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Article

Robust Nitritation of Anaerobic Digester Centrate Using Dual Stressors and Timed Alkali Additions

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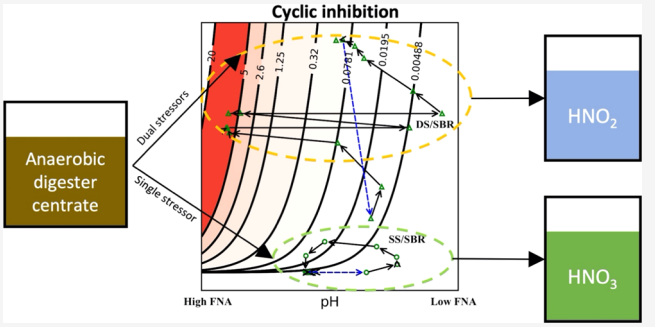
ABSTRACT: Nitrogen is commonly removed from wastewater by nitrification to nitrate followed by nitrate reduction to N_2. Shortcut N removal saves energy by limiting ammonia oxidation to nitrite, but nitrite accumulation can be unstable. We hypothesized that repeated short-term exposures of ammonia-oxidizing communities to free ammonia (FA) and free nitrous acid (FNA) would stabilize nitritation by selecting against nitrite-oxidizing bacteria (NOB). Accordingly, we evaluated ammonium oxidation of anaerobic digester centrate in two bench-scale sequencing batch reactors (SBRs), seeded with the same inoculum and operated identically but with differing pH-control strategies. A single stressor SBR (SS/SBR) using pH set-point control produced HNO_3, while a dual stressor SBR (DS/SBR) using timed alkalinity addition (TAA) produced HNO_2 (ammonium removal efficiency of 97 ± 2%; nitrite accumulation ratio of 98 ± 1%). The TAA protocol was developed during an adaptation period with continuous pH monitoring. After adaptation, automated TAA enabled stable nitritation without set-point control. In the SS/SBR, repeatedly exposing the community to FA (8−10 h/exposure, one exposure/cycle) selected for FA-tolerant ammonia-oxidizing bacteria (*Nitrosomonas* sp. *NM107*) and NOB (*Nitrobacter* sp.). In the DS/SBR, repeatedly exposing the community to FA (2−4 h/exposure, three exposures/cycle) and FNA (4−6 h/exposure, two exposures/cycle) selected for FA- and FNA-resistant AOB (*Nitrosomonas IWT514*) and against NOB, stabilizing nitritation.



INTRODUCTION

Over the past two decades, shortcut biological nitrogen removal (SBNR) processes have received considerable attention for the treatment of anaerobic digester centrate (ADC).[1−8] These processes require NH_4^+ oxidation to NO_2^-, referred to here as nitritation, as per Weißbach et al.[9] Compared to conventional NH_4^+ oxidation to nitrate, nitritation decreases oxygen demand by 25% or more.[10] It also decreases the ratio of biological oxygen demand (BOD_L) to total Kjeldahl nitrogen (TKN) required for denitrification from 6−7 to <4 and reduces residual biomass generated for disposal.[11,12] SBNR processes include the single reactor system for high activity ammonium removal over nitrite (SHARON),[13−15] a process that uses denitrifying heterotrophic bacteria to reduce nitrite to N_2; the coupled aerobic-anoxic nitrous decomposition operation (CANDO), an emerging process that uses heterotrophic bacteria to reduce nitrite N to N_2O for use as a biogas oxidant;[1,16−18] and many processes that use NO_2^- for anammox-mediated anaerobic ammonium oxidation, such as the completely autotrophic nitrogen removal over nitrite process (CANON),[19] DEMON,[20] oxygen-limited autotrophic nitrification/denitrification (OLAND),[5,6] simultaneous partial nitrification, anaerobic ammonium oxidation and denitrification (SNAD),[7] single-stage nitrogen removal using anammox and partial nitritation (SNAP),[8,21] and deammonification in internal-aerated biofilm system (DIB).[22] Compared to conventional nitrification/denitrification, these processes can decrease aeration energy demand by half or more.

All of the above processes require nitritation. Stable nitrite accumulation requires cultivation of ammonia-oxidizing bacteria (AOB) and/or archaea (AOA) and selection against nitrite-oxidizing bacteria (NOB) and *Comammox nitrospira* that oxidize ammonium to nitrate in a single step.[23] To date, inhibition of nitrite oxidation has been accomplished by exploiting differences in the specific growth rates of AOB and NOB as a function of temperature, oxygen affinity, and sensitivity to inhibitors.[24] By manipulating temperature and solids retention time (SRT), AOB can be favored over NOB.[13] At 35 °C, the minimum SRT for NOB (i.e., $\theta_{x,NOB}^{min}$) is greater than the minimum for AOB (i.e., $\theta_{x,AOB}^{min}$).[13] Thus, by operating at SRT values within the range $\theta_{x,AOB}^{min}$ < SRT < $\theta_{x,NOB}^{min}$, NOB are washed out and AOB remain. This approach was successfully

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implemented in the SHARON[14,15] process (Table S1). A disadvantage to the use of temperature as a selecting agent is the energy required for heating, especially in cold climates.[10]

Manipulation of dissolved oxygen (DO) levels can also favor AOB over NOB.[25−29] AOB have oxygen half-saturation coefficients (K_{O_2} ~ 0.033−0.56 mg O_2/L) that are lower than the reported values for NOB (K_{O_2} ~ 0.17−5.3 mg O_2/L). The higher affinity of AOB for DO could enable them to outcompete NOB at low DO levels.[30−34] However, research to date indicates that the optimum DO for nitritation varies from system to system and does not always prevent nitrate formation. Ruiz et al.[26] reported NO_2^- accumulation at DO levels of 0.7−1.7 mg/L in a continuously stirred tank reactor fed high influent NH_4^+−N. Tokutomi[35] reported an optimum DO of ~1 mg/L in an airlift reactor. Others have reported optimum levels <0.5 mg DO/L.[27,30,36] In pure culture experiments, Stenstrom and Poduska[37] observed nitrite production at DO levels near 0.3 mg/L, but groups studying nitrification of low strength wastewater have reported nitrate production at <1 mg DO/L. This was accompanied by a shift in dominant NOB to *Nitrospira* sp.,[38−40] a genus that utilizes oxygen more efficiently than AOB at low DO levels. Such observations suggest that low DO strategies for nitritation can be system- or wastewater-specific. An additional concern with operation at low DO levels is production of greenhouse gas N_2O, an undesirable outcome unless the N_2O is captured and used for energy production.[1,16,17] Tallec et al.[41] reported elevated N_2O emissions at a DO level of 1.0 mg/L.

Production of nitrate can also be prevented by exposing nitrifying microbial biomass to inhibitory levels of free ammonia (FA, NH_3) and free nitrous acid (FNA, HNO_2).[24,42] Even though FA is a base and FNA is an acid, both are uncharged molecules that can pass across cell membranes and enter the cell interior where they can disrupt cell physiology by different mechanisms. In the case of FA, toxicity likely involves multiple mechanisms, as described by Liu et al.[43] One involves activation of potassium-proton exchange pumps that pump H^+ ions into the cell, where FA is protonated while simultaneously transporting K^+ out of the cell. Overall, this reaction increases maintenance energy requirements and decreases cell yield. Other reports indicate that FA can react with and inhibit bacterial enzymes, including proteases, a-glucosidase, phosphotransacetylase, and acetate kinase. FNA likely also has multiple mechanisms of toxicity. Because it can carry a proton, it can act as an uncoupler of oxidative phosphorylation as it diffuses across the cell membrane, and, at high levels, is known to cause loss of cell integrity and lysis.[44] Table S2 summarizes measured K_i values for uncompetitive inhibition by FA and measured K_i values for noncompetitive inhibition by FNA of both ammonia oxidation (AOB) and nitrite oxidation (NOB). For NOB-mediated nitrite oxidation, reported K_i values are lower than the K_i values for ammonia oxidation by AOB, indicating greater sensitivity of NOB to low concentrations of FA and FNA.

A potential limitation to the use of stressors for NOB inhibition is acclimation through shifts in microbial community structure or evolutionary changes. Unacclimated NOB can tolerate up to 4.25 mg FA/L, but acclimated NOB can tolerate levels up to 61 mg FA/L.[45] In one study, acclimated NOB were not suppressed by increasing FA levels and extending FA contact time.[46] At elevated pH, Villaverde et al.[47] observed nitrate production during the treatment of a synthetic influent containing 80−100 mg/L total ammonium nitrogen (TAN).[47] Similarly, Ciudad et al.[48] observed nitrate production during the treatment of a synthetic influent containing 150 mg/L TAN.[48] Because total ammonium levels were low, FA levels were too low to inhibit NOB, even at high pH. Exposure to high FNA levels can also lead to NOB acclimation. Ma et al.[49] observed acclimation of NOB to FNA and increased nitrate production in a bench-scale bioreactor fed with domestic wastewater. In a bench-scale nitrifying sequencing batch reactors (SBRs) treating domestic wastewater, Wang et al.[50] inhibited NOB and achieved nitrite accumulation by recirculating biomass through a selector containing FNA at inhibitory levels. Duan et al.[51] also used a sidestream selector to alternately expose NOB in bench-scale SBRs to inhibitory levels of both FA and FNA. Table S1 summarizes strategies that have been successfully used to achieve nitritation and nitrogen removal from ADC, where nitrogen levels are a factor of ten higher than those of domestic wastewater.

In this work, we demonstrate stable nitritation of ADC in a dual stressor SBR (DS/SBR) with no need for a sidestream selector or for pH- or DO-set-point control. During SBR start-up, timed alkali additions (TAA) were used to "train" a nitrifying microbial community to produce nitrite in pH cycles that oscillate in a stable pattern between 9.5 and 6.5, repeatedly exposing the microbial community to FA and FNA. The TAA pattern was established for the following conditions: (1) stable inputs of TKN and alkalinity, (2) bio-oxidation of ammonium to HNO_2, which causes pH to decrease to a "bottom floor" at pH 6.5 where further ammonia oxidation stops[52] and residual alkalinity is low; and (3) cycle duration is set by the time required for complete oxidation of all of the added ammonia to HNO_2. The result is a DS/SBR operational schedule in which TAA results in changes in pH, ammonia speciation, and nitrite speciation and causes repeated exposures of microbial biomass to FA and FNA at levels that are more inhibitory to NOB than AOB, thus stabilizing nitritation. Performance and community structure of the DS/SBR were compared to a single stressor SBR (SS/SBR) operated side-by-side with the same inoculum, centrate loading, cycle time, recycle ratio, hydraulic retention time (HRT), and SRT, but using pH set-point control, with cycles varying between pH of 9.5 and 7.3, repeatedly exposing the microbial community to FA but providing negligible exposure to FNA.

MATERIALS AND METHODS

Experimental Setup and Operating Parameters. A single stressor SBR (SS/SBR) and a dual stressor SBR (DS/SBR) were operated in parallel. Both SBRs (working volumes of 3-L apiece) were seeded with screened (0.1 mm mesh) activated sludge from the Palo Alto Regional Water Quality Control Plant (PARWQCP, Palo Alto, CA). Both reactors were fed ADC from the Delta Diablo Sanitation District (DDSD, Antioch, CA) on a repeating 24 h cycle. The composition of the ADC feed was total COD of 1088 ± 147 mg/L, soluble COD of 453 ± 40 mg/L, pH of 8.4 ± 0.5, NH_4^+−N of 1435 ± 273 mg/L, and TKN of 1606 ± 254 mg/L. Each cycle consisted of a 0.1 h fill period, 23.3 h react period, 0.5 h settle period, and 0.1 h decant period (total cycle time of 24 h). The recycle ratio was 0.25, resulting in a HRT for each SBR of 32 h. The SRT of each SBR was maintained at 50 days by removing 60 mL of mixed liquor and suspended solids (MLSS) per day. Both reactors were operated side-by-side at room temperature (22 ± 3 °C). Operation of both

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Environ. Sci. Technol. XXXX, XXX, XXX−XXX

SBRs commenced at the same time, and each reactor received the same inoculum. Both reactors passed through three stages of operation with dissolved oxygen between 7 and 9 mg/L: adaptation (stage 1), a 2-day cycle (stage 2), and a 1-day cycle (stage 3). The duration of SBR react period differed in each stage. During stage 1, the react period was adjusted until influent ammonia oxidation was complete. During stages 2 and 3, the react periods were 47.3 and 23.3 h, respectively.

For the SS/SBR, oxidation of ammonia to nitrate occurred at the beginning of each cycle as nitrification decreased pH from 8.5 to 7.3 at which point a further decrease in pH was prevented by alkali additions (0.5 M NaOH plus 1.0 M $NaHCO_3$) with pH set-point control. For each cycle, a pH of 8.5 was reestablished after decanting, settling, and adding a new batch of ADC. Upon aeration, stripping of CO_2 increased pH to ~8.5.

For the DS/SBR, alkali (0.5 M NaOH plus 1.0 M $NaHCO_3$) at a dose near the stoichiometric requirement was added at the start of each cycle to raise the pH from 6.5 to 8.5. Upon initiation of aeration, CO_2 stripping further increased pH to ~9−9.5. Subsequently, AOB-mediated oxidation of ammonia to HNO_2 decreased pH from 9.5 to 6.5, at which point further oxidation stopped likely due to FNA inhibition or limited substrate.[52] Monitoring of pH revealed a repeating cyclic pattern of pH change after community acclimation. For the 2-day cycle, pH was monitored continuously and the optimal time for alkali addition was determined empirically by noting the time in each cycle when pH stabilized. After pH decreased to 6.5, addition of alkali and stripping of CO_2 during aeration increased pH back to starting values >9. Multiple alkali spikes were required per cycle to achieve complete oxidation of influent ammonium to nitrite. For the 1-day cycle, continuous pH monitoring was discontinued to test whether timed alkali additions without pH monitoring could provide reliable operation in the event of a pH probe failure. Different volumes of the alkali stock solution (1.5 N) were added at three time points: (1) the initiation of each reactor cycle, (2) after 12 h, and (3) after 14 h, and the end result was a pH within the reactor that fluctuated between 6.5 and 9.5 in each cycle. A more detailed explanation is provided in Table S3.

Algorithm for Calculating Buffer Requirements. To implement TAA, calculation of the amount of alkali (i.e., volume and concentration of alkali stock) required per cycle to neutralize the strong acid generated by oxidation of ammonium to HNO_2 was critical. Table S4 summarizes the algorithm used to prepare the alkali stock. The calculation can be either computed manually or implemented in Python (available upon request). For the DS/SBR, the algorithm assumes negligible alkalinity after ammonia oxidation to HNO_2 and a pH decrease to near 6.5. This assumption was verified by measuring alkalinity at pH = 6.5 for multiple cycles and batches of ADC. The second assumption, confirmed experimentally, was that oxidation of ammonium is completely inhibited at pH near 6.5.

Analytical Methods. Filtered liquid samples were obtained by filtration through a 0.45 μm nylon membrane filter (Thermo Fisher Scientific). These samples were analyzed for NH_4^+−N, NO_2^-−N, NO_3^-−N. NH_4^+−N and NO_2^-−N were analyzed using a Westco Smartchem 200 discrete analyzer (Brookfield, CT). NO_3^-−N was determined using the Hach method TNT835 after sulfamic acid quenching of nitrite.

Unfiltered samples were analyzed for TSS and VSS. TSS and VSS were measured as per standard methods (APHA, 2006). The nitrite accumulation ratio (NAR) was calculated as:

$$\mathrm{NAR}\ (\%) = \frac{\mathrm{NO_2^-{-}N}}{\mathrm{NO_2^-{-}N} + \mathrm{NO_3^-{-}N}} \times 100\%$$

Concentrations of FA and FNA were calculated as per Anthonisen et al.:[53]

$$\mathrm{FA\ as\ NH_3}\left(\frac{\mathrm{mg}}{\mathrm{L}}\right) = \frac{17}{14} \times \frac{\mathrm{TAN}\left(\frac{\mathrm{mg\ N}}{\mathrm{L}}\right) \times 10^{\mathrm{pH}}}{K_b/K_w + 10^{\mathrm{pH}}}$$

$$\frac{K_b}{K_w} = e^{(6344/273+T)}$$

where TAN is the total ammonium nitrogen, K_b is the base dissociation constant for NH_3, K_w is the ion product constant, and T is the reactor temperature in °C.

$$\mathrm{FNA\ as\ HNO_2}\left(\frac{\mathrm{mg}}{\mathrm{L}}\right) = \frac{47}{14} \times \frac{\mathrm{NO_2^-{-}N}\left(\frac{\mathrm{mg\ N}}{\mathrm{L}}\right)}{K_a \times 10^{\mathrm{pH}}}$$

$$K_a = e^{(-2300/273+T)}$$

where NO_2^-−N is the total nitrite nitrogen, K_a is the acid dissociation constant of HNO_2, K_w is the ion product constant, and T is the temperature of the reactor in °C.

Microbial Community Analysis. Genomic DNA (gDNA) from biomass samples was extracted using FastDNA Spin Kit for Soil (MP Biomedicals, Solon, OH) per the manufacturer's protocol. 16S rRNA gene sequences were amplified using the primer set 8F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-GGTTACCTTGTTACGACTT-3′), and amoA sequences were amplified using the primer set amoA-1F (5′-GGGGTTTCTACTGGTGGT-3′) and amoA-2R (5′-CCCCTCKGSAAAGCCTTCTTC-3′).[54,55] Both polymerase chain reaction (PCR) reactions were conducted with a volume of 25 μL containing 0.4 μM of the respective primers, 1× Fail-Safe PCR buffer, 1.25 units of AmpliTaq LD Taq polymerase (Applied Biosystems, Foster City, CA), and 20−30 ng of gDNA. The PCR thermocycling steps for 16S rRNA were (i) 94 °C for 5 min; (ii) 30 cycles of 94 °C for 45 s, 55 °C for 30 s, and 72 °C for 90 s, and (iii) an extension at 72 °C for 10 min. Similarly, the PCR thermocycling steps for amoA were (i) 94 °C for 5 min; (ii) 30 cycles of 94 °C for 60 s, 60 °C for 90 s, and 72 °C for 90 s, and (iii) an extension at 72 °C for 10 min. The PCR product was verified using 1.5% agarose gel electrophoresis and purified via the PureLink Quick PCR purification kit (Invitrogen, Carlsbad, CA). After purification, the PCR products were cloned using the TOPO TA Cloning kit (Invitrogen, Carlsbad,CA). *Escherichia coli* transformants with *amo*A and 16S rRNA inserts were incubated overnight at 37 °C. These were used as the PCR templates with T7-promoter and SP6-promoter primers and then were digested using *Taq*I (Invitrogen, Carlsbad, CA). All 16S rRNA clones and *amo*A clones were sequenced using ABI 3730XL automated sequencers by MCLab (San Francisco, CA). In total, for 16S rRNA sequences, 93 sequences, 118 sequences, and 101 sequences were obtained for Inoculum, SS/SBR, and DS/SBR, respectively. For amoA, 58 sequences were obtained for inoculum, 68 for the SS/SBR, and 62 for the DS/SBR.

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Environ. Sci. Technol. XXXX, XXX, XXX−XXX

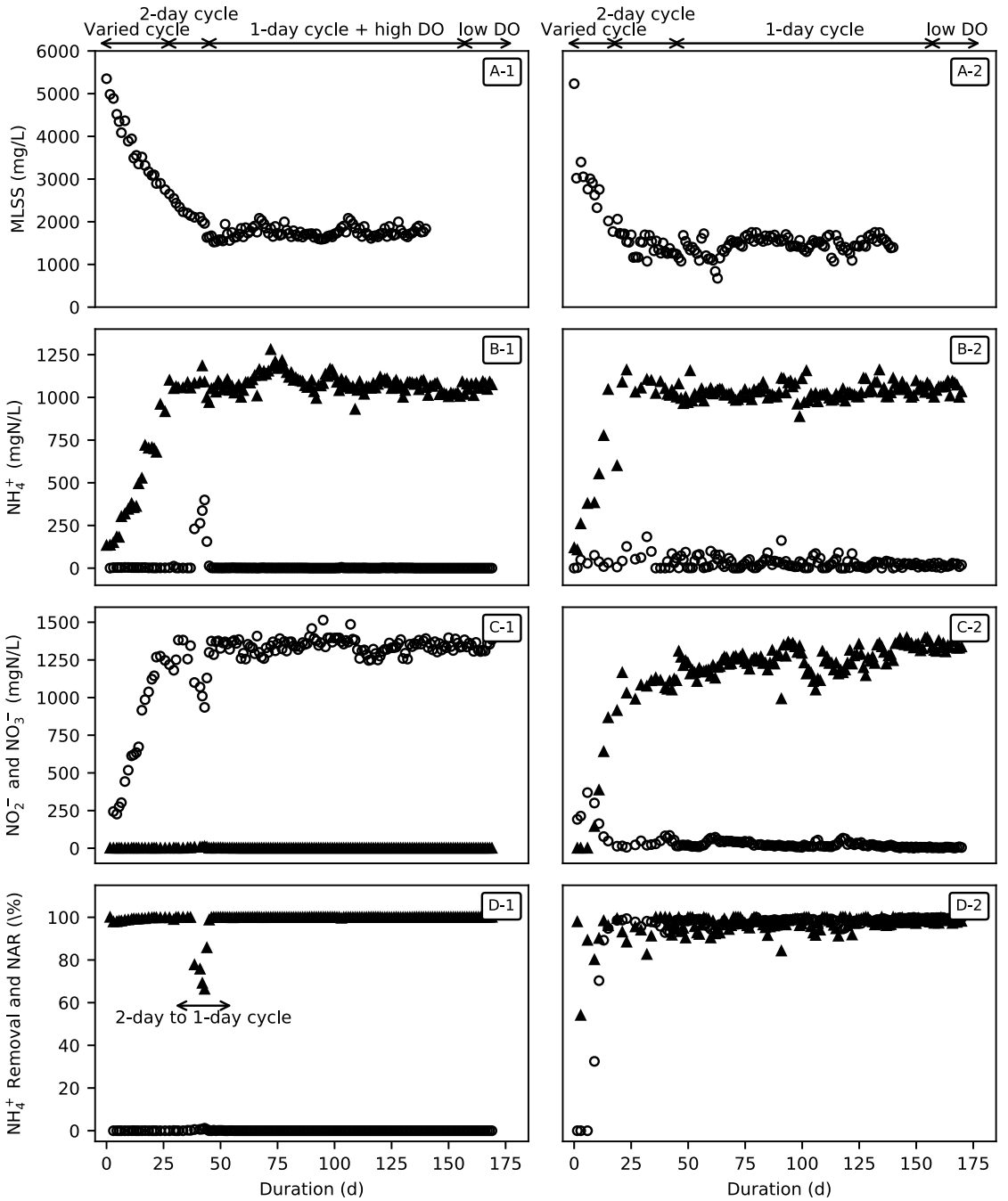

Figure 1. Long-term performance of the SS/SBR (A-1, B-1, C-1, D-1) and DS/SBR (A-2, B-2, C-2, D-2). (A) MLSS concentration (○); (B) NH_4^+−N concentration measured at the beginning (▲) and end of each cycle (○); (C) NO_2^-−N concentration (▲) and NO_3^-−N Concentration at the end of each cycle (○); and (D) NH_4^+−N removal efficiency (▲) and NAR (○).

Oxygen Utilization Rates (OURs). The OURs of autotrophic AOB, NOB, and heterotrophic microorganisms at different pH values were obtained using the following expressions:[56]

$$OUR_{NOB} = OUR_t - OUR_{Auto\ AOB+Het}$$

$$OUR_{Auto\ AOB} = OUR_{Auto\ AOB+Het} - OUR_{Het}$$

Oxygen utilization rates (OURs) for autotrophic AOB, NOB, and heterotrophic microorganisms (including both heterotrophic AOB and heterotrophic bacteria) were assayed for both SBRs by aerating media to oxygen saturation, diluting 30 mL samples of mixed liquor to 276 mL, adding the appropriate substrate (ammonium, nitrite), and monitoring the rate of consumption of dissolved oxygen with a dissolved oxygen probe (Oakton).

To determine OURs for autotrophic AOB, NOB, and heterotrophic microorganisms, selective inhibitors (allylthiourea, ATU;[57−65] $NaClO_3$[66,67]) were added sequentially.[56] Total OUR (OUR_t) was determined in the absence of added inhibitors after adjusting initial NH_4^+−N concentration to 1500 mg/L (20 mL stock at 22.5 g of NH_4^+−N/L). After the DO decreased by 3 mg/L, $NaClO_3$ was added to inhibit NOB (12 mL of a 0.5 M $NaClO_3$ stock solution to give a final $NaClO_3$ concentration of 20 mM) and the combined OUR of autotrophic AOB and heterotroph oxygen utilization rate

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Environ. Sci. Technol. XXXX, XXX, XXX−XXX

Table 1. Changes in Operational Variables for the SS/SBR and DS/SBR

	single stressor SBR			dual stressor SBR		
operational stage	1	2	3	1	2	3
days of operation	1−25	26−39	40−140	1−19	20−39	40−140
cycle time	varied	2 days	1 day	varied	2 days	1 day
influent NH_4^+−N concentration (mg/L)	462 ± 262	1070 ± 20	1114 ± 60	469 ± 309	945 ± 182	1020 ± 25
effluent NH_4^+−N concentration (mg/L)	3 ± 1	2 ± 4	11 ± 31	27 ± 25	63 ± 63	29 ± 24
NH_4^+−N conversion efficiency (%)	99 ± 1	100 ± 0	99 ± 3	88 ± 15	94 ± 6	97 ± 2
effluent NO_2^-−N concentration (mg/L)	0	1 ± 1	2 ± 8	369 ± 392	1085 ± 54	1248 ± 31
effluent NO_3^-−N concentration (mg/L)	753 ± 377	1299 ± 81	1304 ± 185	172 ± 124	33 ± 23	32 ± 13
NAR (%)	0	0	0 ± 1	48 ± 45	97 ± 2	98 ± 1

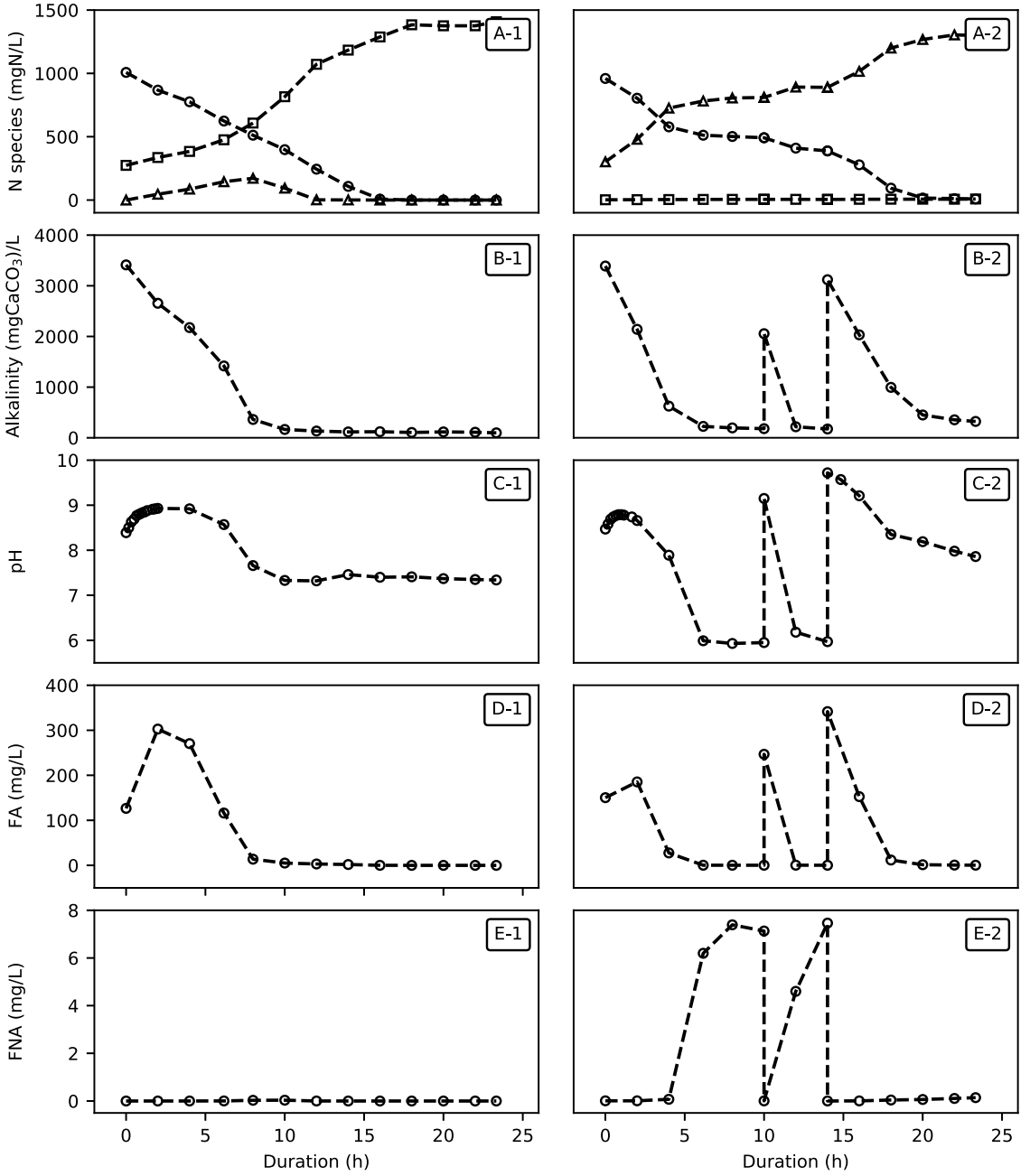

Figure 2. Hourly performance of the SS/SBR (A-1, B-1, C-1, D-1) and the DS/SBR (A-2, B-2, C-2, D-2) on day 100. (A) Nitrogen species concentration (NH_4^+−N (-○-); NO_2^-−N (-△-); NO_3^-−N (-□-)); (B) alkalinity concentration (-○-); (C) pH (-○-); (D) FA concentration (-○-); and (E) FNA concentration (-○-).

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Environ. Sci. Technol. XXXX, XXX, XXX−XXX

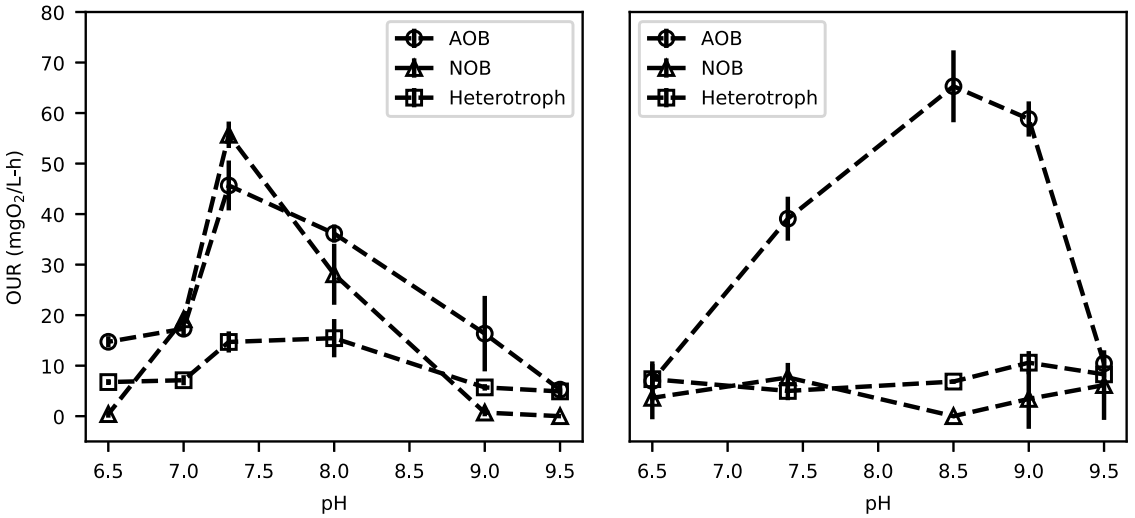

Figure 3. Effects of pH on oxygen utilization rates for autotrophic AOB (-○-), NOB (-△-), and heterotrophs (including heterotrophic AOB) (-□-) for the SS/SBR (left panel) and the DS/SBR (right panel) on day 100.

($OUR_{Auto\ AOB+Het}$) was then determined. After DO decreased by another 2 mg/L, ATU was added (12 mL ATU stock solution (125 mg/L)) to give a final ATU concentration of 5 mg/L to inhibit autotrophic AOB activity,[57,63] and the OUR of heterotrophic microorganisms (OUR_{Het}) was determined. In SS/SBR tests, OURs of NOB (OUR_{NOB}) were assayed after adding 6 mL of nitrite stock solution (10 g NO_2^-−N/L) to give an initial NO_2^-−N concentration of 200 mg N/L. In DS/SBR tests, NOB OURs (OUR_{NOB}) were assayed after adding 42 mL of nitrite stock solution to give an initial NO_2^-−N concentration of 1500 mg/L. Concentrated sulfuric acid (1 M) and sodium hydroxide (1 M) solutions were prepared and used to adjust pH.

RESULTS

Adaptation and Long-Term Performance. Both SBRs were inoculated with the same activated sludge sample from the PARWQCP. Initial MLSS concentrations of SS/SBR and the DS/SBR were 5350 and 5320 mg/L, respectively. During start-up, the MLSS concentration of both SBRs gradually decreased to stable levels (Figure 1A-1,A-2). Mean MLSS concentrations for the SS/SBR and the DS/SBR were 1728 ± 128 mg/L (n = 100) and 1592 ± 185 mg/L (n = 100), respectively. Dual stressors in the DS/SBR appeared to enable a shorter adaptation period. The SS/SBR required 40 days to achieve a consistent and repeating pattern of ammonia oxidation; the DS/SBR required only 20 days to achieve such a pattern, with a NAR of 97%.

To assess ammonia oxidation patterns within each SBR, NH_4^+−N, NO_2^-−N, and NO_3^-−N were monitored throughout each cycle. The performance of the DS/SBR diverged dramatically from the SS/SBR (Figure 1 and Table 1). For the SS/SBR, influent NH_4^+−N concentration was initially 133 mg/L and was slowly increased to 916 mg/L during the adaptation period. The average NH_4^+−N removal efficiency was 99 ± 1%, and average effluent NH_4^+−N concentration was 3 ± 1 mg/L. No NO_2^-−N was observed, and NO_3^-−N levels slowly increased from 132 to 1247 mg/L. After adaptation, the SS/SBR operated on a 2-day cycle from day 26 to 39. During this period, the average influent and effluent NH_4^+−N concentrations were 1070 ± 20 and 2 ± 4 mg/L, respectively, with an average removal efficiency of 99 ± 3%. The average effluent NO_3^-−N concentration was 1304 ± 185 mg/L. On day 40, the cycle duration was reduced to 1 day. NH_4^+−N removal efficiency decreased to 78% then recovered to 99% after five cycles of operation. During the 1-day cycle period, the average influent and effluent NH_4^+−N concentrations were 1054 ± 21 and 2 ± 0 mg/L, respectively, with a NH_4^+−N removal efficiency and a NAR of 100 ± 0 and 0 ± 1%, respectively. The average NO_2^-−N concentration and the average NO_3^-−N concentration were 2 ± 8 and 1304 ± 185 mg/L, respectively.

For the DS/SBR, some NO_3^-−N was initially detected during the adaptation period (days 1−19) but quickly decreased to <20 mg/L. Initially, no NO_2^-−N was detected, but nitrite levels slowly increased to 945 mg/L. After adaptation, the DS/SBR operated on a 2-day cycle for 20 days (days 20−39). During that period, the average influent and effluent NH_4^+−N concentrations were 945 ± 182 and 63 ± 63 mg/L, respectively. The average NH_4^+−N removal efficiency was 94 ± 6%, and the average NAR was 97 ± 2%. Average effluent concentrations for NO_2^-−N and NO_3^-−N were 1085 ± 54 and 33 ± 23 mg/L, respectively. On day 40, the cycle duration was reduced to 1 day for the remainder of the operational period. During this 1-day cycle period, the average NH_4^+−N concentrations of influent and effluent were 1044 ± 54 and 39 ± 32 mg/L, respectively, and the average NH_4^+−N removal efficiency and NAR were 96 ± 3 and 98 ± 1%, respectively. The average NO_2^-−N concentration and the average NO_3^-−N concentrations were 1269 ± 70 and 26 ± 17 mg/L, respectively.

The alkalinity required for addition (i.e., alkalinity needed to neutralize acid released by oxidation of TKN) was equal to the total alkalinity requirement, g as $CaCO_3$/L, based upon the TKN (g N/L oxidized to HNO_2 × 7.13 g $CaCO_3$/g N) minus the measured alkalinity of the ADC.

Hourly Comparison of SS/SBR and DS/SBR Performance. To better understand cycle performance, samples were collected from both SBRs every 2 h on day 100 (Figure 2). In both SBRs, pH increased slowly in the first 2 h after alkali addition. This was attributed to the stripping of CO_2 from the added ADC. For the SS/SBR, the slope of the NH_4^+−N concentration was approximately constant, suggesting a pH-independent zero-order reaction, probably due to negligible FA inhibition of ammonia oxidation and high ammonia levels.

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Environ. Sci. Technol. XXXX, XXX, XXX−XXX

Table 2. Number of Dominant AOB and NOB Clones in 16 rRNA and amoA Analyses on Day 100

		Inoculum	SS/SBR	DS/SBR
16s RNA	*Nitrosomonadaceae*	3	5	15
	Nitrospiraceae	12	0	1
	Bradyrhizobiaceae	0	15	0
	Xanthomonadaceae	2	13	16
	Total number of clones	93	118	101
*amo*A	*Nitrosomonas IWT514*	2	8	61
	Nitrosomonas eutropha	1	6	1
	Nitrosomonas sp. NM107	40	48	0
	Nitrosomonas sp. TK794	2	4	0
	Nitrosomonas sp. GH22	13	2	0
	Total number of clones	58	68	62

Autotrophic AOB

NOB

Putative Heterotrophic AOB

NO_2^-−N concentration steadily increased (Figure 2A-1), peaking at 172 mg NO_2^-−N/L at pH 7.6. As pH decreased to 7.3, the rate of ammonia oxidation did not change as the rate of nitrite oxidation increased. At the end of the 24-h react period, effluent NO_2^-−N concentration was less than 0.1 mg/L. For the DS/SBR, the rate of ammonia oxidation was pH-dependent. Within 6 h of operation, approximately 500 mg/L of NH_4^+−N was oxidized to NO_2^-−N, and bioreactor pH stabilized at ∼6.5, close to the pK_a of H_2CO_3/HCO_3^-, confirming the validity of the second assumption in the control strategy. Nitrition increased after addition of buffer and restoration of pH to 9.5. At the end of the cycle, the NH_4^+−N concentration was 8.8 mg/L, NO_3^-−N concentration was 8.35 mg/L, and NO_2^-−N concentration was 1300 mg/L (Figure 2A-2). To quantify the magnitude of the stressors applied to the SS/SBR and the DS/SBR, we have integrated the data in Figure 2D,E to compute contact-time-dose Ct. For FA, the computed Ct values for the SS/SBR and the DS/SBR were very similar at 1600 and 1500 mg FA/L-h, respectively. For FNA, the computed Ct values for the SS/SBR and the DS/SBR were very different at 0.1 and 50 mg FNA/L-h, respectively. We conclude that the two reactors received a similar stress from FA, and that the second stressor FNA was dominant in the DS/SBR, likely leading to the repression of nitrate production.

Oxygen Utilization Rates at Different pH Values. For both SBRs, oxygen utilization rate (OUR) assays enabled an assessment of respiration at different pH levels by autotrophic AOB, NOB, and aerobic heterotrophs (Figure 3). In the SS/SBR, the highest OURs were obtained at pH 7.3 to 8. At pH 7.3, OUR_{NOB} was maximum and exceeded the autotrophic AOB OUR ($OUR_{Auto\ AOB}$), favoring the production of nitrate from nitrite. At other pH levels, the reverse was true, with $OUR_{Auto\ AOB}$ exceeding OUR_{NOB}. In the DS/SBR, TAA selected for high $OUR_{Auto\ AOB}$ at pH 7.3, 8.5, and 9 (optimum at pH 8.5), indicating rapid ammonia oxidation, with low and stable OURs for NOB and heterotrophic bacteria, consistent with low rates of nitrite oxidation (Figure 3). These results support the conclusion that TAA was selected against NOB.

Microbial Community Analysis. Clone libraries were prepared for the inoculum (day 0) and for samples taken on the final day of operation (day 100). Table 2 summarizes changes in the number of dominant 16S rRNA and *amoA* clones (see Figures S1 and S2 for detailed resolution of the clone libraries). Compared to the inoculum, both the SS/SBR and the DS/SBR were enriched in Nitrosomonadaceae, a family that includes many AOB, and in Xanthomonadaceaee, a family that includes heterotrophic AOB.[68−70] Compared to the day 0 inoculum, the number of AOB clones in the family Nitrosomonadaceae nearly doubled in the SS/SBR (from 3 to 5), but in the DS/SBR, the increase was 5-fold (from 3 to 15) (Table 2). The *amo*A clone library confirmed 16S rRNA data for Nitrosomonadaceae, suggesting selection for *Nitrosomonas* IWT514, a single species that accounted for 61 of the 62 *amoA* clones (98%) in the DS/SBR.

The dominant NOB clones recovered from the SS/SBR shifted from Nitrospiraceae (12 clones in the inoculum and none in the SS/SBR) to Bradyrhizobiaceae (no clones in the inoculum; 15 clones in the SS/SBR), a family that includes *Nitrobacter* species, a well-known NOB genus. In the DS/SBR, Nitrospiraceae was detected, but at greatly reduced abundance, decreasing from 12 clones in the inoculum to one in the DS/SBR.

NOB adaptation and diversity differed dramatically for the SS/SBR and DS/SBR (Table 2). The SS/SBR was selected for FA-tolerance alone, and the dominant NOB shifted from Nitrospiraceae (12 clones in the inoculum; none in the SS/SBR) to Bradyrhizobiaceae (no clones in the inoculum; 15 in the SS/SBR). In the DS/SBR, Nitrospiraceae were the sole NOB but their relative abundance decreased dramatically (12 clones in the inoculum; 1 in the DS/SBR).

DISCUSSION

This work demonstrates that robust nitritation of ADC can be achieved by repeated exposure of the community to FA and FNA without DO- or pH- set-point control. SS/SBR selection based upon pH set-point control with FA as the sole stressor selected for FA-tolerant AOB (*Nitrosomonas eutropha* and *Nitrosomonas* sp. NM107) and FA-tolerant NOB (Bradyrhizobiaceae). Nitrate production was efficient, and nitrite production was negligible.

Adaptation of *Nitrobacter*, a genus of NOB within the Bradyrhizobiace family, has been reported after long-term exposure to FA as the sole stressor.[26] In batch studies with *Nitrobacter* sp. and *Nitrospira* sp., *Nitrobacter* sp. had a higher half-saturation coefficient for nitrite and tolerated higher FA levels.[71] Both these properties would favor *Nitrobacter* in the SS/SBR, where NO_2^-−N level reached ∼200 mg/L during each cycle.

Inhibition of NOB was observed in the DS/SBR, where both FA and FNA were intermittently present at concentrations that exceeded values previously shown to be inhibitory (2.6 and 10 mg N/L, respectively).[53] Repeated exposure of the DS/SBR community to dual stressors—FA and FNA—selected for AOB tolerant of such exposures (notably *Nitrosomonas IWT514*), but NOB largely failed to adapt, and either died

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Environ. Sci. Technol. XXXX, XXX, XXX−XXX

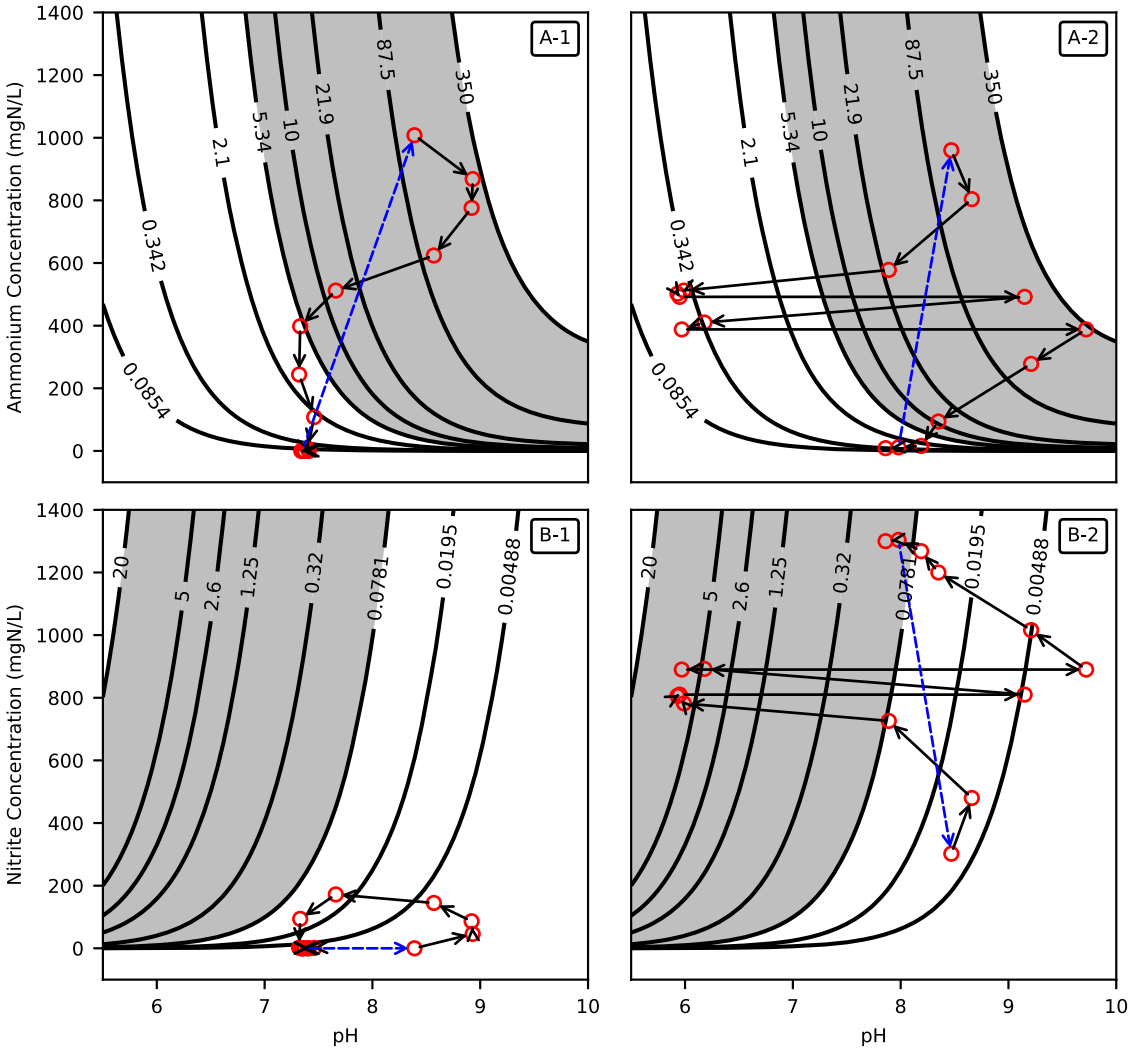

Figure 4. Inhibitory effect of FA and FNA across the cycle of SS/SBR (A-1, B-1) and DS/SBR (A-2, B-2). (A) NH_4^+−N concentration (○). (B) NO_2^-−N concentration (○). Each cycle repeats daily. Contour lines are FA and FNA concentrations. Inhibitory contour lines are shaded. New cycle (blue dashed arrow).

out or were inhibited and removed by wasting, with detection of just a single NOB clone (Nitrospiraceae). Nitrite production was efficient and nitrate production was negligible.

Interestingly, the dominant AOB in the DS/SBR was *Nitrosomonas IWT514*, a species previously isolated in a decolorizing plant operated at high ammonium levels (>4 g N/L) and at temperatures as low as 20 °C.[72] This species grew well in the DS/SBR with NH_4^+−N concentrations at the beginning of each cycle as high as 1 g N/L.

Finally, we note a significant increase in Xanthomonadaceae in both the SS/SBR and the DS/SBR relative to the inoculum. 16S rRNA community analysis results revealed 5 clones in the SS/SBR and 15 clones in the DS/SBR. A possible explanation is heterotrophic nitrification mediated by ammonia-oxidizing Xanthomonadaceae, as previously reported for SBRs operated at low DO[69] and fed with ADC amended with nitrogen-containing coagulants.[70]

On day 100, cycle performance was evaluated for both the SS/SBR and DS/SBR. For the SS/SBR, NOB were initially inhibited, and NO_2^-−N accumulated to 200 mg/L during a period of elevated FA at the initiation of the cycle (Figure 2C-1). As total ammonia levels fell, however, FA levels became negligibly small (Figure 2A-1,B-1), NOB were no longer inhibited, and nitrite was oxidized to nitrate within just 2 h. This observation was supported by OUR tests of biomass from the SS/SBR (Figure 3), showing that $OUR_{Auto\ AOB}$ exceeded OUR_{NOB} at pH values >7.3.

For the DS/SBR, the dominant stressor alternately switched from FA at high pH (9.5) to FNA at pH levels near 6.5 (Figure 4A-2,B-2). OUR data indicated that ammonia oxidation was insensitive to pH, with $OUR_{Auto\ AOB}$ consistently higher than OUR_{NOB} over the pH range 7.3−9.

Throughout the long-term study (Figure 1), the DS/SBR was operated at high DO levels so as to prevent DO impacts on the selection of AOB and NOB. For industrial applications, however, low oxygen consumption is desirable as it decreases aeration costs. To assess DO impacts, we carried out additional tests on the DS/SBR for 30 cycles at low DO levels (0−2.5 mg/L). Low DO operation was achieved by intermittent aeration (30 min on; 30 min off). No difference in performance was observed. Researchers investigating systems with low DO as the sole selection pressure for nitritation have reported perturbations after DO probe failure.[71] This work suggests that such perturbations might be prevented by combining TAA with operation at low DO, enabling simultaneous nitritation and low energy utilization, and

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Environ. Sci. Technol. XXXX, XXX, XXX−XXX

preventing downstream upsets of nitritation (see Table S1). With TAA, failure of a DO probe or exposure of the community to high levels of DO, like those maintained in this study (7−9 mg/L DO), would not result in nitrate production because the selection pressure that is preventing the growth of NOB and nitrate production is repeated exposures to FA and FNA, not low DO.

Comparing across different processes such as DEMON,[20] OLAND,[5,6] SNAD,[7] SNAP,[8,21] and DIB,[22] the main advantage of DS/SBR is stable and complete nitritation without the need for pH set-point control. There is little indication of NOB acclimation to FNA, consistent with prior observations that dual stressors can prevent bacterial adaptation compared to a single stressor.[73] In most existing nitritation processes, pH monitoring and set-point control are required to achieve complete nitritation. System perturbations will occur if a pH probe fails resulting in an alkali overdose that inhibits AOB. Coupling of a DS/SBR with a downstream denitritation process can thus potentially improve the reliability and stability of nitrogen removal. For anammox applications, the use of a DS/SBR could potentially ensure stable production of nitrite and more precise control of the ratio of ammonium to nitrite. For CANDO applications, DS/SBR operation could prevent nitrate perturbations and ensure synchronized nitritation/denitritation. TAA is useful when a wastewater that contains ammonia has insufficient buffer for neutralization of the nitrous acid formed when the ammonia is oxidized, depleting the alkalinity at pH 6.5 where nitrite oxidation to nitrate is inhibited and allowing high pH exposures to NH_3. Research is needed to assess the applicability of this approach for mainstream wastewater flows, industrial wastewater, and landfill leachate.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.est.0c04613.

Strategies for nitritation and nitrogen removal from anaerobic digester centrate; summary of inhibition coefficients by free ammonia and free nitrous acid on AOB and NOB; operational decisions and the rationale for each; algorithms and sample calculation used to calculate the concentration of alkali stocked needed for TAA; nitrogen balance of SS/SBR and DS/SBR at day 100; 16S rRNA gene abundance in clone libraries on day 100; amoA gene abundance in clone libraries on day 100 (PDF)

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Notes

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Environ. Sci. Technol. XXXX, XXX, XXX−XXX