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ABSTRACT

During long-term extra-terrestrial 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 microbial community containing an AOB (*Nitrosomonas europaea)*, NOB (*Nitrobacter winogradskyi)*, and three ureolytic heterotrophs (*Pseudomonas fluorescens*, *Acidovorax delafieldii*, and *Delftia acidovorans)* was compiled and evaluated for these challenges. In reactor 1, salt adaptation of the ammonium-fed AOB and NOB coculture was possible up to 45 mS cm^{-1}, resembling undiluted nitrified urine, while maintaining an 44 ± 10 mg NH_4^+-N L^{-1} d^{-1} removal rate. In reactor 2, the nitrifiers and ureolytic heterotrophs were fed with urine and achieved 15 ± 6 mg NO_3^--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 [21]. 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 [9, 44]. Nitrified urine has good fertilizing properties [5]. Since urine is a complex salt matrix containing a variety of carbon and nitrogen metabolites [37, 39], 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 ($CO(NH_2)_2$)[39] requiring ureolysis (SI, Eq. A.2). The resulting ammonium (NH_4^+) can be oxidized to nitrite (NO_2^-) by ammonium oxidizing bacteria (AOB)(nitritation, SI, Eq. A.3), and NO_2^- can be oxidized by nitrite oxidizing bacteria (NOB) to nitrate (NO_3^-)(nitratation, SI, Eq. A.4).

In a mixed microbial community for urine nitrification, 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, because of their robust operation and resistance to disturbances. In space, however, stringent conditions occur, selecting for a defined (gnotobiotic) synthetic community. These communities, assembled with pure cultures [35], can assure a safe environment for the astronauts because of the absence of potentially pathogenic microbial strains. They further allow to mimic and study fundamental microbial and environmental interactions [13], to develop comprehensive mathematical models.

Drawbacks of synthetic communities are potential contamination and limited metabolic

functionality and flexibility. Contamination, *i.e.*, the invasion of an external species [12, 25], 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 [20].

Synthetic communities require the same stable metabolic functionality as diverse, mixed microbial communities despite physicochemical and microbial variations in the influent [1, 2, 30]. Therefore, synthetic communities need to consist of sufficient species."

This stable metabolic functionality was demonstrated by adapting mixed microbial communities to (un)diluted urine nitrification [10, 47]. The challenges inherent to the urine matrix (high salinity, ureolysis, and organics removal) could be tackled due to the presence of different AOB [49], NOB [40], 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 [17, 36].

As a first challenge, the salinity of fresh urine is about 20 mS cm^{-1} [9], while for nitrified undiluted urine this rises up to 45-75 mS cm^{-1} [10, 11]. The low alkalinity to nitrogen ratio in urine (1:1) only allows 50% nitrification without the addition of alkalinity [27]. This requirement further increases salinity. As a comparison, seawater has a salinity of about 50 mS cm^{-1}. Urine can be diluted for nitrification [15, 24, 28, 42, 43, 47] or the microbial community can be adapted to the high salinities of undiluted urine [10, 11]. Whereas Coppens, Lindeboom, Muys, Coessens, Alloul, Meerbergen, Lievens, Clauwaert, Boon,Vlaeminck [10] confirmed previous findings that the nitritation rate was more affected to short-term salt stress than the nitratation rate, De Paepe, Lindeboom, Vanopen, De Paepe, Demey, Coessens, Lamaze, Verliefde, Clauwaert,Vlaeminck [11] found no effect on the nitritation rate up to 58 mS cm^{-1}

and a linear decline in nitratation 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 [26]. 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 [39]. The required ureolysis tends to be a fast process if the right heterotrophs are selected [14], and not rate limiting for urine nitrification, despite its decrease during short-term salt stress [11]. Therefore, to prevent overloading and NH_4^+ accumulation, urine dilution [15, 24, 28, 42, 43, 47] or high hydraulic retention times (HRT)[3, 10, 16, 33] 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 [6, 37, 39]. The main COD component is creatinine (3.8 g COD L^{-1})[37]. The COD removal efficiencies of 75% for diluted urine [42, 43] to 94% for undiluted urine [10] 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* [45]. Perez, Buchanan, Mellbye, Ferrell, Chang, Chaplen, Bottomley, Arp,Sayavedra-Soto [36] 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, Mellbye, Ferrell, Chang,

Chaplen, Bottomley, Arp,Sayavedra-Soto [36] concluded that *N. europaea* received more benefit than *N. winogradskyi*. Additionally, Tappe, Laverman, Bohland, Braster, Rittershaus, Groeneweg,van Verseveld [45] found that *N. europaea* recovered faster from starvation than *N. winogradskyi*, explaining an NO_2^- peak upon NH_4^+ 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 [23] 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 [22]. 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 *via* two different molecular techniques 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). The salt-adapted nitrifying coculture of reactor 1 was not used in reactor 2 to study the effects of hydrolysis and organics degradation separately from high salinity. 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 NH_4^+ loading rate while increasing the salinity, to decouple salt and nitrogen effects. Ammonium was supplied *via* 3:1 molar ratio solutions of NH_4HCO_3 and $NaHCO_3$, mimicking hydrolysed urea while adding

171 sufficient inorganic carbon substrate. Salinity increase was obtained by supplying a salt
172 matrix solution with varying dilutions, derived from the hydrolysed synthetic urine recipe by
173 Udert [46] with additional trace elements (SI Table C.1). The nitrogen and salt solutions were
174 separately fed to the reactor.
175 In reactor 2, with a full synthetic community, both real and synthetic urine were used. Real
176 urine from healthy men not taking medication was collected *via* a waterfree toilet at Ghent
177 University with permission of the ethical committee (registration number B670201523246)
178 and stored at -20°C until use. At reactor start-up, the thawed urine was filter sterilized (0.22
179 µm) and diluted to 10% with autoclaved demineralized water, with addition of 0.5 mL L^{-1} trace
180 elements (ATCC medium for *Nitrobacter winogradskyi* Nb255, solution D), and $NaHCO_3$ to
181 0.1 g total inorganic carbon (TIC) L^{-1}, which was at least twice the half-saturation constant
182 K_{TIC} [19], to avoid limitation. The pH was adjusted to 11 with NaOH both for sterilization and
183 preliminary pH adjustment of the acidifying nitrification process. The influent bottle connected
184 to the reactor was stored at 4°C. Reactor operation shifted towards synthetic urine with 40
185 mg sodium acetate-COD L^{-1} and 40 mg urea-N L^{-1} to resemble 1% real urine. A urine salt
186 matrix solution was prepared after Udert [46](SI Table C.2). This was also used as a diluting
187 agent to operate the reactor at 1% and 10% fresh real urine, although at 10%, NaCl was
188 replaced by 10 g $NaHCO_3$ L^{-1}. Influent salinity for 1% and 10% urine were similar (SI Table
189 H.2). For both synthetic and the fresh real urine, the pH was no longer adjusted to 11 and the
190 influent bottle was stored at room temperature to avoid precipitation.
191
192 **2.3 Set-up and control of the reactors**
193 Salt adaptation of the AOB and NOB coculture (reactor 1) was conducted in an autoclavable
194 CSTR (Biostat B, Sartorius, Belgium), with a double-walled vessel for temperature control at
195 28°C with a working volume of 1L. The reactor was equipped with a pH probe (Mettler

196 Toledo, Switzerland) allowing pH control at 7.4 ± 0.1 with 1M H_2SO_4 and 1M NaOH, and a
197 dissolved oxygen (DO) probe (Mettler Toledo, Switzerland), monitoring the DO levels at
198 saturation level (8 mg O_2 L^{-1}). An air pump (KNF, Verder, Germany) with a maximum flow
199 rate of 5.8 ± 0.2 L min^{-1} supplied filter sterilized air (0.22 µm; Merck Millipore, Belgium) . Salt
200 and ammonium solutions were filter sterilized inline (0.22 µm; MerckMillipore, Belgium)
201 before being pumped in the reactor. Effluent was discontinuously withdrawn as sample.
202 Urine nitrification at 28°C with the full synthetic community (reactor 2) was conducted in a 2L
203 autoclavable CSTR (Hanna, Belach Bioteknik, Sweden), coupled to a cross-flow ceramic
204 ultrafiltration membrane unit (0.05 µm) in the liquid recirculation line for biomass retention
205 and the generation of a sterile effluent. The influent line was equipped with a series of dead-
206 end hydrophilic PTFE filters (5, 0.22, 0.05 µm; Merck Millipore, Belgium). Peristaltic influent,
207 effluent, and recirculation pumps (Watson Marlow, Belgium) were applied. Discontinuous
208 pumping for influent and effluent was required due to the low flow rate and was controlled by
209 time profiles. The recirculation pump was continuously operating at 58 mL min^{-1}. Filter
210 sterilized (0.22 µm; Merck Millipore, Belgium) and humidified air was bubbled in the reactor
211 and the O_2 concentration in the liquid was controlled at 40% (*i.e.*, 3.2 mg O_2 L^{-1}) by a DO
212 probe (Hamilton, Switzerland). The maximum flow rate of the air pumps was 1.88 ± 0.07 L
213 min^{-1}. The pH (Broadley James, UK) was controlled at 7.6-8.3, depending on the influent pH,
214 by dosing 1M H_2SO_4 and 2M NaOH.
215 Both reactors had gentle stirring (50 rpm) applied by a propeller stirrer and were operated in
216 the dark [4].
217
218 **2.4 Start-up and operational phases of the reactors**
219 Reactor 1 was filled with 1L salt solution, autoclaved, and sterile inoculated with the AOB and
220 NOB coculture near the flame. Initially, the reactor was operated in fed-batch mode (45 ± 43

mg N L^{-1} 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 ± 28 mg N L^{-1} d^{-1}, except for phase VI (3 ± 2 mg N L^{-1} 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} K_2HPO_4, 0.185 g L^{-1} $MgSO_4.7H_2O$, 0.015 g L^{-1} $CaCl_2.2H_2O$, and 0.036 g L^{-1} Na_2CO_3, according to Perez, Buchanan, Mellbye, Ferrell, Chang, Chaplen, Bottomley, Arp,Sayavedra-Soto [36] 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 nitritation and nitratation 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, nitritation, and nitratation 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 [18], 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, Suarez-Diez, Conte, Waliczek, Kaser, Kralova, Junca,Pieper [48], 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} [9]), 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 NH_4^+ 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 NH_4^+ 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 NO_3^- 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 NH_4^+ solution was continuously fed to the reactor. A removal rate of 44 ± 16 mg N L^{-1} d^{-1} with 81 ± 39% removal efficiency was reached and considered as the baseline scenario. Limited biomass concentrations in the coculture probably caused the low volumetric NH_4^+ 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 [33] to 1317 mg N L^{-1} d^{-1} in a SBR [8]. Nitrate production rates could not be calculated as masked by NO_3^- 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 nitritation and nitratation 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 NH_4^+ loading rate of 45 ± 28 mg N L^{-1} d^{-1}. The salt shock in phase III caused a drop in the NH_4^+ 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 NH_4^+ removal efficiencies of 90 ± 39 and 94 ± 31% for phase IV and V, respectively. In these phases, NO_3^- reached concentrations of 264 ± 35 and 289 ± 20 mg N L^{-1}, respectively, compared to NH_4^+ 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 NH_4^+ removal, could be an indication for denitrification in the potentially anoxic biofilm that was observed on the reactor wall or N_2O formation.

At the end of phase V, after 6 days of stable reactor operation at 55 mS cm^{-1}, NH_4^+ started to gradually build up indicating an inhibition or decay of the AOB. The loading rate was reduced close to zero as only then NH_4^+ did no longer accumulate. As a result, hardly any NH_4^+ 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 be not be observed for phase VI and no nitrite built up occurred. Coppens, Lindeboom, Muys, Coessens, Alloul, Meerbergen, Lievens, Clauwaert, Boon,Vlaeminck [10] and Moussa, Sumanasekera, Ibrahim, Lubberding, Hooijmans, Gijzen,van Loosdrecht [32] reported NOB to adapt better to higher salinities than AOB while De Paepe, Lindeboom, Vanopen, De Paepe, Demey, Coessens, Lamaze,

Verliefde, Clauwaert,Vlaeminck [11] found the opposite. Although both strains have the gene encoding for glycine betaine [7, 41], an osmolyte that potentially regulates the osmotic pressure in the cell, *Nitrobacter* was reported to possess genes for a variety of osmolytes [41], 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 [11] or 60% [10] diluted real nitrified urine.

[Fig. 1]

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

The nitritation and nitratation 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 nitritation 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 NH_4^+ removal in a pure culture *N. europaea* chemostat run [36]. 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, Muys, Coessens, Alloul, Meerbergen, Lievens, Clauwaert, Boon,Vlaeminck [10]. At 40 mS cm^{-1}, the NH_4^+ 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 NH_4^+ removal rates of the reactor. Although nitratation 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 nitritation and nitratation 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 nitritation and nitratation 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)[10, 32]. 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 in function of time (dynamics) (Fig. 3). During start-up (phase I), AOB and *Nitrobacter* spp.

increased in abundance due to growth (Fig. 3.A), which was less observed from the illumina data (Fig. 3.B). From day 50 to 60, the sequence data show a higher abundance of NOB compared to AOB. This correlated with a minor NH_4^+ accumulation (Fig. 1). Throughout the different phases of reactor operation, fluctuations occurred for the AOB and NOB. Real-time PCR analysis revealed a relative abundance over all phases 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, Muys, Coessens, Alloul, Meerbergen, Lievens, Clauwaert, Boon,Vlaeminck [10] 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 [38], 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 ± 27 mg N L^{-1}) could provide about 170 mg TIC L^{-1} in the reactor, which matches at least 7 times the half-saturation constant K_{TIC} (22 mg TIC L^{-1})[19] or 3 times the TIC need for AOB (62 mg mg TIC L^{-1})[31] 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^- [31, 34]. To avoid N_2O emissions, $NaHCO_3$ was added from day 55 throughout the entire reactor run to maintain levels of 200-300 mg TIC L^{-1} (SI Fig. H.3). The influent was changed to 1% synthetic urine (48 ± 10 mg N L^{-1} and 90 ± 53 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 ± 7 mg N L^{-1} d^{-1}, resulted in an ureolysis rate of 16 ± 2 mg N L^{-1} d^{-1} (89 ± 7%), a NO_3^- production rate of 15 ± 9 mg N L^{-1} d^{-1} (59 ± 21%), and a TN loss of around 5 mg N L^{-1} 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 ± 2.7 mg N L^{-1} d^{-1}, compared to 19 ± 3.3 mg N L^{-1} d^{-1} for thawed and 22.9 ± 19.1 mg N L^{-1} 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 NO_3^- 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 NO_3^- production rates in the reactor were almost double for both 1% synthetic and real urine compared to the rates obtained in

495 the batch activity test (Fig. 5). Efficiencies reached 81 ± 16% for ureolysis and 84 ± 11% for
496 NO_3^- production. Average effluent NH_4^+ concentrations were 6 ± 3 mg N L^{-1}.
497 After a fed-batch period (phase VI), the reactor was reactivated with 1% fresh real urine in
498 continuous mode during phase VII before switching to 10% fresh real urine (822 ± 57 mg N L^{-1}
499 and 774 ± 93 mg COD L^{-1}, phase VIII)(Fig. 4). To maintain a similar TN and COD loading
500 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
501 increased to 24 ± 2 days (SI Table H.2, SI Fig. H.1). One HRT was sufficient to obtain a
502 stable TN and NO_3^- concentration in the effluent (544 ± 46 mg N L^{-1} and 273 ± 10 mg N L^{-1},
503 respectively), although influent TN levels were not yet reached (Fig. 4), indicating a TN loss
504 of 29 ± 7% (SI Table H.2). For COD, a removal efficiency of 52 ± 7% was obtained (SI Table
505 H.3), which is lower than values reported in mixed microbial communities (75% at lowest for
506 Sun 2012). Volumetric ureolysis rates (21 ± 4 mg N L^{-1} d^{-1}) increased compared to 1%
507 synthetic and fresh real urine, whereas efficiencies decreased till 66 ± 9%. Nitrate production
508 encountered a decreased rate (11 ± 1 mg N L^{-1} d^{-1}) and efficiency (35 ± 3 %). The rate was
509 only one third of what was obtained in the batch activity test for 10% fresh urine (Fig. 5, SI
510 Fig. H.1). However, in the batch test 10% urine equaled a total nitrogen concentration of 398
511 ± 3 mg N L^{-1}. Therefore, the influent was changed to 5% fresh real urine (370 ± 0 mg N L^{-1})
512 during the transition phase IX, to obtain a stable run in phase X (333 ± 26 mg N L^{-1} and 363 ±
513 21 mg COD L^{-1}) with an HRT of 23 ± 9 days (SI Table H.2). Here, the TN and COD loading
514 rates were slightly lower (16 ± 6 mg N L^{-1} d^{-1} and 23 ± 7 mg COD L^{-1} d^{-1}) compared to all
515 previous runs. While ureolysis (16 ± 7 mg N L^{-1} d^{-1}), NO_3^- production (15 ± 6 mg N L^{-1} d^{-1}), and
516 losses (2 ± 2 mg N L^{-1} d^{-1}) remained similar to the 1% synthetic and fresh real urine phases
517 (III and V, respectively), this lower loading rate caused the ureolysis and NO_3^- production
518 efficiencies to increase to 107 ± 8% and 94 ± 8%, respectively. The COD removal rate (16 ±
519 5 mg COD L^{-1} d^{-1}) equaled the one in phase VIII (SI Table H.3), but at 69 ± 15% efficiency,

520 although the remaining COD in the effluent still contained about 62-73% biodegradable
521 organics (SI Table H.4). Average effluent NH_4^+ concentrations in this last phase X were 5 ± 4
522 mg N L^{-1}. Nitrate production rates as obtained in the batch test for 10% fresh real urine could
523 not be achieved in the reactor. Whereas the batch test demonstrated a clear preference for
524 10% fresh real urine, the NO_3^- production rates in the reactor were rather independent of the
525 type of urine (synthetic or fresh real) and the concentration (1 or 10%).
526
527 **3.6 *D. acidovorans* dominated the synthetic community**
528
529 For ureolysis and organics degradation, three heterotrophic strains (*P. fluorescens*, *A.*
530 *delafieldii*, and *D. acidovorans*) were added to the autotrophic nitrifying coculture. A non-
531 pathogenic and ureolytic *Pseudomonas* strain was added, assuming this strain would occupy
532 the same, potential niche that was found in the salt adaptation reactor (*i.e.*, pre-emptive
533 colonization). Community dynamics were followed over time *via* qPCR (Fig. 6.A). During the
534 start-up with 10% thawed real urine (phase I), *N. europaea* and *N. winogradskyi* gradually
535 increased in abundance from day 55, when additional TIC was supplemented to the reactor
536 (200-300 mg L^{-1}). Heterotrophic strains only accumulated when the shift was made to
537 synthetic urine with easily degradable acetate as a carbon source, at the end of phase I. The
538 lower COD removal rates obtained for real urine, and the variety of organics present there,
539 might suggest to include heterotrophic strains that are specialized in degrading certain
540 recalcitrant organic compounds or more generalized in degrading a broad variety of organics
541 [29].
542 *N. europaea* was more abundant than *N. winogradskyi* from phase II on (0-6% and 0-2%,
543 respectively), in contrast to the salt adaptation reactor and literature [10]. For the
544 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.4), 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, Vanopen, De Paepe, Demey, Coessens, Lamaze, Verliefde, Clauwaert,Vlaeminck [11]. 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 [20]. Additional experiments are required to prove this hypothesis.

[Fig. 6]

4. CONCLUSION

For long-term space applications, urine nitrification was successfully achieved with a lean synthetic community in bioreactors. Challenges concerning the matrix (salt, ureolysis, and organics) and the need for a gnotobiotic community were overcome. In a first step, the

569 halotolerance of the nitrifiers *N. europaea* and *N. winogradskyi* could be stretched to 45 mS
570 cm^{-1}, resembling undiluted nitrified urine. Similar nitrate production rates for 1% and 10%
571 urine were achieved with the full synthetic community. Predictive batch activity tests identified
572 maximum rates for 10% fresh real urine. Finally, the long-term combination of ureolysis,
573 nitrification and organics removal was achieved in a stable bioreactor run, although organics
574 removal should be optimized for future space applications. Community dynamics were
575 monitored and showed the dominance of the heterotroph *D. acidovorans.* Additional tests are
576 required to unravel the link between this dominance and the suppression of other (invasive)
577 heterotrophic strains. This would yield valuable information for sterile reactor operation in
578 space or industrial applications.
579
580
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FIGURE 1

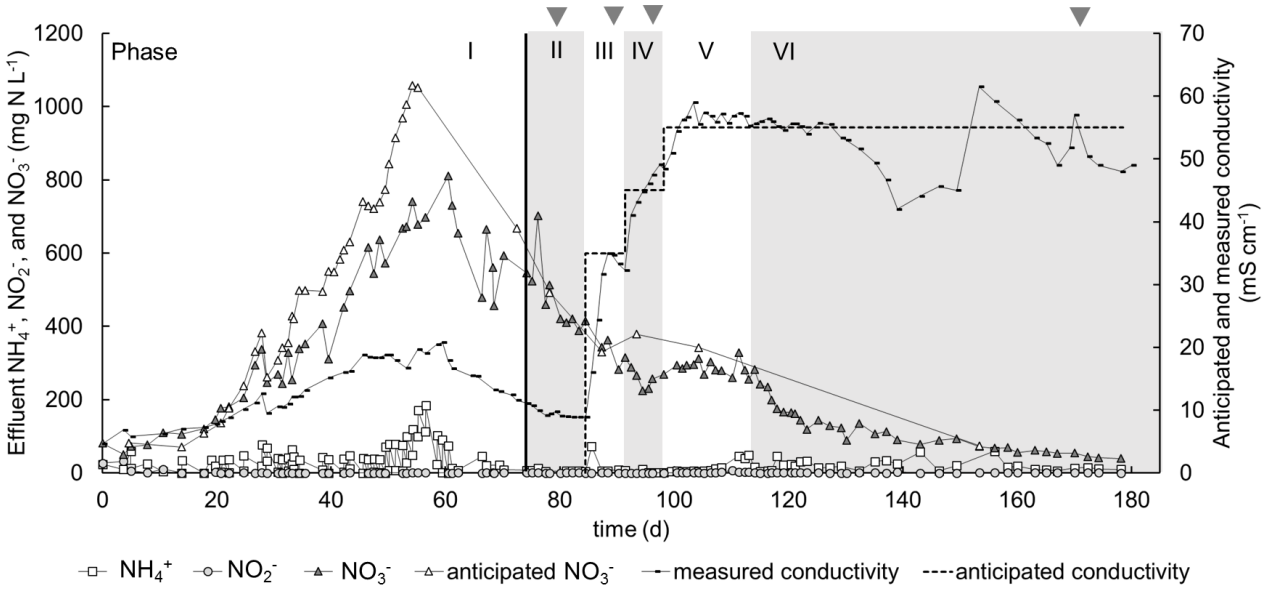


Fig. 1 Reactor 1: Effluent NH_4^+, NO_2^-, and NO_3^- concentrations (mg N L^{-1}), average anticipated NO_3^- concentrations (mg N L^{-1}) per phase based on an assumed 100% NH_4^+ oxidation efficiency (SI section G), and anticipated and measured reactor salinity (as conductivity, in mS cm^{-1}) for 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

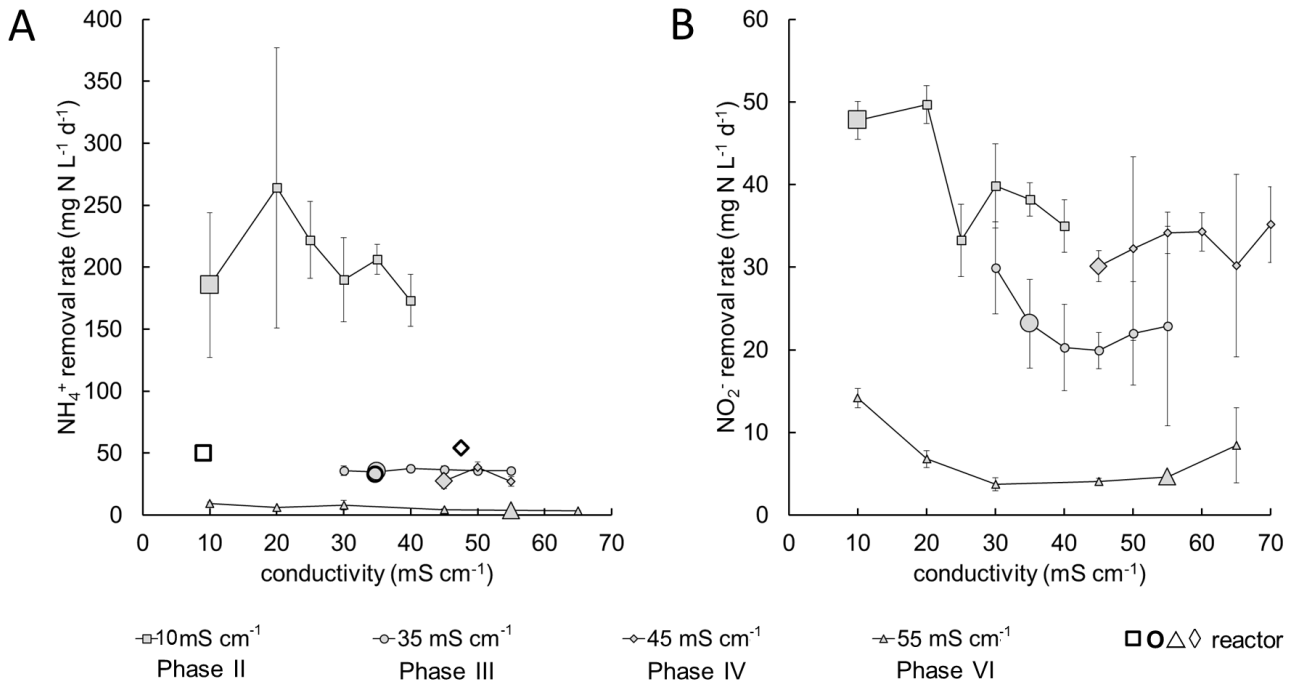
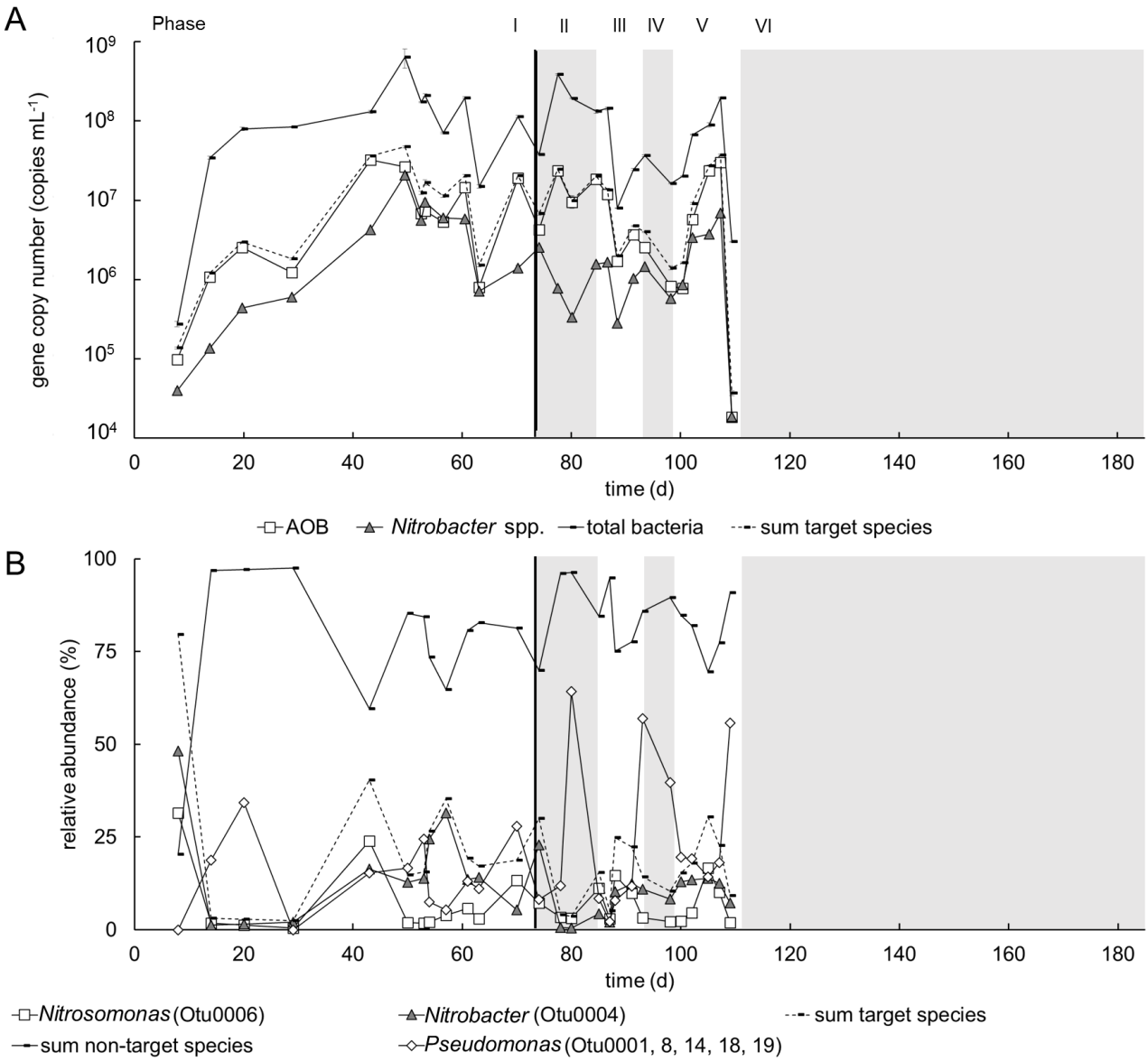


Fig. 2 Batch activity test for reactor 1: Average (± SD) volumetric nitritation (A) and nitratation (B) rates (mg N L^{-1} d^{-1}) measured in function of salinity (as conductivity, mS cm^{-1}) for activity batch tests in 96 well plates. Big filled and unfilled symbols represent the rates measured in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

735 **FIGURE 3**



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737 Fig. 3 Reactor 1: Average (± SD; n = 3) absolute abundance (copies mL^{-1})(A) and relative

738 abundance (%)(B) throughout the different operational phases.

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FIGURE 4

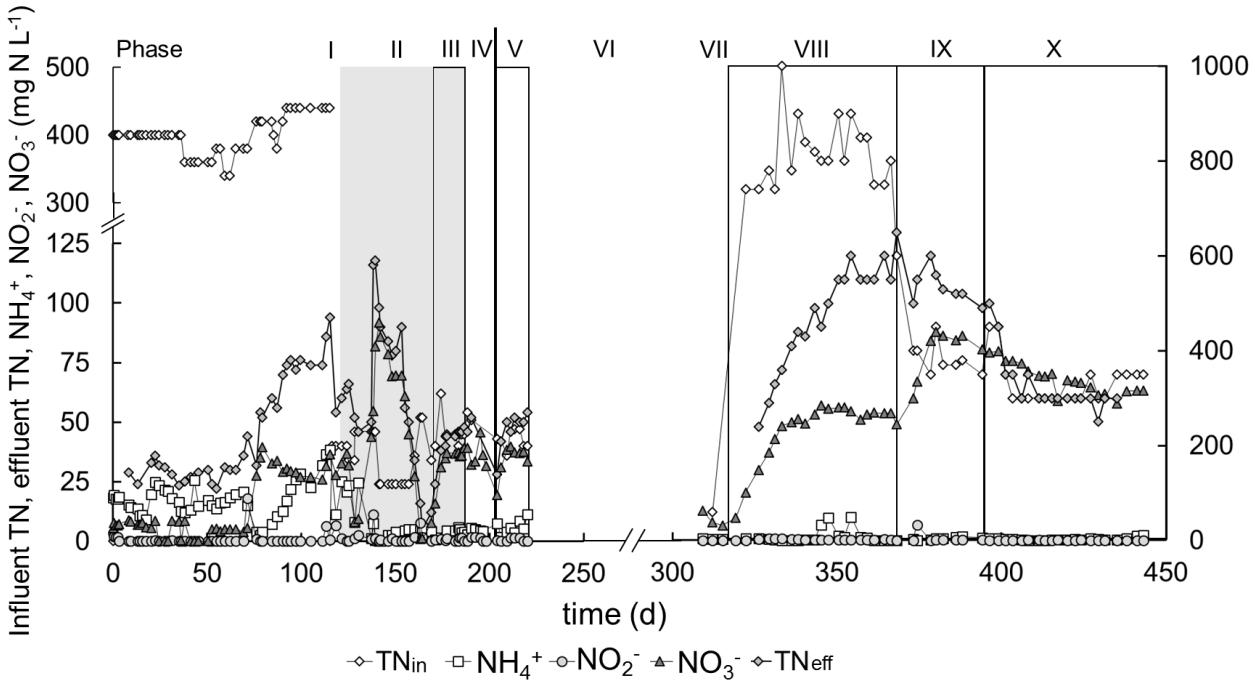

Fig. 4 Reactor 2: Influent total nitrogen (TN) and effluent NH_4^+, NO_2^-, NO_3^-, and TN concentrations (mg N L^{-1}) for the different phases I-X of the reactor with the full synthetic community in function of time (days). The black vertical line separates start-up phases I-IV from continuous reactor 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

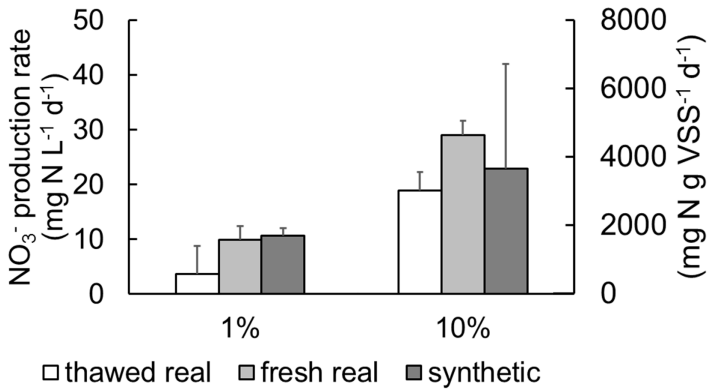

Fig. 5 Batch activity test with biomass from reactor 2: Average (± SD) volumetric (left axis) and specific (right axis) NO_3^- production rates for real, men's urine (thawed and fresh), and synthetic urine, all at 1 and 10%. The same biomass concentrations were used in all test bottles. (n = 2)

FIGURE 6

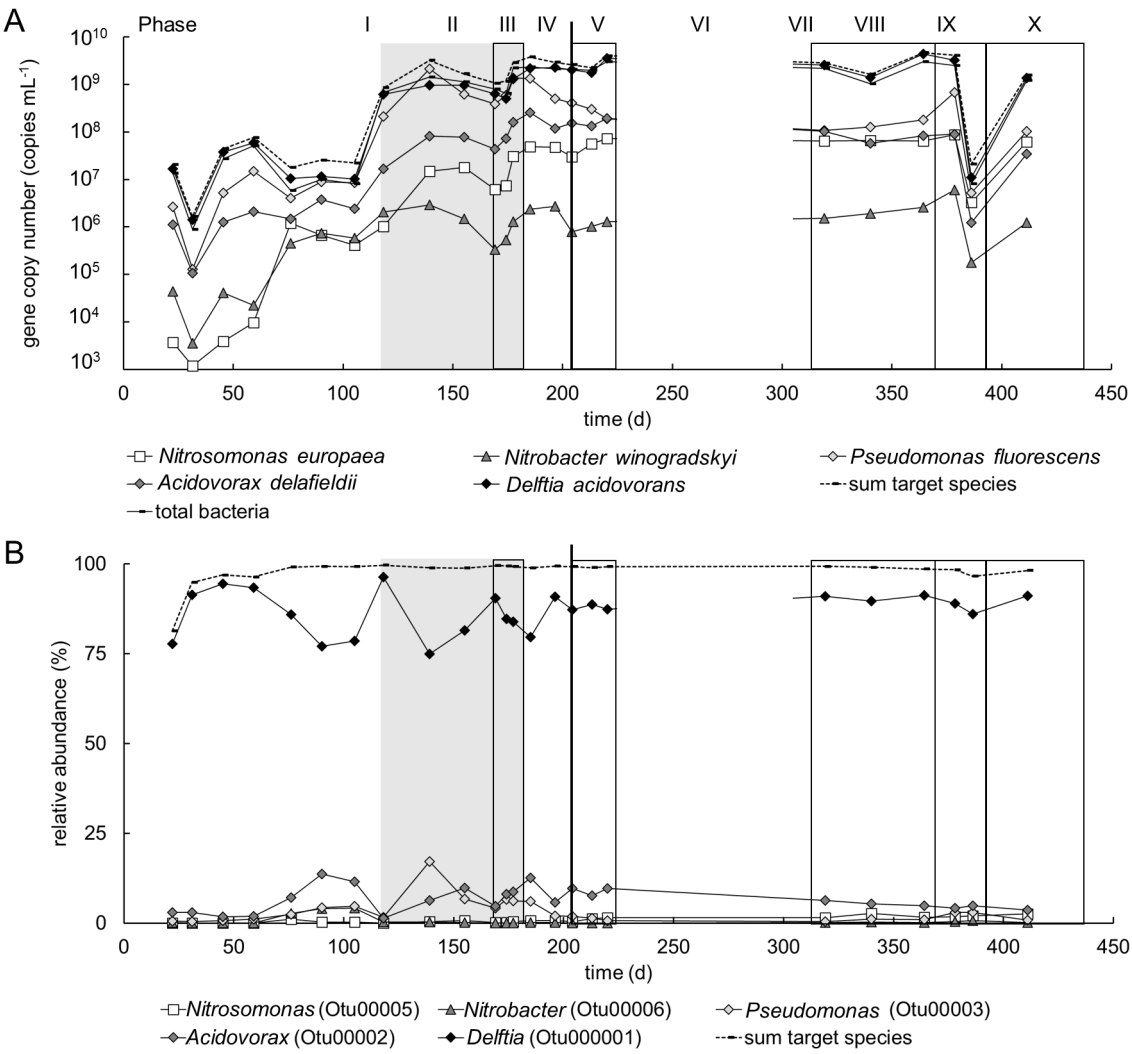

Fig. 6 Reactor 2: Average (± SD; n = 3) absolute abundance results (copies mL^{-1})(A) and relative abundance (%)(B) throughout the different operational phases I-X. The black vertical line separates start-up phases I-IV from continuous reactor operation on fresh real urine (phases V-X). White zones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