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**Comparing Resistance, Resilience, and Stability of Replicate Moving Bed Biofilm and
Suspended Growth Combined Nitrification-Anammox Reactors**

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ABSTRACT

Combined partial nitrification-anammox (PN/A) systems are increasingly employed for sustainable nitrogen removal from wastewater, but process instabilities present ongoing challenges for practitioners. The goal of this study was to elucidate differences in process stability between PN/A process variations employing two distinct aggregate types—biofilm (in moving bed biofilm reactors, MBBRs) and suspended growth biomass. Triplicate reactors for each process variation were studied under baseline conditions and in response to a series of transient perturbations. MBBRs displayed elevated NH_4^+ removal rates relative to suspended growth counterparts over six months of unperturbed baseline operation, but also exhibited significantly greater variability in performance. Transient perturbations led to strikingly divergent yet reproducible behavior in biofilm versus suspended growth systems. A temperature perturbation prompted a sharp reduction in NH_4^+ removal rates with no NO_2^- accumulation and rapid recovery in MBBRs, compared to a similar reduction in NH_4^+ removal rates but strong NO_2^- accumulation in suspended growth reactors. Pulse additions of a nitrification inhibitor (allylthiourea) prompted only moderate declines in performance in suspended growth reactors compared to sharp decreases in NH_4^+ removal rates in MBBRs. Quantitative FISH demonstrated a significant enrichment of anammox in MBBRs compared to suspended growth reactors, and conversely a proportionally higher AOB abundance in suspended growth reactors. Overall, MBBRs displayed significantly increased susceptibility to transient perturbations employed in this study compared to suspended growth counterparts (stability parameter), including significantly longer recovery times (resilience). No significant difference in maximum impact of perturbations (resistance) was apparent. Taken together, our results suggest that aggregate architecture (biofilm versus suspended growth) in PN/A processes exerts an unexpectedly strong influence on process stability.

TOC Art

Biofilm Carriers

Suspended Growth

INTRODUCTION

Combined partial nitrification and anammox in a single reactor (PN/A) is a promising low energy strategy for removing nitrogen (N) from wastewater, thereby limiting detrimental environmental and public health impacts of reactive N release to receiving water bodies. PN/A bioprocesses rely on coordinated activities of two groups of slow-growing microorganisms: 1) aerobic ammonia-oxidizing bacteria (AOB) that oxidize ammonium (NH_4^+) to nitrite (NO_2^-), a process termed nitrification; and 2) anaerobic ammonia-oxidizing bacteria (anammox) that couple NH_4^+ oxidation and reduction of NO_2^- to nitrogen gas (N_2). PN/A processes also rely on robust suppression of unwanted nitrite-oxidizing bacteria (NOB). While full-scale application of PN/A reactors is increasing, particularly for sidestream treatment of high strength anaerobic digester supernatant¹, unexplained process instabilities that occur even after extended periods of stable operation present a key challenge to practitioners²⁻⁸. Two common sources of process instabilities are short-term declines in activity of key microbial populations, particularly AOB, that are thought to be due to transient pulses of inhibitors in reactor influent or to operational instabilities (e.g. aeration or temperature fluctuations), and long-term accumulation of undesirable NOB⁸. Inhibition of anammox by common wastewater constituents, including heavy metals, phosphates, NO_2^- , O_2 , and H_2S , has also been reported to be problematic for PN/A process instability^{2, 9}. At present, we lack a full understanding of both the susceptibility and resilience of different PN/A process variations to sources of instabilities. Addressing this knowledge gap is important to facilitate more predictive strategies to control or avoid process instabilities in sidestream systems, and to facilitate robust implementation of mainstream PN/A processes where strongly fluctuating influent conditions are the norm¹⁰.

Coordinated activity of AOB and anammox is facilitated by growth of these microbial

guilds in close proximity in microbial aggregates, which allows cross-feeding of NO_2^- produced by AOB to anammox bacteria in PN/A reactors. Two common PN/A process variations leverage distinct types of microbial aggregates: 1) suspended growth biomass^{11, 12} and 2) biofilms, including those grown on carriers in moving bed biofilm reactors (MBBRs)¹³ or as granules^{14, 15}. In both process variations, spatial segregation of microbial activities (and possibly populations) mediated by mass transfer limitations are critical for process performance¹⁶, but little is known about the influence of aggregate characteristics—namely, biofilm versus suspended growth biomass—on process stability. Likewise, the reproducibility (and therefore predictability) of fluctuations in process stability often observed in such systems is as yet unclear.

Despite its importance, studies on environmental bioprocess stability are often qualitative, thereby impeding quantitative comparisons of process variations under different conditions¹⁷. Moreover, for practical reasons, comparisons of bioprocess stability generally have included only one replicate per process variation^{18, 19}. Such comparisons contribute to our understanding of different bioprocesses, but limit assessment of generalizability, reproducibility, and statistical significance of any observed differences. Parallel replicated bioprocesses coupled to quantitative assessments of stability differences are strongly desirable to robustly compare PN/A process variations. Quantitative metrics for clarifying both functional (macroscale performance) and compositional (community structure) stability abound in the ecological literature^{20, 21}, and recent work has demonstrated the utility of selected ecological metrics for environmental bioprocess characterization, including in anaerobic reactors²², biofilters²³, and denitrifying reactors²⁴.

The aim of this study is thus to improve our understanding of how PN/A process stability is modulated by aggregate architecture (biofilm versus suspended growth). To this end, we

performed side-by-side comparisons of triplicate lab-scale PN/A reactors with two different types of microbial aggregates: suspended growth biomass and biofilm carriers (MBBRs). Operation of identical independent MBBRs and suspended growth reactors in triplicate, each fed with real anaerobic digester supernatant, is a critical and novel aspect of this study, in that it enables a statistically robust comparison of variations in process stability under real-world conditions. The specific goals of this study were two-fold: 1) to assess differences in stability and reproducibility in process performance between replicate MBBR and suspended growth PN/A systems under unperturbed baseline conditions; and 2) to characterize resistance, resilience, and overall stability of process variations to transient pulse perturbations in the form of a temperature disturbance and dosage of low levels of the nitrification inhibitor allylthiourea (ATU).

MATERIALS AND METHODS

Reactor Setup, Operation, and Monitoring

Six 12L custom-built lab-scale reactors were split into triplicates of two process variations (MBBR and suspended growth) and operated as PN/A reactors for 480 days. Triplicate MBBRs (herein M1, M2, and M3) were started up at 33% fill ratio with colonized AnoxKaldnes K5 biofilm carriers (specific surface area=800 m²/m³, depth=4 mm, diameter=25 mm²⁵) from the ANITA Mox system at the Sundets Wastewater Treatment Plant (WWTP) (Växjö, Sweden). Triplicate suspended growth reactors (herein S1, S2, and S3) were inoculated with biomass from the sidestream PN/A reactor at the Werdhölzli WWTP (Zürich, Switzerland). As is typical for sidestream nitrification/anammox processes^{26,27}, suspended growth biomass consisted of predominantly flocs (~97% total biomass) with a small fraction of reddish granules (~3% total

biomass). Mass fractions are based on sieving and solids (VSS) measurements, as reported by Shi et al. 2016²⁸. Anaerobic digester supernatant from the Werdhölzli WWTP was employed as influent. Supernatant composition is summarized in Table 1. All reactors were temperature controlled to 30°C (typical of sidestream PN/A systems) and operated as sequencing batch reactors (SBRs) with online monitoring and data logging of NH_4^+ , NO_3^- , pH, temperature, conductivity, and redox potential.

All reactors were fed in the initial SBR step to 12L (150-200 mg NH_4^+ -N/L, based on influent composition). Suspended growth reactors were then continuously aerated during the react period to a dissolved oxygen (DO) setpoint of 0.24 mg/L, while MBBRs were operated with a DO setpoint of 0.75 mg/L. DO setpoints were chosen based on typical values in full-scale facilities^{12,13}. With the exception of DO setpoint and a settling phase for suspended growth reactors, all six reactors were operated under equivalent operational conditions. SBR operation was controlled via online NH_4^+ measurement by ion selective electrodes (Endress+Hauser, Switzerland). Reactor DO and cycle time based on online NH_4^+ -N measurements were controlled via a dedicated programmable logic controller. NH_4^+ depletion rates reported herein are based on the aerated react period of SBR operation. A decrease of NH_4^+ to 30 mg NH_4^+ -N/L triggered the end of the aerated react period and the start of a 1 hour anoxic period to draw down residual NO_2^- , followed by a 1 hour settling period in suspended growth reactors, and a 25% volume decantation for both reactors. Under poor settling conditions, two 1-hour stirred anoxic and settling steps were used at times in suspended growth reactors. To select for maximal retention of anammox-laden biomass, no sludge wasting was performed during reactor operation. Solids retention time (SRT) was thus controlled by effluent sludge loss. Based on periodic effluent TSS and VSS measurements, SRT in suspended growth reactors was >50 days

throughout the experimental period. Hydraulic retention time (HRT) varied based on cycle time, and averaged 24-48 hours. pH was not directly controlled due to ample alkalinity (67 ± 10 mmol/L) in reactor influent. pH varied between 7.4 and 8.4 during SBR cycles.

Fluorescence In-Situ Hybridization (FISH) was employed for periodic quantification and visualization of AOB, NOB, and anammox in reactor biomass, as previously described^{29, 30} (Table S2). Detailed descriptions of FISH and reactor monitoring methodologies (including routine biomass monitoring) are provided in the supplementary information.

Operational Phases

The 480 day operational period for PN/A reactors was divided into three distinct phases. *Phase I* (Days 1-33) consisted of inoculation and reactor startup. Phase I was deemed complete upon achievement of stable and high (>90%) NH_4^+ removal and limited accumulation of NO_3^- (<15% of NH_4^+ -N removed) and NO_2^- (<2.5% of NH_4^+ -N removed). *Phase II* (Days 34-222) encompassed a period of baseline operation. The intent of Phase II was to assess differences in long-term process stability between MBBR and suspended growth process variations in the absence of intentional process perturbations, and to assess reproducibility in performance characteristics between replicate bioreactors of the same process variation. In *Phase III* (Days 223-480), we employed a series of transient perturbations (pulse disturbances) to assess differences in resistance, resilience, and overall stability between process variations. We report two sets of perturbations: 1) a transient temperature disturbance (Days 257-264), in which temperature control was discontinued for 24h, during which reactor temperatures declined from $\sim 30^\circ\text{C}$ to 16°C , followed by reinstatement of reactor heating; and 2) a series of pulses of allylthiourea (ATU), a specific inhibitor of AOB activity, to reactors during the aerobic react step

(Days 337-395.) ATU is thought to inhibit AOB by chelating the copper metal cofactor of ammonia monooxygenase³¹. Three concentrations of ATU (500, 800, and 1100 µg/L) were chosen to promote partial, but not complete, AOB inhibition, based on results from Joss et al.⁸ and Munz et al.³². Perturbation 1 (temperature disturbance) was designed to assess susceptibility to temperature fluctuations. Perturbation 2 (pulses of ATU) was designed to clarify variation in resistance and resilience to AOB activity fluctuations.

Resistance, Resilience, and Stability Metrics

We employed the “amplification envelope” method to quantify response of PN/A process variations to transient perturbations. The amplification envelope method originates in the ecology^{22, 33, 34} literature, and is designed to measure functional stability of communities and ecosystems (e.g. microbial bioreactors) in response to disturbances. The method tracks the proportional deviation in an operationally defined response variable to a perturbation³⁴. For the purposes of this study, we define the response variable as percent decrease in NH_4^+ depletion rate relative to the average of the previous five unperturbed reactor cycles (taken here as the referential state). Several metrics can be defined based on the amplification envelope. **Resistance** is a measure of the sensitivity of a system to disturbance²⁴, and quantifies the maximum impact of a perturbation (here the maximum percent decrease in NH_4^+ depletion rate, or maximum inhibition). It should be noted that a high resistance metric indicates low resistance (e.g. increased response to perturbation). **Resilience** (also called Return Time^{33, 34}) is a measure of the temporal impact of a perturbation, and quantifies the time needed for a system to return to a referential state. Similar to the resistance metric, a high resilience metric indicates an extended recovery time, and therefore increased response to a perturbation. Finally, an overall **stability**

parameter incorporates elements of both resistance and resilience. The stability parameter is the area under the amplification envelope²⁴. Analogous to resilience and resistance parameters, a high stability parameter indicates increased response to a perturbation. The reader is directed to Fig. 3 for an illustration of quantification of resistance, resilience, and stability parameters in response to a pulse disturbance in this study.

An additional metric employed to quantify process stability under baseline unperturbed conditions was the coefficient of variation (CV; standard deviation divided by mean) of NH_4^+ depletion rate. Weekly CV values encompassed 20-30 SBR cycles, and are a measure of variability, and therefore stability, in performance of each reactor within each week of operation.

RESULTS AND DISCUSSION

Process Stability Under Baseline Operational Conditions.

All six PN/A reactors displayed excellent overall performance in terms of effluent quality during 189 days of baseline operation (Phase II) with no intentional process perturbations. Reactors maintained >90% NH_4^+ -N removal efficiency and >80% total N removal efficiency (Fig. S2), with limited accumulation of NO_3^- and NO_2^- . Due to the use of actual anaerobic digester supernatant, influent NH_4^+ and sCOD varied substantially over this time period (500-1000 mg NH_4^+ -N/L and 250-700 mg sCOD/L; Table 1 and Fig. S1). Average biomass concentration in replicate MBBRs increased over the baseline period of operation from 3570 to 5250 mgTSS/L (13.7 to 20.2 gTSS/m²), while average suspended growth reactor biomass concentration decreased from 4370 to 2870 mgTSS/L (Fig. S3). Concentrations of NO_2^- during the aerobic react period averaged 4 and 0.7 mgN/L for MBBRs and suspended growth reactors, respectively (Fig. S4). These values are in line with observations in full-scale sidestream PN/A reactors^{12, 13}.

Effluent NO_2^- measurements were low ($1.7 \pm 1.1 \text{ mgN/L}$, or on average $\sim 0.3\%$ of $\text{NH}_4^+\text{-N}$ removed) for all reactors. NO_3^- effluent levels averaged 30 mgN/L , or approximately 5% of total NH_4^+ removal (Fig. S5). Theoretical NO_3^- production from PN/A stoichiometry is 11.1% of $\text{NH}_4^+\text{-N}$ depletion¹⁵. NO_3^- accumulation well under this theoretical value, as in this study, has been observed previously in PN/A reactors^{12, 19}, and has been attributed to denitrification employing either external or endogenous organic carbon.

Measured NH_4^+ depletion rates in both MBBRs and in suspended growth reactors across the baseline period of operation were comparable to those documented previously for both process variations^{11-13, 19, 35}. NH_4^+ depletion rates in MBBRs were significantly higher than in suspended growth reactors ($p < 0.001$, Student's t-test) (Table S2), as has been observed in previous studies^{12, 13}. MBBRs averaged $0.65 \pm 0.18 \text{ kg-N/m}^3 \cdot \text{d}$ ($0.14 \pm 0.04 \text{ kgN/kgTSS} \cdot \text{d}$), with maximum observed single SBR cycle rate of $1.15 \text{ kg-N/m}^3 \cdot \text{d}$. Suspended growth reactors displayed notably slower removal rates, with an average of $0.36 \pm 0.08 \text{ kg-N/m}^3 \cdot \text{d}$ ($0.11 \pm 0.04 \text{ kgN/kgTSS} \cdot \text{d}$) and maximum observed rate of $0.58 \text{ kg-N/m}^3 \cdot \text{d}$.

In addition to superior average NH_4^+ depletion rates, MBBRs also displayed significantly greater variability in performance over time compared to suspended growth reactors during the baseline period of operation, even when depletion rates were averaged over replicate reactors and across distinct weeks of operation (Fig. 1, Fig. S6). All replicate MBBRs experienced a pronounced decline in N removal rate between days 82 and 152. Day 110 represented the nadir in this decline, with weekly average NH_4^+ removal rates sinking to $0.31\text{-}0.41 \text{ kg-N/m}^3 \cdot \text{d}$. MBBR performance subsequently recovered to initial rates. In contrast, suspended growth reactor performance, while exhibiting lower NH_4^+ removal rates on average than their MBBR counterparts, was relatively stable over time.

Within-Week Variability and Co-Variation in Process Performance During the Baseline Period of Operation. To quantify stability in reactor performance over time, we calculated the weekly coefficient of variation (CV) in NH_4^+ depletion rate individually for all six reactors (Fig. S7, Table S2). Variability within MBBR reactors (M1-M3) was higher in most time points than suspended growth reactors (S1-S3), despite sporadic elevated values in the latter. This observation is highly statistically significant ($p < 0.001$, Student's t-test). The weekly average CV for MBBR reactors was 0.14, versus 0.09 for suspended growth reactors (Table S2).

In addition to greater performance variability, dynamics in process performance co-varied to a greater extent between replicate MBBRs compared to replicate suspended growth reactors over the baseline period of operation. Pearson correlation coefficients for association of weekly NH_4^+ depletion rates between reactors are shown in Table S3. MBBR performance correlated strongly and significantly ($p < 0.001$, Student's t-test) to other MBBRs in all pairwise comparisons between M1, M2, and M3. This result suggests that MBBR PN/A process performance and dynamics are reproducible under the conditions employed in this study. In contrast, results for suspended growth reactors were more ambiguous. NH_4^+ depletion rates in S1 were not significantly correlated to those in S3, but the two other possible comparisons (S1 to S2, and S2 to S3) were significant ($p < 0.001$, Student's t-test). As discussed above, suspended growth reactors also displayed less variability in process performance over time than MBBRs, so it is possible that the lack of statistically significant co-variation in selected reactor pairings could simply be due to the small observed variations in average weekly NH_4^+ depletion rate around a relatively stable mean performance.

Process Stability in Response to a Transient Temperature Perturbation.

Following assessment of process stability during the baseline period of operation (Phase II), we employed a series of simulated transient perturbations to further clarify differences in resistance, resilience, and overall stability between MBBR and suspended growth PN/A systems (Phase III). First, susceptibility to temperature fluctuations was characterized via a sudden cessation in reactor temperature control (Fig. 2). From an applied standpoint, clarifying susceptibility of process variations to temperature fluctuations is relevant given that sidestream systems often experience operational instabilities or dynamics that transiently impact temperature^{12, 19}. It is also an important consideration for emerging strategies for semi-continuous bioaugmentation of low temperature mainstream PN/A processes with anammox-enriched biomass from high temperature sidestream reactors³⁶.

Temperature in reactors M1-M3 and S1-S3 normally ranged between 25°C (just after reactor filling) and 30°C (middle and end of SBR cycles). Absent reactor heating, temperature declined over 24 hours from 30 to 16°C (Fig. 2, top). Reactor heating was then reinstated, and normal operating temperatures were achieved within 12 hours. As expected, NH_4^+ depletion rates dropped in all reactors during the temperature decline (Fig. 2, middle). Average NH_4^+ depletion rates fell in MBBR replicates from $0.55 \pm 0.09 \text{ kgN/m}^3 \cdot \text{d}$ just prior to the perturbation to a minimum of $0.30 \pm 0.05 \text{ kgN/m}^3 \cdot \text{d}$. In suspended growth reactors, average NH_4^+ depletion rate just prior to the perturbation was $0.17 \pm 0.05 \text{ kg-N/m}^3 \cdot \text{d}$, and declined to a minimum of $0.11 \pm 0.02 \text{ kg-N/m}^3 \cdot \text{d}$. All reactors regained previous NH_4^+ depletion rates within 1 day of reinstated temperature control. The average decline in NH_4^+ depletion rate (percent inhibition) was higher in MBBRs than in suspended growth reactors (43% versus 32% inhibition), but the difference in means was not statistically significant ($p=0.05$ level, Student's t-test). Similarly, MBBRs

displayed elevated average resistance, resilience, and stability metrics (elevated average response to perturbation, based on 2 hour rolling averages of NH_4^+ depletion rate) compared to suspended growth reactors (Fig. S8), but differences in means were not statistically significant ($p=0.05$ level, Student's t-test) based on this single perturbation.

MBBR and suspended growth reactors displayed dramatically different patterns of accumulation of the key intermediate NO_2^- during the temperature perturbation (Fig. 2, bottom). NO_2^- did not significantly accumulate relative to baseline levels in MBBRs ($p=0.05$ level, Student's t-test). In contrast, strong and significant NO_2^- accumulation was apparent in all suspended growth reactors ($p<0.05$ for S1, $p<0.001$ for S2 and S3, Student's t-test). S1 displayed a relatively subtle response, with NO_2^- levels rising to maximum of 8 mgN/L. S2 and S3 displayed far greater NO_2^- accumulation, with maximum measured levels of 54 and 76 mgN/L, respectively. Interestingly, NO_2^- drawdown was rapid in all reactors following reintroduction of temperature control. Despite numerous reports of inhibitory impacts of NO_2^- on anammox activity^{2, 37, 38}, no adverse impacts on performance were apparent. Recent reports provide estimates of 50% inhibitory concentrations of NO_2^- on anammox activity of between 100 and 400 mgN/L^{37, 39}, while earlier studies documented strong anammox inhibition at NO_2^- concentrations as low as 5 mgN/L¹¹. It is possible that a more severe temperature perturbation coupled to increased NO_2^- accumulation would lead to inhibition of this process⁹.

Few assessments of PN/A performance in response to short-term temperature disturbances are available in the literature. Lackner and Horn¹⁹ also compared response to a temperature perturbation in a single lab-scale MBBR and a suspended growth PN/A system, with volumetric N loading rates similar to those employed in this study. While the range of temperatures employed was similar to this study, the use of larger reactors (115 and 200L)

necessitated increased time (~7 days) for both cooling and warming. In contrast to our results, Lackner and Horn measured extremely high NO_2^- accumulation (100-600 mgN/L) coupled with almost complete loss of NH_4^+ removal capacity in both MBBR and suspended growth reactors. Recovery of system performance took significantly longer than observed in our study—namely, 20 and 40 days for MBBR and suspended growth reactors, respectively, putatively due to NO_2^- toxicity. Notably, both reactors maintained significantly higher O_2 concentrations (1.5 mg/L in the MBBR and 0.4 mg/L in the suspended growth reactor) than employed in our study. The difference in O_2 concentrations coupled to the longer exposure time to elevated NO_2^- may be at least partially responsible for the starkly different responses and recovery times observed by Lackner and Horn compared to our work. It should be noted that the relationship between NO_2^- concentration, contact time, degree of anammox inhibition, and requisite recovery time in PN/A systems is still poorly understood and warrants further study.

Lotti et al.³⁶ employed ex-situ batch activity assays of anammox and PN/A biomass to assess the impact of short-term temperature changes on anammox activity. Intriguingly, Lotti and colleagues suggest that AOB displayed less temperature dependence (based on literature values) than anammox, thus providing a possible explanation for NO_2^- accumulation in some PN/A systems undergoing temperature fluctuations via an excess of AOB relative to anammox activity. Biomass from only one non-granular system was explored by Lotti, but results from this free cell suspension suggested that anammox activity in suspension was more influenced by a temperature decrease than biofilm (granular) biomass. Lotti's results are in broad agreement with reproducible patterns of activity decline coupled to NO_2^- accumulation in suspended growth reactors in our study, compared to limited NO_2^- accumulation (presumably linked to elevated anammox activity or capacity, and/or limited AOB activity) in MBBR reactors. Our results also

corroborate observations by Gilbert et al.¹⁸ of increased propensity for NO_2^- accumulation in a single suspended growth reactor compared to biofilm (MBBR or granular) PN/A reactors subjected to long-term (20 week) declines in temperature, though it should be noted that Gilbert et al. employed substantially lower N loading rates than those employed in this study.

Process Stability in Response to Pulses of an Inhibitor of Aerobic Ammonia Oxidation

Our results demonstrate pronounced differences in response of MBBR versus suspended growth PN/A systems to transient temperature perturbations, with unexpected and highly significant between-process variations in propensity for NO_2^- accumulation. NO_2^- is the product of AOB (nitrification) activity. Our results thus suggested differing susceptibilities to variations in AOB activity, with excess activity in suspended growth reactors leading to elevated de-coupling of AOB and anammox metabolisms compared to MBBRs. Such variations in AOB activity have been proposed to be a potentially important source of process instabilities in PN/A systems. Indeed, Joss and colleagues⁸ demonstrated that process failures in suspended growth PN/A systems can be initiated by partial inhibition of AOB activity, which in turn leads to the secondary effect of anammox inhibition due to temporarily reduced O_2 depletion and corresponding NO_2^- accumulation.

To clarify potential variations in resistance to such AOB activity fluctuations between biofilm and suspended growth systems, we subjected all reactors to a series of pulses of the nitrification inhibitor ATU. Three concentrations of ATU (500, 800, and 1100 $\mu\text{g/L}$) were employed in independent perturbation experiments to promote partial, but not complete, AOB inhibition. ATU has been shown to have no significant impact on anammox activity^{40, 41}. As expected, NH_4^+ depletion rates declined in all reactors for all ATU pulses (Fig. 3). Complete

recovery of pre-perturbation performance was achieved in all reactors within 1.5 days.

The amplification envelope method revealed substantial and significant differences in response to ATU perturbations in MBBR versus suspended growth reactors. For illustrative purposes, application of the amplification envelope method to quantify resistance, resilience, and overall stability metrics in response to the 500 $\mu\text{g/L}$ ATU pulse is shown in Fig. 3. Resilience, resistance, and overall stability metrics for all reactors in response to the three levels of ATU pulses are summarized in Fig. 4. At the 500 $\mu\text{g/L}$ level, MBBRs displayed significantly elevated resilience ($p < 0.005$, Student's t-test) and overall stability ($p < 0.05$, Student's t-test) metrics, indicating substantially enhanced response to this perturbation. Interestingly, the resistance metric was not significantly different between MBBR and suspended growth process variations at the $p = 0.05$ level, suggesting that the maximum impact of AOB inhibition was similar, and that the observed enhanced overall stability in suspended growth reactors is due in large part to faster return times (resilience metric). The average resistance, resilience, and stability metrics for MBBRs in response to 800 and 1100 $\mu\text{g/L}$ ATU pulses were higher than those for suspended growth reactors, again suggesting potentially elevated response to perturbations, but differences in means were not significant at the $p = 0.05$ level (Student's t-test). However, when responses to perturbations were considered across all ATU levels, suspended growth reactors demonstrated statistically significant reduced overall response (stability parameter; $p < 0.005$, Student's t-test) and recovery times (resilience metric; $p < 0.001$, Student's t-test) relative to MBBRs. Similarly, consideration of all perturbations (temperature and ATU) together indicated highly statistically significant reduced response (stability parameter; $p < 0.001$, Student's t-test) and recovery times (resilience metric; $p < 0.01$, Student's t-test) in suspended growth reactors compared to MBBRs (Fig. S9). Mirroring results for 500 $\mu\text{g/L}$ ATU pulses, the resistance metric was not significantly

different between suspended growth and MBBR reactors.

Joss and colleagues⁸ spiked a lab-scale suspended growth PN/A reactor (analogous to the suspended growth reactors in this study) with 1250 $\mu\text{g/L}$ ATU, leading to a strong decrease in NH_4^+ removal rate, with associated NO_2^- accumulation due to a putative “secondary effect” of anammox inhibition from exposure to excess O_2 supply resulting from decreased AOB activity. Contrary to our expectations, in response to pulses of ATU, NO_2^- did not accumulate in either suspended growth or MBBR systems in the current study ($p=0.05$ level, Student’s t-test). These results may contrast to those reported by Joss due to lower ATU doses employed here. Nonetheless, the patterns of NO_2^- accumulation observed in our study suggests that anammox activity was not adversely impacted in either process variation. The enhanced stability of suspended growth reactors to ATU pulses also suggests that nitrification activity (the source of NO_2^-) was somewhat less impacted in suspended growth systems relative to MBBRs.

Taken as a whole, our results demonstrate strikingly divergent behavior in response to transient perturbations in biofilm versus suspended growth PN/A reactors. MBBRs displayed enhanced susceptibility (decreased NH_4^+ depletion rate) to disturbances under the scenarios assessed here, and suspended growth reactors displayed a marked propensity under some conditions for NO_2^- accumulation. These differences are statistically significant and reproducible across replicate independent reactors.

Population Abundance and a Conceptual Model Linking Aggregate Architecture to Resistance, Resilience, and Stability in PN/A Process Variations.

In addition to distinct patterns of response and recovery to multiple transient perturbations, MBBR and suspended growth reactors displayed strong and reproducible differences in

abundance of key microbial guilds (Table 2). Anammox were more abundant in MBBRs than in suspended growth reactors across the experimental period, based on quantitative FISH ($p < 0.05$, Student's *t*-test). Conversely, AOB were highly enriched in suspended growth reactors compared to MBBRs ($p < 0.001$, Student's *t*-test). NOB were near or below the detection limit (0.5%) in all samples measured, demonstrating robust outcompetition of this unwanted microbial guild. These results suggest at least the potential for additional AOB capacity in suspended growth reactors compared to MBBRs, and conversely suggest elevated anammox capacity in MBBRs. The measured relative abundances of AOB in MBBRs were substantially lower than predicted by consideration of stoichiometry and previously estimated growth yields alone⁴²⁻⁴⁴. The apparent "excess" anammox abundance relative to AOB in MBBRs parallels observations from previous experimental studies^{18, 42} and from 1-D mathematical biofilm models that predict a layered biofilm structure comprised of a thick inner layer dominated by anammox overlain by a thin outer layer dominated by AOB in PN/A biofilms⁴⁵⁻⁴⁷. The outer biofilm layers are subject to elevated erosion and biomass loss, and therefore maintain a substantially lower SRT than inner anammox-dominated layers, thus providing a likely explanation for the disparity in population abundances. Importantly, only a fraction of the thick anammox-dominated inner layer in typical deep biofilms is predicted to be active in 1-D biofilm models, suggesting that a "reservoir" of inactive anammox may be present in most biofilm systems. This apparent reservoir of anammox capacity coupled to low relative abundance of AOB in MBBRs may have critical implications for resistance, resilience, and stability in response to dynamic conditions and transient perturbations in PN/A reactors.

In order to synthesize our results and highlight putative connections between macroscale patterns of resistance, resilience, and stability, abundance of key functional groups, and

mesoscale aggregate characteristics, a conceptual model of aggregate architecture in biofilm (MBBR) and suspended growth PN/A reactors is presented in Fig. S10. This conceptual model is based on accumulated evidence presented here and in previous studies, including a combination of qualitative and quantitative FISH observations^{27,48-50} (Fig. S11 and S12), patterns of resistance, resilience, and stability of process performance, and predictions from mathematical biofilm models^{45,47,51}. In the MBBR (biofilm) environment, our conceptual model includes a highly heterogeneous environment with a clear layering of both microbial populations and activities, with a thick under layer predominated by anammox and a thin overlying layer of AOB at low relative abundance directly exposed to the bulk liquid (and to any nitrification inhibitors [e.g. ATU] therein) (Fig. S10 left, Fig S11). In the suspended growth environment, in contrast, our conceptual model depicts a much more evenly distributed microbial environment, with a complex and relatively homogenous mixture of AOB and anammox, and significantly greater percentage of AOB as a fraction of the biomass (Fig. S10 right, Fig. S12). This conceptual model coupled to our results suggest that MBBR performance may be strongly limited by AOB activity, and thus that MBBRs may display low resistance and stability in response to transient perturbations that strongly impact AOB activity (e.g. toxic inhibitors of nitrification, including ATU, as observed in this study). Conversely, this model also implies that the apparent excess “reservoir” of anammox capacity in PN/A MBBRs, coupled to increased mass transfer limitations in biofilms (such as on biofilm carriers) relative to floccular growth, may provide a degree of shielding against anammox inhibition events that can be caused by exposure to excess O₂ in response to decreased AOB activity⁸, thus limiting propensity for NO₂⁻ accumulation (as observed in this study). In contrast, this model coupled to our results suggests that, in suspended growth PN/A systems, excess AOB capacity (mirrored by higher AOB abundance) relative to

MBBRs apparently limits the impact of small doses of nitrification inhibitors (such as ATU) but also increases propensity for rapid NO_2^- accumulation (as observed in this study). Taken together, these results suggest that suspended growth systems may be more resistant, resilient, and stable in response to perturbations that decrease aerobic ammonia oxidation activity (e.g. inhibitory toxics [represented in this study by ATU] in the influent), while MBBR systems may be more resistant and resilient to perturbations that predominantly impact anammox activity (such as a decrease in O_2 consumption or an increase in O_2 supply rate). Further testing of this hypothesis will require additional replicated perturbation experiments under a variety of conditions (particularly those that predominantly impact anammox activity), coupled with characterization of microscale variations in aggregate morphology, stratification in microbial populations and activities, and mass transfer limitations in biofilm carriers and suspended growth biomass.

We also wish to stress that the common process variations explored in this study constitute opposite ends of a spectrum of bioprocesses that combine elements of biofilm and suspended growth aggregates. Indeed, hybrid systems that incorporate both biofilm-laden carriers and substantial floccular biomass are now undergoing testing for PN/A process implementation⁵². Based on our results, it is tempting to speculate that combining suspended growth and biofilms in hybrid systems may be beneficial to both limiting propensity for NO_2^- accumulation in suspended growth systems, and also mitigating susceptibility to sharp decreases in substrate (NH_4^+) removal rates in MBBRs in response to transient perturbations. In effect, hybrid systems may offer the “best of both worlds” with regards to process stability. The validity of this hypothesis remains to be tested.

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SUPPORTING INFORMATION

Details on analytical methods and reactor biomass monitoring, FISH and associated imaging (CLSM) methodology, MBBR process variability during baseline period of operation, and population segregation in PN/A MBBRs. Tables showing FISH probes, average and maximum ammonium depletion rate and average weekly CV in ammonium depletion rate by reactor, Pearson correlation coefficients for co-variation in ammonium depletion rates between reactors, and FISH-based relative abundance of microbial populations in MBBR biofilm and suspended growth biomass. Figures showing influent sCOD and ammonium, percent N removal in reactors, reactor biomass concentrations, reactor nitrite concentrations, percent ammonium converted in reactors to nitrate, ammonium depletion rate normalized to biomass, weekly CV of ammonium depletion rates, amplification envelope analysis of response of PN/A bioreactors to transient temperature perturbation, amplification envelope analysis of response of PN/A bioreactors to all transient perturbations, conceptual model of aggregate architecture in PN/A biofilm carriers and suspended growth flocs, and representative FISH micrographs of key microbial populations in PN/A biofilm carriers and in suspended growth.

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List of Figures and Tables

Figure 1. NH_4^+ depletion rates over 189 days of baseline operation in MBBR and suspended growth reactors. NH_4^+ depletion rates are shown averaged weekly over triplicate reactors in each process variation. One week for each process variation encompasses 20-30 SBR cycles in each of three separate reactors.

Figure 2. Temperature, average cycle NH_4^+ depletion rate, and with-in reactor NO_2^- accumulation during transient temperature perturbation. Temperature (top) is shown for reactor S3, but is representative of all reactors. Black arrows represent cessation and restart of reactor heating (setpoint 30°C). NH_4^+ depletion rates (middle) represent full SBR cycle averages. M1, M2, and M3 are replicate MBBRs; S1, S2, and S3 contain suspended growth biomass. NO_2^- measurements were all taken during react steps of SBR cycles. NO_2^- accumulation relative to baseline reactor operation was statistically significant in all suspended growth reactors (S1: $p < 0.05$, S2 and S3: $p < 0.001$, Student's t-test), but was not statistically significant in all MBBRs (M1-M3).

Figure 3. Illustration of amplification envelope method for 500 $\mu\text{g/L}$ ATU pulse for reactors M1 (MBBR) and S1 (suspended growth). Only two reactors are shown for ease of interpretation; the remaining 4 reactors qualitatively matched patterns of response to perturbations illustrated here. The black vertical arrow indicates start of perturbation. A) 2 hour rolling average NH_4^+ depletion rate for M1 and S1 in response to pulse perturbation of ATU (500 $\mu\text{g/L}$). B) Percent inhibition (decrease) in NH_4^+ depletion rate relative to previous five unperturbed cycles. Percent inhibition in NH_4^+ depletion rate is employed as the response variable to perturbations for quantification of resistance, resilience, and stability parameters using the amplification envelope method. Quantification of resistance (%) and resilience (d) is illustrated here. The area of the amplification envelope (integral of the response to perturbation) was used as an overall stability metric.

Figure 4. Resistance (Top), Resilience (Middle), and Stability Parameter (bottom) metrics in MBBR and suspended growth reactors in response to pulses of the specific inhibitor of aerobic ammonia oxidation ATU. Average values across triplicate reactors are shown. Error bars

represent standard deviations. MBBR replicates displayed significantly elevated stability parameters ($p < 0.05$, Student's t-test) and resilience ($p < 0.005$, Student's t-test) (significantly greater response to perturbation) for the 500 $\mu\text{g/L}$ ATU pulse relative to suspended growth reactors. Resistance was not significantly different between process variations. Average stability parameter and resilience metric were also higher for MBBRs than suspended growth reactors for pulses of 800 and 1100 $\mu\text{g/L}$ ATU, but differences in means were not statistically significantly different at the $p = 0.05$ level (Student's t-test) when perturbations were assessed independently.

Table 1. Summary of the composition of anaerobic digester supernatant supplied as influent to the PN/A reactors. All parameters except alkalinity were measured weekly over the experimental period. Alkalinity was measured periodically to verify stability over time, particularly in response to new batches of supernatant.

Table 2. Relative abundance based on 16S rRNA-targeted FISH of anammox (oligonucleotide probes Amx820 and Bfu613), AOB (NEU, Nso1225, and Cluster 6a 192), and NOB (Ntspa662 and Ntspa712) normalized to total bacteria (EUB mix) in inocula for MBBRs and for suspended growth reactors and during transient perturbation experiments with ATU (day 370).

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Parameter	Average value (± Standard Deviation)
NH ₄ ⁺ (mgN/L)	710±123
NO ₂ ⁻ (mgN/L)	<1
NO ₃ ⁻ (mgN/L)	<1
N _{tot} (mgN/L)	747±117
PO ₄ ³⁻ (mgP/L)	18±8
Alkalinity (mmol/L)	67±10
sCOD (mg/L)	378±103
sCOD/N _{tot}	0.55±0.15
tCOD (mg/L)	712±236
tCOD/N _{tot}	0.98±0.23

Table 2. Relative abundance based on 16S rRNA-targeted FISH of anammox (oligonucleotide probes Amx820 and Bfu613), AOB (NEU, Nso1225, and Cluster 6a 192), and NOB (Ntspa662 and Ntspa712) normalized to total bacteria (EUB mix) in inocula for MBBRs and for suspended growth reactors and during transient perturbation experiments with ATU (day 370).

		Anammox (%)	AOB (%)	NOB (%)
MBBRs	Inoculum	48.6±8.8	2.1±0.8	ND*
	M1 (Day 370)	55.6±7.4	0.5±.3	ND*
	M2 (Day 370)	29.6±9.5	0.6±.1	ND*
	M3 (Day 370)	46.1±17.9	0.8±0.2	ND*
Suspended Growth Reactors	Inoculum	36.5±2.2	4.9±1.6	ND*
	S1 (Day 370)	24.2±5.8	6.0±1.4	ND*
	S2 (Day 370)	15.6±6.9	7.3±1.0	ND*
	S3 (Day 370)	16.6±2.8	6.4±1.7	ND*

* ND=below detection limit (0.5%)