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Influence of pH on Urine Nitrification: Community Shifts of Ammonia-Oxidizing Bacteria and Inhibition of Nitrite-Oxidizing Bacteria

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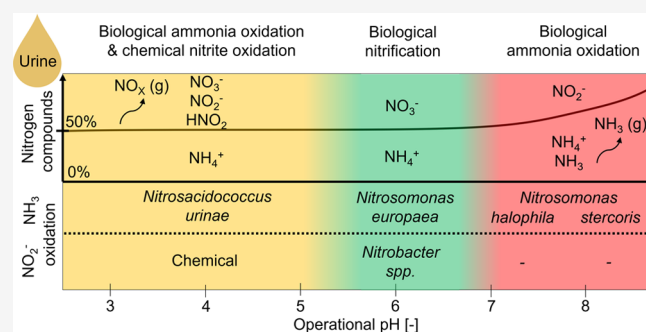
ABSTRACT: Urine nitrification is pH-sensitive due to limited alkalinity and high residual ammonium concentrations. This study aimed to investigate how the pH affects nitrogen conversion and the microbial community of urine nitrification with a pH-based feeding strategy. First, kinetic parameters for NH_3, HNO_2, and NO_2^- limitation and inhibition were determined for nitrifiers from a urine nitrification reactor. The turning point for ammonia-oxidizing bacteria (AOB), i.e., the substrate concentration at which a further increase would lead to a decrease in activity due to inhibitory effects, was at an NH_3 concentration of 12 mg-N L^{-1}, which was reached only at pH values above 7. The total nitrite turning point for nitrite-oxidizing bacteria (NOB) was pH-dependent, e.g., 18 mg-N L^{-1} at pH 6.3. Second, four years of data from two 120 L reactors were analyzed, showing that stable nitrification with low nitrite was most likely between pH 5.8 and 6.7. And third, six 12 L urine nitrification reactors were operated at total nitrogen concentrations of 1300 and 3600 mg-N L^{-1} and pH values between 2.5 and 8.5. At pH 6, the AOB *Nitrosomonas europaea* was found, and the NOB belonged to the genus *Nitrobacter*. At pH 7, nitrite accumulated, and *Nitrosomonas halophila* was the dominant AOB. NOB were inhibited by HNO_2 accumulation. At pH 8.5, the AOB *Nitrosomonas stercoris* became dominant, and NH_3 inhibited NOB. Without influent, the pH dropped to 2.5 due to the growth of the acid-tolerant AOB "Candidatus Nitrosacidococcus urinae". In conclusion, pH is a decisive process control parameter for urine nitrification by influencing the selection and kinetics of nitrifiers.

KEYWORDS: *source separation, resource recovery, nutrient recovery, decentralized treatment, fertilizer production*

1. INTRODUCTION

Urine contributes about 80% of nitrogen and 50% of phosphorus to domestic wastewater, while it accounts for less than 1% of its volume.[1] Separation of urine at the source allows efficient recovery of these nutrients and reduces the nitrogen and phosphorus loads to wastewater treatment plants.[2,3] During urine collection and storage, urea, the main source of nitrogen in urine, is rapidly hydrolyzed to free ammonia (NH_3), ammonium (NH_4^+), and bicarbonate, raising the pH above 9.[4] Ammonia is highly volatile and causes odor problems, human health hazards, environmental pollution, and nitrogen losses. To prevent ammonia volatilization, a treatment step for nitrogen stabilization such as nitrification is required before urine can be used as a fertilizer.[2] The total ammoniacal-nitrogen (TAN = NH_3-N + NH_4^+-N) concentration in stored urine can be as high as 8000 mg-N L^{-1}.[5] In reality, source-separated urine is diluted by flushing water,[6] resulting in lower TAN concentrations of 2000−4000 mg-N L^{-1}.[7]

Ammonia can be biologically converted to nitrate by nitrification. Nitrification of urine differs greatly from conventional sewage nitrification because of the high pH, TAN concentration, and salt content of stored urine.[8] In addition, the ratio of alkalinity to TAN in urine is only about one mole of alkalinity per mole of TAN.[5] Approximately 50% of the TAN is converted to nitrate if no alkalinity is added, and the system is operated at a pH between 6 and 7.[9] Despite this partial conversion, ammonia volatilization is prevented because nitrification causes a pH drop that equilibrates the remaining TAN to nonvolatile NH_4^+. However, the low alkalinity and high residual TAN concentration in the reactor make the system susceptible to pH changes that affect the activity of ammonia-oxidizing bacteria (AOB) and nitrite-oxidizing bacteria (NOB) directly or through the acid−base equilibria of $NH_4^+ \rightleftharpoons NH_3 + H^+$ and nitrous acid (HNO_2) ⇌ nitrite

(NO_2^-) + H^+. Alkalinity addition allows complete conversion of ammonia to nitrate but requires additional oxygen and either an electrochemical unit that produces hydroxide anions or the dosing of, e.g., sodium hydroxide, which increases salinity.[10] This publication focuses on urine nitrification without alkalinity addition, hereafter termed urine nitrification.

Higher pH results in higher concentrations of NH_3 ($pK_{a,25\,°C} \approx 9.25$), the substrate of AOB, which increases the ammonia oxidation rate.[11] However, as ammonia is also known to inhibit AOB, this is only true up to a certain concentration, where further increases in ammonia concentration lead to decreased AOB activity.[12] While Jubany et al.[13] found this turning point, i.e., the substrate concentration at which a further increase would lead to a decrease in activity due to inhibitory effects, at an NH_3 concentration of 5 mg-N L^{-1}, the value found by Pambrun et al.[14] was twice as high at 10 mg-N L^{-1}. Higher ammonia oxidation rates are beneficial because they allow for smaller reactor volumes, but if ammonia is oxidized faster than nitrite, then the latter will accumulate. Nitrite instead of nitrate in the effluent is problematic for fertilizer production because nitrite is toxic to plants,[15] can potentially increase N_2O emissions during the nitrification process,[16] and can be lost to the gas phase during downstream treatment, such as distillation for sanitization and concentration.[9] A slight increase in the NO_2^- concentration promotes nitrite oxidation, as it is the actual substrate of NOB.[14] This is only true up to a certain total nitrite-nitrogen ($TNN = HNO_2^- \text{-}N + NO_2^- \text{-}N$) concentration because the conjugated acid of nitrite, nitrous acid ($pK_{a,25\,°C} \approx 3.25$), inhibits NOB.[12] Since NOB are often more sensitive to HNO_2 inhibition than AOB,[17] further nitrite accumulation occurs when this pH-dependent turning point is exceeded, resulting in a positive feedback loop.[18]

Due to the low alkalinity-to-TAN ratio, the pH value can decrease strongly during nitrification, reducing the ammonia oxidation rate due to the lower availability of NH_3. At pH values around 5.4, ammonia oxidation usually ceases completely due to a direct pH effect related to the energy available from the proton motive force.[19] However, a prolonged period at pH values around 5.4, for example, due to limited urine influent, can lead to the growth of acid-tolerant AOB, resulting in a further pH drop to values as low as 2.2 and the release of harmful nitrogen oxide gases.[20] In a urine nitrification reactor operated at pH 5, a novel acid-tolerant AOB "*Candidatus* Nitrosacidococcus urinae" was found that can grow at low NH_3 concentrations of 0.04 mg-N L^{-1} and survive high HNO_2 concentrations of 15 mg-N L^{-1}.[21] Once nitrite accumulates at low pH, biological nitrite oxidation is inhibited by HNO_2, and chemical nitrite oxidation becomes dominant.[21,22]

Urine nitrification reactors operated at a continuous loading rate were subject to large variations in pH and nitrite and nitrate concentrations.[23] To avoid large pH fluctuations during urine nitrification, Udert and Wächter[9] suggested controlling the pH with the influent within a narrow range using an on/off controller. Stable urine nitrification, defined in this publication as the conversion of ammonia to nitrate with little nitrite accumulation, was observed at least transiently at pH set-points between 6.1 and 6.9 in a membrane-aerated biofilm reactor,[9] 5.8 and 6.2 in a moving-bed biofilm reactor,[7] 5.75 and 6.4 in a fed-batch reactor,[16] and at 6.2 in a membrane bioreactor.[24] Nevertheless, nitrite accumulation occurred frequently, primarily related to changes in pH set-points[9] or influent concentrations.[25] Process instability is, therefore, one of the

most critical aspects of urine nitrification without alkalinity addition,[10] and stable nitrification is only possible within a narrow range of reactor conditions.[17] Zuo et al.[26] even suggested separating ammonia and nitrite oxidation in two different reactors to increase the process stability of urine nitrification.

Only a few studies have identified the nitrifiers involved in stable urine nitrification. AOB of the lineage *Nitrosomonas europaea*[20] or *Nitrosomonas eutropha*[19] were observed at pH 6 and *Nitrosospira* spp. at pH 5.8.[7] *N. europaea* was also found by Faust et al.[21] for stable urine nitrification. A *Nitrosomonas* sp. was also found in a reactor operated at a continuous flow rate during periods of low nitrite concentration.[17] In urine nitrification with added alkalinity, AOB closely related to *Nitrosomonas aestuarii* or *Nitrosomonas marina* were found.[27] NOB of the genus *Nitrobacter* were suspected in urine nitrification, but the NOB could not be uniquely attributed to this genus.[7,27]

Despite several reports on urine nitrification, the influence of operational pH on nitrification performance and nitrifier species is still poorly understood, especially at neutral and alkaline pH, and the operational pH range for stable urine nitrification has not yet been systematically investigated. While Chipako and Randall[28] conducted a review that focused on the importance of pH in urine treatment technologies, the influence of pH on urine nitrification was not discussed. Switching functions, such as the Monod equation, are commonly used to describe the effect of NH_3, NO_2^- and HNO_2 on nitrification and to calculate turning point concentrations. However, kinetic parameters vary widely,[29] and a complete set of parameters for urine nitrification has not yet been determined.

This study aimed to investigate how pH, NH_3, NO_2^-, HNO_2, and salinity affect the effluent composition, i.e., nitrite and nitrate concentrations, and the selection of microbial species for urine nitrification. First, biomass from a urine nitrification reactor was used to determine the short-term effects of salinity, NH_3 and HNO_2 inhibition, and NH_3 and NO_2^- limitation, specifically to determine kinetic parameters and the turning point concentrations of NH_3 and TNN for AOB and NOB, respectively. Second, four years of data from a urine nitrification and fertilizer production plant were analyzed to evaluate the most likely pH for stable nitrification in practice. Finally, six lab-scale reactors were operated at pH values ranging from 2.5 to 8.5 and for two different urine dilutions to evaluate the long-term influence of different pH set-points outside the usual range, and the kinetic parameters were used to see if they could explain the changes in nitrification performance.

2. MATERIALS AND METHODS

2.1. Short-Term Influence of Ammonia, Nitrite, Nitrous Acid, and Salinity on Nitrifier Activity.

The effect of NH_3, HNO_2, and NO_2^- on AOB and NOB activity was investigated by using short-term activity assays in a 3 L respirometer. Understanding the effects of these nitrogen compounds on AOB and NOB is crucial to understanding the influence of pH changes on nitrification. The respirometer was operated as either a two-chamber LSF respirometer (LSF: static gas, flowing liquid) or a two-chamber LSS respirometer (LSS: static gas, static liquid).[30] Activated sludge from a 120 L urine nitrification reactor for fertilizer production at Forum Chriesbach (Dübendorf, Switzerland) operated at a pH value

Table 1. Overview of Experiments with 12 L Reactors

run	origin of inoculum and urine	TAN[a] influent [mg-N L^{-1}]	salinity[b] [mS cm^{-1}]	pH set-points before day 25	pH set-points after day 25
Low-Concentration Urine					
1.1	NEST	1270 ± 120	11.2 ± 0.9	6.0/6.05	7.00/7.05
1.2	NEST	1270 ± 120	11.2 ± 0.9	6.0/6.05	8.50/8.55
1.3	NEST	1270 ± 120	11.2 ± 0.9	6.0/6.05	influent stop
High-Concentration Urine					
2.1	Forum Chriesbach	3610 ± 50	26.3 ± 0.5	6.0/6.05	7.00/7.05
2.2	Forum Chriesbach	3610 ± 50	26.3 ± 0.5	6.0/6.05	8.50/8.55
2.3	Forum Chriesbach	3610 ± 50	26.3 ± 0.5	5.8/5.85	influent stop

[a]TAN = NH$_3$-N + NH$_4^+$-N. [b]Electrical conductivity was used as a proxy for salinity. The influent used in Run 1 is referred to as "low-concentration urine", and the influent used in Run 2 is called "high-concentration urine".

of about 6.3, TAN influent concentrations of 3500 mg-N L^{-1}, and a salinity of 26 mS cm^{-1} was used. The data were used to determine the substrate limitation and noncompetitive inhibition constants for AOB and NOB according to the switching functions in eqs 1−4,[29]

$$\text{AOB: } \frac{S_{NH_3}}{S_{NH_3} + K_{S,NH_3}} \tag{1}$$

$$\text{NOB: } \frac{S_{NO_2^-}}{S_{NO_2^-} + K_{S,NO_2^-}} \tag{2}$$

$$\text{AOB, NOB: } \frac{K_{I,NH_3}}{S_{NH_3} + K_{I,NH_3}} \tag{3}$$

$$\text{AOB, NOB: } \frac{K_{I,HNO_2}}{S_{HNO_2} + K_{I,HNO_2}} \tag{4}$$

$$\text{AOB: } \frac{S_{NH_3}}{K_{S,NH_3} + S_{NH_3} + \frac{S_{NH_3}^2}{K_{I,NH_3}}} \tag{5}$$

where S_{NH_3} is the ammonia concentration, $S_{NO_2^-}$ is the nitrite concentration, S_{HNO_2} is the nitrous acid concentration, K_{S,NH_3} is the ammonia substrate limitation constant for AOB, K_{S,NO_2^-} is the nitrite substrate limitation constant for NOB, K_{I,NH_3} is the ammonia inhibition constant for AOB or NOB, and $K_{I,HNO2}$ is the nitrous acid inhibition constant for AOB or NOB. For AOB, ammonia is both substrate and inhibitor. This is often described by multiplying eqs 1 and 3 or by using the Haldane function in eq 5.[29] Han and Levenspiel[31] reported that these equations underestimate substrate inhibition at high concentrations, and eq 6 has been proposed as an alternative,

$$\text{AOB: } \left(1 - \frac{S_{NH_3}}{K_{I,NH_3}^*}\right)^n \frac{S_{NH_3}}{S_{NH_3} + K_{S,NH_3}\left(1 - \frac{S_{NH_3}}{K_{I,NH_3}^*}\right)^m} \tag{6}$$

where k_{I,NH_3}^* is the critical concentration above which the reaction stops, and n and m are dimensionless constants.

In addition, the influence of salinity on AOB and NOB activity was investigated using the same respirometer since different urine dilutions, and thus, different salinities may occur depending on the source separation system, i.e., the dilution with flushing water. Sodium chloride (NaCl) was added stepwise, and the electrical conductivity was used as a proxy for the salinity. The temperature in the respirometer was set at 25

°C, and the pH was controlled with 0.4 M NaOH and 0.4 M HCl. More details about the setup and the experimental procedure can be found in the Supporting Information, SI 1.

2.2. Stable Nitrification in a Decentralized Urine Nitrification Reactor. Four years of data from two 120 L urine nitrification reactors for fertilizer production at Forum Chriesbach (Dübendorf, Switzerland) were analyzed from 2017 to 2021 to investigate the conditions, i.e., pH range, for stable nitrification. The reactors were operated with suspended activated sludge in parallel first as continuous flow stirred-tank reactors (CSTR) and then in fed-batch mode.[16] In the CSTR the sludge retention time (SRT) was equal to the hydraulic retention time (HRT). The fed-batch mode consisted of an aerated feeding phase, a settling phase for sludge retention, and a decanting phase. The pH in the reactor was controlled with the urine influent via a narrow two-point on/off controller with a pH control band of 0.05 units.[32] Once the pH reached the lower set-point due to protons released during ammonia oxidation, the influent was turned on, causing the pH to increase due to the higher pH (pH of stored urine ≈ 9) and alkalinity of the urine influent. Nitrite was measured with grab samples or continuously with an electrochemical nitrite sensor.[33] The 120 L reactors were operated without fixed pH set-points; instead, the pH set-points were adjusted frequently. In general, the operational strategy was to maximize the nitrification rate by incrementally increasing the pH set-point while simultaneously avoiding nitrite accumulation. The pH set-point was reduced when nitrite accumulation was observed to prevent process breakdown. Consequently, the empirically collected data provide a representative overview of the pH range within which stable nitrification occurred while simultaneously maximizing the nitrification rates.

2.3. Long-Term Influence of pH Ranges Outside of Stable Urine Nitrification. To understand the long-term impact of pH values beyond the typical pH range for stable urine nitrification found in Section 2.2, six 12 L CSTR were operated at different pH values and with different TAN concentrations in the influent (Table 1). The CSTR were operated without sludge retention to have a dynamic but simple system (see SI 2 for more details). The pH was controlled with the urine influent using a narrow two-point on/off controller with a pH control band of 0.05 units. The temperature in the reactors was controlled at 25 °C, and the dissolved oxygen (DO) concentration was controlled with a two-point controller between 4 and 6 mg L^{-1}.

Two runs were performed with activated sludge and urine from two different urine treatment systems. In the first run, three reactors were inoculated with activated sludge and fed with urine from the urine treatment system at the NEST

building (Dübendorf, Switzerland), which was operated as a CSTR without sludge retention. The urine collected at the NEST building had TAN concentrations of about 1300 mg-N L^{-1} and salinities, measured as electrical conductivity, of about 11 mS cm^{-1}. Due to the source separation system at NEST, the urine was rather heavily diluted with flushing water, i.e., rainwater. Therefore, the urine used in the first run is called "low-concentration urine".

In the second run, the three reactors were inoculated with activated sludge and fed with urine from the nitrification reactor of the fertilizer production plant at Eawag's main building, Forum Chriesbach (Dübendorf, Switzerland), which was operated in fed-batch mode with sludge retention.[16] The urine collected at Forum Chriesbach was more concentrated with TAN concentrations of about 3600 mg-N L^{-1} and salinities of about 26 mS cm^{-1} and is referred to as high-concentration urine.

In both runs, the three reactors were operated similarly. Initially, all three reactors were operated at pH 6 or 5.8 (Run 2.3) to establish stable nitrification, and after 25 days, the pH control was changed. In the first reactors (Run 1.1 and 2.1), the pH set-point was increased to 7. In the second reactors (Run 1.2 and 2.2), the pH set-point was changed to 8.5. In the third reactor (Run 1.3 and 2.3), the influent and, therefore, the pH control were turned off. The initial pH set-point of 6 was chosen to ensure stable nitrification, which is very likely at this pH (see Section 3.2). In Run 2.3, the reactor was operated at 5.8 to see if this could already promote the growth of acid-tolerant AOB, as in Fumasoli et al.[7]

2.4. Simulation of Proton Release during Nitrification. A model was used to investigate how protons released during nitrification lower the pH of stored urine. The objective was to determine how much TAN would theoretically be nitrified at a given pH without considering whether the nitrifiers are actually active at that pH. The modeling software SUMO 19 (Dynamita, France) was used for this purpose. An aerated batch reactor with suspended sludge was simulated using the concentration of stored urine according to Fumasoli et al.[7] for the initial solution. The switching functions for AOB and NOB were disabled, so they were neither limited nor inhibited by any substances. The charge balance is used to calculate the proton concentration and pH in SUMO 19. The chemical equilibria were corrected for the temperature and ionic strength. Volatilization of ammonia was not considered for simplicity. More information and input parameters can be found in SI 3.

2.5. Chemical and Physical Analyses. Samples for the analyses of dissolved compounds were filtered through a 0.45 μm GF/PET filter (Chromafil, Macherey-Nagel). Ion chromatography (881 compact IC pro, Metrohm) was used to measure cations (ammonium, sodium, and potassium) and anions (nitrate, nitrite, chloride, phosphate, and sulfate). The acid–base equilibrium of HNO_2 and NO_2^-, and NH_4^+ and NH_3 were calculated according to Crittenden et al.[34] using the dissociation constants of Anthonisen et al.[12] and corrected for ionic strength according to Davies[35] (see SI 4 and SI 5). Dissolved COD in the influent was measured with a spectrophotometer (DR 2800, Hach Lange) using photometric cuvette tests (LCK114, Hach Lange). Nitrite and nitrate concentrations in the influent were measured with semi-quantitative colorimetric strips (110,007 and 110,020 MQuant, Merck). Total suspended solids and volatile suspended solids were measured according to the APHA (2012) standard

protocol. Electrical conductivity was measured using a standard conductivity cell (TetraCon 325, WTW) with automatic temperature correction to 25 °C as a proxy for salinity. DO was measured with optical oxygen sensors (Oxymax COS61D and Memosens COS81D, Endress + Hauser). The pH was measured with glass electrodes (Orbisint CPS11D, Endress + Hauser), which were calibrated weekly.

2.6. Molecular Analyses of the Biomass. Biomass samples were collected from all six 12 L reactors (Section 2.3) after 25 days, just before the pH control change and at the end of the experiment. In addition, a sample was collected from Run 1.1 after 40 days. Samples were stored at −80 °C before further processing. Genomic DNA was extracted using the FastDNA Spin Kit for Soil (MP Biomedicals) with an adjustment to the manufacturer's protocol: to lyse the matrix, bead-beating steps (Bead Ruptor Elite, OMNI) were performed under conditions close to the MIDAS field guide[36] in series of 4 × 20 s at 6 m s^{-1} separated by 2 min on ice. The quality and concentration of the purified DNA extracts were assessed using a Qubit 4 fluorometer (dsDNA assay kit, Thermo Fischer Scientific Inc.) and NanoDrop Eight UV/vis spectrophotometer (Thermo Fischer Scientific Inc.). DNA extracts were sent to LGC Genomics (Berlin, Germany) for 16S rRNA gene-based amplicon sequencing, library preparation, and sequencing on an Illumina Miseq platform. The primer pair 341F (5′-CCTACGGGNGGCWGCAG-3′)/785Rmod (5′-GACTACHVGGGTATCTAAKCC-3′) was used, targeting the V3−V4 hypervariable region of bacterial 16S rRNA gene sequences. Data were processed using the Mothur software package (v.1.40.5), as De Paepe et al.[37] described. Operational taxonomic units (OTU) were defined as a collection of sequences with a length between 393 and 429 nucleotides that were found to be more than 97% similar to each other in the V3−V4 region of their 16S rRNA gene after applying OptiClust clustering.[38] Taxonomy was assigned using the Silva.nr_v138_1 database.[39] The OTU table with taxonomy assignments was loaded into R, version 4.0.4 (2021-02-15), and singletons were removed.[40] Additional analyses were performed using NCBI BLAST and MEGA (version 11) software to construct a neighbor-joining tree, including bootstrapping (n = 500) using the maximum composite likelihood method.

3. RESULTS AND DISCUSSION

3.1. Inhibition of AOB and NOB due to NH_3, HNO_2, and Salinity. The NOB in urine nitrification were more sensitive to high NH_3 and HNO_2 concentrations than the AOB (Tables 2 and 3). The kinetic parameters were within the range of reported literature values for high-strength nitrogen wastewater, except for the ammonia affinity constant for AOB, which was higher than previously reported values, and the ammonia inhibition constant for AOB, which was lower than in other reports. The two-term switching function (eqs 1 and 3) and the Haldane function (eq 5) could not accurately describe the effect of ammonia on the AOB activity, especially at high ammonia concentrations (Figure 1). The Han and Levenspiel[31] equation (eq 6), which considers stronger activity decrease at high substrate concentrations, provided a much better fit. Using this equation, the turning point, i.e., the NH_3 concentration at which a further increase in concentration would lead to a decrease in AOB activity, was estimated to be 12 mg-N L^{-1}. At a TAN concentration of 1800 mg-N L^{-1} in the reactor, corresponding to the high-concentration urine, this

Table 2. Kinetic Constants for AOB of the *N. europaea* Lineage from Stable Urine Nitrification Reactors Operated at pH Values of about 6.3[a]

K_{I,HNO_2} [mg-N L^{-1}]	K_{I,NH_3} [mg-N L^{-1}]	K_{S,NH_3} [mg-N L^{-1}]	NH_3 turning point [mg-N L^{-1}]	source
0.34 ± 0.07	49 ± 17	1.6 ± 0.5	9	this study, two-term eqs 1, 3, and 4
0.34 ± 0.07	51 ± 17	1.5 ± 0.5	9	this study, Haldane[b] eqs 4 and 5
0.34 ± 0.07			12	this study; Han and Levenspiel[c], eqs 4 and 6
0.164	77	0.28	5	Jubany et al.[13b]
0.053	241	0.5	11	Pambrun et al.[14b]
2.04	600	0.75	21	Van Hulle et al.[41]
0.203	70	0.47	6	Hellinga et al.[42]
2.8	3000			Wett and Rauch[43]
0.07		0.7		Jones[44]

[a]For comparison, kinetic constants of AOB reported for high-strength nitrogen wastewater are shown. The turning point is the NH_3 concentration, at which a further increase in concentration would lead to a decrease in AOB activity. [b]The Haldane function was used for ammonia limitation and inhibition. [c]The parameters for the switching function according to Han and Levenspiel[31] were $K_{S,NH3}$ = 1.2 mg-N L^{-1}, $K_{I,NH3}$* =377 mg-N L^{-1}, n = 2.8, and m = 0.7.

Table 3. Kinetic Constants for NOB of the *Nitrobacter* Genus from Urine Nitrification Reactors Operated at pH Values of about 6.3[a]

K_{I,NH_3} [mg-N L^{-1}]	K_{I,HNO_2} [mg-N L^{-1}]	K_{S,NO_2} [mg-N L^{-1}]	TNN turning point at pH 6.3 [mg-N L^{-1}]	source
33 ± 6	0.17 ± 0.02	1.5 ± 0.2	18	this study, eqs 2–4
1.6−20	2.8	0.3	32	Wett and Rauch[43]
252	0.4	1.25	25	Blackburne et al.[45]
	1.05	0.05	8	Jones[44]
	0.2	4.0	31	Hunik et al.[46]
3.9−11.1		1.6		Pambrun et al.[14]
0.8	0.13			Jubany et al.[13]

[a]For comparison, the kinetic constants of NOB reported for high-strength nitrogen wastewater are shown. The turning point is the TNN (HNO_2-N + NO_2^--N) concentration, at which further increases in concentration would lead to a decrease in NOB activity. Since the turning point depends on the concentration of HNO_2 and NO_2^-, it is pH-dependent, and the value at a pH of 6.3 is an example.

NH_3 concentration would be reached at a pH of 7.2 (see SI 6 for a detailed figure).

The AOB were more resistant to HNO_2 inhibition than NOB. This higher HNO_2 tolerance of AOB is often used in partial nitritation/anammox (PN/A) systems to suppress the growth of NOB.[47] In the case of urine nitrification for nitrogen recovery, the higher HNO_2 sensitivity of NOB is a problem because once a certain TNN concentration is reached, the NOB activity decreases relative to the AOB activity, favoring
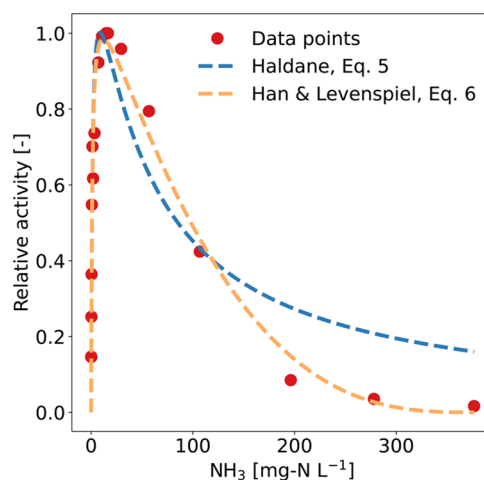

Figure 1. Haldane switching function and the two-term switching function overlap. The best fit was found for eq 6, suggested by Han and Levenspiel[31] To combine the ammonia limitation and inhibition experiments, the activities in both experiments were normalized as relative activities by dividing the ammonia oxidation rate by the ammonia oxidation rate at the ammonia concentration of 15 mg-N L^{-1}.

further TNN accumulation and thus causing positive feedback. Figure 2 shows this pH-dependent turning point along with
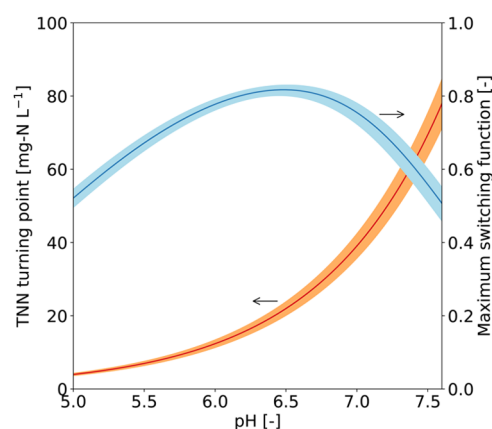

Figure 2. Turning point, i.e., the TNN (HNO_2-N + NO_2^--N) concentration at which the nitrite oxidation rate is maximized. The maximum switching function was calculated by multiplying the terms for nitrite substrate limitation, nitrous acid inhibition, and ammonia inhibition using the turning point at a given pH. The calculations were performed for the high-concentration urine with a TAN concentration of 1800 mg-N L^{-1} in the reactor. The results for the low-concentration urine are very similar and are shown in SI 7, along with the turning point curves for other literature values.

the relative activity including NH_3 inhibition. While the turning point increases with pH, the maximum nitrite oxidation rate is reached at a pH of 6.5.

AOB were more sensitive to high salt concentrations than NOB (Figure 3). Since the salt concentrations in the influent and the reactor do not usually change rapidly due to the buffering of the collection tanks, the higher sensitivity of AOB does not affect the short-term process stability during the operation. However, due to the salinity effect, higher concentrated urine will likely result in lower nitrification rates at the same ammonia concentration. The AOB of the
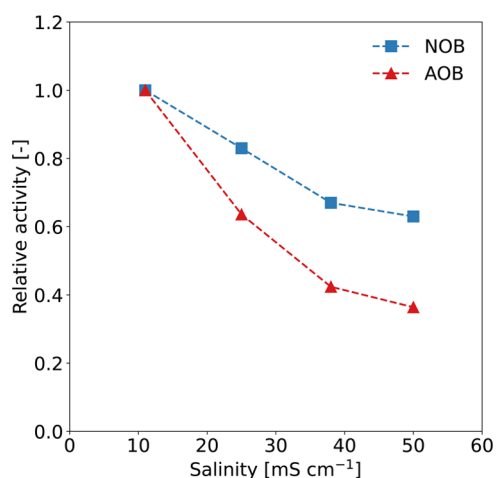
Figure 3. Effect of salinity on the activity of AOB from the *N. europaea* lineage and NOB from the Nitrobacter genus. Electrical conductivity was used as a proxy for salinity. To compare AOB and NOB, the activity was normalized as relative activity by dividing the oxygen uptake rate (OUR) by the OUR at a salinity of 11 mS cm^{-1}, which is the lowest salinity tested. The activated sludge was obtained from a reactor operated at a salinity of approximately 26 mS cm^{-1}.

short-term activity tests most likely belonged to the *N. europaea* lineage, and the NOB to the genus *Nitrobacter*, as these were the nitrifiers found in stable urine nitrification (see Section 3.3). Therefore, the kinetic parameters apply only to these nitrifiers. Details of the short-term activity tests are given in SI 8.

3.2. Stable Urine Nitrification between pH 5.8 and 6.7. Stable urine nitrification, i.e., without nitrite accumulation, was observed under mildly acidic conditions. The 120 L reactors were mainly operated in a pH range between 5.8 and 6.7 (5th and 95th percentile of both reactors, Figure 4A), resulting in a nitrification rate of 430 ± 190 mg-N L^{-1} day^{-1} (see boxplot of nitrification rates in SI 9). Because the pH set-points in the reactors were frequently adjusted to avoid nitrite accumulation while maximizing the nitrification rate, this was the pH range in which stable urine nitrification was most likely to occur. At pH values below 5.8, nitrification was generally very slow due to ammonia limitation. In addition, the

operators avoided such low pH values due to the potential risk of acid-tolerant AOB.[21] Therefore, such low pH values were less frequent during operation except when needed to counteract nitrite accumulation. Although both reactors were operated independently, the boxplot distributions are very similar. Switching from a CSTR to a sludge retention system, i.e., a fed-batch reactor, increased the nitrification rate but not the pH range for stable urine nitrification (SI 9).

Nitrite accumulation was very likely at pH values of 6.7 or higher, as shown in Figure 4B, after 30 days, but nitrite accumulation was also possible at lower pH values, such as 6.4 after 75 days. In both cases, nitrite concentrations decreased rapidly after the pH was lowered. Lowering the pH set-point generally worked well to reduce nitrite concentrations as AOB activity decreased due to lower NH$_3$ availability, giving NOB an advantage. However, this can only be done while nitrite concentrations are still low, as a decrease in pH will result in a greater amount of the TNN being present as HNO$_2$, which inhibits NOB stronger than AOB. By keeping the TNN concentration below the turning point, a positive feedback loop that quickly leads to very high TNN concentrations can be avoided in the first place (Figure 2). Therefore, continuous nitrite measurements, for example, with an electrochemical nitrite sensor,[33] are necessary to detect nitrite accumulation early on. It should be noted that other factors such as high temperatures or low DO can also cause nitrite accumulation.[16]

Due to the limited alkalinity in the influent, 50 ± 1% of the TAN was oxidized (Figure 5). Within the operational pH range for stable urine nitrification, the ratio of TAN to TNN and nitrate was independent of pH. The simulation in SUMO confirmed that the amount of TAN that can be oxidized due to the limited alkalinity does not change in the pH range of 2.5−7 due to the low buffer capacity of nitrified urine in this pH range. It is only when the pH is operated above 7 that the amount of ammonia oxidized is lower.

3.3. Growth of *N. europaea* and *Nitrobacter* spp. at pH 5.8 and 6. In the 12 L reactors, stable nitrification was observed at pH 6 and 5.8 in all six reactors during the first 25 days, regardless of the influent TAN concentration (Figures 6−8). Nitrite concentrations were low in all reactors at 0.3 ± 0.15 mg-N L^{-1}. Regardless of the urine concentration, 50.5 ± 0.5% of the TAN was oxidized. Since no nitrite accumulated,
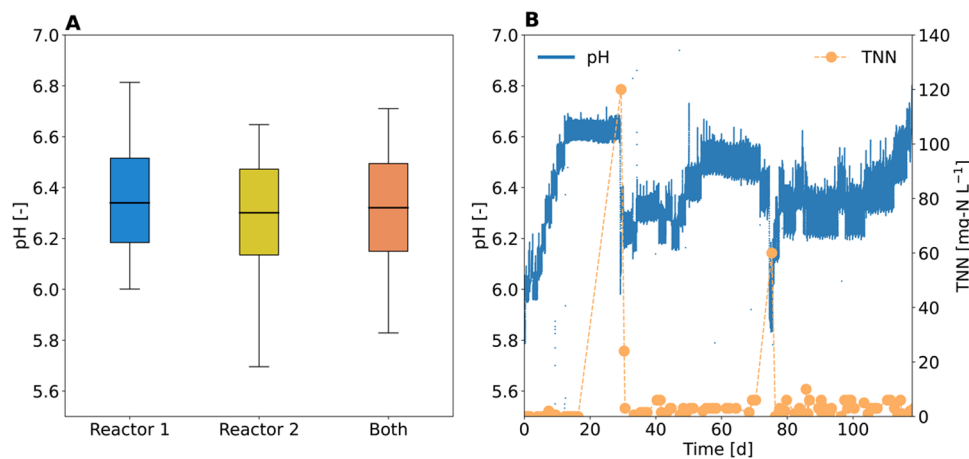

Figure 4. (A) Boxplot of pH values in two 120 L urine nitrification reactors from a fertilizer production plant. The average operational pH of both reactors was 6.3, with a standard deviation of 0.25. Whiskers indicate the 5th and 95th percentiles. (B) TNN (NO$_2^-$-N + HNO$_2^-$-N) and pH values in Reactor 2 over a 130-day period as an example of pH variability during long-term urine nitrification.
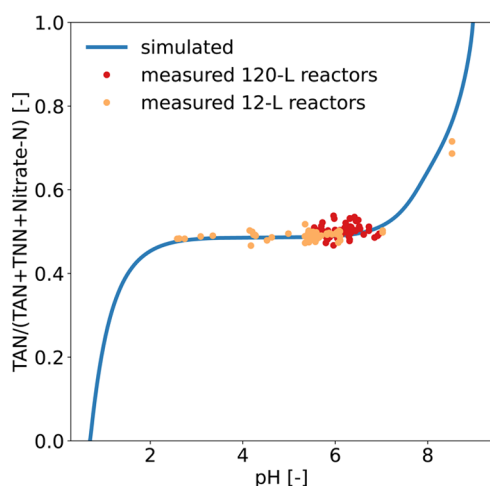
Figure 5. Measured and simulated ratio of TAN (NH_3-N + NH_4^+-N) compared to the sum of TAN, TNN (NO_2^--N + HNO_2-N), and nitrate-N as a function of the pH in the reactor. At a pH of 2.5 to 7, approximately 50% of the TAN is oxidized due to the limited alkalinity in urine. It was not possible to validate the simulated values below 2.5 because the ammonia oxidation stopped before that.

ammonia oxidation was the limiting nitrification step at pH 6 and 5.8. According to the kinetic parameters in Section 3.1, both the maximum ammonia oxidation and the nitrite oxidation were reduced by more than 80% due to the limited availability of the substrate ammonia and nitrite, respectively (see SI 10 for switching functions). Lower specific and volumetric rates were measured in the reactor with the high-concentration urine, despite the higher NH_3 concentration in the reactors (see SI 11 for the NH_3 concentration and SI 12 for volumetric rates). This can be partly explained by the higher salinity in the high-concentration urine of 26 mS cm^{-1} compared to 11 mS cm^{-1}, which, according to the activity

assays, causes a 40% reduction in AOB activity compared to the low-concentration urine (Figure 3). The lower pH of 5.8 resulted in 40% lower rates than the reactor at pH 6 due to lower NH_3 concentrations at the same salinity (Figure 8). The removal of soluble COD was slightly higher at 90% for the high-concentration urine compared to 83% for the low-concentration urine. This is probably related to the higher HRT in the reactors fed with high-concentration urine (see SI 13 for HRT). Nevertheless, both values are close to the value of about 90% previously reported for urine nitrification.[9,23]

Despite different salinities, TAN concentrations, and inocula, OTU 14 *Nitrosomonas* sp. was the dominant AOB in all reactors after 25 days, regardless of the dilution with relative read abundances ranging from 0.7 to 10% (see SI 14 for relative read abundance of AOB). OTU 14 *Nitrosomonas* sp. clustered strongly with *N. europaea* (see SI 15 for the phylogenetic tree of *Nitrosomonas* spp.). Although biological nitrite oxidation was observed, no NOB species could be unambiguously identified. However, several unknown species of the family *Xanthobacteraceae* were observed (see SI 16 for the relative read abundance of NOB). This family includes NOB of the genus *Nitrobacter* (see SI 17 for phylogenetic tree of unclassified *Xanthobacteraceae* spp.). The majority of the microbial community consisted of heterotrophic bacteria (see SI 18 for relative read abundance of the top 12 OTU).

3.4. Growth of *Nitrosomonas halophila* and Nitrite Accumulation at pH 7. Increasing the pH to 7 resulted in nitrite accumulation and a shift of the AOB community to *N. halophila*, regardless of the TAN concentration in the influent (Figure 6). The nitrite concentration increased continuously after the pH switch and the nitrate concentration decreased inversely. In the reactor with the low-concentration urine, the nitrite accumulation ratio, i.e., the amount of nitrite that was not converted to nitrate, was already 95% at the end of the experiment. Despite the higher pH, still 49.7 ± 1.0% of the
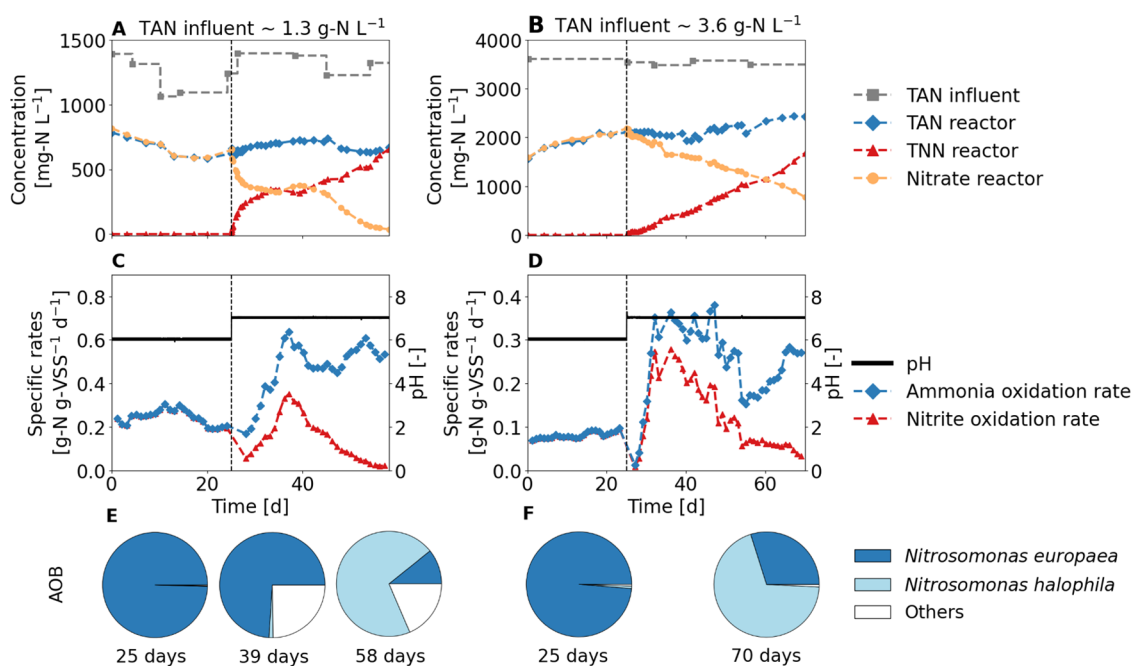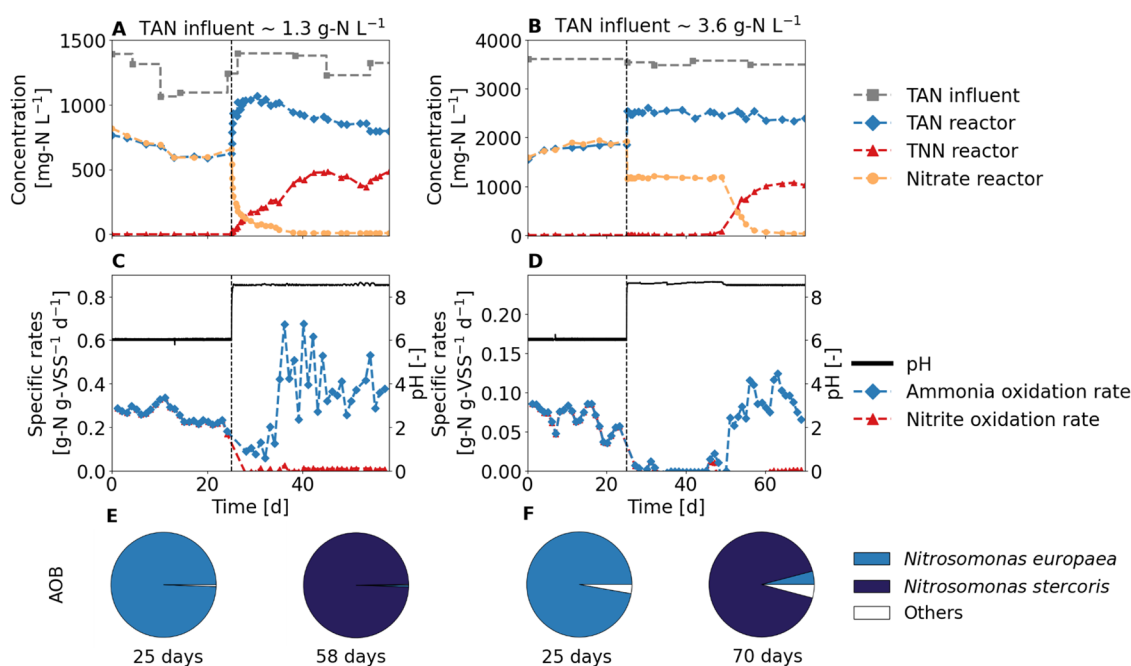

Figure 6. Reactors were initially operated at pH 6, and after 25 days, the pH set-points were increased to pH 7. (A, B) Concentrations of nitrogen compounds in the influent and the reactors fed with TAN (NH_3-N + NH_4^+-N) concentrations of 1.3 and 3.6 g-N L^{-1}. (C, D) Specific rates and pH, (E, F) Distribution of identified AOB species.

Figure 7. Reactors were initially operated at pH 6, and after 25 days, the pH set-points were increased to pH 8.5. (A, B) Concentrations of nitrogen compounds in the influent and the reactors fed with TAN (NH_3-N + NH_4^+-N) concentrations of 1.3 and 3.6 g-N L^{-1}. (C, D) Specific rates and pH. (E, F) Distribution of identified AOB species.

TAN was oxidized, which is only slightly less than that at pH 6, confirming again that the buffer capacity of nitrified urine between pH 6 and 7 is low.

OTU 65 *Nitrosomonas* sp., which was closely related to *N. halophila* was the dominant AOB at pH 7 at the end of the experiment, regardless of the influent or inocula. The relative read abundance of OTU 65 *Nitrosomonas* sp. of the OTU increased from 0.02 and 0.01% before the change to 3.8 and 1.4% at the end of the experiment for the low-concentration and the high-concentration urine, respectively. After the pH change, the ammonia and nitrite oxidation rates decreased briefly in both reactors, followed by a rapid recovery and increase in rates. Around day 40 and day 30 in the low-concentration urine and high-concentration urine, respectively, ammonia and nitrite oxidation rates began to decrease. While nitrite oxidation decreased to almost zero, the ammonia oxidation rate increased again.

Presumably, the ammonia oxidation rate increased initially due to the higher NH_3 concentration, as *N. europaea* is strongly ammonia limited at pH 6, but decreased again due to continuous HNO_2 accumulation (see SI 10 for switching functions). The accumulation of HNO_2 most likely forced a population shift of the AOB community toward a more HNO_2-tolerant species, as the final HNO_2 concentrations of 0.1 and 0.25 mg-N L^{-1} would inhibit *N. europaea* by 25 and 45% for the low-concentration and the high-concentration urine, respectively, according to Section 3.1. Ammonia inhibition had no significant effect on AOB. The community shift could explain why the ammonia oxidation rate increased again after 48 and 58 days, despite the increase in HNO_2. A sample after 40 days from the low-concentration urine reactor confirms this, as *N. europaea* was still predominant at this time, while *N. halophila* was barely present (0.04%). *N. halophila* has been reported to be halophilic and alkali-tolerant with optimum growth conditions at salt concentrations of about 30 mS cm^{-1},[48] but no data on HNO_2 tolerance were found. *N.*

halophila has also been selected in a partial nitritation reactor at pH 7 with TNN concentrations of 1000 mg-N L^{-1},[21] and in a long-term experiment at elevated salinity of 50 mS cm^{-1} for stable urine nitrification (see experimental details in SI 19).

The nitrite oxidation rate initially decreased after the pH change but then increased, probably due to an increase in the nitrite concentration. However, this increase was short-lived, as the continuous accumulation of HNO_2 led to a subsequent decrease in activity. Based on the parameters determined in Section 3.1, the HNO_2 concentration of 0.1 and 0.25 mg-N L^{-1} at the end of the experiment strongly inhibited NOB, by 40% for the low-concentration and 60% for the high-concentration urine, and was most likely the cause of the decrease in nitrite oxidation (see SI 10 for switching functions). The NH_3 concentrations increased to 3 and 11 mg-N L^{-1}, additionally inhibiting NOB, but to a lesser extent of 10 and 25% for the low-concentration urine and the high-concentration urine, respectively. The initial drop in nitrite oxidation rate and also in ammonia oxidation rate for the high-concentration urine immediately after the pH change cannot be explained by the kinetic parameter and may be due to some additional short-term effect. Again, NOB were not clearly identified in the sample, but the relative read abundance of unknown *Xanthobacteraceae* spp. that are potentially NOB decreased toward the end of the experiment.

3.5. Growth of *Nitrosomonas stercoris* and Near Cessation of Nitrification Followed by Nitrite Accumulation at pH 8.5. Increasing the pH to 8.5 resulted in a near cessation of nitrification for the high-concentration urine, followed by nitrite accumulation and a shift of the AOB community to *N. stercoris* for both urine influents (Figure 7). The nitrite accumulation ratio at the end of the experiment was 98% for both reactors, but the nitrite accounted for only 29.7 ± 5.7% of the inorganic nitrogen in the reactor, which was slightly higher than the simulated value of 25%.
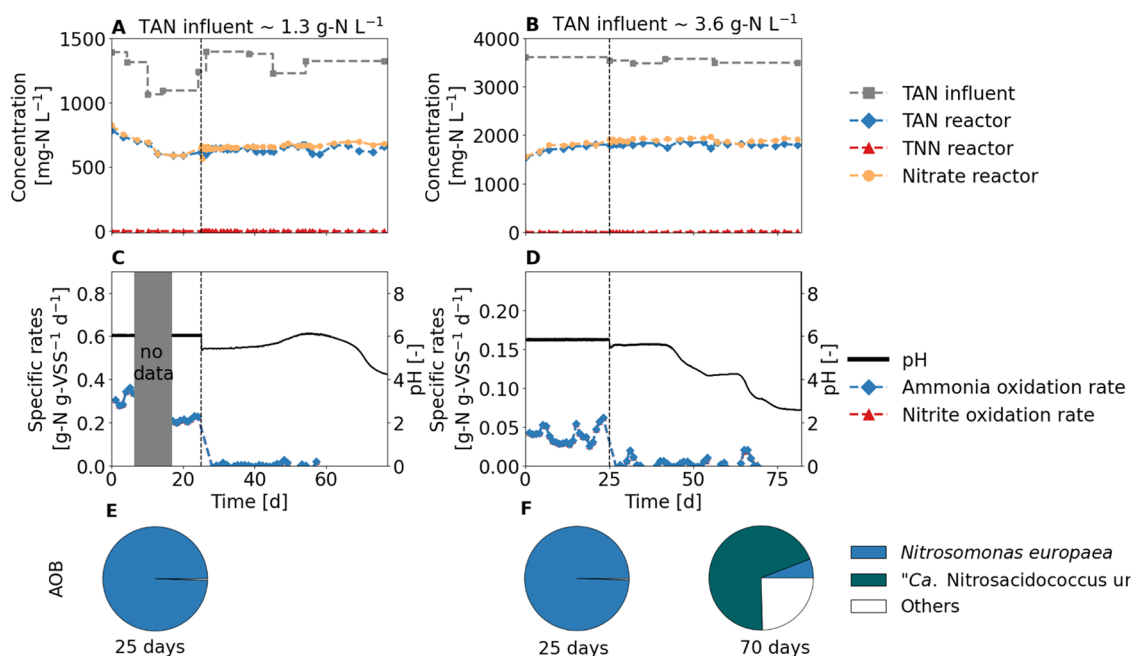
Figure 8. Reactors were initially operated at pH 6 (low-concentration urine) and pH 5.8 (high-concentration urine), and after 25 days, the influent, and therefore the pH control, was turned off, resulting in a pH decrease. (A, B) Concentrations of nitrogen compounds in the influent and the reactors fed with TAN (NH_3N + NH_4^+-N) concentrations of 1.3 and 3.6 g-N L^{-1}. (C, D) Specific rates and pH, (E, F) Distribution of identified AOB species. The DNA sample at the end of the low-concentration urine experiment did not amplify.
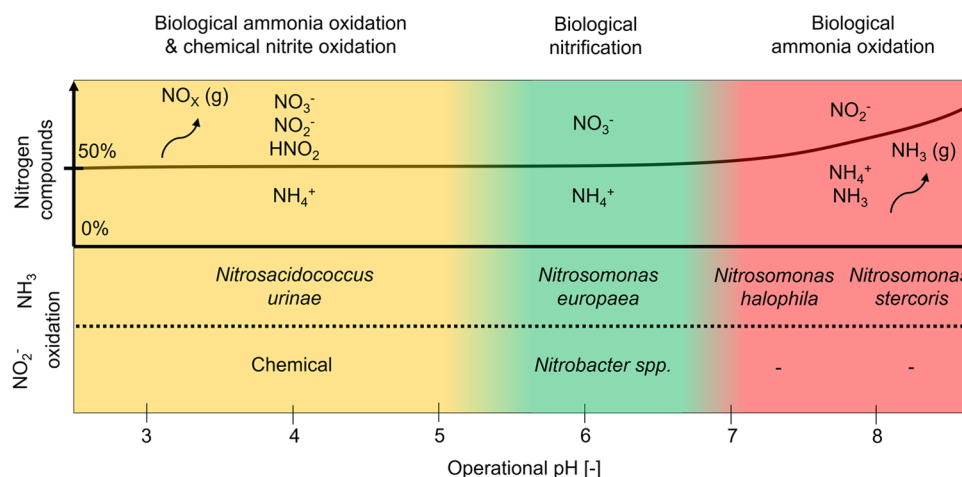

Figure 9. Observed influence of operational pH on effluent composition and microbial community composition during urine nitrification without alkalinity addition for urine influent with TAN (NH_3-N + NH_4^+-N) concentrations ranging from 1300 to 3600 mg-N L^{-1}.

OTU 74 *Nitrosomonas* sp., which was closely related to *N. stercoris*, was the dominant AOB at pH 8.5 at the end of the experiment, regardless of influent or inocula. The relative read abundance of OTU 74 *Nitrosomonas* sp. before the pH change was about 0.01% in both reactors but increased to 2 and 0.2% in the low-concentration urine and high-concentration urine, respectively. In both reactors, the ammonia and nitrite oxidation rates decreased immediately after the change to pH 8.5. While the ammonia oxidation rate increased again, the nitrite oxidation rate did not recover in either reactor.

Presumably, the ammonia oxidation rate initially decreased after the pH change due to the high NH_3 concentrations of about 105 and 305 mg-N L^{-1} for the low-concentration and high-concentration urine, respectively. It is likely that the high NH_3 concentration forced a population shift in the AOB community, which could explain why ammonia oxidation

increased around days 35 and 50. *N. stercoris* was initially isolated from composted cattle manure and has been reported to tolerate ammonia concentrations greater than 900 mg-N L^{-1} and high salinity.[49]

The nitrite oxidation rate most likely ceased due to the higher NH_3 concentrations. The activity assays (Section 3.1) showed that NOB activity was strongly reduced by 80 and 90% at these concentrations in low- and high-concentration urine, respectively (see SI 10 for switching functions). The relative read abundance of unknown *Xanthobacteraceae* spp. that are potentially NOB strongly decreased toward the end of the experiment.

3.6. Growth of "*Candidatus* Nitrosacidococcus Urinae" and Chemical Nitrite Oxidation at and below pH 5.5. The influent stop resulted in a pH drop to about 5.5, and a long idle phase followed by a second pH drop due to the

growth of the acid-tolerant AOB "*Candidatus* Nitrosacidococcus urinae" (Figure 8). The second pH drop occurred more rapidly in the reactor operated at pH 5.8 and with high-concentration urine. Although the pH decreased to 2.5, the ammonium concentration in the reactor hardly decreased, which means that the buffer capacity of the nitrified urine is low between 2.5 and 6 (see Figure 5). Since the activity of known acid-tolerant AOB ceases at a pH of about 2.5,[50] no more than about 50% of TAN can be biologically oxidized without adding alkalinity. No nitrite accumulated in the low-concentration urine. The nitrite concentration for the high-concentration urine increased to 17 mg-N L^{-1} but decreased again toward the end of the experiment.

OTU 41 "*Candidatus* Nitrosacidococcus urinae" was the dominant AOB at pH 2.5 for the reactor with the high-concentration urine. In the biomass from the low-concentration urine, the DNA sample at the end of the experiment did not amplify due to insufficient DNA quantity. "*Candidatus* Nitrosacidococcus urinae" was not found in any of the samples from the reactors operated at pH 5.8 to 8.5. The relative read abundance of unknown *Xanthobacteraceae* spp. that are potentially NOB was very low in the high-concentration urine at the end. Presumably, the HNO$_2$ concentration of up to 7 mg-N L^{-1} completely inhibited the NOB, i.e., by 98% according to the activity assays (see SI 10 for switching functions), and nitrite was chemically oxidized instead.[21]

3.7. Practical Implication. To produce an ammonium nitrate fertilizer, urine nitrification reactors should be operated in the pH range of 5.8–6.7 (Figure 9). However, even within this pH range, nitrite accumulation can occur and can jeopardize the process. Nitrite accumulation can be counteracted by lowering the pH, resulting in lower ammonia oxidation rates due to reduced NH$_3$ substrate availability. This is only possible while the TNN concentration is still low because lowering the pH will also increase the HNO$_2$ concentration, decreasing the nitrite oxidation rates relative to the ammonia oxidation rates since NOB are more sensitive to HNO$_2$ inhibition than AOB. A pH-dependent TNN threshold should not be exceeded to avoid a positive feedback loop, e.g., a TNN concentration of about 10 mg-N L^{-1} at a pH of 5.8 and about 30 mg-N L^{-1} at a pH of 6.7. Therefore, the nitrite concentration should be measured continuously, e.g., with an electrochemical nitrite sensor to detect nitrite accumulation early on.

AOB can grow over a wide pH range because different species were selected, depending on the pH, HNO$_2$, and NH$_3$ concentrations. A pH of 7 may be appropriate to produce nitrite for sulfide control in sewers[51] or for two-stage partial nitritation/anammox (PN/A). However, other aspects, such as N$_2$O emissions due to high nitrite concentrations,[16] would need to be considered. Single-stage PN/A of urine should also be considered as it avoids high nitrite concentrations and may result in lower N$_2$O emissions.[52]

The operational pH of 8.5 resulted in partial nitrition, but is less suitable for nitrogen removal by PN/A than pH 7 because the ammonia oxidation rates were lower, and the TNN-to-TAN ratio was even further below optimal values of 1.15 to 1.32 g-N g-N^{1-},[53] which means that not enough ammonia is oxidized. In addition, the pH of 8.5 may be slightly higher than the optimal pH of 6.7 to 8.3 reported for anammox bacteria.[54]

An operational pH of 5 or less may be suitable for combining biological ammonia and chemical nitrite oxida-

tion.[22] However, biological ammonia oxidation at low pH has been reported to be very sensitive to process disturbances and can cause high nitrogen losses as harmful nitrogen oxide gases.[21] Nevertheless, Li et al.[55] suggested that the inhibition of NOB at low pH for PN/A could be used for highly diluted urine. The amount of TAN that can be oxidized does not change significantly between pH 2.5 and 7. Since no AOB are known to be active below pH 2.5,[50] obtaining more than 50% of the nitrogen in the fertilizer as nitrate without adding alkalinity is not possible.

The results apply only to systems without alkalinity addition for pH control. The addition of alkalinity would allow all of the ammonia to be converted to nitrate[56] but would require additional oxygen for nitrification and either an electro-chemical unit to produce hydroxide ions on-site[10] or the dosing of a chemical, such as NaOH, which would further increase the salinity of the urine.[27] The addition of alkalinity would allow higher operational pH due to the lower TAN concentration and would avoid the growth of acid-tolerant AOB. Therefore, De Paepe et al.[10] argued that nitrification with alkalinity addition has a higher process stability. However, the main process failure, nitrite accumulation, has also been repeatedly observed for urine nitrification with alkalinity addition.[57,58]

4. CONCLUSIONS

- Biological ammonia oxidation of urine was possible over a wide pH range of 2.5–8.5.
- As a function of pH, one of four distinct AOB species became dominant in different niches of the challenging urine matrix to cope with pH, NH$_3$, and HNO$_2$.
- The amount of ammonia that can be oxidized does not change significantly between pH 2.5 and 7. Without alkalinity addition, it is not possible to oxidize more than 50% of the ammonia.
- Stable nitrification without nitrite accumulation was only possible if the pH was low enough to limit the nitrite production by *Nitrosomonas* spp. and high enough to prevent the growth of acid-tolerant AOB "*Candidatus* Nitrosacidococcus urinae". In practice, this was usually within a narrow pH range of 5.8–6.7.
- Outside the pH range of 5.8–6.7, biological nitrite oxidation was inhibited by HNO$_2$ or NH$_3$, which affected NOB more than AOB, causing nitrite accumulation.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsestengg.3c00320.

> Details of the experimental setup, description of the model, supplementary results from the short-term respirometry tests and the long-term experiment, and the relative abundance and phylogenetic tree of the nitrifiers (PDF)

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CRediT: **Valentin Faust** conceptualization, data curation, formal analysis, investigation, methodology, software, validation, visualization, writing-original draft, writing-review & editing; **Siegfried E Vlaeminck** conceptualization, methodology, supervision, writing-review & editing; **Ramon Ganigue** conceptualization, methodology, supervision, writing-review & editing; **Kai M Udert** conceptualization, funding acquisition, methodology, project administration, resources, supervision, writing-review & editing.

Notes

The authors declare no competing financial interest.

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