containing octopus, the other from a freshwater aquarium containing piranhas. Both filter materials were scrubbed with soft brushes in order to detach the biofilm and obtain suspended biomass. These respective consortia will be referred to as Octopus and Piranha. An overview of the consortia and the operation conditions is given in Table 8.

Plastic containers were used to transport and store collected consortia. The normal operating EC was measured in the supernatant from the collected samples. Electrical conductivity (EC) was measured as indication for ionic strength and osmotic pressure. The containers were stored in the dark and at 4°C until experiments were started.

1.2 Nitrification activity test

Batch experiments were conducted to determine nitrification activity. Ammonia oxidation and nitrite oxidation were tested in separate 250 mL Erlenmeyer flasks with 100 mL working volume. The experiments are performed at the respective normal EC and at 45 mS cm^{-1} of the tested consortium. Each conductivity was tested in triplicate.

Reactivation was required before the start of the experiment in case of long duration storage (one week or longer). Reactivation was completed on an orbital shaker (120 rpm) at 20°C in Erlenmeyer flasks (2L) which were made light-tight with aluminum foil. Ammonia chloride (NH_4Cl) and sodium nitrite ($NaNO_2$) were added both to a concentration of 50 mg N L^{-1}. DO was monitored daily during reactivation to assure aerobic conditions. Test strips were used to indicate success of reactivation.

Biomass was washed two times with a phosphate buffer solution on the original EC (physiological conditions) prior to the test. Next, the biomass was washed with a buffer solution at the desired EC for the activity test. Buffer solution was prepared by dissolving 4.158 g KH_2PO_4, 7.258 g K_2HPO_4 and 1.111 g $NaHCO_3$ per liter and was corrected to pH 7 by addition of HCl (1M) or NaOH (1M). The buffer solution was adjusted to the desired EC with sodium chloride (NaCl). A centrifuge was used 10 minutes at 3000 rpm (1540 g) to separate the sludge from the washing solution.

Directly after washing the biomass, the activity test was initiated. Washed biomass (50 mL) was added to 45 mL of buffer solution adjusted to the desired EC. The activity test was started by adding 5 mL of substrate solution (NH_4Cl or $NaNO_2$). At this point the EC of the mixture was measured to confirm that the mixed liquor was at the desired EC. This was always the case.

Samples of mixed liquor (2 mL) were collected and filtered using a syringe filter (0.45 µm). Collection of samples was repeated during monitored time intervals. The required frequency of sampling was assessed using test strips indicating ammonium or nitrite levels. Samples were stored at 4°C and analyzed within two days. Dissolved oxygen (DO) and pH were monitored during the test. If DO was to low (<2 mg O_2 L^{-1}) the test was repeated with less biomass.

2 Membrane bioreactor

2.1 Reactor setup

A continuous urine nitrification experiment was conducted with the ABIL consortium in a submerged membrane bioreactor (MBR). The working volume in the MBR was controlled at 8.0 L. The MBR was used to maximize sludge retention time and withhold potential pathogens from the effluent. A commercially available flat sheet membrane (Kubota) was used to extract effluent. The pore diameter is 0.4 µm and surface area 0.8 m^2. A vacuum pump (KNF, type PM14469-88) connected to the bottom of the reactor realized air supply and kept the biomass in suspension. Four additional air diffuser stones connected to two aquarium pumps (Rena Air 300, Rena Air 600) optimized mixing and oxygen supply. The intense mixing prevented biofilm formation on the membrane. A Watson Marlow pump (Watson-Marlow 323 S) was used to dose influent. This peristaltic pump was connected to a timer to deliver a flow rate between 100 and 500 mL d^{-1}. The effluent was extracted from the MBR through the flat sheet membrane by use of a peristaltic pump (Prominent 1.6). A pH controller (Prominent Dulcometer; Prominent dulcotest pH electrode PHEP-112) measured pH directly in the reactor and adjusted the pH to values between 6.9 and 7.1 by dosage of 1 M sodium hydroxide (NaOH) or 1 M hydrogen chloride (HCl). The pH was monitored daily by means of the pH controller display. DO was checked regularly with a portable Hach HQ40d oxygen probe.

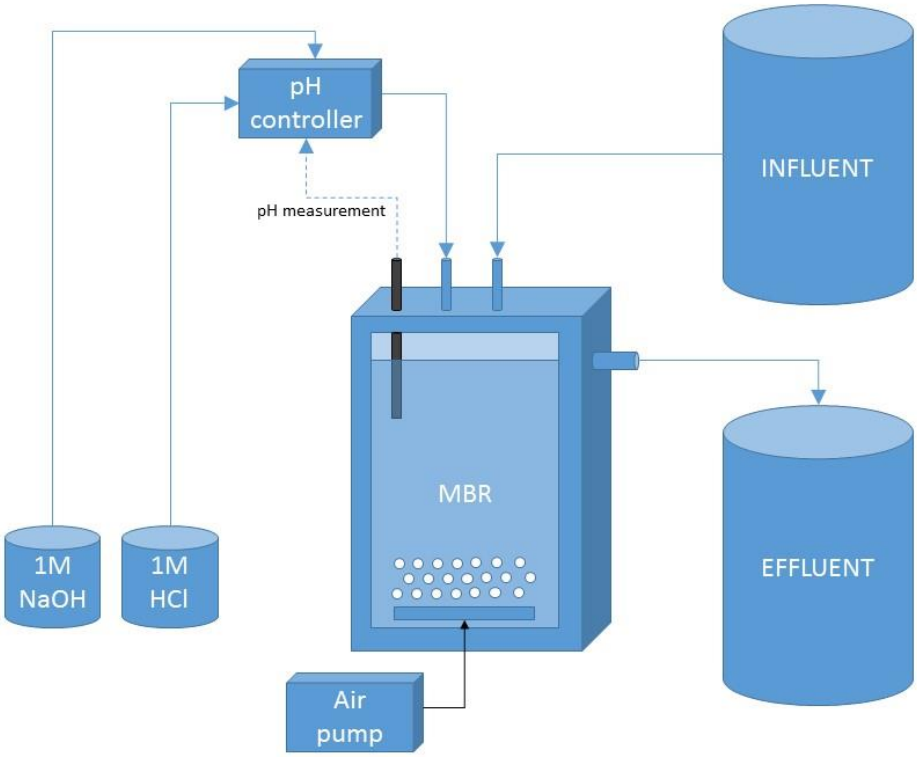

Figure 8: Setup of the membrane bioreactor (MBR). Influent and effluent flow rates are regulated by a peristaltic pump coupled to a timer (not depicted).

2.2 Inoculum

Based on the results of the nitrification activity screening, the best performing consortium at projected conditions was selected. The ABIL consortium (Avecom, Wondelgem) was used to inoculate the MBR at 1.32 g VSS L^{-1}.

2.3 Operation

Throughout the entire operation, the effluent was sampled daily and was analyzed for ammonium, nitrite and nitrate as well as EC. The VSS and TSS concentrations were determined on a weekly basis by sampling 30 mL mixed liquor from the center of the MBR.

The MBR was operated in three distinct phases. Synthetic urine was dosed in increasing concentrations of 10-100% at a constant flow rate of 500 mL d^{-1} during phase I. Phase I ended when a concentration of 100% synthetic urine was reached. During phase II the concentration of the synthetic urine was kept constant at 100% and the influent flow rate was used to control the nitrogen loading rate of the reactor. To end phase II, the reactor should be operated on a stable nitrogen loading rate during 2 times the HRT. The HRT is dependent on the influent flow rate and the addition of base or acid by the pH controller. At the start of phase III the synthetic influent was replaced by real urine. The objective of the third and last phase was to retain stable operation on the same nitrogen loading rate as achieved during phase II.

Nitrification activity tests were performed at the start of phase I (day 0), at the end of phase II (day 118) and at the end of phase III (day 145). The experiments aimed to determine the specific nitrification activity of the sludge present in the reactor at different salinities. The protocol described in section 1.2 was expanded by testing more EC values.

2.4 Urine

Synthetic urine was used to adapt the biomass to the salinity conditions and nitrogen loading rate at steady state operation. The composition of synthetic urine (100%) used in phase I and phase II is given in Table 6.

Real urine was collected at Ghent University between 9-12 a.m. from healthy male scientific staff members. Before donation, the urine donors were asked to confirm that they were healthy and that they were not taking any antibiotics or medication. A total of 10 L fresh urine was collected, characterized and mixed. Batches of mixed urine (6.33 g TKN L^{-1}, pH 6.5, 17 mS cm^{-1}) were prepared and frozen separately before use. The batches were sized to feed the MBR for two days at the nitrogen loading rate achieved at the end of phase II.

Table 6: Composition of synthetic hydrolyzed urine (8.0 g N L^{-1}). Add 1 mL trace elements A and B to 1 L.

Component	Concentration (g L^{-1})
Hydrolyzed urine	
$Na_2SO_4.10H_2O$	5.21
$NaH_2PO_4.H_2O$	2.42
NaCl	3.6
KCl	4.2
NH_4Acetate	9.6
NH_4Cl	9.2153
NaOH	6.89
NH_4HCO_3	21.4
Trace elements A (1 mL L^{-1})	
$FeSO_4.7H_2O$	9.1513
Na_2EDTA	6.3689
Trace elements B (1 mL L^{-1})	
$Na_2EDTA.xH_2O$	11.7767
$ZnSO_47H_2O$	0.3376
$CoCl_2.6H_2O$	0.1884
$MnCl_2$	0.4938
$CuSO_4.5H_2O$	0.1963
$Na_2MoO_4.2H_2O$	0.1727
$NiCl_2.6H_2O$	0.1492
$Na_2SeO_4.10H_2O$	0.1649
H_3BO_3	0.0110
$NaWo_4.2H_2O$	0.0393

3 Microalgae cultivation

Following the urine nitrification urine in the MBR, growth experiments were conducted in order to determine the suitability of nitrified urine as a cultivation medium. *Arthrospira platensis* was used as a model organism because it is extensively characterized in literature and is also commercially

cultivated for several applications. The *A. platensis* culture used in this study was provided by SCK•CEN (Mol).

The growth tests were conducted under axenic conditions in 96 well plates on an orbital shaker (700 rpm) in a temperature controlled room at 28°C. The algae were exposed to 2 TL lamps (Philips TL-D 90 Deluxe 36W) at 200 µmol m^{-2} s^{-1}.

Tests were performed in quintuplicate (n=5). Biomass growth was determined by measuring the optical density at 680 nm (OD_{680}). The OD_{680} measurement was performed with a Platereader Tecan Infinity 200 PRO NanoQuant.

3.1 Nitrogen source

An experiment was set up to assess the effect of different nitrogen sources on growth of *A. platensis*. Standard Zarrouk medium (Zarrouk 1966) was used during the experiment to represent the nitrate nitrogen source. Sodium nitrate ($NaNO_3$) in standard Zarrouk medium was replaced by NH_4Cl or urea to represent respectively the ammonium nitrogen source or the urea nitrogen source.

The nitrogen concentration was varied to assess stimulating or toxic effects of the nitrogen sources tested. Concentrations of nitrate varied between the standard Zarrouk concentration (410 mg NO_3-N L^{-1}) and the nitrate concentration of the MBR effluent (phase III) at the moment of medium preparation (5300 mg NO_3-N L^{-1}). Tested ammonia concentrations were in a lower range (100-2000 mg NH_4-N L^{-1}) because literature suggests ammonia is toxic above 1400 mg NH_4-N L^{-1} at pH 8.2. Urea was tested in the same nitrogen concentrations as the ammonium nitrogen source. Four conductivities were tested for every nitrogen concentration of each nitrogen source. The tested EC values were 20 mS cm^{-1} (standard EC of Zarrouk medium), 30 mS cm^{-1}, 45 mS cm^{-1} and 60 mS cm^{-1}. The pH of each medium was adjusted to pH 8.2 with 5 M NaOH or 5 M HCl.

3.2 Urine matrix

Synthetic and real urine were used to investigate matrix effects on the growth of *A. platensis*. The composition of the synthetic equivalent of non-hydrolyzed real urine is given in TABLE X. Hydrolyzed synthetic urine was the same as used during phase I and phase II of MBR operation (Table 6). The effluent of the MBR, nitrified (synthetic) urine, was used as a third synthetic medium for microalgae growth. Real non-hydrolyzed urine (MBR influent during phase III), hydrolyzed urine (provided by the research group PaInT at Ghent University) and nitrified real urine (MBR effluent at the end of phase III) were tested for suitability as cultivation medium for *A. platensis*, along with their synthetic counterparts which are described above.

Portions of the synthetic and real hydrolyzed urine were stripped at 30°C and pH 12 (10 M NaOH) by intensively mixing by means of an aquarium air pump coupled to a diffuser stone. Complete ammonia volatilization was checked with strip tests. A dilution series for both liquids was made (100%, 1/2 diluted, 1/3 diluted, 1/5 diluted and 1/10 diluted). Each dilution was subsequently brought to an ammonium concentration of 100 mg NH_4-N L^{-1} with a highly concentrated NH_4Cl-solution (20 g N L^{-1}).

Dilution series were made of non-hydrolyzed, hydrolyzed and nitrified urine (for both synthetic and real) to concentrations 100%, 3.0 g TKN L^{-1}, 2.0 TKN L^{-1}, 1.0 TKN L^{-1}, 0.5 TKN L^{-1} and 0.1 TKN L^{-1}.

The measurements in this experiment were corrected with blanks because the absorbance of the matrix in several real urine treatments is significantly higher than other treatments.

Table 7: Composition of synthetic non-hydrolyzed urine (Brooks & Keevil 1997).

Component	Concentration g L^{-1}
Peptone L37	1
Yeast extract	0.005
Lactic acid	0.1
Citric acid	0.4
Sodium bicarbonate	2.1
Urea	10
Uric acid	0.07
Creatinine	0.8
Calcium chloride.$2H_2O$	0.37
Sodium chloride	5.2
Iron(II) sulphate.$7H_2O$	0.0012
Magnesium sulphate.$7H_2O$	0.49
Sodium sulphate.$10H_2O$	3.2
Potassium dihydrogen phosphate	0.95
Di-potassium hydrogen phosphate	1.2
Ammonium chloride	1.3

4 Analysis techniques

4.1 Test strips

Test strips (MQuant) were used as an indication of ammonium and nitrite levels. Ammonium test strips have a range between 0 and 310 mg N L^{-1}, while nitrite has a range between 0. and 24 mg NO_2^-N L^{-1}. Samples (5mL) were taken from the reactor and filtered over a 0.45µm Chromafil Xtra filter (Machery- Nagle, PA, USA). Nitrite strips are used directly in the filtered liquid, while ammonium test strips require addition of 10 drops NaOH (provided in the test kit) to the sample, after which the strip is immersed in the solution.

4.2 High throughput

The ammonium concentrations were determined spectrophotometrically by the Berthelot reaction (Bucur et al. 2006). Nitrite concentrations were determined spectrophotometrically by the Montgomerey reaction (Montgomery & Dymock 1961). Both measurements were performed by a Platereader Tecan Infinite F50.

4.3 Ion chromatography

Ion chromatography (IC) was used to determine nitrite and nitrate concentrations. Samples were diluted with milliQ water in sample tubes (10mL) to reach an estimated concentration between 1 mg L^{-1} and 50 mg L^{-1}. Subsequently, nitrite and nitrate concentrations were determined on a 761 Compact Ion Chromatograph (Metrohm, Switzerland) equipped with a conductivity detector.

4.4 Total Kjeldahl nitrogen

Kjeldahl nitrogen was analysed according to Standard methods (4500-Norg B; APHA 1992). The sample was diluted in a Kjeldahl tube so that the concentration was located between the detection limits (9 - 250 mg NH_4-N/L). The organic nitrogen present in the sample is transformed into ammonium nitrogen $(NH_4)_2SO_4$ by means of destruction at 400°C for 1.5 hours with sulphuric acid

(98% H_2SO_4), potassium sulphate (K_2SO_4) and copper sulphate ($CuSO_4$) as a catalyst. The digested sample is distillated and ammonia is captured in a boric acid indicator. The captured ammonia (as $(NH_4)_3BO_3$) is titrimetrically determined with hydrochloric acid 0.02 M.

4.5 Suspended solids

Volatile suspended solids (VSS) and total suspended solids (TSS) were determined according to the Standard Methods 2540D and E (APHA 1992). Knowledge on the VSS concentration was necessary to determine specific oxidation rates and could indicate trends in reactor operation.

4.6 Electrical conductivity

Electrical conductivity was measured with either a glass conductivity probe (Consort SK20T) or a plastic conductivity probe (Metrohm conductometric cell Pt1000). Conductivity was corrected automatically to standard 25°C conductivity by the conductivity meter (Consort C833 Multi-channel analyser).

4.7 Microscopy

A sample from the reactor at day 118 (end of phase II) was analyzed with light microscopy. A Zeiss microscope (type Axioskop 2 plus) was used. A sample of the culture of *A. platensis* was also analyzed with light microscopy.

4.8 Dissolved oxygen

The dissolved oxygen (DO) concentration was measured directly in the mixed liquor with a portable digital oxygen probe (Hach HQ40d portable meter).

PART 3: Results

1 Screening nitrifying consortia

The goal of this screening was to determine the nitrification activity of different microbial consortia and assess the influence of salinity on nitrification. Inocula were collected from wastewater treatment sites with a broad range of salinity and nitrogen load. An overview of the operating conditions of the different tested inocula can be found in Table 8.

The specific ammonia and nitrite oxidation rates were determined at the conductivity to which the community was conditioned, as well as a conductivity of 45 mS cm^{-1}. A complete overview of the results can be found in Table A1.

1.1 Ammonia oxidation activity

Ammonia oxidation and the effect of salinity was investigated to test the resilience to salt stress. Results are graphically presented in Figure 9. Results of sludge types which did not show any ammonium nor nitrite oxidation are omitted from the figures.

ABIL sludge oxidized ammonia at the highest specific rate (143mg N (g VSS)$^{-1}$ d^{-1}). Other consortia showing high ammonia oxidation activity were OLAND, Imog and Breda with specific rates of respectively 124, 101 and 59 mg N (g VSS)$^{-1}$ d^{-1}. The consortia HANDS, Decoster, Danis had lower specific oxidation rates (4, 10 and 26 mg N (g VSS)$^{-1}$ d^{-1} respectively). OLAND sludge had the lowest relative ammonia oxidation inhibition (8%), followed by Decoster (30%), ABIL (33%), Imog (70%) and Danis (77%). Ammonia oxidation of other consortia (Breda, HANDS, Piranha) was 100% inhibited at 45 mS cm^{-1}. DOW sludge was inactive at a normal conductivity as well as at 45 mS cm^{-1}. The same was observed for most consortia originating from aquaculture (Inagro, Shrimp, Octopus), except the Piranha consortium.

Imog sludge is the consortium with the highest conductivity difference between the normal conductivity and 45 mS cm^{-1} (ΔEC = 36.2 mS cm^{-1}) which retains a significant ammonia oxidation activity.

OLAND ammonia oxidation rate at normal EC (124 mg N (g VSS)$^{-1}$ d^{-1}) is not significantly different from the oxidation rate at 45 mS cm^{-1} (114 mg N (g VSS)$^{-1}$ d^{-1}). The Imog, Danis, Decoster and ABIL consortia showed a relative ammonia oxidation inhibition of 70%, 77%, 30% and 33%, respectively.

1.2 Nitrite oxidation

Figure 10 gives an overview of the nitrite oxidation rates. The numeric results with relative inhibition and p-value are given in Table A1.

Nitrite oxidation rate at normal conductivity was the highest for the ABIL sludge (555 mg N (g VSS)$^{-1}$ d^{-1}), followed by Imog and Breda (respectively 131 and 86 mg N (g VSS)$^{-1}$ d^{-1}). Other consortia had relatively low specific oxidation rates (lower than 20 mg N (g VSS)$^{-1}$ d^{-1}).

Danis, ABIL, HANDS, Imog, Piranha, Breda, Inagro showed relative inhibition percentages of 7%, 18%, 19%, 57%, 74%, 97% and 100%, respectively. No nitrite oxidation activity was present in the OLAND sludge.

Decoster and DOW showed a significantly higher specific nitrite oxidation rate at 45 mS cm^{-1} than at their respective normal EC (respective p-values are 3.81 10^{-3} and 0.028). Nitrite oxidation rate of DOW sludge increased by 55% while Decoster nitrite oxidation increased by 23%.

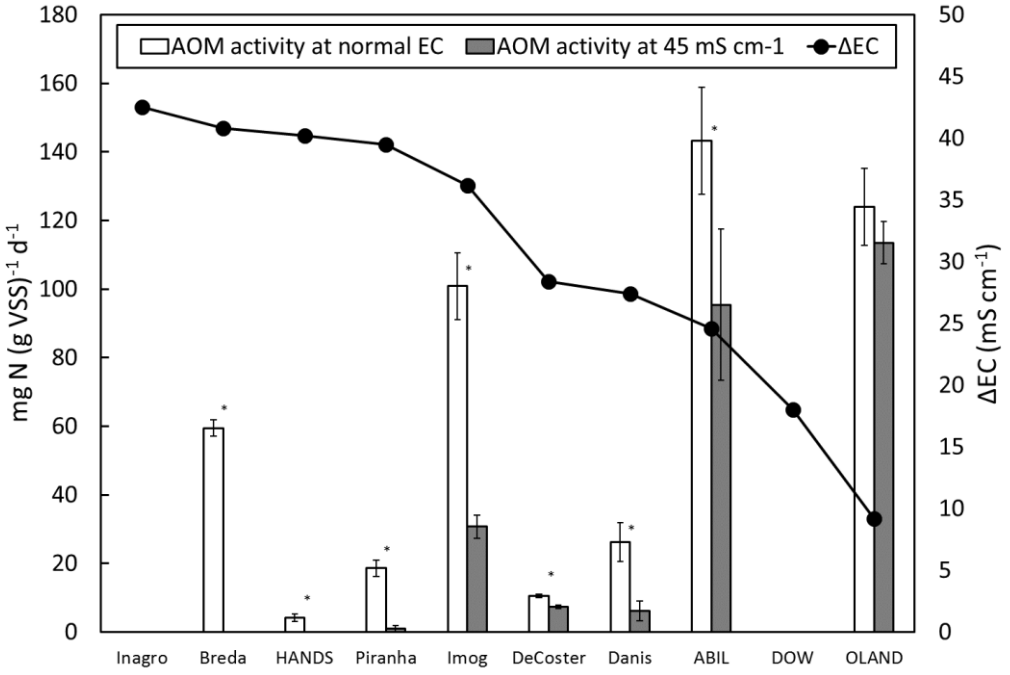

Figure 9: Specific activity of Ammonia Oxidizing Microorganisms (AOM) of different consortia at their respective normal conductivity and at 45 mS cm^{-1} (pH 7, 20°C). The absolute difference between the normal conductivity and 45 mS cm^{-1} (ΔEC) is shown on the secondary y-axis. Significant difference is indicated by an asterisk (p<0.05).

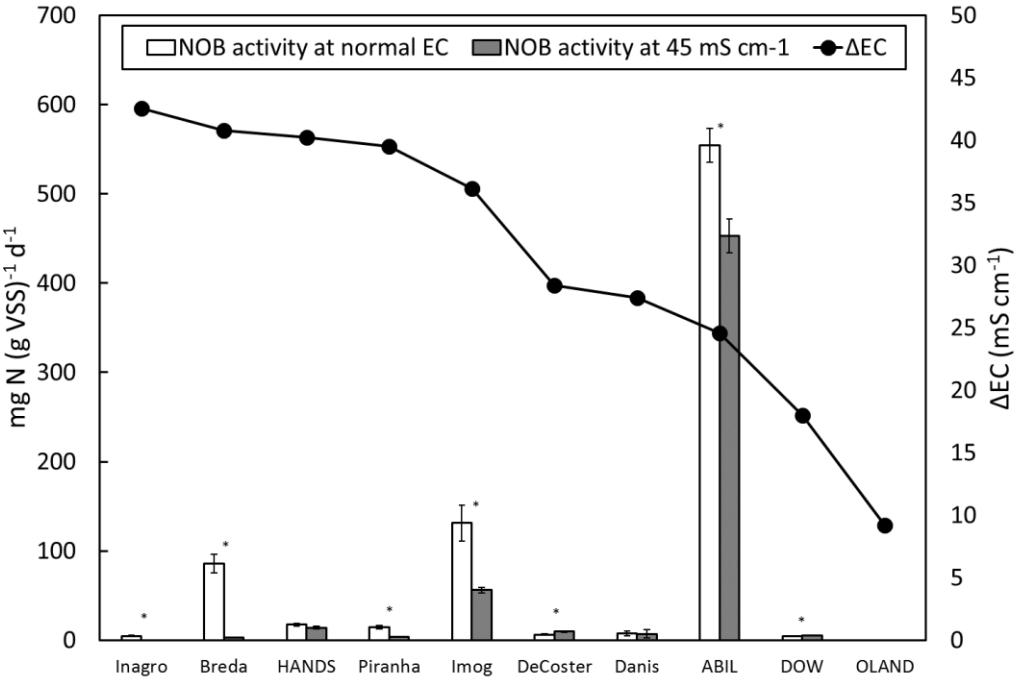

Figure 10: Specific activity of Nitrite Oxidizing Bacteria (NOB) of different consortia at their respective normal conductivity and at 45 mS cm^{-1} (pH 7, 20°C). The absolute difference between the normal conductivity and 45 mS cm^{-1} (ΔEC) is shown on the secondary y-axis. Significant difference is indicated by an asterisk (p<0.05).

Table 8: Operating conditions of nitrifying consortia at the site and time of collection.

Company - location	Consortium	Process configuration	Wastewater type	EC	Influent N conc.	vNLR [5]	sNLR [6]	pH	Temp.
				mS cm^{-1}	mg N L^{-1}	mg N L^{-1} d^{-1}	mg N g VSS^{-1} d^{-1}	-	°C
Inagro - Rumbeke	Inagro	MBBR [1]	Pikeperch aquaculture	1	0.1	7.2	36	8.5	22
SeaLife - Blankberge	Piranha	MBBR [1]	Fresh water aquarium	1	< 0.1	N.A. [2]	N.A. [2]	6.0-7.0	20
RWZI dokhaven - Nieuwveer	Breda	AB-process	Domestic wastewater	2	17	125	37	7.6	14
Hooge Maey - Antwerp	HANDS	Circuit reactor	Landfill leachate	9	400	N.A. [2]	N.A. [2]	N.A. [2]	25-30
Imog - Moen	Imog	N.A. [2]	Landfill leachate	9	330	69	17	7.0-8.5	20-30
Avecom - Wondelgem	ABIL	Fed batch	Synthetic nitrification medium	23	N.A. [2]	N.A. [2]	300-500	7.0	24
DANIS NV - Izegem	Danis	N.A. [2]	Liquid pig manure	25	4500	162	41	7.7	26-27
Decoster - Kortemark	DeCoster	N.A. [2]	Liquid pig manure	25	N.A. [2]	N.A. [2]	N.A. [2]	7.7	N.A. [2]
UGent - Ghent	OLAND	SBR [3]	Synthetic wastewater	37	N.A. [2]	250	133	7.8	26
SeaLife – Blankberge	Octopus	MBBR [1]	Marine aquarium	46	0.1	N.A. [2]	N.A. [2]	8.2	15
DOW - Terneuzen	DOW	Carrousel	Chemical wastewater	70	15	N.A. [2]	6	7.2-7.5	23-28
UGent - Ghent	Shrimp	FBBR [4]	N.A. [2]	90	<0.1	N.A. [2]	N.A.	8.2	28

[1] N.A.: Not available

[2] MBBR: Moving Bed Biofilm Reactor

[3] SBR: Sequencing Batch Reactor

[4] FBBR: Fixed Bed Biofilm Reactor

[5] vNLR: Nitrogen loading rate

[6] sNLR: specific nitrogen loading rate

2 Urine composition

Human urine was collected for use during MBR operation. The EC distribution of the collected samples was characterized (Figure 11). The samples were mixed to yield a single stock solution for usage as influent. The TKN concentration and COD concentration of the stock solution were determined and were 6.3 g TKN L^{-1} and 7.9 g COD L^{-1} respectively. The EC of the stock solution was 17 mS cm^{-1} and the pH was 6.5.

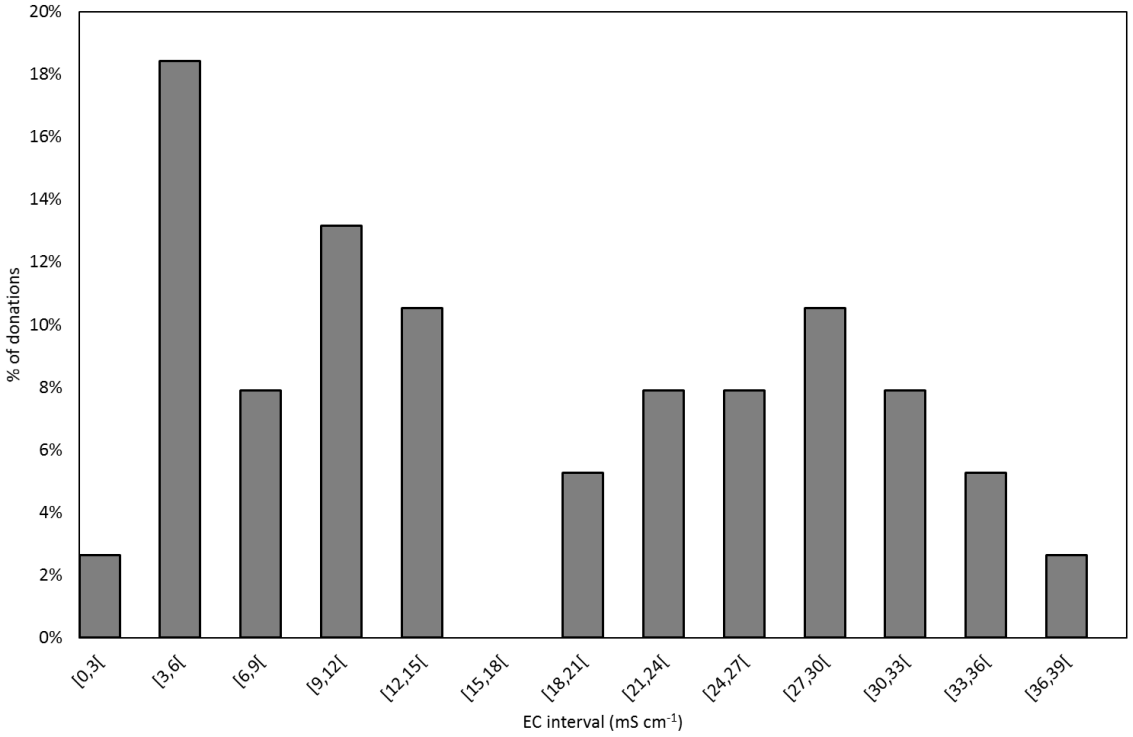

Figure 11: EC distribution of collected urine samples.

3 Start-up of a urine nitrification MBR

The MBR was fed with increasing concentration of 10-100% diluted synthetic urine at a constant flow rate (500 mL d^{-1}) during phase I. Phase II aimed to maintain the volumetric loading rate reached at the end of phase I (500 mg N L^{-1} d^{-1}) over a period spanning two times the hydraulic retention time (HRT). After the steady state operation (phase II), the influent was switched from synthetic medium to real human urine (phase III).

3.1 Phase I: startup with diluted synthetic urine

ABIL sludge was used to inoculate the MBR with 1.32 g VSS L^{-1} at 23.0 mS cm^{-1}, which is the normal EC to which ABIL is adapted at Avecom. This is the VSS concentration to nitrify 25% of the final loading rate at 45 mS cm^{-1}, based on results of the nitrification activity screening. Start-up during phase I was completed in 12 days.

Before the start of MBR inoculation and operation, a nitrification activity test was performed on several conductivities (3.3, 23.0, 33.8 and 47.6 mS cm^{-1}). The results are graphically presented in Figure 12. Ammonia oxidation ranged between 72 and 143 mg NH_4^+-N (g VSS)$^{-1}$ d^{-1}. The optimum conductivity for ammonia oxidation in this test was 23.0 mS cm^{-1}, which was significantly higher than

ammonia oxidation rates at other conductivities. Nitrite oxidation ranged between 561 and 397 mg NO_2^--N (g VSS)$^{-1}$ d^{-1}. A single significant optimal conductivity could not be determined. The nitrite oxidation rate at 47.6 mS cm^{-1} (397 mg NO_2^--N (g VSS)$^{-1}$ d^{-1}) was significantly lower than those at lower conductivities.

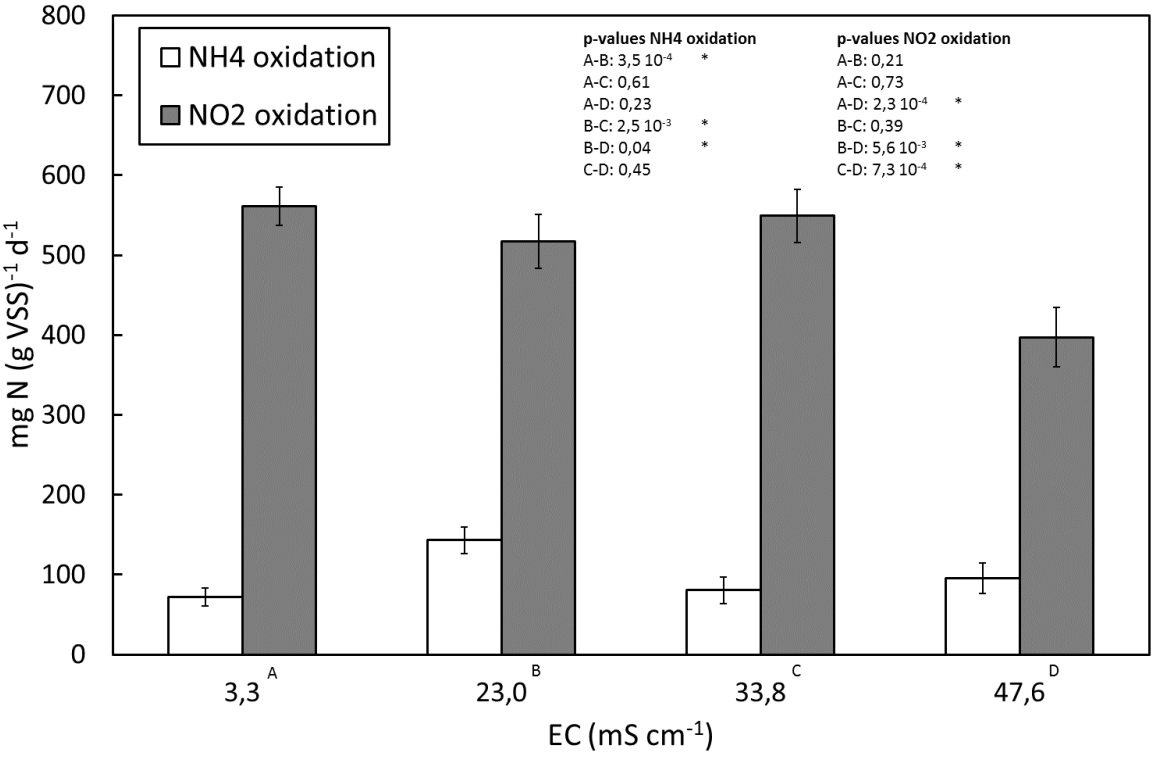

Figure 12: Ammonia and nitrite oxidation rates at several electrical conductivities (EC). Test performed on day 0 of reactor operation. Significant differences are indicated by asterisks.

At the start of the operation (day 0), a low volumetric nitrogen loading rate (Figure 14) caused a corresponding low sNLR of 58 mg N (g VSS)$^{-1}$ d^{-1} (Figure 16). The sNLR was increased in the following days as the dilution of the feed could be lowered. After 12 days of operation undiluted feed was fed to the MBR and the sNLR reached 175 mg N (g VSS)$^{-1}$ d^{-1} due to the slowly increasing VSS concentration and rapidly increased influent concentration. The biomass achieved a higher specific ammonia oxidation rate at 38.9 mS cm^{-1} in the bioreactor than observed from the activity test at a 33.8 mS cm^{-1} (81 mg NH_4-N (g VSS)$^{-1}$ d^{-1}).

Apart from ammonia accumulations on day 4 and day 7 (17 and 52 mg NH_4-N L^{-1} respectively), no detectable residual ammonium (NH_4^+) or nitrite (NO_2^-) was observed. The dilution of the influent could be reduced until at day 12 undiluted influent was used (end of phase I). The electrical conductivity (EC) in the reactor increased from 23.0 mS cm^{-1} at day 0 to 33.7 mS cm^{-1} at day 12.

3.2 Phase II: concentrated synthetic urine

The concentration of the feed was kept constant at 8.0 g NH_4-N L^{-1} during phase II. Several ammonium peaks were observed as the EC in the reactor rose to 45 mS cm^{-1}. The volumetric loading was therefore decreased by reducing the influent flow rate. A stable volumetric loading rate of around 485 mg N L^{-1} d^{-1} was achieved and maintained from day 43 on. The loading rate was decreased to 145 mg N L^{-1} d^{-1} on day 69 after a hitch with the pH controller on day 68 and a power shutdown on day 69, which induced an ammonia peak in the reactor of 140 mg N L^{-1} on day 69. The

salinity further increased from 63.9 mS cm^{-1} to 75.7 mS cm^{-1} between day 61 and day 82. Concurrently, ammonia peaks were observed (Figure 15). The reactor volume was diluted with 2.5L demineralized water on day 82 of operation while extracting the same volume of water. The nitrate concentration dropped from 5542 mg NO_3-N L^{-1} to 3930 mg NO_3-N L^{-1} and the EC from 75.7 mS cm^{-1} to 59.2 mS cm^{-1} as a result of the dilution. From day 85 onwards, the volumetric loading rate was increased again in an attempt to recover the previous loading rate. A maximum volumetric loading rate of 250 mg N L^{-1} d^{-1} could be reached with no ammonium accumulation. At the end of phase II the EC had risen again to 77.6 mS cm^{-1}.

Before day 69, nitrite levels were not detectable (<3.0 mg NO_2^--N L^{-1}). From day 69 onwards, nitrite concentrations were detected (>3.0 mg NO_2^--N L^{-1}). These nitrite concentrations ranged between 3.0 and 14.6 mg NO_2^--N L^{-1} and remained present until the end of phase III.

Between 30 and 60 days of operation, the sNLR was stable around 60 mg N (g VSS)$^{-1}$ d^{-1} Figure 16. A maximum VSS concentration of 9.1 g VSS L^{-1} was reached on day 77. On day 69 the volumetric loading rate was lowered from 480 to 158 mg N L^{-1} d^{-1}. The vNLR was increased again from 110 mg N L^{-1} d^{-1} at day 83 to 250 mg N L^{-1} d^{-1} at day 118. The VSS concentration increased from 5.5 g VSS L^{-1} to 7.7 g VSS L^{-1} between day 95 and 118.

During the operation of the MBR, the utilized EC probe became inconsistent. This was noticed because the EC was fluctuating between 60 and 90 mS cm^{-1} for no apparent reason. When the same sample was measured two days in a row, the EC was not the same at all, even after (re)calibration. All available samples of the MBR effluent (samples from day 61 onwards) were measured again with the new EC-probe because it is not known from which day exactly the measurements were no longer reliable. The EC measurements are depicted as a broken line in Figure 14 because of a different response between the used EC probes (Consort SK20T; Metrohm Pt1000).

From day 91 to day 102 the vNLR could be kept constant around 250 mg N L^{-1} d^{-1}. The EC had risen from 61.1 mS cm^{-1} to 69.3 mS cm^{-1} during this period. Nitrite was present but remained stable and FNA reached a maximum concentration of 0.0044 mg HNO_2-N L^{-1} (day 102) calculated at pH 6.9, the lower bound of the pH interval, and 20°C. This concentration of FNA is not considered harmful to either AOM or NOB (Table 3; Anthonisen et al. 1976). Ammonium concentrations were observed corresponding to 0.109 mg NH_3 L^{-1} (calculated at the upper bound of the pH interval, pH 7.1, and 20°C). Starting from day 104, the vNLR was lowered to 230 mg N L^{-1} d^{-1} and remained on this level until the end of phase II (day 118). During this period, the EC increased to 77.6 mS cm^{-1} and nitrite concentrations were decreasing from 13.6 to 5.2 mg NO_2^--N L^{-1}. The average nitrification efficiency during this period is 97%. No further instability was observed in the reactor.

At the end of phase II (day 118) another nitrification activity test was performed at different salinities (Figure 13).

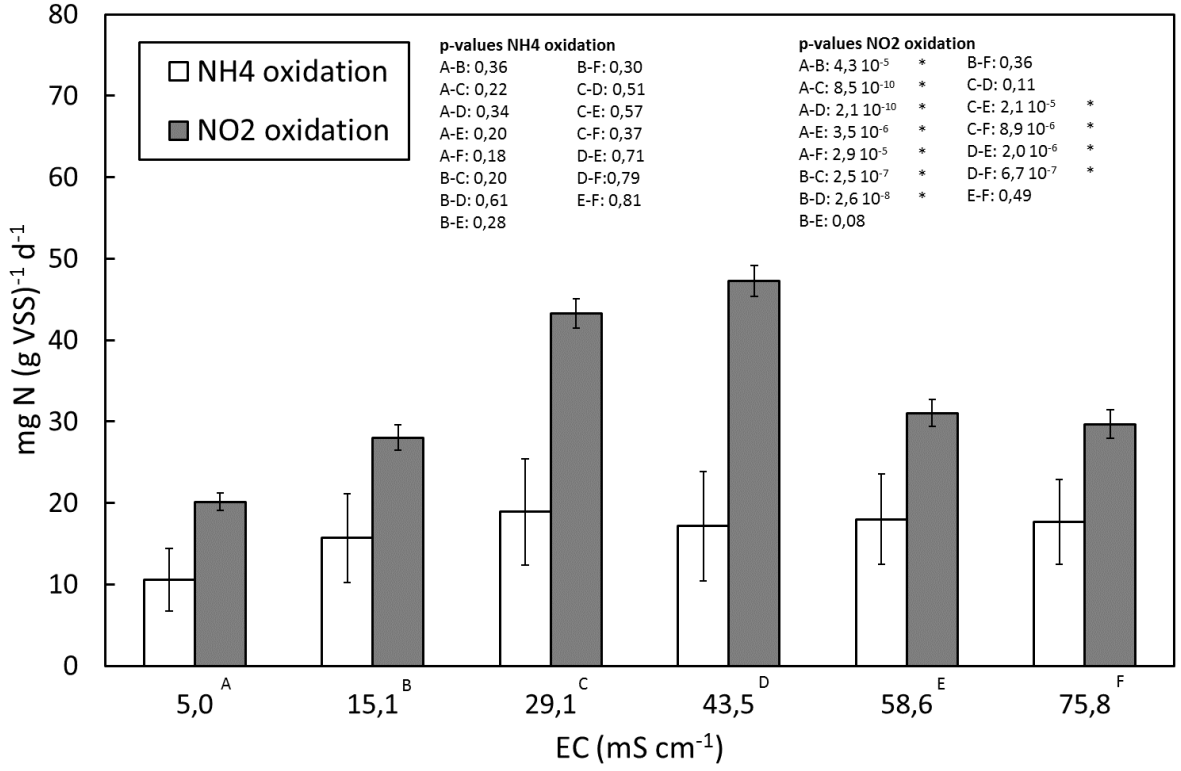

Figure 13: Nitrification activity profile at several electrical conductivities (EC). Both Ammonia oxidation and nitrite oxidation rates are determined on day 118 of reactor operation. Significant differences are indicated by asterisks.

Specific ammonia and nitrite oxidation rates at 45 mS cm^{-1} were respectively 81% and 88% lower compared to the previous activity test. Specific ammonia oxidation rates are in the range of 10.6 to 18.9 mg NH_4-N (g VSS)$^{-1}$ d^{-1}, while specific nitrite oxidation range from 20.2 to 47.3 mg NO_2^--N (g VSS)$^{-1}$ d^{-1}. The highest specific ammonia oxidation rate is observed at a conductivity of 29.1 mS cm^{-1} (18.9 mg NH_4-N (g VSS)$^{-1}$ d^{-1}) but no significant difference could be shown between any of the tested conductivities. The maximum specific nitrite oxidation rate was 47.3 mg (NO_2^--N (g VSS)$^{-1}$ d^{-1} at 43.5 mS cm^{-1}. No significant difference was present between the nitrite oxidation rates of 29.1 and 43.5 mS cm^{-1} (p=0.11) but both conductivities yielded significantly higher rates than lower or higher conductivities.

Samples of the feed and effluent of the MBR were analyzed to estimate the COD removal efficiency. The synthetic hydrolyzed urine contained 7520 mg COD L^{-1} in the form of acetate. The reactor effluent contained 270, 320, and 290 mg COD L^{-1} at respectively day 61, day 81, day 113. The average COD removal is 96.1%.

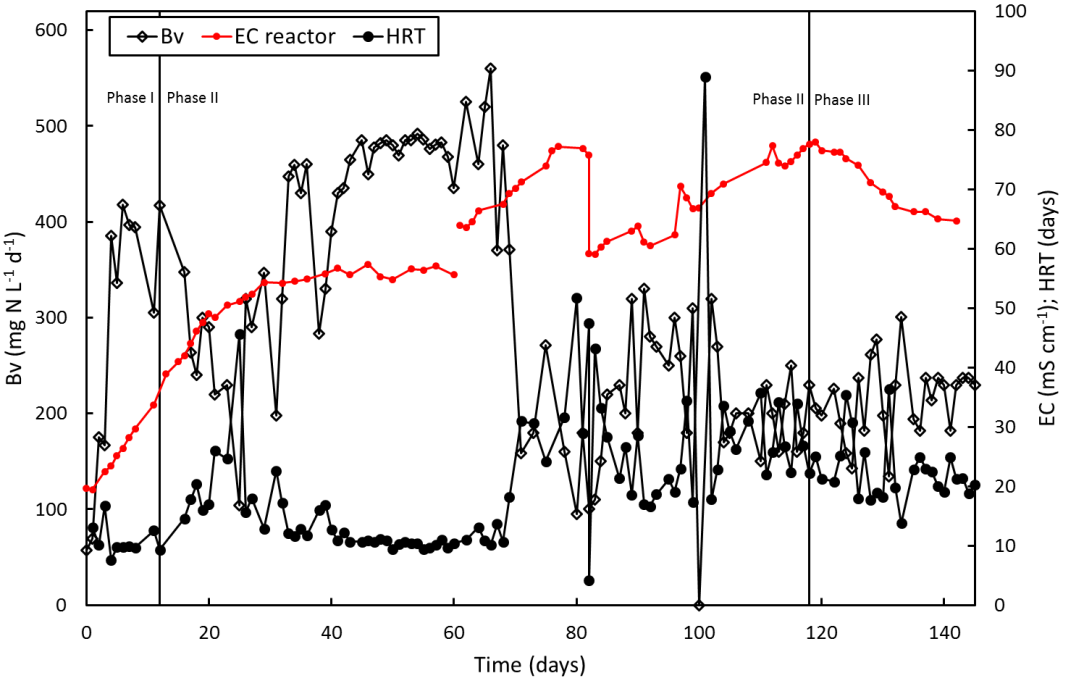

Figure 14: Volumetric loading rate (Bv), hydraulic retention time (HRT) and the electrical conductivity (EC) of the MBR during operation. Phase I: diluted synthetic hydrolyzed urine (10-100%). Phase II: undiluted synthetic hydrolyzed urine (100%). Phase III: undiluted non hydrolyzed human urine.

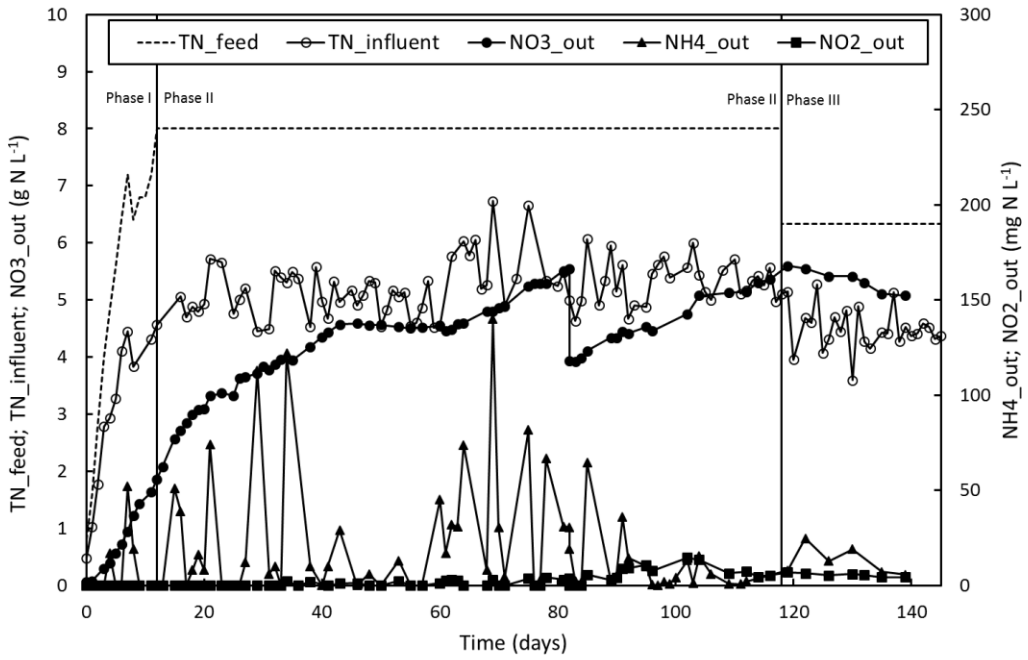

Figure 15: Nitrogen species of the feed (synthetic urine or real urine), the total influent (corrected with volume of the added base) and the effluent during MBR operation. Phase I: diluted synthetic hydrolyzed urine (10-100%). Phase II: undiluted synthetic hydrolyzed urine (100%). Phase III: undiluted non hydrolyzed human urine.

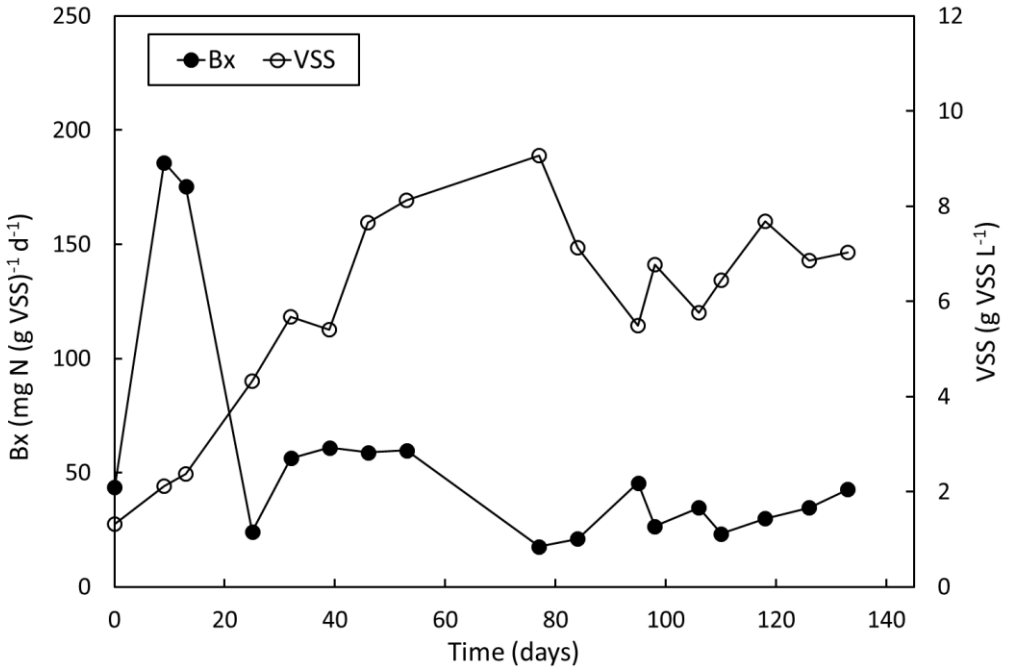

Figure 16: Specific loading rate (Bx) and volatile suspended solids concentration (VSS) throughout the operation period of the MBR.

3.3 Phase III: real non-hydrolyzed urine

Before switching to real non-hydrolyzed urine, a urease activity test was performed to estimate the capability of the sludge to hydrolyze urea. It was found that the sludge from the reactor had a urease activity of 202 ± 23 mg urea-N (g VSS)$^{-1}$ d^{-1}. This is sufficiently high as it is higher than the specific nitrogen loading rates achieved in the MBR (Figure 16).

Phase III was started from day 118 on and aimed to assess the influence of the real urine matrix on the performance of the nitrification. The feeding regime was constantly 250 mg N L^{-1} d^{-1} and the influent was non-hydrolyzed human urine (6.3 g TKN L^{-1}). Nitrate levels decreased from 5598 to 5078 mg NO_3^--N L^{-1} during phase III. The EC decreased from 77.6 mS cm^{-1} to 64.7 mS cm^{-1}. On day 133 of operation the sNLR amounted to 42.8 mg N (g VSS)$^{-1}$ d^{-1}.

The last nitrification activity test (Figure 17) was performed at the end of phase III. The MBR had been fed with non-hydrolyzed human urine for 28 days (day 118 to day 145). Ammonia oxidation rates ranged from 20.4 to 35.6 mg NH_4-N (g VSS)$^{-1}$ d^{-1}. It can be seen that the specific ammonia oxidation rates increased compared to the previous activity nitrification test which ranged from 10.6 to 18.9 mg NH_4-N (g VSS)$^{-1}$ d^{-1}. A conductivity of 29.0 mS cm^{-1} yielded the maximum specific ammonia oxidation rate (35.6 mg NH_4-N (g VSS)$^{-1}$ d^{-1}). This did not differ significantly from the ammonia oxidation rates at 5.6 mS cm^{-1}, 16.9 mS cm^{-1} or 44.3 mS cm^{-1} (respectively 31.0, 31.7 and 29.3 mg NH_4-N (g VSS)$^{-1}$ d^{-1}) but did show a significant difference with the specific rates at 59.4 mS cm^{-1} and 73.9 mS cm^{-1} (28.5 and 20.4 mg NH_4-N (g VSS)$^{-1}$ d^{-1} respectively). Specific nitrite oxidation also increased compared to the activity test at the end of phase II. The range of nitrite oxidation was between 51.5 and 67.5 mg NO_2^--N (g VSS)$^{-1}$ d^{-1} at the end of phase III, with the maximum value at 29.0 mS cm^{-1}. No significant difference was observed between nitrite oxidation rates at 29.0 and 16.9 mS cm^{-1}.

Non-hydrolyzed human urine contained 7880 mg COD L^{-1} and at the end of phase III a COD concentration in the MBR effluent was measured of 340 mg COD L^{-1}. To determine the COD removal efficiency, the volume of the added base and acid has to be taken into account. This means that COD was removed with an efficiency of 93.8%.

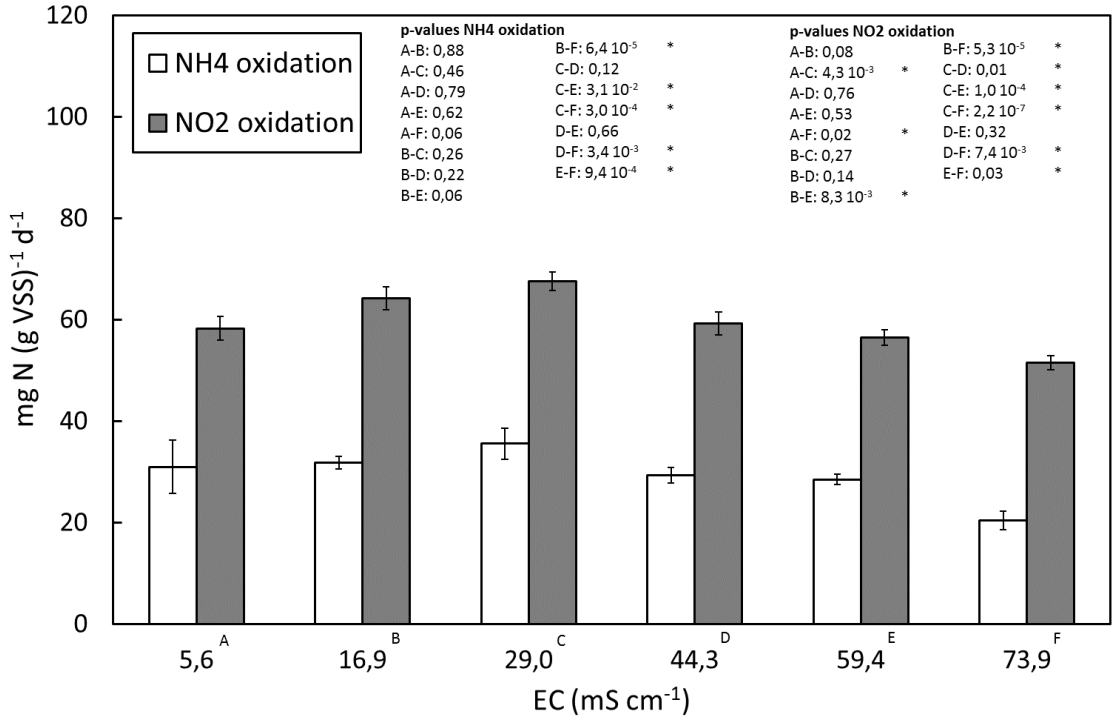

Figure 17: Nitrification activity profile at several electrical conductivities (EC). Both Ammonia oxidation and nitrite oxidation rates are determined on day 145 of reactor operation. Significant differences are indicated by asterisks.

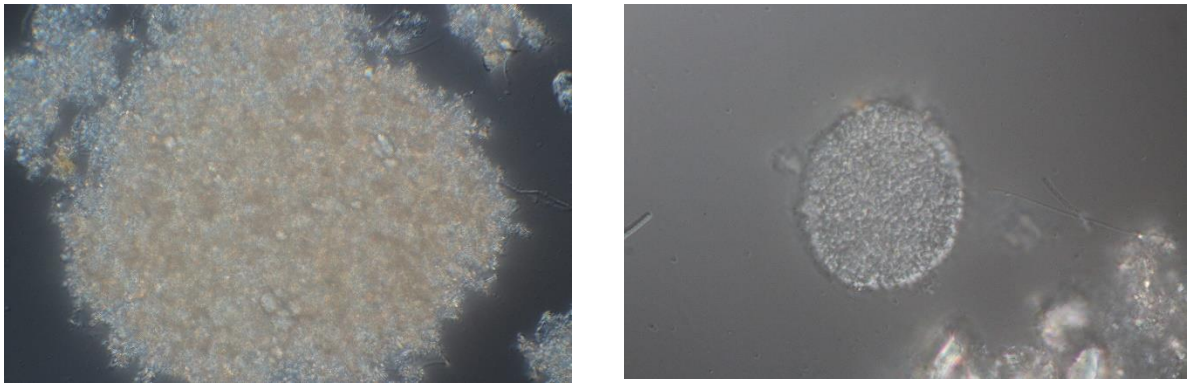

Figure 18: Microscopy images of a sample of the urine nitrification MBR (Left: 400x magnification, Right: 1000x magnification)

The biomass of the MBR was analyzed with a microscope under several magnifications. The flocs of the sludge consisted mostly of aggregated salts (Figure 18, Left). The sludge contains a large number of cauliflower-like structures (Figure 18, Right). These formations have the typical morphology of AOB clusters.

4 Cultivation of *Spirulina*

A follow-up set of experiments to the urine nitrification MBR was conducted to determine the suitability of the nitrified urine for microalgae cultivation. The microalgae used was *Arthrospira platensis*.

In a first stage, the effect of N-source, N concentration and salinity was examined in a high-throughput growth experiment. In a second stage, the influence of the urine matrix on the growth of *A. platensis* was assessed. More specifically, a high-throughput growth experiment was performed to assess the growth on non-hydrolyzed urine, hydrolyzed urine and nitrified urine.

4.1 Growth of *Arthrospira platensis* on modified Zarrouk medium

4.1.1 Effect of salinity

Figure 19 shows the effect of salinity on the growth of *A. platensis* with a nitrate N source. Increase of OD_{680} indicates the increase of biomass. Growth on standard Zarrouk medium modified to 60 mS cm^{-1} shows a prolonged lag phase compared to lower salinities. The salinity effect is more pronounced when the nitrate N source of standard Zarrouk medium was replaced by ammonium N source; media at 30, 45 or 60 mS cm^{-1} with the ammonium N source lagged behind on the medium at 20 mS cm^{-1} (Figure 20). All mediums with ammonium N source at elevated salinities (30, 45 and 60 mS cm^{-1}) showed an increased lag phase compared to the medium at 20 mS cm^{-1}.

However the opposite was true for urea as N source. Growth of *A.platensis* urea N source at 20 mS cm^{-1} was largely inhibited, while the increased salinities (30, 45 and 60 mS cm^{-1}) grew well (Figure 21). After 66 hours the color of the biomass grown on urea changed from green to orange. Despite this color change, OD_{680} increased even after 66 hours.

The OD_{680} at the end of experiments are given in Table A2Table A5.

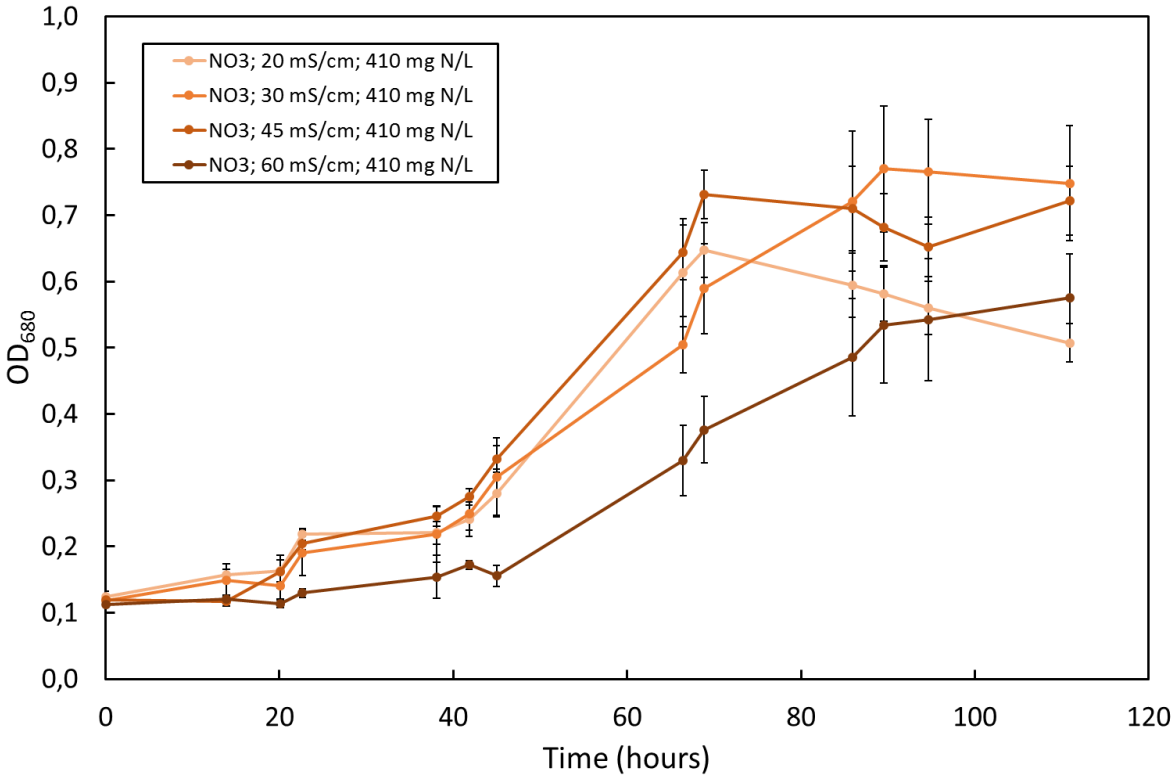

Figure 19:The effect of salinity (indicated by EC in mS cm^{-1}) on growth of *Arthrospira platensis* in standard Zarrouk medium.

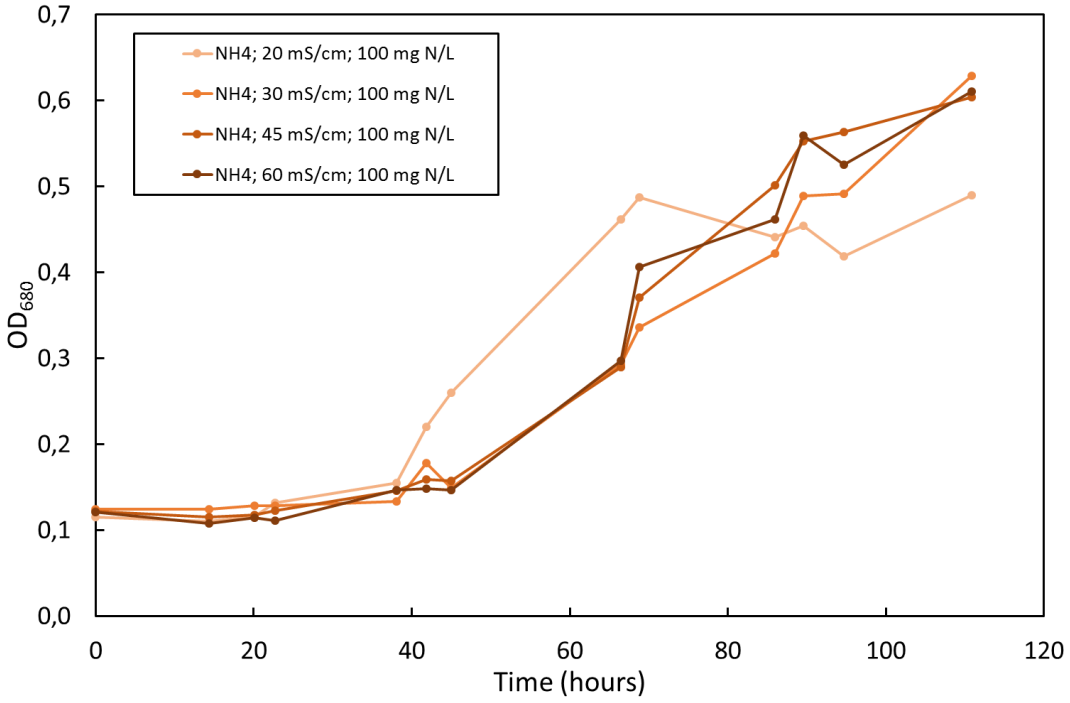

Figure 20: The effect of salinity (indicated by EC in mS cm^{-1}) on growth of *Arthrospira platensis* in modified Zarrouk medium (N source: NH_4^+).

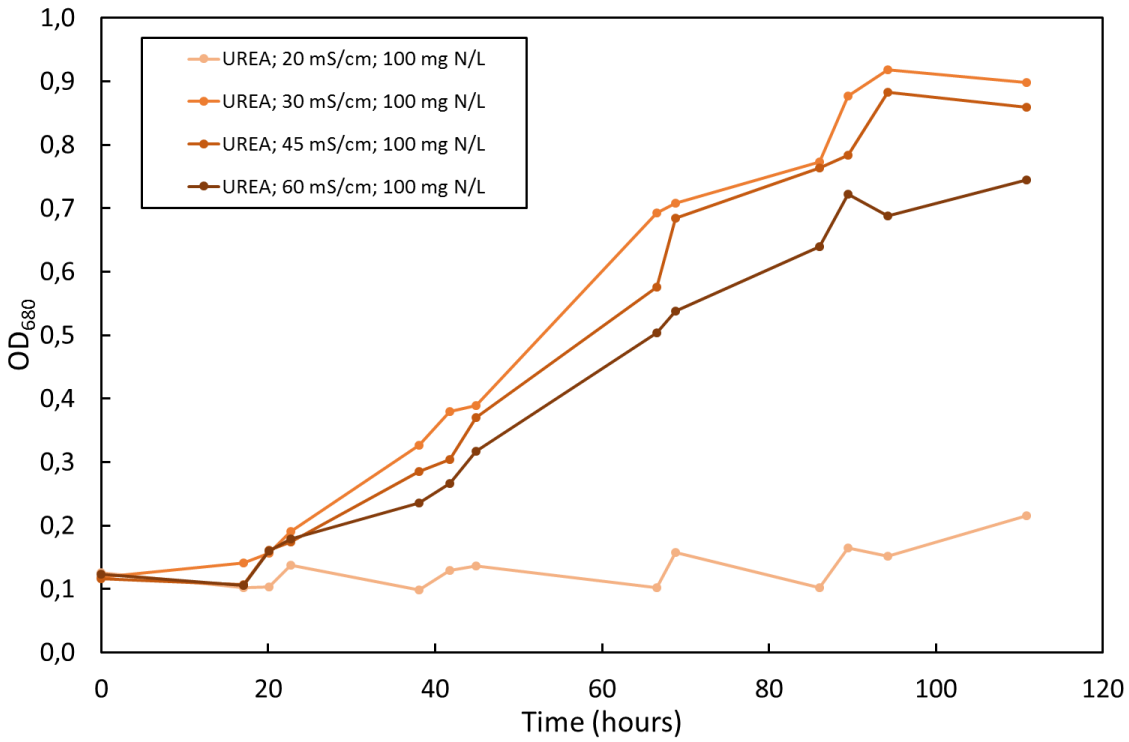

Figure 21: The effect of salinity (indicated by EC in mS cm^{-1}) on growth of *A. platensis* in modified Zarrouk medium (N source: Urea).

4.1.2 Effect of the nitrogen source

Standard Zarrouk medium (N source: nitrate) was adjusted with different N sources. Urea and ammonium were tested besides the non-adjusted Zarrouk medium.

From Figure 22 it can be seen that the standard Zarrouk medium with nitrate N source has the highest growth rate, reaching its maximum OD_{680} (0.648) after only 69 hours. The ammonium and urea N sources lag longer than the nitrate N source and at the end of the experiment (111 hours), OD_{680} values of 0.440 and 0.532, respectively were reached.

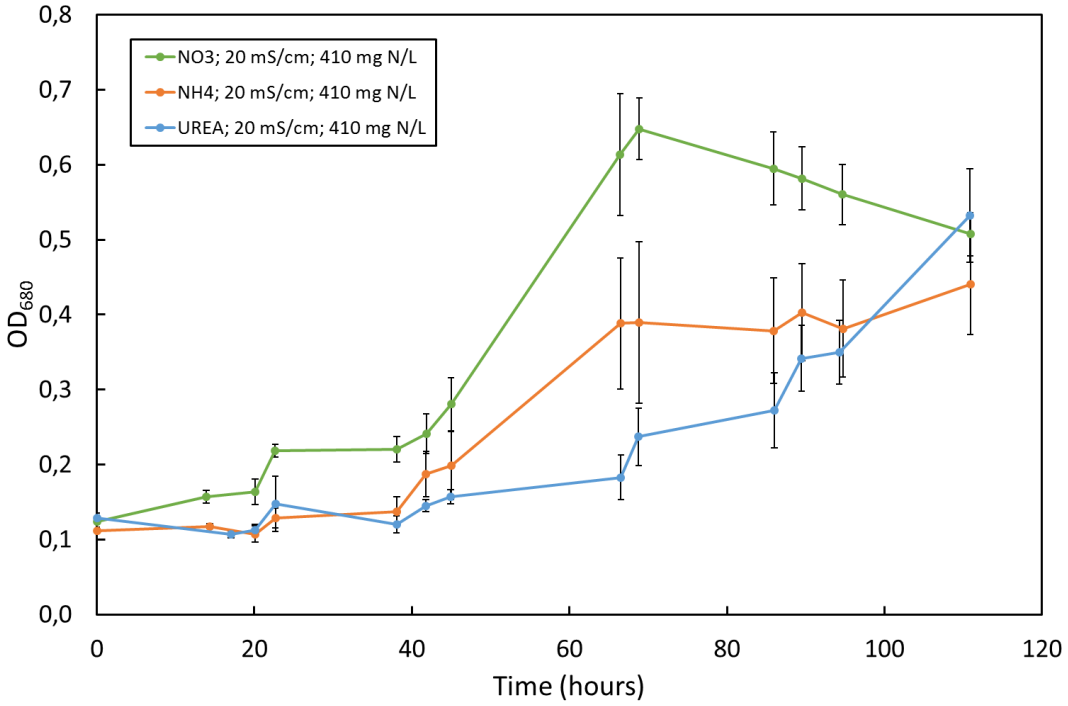

Figure 22: Effect of N source in (adjusted) standard Zarrouk medium. Nitrate, ammonium and urea are compared on standard Zarrouk EC (20 mS cm^{-1}) and N concentration (410 mg N L^{-1}).

The effect of the nitrogen source on other N concentrations and conductivities are consistent with the data from Figure 22, figures at higher concentrations are omitted. Growth curves on ammonium N source is always lagging behind on the nitrate N source. Urea N sources on higher conductivities lag less or not on nitrate N sources, however after 66 hours the biomass on urea N source appeared to have lost its photosynthetic capacity (orange color).

4.1.3 Effect of the nitrogen concentration

The effect of N concentration is dependent on the N source used in the cultivation medium.

In standard Zarrouk medium (N source: nitrate), the growth rate is inhibited by increased nitrogen concentrations. Nitrate concentrations up to 1 g N L^{-1} yield a high growth rate (Figure 23), reaching maximum OD_{680} after only 69 hours, while higher N concentrations show reduced growth rates. This is consistently observed over nitrate N source mediums at all conductivities (Figure A1-Figure A3).

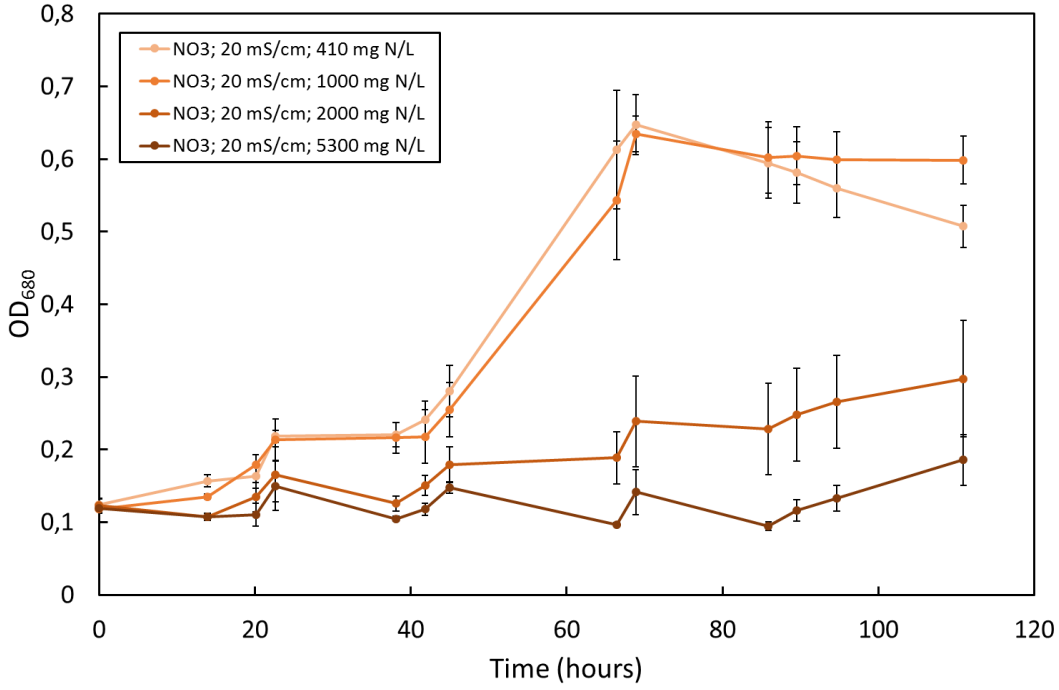

Figure 23: Effect of NO_3^--N concentration on the growth of *Arthrospira platensis* in standard Zarrouk medium (20 mS cm^{-1}).

When ammonia is the selected N source, significant growth is observed for concentrations of 100 and 410 mg NH_4^+-N L^{-1} at a conductivity of 20 mS cm^{-1} (Figure 24). At higher conductivities growth is observed for concentrations of 100, 410 but also at 1000 mg NH_4-N L^{-1} (Figure A4-Figure A6).

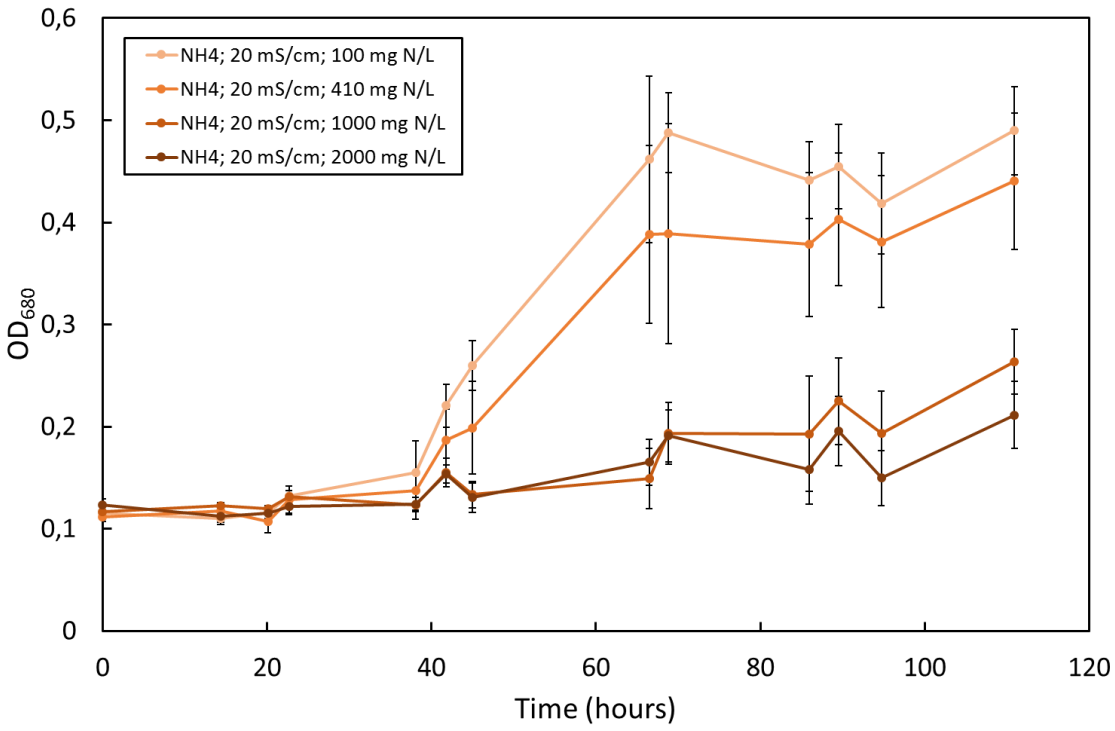

Figure 24: Effect of NH_4^+-N concentration on the growth of *Arthrospira platensis* in modified Zarrouk medium (20 mS cm^{-1}).

Initial growth on urea-N was good but after 66 hours of light exposure *A. platensis* visibly changed color from green to orange (lower right on Figure 25). This color change was observed on every urea based medium, except on urea-based medium at 20 mS cm^{-1}. The evolution of the OD_{680} was hereafter not visibly affected by the change of color.

Urea N concentrations had a different effect on growth at 20 mS cm^{-1}, in contrast to urea concentrations at media on 30, 45 or 60 mS cm^{-1}. At 20 mS cm^{-1}, increased growth rates were observed with increasing urea nitrogen concentrations (Figure A7). At higher conductivities, no effect of the nitrogen concentration was observed (Figure A8Figure A10).

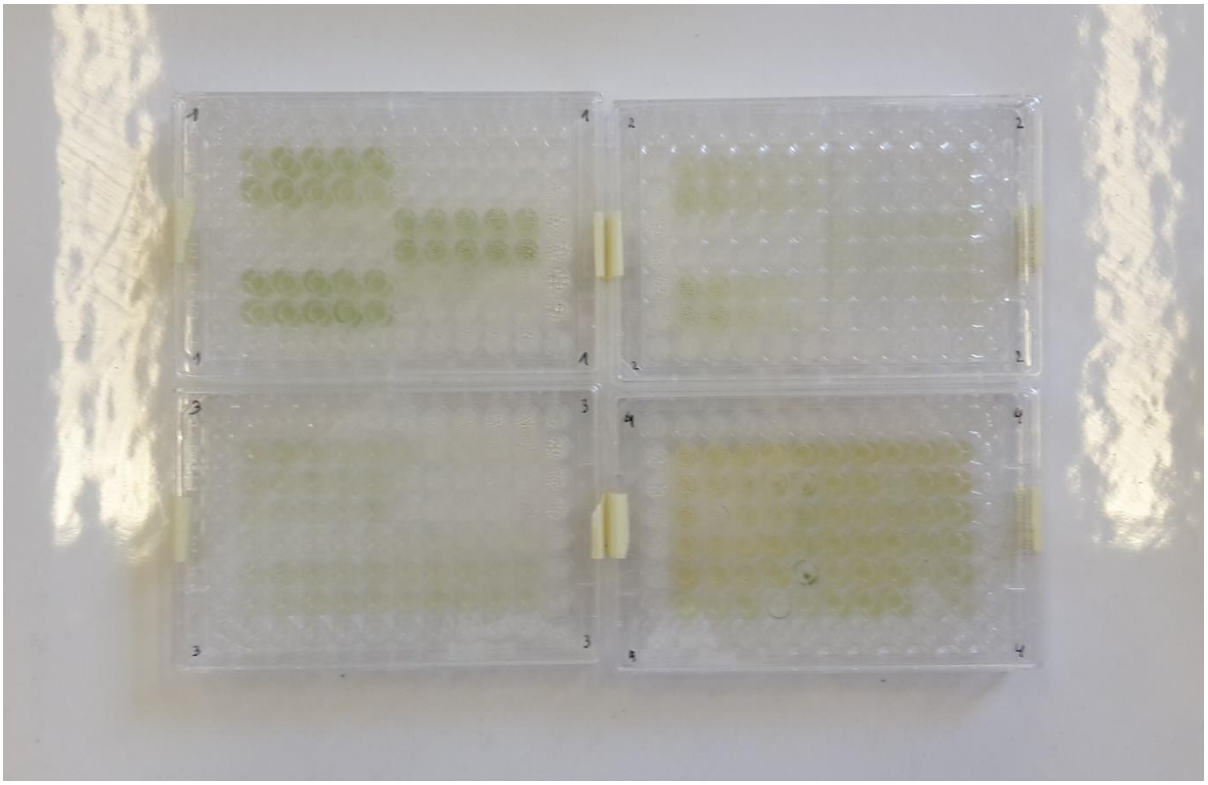

Figure 25: High throughput 96 well plate microalgae growth experiment on modified standard medium. Upper left: NO_3^- at 20, 30 and 45 mS cm^{-1}. Upper right: NO_3^- at 60 mS cm^{-1} + NH_4^+ at 20 and 30 mS cm^{-1}. Lower left: NH_4^+ at 45 and 60 mS cm^{-1} and Urea at 20 mS cm^{-1}. Lower right: Urea at 30, 45 and 60 mS cm^{-1}.

4.2 Growth feasibility in a urine matrix

An experiment was set up to assess the growth feasibility of *A. platensis* in synthetic and a real urine matrices, in contrast to the previous experiment in which the medium was based on Zarrouk medium.

4.2.1 Growth on non-hydrolyzed urine

Among the treatment dilutions with synthetic non-hydrolyzed urine, the maximum $OD_{680,end}$ is observed in the dilution at 0.5 g urea-N L^{-1} ($OD_{680,end}$ = 0.1581). Less diluted treatments with concentrations of 1.0, 2.0 and 3.0 g urea-N L^{-1} also grew well with 1.422, 1.465 and 1.269 respective $OD_{680,end}$. The undiluted and most diluted treatment both did not grow well ($OD_{680,end}$ 0.592 and 0.466 respectively).

Real non-hydrolyzed urine showed different results, as the maximum $OD_{680,end}$ is only 0.627 and is encountered on the lowest dilution treatment (Figure 26). $OD_{680,end}$ of all treatments with (synthetic and real) non-hydrolyzed urine are shown in Table A6.

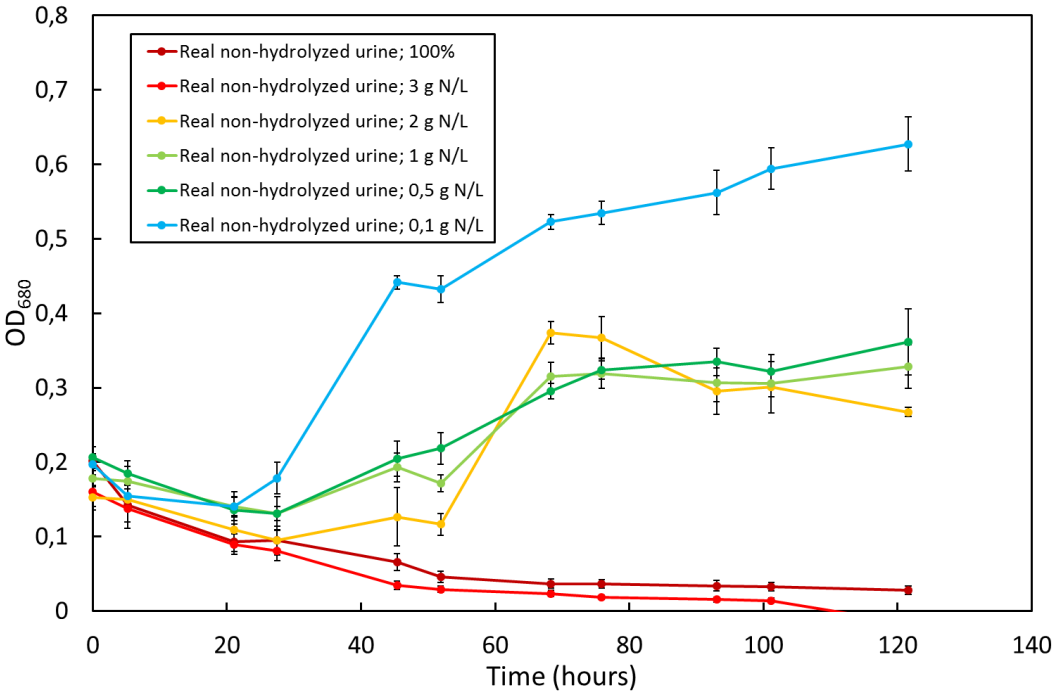

Figure 26: Growth experiment of *Arthrospira platensis* on dilutions of real non-hydrolyzed urine.

4.2.2 Growth on hydrolyzed urine and stripped urine

Hydrolyzed urine treatment results are shown in Table A7 (synthetic and real). Two synthetic urine treatments (2.0 and 3.0 g NH_4-N L^{-1}) and a real urine treatment (3.0 g NH_4 L^{-1}) show an increased $OD_{680,end}$. These false positive results are rejected based on visible judgement, which revealed no microalgae growth (no green biomass in the wells) occurred. No growth was observed in treatments with hydrolyzed urine (Figure 27).

The effect of the matrix on growth was investigated by stripping hydrolyzed urine via air bubbling at high temperature and high pH. Several dilutions of this matrix were made after NH_3 was completely stripped and pH was corrected to 8.2. Ammonium chloride (NH_4Cl) was added to each of the matrix dilutions until a final nitrogen concentration of 100 mg NH_4^+-N L^{-1} was reached. The optimal treatment with stripped synthetic hydrolyzed urine was the undiluted matrix (100%). The OD_{680} of this treatment reached 0.910 by the end of the experiment (120 hours). Moderate growth was observed in the other synthetic dilutions ($OD_{680,end}$ ranged from 0.288 to 0.541).

The undiluted stripped real hydrolyzed urine treatment (100%) did not show any growth (Figure 28). The treatment with 50% diluted matrix (1/2) showed the highest $OD_{680,end}$ (0.476). Diluted urine matrices 1/3 and 1/5 also supported growth. The final OD_{680} of these treatments are 0.401 and 0.275 respectively. The most diluted matrix treatment (1/10) did not show any significant growth (0.079). Results with stripped hydrolyzed urine are presented in Table A9.

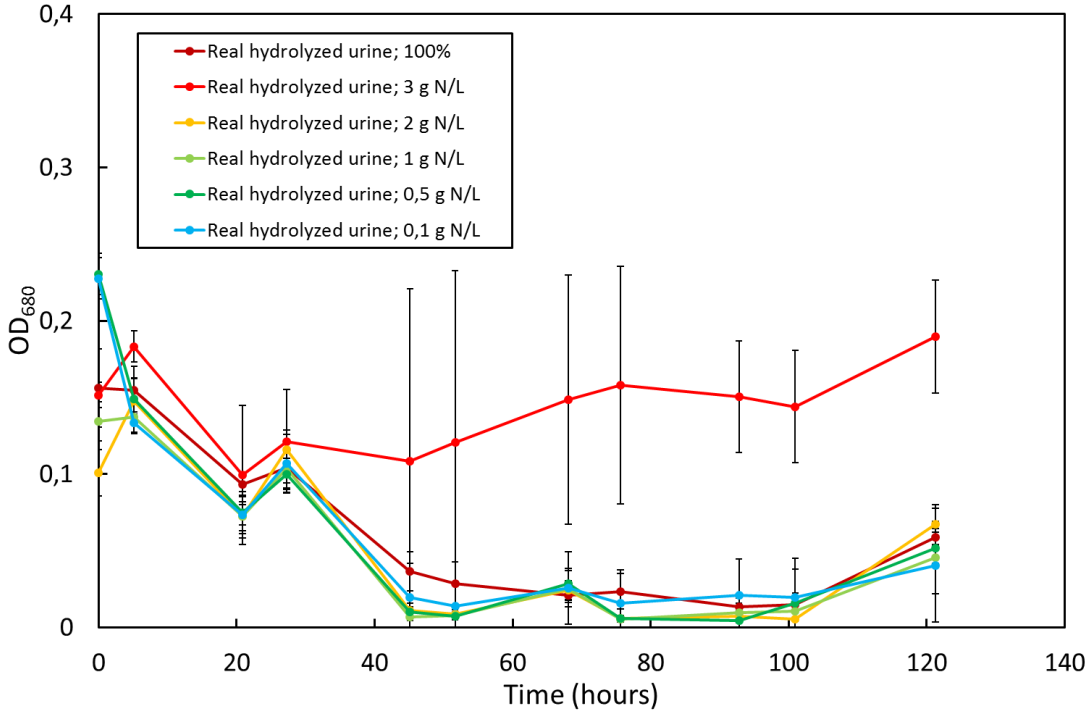

Figure 27: Growth experiment of *Arthrospira platensis* on dilutions of real hydrolyzed urine.

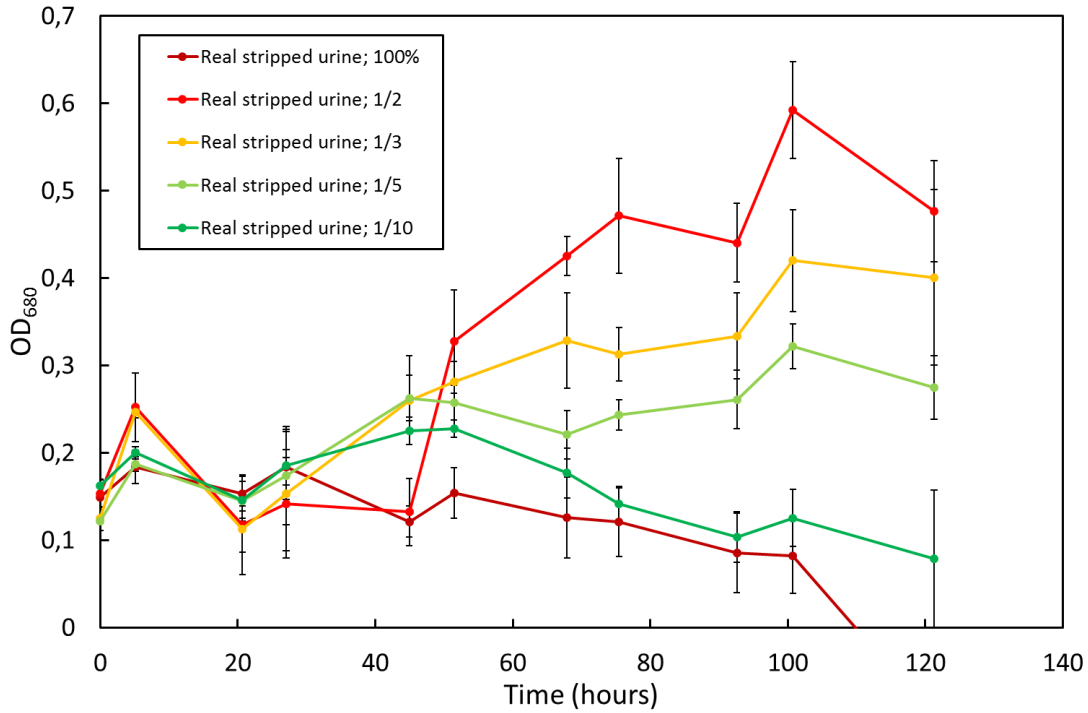

Figure 28: Growth experiment of *Arthrospira platensis* on dilutions of real hydrolyzed stripped urine with a NH_4^+-N concentration of 100 mg NH_4^+-N L^{-1}.

4.2.3 Growth on nitrified urine

Effluent of the urine nitrification MBR was collected at the end of phase II and phase III and are labelled as 'nitrified synthetic urine' and 'real nitrified urine' respectively. This collected effluent was used to perform a growth experiment with *A. platensis*. The results are shown in Table A8. Nitrified synthetic urine yielded a modest maximal $OD_{680,end}$ of 0.335 at a medium concentration of 1.0 g N L^{-1}.

Diluted nitrified synthetic urine with concentrations of 0.5 and 0.1 g N L^{-1} showed also modest $OD_{680,end}$ (respectively 0.194 and 0.253).

The nitrified urine tests confirmed the results obtained: maximum OD_{680} at 1.0 g NO_3^--N L^{-1} for both synthetically based nitrified urine as human based nitrified urine. Undiluted nitrified real urine and diluted nitrified real urine concentrations of 3.0 and 2.0 g N L^{-1} did not show any growth (Figure 29). Like the nitrified synthetic urine, nitrified real urine showed a maximum $OD_{680,end}$ at a concentration of 1.0 g N L^{-1}. The magnitude however was not in the same range, as the nitrified real urine 1.0 g N L^{-1} reached an $OD_{680,end}$ of 1.653. Comparing this to standard medium for *A. platensis* (Zarrouk medium, 500 mg NO_3-N L^{-1}) shows that the real nitrified urine has a similar but slightly slower growth curve as the standard Zarrouk medium. The OD_{680} in the Zarrouk medium at the end of the experiment reached 2.024.

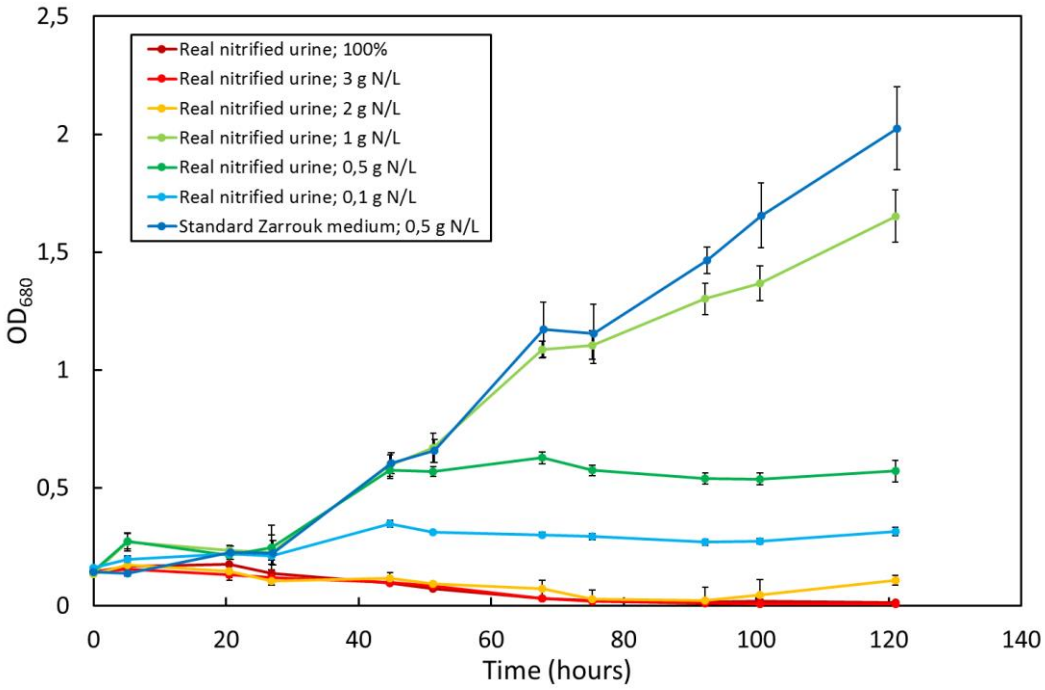

Figure 29: Growth experiment of *Arthrospira platensis* on real nitrified urine (= nitrification MBR effluent). Standard Zarrouk medium (500 mg NO_3-N L^{-1}) is used as a control.

PART 4: Discussion

1 Screening nitrifying consortia

The preliminary screening of nitrifying consortia was performed to characterize ammonia and nitrite oxidation rates at the normal conductivity of the sludge and at 45 mS cm^{-1}, the prospected conductivity of nitrified urine (Muys 2014). The prospected conditions on which the MBR will operate are pH 7, 20°C and a conductivity of 45 mS cm^{-1} at dynamic steady state. Nitrification rate performance and resilience to an osmotic shock (relative inhibition %) were characterized. The best performing nitrifying inoculum was selected and used to inoculate the urine nitrification MBR. Adaptation over time to specified conditions was not examined with these screening experiments, only a shock treatment to the projected MBR conditions was performed. No quantitative DGGE analysis was performed, this means the abundance of nitrifying organisms and heterotrophic microbiota was not quantified. The obtained specific oxidation rates may not be compared as such because …. The potential to ensure an effortless startup of the MBR with each inoculum is estimated, not depending on relative abundancies of AOM or NOB.

1.1 Influence of inoculum on nitrification activity

Because ammonia and nitrite are interfering with growth of aquatic fauna at low concentrations (Frances et al. 1998; Frances et al. 2000; Siikavuopio & Sæther 2006) aquaculture systems demand fast removal of low ammonium and nitrite concentrations. Aquaculture nitrification systems therefore control ammonium concentrations below 0.1 mg N L^{-1}. The dominant AOM and NOB in aquaculture are scavengers (K-strategists), utilize low concentrations of ammonium and nitrite (Schramm et al. 1999) and reach only modest maximum growth rates (Kim & Kim 2006). The low N concentrations can result in higher sensitivity towards FA and FNA and it is therefore not likely the consortia retain nitrification activity should ammonia or nitrite accumulations occur. This indicates that aquaculture activated sludge is not able to reach high specific oxidation rates and this was confirmed in our tests. In conclusion, nitrifying biomass from aquaculture is likely to have trouble coping with the projected high N-load during MBR operation. The sludge types originating from aquaculture do not seem to be ideal for inoculating the MBR.

Danis and Decoster sludge originate from the treatment of manure leachate from piggeries. The leachate is a highly concentrated liquid waste product and contains high nitrogen concentrations (Table 8) up to 4500 mg N L^{-1} as well as high COD concentrations. The normal EC value of both Danis and Decoster 25 mS cm^{-1}. The conditions under which the consortia are operated are more closely related to the operating conditions of the urine nitrification MBR than the consortia originating from aquaculture discussed previously. Comparing the sNLR of Danis and Decoster (Table 8) to the specific oxidation rates achieved during the nitrification activity test (Figure 9; Figure 10), the results obtained in the tests are lower. This might be due to the lower temperature (20°C) compared to the normal operating conditions (25-30°C). Also the pH during the test was buffered at pH 7, while the operating pH of the manure leachate treatment is pH 7.7.

The Breda activated sludge was tested at the normal operating EC and showed specific oxidation rates of 59 mg NH_4^+-N (g VSS)$^{-1}$ d^{-1} and 86 mg NO_2^--N (g VSS)$^{-1}$ d^{-1}. This is almost double the specific nitrogen loading rate (37 mg N (g VSS)$^{-1}$ d^{-1}) to which the activated sludge is exposed at the Breda

WWTP. This can be explained by the higher temperature (20°C) during the batch tests, while the sludge in Breda was operating at a range of 10-15°C.

Industrial WWTP nitrifying consortium did not show any ammonia oxidation activity but nitrite oxidation was 4.3 mg N (g VSS)$^{-1}$ d^{-1} at normal EC. No literature was found describing an industrial WWTP operated on a similar high salinity wastewater as DOW sludge (70 mS cm^{-1}), but a study (Moussa, Fuentes, et al. 2006) conducted with activated sludge from several industrial WWTP (3) and a domestic WWTP investigated the effect of wastewater salinity on specific oxidation rates. The obtained sNLR's show a negative correlation with the salinity. The highest ammonia and nitrite oxidation rates (4.3 mg NH_4-N (g VSS)$^{-1}$ h^{-1} and 2.4 mg NO_2^--N (g VSS)$^{-1}$ h^{-1}) were found in the domestic WWTP with a salinity of 0.13 g Cl^- L^{-1} (corresponding to 1 mS cm^{-1}), while the most saline industrial WWTP had a salinity of 16 g Cl^- L^{-1} (corresponding to 44 mS cm^{-1}) and showed the lowest oxidation rates (1.1 mg NH_4-N (g VSS)$^{-1}$ h^{-1} and 0.5 mg NO2--N (g VSS)$^{-1}$ h^{-1}). More information was given later by DOW company, which stated the provided biomass was collected on a moment the wastewater treatment was having serious trouble in terms of performance. The exact cause of this malfunctioning was not reported. From the results of specific NO2- oxidation, it can be hypothesized that the activated sludge was not adapted to 70 mS cm^{-1}. A lack of information prevents any further conclusions with regards to the DOW activated sludge.

The Imog sludge showed very high specific activity of 101 mg NH_4-N (g VSS)$^{-1}$ d^{-1} and 131 mg NO2--N (g VSS)$^{-1}$ d^{-1} at its normal EC (9 mS cm^{-1}). The obtained results at 9 mS cm^{-1} are much higher compared to the specific nitrogen load in the landfill site (17 g N (g VSS)$^{-1}$ d^{-1}). This large difference in activity cannot be explained by operating parameters such as pH or temperature (Table 8). Nonetheless the medium used during the nitrification activity tests is most likely a better environment for growth and activity of the microbiota present in the sludge. The operators reported presence of heavy metals such as lead (Pb), arsenic (As), cadmium (Cd), mercury (Hg), among others. Heavy metals can inhibit nitrification performance significantly and reversibly, according to literature (S. J. You et al. 2009; Kelly et al. 2004). Thus the nitrification capacity was reduced by the heavy metals in the landfill leachate and not in the nitrification activity tests, explaining why a higher oxidation rate is observed compared to the specific loading rate in the landfill leachate treatment. Another less exotic possibility is that the biomass at the moment of collection was loaded under capacity, no information on this behalf is provided.

The HANDS sludge is also a landfill activated sludge, but is found to be much less active (4.07 mg NH_4-N (g VSS)$^{-1}$ d^{-1} and 17.52 mg NO_2^--N (g VSS)$^{-1}$ d^{-1}). HANDS originates from landfill 'Hoge Maey', a category 2 landfill for non-hazardous solid wastes. No information on the loading rates or heavy metal concentrations at the landfill wastewater treatment is reported. It can be assumed that less toxic or inhibiting compounds are present in the leachate from Hoge Maey compared to the Imog landfill which is a category 1 and 2 landfill site for hazardous and non-hazardous solid wastes. It is possible the sludge was not inhibited and thus not selected for high intrinsic activity, compared to Imog. No definite conclusions can be drawn.

The OLAND reactor at Ghent University is operated under oxygen limited conditions, allowing only a partial nitrification process and subsequent (autotrophic) ammonia oxidation with nitrite as the electron acceptor (Anammox activity). An aerobic ammonia oxidation rate of 124 mg NH_4^+-N (g VSS)$^{-1}$ d^{-1} could be determined on normal EC (37 mS cm^{-1}). This is similar to the reported specific loading rate of the OLAND reactor (131 mg NH_4 (g VSS)$^{-1}$ d^{-1}) despite a difference in pH and operating

temperature (Table 8). Comparing the ammonium oxidation rate in the batch tests with the ammonium removal rate in an OLAND system (Kuai & Verstraete 1998), the specific ammonium removal rate in the conventional OLAND system is only in the range of 11 to 16 mg NH_4-N (g VSS)$^{-1}$ d^{-1}. This can be attributed to the limited oxygen conditions in Kuai's study. The DO concentration in the batch tests were monitored and were never rate limiting (> 7.5 mg O_2 L^{-1}) whereas DO concentrations in the OLAND system were in the range of 0.1 to 0.8 mg O_2 L^{-1}, but mostly below 0.5 mg O_2 L^{-1}. Peng & Zhu (2006) reported oxygen half saturation constants for AOM in the range of 0.2 to 0.4 mg O_2 L^{-1}, explaining the poor performance of OLAND ammonium removal rate in comparison with this study. Due to the operation conditions of the reactor (low DO) from which the sludge is originated, no nitrite oxidation was expected and this hypothesis was confirmed (**Fout! Verwijzingsbron niet gevonden.**).

ABIL sludge is a synthetic inoculum. It is cultivated and enriched on a synthetic medium for commercial goals (Grommen et al. 2002). The synthetic medium is used to increase the performance of the nitrite oxidizing microorganisms in the community. Specific ammonia oxidation is very high while nitrite oxidation rates are even higher due to the selective feeding regime at Avecom. The results of NO_2^- oxidation are in line with Grommen et al. (2002), who reported oxidation rates between 300 and 500 mg N (g VSS)$^{-1}$ d^{-1}. Ammonia oxidation is lower than reported by Grommen et al. (2002), no explanation for this can be provided.

1.2 Influence of salt stress on nitrification activity

The aquaculture consortia were not able to cope with the combined effect of high nitrogen concentrations (discussed in section 1.1) and a salt shock to 45 mS cm^{-1}. The only consortium retaining any oxidation capacity is the Piranha consortium. It retained 26% of nitrite oxidation while ammonia oxidation was fully inhibited. The nitrification activity tests at 45 mS cm^{-1} with Danis and Decoster showed a 70% and 30% decrease of specific ammonia oxidation rate compared to the tests at normal EC (6.11 mg NH_4-N (g VSS)$^{-1}$ d^{-1} and 7.35 mg NH_4-N (g VSS)$^{-1}$ d^{-1} respectively). Specific nitrite oxidation rates decreased by 7% to 7.2 mg NO2- (g VSS)$^{-1}$ d^{-1} for Danis sludge. Decoster sludge at 45 mS cm^{-1} exhibited a 55% increased specific nitrite oxidation rate (9.77 mg NO2--N (g VSS)$^{-1}$ d^{-1}) compared to the normal operating EC (25 mS cm^{-1}). No confident explanation could be proposed regarding the increase in activity at elevated EC. At an increased EC to 45 mS cm^{-1}, no significant ammonia oxidation by Breda activated sludge could be observed and nitrite oxidation was inhibited by 97% to 2.9 mg NO2- (g VSS)$^{-1}$ d^{-1}. This showed the Breda activated sludge was not suitable to use as a rapid start-up inoculum for the urine nitrification MBR, a hypothesis confirmed by Muys (2014). Activated sludge from a domestic WWTP (Rio de Janeiro) was adapted by Bassin et al. (2012) in a sequencing batch reactor (SBR) to subsequently 5, 10, 15 and 20 g NaCl L^{-1}. The SBR had an average specific loading rate of 2 mg N (g VSS)$^{-1}$ h^{-1} but the maximum oxidation rate measured in a batch test was 14 mg N (g VSS)$^{-1}$ h^{-1} at a salinity of 5 g NaCl L^{-1}. The temperature was 24.2°C, which could explain part of the reason why much higher specific oxidation rates are achieved in Bassin's study than the activated sludge from Breda in the screening tests. The Imog sludge showed very high specific activity of 101 mg NH_4-N (g VSS)$^{-1}$ d^{-1} and 131 mg NO2--N (g VSS)$^{-1}$ d^{-1} on its normal EC (9 mS cm^{-1}). This dropped to 31 mg NH_4-N (g VSS)$^{-1}$ d^{-1} and 56 mg NO2--N (g VSS)$^{-1}$ d^{-1} when increasing the conductivity to 45 mS cm^{-1} (a respective decrease by 70% and 57%). The nitrification activity test on 45 mS cm^{-1} revealed a high resilience of OLAND sludge to salinity, as the oxidation rate of ammonia decreased only by 8%. The ABIL consortium was very resilient to salt stress: the ammonia oxidation rate decreased by only 33% at 45 mS cm^{-1} while the nitrite oxidation was even more resilient (18% decrease of activity on 45 mS cm^{-1}).

In the experiments, generally, ammonia oxidizing microorganisms are found to be more sensitive to a shock of salinity than nitrite oxidizing bacteria. When comparing specific oxidation activity at the normal EC and the specific oxidation activity at the targeted EC, all sludge types tested showed a higher relative decrease of the ammonia oxidation rate compared to the nitrite oxidation rate. Nitrite accumulations under increased salinity conditions reported by literature (Cortés-Lorenzo et al. 2015; Natori et al. 2012) suggest that nitrite oxidizing organisms are more sensitive to salt stress than ammonia oxidizing organisms, in contrast to the findings in Moussa, Sumanasekera, et al. (2006). Moussa et al. suggest that nitrite accumulation is due to the decreased availability of dissolved oxygen at higher salinities and the lower K_s value for oxygen of the NOB compared to the AOM. Moussa also found that NOB are less affected by salinity concentrations up to 10 g NaCl L^{-1} than AOM.

In this research, consortia which experience a higher salt shock (higher ΔEC) were generally (with some exceptions) more inhibited than consortia which experienced a lower salt shock in their original environment. This is logic and is confirmed by a study on industrial WWTPs on different salinities and a domestic WWTP (Moussa, Fuentes, et al. 2006).

1.3 Optimal inoculum for urine nitrification

The desired inoculum has a high oxidation rate at 45 mS cm^{-1} and a higher ammonia oxidation rate than nitrite oxidation rate to avoid nitrite buildup in the reactor.

The use of sludge from aquaculture as inoculum in a high salinity MBR and a high loading rate is not recommended because high nitrogen concentrations seem to be problematic, and in combination with a high salinity, no nitrification activity is retained. Sludge originating from the treatment of manure leachate showed in most batch tests low activity. For application in a nitrification reactor aiming to recover nitrogen in food or feed, this type of inoculum is not preferred because of the lower specific nitrite oxidation rates than ammonia oxidation rates, as well as because of the association with manure. Activated sludge originating from domestic WWTP had good nitrification activity on low conductivities, but is not resilient at all to a conductivity of 45 mS cm^{-1}. No conclusions could be drawn for sludge originating from industrial WWTP (DOW) since …. Activated sludge originating from landfill categories 1 (Imog) is shows very high oxidation rates, but is not recommended due to possible adsorption of heavy metals or hazardous substances to the sludge. ABIL nitrifying biomass showed very high ammonia and nitrite oxidation rates at both high and low salinity, indicating a good resilience to salt stress and high nitrification capacity. The lack of any nitrite oxidation makes the OLAND sludge less interesting to use as inoculum for the MBR, as full nitrification is desired. The OLAND sludge is however a good candidate as supplement to an inoculum with less resilient ammonia oxidation to high salinity but more resilient nitrite oxidation. The high specific rate ammonia and nitrite oxidation combined with the high resilience towards salinity make the ABIL sludge a very good candidate as inoculum of the urine nitrification MBR.

In conclusion, ABIL was selected as inoculum in the following experiments because of the high ammonia oxidation rates at both normal EC and 45 mS cm^{-1}, as well as the high nitrite oxidation rates on the projected conditions. Another advantage in comparison with sludge types originating from manure or landfill leachate treatment is that ABIL is a synthetic inoculum, and no association to pathogens or hazardous substances is apparent, making subsequent recovery to single cell protein (SCP) safer.

2 Urine composition

The urine composition (TKN, COD, EC) of synthetic and real urine in this study was analyzed to enable operation at a predetermined vNLR.

The synthetic hydrolyzed urine used to feed the MBR had a concentration of 8.0 g TKN L^{-1}. The collected real human urine (non-hydrolyzed) had a concentration of 6.3 g TKN L^{-1}. Both these values are lower but in a range comparable to reported values from literature. Maurer et al. (2006) reported nitrogen concentrations of 8.8 and 9.2 g TN L^{-1}. Udert, Larsen, Biebow, et al. (2003) reported lower values in stored urine of 1.79 g TN L^{-1}, but this is attributed to urea hydrolysis and subsequent ammonia volatilization. Ammonia volatilization is undesirable and must be controlled as much as possible when aiming for nutrient recovery.

The COD concentrations in real and synthetic urine were 7.9 g COD L^{-1} and 7.5 g COD L^{-1} respectively, which is lower than reported values of 10 g COD L^{-1} (Maurer et al. 2006). Udert, Larsen, Biebow, et al. (2003) report a lower COD concentration (1.65 g COD L^{-1}) in stored urine. In another study, COD removal in a urine storage tank was confirmed to take place (Udert et al. 2006).

3 Start-up of a urine nitrification MBR

The nitrification of urine aims to transform the nitrogen compounds of urine via ammonium and nitrite to nitrate, a non-volatile and generally non-toxic form of nitrogen. In MBR operation three phases are distinguished: the influent concentration of synthetic urine was increased (phase I) followed by stable operation on synthetic urine (phase II) after which the influent was switched to real human urine (phase III).

Several activity tests were performed over the course of the MBR operation. The objective was to determine the oxidation rates of ammonia and nitrite as well as to determine the optimal conductivity. By repeatedly performing activity tests at specific time points, possible changes in activity and optimum conductivity can be revealed.

3.1 Effect of inoculum on startup of urine nitrification

The current study achieved startup of the MBR in only 12 days. From day 12 on, undiluted urine (8.0 g N L^{-1}) was fed to the reactor at a volumetric loading rate of 500 mg N L^{-1} d^{-1} (and COD loading rate of …). Comparison to literature shows that the synthetic ABIL inoculum has a positive effect on the duration of start-up. Muys (2014) studied the start-up of a urine MBR inoculated with Breda activated sludge. In the study of Muys, a concentration of 100% synthetic urine (8.0 g N L^{-1}) was reached after 43 days of operation. The inoculation with ABIL sludge thus offers a significant reduction in startup duration. In the study of Muys, startup was significantly delayed by a decrease in ammonia oxidation activity of the biomass due to salinity stress from 21 to 11 mg NH_4-N (g VSS)$^{-1}$ d^{-1}. In addition, the biomass concentration decreased from 5.7 g VSS L^{-1} after inoculation to 3.0 g VSS L^{-1} after only 13 days of operation. In the present study, the reactor biomass did not experience this delay due to salt stress. The sNLR and absence of residual ammonia and nitrite concentrations at the end of phase I indicate that the ammonium oxidation activity of the biomass increased from 143 mg NH_4-N (g VSS)$^{-1}$ d^{-1} to approximately 175 mg NH_4-N (g VSS)$^{-1}$ L^{-1}. Specific nitrite oxidation is equal or higher than specific ammonia oxidation. The preliminary screening of nitrifying inoculum (**Fout! Verwijzingsbron niet gevonden.**) in the present study confirmed that Breda activated sludge has difficulties with increased salinity. Muys (2014) and the current study did not observe residual

ammonia and nitrite in the reactor effluent, indicating that nitrification was successful at the end of MBR startup in both studies. Due to low observed concentrations and pH 7, ammonia stripping is unlikely to have an impact on the efficiency of nitrogen conversion. Denitrification is considered unlikely due to the high DO concentrations (> 8.0 mg O_2 L^{-1}) and intensive mixing. As previously mentioned, the achieved specific nitrogen loading rates (Figure 16) and absence of residual ammonia or nitrite in the effluent indicate that ABIL was able to achieve higher specific oxidation rates than observed during the screening. This is in agreement with the study of Grommen et al. (2002), which reports specific nitrification rate of 300-500 mg N (g VSS)$^{-1}$ d^{-1}. The observations and literature indicate that ABIL inoculum experienced a reduced specific oxidation activity during the screening but adjusted well to the conditions in the MBR during the 12 day startup period. It is unknown what caused a reduced ammonia oxidation rate during the nitrifying activity test.

3.2 Reactor performance

A stable vNLR was reached of 500 mL L^{-1} d^{-1}, but due to abnormalities in pH control and a power shutdown in the building, the biomass was shocked and the reactor performance dropped sharply as it was impossible to increase the vNLR beyond 250 mg N L^{-1} d^{-1}. The difficulties to recover the loading rate were due to increasing salinity. The lower influent flow rate resulted in a longer HRT and a higher fraction of water which evaporated from the MBR. The increased evaporation was the cause of the increasing salinity. Ammonia levels were observed of 0.109 mg NH_3-N L^{-1}. This is not toxic to AOB or NOB according to Table 3 (Anthonisen et al. 1976). The loss of ammonia oxidation capacity was thus not due to FA levels, but most likely due to the increased salinity. This setback stresses the importance of consistent pH and DO control in a high rate nitrification reactor at high salinity. A change of flow rate can result in non-anticipated difficulties such as increased evaporation and increasing salinity. Despite the setback, close to 100% nitrification efficiency could be achieved at the end of phase II. The maximum loading rate of undiluted synthetic urine in this study was 500 mg N L^{-1} d^{-1}. The maximum vNLR achieved with undiluted real human urine was 250 mg N L^{-1} d^{-1}.

Feng et al. (2008) studied urine nitrification in a packed bed reactor applying two operating strategies in series on the same reactor. A vNLR of only 50 mg N L^{-1} d^{-1} was reached and the influent was 10-fold diluted real human urine. The pH of the reactor was uncontrolled in the first strategy, and only 50% nitrification was observed. Another strategy controlled the pH daily to pH 8.0, and a 95% nitrification efficiency was achieved.

Udert & Wächter (2012) operated a membrane aerated biofilm reactor for urine nitrification between pH 6.2 and 7.0. The authors observed a nitrogen loss of 24%. Due to the low shear of aeration, a thick biofilm was formed. Ammonia volatilization during storage (influent pH 8.7) caused an increased COD/N ratio. In the anoxic zone of the thick biofilm, conditions were thus favorable for heterotrophic denitrification. The DO concentration in the bulk liquid was between 3.0 and 5.5 mg O_2 L^{-1}. The maximum vNLR was 180 mg N L^{-1} d^{-1}.

In the MBR setup of the present study a high shear by aeration prevented biofilm formation. The DO concentration was always above 8.0 mg O_2 L^{-1} in the mixed liquor, reducing the probability of diffusion limitation and anoxic zones in biomass flocs. Denitrification is unlikely to have occurred.

3.3 Adaptation to salinity

Activity tests have been performed day 0, day 118 (end of phase II) and day 145 (end of phase III) to determine the specific nitrification capacity of the sludge and reveal shifts in specific oxidation rates

and optimal salinity.

From the results (Figure 12, Figure 13 and Figure 17), it can be seen that the specific nitrification activity dropped between day 0 and day 118. Optimal salinity for ammonia oxidation in the nitrification activity test was 29.1 mS cm^{-1}, while optimal salinity for nitrite oxidation had shifted from 23.0 to 47.6 mS cm^{-1}. The EC in the reactor at the moment of the test was 77.9 mS cm^{-1}. The specific ammonium and nitrite oxidation rates had dropped considerably. This is likely due to the abnormalities encountered around day 69 or due to prolonged exposure to high salinity. Muys (2014) encountered a drop in specific nitrification activity due to the salt stress. However the decrease in activity was resolved and even reversed after 55 days of operation, as an increased specific activity was observed. Adaptation of the biomass in the study of Muys was successful.

At the end of phase III the nitrification activity of the biomass was tested again. At this time the EC in the reactor had dropped to 64.7 mS cm^{-1}. The oxidation rates had enhanced compared to the activity test on day 118. This is likely due to the decreased EC in the reactor, indicating the biomass was severely stressed at 77.9 mS cm^{-1}. The tests also indicate that neither ammonia or nitrite oxidation recovered rapidly from the salt stress, as the oxidation rates at low salinity during the activity tests were not near the initial oxidation rates.

Studies on biomass adaptation strategies to higher salinities have been found in literature (Uygur 2006). Uygur tested the salinity adaptation of a mixed culture originating from freshwater wastewater treatment processes. A decrease in specific oxidation rates was recorded rather than a shift towards an increased optimal salinity.

3.4 Effect of real human urine

Foam formation occurred from the start of phase III. Foaming in a nitrification process can occur due to several reasons. Gas formation in anoxic zones, extracellular polymeric substances (EPS) or filamentous organisms (Pujol et al. 1991). Stress factors (famine, salt stress) have been described to induce foaming due to excess production of extracellular polymeric substances (EPS) (Di Bella et al. 2011). Reid et al. (2006) described that high salinity increased the EPS concentrations in the bulk phase of the reactor. The foaming was however not present in phase II at higher conductivities. Foaming during phase II did occur however after the pH controller had hitched on day 68 and the power shutdown on day 69. This indicates that the salinity was probably not the factor inducing foam formation during phase III. Anoxic zones in the MBR or stress due to famine or salinity are not likely to have caused the foaming. The microorganisms are fed continuously with high concentration influent, so it is also unlikely that famine has been the cause for foaming. The foam formation may be either a biological or a physical consequence of the urine matrix composition. No literature on these topic was found. The sludge was examined under a microscope, however, little or no filamentous bacteria were found (Figure 18).

Nitrification of real human urine was performed at a vNLR of 250 mg N L^{-1} d^{-1} despite the foam formation. The unaltered nitrification activity suggests that the foam formation was likely an effect of the urine matrix combined with intensive bubble aeration. Biomass concentrations decreased from 7.7 to 7.0 g VSS L^{-1}. The decrease of biomass is likely due to the steady decrease of EC in the reactor. A lower EC induced higher specific oxidation rates, conform the latest nitrification activity test. The ammonia and nitrite oxidizing organisms in the nitrifying community in the reactor became more competitive, resulting in less biomass with higher specific oxidation rates.

4 Microalgae cultivation on urine

Urine is characterized by high N concentrations and high salinities. The high N concentrations make it an interesting stream for nutrient recovery. The multitude of studies conducted in this field (Feng et al. 2007; Feng et al. 2008; Yang et al. 2008; Rodrigues et al. 2010; Chang et al. 2013; Tuantet et al. 2014) indicate that cultivation of microalgae on urine is a promising concept in nutrient recovery.

However, the high urea and ammonia concentrations also induce limitations for batch-fed algae cultivation. In a two-stage experiment the growth characteristics of *Arthrospira platensis* were assessed for different N species, concentrations and salinities. Based on these results, the growth of *A. platensis* was afterwards determined for different types and concentrations of urine.

4.1 *Arthrospira platensis* growth on non-hydrolyzed urine

Urea is the dominant nitrogen source in fresh human urine. Because it can easily hydrolyze, it is not advised to keep urine as such in a storage tank. With the aim of final nitrogen recovery, it is assessed whether fresh urine can serve as a direct nitrogen source in *A. platensis* cultivation.

Undiluted non-hydrolyzed human urine was unable to sustain *A. platensis* growth. The best growth on non-hydrolyzed urine was observed in the most diluted medium (with 0.1 mg urea-N L^{-1}). This is in agreement with 85-98% urea removal from 120-fold diluted human urine (75 mg urea L^{-1}) reported in literature (Chang et al. 2013). Chang et al. also reported an increased growth when adding organic carbon. The authors suggested mixotrophic growth reduced ammonia inhibition and increased biomass growth compared to autotrophic growth.

4.2 Effect of urine hydrolysis

Ammonia toxicity was observed in all dilutions of real hydrolyzed urine, as biomass growth was not going on. From the experiments on modified Zarrouk medium with NH_4Cl as N source, it could be concluded the ammonia toxicity is only apparent at 2.0 g NH_4-N L^{-1} or higher concentrations (pH 8.2). This agreed to the findings of Tuantet et al. (2014) who reported growth inhibition of the microalgae *Chlorella sorokiniana* at ammonium concentrations of 1.4 g NH_4-N L^{-1} at pH 8.0.

Together with the volatility of ammonia and possible N losses at high pH, ammonia toxicity obstructs the practicality of hydrolyzed urine as algae growth medium. A lowered pH to reduce NH3 volatility may affect the growth of *A. platensis* because the optimal conditions for growth are between pH 8 and pH 11.

4.3 Effect of urine nitrification

The growth experiments with real urine show that nitrified urine up to 1 g NO_3-N L^{-1} yields good growth rates of *A. platensis*. This is confirmed by Feng et al. (2007), who concluded from their study that nitrate was the best performing nitrogen source. Considering the stable character of nitrified urine compared to fresh urine (which will hydrolyze during storage), nitrification of urine is a good strategy to stabilize urine and prepare a suitable medium for the microalgae.

Feng et al. (2008) used nitrified real human urine as medium for the growth of *A. platensis*. The human urine fed to a nitrification reactor was diluted 10 times. The effluent was then used as a culture medium. The effluent nitrogen concentration was 600 mg NO_3-N L^{-1}. *A. platensis* was reported to grow well on this nitrate concentration. This agrees with our experiments, in which good growth of *A. platensis* was observed up to nitrate concentrations of 1 g NO_3-N L^{-1}.

4.4 Optimal cultivation conditions

All previous arguments condensed, it can be concluded that nitrate is a stable nitrogen compound yielding good growth rates in nitrified urine as well as standard Zarrouk medium. The optimal nitrate concentration is 1.0 g NO_3-N L^{-1}. Ammonium concentrations of 2.0 g NH_4-N L^{-1} or are toxic at a pH of 8.2 in modified Zarrouk medium. Tuantet et al. (2014), found that concentrations of 1.4 g NH_4^+-N $L^{-1.}$ are already toxic to another microalgae: *Chlorella sorokiniana*. Hydrolyzed human urine showed no growth rate however, indicating this is not a suitable medium to grow *A. platensis* (without pH correction).

Nutrient recovery via source separated urine, urine nitrification and subsequent microalgae cultivation is thus a promising pathway in sustainable wastewater treatment. The concentrations in the reactor effluent are around 5.3 g NO_3-N L^{-1}, but in a continuous photobioreactor the projected effluent concentration is much lower. Nutrient recovery in biomass can be achieved in a continuous reactor system where the reduced growth rate at high nitrate concentrations is bypassed.

PART 5: Conclusions

1 Conclusion

Commercially available inoculum for aquaculture applications (ABIL) has a high resilience towards salinity as well as a high nitrification rate and was therefore the most optimal inoculum from inoculum screening.

The utilized ABIL inoculum had a major positive effect on the duration of startup of a urine nitrification reactor. High nitrogen loading rates were obtained (500 mg N L^{-1} d^{-1}) on synthetic urine. Disturbances of aeration due to external factors and failure of the pH controller lead to a reduced nitrification performance. The nitrogen loading rate could be recovered to 250 mg N L^{-1} d^{-1}. Substitution of the synthetic influent by real human urine was successful. The nitrogen loading rate could be maintained at 250 mg N L^{-1} d^{-1} despite foam formation. Urine was successfully stabilized by nitrification. The nitrification MBR produced a nitrate-rich solution with a low COD content.

Growth of *A. platensis* on nitrate was optimal at a nitrate concentration of 1 g N L^{-1}. Nitrate concentrations of 2 g N L^{-1} and higher had an inhibitory effect on *A*. *platensis*. The tested microalgae did not show reduced growth at high salinities. Growth on real urine matrix confirmed optimal nitrate concentrations of 1 g N L^{-1} and showed inhibition at concentrations from 2 g N L^{-1} onwards. Nutrient valorization by means of *A. platensis* cultivation on urine is a promising strategy in combination with urine nitrification.

2 Future prospects

The ABIL inoculum was selected as the best performing under given high salinity conditions. Species distribution in the microbial nitrification community during actual operation of a urine nitrification reactor may indicate the favored species of ammonia or nitrite oxidizing microorganisms. This knowledge could then be used to enrich a specific synthetic inoculum and decrease the startup period of new urine nitrification systems as well as increase the performance in terms of maximum nitrogen loading rates.

The startup of a urine nitrification system for the stabilization of urine was examined. Batch tests were performed to indicate feasibility on the nutrient valorization. Further research is proposed to maximize knowledge on nutrient recovery and process stability.

From the reduction in nitrification performance following flawed operation, it is clear that disturbances of aeration and pH control can have a major negative effect on process performance. Insight in shifts of species distribution in the microbial community induced by disturbed operation may prove crucial to develop robust and trustworthy nitrification systems, which are a “conditio sine qua non” in space applications.

Cultivation of *A. platensis* on (nitrified) urine seems feasible from the axenic batch experiments and literature, but further research is necessary to get closer to actual real life applications. Because contamination of a continuous system is likely to occur, the stability of a non-axenic photobioreactor should be investigated by operation of a continuous microalgae cultivation system.

PART 5

The integration of microalgae cultivation with the nitrification of human urine is an important next step in developing a complete nutrient recovery system from waste stream to recovered product.

Besides operational feasibility, the accumulation of micropollutants in microalgae biomass need to be investigated before unrestricted use as a supplement in food or feed is allowed. In space applications this is of particular interest for operational control. The production of biomass for nutritional purposes would no longer be interesting should micropollutants accumulate in the biomass. Instead the photosynthetic compartment should then be used solely as oxygen supplier.

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Appendix

1 Screening nitrifying consortia

Table A1: Overview table of oxidation rates from different tested nitrifying consortia at normal conductivity and 45mS cm^{-1}. Relative inhibition

Consortium	ΔEC	Ammonia Oxidation				Nitrite Oxidation			
		normal EC	45 mS cm^{-1}	Relative inhibition	p-value	normal EC	45 mS cm^{-1}	Relative inhibition	p-value
	mS cm^{-1}	mg N g VSS^{-1} d^{-1}	mg N g VSS^{-1} d^{-1}			mg N g VSS^{-1} d^{-1}	mg N g VSS^{-1} d^{-1}		
Inagro	42.5	- [(a)]	- [(a)]	-	-	4.88 ± 1.08	- [(a)]	100%	0.011
Breda	40.8	59.41 ± 2.38	- [(a)]	100%	5.01 10^{-11}	85.71 ± 10.70	2.93 ± 0.33	97%	3.80 10^{-3}
HANDS	40.2	4.07 ± 1.12	- [(a)]	100%	0.035	17.52 ± 1.67	14.17 ± 1.91	19%	0.209
Shrimp	40.1	- [(a)]	- [(a)]	-	-	- [(a)]	- [(a)]	-	-
Piranha	39.5	18.55 ± 2.23	- [(a)]	100%	1.27 10^{-9}	14.75 ± 1.90	3.80 ± 0.55	74%	1.61 10^{-6}
Imog	36.2	100.85 ± 9.72	30.67 ± 3.39	70%	9.14 10^{-6}	131.20 ± 20.06	56.08 ± 3.34	57%	2.38 10^{-4}
Decoster	28.4	10.49 ± 0.57	7.35 ± 0.49	30%	1.41 10^{-4}	6.31 ± 0.84	9.77 ± 0.75	-55%	3.81 10^{-3}
Danis	27.4	26.13 ± 5.64	6.11 ± 2.93	77%	0.008	7.68 ± 2.53	7.17 ± 4.41	7%	0.864
ABIL	24.6	143.28 ± 15.56	95.44 ± 22.01	33%	0.042	554.68 ± 19.01	452.64 ± 18.98	18%	8.25 10^{-4}
DOW	18.0	- [(a)]	- [(a)]	-	-	4.31 ± 0.26	5.32 ± 0.34	-23%	0.028
OLAND	9.2	123.93 ± 11.23	113.52 ± 6.10	8%	0.390	- [(a)]	- [(a)]	-	-
Octopus	0.0	- [(a)]	- [(a)]	-	-	- [(a)]	- [(a)]	-	-

[(a)] no significant oxidation rate

[(b)] no significant difference ($p > 0.05$)

2 Growth of *Arthrospira platensis* on modified Zarrouk medium

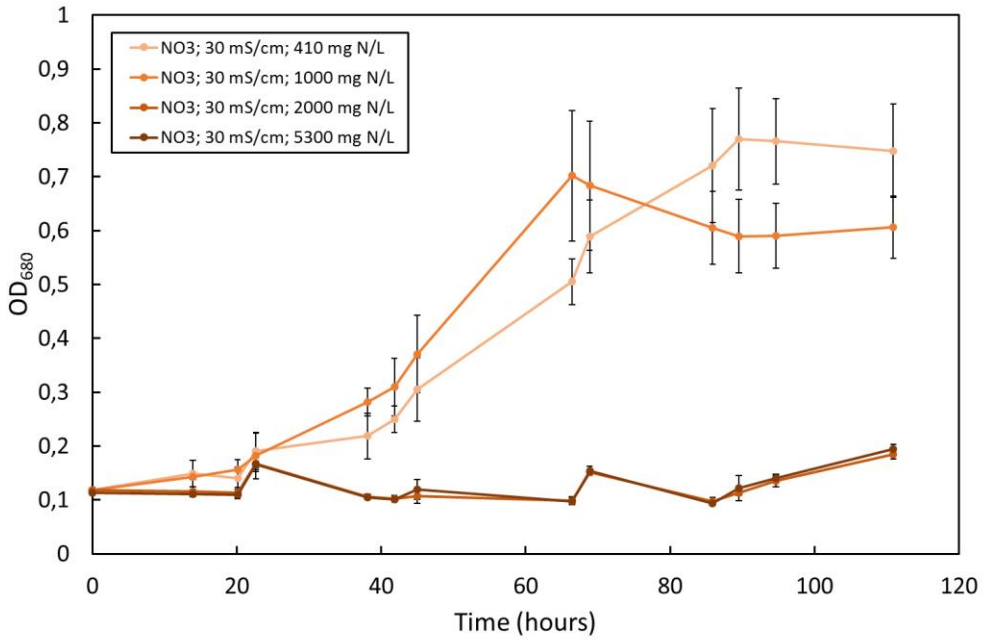

Figure A1: *Arthrospira platensis* growth curves on standard Zarrouk medium with different NO_3-N concentrations. The electrical conductivity of the medium is adjusted to 30 mS cm^{-1}. The pH was adjusted to 8.2.

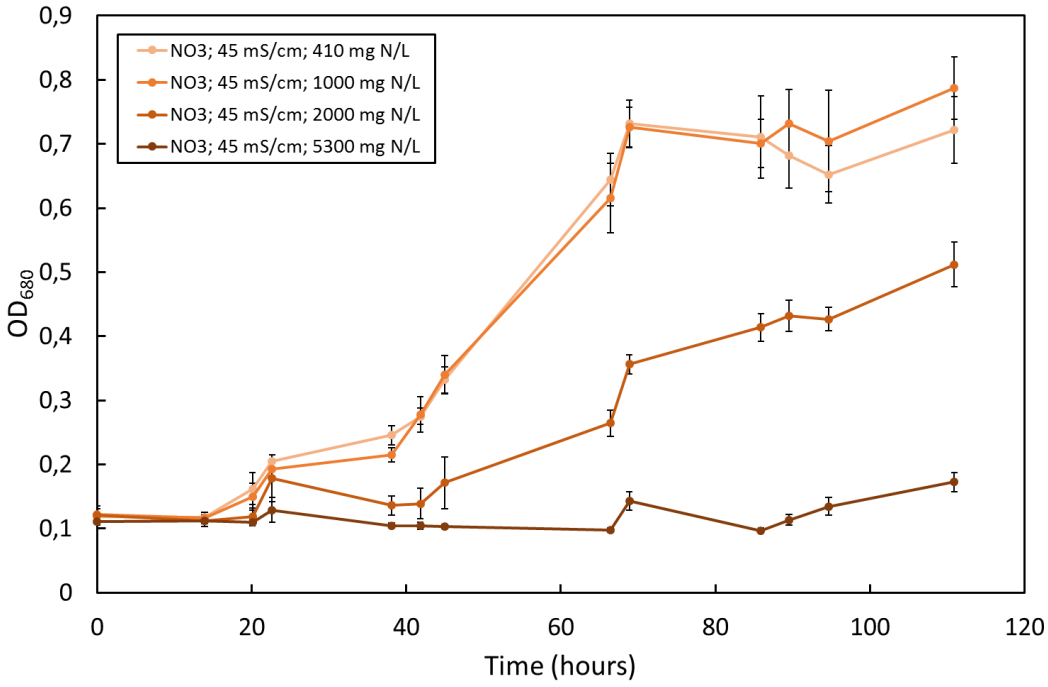

Figure A2: *Arthrospira platensis* growth curves on standard Zarrouk medium with different NO_3-N concentrations. The electrical conductivity of the medium is adjusted to 45 mS cm^{-1}. The pH was adjusted to 8.2.

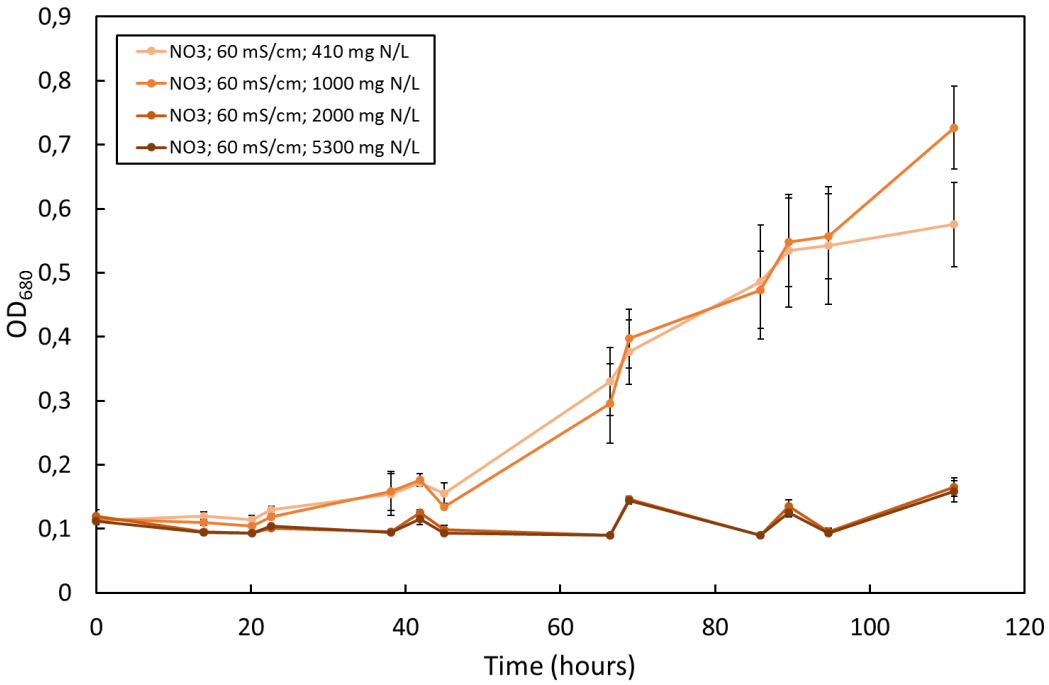

Figure A3: *Arthrospira platensis* growth curves on standard Zarrouk medium with different NO_3-N concentrations. The electrical conductivity of the medium is adjusted to 60 mS cm^{-1}. The pH was adjusted to 8.2.

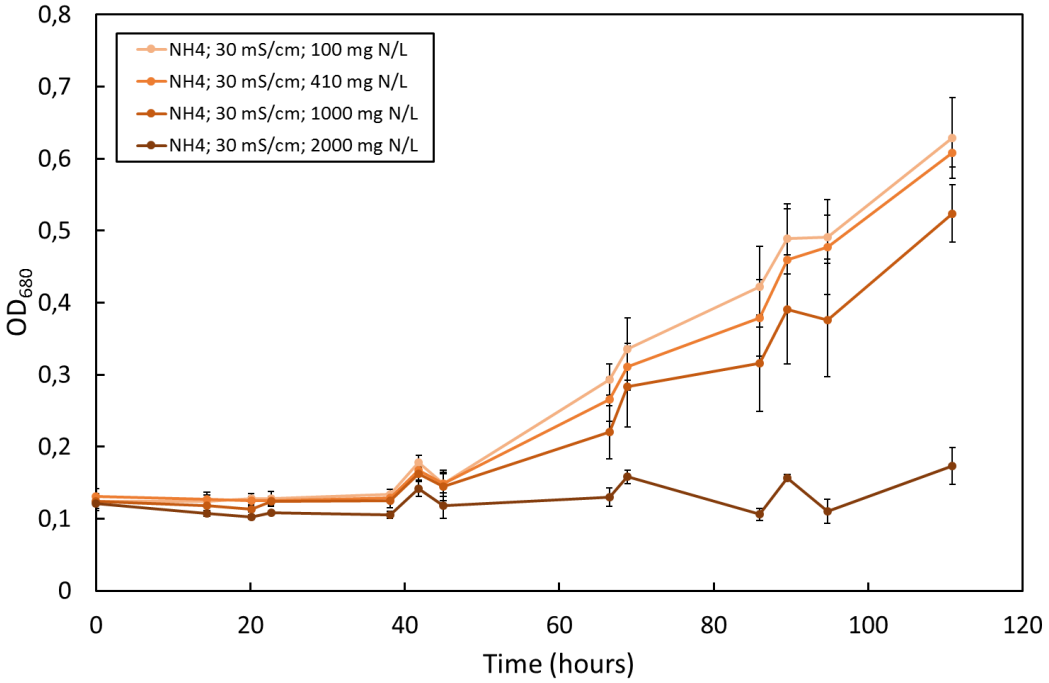

Figure A4: *Arthrospira platensis* growth curves on standard Zarrouk medium with different NH_4-N concentrations. The electrical conductivity of the medium is adjusted to 30 mS cm^{-1}. The pH was adjusted to 8.2.

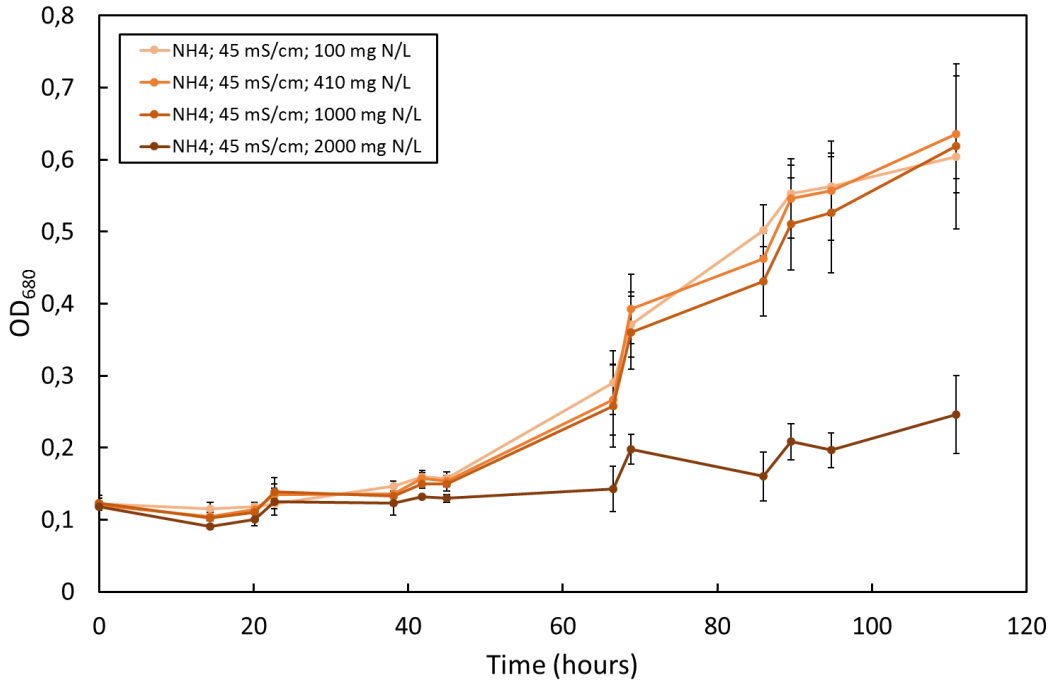

Figure A5: *Arthrospira platensis* growth curves on standard Zarrouk medium with different NH_4-N concentrations. The electrical conductivity of the medium is adjusted to 45 mS cm^{-1}. The pH was adjusted to 8.2.

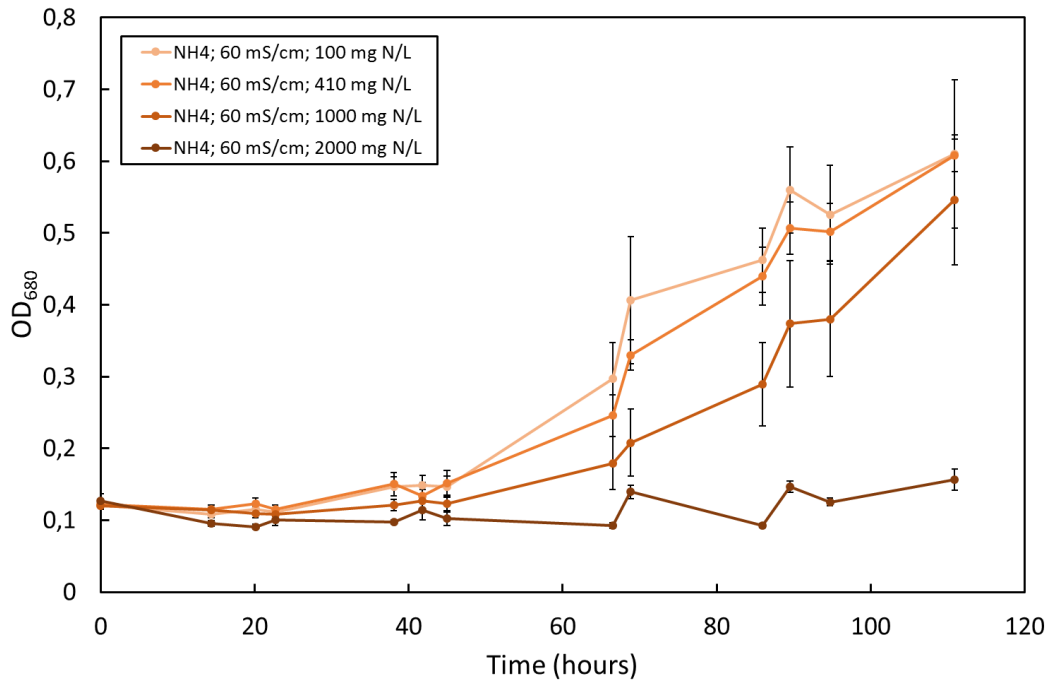

Figure A6: *Arthrospira platensis* growth curves on standard Zarrouk medium with different NH_4-N concentrations. The electrical conductivity of the medium is adjusted to 60 mS cm^{-1}. The pH was adjusted to 8.2.

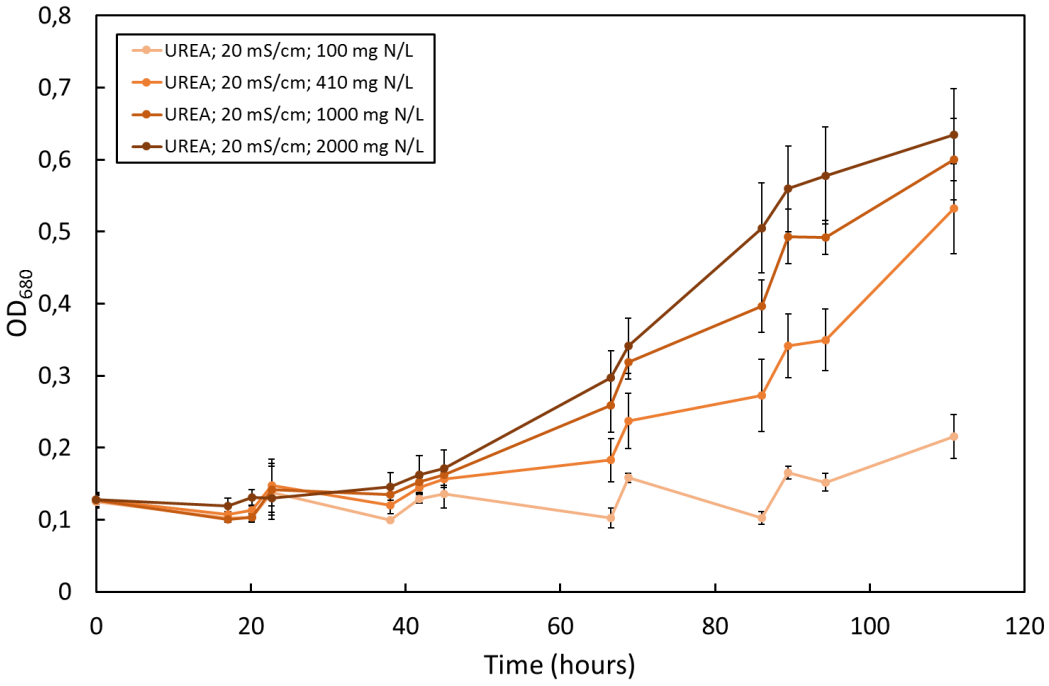

Figure A7: *Arthrospira platensis* growth curves on standard Zarrouk medium with different urea-N concentrations. The electrical conductivity of the medium is adjusted to 20 mS cm^{-1}. The pH was adjusted to 8.2.

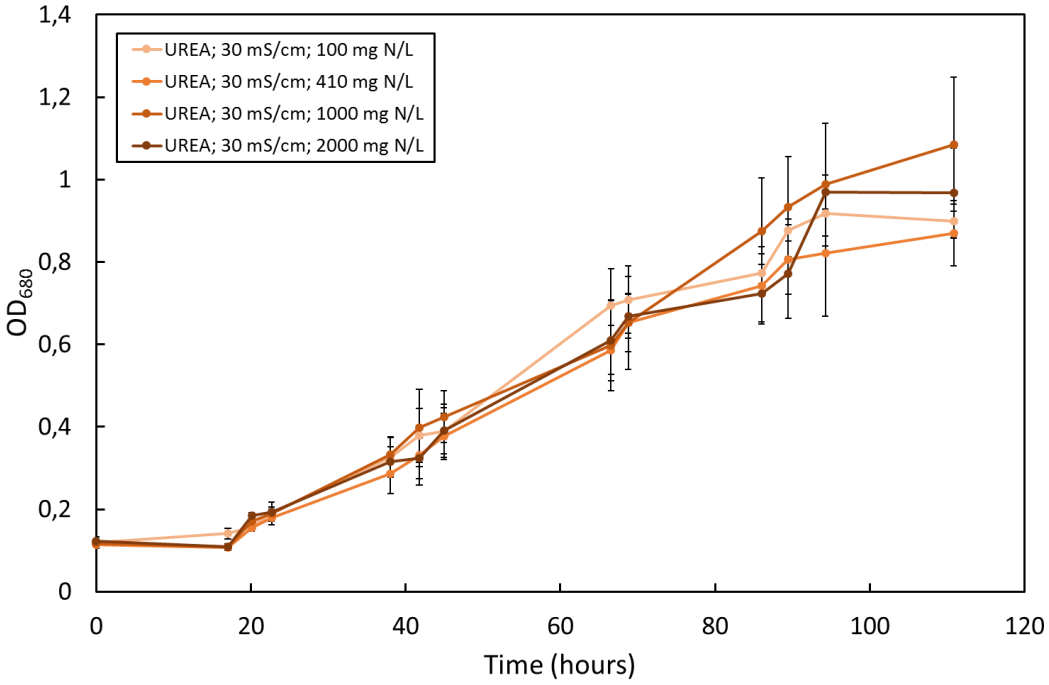

Figure A8: *Arthrospira platensis* growth curves on standard Zarrouk medium with different urea-N concentrations. The electrical conductivity of the medium is adjusted to 30 mS cm^{-1}. The pH was adjusted to 8.2.

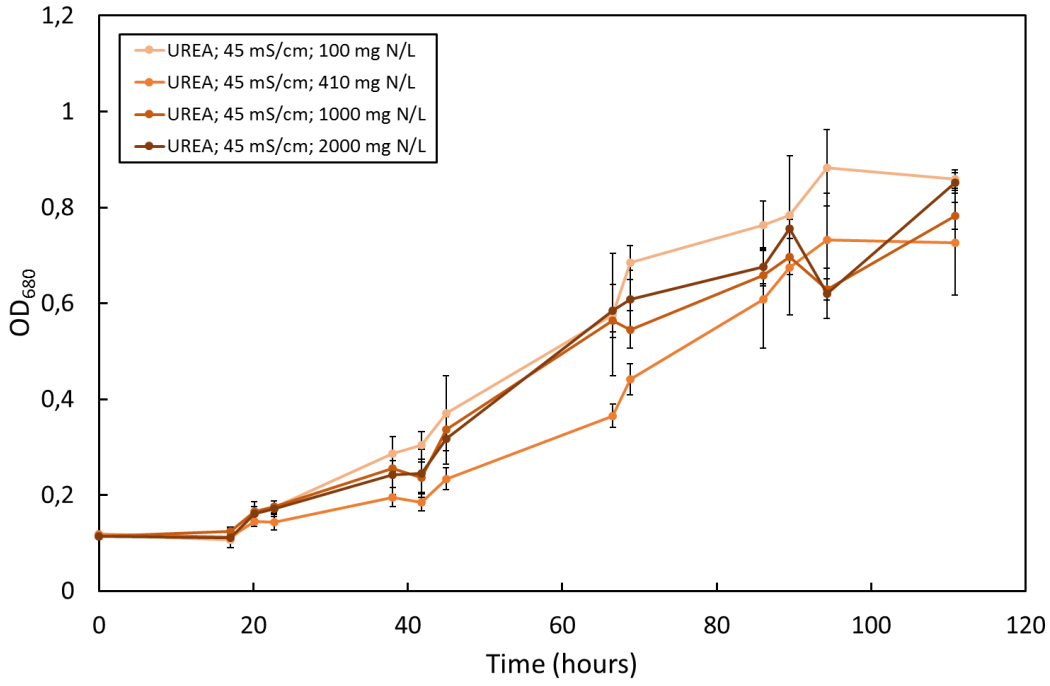

Figure A9: *Arthrospira platensis* growth curves on standard Zarrouk medium with different urea-N concentrations. The electrical conductivity of the medium is adjusted to 45 mS cm^{-1}. The pH was adjusted to 8.2.

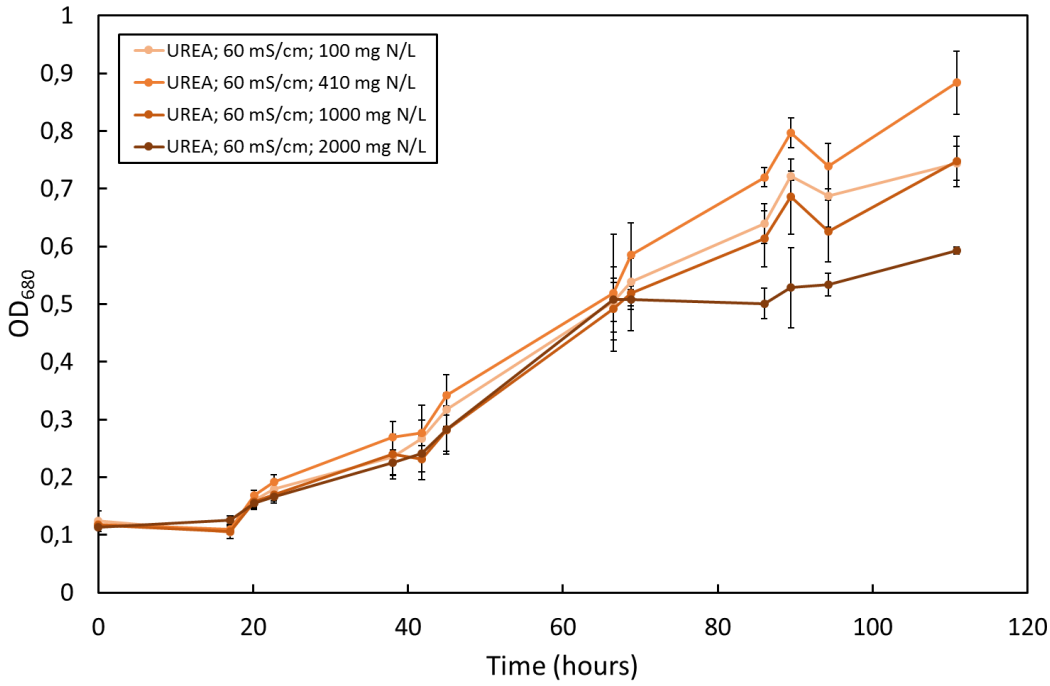

Figure A10: *Arthrospira platensis* growth curves on standard Zarrouk medium with different urea-N concentrations. The electrical conductivity of the medium is adjusted to 60 mS cm^{-1}. The pH was adjusted to 8.2.

Table A2: The effect of nitrate, ammonium or urea as nitrogen source in different concentrations. Final optical densities (OD) of *Arthrospira platensis* cultivated at 20 mS cm^{-1}. OD measured at 680 nm.

Concentration	$OD_{680,end}$ (20 mS cm^{-1})		
mg N L^{-1}	Nitrate	Ammonium	Urea
100	-	0.490 ± 0.043	0.216 ± 0.030
410	0.508 ± 0.029	0.440 ± 0.067	0.532 ± 0.063
1000	0.598 ± 0.033	0.264 ± 0.031	0.601 ± 0.056
2000	0.298 ± 0.080	0.211 ± 0.033	0.635 ± 0.064
5300	0.186 ± 0.035	-	-

Table A3: The effect of nitrate, ammonium or urea as nitrogen source in different concentrations. Final optical densities (OD) of *Arthrospira platensis* cultivated at 30 mS cm^{-1}. OD measured at 680 nm.

Concentration	$OD_{680,end}$ (30 mS cm^{-1})		
mg N L^{-1}	Nitrate	Ammonium	Urea
100	-	0.629 ± 0.056	0.899 ± 0.041
410	0.748 ± 0.087	0.609 ± 0.020	0.870 ± 0.080
1000	0.606 ± 0.057	0.524 ± 0.040	1.085 ± 0.162
2000	0.184 ± 0.008	0.173 ± 0.025	0.968 ± 0.108
5300	0.195 ± 0.009	-	-

Table A4: The effect of nitrate, ammonium or urea as nitrogen source in different concentrations. Final optical densities (OD) of *Arthrospira platensis* cultivated at 45 mS cm^{-1}. OD measured at 680 nm.

Concentration	$OD_{680,end}$ (45 mS cm^{-1})		
mg N L^{-1}	Nitrate	Ammonium	Urea
100	-	0.604 ± 0.030	0.859 ± 0.019
410	0.722 ± 0.052	0.635 ± 0.081	0.727 ± 0.109
1000	0.787 ± 0.049	0.619 ± 0.115	0.783 ± 0.028
2000	0.512 ± 0.035	0.246 ± 0.054	0.851 ± 0.021
5300	0.172 ± 0.014	-	-

Table A5: The effect of nitrate, ammonium or urea as nitrogen source in different concentrations. Final optical densities (OD) of *Arthrospira platensis* cultivated at 60 mS cm^{-1}. OD measured at 680 nm.

Concentration	$OD_{680,end}$ (60 mS cm^{-1})		
mg N L^{-1}	Nitrate	Ammonium	Urea
100	-	0.611 ± 0.103	0.744 ± 0.030
410	0.576 ± 0.066	0.608 ± 0.023	0.884 ± 0.052
1000	0.727 ± 0.065	0.546 ± 0.091	0.748 ± 0.041
2000	0.165 ± 0.014	0.156 ± 0.015	0.593 ± 0.006
5300	0.158 ± 0.016	-	-

3 Growth of *Arthrospira platensis* on synthetic and real urine

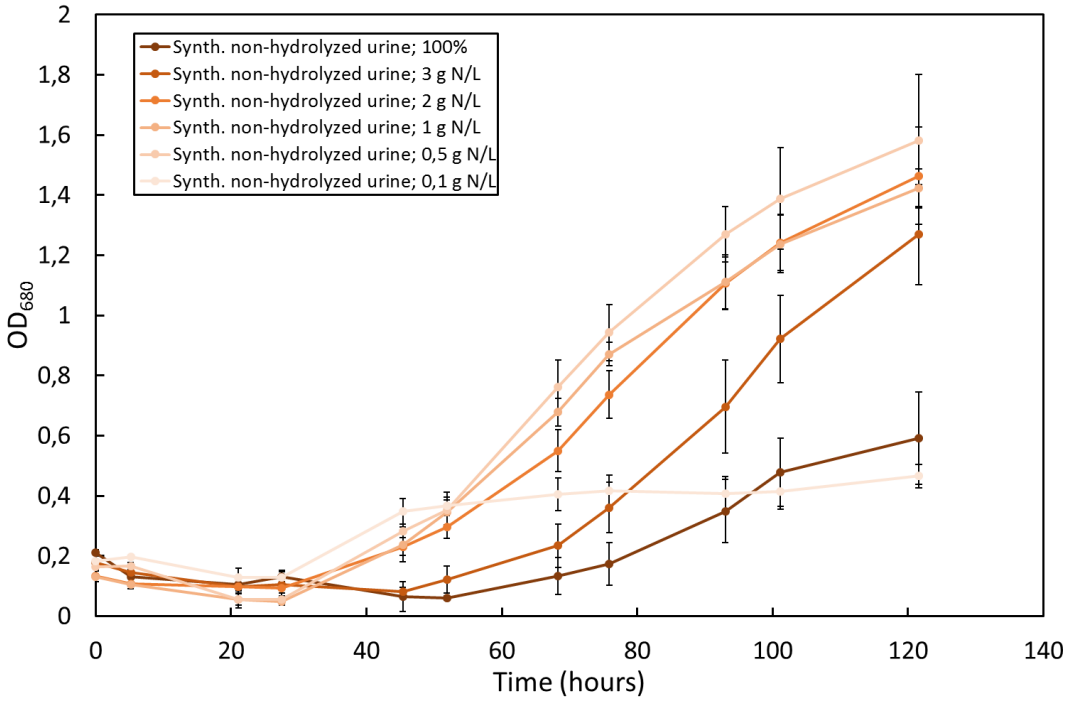

Figure A11: *Arthrospira platensis* growth curves on synthetic non-hydrolyzed urine, diluted to different nitrogen concentrations. The pH was adjusted to 8.2.

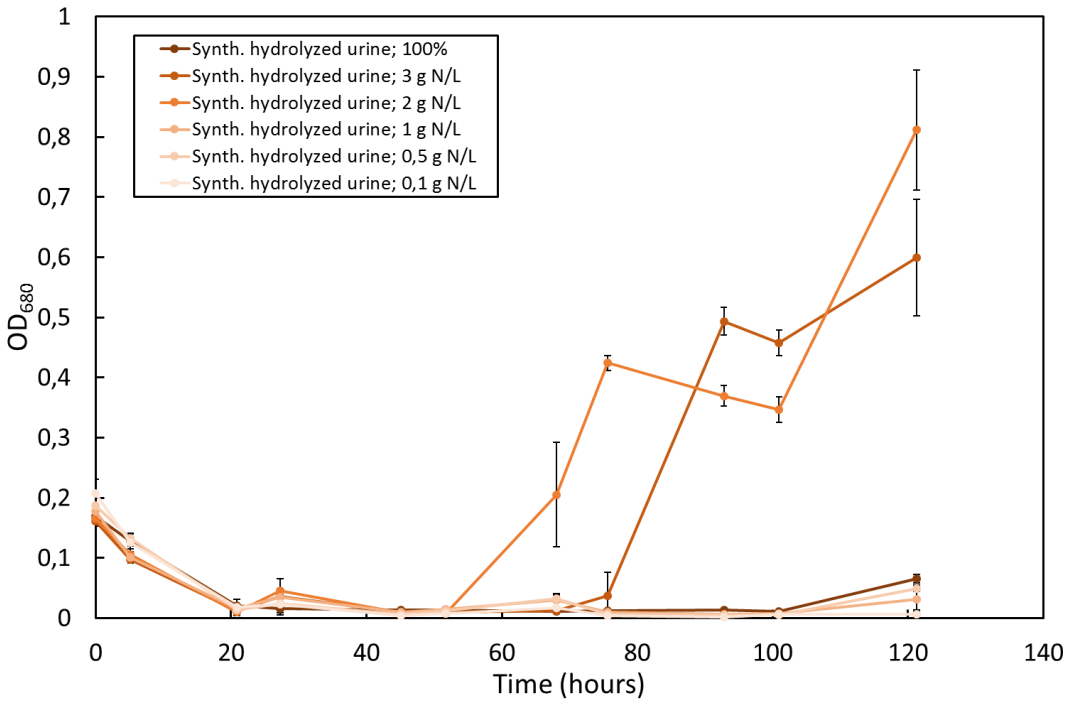

Figure A12: *Arthrospira platensis* growth curves on synthetic hydrolyzed urine, diluted to different nitrogen concentrations. The pH was adjusted to 8.2.

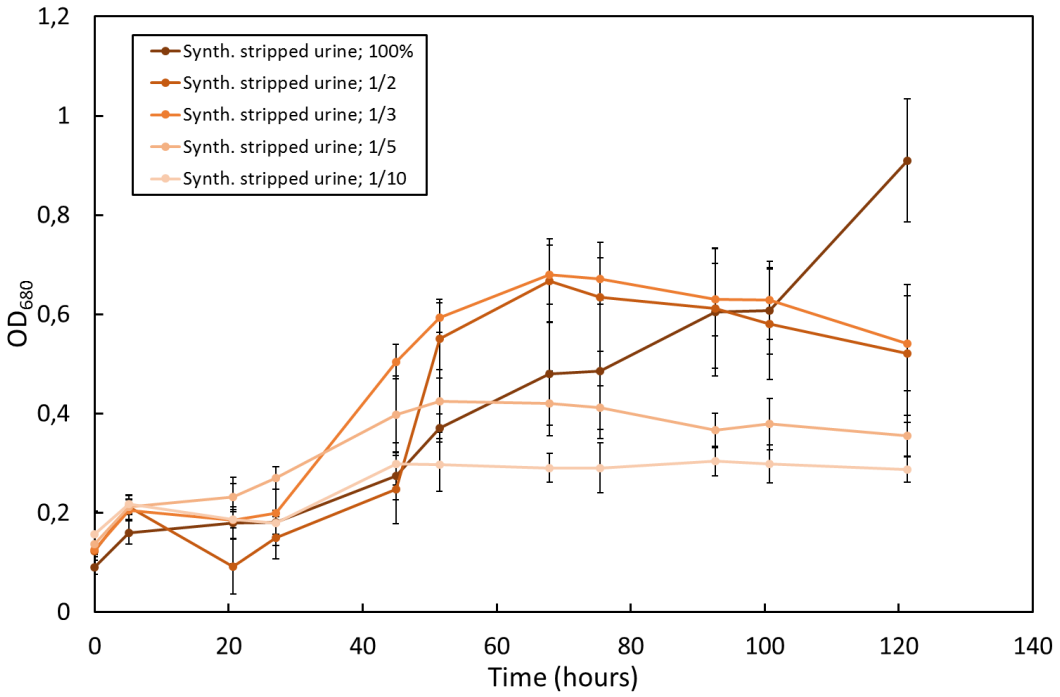

Figure A13: *Arthrospira platensis* growth curves on stripped synthetic hydrolyzed urine. A dilution series of the matrix was made and each dilution spiked with NH_4Cl to a final concentration of 100 mg NH_4-N L^{-1}. The pH was adjusted to 8.2.

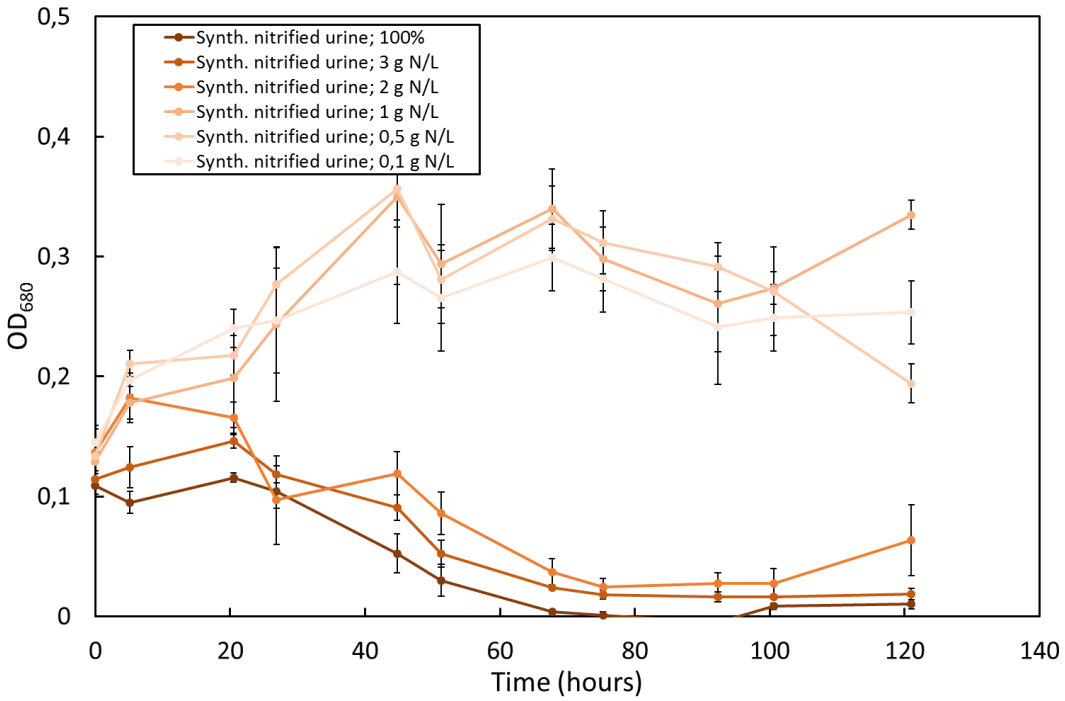

Figure A14: *Arthrospira platensis* growth curves on the effluent of a urine nitrification MBR fed with human urine, diluted to different nitrogen concentrations. The pH was adjusted to 8.2.

Table A6: Growth of *A. platensis* on non-hydrolyzed urine (synthetic and real) undiluted (100%) and diluted to 3.0, 2.0, 1.0, 0.5 and 0.1 g N L^{-1}. Optical densities (OD) at the end of the experiment. OD measured at 680 nm.

N-concentration	$OD_{680,end}$ (Non-hydrolyzed urine)	
g N L^{-1}	Synthetic (5.0 g urea-N L^{-1})	Real (6.3 g urea-N L^{-1})
100%	0.592 ± 0.154	0.028 ± 0.005
3.0	1.269 ± 0.166	-0.017 ± 0.003
2.0	1.465 ± 0.161	0.267 ± 0.006
1.0	1.422 ± 0.065	0.328 ± 0.029
0.5	1.581 ± 0.220	0.362 ± 0.044
0.1	0.466 ± 0.040	0.627 ± 0.036

Table A7: Growth of *A. platensis* on hydrolyzed urine (synthetic and real) undiluted (100%) and diluted to 3.0, 2.0, 1.0, 0.5 and 0.1 g N L^{-1}. Optical densities (OD) at the end of the experiment. OD measured at 680 nm.

N-concentration	$OD_{680,end}$ (Hydrolyzed urine)	
g N L^{-1}	Synthetic (8.0 g NH_4-N L^{-1})	Real (5.7 g NH_4-N L^{-1})
100%	0.066 ± 0.007	0.059 ± 0.006
3.0	0.600 ± 0.097	0.190 ± 0.037
2.0	0.812 ± 0.100	0.067 ± 0.013
1.0	0.032 ± 0.018	0.045 ± 0.024
0.5	0.050 ± 0.005	0.052 ± 0.010
0.1	0.007 ± 0.005	0.041 ± 0..037

Table A8: Growth of *A. platensis* on nitrified urine (synthetic and real), undiluted (100%) and diluted to 3.0, 2.0, 1.0, 0.5 and 0.1 g N L^{-1}. Optical densities (OD) at the end of the experiment. OD measured at 680 nm.

N-concentration	$OD_{680,end}$ (Nitrified urine)	
g N L^{-1}	Synthetic (5.0 g NO_3-N L^{-1})	Real (5.4 g NO_3-N L^{-1})
100%	0.010 ± 0.004	0.015 ± 0.003
3.0	0.019 ± 0.005	0.006 ± 0.004
2.0	0.064 ± 0.029	0.109 ± 0.021
1.0	0.335 ± 0.012	1.653 ± 0.110
0.5	0.194 ± 0.016	0.572 ± 0.046
0.1	0.253 ± 0.026	0.315 ± 0.018

Table A9: Growth of *A. platensis* on stripped hydrolyzed urine (synthetic and real) at undiluted (100%) and different dilutions (1/2, 1/3, 1/5 and 1/10).Each treatment was spiked to a concentration of 0.1 g N L^{-1}. Optical densities (OD) at the end of the experiment. OD measured at 680 nm.

Matrix dilution	$OD_{680,end}$ (Stripped hydrolyzed urine)	
	Synthetic (0.1 g NH_4-N L^{-1})	Real (0.1 g NH_4-N L^{-1})
100%	0.910 ± 0.124	-0.103 ± 0.046
1/2	0.521 ± 0.139	0.476 ± 0.058
1/3	0.541 ± 0.095	0.401 ± 0.100
1/5	0.355 ± 0.042	0.275 ± 0.036
1/10	0.288 ± 0.027	0.079 ± 0.078