Urine nitrification with a synthetic microbial community

Marlies E.R. Christiaens^a, Jolien De Paepe^{a,b}, Chiara Ilgrande^a, Jo De Vrieze^a, Justyna Barys^{a,b}, Pieter Teirlinck^a, Ken Meerbergen^{c,1}, Bart Lievens^c, Nico Boon^a, Peter Clauwaert^a, Siegfried E. Vlaeminck^{a,d*}

^aCenter for Microbial Ecology and Technology (CMET), Ghent University, Coupure Links 653, B-9000 Gent, Belgium

^bDepartment of Chemical, Biological and Environmental Engineering, School of Engineering, Universitat Autònoma de Barcelona, Bellaterra 08193 Barcelona, Spain

^cLaboratory for Process Microbial Ecology and Bioinspirational Management (PME&BIM), Technology cluster Bioengineering Technology (CBeT), Campus De Nayer Sint-Katelijne-Waver, KULeuven, Jan De Nayerlaan 5, B-2860 Sint-Katelijne-Waver, Belgium

^dResearch Group of Sustainable Energy, Air, and Water Technology, Department of Bioscience Engineering, University of Antwerp, Groenenborgerlaan 171, B-2020 Antwerpen, Belgium

¹Technology cluster Materials Technology, Campus Groep T, KULeuven, Andreas Vesaliusstraat 13 – bus 2600, B-3000 Leuven, Belgium

*Correspondence to: siegfried.vlaeminck@uantwerpen.be

ABSTRACT

During long-term extraterrestrial missions, food is limited and waste is generated. By recycling valuable nutrients from this waste *via* regenerative life support systems, food can be produced in space. Astronauts' urine can for instance be nitrified by micro-organisms into a liquid nitrate fertilizer for plant growth in space. Due to stringent conditions in space, microbial communities should be defined, gnotobiotic. Therefore, synthetic over mixed microbial communities are preferred. For urine nitrification, synthetic communities face challenges, *i.e.*, salinity, ureolysis, and organics.

In this study, a synthetic community containing an AOB (*Nitrosomonas europaea*), NOB (*Nitrobacter winogradskyi*), and three ureolytic heterotrophs (*Pseudomonas fluorescens*, *Acidovorax delafieldii*, and *Delftia acidovorans*) was tested for these challenges. In reactor 1, salt adaptation of the ammonium-fed AOB and NOB coculture was possible to 45 mS cm⁻¹, resembling undiluted nitrified urine, while maintaining an 44 ± 10 mg NH₄⁺-N L⁻¹ d⁻¹ removal rate. In reactor 2, the nitrifiers and ureolytic heterotrophs were fed with urine and achieved 15 ± 6 mg NO₃⁻-N L⁻¹ d⁻¹ production rate for 1% and 10% synthetic and fresh real urine. Batch activity tests with this community on fresh real urine even reached 29 ± 3 mg N L⁻¹ d⁻¹. Organics removal in the reactor (69 ± 15%) should be optimized to generate a nitrate fertilizer for future space applications.

KEY WORDS

nitrification; resource recovery; space; sterile reactor; synthetic community; urine

1. INTRODUCTION

In regenerative life support systems for application during long-term space missions, e.g. the Micro-Ecological Life Support System Alternative (MELiSSA) developed by the European Space Agency, waste is biologically converted into useful products [11]. Astronauts' urine, for instance, contains 50-64% of the nitrogen used for food production and can be a precursor for a nitrate liquid fertilizer in hydroponic plant production units [12, 13]. Nitrified urine has good fertilizing properties [14]. Since urine is a complex salt matrix containing a variety of carbon and nitrogen metabolites [15, 16], several metabolic functions are required in a microbial community to achieve complete urine nitrification. Organic compounds need to be oxidized to CO_2 by heterotrophic bacteria (SI, Eq. A.1). About 90% of urine's nitrogen is present as urea ($\text{CO}(\text{NH}_2)_2$) [16] requiring ureolysis (SI, Eq. A.2). The resulting ammonium (NH_4^+) can be oxidized to nitrite (NO_2^-) by ammonium oxidizing bacteria (AOB) (nitrification, SI, Eq. A.3), and NO_2^- can be oxidized by nitrite oxidizing bacteria (NOB) to nitrate (NO_3^-) (nitrification, SI, Eq. A.4).

In a mixed microbial community nitrifying urine, these groups of bacteria are present besides many other (unknown) micro-organisms. Mixed communities, open to competition and selection, are the standard in microbial processes, e.g. activated sludge in wastewater treatment plants. In space, however, stringent conditions occur, selecting for a defined (gnotobiotic) synthetic community. These communities, assembled with pure cultures [9], can assure safety because of the absence of pathogenic microbial strains. They further allow to mimic and study fundamental microbial and environmental interactions [10], to develop comprehensive mathematical models.

Drawbacks of synthetic communities are contamination and limited metabolic functionality. Contamination, i.e., the invasion of an external species [6, 7], influencing the productivity and

economics of the microbial process, can occur. Especially communities with limited microbial strain diversification, leaving niches uncolonized, are susceptible to contamination. A potential solution could be pre-emptive colonization with strains not interfering with the required microbial process [8].

Synthetic communities need to be sufficiently rich to achieve the same stable metabolic functionality that rich, mixed microbial communities display, despite physicochemical and microbial variations in the influent [1-3].

This stable metabolic functionality was demonstrated by adapting mixed microbial communities to (un)diluted urine nitrification [17, 18]. The challenges inherent to the urine matrix (high salinity, ureolysis, and organics removal) could be tackled due to the presence of different AOB [19], NOB [20], and heterotrophs that can fulfill the same metabolic functionality but often under different conditions. With gnotobiotic communities, nitrification was only shown with an autotrophic ammonium medium [21, 22].

As a first challenge, the salinity of fresh urine is about 20 mS cm⁻¹ [12], while for nitrified undiluted urine this rises up to 45-75 mS cm⁻¹ [17, 23]. The low alkalinity to nitrogen ratio in urine (1:1) only allows 50% nitrification without the addition of alkalinity [24]. This requirement further increases salinity. As a comparison, seawater has a salinity of about 50 mS cm⁻¹. Urine can be diluted for nitrification [18, 25-29] or the microbial community can be adapted to the high salinities of undiluted urine [17, 23]. Whereas Coppens, Lindeboom [17] confirmed previous findings that the nitrification rate was more affected to short-term salt stress than the nitrification rate, De Paepe, Lindeboom [23] found no effect on the nitrification rate up to 58 mS cm⁻¹ and a linear decline in nitrification rates. For pure cultures, *Nitrosomonas europaea* (AOB) was described to be halotolerant to moderately halophilic (upper limit 17.5 g NaCl L⁻¹ or about 40 mS cm⁻¹ at 25°C), while the salt tolerance of *Nitrobacter winogradskyi* (NOB) is

not known [30]. However, to the authors' knowledge, pure (co)cultures of these strains were never demonstrated to nitrify (synthetic) urine or to be salt adapted to enlarge their haloplasticity.

Secondly, nitrogen concentrations in real urine range from 4 to 14 g N L⁻¹ and are 90% present as urea [16]. The required ureolysis tends to be a fast process if the right heterotrophs are selected [31], and not rate limiting for urine nitrification, despite its decrease during short-term salt stress [23]. Therefore, to prevent overloading and NH₄⁺ accumulation, urine dilution [18, 25-29] or high hydraulic retention times (HRT)[17, 32-34] should be applied.

Thirdly, urine has a low total chemical oxygen demand (COD) over nitrogen ratio of about 1.5:1 and consists of a wide range of lowly concentrated organic compounds [15, 16, 35]. The main COD component is creatinine (3.8 g COD L⁻¹)[15]. The COD removal efficiencies of 75% for diluted urine [28, 29] to 94% for undiluted urine [17] were reported for mixed communities.

For gnotobiotic, nitrifying reactors, only limited research has been conducted and only on synthetic growth media. The challenges inherent to real human urine remain unexplored. Nitrification-denitrification was demonstrated in a multi-stage retentiostat with a cascade of pure cultures of *Nitrosomonas europaea*, *Nitrobacter winogradskyi*, and *Pseudomonas fluorescens* [36]. Perez, Buchanan [22] found that a coculture of *N. europaea* and *N. winogradskyi* achieved higher cell densities than pure cultures with an interesting 80-20% AOB-NOB balance. Combining this with transcriptome analysis, Perez, Buchanan [22] concluded that *N. europaea* received more benefit than *N. winogradskyi*. Additionally, Tappe, Laverman [36] found that *N. europaea* recovered faster from starvation than *N. winogradskyi*, explaining an NO₂⁻ peak upon NH₄⁺ oxidation.

In this study, a synthetic community was challenged to nitrify real human urine (taking into account ureolysis and salinity) and remove organics in a bioreactor, to establish the proof of concept to treat a complex matrix with a lean synthetic community in a stable reactor run for long-term space applications. In a first reactor, adaptation to increasing salt concentrations as determined in batch activity tests, was tested with the selected ammonium and nitrite oxidizing strains *Nitrosomonas europaea* ATCC 19718 and *Nitrobacter winogradskyi* Nb-255 ATCC 25391 [37] in a continuous stirred tank reactor (CSTR) operated with synthetic hydrolyzed urine (*i.e.*, NH_4^+ instead of urea). The maximum salt level at which nitrification activity could be maintained indicated the minimum urine dilution. In a second reactor, ureolysis and organics removal was incorporated in urine nitrification by adding the heterotrophs *Pseudomonas fluorescens* LMG 1794, *Acidovorax delafieldii* LMG 5943, and *Delftia acidovorans* SPH-1 DSMZ 14801 to the aforementioned AOB and NOB, as selected by Ilgrande, Christiaens [37]. This community was operated with diluted synthetic and real urine in a CSTR coupled to a cross-flow ultrafiltration membrane module to maximize biomass retention and to obtain a sterile effluent for the hydroponic plant compartment of MELiSSA. The microbial community in the reactor was monitored to determine the dynamics among the heterotrophic strains.

2. MATERIAL AND METHODS

2.1 Inocula

Reactor 1 was inoculated with 34 mL AOB, *Nitrosomonas europaea* ATCC 19718, at an optical density (OD) of 0.1, and 20 mL NOB, *Nitrobacter winogradskyi* Nb255 ATCC 25391, with an OD of 0.2 obtained from the MELiSSA pilot plant, Universitat Autònoma de

Barcelona, Spain.

Reactor 2 was inoculated with the selected pure cultures of *Nitrosomonas europaea* ATCC 19718, *Nitrobacter winogradskyi* Nb255 ATCC 25391, and heterotrophs *Pseudomonas fluorescens* LMG 1794, *Acidovorax delafieldii* LMG 5943, and *Delftia acidovorans* SPH-1 DSMZ 14801, which were previously tested for growth on urine but for inoculation grown from commercial -80°C stocks in the dark on their recommended media at 28°C and 150 rpm for the heterotrophs (SI Table B.1). After quantifying the intact cell counts with flow cytometry (FACSVerse, BD Biosciences, Belgium), the reactor was inoculated to intact cell concentrations in the reactor of $2.65 \cdot 10^5$ *N. europaea* mL⁻¹, $3.24 \cdot 10^5$ *N. winogradskyi* mL⁻¹, $1.19 \cdot 10^5$ *P. fluorescens* mL⁻¹, $1.19 \cdot 10^5$ *A. delafieldii* mL⁻¹, and $1.17 \cdot 10^5$ *D. acidovorans* mL⁻¹. Before inoculation, purity of all cultures was verified by Sanger sequencing (see 2.7 Molecular analyses).

2.2 Media and urine

In reactor 1, salt adaptation experiments were fed at a stable NH₄⁺ loading rate while increasing the salinity, to decouple salt and nitrogen effects. Ammonium was supplied *via* 3:1 molar ratio solutions of NH₄HCO₃ and NaHCO₃, mimicking hydrolysed urea while adding sufficient inorganic carbon substrate. Salinity increase was obtained by supplying a salt matrix solution with varying dilutions, derived from the hydrolysed synthetic urine recipe by Udert [38] with additional trace elements (SI Table C.1). The nitrogen and salt solutions were separately fed to the reactor.

In reactor 2, with a full synthetic community, both real and synthetic urine were used. Real urine from healthy men not taking medication was collected *via* a waterfree toilet at Ghent University with permission of the ethical committee (registration number B670201523246) and stored at -20°C until use. At reactor start-up, the thawed urine was filter sterilized (0.22

µm) and diluted to 10% with autoclaved demineralized water, with addition of 0.5 mL L⁻¹ trace elements (ATCC medium for *Nitrobacter winogradskyi* Nb255, solution D), and NaHCO₃ to 0.1 g total inorganic carbon (TIC) L⁻¹, which was at least twice the half-saturation constant K_{TIC} [39], to avoid limitation. The pH was adjusted to 11 with NaOH both for sterilization and preliminary pH adjustment of the acidifying nitrification process. The influent bottle connected to the reactor was stored at 4°C. Reactor operation shifted towards synthetic urine with 40 mg sodium acetate-COD L⁻¹ and 40 mg urea-N L⁻¹ to resemble 1% real urine. A urine salt matrix solution was prepared after Udert [38](SI Table C.2). This was also used as a diluting agent to operate the reactor at 1% and 10% fresh real urine, although at 10%, NaCl was replaced by 10 g NaHCO₃ L⁻¹. Influent salinity for 1% and 10% urine were similar (SI Table G.2). For both synthetic and the fresh real urine, the pH was no longer adjusted to 11 and the influent bottle was stored at room temperature to avoid precipitation.

2.3 Set-up and control of the reactors

Salt adaptation of the AOB and NOB coculture (reactor 1) was conducted in an autoclavable CSTR (Biostat B, Sartorius, Belgium), with a double-walled vessel for temperature control at 28°C with a working volume of 1L. The reactor was equipped with a pH probe (Mettler Toledo, Switzerland) allowing pH control at 7.4 ± 0.1 with 1M H₂SO₄ and 1M NaOH, and a dissolved oxygen (DO) probe (Mettler Toledo, Switzerland), monitoring the DO levels at saturation level (8 mg O₂ L⁻¹). An air pump (KNF, Verder, Germany) with a maximum flow rate of 5.8 ± 0.2 L min⁻¹ supplied filter sterilized air (0.22 µm; Merck Millipore, Belgium) . Salt and ammonium solutions were filter sterilized inline (0.22 µm; MerckMillipore, Belgium) before being pumped in the reactor. Effluent was discontinuously withdrawn as sample. Urine nitrification at 28°C with the full synthetic community (reactor 2) was conducted in a 2L autoclavable CSTR (Hanna, Belach Bioteknik, Sweden), coupled to a cross-flow ceramic

ultrafiltration membrane unit (0.05 μm) in the liquid recirculation line for biomass retention and the generation of a sterile effluent. The influent line was equipped with a series of dead-end hydrophilic PTFE filters (5, 0.22, 0.05 μm ; Merck Millipore, Belgium). Peristaltic influent, effluent, and recirculation pumps (Watson Marlow, Belgium) were applied. Discontinuous pumping for influent and effluent was required due to the low flow rate and was controlled by time profiles. The recirculation pump was continuously operating at 58 mL min^{-1} . Filter sterilized (0.22 μm ; Merck Millipore, Belgium) and humidified air was bubbled in the reactor and the O_2 concentration in the liquid was controlled at 40% (*i.e.*, 3.2 $\text{mg O}_2 \text{ L}^{-1}$) by a DO probe (Hamilton, Switzerland). The maximum flow rate of the air pumps was $1.88 \pm 0.07 \text{ L min}^{-1}$. The pH (Broadley James, UK) was controlled at 7.6-8.3, depending on the influent pH, by dosing 1M H_2SO_4 and 2M NaOH .

Both reactors had gentle stirring (50 rpm) applied by a propeller stirrer and were operated in the dark [40].

2.4 Start-up and operational phases of the reactors

Reactor 1 was filled with 1L salt solution, autoclaved, and sterile inoculated with the AOB and NOB coculture near the flame. Initially, the reactor was operated in fed-batch mode ($45 \pm 43 \text{ mg N L}^{-1} \text{ d}^{-1}$) allowing biomass acclimation to the salt solution matrix (phase I, SI Table G.1). At the end of phase I, the salt solution was continuously supplied, establishing an HRT of 9.3 ± 8.2 days. Phase II, lasting for 1 HRT, was set as the baseline for nitrification activity. During phase III-V, the reactor salinity was stepwise increased to the maximum salinity at which activity could be maintained, *i.e.*, 35, 45, and 55 mS cm^{-1} , as tested in batch activity tests (see 2.5 Batch activity tests). Throughout these steps, the reactor was operated at a stable HRT of 7.8 ± 3.6 days and an NH_4^+ loading rate (B_v) of $45 \pm 28 \text{ mg N L}^{-1} \text{ d}^{-1}$, except for phase VI ($3 \pm 2 \text{ mg N L}^{-1} \text{ d}^{-1}$). Sterile sampling was done daily to prevent NH_4^+ or NO_2^- accumulation

(SI Fig. D.1).

After reactor 2 was sterilized by autoclaving, 1L of sterile AOB NOB growth medium (0.87 g L⁻¹ K₂HPO₄, 0.185 g L⁻¹ MgSO₄·7H₂O, 0.015 g L⁻¹ CaCl₂·2H₂O, and 0.036 g L⁻¹ Na₂CO₃, according to Perez, Buchanan [22] with 0.5 mL L⁻¹ filter sterilized trace elements (ATCC medium for *Nitrobacter winogradskyi* Nb255, solution D) was pumped in the reactor to provide optimal conditions for the autotrophic strains. Sterile inoculation occurred at the flame. Initially, the reactor was operated in fed-batch mode until activity increased (phase I, days 0-55, SI Table H.1). As this did not occur, a shift towards continuous operation replaced the medium for urine (phase I, days 55-116). Changing from 10% real to 1% synthetic urine initiated nitrification activity but technical issues (phase II) delayed a stable run, *i.e.*, effluent concentrations remained stable, until phase III. Proven for 3 HRTs on synthetic urine, the shift was made back to real urine and after some technical issues (phase IV) a stable run on 1% was achieved (phase V). After a fed-batch period (phase VI), this was repeated (phase VII) before shifting to 10% real urine for 3 HRTs (phases VIII-X). Every change in urine was done in batch mode to allow nitrate to build up and clearly detect activity. During longer periods of technical malfunction, urine spikes kept the biomass active. No excess biomass was wasted throughout the entire experimental period. Sampling of the reactor occurred *via* air-tight glass sampling ports that were built in the influent, effluent, and recirculation lines. Sterile syringes and needles were additionally disinfected with ethanol and used close to the flame to withdraw influent and effluent samples, 3 times a week, and biomass samples, 2 times a week, through the rubber caps of the ports.

For both reactors, liquid samples were filter sterilized (0.22 µm) prior to storage at 4°C while biomass samples were immediately processed.

2.5 Batch activity tests

Ex situ salt adaptation batch tests for reactor 1 were performed in 96 well plates to separately quantify nitrification and nitrification rates at lower and higher salinities than present in reactor 1 and to identify the highest salinity at which reactor activity could be maintained (SI Table E.1 and 2). Each test well was axenically filled with 145 μL of salt matrix solution with different salinities, spiked with 5 μL of NH_4HCO_3 or NaNO_2 with concentrations matching the current reactor activities, and 100 μL of a biomass/buffer solution. This solution was prepared by sampling 50 mL reactor biomass, centrifugation at 6000 rpm for 10 min, and concentration of the biomass by resuspension in 20 mL 0.045M NaHCO_3 buffer (pH 7-7.5) to obtain a final well biomass concentration similar as in the reactor. Test wells were conducted in quadruplicates, while positive (*i.e.*, only reactor salinity) and negative (*i.e.*, no biomass or no substrate) controls were tested in duplicate. Plates were sterile incubated at 28°C at 600 rpm in a thermoshaker (Allsheng, China), covered with parafilm. At least 4 time points were collected during 1-3 days by sterile sampling of 2 μL for both NH_4^+ and NO_2^- spectrophotometric analysis.

For the full synthetic community reactor (reactor 2), a batch activity test was set up with larger volumes to quantify ureolysis, nitrification, and nitrification rates at varying urine types and concentrations: thawed, real, men's urine used in the reactor in phase I; fresh, real, men's urine; and synthetic urine with urea, sodium acetate, and a salt matrix (see 2.2 Media and urine); all at 1% and 10%. Reactor biomass sampled at day 197 was used and all conditions were tested in duplicate. Glass bottles of 0.5L were inoculated with 200 mL urine and 0.006 g volatile suspended solids (VSS) L^{-1} , which was 10 times lower than the biomass concentration in the reactor. For the reactor biomass, sterility was required throughout the test. Hence, these glass bottles were equipped with a tightly sealing metal cap having a connection for an air filter and one for sampling. These bottles were autoclaved, and contamination was avoided by preparing and sampling them near the flame. The sampling

volume was below 10% of the total volume available and analyses involved total ammonium nitrogen (TAN), NO_2^- , NO_3^- , COD, and total nitrogen (TN) at the beginning and end of the experiment. Bottles were incubated at 28°C covered in aluminum foil and shaken at 125 rpm.

2.6 Chemical analyses

Liquid samples were analysed for electrical conductivity. For reactor 1, TAN was quantified by the Nessler method [41], while for the salt adaptation batch tests and reactor 2, this was spectrophotometrically measured *via* the Berthelot reaction in 96 well plates. A 930 Compact IC Flex with a Metrosep A supp 5 guard and A supp 5 150/4.0 main column equipped with a conductivity detector (Metrohm, Switzerland) was used to quantify anions (Cl^- , NO_2^- , NO_3^- , PO_4^{3-} , SO_4^{2-}) in both reactors (LOQ: 5-100 mg L⁻¹). For the salt adaptation batch tests, NO_2^- could be spectrophotometrically quantified by the Montgomery reaction. For measurements in 96 well plates, a triplicate standard curve (0-80 mg N L⁻¹) per analysis plate was included and a Tecan infinite plate reader (Tecan, Switzerland) was used. COD and TN were analysed with nanocolor COD40 or 160 and nanocolor TN60 tube test kits (Machery-Nagel, Düren, Germany), respectively. Total inorganic carbon (TIC) was quantified by a Total Organic Carbon analyser TOC-V CPN (Shimadzu, Belgium)(LOD: 50 µg L⁻¹).

2.7 Microbial and molecular analyses

The presence of heterotrophic microorganisms in the autotrophic reactor 1, or the sterile influent and effluent lines of reactor 2, was checked *via* plating of 50 µL unfiltered liquid samples on potato dextrose agar (28°C) and plate count agar (37°C) plates. Colonies were picked up and directly processed for DNA extraction. Liquid reactor biomass samples were centrifuged at 12 000 rpm for 10 min prior to storage at -20°C. Total DNA extraction was carried out according to Vilchez-Vargas, Geffers [42], except for samples of reactor 2, where

the ZymoBIOMICS DNA Microprep Kit was used (BaseClear, the Netherlands). Sanger sequencing (LGC Genomics GmbH, Germany) was used to check the purity of the monocultures used for inoculation of both reactors and to identify the colonies picked up from the incubated plates of reactor 1. After quality control (SI section F.2), the PCR products were purified with the innuPREP PCR pure kit (Analytik Jena, Jena, Germany) before Sanger sequencing. Results were blasted *via* NCBI. DNA extracts of both reactors were analysed *via* real-time PCR (qPCR)(SI section F.1) and sent for 16S rRNA gene illumina amplicon sequencing *via* the MiSeq platform (SI section F.3). For reactor 1, samples were sent to LGC Genomics GmbH (Germany) while for reactor 2, samples were analysed by BaseClear (the Netherlands). The raw fastq files that were used to create the OTU tables, which served as a basis for the microbial community analysis, have been deposited in the National Center for Biotechnology Information (NCBI) database (accession numbers: SRP158326 and SRP158326, for reactor 1 and 2, respectively). Data processing is described in SI (section F.4).

3. RESULTS AND DISCUSSION

3.1 Halotolerance of the coculture *N. europaea* and *N. winogradskyi* can be stretched to 45 mS cm⁻¹ for nitrification of hydrolysed synthetic urine

The high salinity of fresh human urine (about 20 mS cm⁻¹ [12]), combined with the additional alkalinity required for complete nitrification and nitrate production itself, pose a challenge to nitrifying microorganisms. To determine the minimum dilution required to achieve urine nitrification with a synthetic community, the halotolerance of the selected nitrifiers grown in coculture, *N. europaea* and *N. winogradskyi*, was stretched in a CSTR and verified in batch activity tests.

During start-up (phase I), the CSTR was operated in fed-batch mode to let the coculture acclimatize to the urine salt matrix (9.7 ± 2.8 mS cm⁻¹)(SI Table G.1). An average NH₄⁺ removal rate of 45 ± 37 mg N L⁻¹ d⁻¹ was achieved, resulting in nearly 100% removal efficiency (SI Fig. G.1). Nitrate accumulated in the reactor to 600 ± 92 mg N L⁻¹ while 777 ± 680 mg N L⁻¹ was expected based on the influent NH₄⁺ concentration (Fig. 1). Subsequently, salinity increased till 17.9 ± 1.1 mS cm⁻¹. At the end of phase I (day 55), the salt solution was continuously fed to the reactor, washing out NO₃⁻ to the original salinity level of 9.6 ± 0.8 mS cm⁻¹. This was reached after 3 HRTs, during phase II. During this phase, also the NH₄⁺ solution was continuously fed to the reactor. A removal rate of 44 ± 16 mg N L⁻¹ d⁻¹ with $81 \pm 39\%$ removal efficiency was reached and considered as the baseline scenario. Limited biomass concentrations in the coculture probably caused the low volumetric NH₄⁺ removal rate compared to mixed culture nitrification of real urine in literature, ranging from 56 mg N L⁻¹ d⁻¹ in a biofilm-CSTR [34] to 1317 mg N L⁻¹ d⁻¹ in a SBR [43]. Nitrate production rates could not be calculated as masked by NO₃⁻ wash-out until phase IV.

To determine the level to which salinity could be increased in the reactor, biomass was sampled on day 80 (phase II), 89 (phase III), and 96 (phase IV), and used to quantify nitrification and nitrification rates at reactor and higher salinities (see below, paragraph 3.2). The selected salinity was gradually applied in the reactor during a period of 3-4 days. For phases III-V, these salinities were 35, 45, and 55 mS cm⁻¹. The reactor was operated at an HRT of 7.8 ± 3.6 days and an NH₄⁺ loading rate of 45 ± 28 mg N L⁻¹ d⁻¹. The salt shock in phase III caused a drop in the NH₄⁺ removal rate to 33 ± 42 mg N L⁻¹ d⁻¹ but was quickly recovered in phase IV and V to 44 ± 10 and 43 ± 12 mg N L⁻¹ d⁻¹, respectively. This resulted in NH₄⁺ removal efficiencies of 90 ± 39 and 94 ± 31% for phase IV and V, respectively. In these phases, NO₃⁻ reached concentrations of 264 ± 35 and 289 ± 20 mg N L⁻¹, respectively, compared to NH₄⁺ influent concentrations of 379 ± 199 and 342 ± 122 mg N L⁻¹, respectively (Fig. 1). Nitrification rates of 37 ± 45 and 39 ± 28 mg N L⁻¹ d⁻¹ and nitrification efficiencies of 77 ± 96 and 87 ± 63% were achieved for phase IV and V, respectively. These lower values compared to NH₄⁺ removal, could be an indication for denitrification in the potentially anoxic biofilm that was observed on the reactor wall or N₂O formation.

At the end of phase V, after 6 days of stable reactor operation at 55 mS cm⁻¹, NH₄⁺ started to gradually build up indicating an inhibition or decay of the AOB. The loading rate was reduced close to zero as only then NH₄⁺ did no longer accumulate. As a result, hardly any NH₄⁺ was removed during phase VI. This indicated an activity loss of 93 ± 52% compared to the baseline scenario (phase II). A final biomass sample was taken to verify these rates in a batch activity test at day 170. Nitrification could not be observed for phase VI and no nitrite built up occurred. Coppens, Lindeboom [17] and Moussa, Sumanasekera [44] reported NOB to adapt better to higher salinities than AOB while De Paepe, Lindeboom [23] found the opposite. Although both strains have the gene encoding for glycine betaine [45, 46], an osmolyte that potentially regulates the osmotic pressure in the cell, *Nitrobacter* was reported

to possess genes for a variety of osmolytes [46], therefore increasing his potential to cope with salt stress. Overall, the coculture could maintain its nitrification activity to a salinity of 45 mS cm⁻¹ which resembles undiluted [23] or 60% [17] diluted real nitrified urine.

[Fig. 1]

3.2 Batch activity tests as a predictive tool for the nitrifiers' halotolerance in the reactor

The nitrification and nitrification rates of the coculture in the salt adaptation reactor were verified, *i.e.*, at the same salinity as in the reactor, and predicted, *i.e.*, at higher salinities compared to the reactor, in batch activity tests (Fig. 2). The nitrification rate for biomass sampled at phase II (10 mS cm⁻¹) was 186 ± 58 mg N L⁻¹ d⁻¹ at 10 mS cm⁻¹, which is four times higher compared to the reactor and more in line with literature values for mixed culture urine nitrification and NH₄⁺ removal in a pure culture *N. europaea* chemostat run [22]. Although this indicates that the reactor was not operated at its maximum loading rate, it allowed the biomass to adapt to the salinities to come. At 20 mS cm⁻¹, an optimum was reached of 264 ± 113 mg N L⁻¹ d⁻¹, potentially due to the unintended salt adaptation to a maximum of 21 mS cm⁻¹ during phase I in the reactor. A similar shift in optimum salinity after salt adaptation of a mixed culture was also observed by Coppens, Lindeboom [17]. At 40 mS cm⁻¹, the NH₄⁺ removal rate was still 173 ± 21 mg N L⁻¹ d⁻¹. Probably, this could only be maintained for the short time period of the batch test because the tests with biomass adapted to 35 or 45 mS cm⁻¹ could not achieve these high rates and resembled NH₄⁺ removal rates of the reactor.

Although nitrification rates could not be quantified in the reactor, the rates achieved in the

batch test were in line with the reactor NH_4^+ removal and NO_3^- production rates, except for the first two data points (10 and 20 mS cm^{-1}) of the biomass of phase II (10 mS cm^{-1}) where the reactor might not have been operated at full capacity and the biomass was already adapted to 20 mS cm^{-1} during phase I.

Since the nitrification and nitrification rates at higher salinities than in the reactor, were rather steady throughout all the batch tests, the salinities selected for application in the reactor were not the highest salinities tested in each batch test but the one or two before that. For phases III, IV, and V, the selected salinities were 35, 45, and 55 mS cm^{-1} , respectively. During the reactor crash (phase VI), the last batch test indicated the biomass was able to (partially) recover nitrification and nitrification rates at 10 and 20 mS cm^{-1} . Consequently, a shift in the optimal salinity did not occur, perhaps due to the fast salt increase and the short adaptation periods applied in the reactor (max. 7 days)[17, 44]. These findings also indicate that AOB can cope with salt stress equally well as NOB.

[Fig. 2]

3.3 The autotrophic salt reactor displayed similar relative abundances for AOB and NOB, but allowed a niche for heterotrophs

The microbial community in reactor 1 was monitored by real-time PCR and 16S rRNA gene illumina amplicon sequencing to track the relative abundance and potential contamination (Fig. 3). During start-up (phase I), AOB and *Nitrobacter* spp. increased in abundance due to growth (Fig. 3.A), which was also observed from the illumina data (Fig. 3.B). Throughout the different phases of reactor operation, fluctuations occurred but no major differences in relative abundance arose between the AOB and NOB. Real-time PCR analysis revealed a

relative abundance of 1-36% AOB and 0-15% *Nitrobacter* spp., while illumina amplicon sequencing confirmed this with 1-31% for *Nitrosomonas* (Otu0006) but gave a more fluctuating value of 1-48% for *Nitrobacter* (Otu0004). The calculated time-normalized Bray-Curtis dissimilarity index was similar for both methods (SI Fig. G.2). A Spearman's Rank correlation analysis verified a statistically significant correlation of these trends for both methods ($P < 0.0001$, $\rho = 0.79$). Coppens, Lindeboom [17] reported AOB and *Nitrobacter* spp. to be relatively equally abundant in mixed cultures. At the end of phase V, AOB and NOB encountered a sudden drop in abundance, potentially induced by the continued salt stress. This could have caused the reactor to crash in phase VI.

Although the reactor was operated with sterile hydrolysed synthetic urine without organics, both qPCR and 16S rRNA gene illumina amplicon sequencing data displayed the presence of other microorganisms than AOB and NOB. Sequencing data identified heterotrophs, more specifically, several operational taxonomic units (OTU), including the most abundant one, were identified as *Pseudomonas* (Fig. 3.B). Heterotrophs are reported to grow on organic carbon leaked by autotrophs [47], which could have created a niche for heterotrophs and could explain their high abundance.

[Fig. 3]

3.4 Nitrification with a full synthetic community started on fresh rather than frozen/thawed real urine

The coculture of *N. europaea* and *N. winogradskyi* was shown here to adapt to salinity levels of 100%, or at least 60%, nitrified urine. For reactor operation with the full synthetic community, *i.e.*, the aforementioned AOB, NOB, and three heterotrophs, a safe operating

urine percentage of 10% was used to eliminate salt stress. Thawed urine was diluted and spiked in the reactor during start-up (phase I, Fig. 4). After ureolysis, 10% real urine ($399 \pm 27 \text{ mg N L}^{-1}$) could provide about $170 \text{ mg TIC L}^{-1}$ in the reactor, which matches at least 7 times the half-saturation constant K_{TIC} (22 mg TIC L^{-1})[39] or 3 times the TIC need for AOB ($62 \text{ mg mg TIC L}^{-1}$)[48] and would therefore not be limiting as a carbon source. However, operating the reactor in fed-batch yielded TIC concentrations far below this threshold (SI Fig. H.3). This could potentially result in NH_4^+ oxidation to N_2O instead of NO_2^- [48, 49]. To avoid N_2O emissions, NaHCO_3 was added from day 55 throughout the entire reactor run to maintain levels of $200\text{-}300 \text{ mg TIC L}^{-1}$ (SI Fig. H.3). The influent was changed to 1% synthetic urine ($48 \pm 10 \text{ mg N L}^{-1}$ and $90 \pm 53 \text{ mg COD L}^{-1}$) during phase II, which immediately showed NO_3^- production (Fig. 4). A stable continuous run on 1% synthetic urine during phase III for 3 HRTs (2 ± 1 days) with a TN load of $24 \pm 7 \text{ mg N L}^{-1} \text{ d}^{-1}$, resulted in an ureolysis rate of $16 \pm 2 \text{ mg N L}^{-1} \text{ d}^{-1}$ ($89 \pm 7\%$), a NO_3^- production rate of $15 \pm 9 \text{ mg N L}^{-1} \text{ d}^{-1}$ ($59 \pm 21\%$), and a TN loss of around $5 \text{ mg N L}^{-1} \text{ d}^{-1}$ (23%)(SI Table H.2). Ammonium effluent levels were close to zero. A shift was made to 1% real urine during phase IV, although now freshly collected urine was used. While the biomass could acclimatize to the new feed in a fed-batch regime, biomass was sampled on day 197 to start a batch test in which thawed and fresh real urine, and synthetic urine at 1 and 10% were compared. Synthetic urine at 10% showed NH_4^+ accumulation and decrease, but this was not observed for thawed urine, although both treatments reached similar NO_3^- concentrations after 13 days (SI Fig. H.2). Fresh real urine at 10% followed the same trend as thawed urine, but achieved the highest maximum NO_3^- production rate of $29 \pm 2.7 \text{ mg N L}^{-1} \text{ d}^{-1}$, compared to $19 \pm 3.3 \text{ mg N L}^{-1} \text{ d}^{-1}$ for thawed and $22.9 \pm 19.1 \text{ mg N L}^{-1} \text{ d}^{-1}$ for synthetic urine (Fig. 5). The freezing/thawing process seemed to make the urine less suitable for faster NO_3^- production. This was confirmed for 1% urine, where synthetic and fresh urine achieved higher final NO_3^- concentrations and maximum

production rates (10.6 ± 1.5 and 9.9 ± 2.5 mg N L⁻¹ d⁻¹, respectively) compared to thawed urine (3.7 ± 5.1 mg N L⁻¹ d⁻¹). Following these findings, a gradual shift from 1% synthetic over 1% to 10% fresh real urine was applied in the reactor. Fresh urine would also resemble real life conditions better than thawed urine.

[Fig. 4]

[Fig. 5]

3.5 Ureolysis and nitrification rates with a full synthetic community were similar for 1% or 10% synthetic or fresh real urine

Following the stable run on 1% synthetic urine (phase III) and the transition phase IV to fresh real urine at 1%, a stable run was reported for phase V on 1% fresh real urine (43 ± 4 mg TN L⁻¹ and 67 ± 32 mg COD L⁻¹) at the same TN loading rate and HRT (22 ± 3 mg N L⁻¹ d⁻¹ and 2 ± 0 days, respectively) as the synthetic urine (Fig. 4, SI Table H.2). After 3 HRTs, similar ureolysis (12 ± 4 mg N L⁻¹ d⁻¹) and NO₃⁻ production rates (18 ± 2 mg N L⁻¹ d⁻¹) were achieved as with synthetic urine (SI Table H.2, SI Fig. H.1). The NO₃⁻ production rates in the reactor were almost double for both 1% synthetic and real urine compared to the rates obtained in the batch activity test (Fig. 5). Efficiencies reached $81 \pm 16\%$ for ureolysis and $84 \pm 11\%$ for NO₃⁻ production. Average effluent NH₄⁺ concentrations were 6 ± 3 mg N L⁻¹.

After a fed-batch period (phase VI), the reactor was reactivated with 1% fresh real urine in continuous mode during phase VII before switching to 10% fresh real urine (822 ± 57 mg N L⁻¹ and 774 ± 93 mg COD L⁻¹, phase VIII)(Fig. 4). To maintain a similar TN and COD loading rate (33 ± 5 mg N L⁻¹ d⁻¹ and 31 ± 5 mg COD L⁻¹ d⁻¹) as in the 1% runs, the HRT was

487 increased to 24 ± 2 days (SI Table H.2, SI Fig. H.1). One HRT was sufficient to obtain a
488 stable TN and NO_3^- concentration in the effluent (544 ± 46 mg N L^{-1} and 273 ± 10 mg N L^{-1} ,
489 respectively), although influent TN levels were not yet reached (Fig. 4), indicating a TN loss
490 of $29 \pm 7\%$ (SI Table H.2). For COD, a removal efficiency of $52 \pm 7\%$ was obtained (SI Table
491 H.3), which is lower than values reported in mixed microbial communities (75% at lowest for
492 Sun 2012). Volumetric ureolysis rates (21 ± 4 mg N $\text{L}^{-1} \text{d}^{-1}$) increased compared to 1%
493 synthetic and fresh real urine, whereas efficiencies decreased till $66 \pm 9\%$. Nitrate production
494 encountered a decreased rate (11 ± 1 mg N $\text{L}^{-1} \text{d}^{-1}$) and efficiency ($35 \pm 3\%$). The rate was
495 only one third of what was obtained in the batch activity test for 10% fresh urine (Fig. 5, SI
496 Fig. H.1). However, in the batch test 10% urine equaled a total nitrogen concentration of 398
497 ± 3 mg N L^{-1} . Therefore, the influent was changed to 5% fresh real urine (370 ± 0 mg N L^{-1})
498 during the transition phase IX, to obtain a stable run in phase X (333 ± 26 mg N L^{-1} and $363 \pm$
499 21 mg COD L^{-1}) with an HRT of 23 ± 9 days (SI Table H.2). Here, the TN and COD loading
500 rates were slightly lower (16 ± 6 mg N $\text{L}^{-1} \text{d}^{-1}$ and 23 ± 7 mg COD $\text{L}^{-1} \text{d}^{-1}$) compared to all
501 previous runs. While ureolysis (16 ± 7 mg N $\text{L}^{-1} \text{d}^{-1}$), NO_3^- production (15 ± 6 mg N $\text{L}^{-1} \text{d}^{-1}$), and
502 losses (2 ± 2 mg N $\text{L}^{-1} \text{d}^{-1}$) remained similar to the 1% synthetic and fresh real urine phases
503 (III and V, respectively), this lower loading rate caused the ureolysis and NO_3^- production
504 efficiencies to increase to $107 \pm 8\%$ and $94 \pm 8\%$, respectively. The COD removal rate ($16 \pm$
505 5 mg COD $\text{L}^{-1} \text{d}^{-1}$) equaled the one in phase VIII (SI Table H.3), but at $69 \pm 15\%$ efficiency,
506 although the remaining COD in the effluent still contained about 62-73% biodegradable
507 organics (SI Table H.4). Average effluent NH_4^+ concentrations in this last phase X were 5 ± 4
508 mg N L^{-1} . Nitrate production rates as obtained in the batch test for 10% fresh real urine could
509 not be achieved in the reactor. Whereas the batch test demonstrated a clear preference for
510 10% fresh real urine, the NO_3^- production rates in the reactor were rather independent of the
511 type of urine (synthetic or fresh real) and the concentration (1 or 10%).

3.6 *D. acidovorans* dominated the synthetic community

For ureolysis and organics degradation, three heterotrophic strains (*P. fluorescens*, *A. delafieldii*, and *D. acidovorans*) were added to the autotrophic nitrifying coculture. A non-pathogenic and ureolytic *Pseudomonas* strain was added, assuming this strain would occupy the same, potential niche that was found in the salt adaptation reactor (*i.e.*, pre-emptive colonization). Community dynamics were followed over time *via* qPCR (Fig. 6.A). During the start-up with 10% thawed real urine (phase I), *N. europaea* and *N. winogradskyi* gradually increased in abundance from day 55, when additional TIC was supplemented to the reactor (200-300 mg L⁻¹). Heterotrophic strains only accumulated when the shift was made to synthetic urine with easily degradable acetate as a carbon source, at the end of phase I. The lower COD removal rates obtained for real urine, and the variety of organics present there, might suggest to include heterotrophic strains that are specialized in degrading certain recalcitrant organic compounds or more generalized in degrading a broad variety of organics [50].

N. europaea was more abundant than *N. winogradskyi* from phase II on (0-6% and 0-2%, respectively), in contrast to the salt adaptation reactor and literature [17]. For the heterotrophs, *D. acidovorans* dominated the community with 58 up to 100%. These trends were also present in the 16S rRNA gene amplicon sequencing data (Fig. 6.B). Here, *Nitrosomonas* (Otu0005) and *Nitrobacter* (Out0006) had a relative abundance of 0.01-3% and 0.03-0.7%, respectively, and 75-96%% for *D. acidovorans*. A time-normalized Bray-Curtis dissimilarity index emphasized these similar trends between both methods (SI Fig. H.5), and was proven to be statistically significant by a Spearman's rank correlation ($P = 0.0017$, $\rho = 0.63$).

P. fluorescens seemed to be in competition with *D. acidovorans* during the first phases, but was outcompeted upon the shift to 1% fresh real urine (phase IV)(Fig. 6.A). A shift in strains upon switching from synthetic to real urine was also observed by De Paepe, Lindeboom [23]. The abundance of *D. acidovorans* was similar to total bacteria which could indicate that the growth of potentially invasive strains was suppressed as the niche was occupied. Although *P. fluorescens* did not fulfill the potential role of pre-emptive colonizer, potentially due to the different matrix of reactor 2 compared to reactor 1, the dominance of the right heterotroph could prove the hypothesis and use of pre-emptive colonization to maintain synthetic communities gnotobiotic [8]. Additional experiments are required to prove this hypothesis.

[Fig. 6]

4. CONCLUSION

For long-term space applications, urine nitrification was successfully tested with a lean synthetic community in bioreactors. Challenges were posed by the matrix (salt, ureolysis, and organics) and by the application (gnotobiotic community). In a first step, the halotolerance of the nitrifiers *N. europaea* and *N. winogradskyi* could be stretched to 45 mS cm⁻¹, resembling undiluted nitrified urine. Next, different types of urine were tested for ureolysis and nitrification with the full synthetic community. Similar nitrate production rates for 1% and 10% urine were achieved. Predictive batch activity tests identified maximum rates for 10% fresh real urine. Finally, the long-term combination of ureolysis, nitrification and organics removal was achieved in a stable bioreactor run, although organics removal should be optimized for future space applications. Community dynamics and potential contamination were monitored and

showed the dominance of the heterotroph *D. acidovorans*. Additional tests are required to unravel the link between this dominance and the suppression of (invasive) heterotrophic strains. This would yield valuable information for sterile reactor operation in space or industrial applications.

E-supplementary data of this work can be found in the online version of the paper.

Declarations of interest: none.

ACKNOWLEDGEMENTS

This article has been made possible through the involvement of M.C., C.I., N.B., J.DP, J.B, P.C., and S.E.V. in the MELiSSA project, ESA's life support system research program (http://www.esa.int/Our_Activities/Space_Engineering_Technology/Melissa). We kindly acknowledge the financial support of the Belgian Science Policy (BELSPO) in the UNICUM project (ESA contract number 4000109948/13/NL/CP) and the MELiSSA foundation to support J.DP and J.B via the POMP1 (Pool Of MELiSSA PhD) program. We acknowledge financial support for J.DV. as postdoctoral fellow by Research Foundation Flanders (FWO), K.M. by the Industrial Research Council of KU Leuven (KP/10/006) and the Research Council of KU Leuven (OT/13/063). We gratefully thank Francesc Gòdia and Carolina Arnau (Universitat Autònoma de Barcelona) to provide pure cultures for the salt adaptation reactor, Ramon Ganigué for fruitful discussions, Frederiek-Maarten Kerckhof, Tim Lacoere, and Bert Schulpen for the primer design, Leonardo Gutierrez, Greet Van de Velde, Jana De Bodt, and Tim Lacoere for lab assistance, and Jan B.A. Arends for critically reading and commenting on the manuscript.

586 REFERENCES

- 587 1. Allison, S.D. and J.B.H. Martiny, *Resistance, resilience, and redundancy in microbial*
588 *communities*. Proceedings of the National Academy of Sciences of the United States of
589 America, 2008. **105**: p. 11512-11519.
- 590 2. Bell, T., et al., *The contribution of species richness and composition to bacterial services*.
591 Nature, 2005. **436**(7054): p. 1157-1160.
- 592 3. Meerburg, F.A., et al., *High-rate activated sludge communities have a distinctly different*
593 *structure compared to low-rate sludge communities, and are less sensitive towards*
594 *environmental and operational variables*. Water Research, 2016. **100**: p. 137-145.
- 595 4. Lee, J.W., et al., *Systems metabolic engineering of microorganisms for natural and non-*
596 *natural chemicals*. Nature Chemical Biology, 2012. **8**(6): p. 536-546.
- 597 5. Matsumoto, T., T. Tanaka, and A. Kondo, *Engineering metabolic pathways in Escherichia coli*
598 *for constructing a "microbial chassis" for biochemical production*. Bioresource Technology,
599 2017. **245**: p. 1362-1368.
- 600 6. De Roy, K., et al., *Environmental conditions and community evenness determine the outcome*
601 *of biological invasion*. Nature Communications, 2013. **4**.
- 602 7. Kinnunen, M., et al., *A conceptual framework for invasion in microbial communities*. Isme
603 Journal, 2016. **10**(12): p. 2773-2779.
- 604 8. He, X.S., et al., *The social structure of microbial community involved in colonization*
605 *resistance*. Isme Journal, 2014. **8**(3): p. 564-574.
- 606 9. Peng, X.F., S.P. Gilmore, and M.A. O'Malley, *Microbial communities for bioprocessing:*
607 *lessons learned from nature*. Current Opinion in Chemical Engineering, 2016. **14**: p. 103-109.
- 608 10. De Roy, K., et al., *Synthetic microbial ecosystems: an exciting tool to understand and apply*
609 *microbial communities*. Environmental Microbiology, 2014. **16**(6): p. 1472-1481.
- 610 11. Hendrickx, L., et al., *Microbial ecology of the closed artificial ecosystem MELiSSA (Micro-*
611 *Ecological Life Support System Alternative): Reinventing and compartmentalizing the Earth's*
612 *food and oxygen regeneration system for long-haul space exploration missions*. Research in
613 Microbiology, 2006. **157**(1): p. 77-86.
- 614 12. Clauwaert, P., et al., *Nitrogen cycling in Bioregenerative Life Support Systems: Challenges for*
615 *waste refinery and food production processes*. Progress in Aerospace Sciences, 2017. **91**: p.
616 87-98.
- 617 13. Tan, X.W., H. Ikeda, and M. Oda, *The absorption, translocation, and assimilation of urea,*
618 *nitrate or ammonium in tomato plants at different plant growth stages in hydroponic culture*.
619 Scientia Horticulturae, 2000. **84**(3-4): p. 275-283.
- 620 14. Bonvin, C., et al., *Plant uptake of phosphorus and nitrogen recycled from synthetic source-*
621 *separated urine*. Ambio, 2015. **44**: p. S217-S227.
- 622 15. Putnam, D.F., *Composition and concentrative properties of human urine*. 1971, National
623 Aeronautics and Space Administration.
- 624 16. Rose, C., et al., *The Characterization of Feces and Urine: A Review of the Literature to Inform*
625 *Advanced Treatment Technology*. Critical Reviews in Environmental Science and Technology,
626 2015. **45**(17): p. 1827-1879.
- 627 17. Coppens, J., et al., *Nitrification and microalgae cultivation for two-stage biological nutrient*
628 *valorization from source separated urine*. Bioresource Technology, 2016. **211**: p. 41-50.
- 629 18. Udert, K.M., et al., *Nitrification and autotrophic denitrification of source-separated urine*. Water
630 Science and Technology, 2003. **48**(1): p. 119-130.
- 631 19. Wittebolle, L., W. Verstraete, and N. Boon, *The inoculum effect on the ammonia-oxidizing*
632 *bacterial communities in parallel sequential batch reactors*. Water Research, 2009. **43**(17): p.
633 4149-4158.
- 634 20. Seuntjens, D., et al., *Pinpointing wastewater and process parameters controlling the AOB to*
635 *NOB activity ratio in sewage treatment plants*. Water Research, 2018. **138**: p. 37-46.

21. Godia, F., et al., *MELISSA: a loop of interconnected bioreactors to develop life support in space*. Journal of Biotechnology, 2002. **99**(3): p. 319-330.
22. Perez, J., et al., *Interactions of Nitrosomonas europaea and Nitrobacter winogradskyi grown in co-culture*. Archives of Microbiology, 2015. **197**(1): p. 79-89.
23. De Paepe, J., et al., *Refinery and concentration of nutrients from urine with electrodialysis enabled by upstream precipitation and nitrification*. Water Research, 2018. **144**: p. 76-86.
24. Larsen, T.A., K.M. Udert, and J. Lienert, *Source Separation and Decentralization for Wastewater Management*, ed. K.M. Udert and J. Lienert. 2013, London, UK: IWA Publishing.
25. Feng, D.L., Z.C. Wu, and S.H. Xu, *Nitrification of human urine for its stabilization and nutrient recycling*. Bioresource Technology, 2008. **99**(14): p. 6299-6304.
26. Jiang, F., et al., *Urine nitrification and sewer discharge to realize in-sewer denitrification to simplify sewage treatment in Hong Kong*. Water Science and Technology, 2011. **64**(3): p. 618-626.
27. Mackey, H.R. and G.H. Chen. *Urine Separation, Nitrification and Sewer-Discharge for Sewer Gas Control – Performance and Adaptation of a Urine Nitrifying Batch Reactor*. in Qatar Foundation Annual Research Conference. 2016.
28. Sun, F.Y., et al., *Stabilization of source-separated urine by biological nitrification process: treatment performance and nitrite accumulation*. Water Science and Technology, 2012. **66**(7): p. 1491-1497.
29. Sun, F.Y., et al., *Granulation of Nitrifying Bacteria in a Sequencing Batch Reactor for Biological Stabilisation of Source-Separated Urine*. Applied Biochemistry and Biotechnology, 2012. **166**(8): p. 2114-2126.
30. Koops, H.P. and A. Pommerening-Roser, *Distribution and ecophysiology of the nitrifying bacteria emphasizing cultured species*. Fems Microbiology Ecology, 2001. **37**(1): p. 1-9.
31. Defoirdt, T., et al., *Ureolytic Activity and Its Regulation in Vibrio campbellii and Vibrio harveyi in Relation to Nitrogen Recovery from Human Urine*. Environmental Science & Technology, 2017. **51**(22): p. 13335-13343.
32. Bischel, H.N., et al., *Inactivation kinetics and mechanisms of viral and bacterial pathogen surrogates during urine nitrification*. Environmental Science-Water Research & Technology, 2015. **1**(1): p. 65-76.
33. Fumasoli, A., et al., *Operating a pilot-scale nitrification/distillation plant for complete nutrient recovery from urine*. Water Science and Technology, 2016. **73**(1): p. 215-222.
34. Oosterhuis, M. and M.C.M. van Loosdrecht, *Nitrification of urine for H₂S control in pressure sewers*. Water Practice and Technology, 2009. **4**(3).
35. Bouatra, S., et al., *The Human Urine Metabolome*. PLOS ONE, 2013. **8**(9): p. e73076.
36. Tappe, W., et al., *Maintenance energy demand and starvation recovery dynamics of Nitrosomonas europaea and Nitrobacter winogradskyi cultivated in a retentostat with complete biomass retention*. Applied and Environmental Microbiology, 1999. **65**(6): p. 2471-2477.
37. Ilgrande, C., et al., *Can nitrification bring us to Mars? The role of microbial interactions on nitrogen recovery in Life Support Systems*. Communications in Agricultural and Applied Biological Sciences, 2016. **81**(1): p. 74-79.
38. Udert, K.M., *The fate of nitrogen and phosphorus in source-separated urine*, in Eawag, T.A. Larsen and M.C.M. van Loosdrecht, Editors. 2002, ETH Zürich: Zürich, Switzerland.
39. Guisasola, A., et al., *Inorganic carbon limitations on nitrification: Experimental assessment and modelling*. Water Research, 2007. **41**(2): p. 277-286.
40. Bock, E., *Vergleichende Untersuchungen über die Wirking sichtbaren Lichtes auf Nitrosomonas europaea und Nitrobacter winogradskyi*. Archiv für Mikrobiologie, 1965. **51**: p. 18-41.
41. Greenberg, A.E., *Standard methods for the examination of water and wastewater*. 1992, Washington DC: American Public Health Association.
42. Vilchez-Vargas, R., et al., *Analysis of the microbial gene landscape and transcriptome for aromatic pollutants and alkane degradation using a novel internally calibrated microarray system*. Environmental Microbiology, 2013. **15**(4): p. 1016-1039.
43. Chen, L.P., et al., *Partial nitrification of stored source-separated urine by granular activated sludge in a sequencing batch reactor*. Amb Express, 2017. **7**: p. 1-10.

- 691 44. Moussa, M.S., et al., *Long term effects of salt on activity, population structure and floc*
692 *characteristics in enriched bacterial cultures of nitrifiers*. Water Research, 2006. **40**(7): p.
693 1377-1388.
- 694 45. Chain, P., et al., *Complete genome sequence of the ammonia-oxidizing bacterium and*
695 *obligate chemolithoautotroph Nitrosomonas europaea*. Journal of Bacteriology, 2003. **185**(9):
696 p. 2759-2773.
- 697 46. Starkenburg, S.R., et al., *Genome sequence of the chemolithoautotrophic nitrite-oxidizing*
698 *bacterium Nitrobacter winogradskyi Nb-255*. Applied and Environmental Microbiology, 2006.
699 **72**(3): p. 2050-2063.
- 700 47. Rittmann, B.E., J.M. Regan, and D.A. Stahl, *Nitrification as a source of soluble organic*
701 *substrate in biological treatment*. Water Science and Technology, 1994. **30**(6): p. 1-8.
- 702 48. Mellbye, B.L., et al., *Steady-State Growth under Inorganic Carbon Limitation Conditions*
703 *Increases Energy Consumption for Maintenance and Enhances Nitrous Oxide Production in*
704 *Nitrosomonas europaea*. Applied and Environmental Microbiology, 2016. **82**(11): p. 3310-
705 3318.
- 706 49. Peng, L., et al., *N₂O production by ammonia oxidizing bacteria in an enriched nitrifying sludge*
707 *linearly depends on inorganic carbon concentration*. Water Research, 2015. **74**: p. 58-66.
- 708 50. Matias, M.G., et al., *Ecological strategies shape the insurance potential of biodiversity*.
709 Frontiers in Microbiology, 2013. **3**.
710

711

FIGURE 1

Fig. 1 Reactor 1: Effluent NH_4^+ , NO_2^- , and NO_3^- concentrations (mg N L^{-1}), average anticipated NO_3^- concentrations (mg N L^{-1}) per phase based on an assumed 100% NH_4^+ oxidation efficiency (SI section G), and anticipated and measured reactor salinity (as conductivity, in mS cm^{-1}) for the different phases I-VI of the salt adaptation reactor in function of time (days). In phase I, NH_4^+ was fed *via* spikes to the reactor. From phase II on, the reactor was continuously fed (indicated by the black vertical line). The dark grey arrows indicate biomass sampling for the batch activity tests.

FIGURE 2

Fig. 2 Batch activity test for reactor 1: Average ($\pm$ SD) volumetric nitrification (A) and nitrification (B) rates (mg N L⁻¹ d⁻¹) measured in function of salinity (as conductivity, mS cm⁻¹) for activity batch tests in 96 well plates. Big filled and unfilled symbols represent the rates measured in the batch test and the reactor, respectively, at reactor salinity at the time of biomass sampling. (n = 4)

FIGURE 3

Fig. 3 Reactor 1: Average ($\pm$ SD; $n = 3$) absolute abundance (copies mL⁻¹)(A) and relative abundance (%) (B) throughout the different operational phases.

FIGURE 4

Fig. 4 Reactor 2: Influent total nitrogen (TN) and effluent NH_4^+ , NO_2^- , NO_3^- , and TN concentrations (mg N L⁻¹) for the different phases I-X of the reactor with the full synthetic community in function of time (days). The black vertical line separates start-up phases I-IV from continuous reactor operation on fresh real urine (phases V-X). At that time point, biomass was sampled to start up the batch activity test. White zones indicate operation on real urine, grey zones on synthetic urine. Zones surrounded by a black border were successful runs (III, V, VIII, IX, X).

FIGURE 5

Fig. 5 Batch activity test with biomass from reactor 2: Average ($\pm$ SD) volumetric (left axis) and specific (right axis) NO_3^- production rates for real, men's urine (thawed and fresh), and synthetic urine, all at 1 and 10%. The same biomass concentrations were used in all test bottles. (n = 2)

FIGURE 6

Fig. 6 Reactor 2: Average ($\pm$ SD; $n = 3$) absolute abundance results (copies mL⁻¹)(A) and relative abundance (%) (B) throughout the different operational phases I-X. The black vertical line separates start-up phases I-IV from continuous reactor operation on fresh real urine (phases V-X). White zones indicate operation on real urine, grey zones on synthetic urine. Zones surrounded by a black border were successful runs (III, V, VIII, IX, X).