Growth of \textit{Nitrosococcus}-Related Ammonia Oxidizing Bacteria Coincides with Extremely Low pH Values in Wastewater with High Ammonia Content

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**Supporting Information**

**ABSTRACT:** Ammonia oxidation decreases the pH in wastewaters where alkalinity is limited relative to total ammonia. The activity of ammonia oxidizing bacteria (AOB), however, typically decreases with pH and often ceases completely in slightly acidic wastewaters. Nevertheless, nitrification at low pH has been reported in reactors treating human urine, but it has been unclear which organisms are involved. In this study, we followed the population dynamics of ammonia oxidizing organisms and reactor performance in synthetic fully hydrolyzed urine as the pH decreased over time in response to a decrease in the loading rate. Populations of the \(\beta\)-proteobacterial \textit{Nitrosomonas europaea} lineage were abundant at the initial pH close to 6, but the growth of a possibly novel \textit{Nitrosococcus}-related AOB genus decreased the pH to the new level of 2.2, challenging the perception that nitrification is inhibited entirely at low pH values, or governed exclusively by \(\beta\)-proteobacterial AOB or archaea. With the pH shift, nitrite oxidizing bacteria were not further detected, but nitrous acid (HNO₂) was still removed through chemical decomposition to nitric oxide (NO) and nitrate. The growth of acid-tolerant \(\gamma\)-proteobacterial AOB should be prevented, by keeping the pH above 5.4, which is a typical pH limit for the \textit{N. europaea} lineage. Otherwise, the microbial community responsible for high-rate nitrification can be lost, and strong emissions of hazardous volatile nitrogen compounds such as NO are likely.

1. **INTRODUCTION**

Ammonia oxidation to nitrite, the first step of nitrification, is a biological process that releases protons. Ammonia oxidation can substantially decrease the pH in terrestrial and aquatic systems that do not contain sufficient alkalinity to buffer the proton release. This can for instance happen in acidic soils or wastewaters with a low alkalinity to total ammonia ratio.

Ammonia oxidizing bacteria (AOB) in wastewater treatment, however, are typically found to be acid-sensitive: the activity of AOB was found to decrease with pH and to completely cease at pH values slightly below pH 6.† Occasional reports indicate that ammonia oxidation can still occur at lower pH. It was observed that ammonia oxidation proceeds at pH values of around 4 in engineered reactors containing synthetic wastewaters.‡−§ In nitrified urine, the pH dropped to values as low as 2.5.∥ The minimal pH value of 2.5 is stunning, as a lower pH limit of 2.9 was demonstrated for ammonia oxidation in acidic tea soils¶ and as nitrification is not expected at pH values below 3 in acidic lakes.¶ Ammonia oxidation in urine was shown to be due to biological activity.¶ However, it is not clear which organisms were involved.

Low pH values can be reached during nitrification of urine. Stored human urine contains an alkalinity to total ammonia ratio of 1 mol-mol⁻¹.¶ A minimal molar ratio of 2 mol-mol⁻¹ would be required for complete ammonia oxidation. Con-
Nitrosomonas europaea the pH dropped from above 6 to 4.5.5 However, the wastewater environments15 and have not been detected in wastewater proteobacterial AOB are predominantly found in marine sococcus γ for example, the ammonia concentrations, such as urine.9

Whether these AOB are also selected in wastewater with high nitrogen was lost by volatilization, partially in the acidification processes is important in order to prevent these strong emissions.

The main population of AOB in urine nitrification reactors at neutral pH values was found to be affiliated with the Nitrosomonas europaea lineage.12 Their activity was shown to cease at pH values close to 5.4.12 Hence, it has to be expected that a population shift from this acid-sensitive to other, acid-tolerant, AOB is responsible for low-pH nitrification in urine. A complete population shift from Nitrosomonas europaea to Nitrosomonas oligotropha has been observed in a reactor operated with synthetic low-strength nitrogen wastewater as the pH dropped from above 6 to 4.5.12 However, the wastewater used in these experiments contained far lower salt and total ammonia concentrations than the concentrations expected in urine. Nitrosomonas oligotropha have a high ammonia affinity, but also a high salt sensitivity.13 Hence, it remains unclear whether these AOB are also selected in wastewater with high ammonia concentrations, such as urine.

Several AOB are better adapted to high salt concentrations, for example, the γ-proteobacterial AOB (e.g., genus Nitroscoccus).14 Based on morphological observations, AOB were hypothesized to be active at a pH value as low as 2.9 in acidic tea soils belong to the genus of Nitroscoccus.15 However, γ-proteobacterial AOB are predominantly found in marine environments15 and have not been detected in wastewater treatment reactors.16 Recent studies showed that ammonia oxidizing archaea (AOA) outnumber AOB at low pH values in the soil,17 and play a more important role than AOB in strongly acidic soils.18 While the relative abundance of AOA is low compared to the relative abundance of AOB in municipal wastewater treatment,19 the occurrence of AOA in wastewater reactors at low pH values has, to our knowledge, so far not been investigated.

The growth of bacteria in acidic environments requires specific adaptation mechanisms: bacteria need to keep their cell internal pH values close to neutrality against the extracellular pH, a phenomenon known as pH homeostasis.20 One known mechanism of pH homeostasis is the uptake of potassium ions, which allows for the inversion of the membrane potential and decreases the proton pressure on the cytoplasmic membrane.21

The aim of this study was to select for the ammonia oxidizing organisms that drive the pH in wastewater with high ammonia concentrations to very low values and to investigate how the selection of these organisms affect the reactor performance and the overall bacterial community structure. The bacterial population dynamics and reactor performance in wastewater with high ammonia concentrations were compared with parallel reactors operating at low ammonia concentrations. The availability of potassium ions was altered to test its importance for bacterial survival at low pH.

### 2. MATERIALS AND METHODS

#### 2.1. Reactor Operation under Continuous-Flow Regime. Reactor Configurations

Four moving bed biofilm reactors (MBBR) with a volume of 2 L each were operated under continuous-flow conditions. Each reactor was filled with 40% (volumetric ratio) K1 Kaldnes biofilm carriers with a specific surface area of 500 m²·m⁻³.22 The reactor temperature was adjusted to 25.4 ± 0.1 °C with a thermostat (F32, Julabo Labortechnik GmbH, Seelbach, Germany). To maintain constant nitrogen loading rates, as detailed below, reactors were supplied with influent at specific volumetric flow rates (REGLO Digital, ISMATEC, Wertheim, Germany). A sufficient mixing of biofilm carriers was ensured by aeration with pressurized, premoistened, ambient air at 35 NL·h⁻¹ (22R1411/01807, Wisag, Fällanden, Switzerland). In combination with low nitrification rates, the high air flow maintained the dissolved oxygen close to saturation. Online pH monitoring, the setup for batch experiments, and the characteristics of the inoculum are described in the Supporting Information.

#### Influent Compositions

The experimental design consisted of four reactors fed with different synthetic influent solutions to investigate the effects of urine and wastewater matrices, and of potassium and sodium cations (Table 1). Two so-called urine reactors (UR) were supplied with influent that contained total ammonia and total salt concentrations similar to women’s urine,23 but varied in their potassium and sodium concentrations. Ammonia rather than urea was added to the synthetic solutions, because urea decomposes very quickly in urine as it enters a urine treatment facility, decomposed in most cases as it enters a urine treatment facility, and has not been detected in wastewater environments.15

| Table 1. Average Measured Concentrations of Ammonium and Accompanying Salts in the Reactor Influent Solutions | UR-K | UR-Na | WWR-K | WWR-Na |
|-----------------------------------------------|-----|-----|-----|-----|
| pH | 9.18 ± 0.06 | 9.32 ± 0.07 | 8.09 ± 0.34 | 8.16 ± 0.33 |
| NH₄−N (mg L⁻¹) | 1710 ± 140 | 1630 ± 90 | 149 ± 8 | 145 ± 16 |
| TIC (mg C L⁻¹) | 753 ± 60 | 695 ± 123 | 219 ± 10 | 211 ± 24 |
| PO₄³−P (mg L⁻¹) | 146 ± 6 | 138 ± 13 | 11.0 ± 1.5 | 11.4 ± 1.6 |
| Cl⁻ (mg L⁻¹) | 1740 ± 100 | 1550 ± 130 | 387 ± 22 | 381 ± 33 |
| Na⁺ (mg L⁻¹) | 5.59 ± 0.40 | 1160 ± 130 | 6.20 ± 0.62 | 424 ± 130 |
| K⁺ (mg L⁻¹) | 2100 ± 260 | <1 | 799 ± 35 | <1 |
| Alkalinity (meq L⁻¹) | 123 | 130 | 19 | 19 |

*The urine reactors (UR-K and UR-Na) contained high salts and high total ammonia concentrations; the wastewater reactors (WWR-K and WWR-Na) contained low salts and low total ammonia concentrations. Influent solutions to the urine reactors as well as the wastewater reactors varied also in their sodium and potassium content. All influent solutions had alkalinity to ammonia ratios of less than 2 mol·mol⁻¹. Calculated.*
(WWR) were fed with a synthetic substrate containing lower total ammonia and total salt concentrations, and high potassium (WWR-K) or sodium (WWR-Na) concentrations. Influent with high potassium (UR-K, WWR-K) or sodium concentrations (UR-Na, WWR-Na) should provide information on the necessity of potassium for AOB growth at low pH values. The recipes of all synthetic influent solutions are given in Table S1. Micro- and macronutrients were added as specified in Table S2. The influent solutions did not contain organic substances. The liquid phase sampling and chemical analyses are described in the Supporting Information. The relative standard deviation for liquid phase analysis was below 4% for all compounds.

Operational Conditions. All operational conditions were kept the same throughout the whole experimental duration, except for the nitrogen loading rates. During a start-up phase of 9 days, the urine and wastewater reactors were fed with a nitrogen loading rate of 355 ± 15 and 95 ± 5 mg NH₄⁻N·L⁻¹·d⁻¹, respectively. The experiment was initiated (time point zero) by a decrease in the influent rates to 22.8 mL·d⁻¹ (UR) and 101 mL·d⁻¹ (WWR), resulting in nitrogen loading rates of 19 ± 2 (UR) and 8 ± 2 mg NH₄⁻N·L⁻¹·d⁻¹.

Figure 1. For each experimental condition pH and nitrogen species in the reactor (total ammonia, total nitrite, total nitrate, and total nitrogen) are shown together with the relative abundance of AOB. Results for the synthetic urine reactors (UR-K and UR-Na), and the synthetic wastewater reactors (WWR-K and WWR-Na) are presented in panels A–C, D–F, G–I, and J–L, respectively. Experimental conditions are described in more detail in the text and in Table 1. Sequencing samples from day 131 (both urine reactors), as well as 90 and 154 (UR-Na) were excluded due to the low sequencing depth.
A Neighbor Joining phylogenetic tree was constructed in MEGA (version 6.0) using the Maximum Composite Likelihood model on a ClustalW alignment of OTU reference sequences best BLAST matches from NCBI and reference organism sequences obtained from RDP; 500 bootstrap resamplings were carried out to test the tree topology.

Analyses of variance (ANOVA) were conducted to assess the extent and significance of the effects of the two main factors of feed composition (synthetic urine versus synthetic wastewater) and monovalent cationic specie (K⁺ vs Na⁺) on microbial population dynamics, by analogy to Weissbrodt et al. Heatmaps of Spearman’s rank-order correlation coefficients were computed according to Weissbrodt et al. in order to delineate clusters of predominant OTUs (>5%) sharing similar dynamics in relationship with operational conditions and process responses.

3. RESULTS

3.1. Nitrification Performance of MBBRs with Synthetic Urine and Synthetic Wastewater. Urine Reactors. After the decrease in the influent loading (time point zero) the pH started to drop to a level of 4.3 after 30 (UR-K) and 25 days (UR-Na, Figure 1), respectively. As the reactor was continuously supplied with synthetic urine, such a pH drop can only be explained by an increased rate of NH₃ oxidation and proton production by AOB.

In parallel to the pH drop, the total nitrite (NO₂⁻ and HNO₂) concentrations increased. Subsequently, the pH increased again, which is a sign that AOB growth was slower due to an inhibition effect. HNO₂ is a known inhibitor for AOB. Despite the high HNO₂ concentrations (Figure S1) a second decrease of pH was observed after 52 (UR-K) and 46 days (UR-Na) to average pH values of 2.2 ± 0.1 (UR-K) and 2.3 ± 0.3 (UR-Na). During this phase, the total nitrite concentrations decreased from around 100 mgN·L⁻¹ to 3.7 ± 0.8 (UR-K) and 5.9 ± 1.4 mgN·L⁻¹ (UR-Na) and remained stable for the rest of this study. The pH increased only slightly after an aeration failure on days 68 and 98. Despite the low pH values, average ammonia oxidation rates of 13.8 ± 0.3 (UR-K) and 14.5 ± 0.8 mgN·L⁻¹·d⁻¹ (UR-Na) were maintained until day 160. These rates were slightly higher than the nitrification rates of 12.0 ± 0.8 (UR-K) and 11.8 ± 1.0 mgN·L⁻¹·d⁻¹ (UR-Na) observed before the second pH drop. After the well-controlled reactor operation of 160 days, the reactors were run for another 120 days. In this phase the reactor pH remained constant at the very low levels (results not shown), proving that AOB could also grow over long time-periods at such low pH values.

After the second pH drop, the total nitrogen concentration (sum of total ammonia, total nitrite, and nitrate) in the reactor decreased. Nitrogen losses accounted to 9.2 (UR-K) and 9.4 mgN·L⁻¹·d⁻¹ (UR-Na) corresponding to 53 and 50%, respectively (Figure 1). Off-gas measurement for NO, NO₂, and N₂O revealed that the losses from the reactor solution were mainly due to the volatilization of NO: 8.7 (UR-K) or 7.1 mgN·L⁻¹·d⁻¹ (UR-Na) were detected. NO₂ and N₂O were also detectable: NO₂ was 1.3 or 1.6 mgN·L⁻¹·d⁻¹ in UR-K and UR-Na, whereas N₂O accounted for 0.4 or 0.2 mgN·L⁻¹·d⁻¹, respectively. Total emissions of analyzed nitrogen compounds in the off-gas were 10.4 mgN·L⁻¹·d⁻¹ and 8.9 mgN·L⁻¹·d⁻¹, which corresponds well to the nitrogen losses in the liquid phase (Table S3). HNO₂ emissions were not analyzed, but are expected to be small estimated from Henry’s Law. NO was thus
the major compound produced at low pH in the urine reactors, followed by \( \text{NO}_2^- \) (Table S3).

**Wastewater Reactors.** In the wastewater reactors the pH decreased after around 40 days (Figure 1). Total nitrite concentrations in the reactor remained below the detection limit of 0.015 mgN-L\(^{-1}\) in almost all samples and were thus clearly lower than in the urine reactors. In contrast to urine reactors, nitrogen losses from the liquid phase were negligible (Table S3). A new pH level of 4.2 ± 0.4 (WWR-K) and 4.0 ± 0.4 (WWR-Na) was reached. Nitrification rates of 8.2 ± 0.6 (WWR-K) and 8.0 ± 0.5 mgN-L\(^{-1}\).d\(^{-1}\) (WWR-Na) were retained, which is similar to the nitrification rate before the pH drop (7.9 ± 1.1 and 8.7 ± 0.8 mgN-L\(^{-1}\).d\(^{-1}\)).

The chemical speciation model PhreeqC was used to calculate the minimal pH values, which would be reached, if all ammonia was converted to nitrate. In the synthetic wastewater solutions, the minimal pH value would be 2.6, while the synthetic urine solutions would allow the pH to decrease to a minimal value of 0.9 (see the Supporting Information for further details). The buffer capacity of the influent is therefore sufficiently low in both solutions to allow for reaching very low pH values during nitrification.

**Low Impact of Monovalent Cations.** The two urine reactors showed very similar reactor behavior, as did the two wastewater reactors: the difference in K\(^+\) and Na\(^+\) content had little effect (Figure 1). Potassium concentrations in the reactors fed with sodium-rich influent were higher than expected from the influent composition (Table 1): 25.8 ± 21.3 mg-L\(^{-1}\) and 13.2 ± 4.4 mg-L\(^{-1}\) in UR-Na and WWR-Na, respectively (Table S4), likely due to the leakage of potassium ions from the pH electrodes. The potassium levels were, however, still more than 80 and 60 times lower compared to the potassium reactors UR-K and WWR-K, respectively.

### 3.2. Shifts in Nitrifying Populations.

**Urine Reactors.** *Nitrosomonas* OTU 3 was the most abundant AOB in the beginning of the experiment (>15% relative abundance according to 16S rRNA gene sequencing results) and the relative abundance of all other AOB was below 0.2%. According to BLAST, *Nitrosomonas* OTU 3 affiliates with the *Nitrosomonas europaea* lineage. As soon as the pH in the urine reactors decreased, the relative abundance of *Nitrosomonas* OTU 3 declined to values below 0.5% (Figure 1). Concomitantly with this first pH decrease, the relative abundance of OTU 66 sequence, which according to BLAST showed the greatest similarity to *Nitrosococcus oceanii* (95% identity) increased to above 1%. However, the relative abundance of OTU 66 decreased again with the second pH drop, whereas the closely related OTUs 1 and 187, with 93% BLAST similarity to *Nitrosococcus halophilus* strain Nc4, increased strongly. OTU 1 reached maximal relative abundances of 94% and remained the only AOB with relative abundance of more than 0.5% until the end of the experiment. OTU 187 is not shown in Figure 1, because its abundance was considerably lower than the abundance of OTU 1. The dynamics of the *Nitrosococcus*-related OTU 1 was also confirmed by a TaqMan qPCR assay designed to specifically quantify this OTU (Figure S2). A de novo phylogenetic tree indicated that *Nitrosococcus* OTU 1 clustered separately from known *Nitrosococcus* sequences, while the rare OTU 187 was 99% similar to an environmental sequence retrieved from leaf cutter ant nests (Figure 2). Although these results would have to be confirmed, for example, by full-length 16S rRNA gene sequences and other indicators, this suggests that the sequences of OTU 1 belong to an undescribed species, possibly even a new genus.

**Bradyrhizobiaceae OTU 2,** an abundant sequence that was assigned by our pipeline to the family of *Bradyrhizobiaceae*, showed 100% identity to *Nitrobacter* (Nitrobacter sp. 219, AM286375.1). OTU 2 was abundant at the beginning of the experiment, but disappeared in the urine reactors after the second pH drop (Figure 3, and S3). The absence of nitrite oxidizing bacteria (NOB) in the urine reactors was confirmed with batch experiments demonstrating no nitrite oxidation (Figure S4).

The DNA yield per carrier was determined as an estimator for total biomass. The overall DNA yield from urine reactor carriers decreased very strongly after the second pH drop (Figure S3). The high relative abundance of *Nitrosococcus* OTU 1 was thus at least partly due to a strong biomass decay. However, when using the DNA yield and the relative abundance of OTU 1 to estimate the total abundance of this group, then this value increased from below 0.01 to average values of 0.8 \(\mu\)g DNA-carrier\(^{-1}\) after the second pH drop,
indicating that OTU 1 was actually growing. This was further confirmed by qPCR analysis of OTU 1 abundance (Figure S2).

Wastewater Reactors. Similar to the urine reactors, the relative abundance of Nitrosomonas OTU 3 decreased below 0.5% as the pH in the wastewater reactors started to drop (Figure 1). Instead, the relative abundance of Nitrosospira sp. (OTU 18) increased to maximal values of 8%. Nitrobacter-like sequences from the family of Bradyrhizobiaceae remained constant over the whole experimental duration (Figure 3, and S3), indicating that NOB remained viable under the low pH conditions in the wastewater reactors, which was also confirmed in batch experiments (Figure S4). DNA yield per carrier remained relatively constant in the wastewater reactors (Figure S3).

Low Abundance of Archaea. AOA were not detected in any of the low pH reactors by any of the primer pairs used for the 16S rRNA gene-based amplicon sequencing. AOA were also not detected with the AOA-specific PCR assay38 (Figure S5). qPCR for overall abundance of archaea compared to bacteria also failed to detect archaea in the low pH urine reactors, and showed that archaea never exceeded a relative abundance of more than 0.7% at any time in any of the reactors (Figure S6).

3.3. Shifts in Overall Bacterial Community Compositions. The estimated Chao1 richness of the sequencing data sets was correlated to the pH ranges in the reactors (Figure 4). Whereas the richness remained at around 280 OTUs during the first pH drop to 4.3 in the urine reactors, it decreased dramatically to 110 OTUs as the pH dropped to average values of 2.2. The richness in the wastewater reactors decreased only slightly from around 340 to 280 OTUs as the pH regime shifted from above pH 5.5 to average values of 4.1, which corresponds well with the richness in the urine reactors in the same pH range (pH 5.5–3.5).

Urine and wastewater reactors originally contained very similar microbial communities that differentiated increasingly over the course of the experiment, as represented in the nonmetric multidimensional scaling analysis (Figure 5). pH and HNO2 showed the best correlation of the tested environmental variables (pH, HNO2, NO2−, NH3, NH4+, and total salts) with community structure (spearman correlation coefficients: 0.74 for pH, 0.59 for HNO2).

The heatmap of Spearman’s rank-order correlations delineated three major clusters of coevolving predominant OTUs (>5%). Nitrososoccus OTU 1, Nitrosomonas OTU 3, Nitrosospira OTU 18 belonged to one cluster each. Hardy any OTUs clustered together with Nitrososoccus OTU 1, except of the two OTUs 21 and 684 affiliated with the genus-
The salinity of 300 mmol·L⁻¹ for Nitrosospira optimally at salt concentrations of 300 mmol·L⁻¹ and 8.1 mmol·L⁻¹ for Nitrosococcus oceanus stress salt tolerance as a major selection criterion.

The shift from Nitrosococcus OTU 66 to OTU 1 corresponds to an increase in the HNO₂ concentrations (Figure S1) and is thus likely due to a higher HNO₂ tolerance of OTU 1. These traits, in particular acid and HNO₂ tolerance, ultimately allowed Nitrosococcus OTU 1 to drive the system to a new stable state in which it dominated the bacterial community. Nitrosospira OTU 18 may be less resistant to extreme environments and did therefore not cause such strong acidification.

4.4. Nitrosococcus OTU 1 Causes, and Grows in, Environments with Low pH Values and High HNO₂ Concentrations. The decrease in pH and increase in HNO₂ levels caused by the growth of Nitrosococcus OTU 1 corresponded with the decrease in microbial richness and overall DNA yields per carrier (Figures 4, and S3). A strong influence of pH on microbial diversity has been reported for soils: soil pH was the major factor determining the richness of soil bacterial communities. Low environmental pH values decrease the intracellular pH value in bacteria, which in turn compromises enzyme activity, as well as protein and DNA stability. Low intracellular pH values also hamper the energy generation in certain bacteria, for example, AOB affiliating with the Nitrosomonas europaea lineage (Section 4.1). pH homeostasis is therefore an essential requirement for the survival of bacteria at low pH values. HNO₂ impedes pH homeostasis under acidic conditions as it diffuses passively across the cytoplasmic membrane and decreases the intracellular pH value. HNO₂ also inhibits enzymes and it decomposes to NO (Section 4.3), which is another toxic compound for bacteria. It is therefore not surprising that most of the bacteria did not survive these toxic conditions.

Nitrosococcus OTU 1 and Mycobacterium OTUs 21 and 684, however, still managed to grow (Figure 3, and Figure S3). The uptake of potassium ions to inverse the membrane potential is a known pH homeostasis mechanism. The potassium concentration, however, did not have a significant impact on the reactor performance or the microbial community in our experiments (Figures 1, and 3), indicating that either still sufficient potassium was available in the reactors fed with sodium-rich influent or that sodium ions were used instead. Sodium ions have been found to increase the activity of Thiobacillus thiooxidans at low pH values, but the positive influence of sodium was less pronounced than the one for potassium. The Gram-positive bacteria of the genus Mycobacterium are also known to have lipid-rich cell walls, which play an important role in their resistance to acids. Highly impermeable cell membranes are another prerequisite for bacterial growth at low pH values as they reduce the leakage of protons. Thus, acid tolerance can be due to a large variety of factors and the presence of potassium or possibly sodium alone does not determine, whether the acid tolerant bacteria grow in.

4.3. Biological versus Chemical Nitrite Oxidation. The NOB of the genus Nitrobacter have been reported to be active in engineered reactors at minimum pH values between 3.2 and 4.5. The NOB of the genus Nitrospira have been widely detected in acidic soils (pH values as low as 3) and were also observed at average pH values of 4.1 in the synthetic wastewater in this study (Figure 1). It is possible that in...
some studies, chemical nitrite oxidation was wrongly interpreted as NOB activity. Nevertheless, it is likely that accumulated HNO₂ rather than pH alone inhibited *Nitrobacter* sp. in the urine reactors with the first pH drop to 4.3 and caused the accumulation of total nitrite.

Despite the apparent absence or inactivity of NOB, total nitrite remained low once the pH dropped to pH levels below 2.5 (Figure 1), indicating nitrite conversion. At low pH values, HNO₂ is chemically converted to NO₃⁻, involving several volatile intermediates, such as NO, NO₂, and N₂O₃.⁶ Van Cleemput and Baert⁵⁹ observed experimentally that NO is the major gaseous decomposition product, while NO₃⁻ production was favored under conditions in which NO was not stripped, which corresponds very well with the results in this study: strong emissions of NO were observed due to the strong aeration in the MBBR, while NO₃⁻ concentrations decreased during the course of the experiment. NO can also be produced by AOB via the nitriﬁcation pathway,⁶⁰ however, McKenney et al.⁵¹ found that emissions due to the chemical process are dominant at pH values below 4.5. NO is an unwanted nitriﬁcation byproduct as it impacts human health and is considered to be the main precursor of ground-level tropospheric ozone in rural areas.⁶¹

4.4. Implications for Wastewater Treatment. With our results we show that γ-proteobacterial AOB and *Nitrosospira* sp. are important players in wastewaters with high and low ammonia content, respectively, and can cause strong pH decreases. This finding challenges the perception that low pH nitriﬁcation is either not possible or dominated by AOA. The growth of γ-proteobacterial AOB is more critical than the growth of *Nitrosospira* sp., as γ-proteobacterial AOB acidify the wastewater more strongly allowing for the chemical decomposition of HNO₂ (Figure 1). The selection of γ-proteobacterial AOB may not only be a risk in urine nitriﬁcation reactors, but also during the treatment of other wastewaters with high ammonia concentrations with limited alkalinity, e.g., digester supernatant, animal wastewaters, or landfill leachate. Besides reports on low pH nitriﬁcation with human urine in bioﬁlm systems⁶² and with suspended biomass systems,⁵³,⁶³ nitriﬁcation at pH values below 5 has also been observed in poultry manure.⁵⁴ This study shows that nitriﬁcation of urine, manure, digester supernatant, or another wastewater with high ammonia content is prone to low pH values, if the ratio of alkalinity to total ammonia is less than 2. When nitriﬁying such solutions, any decrease of the pH far below the typical limit of 5.4 must be prevented. Otherwise, acid-tolerant γ-proteobacterial AOB will grow in, which has two detrimental consequences: first, the loss of the microbial community, which is responsible for high-rate nitriﬁcation at neutral pH, and, second, the emission of hazardous volatile nitrogen compounds such as NO, N₂O, NO₂, and HNO₂.

**ASSOCIATED CONTENT**

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.est.7b00392.

Additional materials and methods; recipes for synthetic inﬂuent solutions; nitrogen balance for urine and wastewater reactors; chemical concentrations in the reactors; batch experiments for NOB; DNA mass and average copy numbers of AOB; microbiological measurements of AOA; heatmaps of Spaearman’s rank-order correlation of phylotypes and environmental conditions (PDF)

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**Notes**

The authors declare no competing ﬁnancial interest.

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