Sustained organic loading perturbation inhibits nitrite oxidation in bioreactors, unveiling variable resistance and resilience of nitrification and nitrifiers

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Abstract

Sustained perturbations are of interest in environmental biotechnology as they can lead systems to alternative stable states that may not be reversible. Studies assessing these concerns are scarce as robust replication is required. Here, we tested the effect of sustained organic loading variations (food-to-biomass ratio, F:M; carbon-to-nitrogen ratio, C:N) on both structure and function of activated sludge bacterial communities, focusing on nitrifiers. Two sets of four replicate 5-liter sequencing batch reactors were operated at two different, low and high, F:M (0.19 and 0.36) and C:N (3.5 and 6.3) conditions for a period of 74 days, following 53 days of sludge acclimation at low F:M and C:N (F:M-C:N) from a full-scale plant inoculum. Resilience was tested during the last 14 days by operating all eight reactors at low F:M-C:N. Samples were analyzed using metagenomics, 16S rRNA gene amplicon sequencing, and effluent characterization. High F:M-C:N reactors exhibited different ecosystem functions and nitrifier abundance compared to the ones at low F:M-C:N. Perturbed high F:M-C:N reactors displayed quantifiable and initially variable functional resistance. Stable nitrite accumulation (77%) was achieved through high F:M-C:N loading with concurrent suppression of *Nitrospira*, revealing a new partial nitrification strategy for nitritation-denitritation systems. Subsequently, only two of the four reactors experiencing a switch back from high to low F:M-C:N recovered the nitrification function, with an increase in *Nitrobacter* (r-strategist) abundance as the predominant NOB replacing the niche initially occupied by *Nitrospira* (K-strategist). Overall, the AOB community was more diverse and resilient than the NOB community. We showed that functional resistance and resilience can vary across replicate reactors in a closed system, and that nitrification resilience need not coincide with a return to the initial nitrifying community structure.

1. Introduction

Improving stability and optimizing performance of wastewater treatment processes is a central aim of environmental engineering and biotechnology, as it would help to achieve the sustainable development goal of guaranteeing availability and sustainable management of water and sanitation for everyone (Osborn et al. 2015). In ecology, disturbances are believed to have direct effects on the stability of ecosystems by altering community diversity and function (Cain et al. 2014). For engineered systems like activated sludge bioreactors, it is important to identify the effect of different disturbances on the microbial community diversity so as to relate them to changes in process performance (Briones and Raskin 2003). Sustained perturbations or press disturbances that impose a long-term continuous change of species densities through an alteration of the environment (Bender et al. 1984, Shade et al. 2012a) can lead to alternative stable states that may or may not be reversible in terms of both community composition and function (Botton et al. 2006). So far press disturbance studies on wastewater treatment systems have employed harsh perturbations like toxic aromatic pollutants (Falk and Wuertz 2010) or washout of organisms through sludge discharge (Bagchi et al. 2015, Vuono et al. 2015). Moreover, such perturbations are often beyond normal operational or environmental ranges, which hinders the practical application of their findings in biotechnology. Hence, the effect of sustained and practically relevant perturbations on sludge communities remains uncertain.

Alterations in the environment that are not directly detrimental for organisms but still provide opportunities for low abundance members within the community are also considered a disturbance (Rykiel 1985). In bioreactors, a switch in the substrate feeding scheme employed could then elicit changes in community structure and functionality. Such perturbations can occur in the form of organic shocks within activated sludge (Bassin et al. 2016, Hu et al. 2011, Seetha et al. 2010) and anaerobic reactor systems (Ketheesan and Stuckey 2015, Senturk et al. 2014). The food-to-biomass ratio (F:M) or sludge loading rate is an important parameter in wastewater treatment as it determines the growth type and settleability of sludge microorganisms (Gray 2004). Moreover, the F:M is also related to the carbon-to-nitrogen ratio (C:N) as both depend on the amount of organic carbon in the feed. However, the few studies describing the effect of alterations in F:M on sludge microbial communities often neglect

uncontrolled covariations in C:N (Cardinali-Rezende et al. 2013, Monsalvo et al. 2012), despite the fact that C:N variations are known to affect nitrification (Okabe et al. 1996). This is also overlooked in studies where F:M values change through modifications in solids retention time (SRT) (Bagchi et al. 2015, Vuono et al. 2015) and organic carbon loading rate (OLR) (Meerburg et al. 2016, Pholchan et al. 2010).

Partial nitrification is an important stage in biological nitrogen removal from wastewater via nitritation/anammox (Laureni et al. 2016), for which the accumulation of nitrite is desired by promoting the growth of ammonia oxidizing bacteria (AOB) while suppressing nitrite oxidizing bacteria (NOB) (Ma et al. 2015). Different strategies have been employed with this objective in mind like varying temperature, dissolved oxygen (DO), pH, SRT, and substrate concentrations (Sinha and Annachhatre 2007). Influent manipulation in particular has been reported to yield contrasting results in terms of C:N (Ge et al. 2015). Low C:N values (2-3 mg COD mg TN^{-1}) have been shown to promote partial (Tan et al. 2012), but also complete (Ma et al. 2013) nitrification in different sludge systems. Additionally, although adjustments in C:N also impact F:M values by altering the available carbon for heterotrophic growth, there are no controlled studies assessing the effect of both factors simultaneously.

Disturbance and community stability are essentially related, as stability is defined as the response of a community to disturbance (Rykiel 1985). Stability is comprised of resistance and resilience, which are two quantifiable metrics useful for comparing community disturbance responses as previously shown for microbial ecology (Allison and Martiny 2008, Griffiths and Philippot 2013). Resistance is defined as the degree to which a community is insensitive to a disturbance, and resilience as the capacity of a community to return to a pre-disturbance condition (Ingrisch and Bahn 2018). The stability of a community can be studied in terms of functional or compositional parameters (Shade et al. 2012a). For functions that are carried out by many taxa, variations in community composition might not match variations in function performance (Allison and Martiny 2008). Alternatively, for functions performed by only a few taxa (such as nitrification), the sensitivity and resilience of these particular functions may closely follow changes in the abundance of those taxa (Shade et al. 2012a). Studies of resistance and resilience in complex engineered microbial systems are scarce (Cabrol et al. 2016, Vuono et al. 2015)

and often lack the replication required to ensure reproducibility and capture variability that could be associated with perturbations.

The aim of this work was to test the effect of a sustained perturbation (press disturbance) of doubling F:M and C:N values within typical wastewater treatment operational ranges in a set of replicated lab-scale bioreactors after an acclimation period, focusing on nitrification and nitrifiers. Dynamics in community function were monitored throughout the study by periodic analysis of reactor effluent, as well as detailed temporal studies of reactor cycles at seven different time points. Changes in nitrifying organisms were assessed by metagenomics and 16S rRNA gene amplicon sequencing. The resistance of the ecosystem function was evaluated by monitoring its transition to a different functional steady state after the perturbation. We further tested the functional resilience of the perturbed reactors by shifting the F:M and C:N back to the original state.

2. Materials and Methods

2.1. Experimental design

The experiment was conducted using eight 5-liter bioreactors inoculated with activated sludge from a water reclamation plant in Singapore and operated as sequencing batch reactors (SBR) on continuous 12-h cycles. Initially, four reactors were acclimated to lab conditions and fed with complex synthetic wastewater for 53 days (for details see Supplementary Information). At the start of the experiment (d54), the sludge of the acclimation reactors was thoroughly mixed and redistributed across eight reactors. From these, four were randomly selected and designated as high F:M and C:N reactors, receiving double the carbon substrate in terms of chemical oxygen demand (COD) amount in its feed as a press disturbance for 60 days. The remaining four reactors were operated as before at low F:M and C:N. During the last two weeks of the study (d114-d127), the feed for the high F:M and C:N reactors was adjusted to equal that of low F:M and C:N reactors (Table 1). For the sake of simplicity, we will refer to both F:M and C:N as F:M-C:N.

2.2. Functional parameters

In each cycle, the DO concentration was controlled at 2 – 6 mg/L during the aerobic phase, and the pH ranged from 6 – 9, owing to alkalinity provided in the feed. The complex synthetic feed was adapted from (Hesselmann et al. 1999) (Supplementary Information). The reactor temperature was maintained at 30°C and sludge was continuously mixed with a magnetic stirrer. SBR phases were: 5 min feed, 200 min anoxic/anaerobic react, 445 min aerobic react, 50 min sludge settle, and 20 min supernatant drain. Two cycles per day accounted for an hydraulic retention time (HRT) of 24 h. Effluent and influent compositions were measured 2-3 times per week in accordance with Standard Methods (APHA-AWWA-WEF 2005). The targets were soluble COD, total alkalinity, and nitrogen species (ammonium, nitrite and nitrate) in the liquid phase using colorimetric tests and ion chromatography. Nitrite accumulation percentage in the effluent was calculated as the ratio of nitrite concentration and the sum of nitrate and nitrite concentrations. Total organic carbon and total Kjeldahl nitrogen (TKN) were also measured in the influent. To control the F:M, sludge biomass was measured as total (TSS)

and volatile suspended solids (VSS) twice a week, after which sludge wastage was done to target 1500 mg/L of TSS. Sludge volume index (SVI) was calculated from the liquid and sludge volumes measured in the reactors after 30 min settling and the TSS values obtained in the same cycle. Microbial community function was also investigated in the form of intensive sampling (every 30 to 60 min) over seven 12-h cycle studies conducted throughout the experiment.

2.3. Bioreactor arrangement

Each of the SBRs employed in this study was equipped with: a magnetic stir plate to ensure mixed liquor homogeneity, a pair of EasySense pH and DO probes with their corresponding transmitters (Mettler Toledo), a dedicated air pump, a dedicated feed pump, a solenoid valve for supernatant discharge, and a surrounding water jacket connected to a re-circulating water heater. The different portions of the cycle were controlled by a computer software specifically designed for these reactors (VentureMerger, Singapore).

2.4. 16S rRNA amplicon sequencing and reads processing

Bacterial 16S rRNA amplicon sequencing was done in two steps (for details see Supplementary Information). Primer set 341f/785r targeted the V3-V4 variable regions of the 16S rRNA gene (Thijs et al. 2017). The libraries were sequenced on an Illumina MiSeq platform (v.3) with 20% PhiX spike-in and at a read-length of 300 bp paired-end. Sequenced sample libraries were processed following the DADA2 bioinformatics pipeline (Callahan et al. 2016) using the version 1.3.3 of the *dada2* R-package. DADA2 allows inference of exact amplicon sequence variants (ASVs) providing several benefits over traditional OTU clustering methods (Callahan et al. 2017). Illumina sequencing adaptors and PCR primers were trimmed prior to quality filtering. Sequences were truncated after 280 and 255 nucleotides for forward and reverse reads, respectively, length at which average quality dropped below a Phred score of 20. After truncation, reads with expected error rates higher than 3 and 5 for forward and reverse reads were removed. After filtering, error rate learning, ASV inference and denoising, reads were merged with a minimum overlap of 20 bp. Chimeric sequences (0.18% on average) were identified and removed. For a total of 104 samples, 19679 reads were kept on average per sample after processing,

representing 49.2% of the average input reads. Taxonomy was assigned using the SILVA database (v.132) (Glöckner et al. 2017). Adequacy of sequencing depth after reads processing was corroborated with rarefaction curves at the ASV level.

2.5. Metagenomics sequencing and reads processing

Libraries were sequenced in one lane on an Illumina HiSeq2500 sequencer in rapid mode at a final concentration of 11pM and a read-length of 250 bp paired-end. In total, around 325 million paired-end reads were generated, with 3.4 ± 0.4 million paired-end reads on average per sample (total 48 samples). Illumina adaptors, short reads, low quality reads or reads containing any ambiguous bases were removed using *cutadapt* (Chen et al. 2014). High quality reads (91.0 ± 1.4% of the raw reads) were randomly subsampled to an even depth of 4,678,535 for each sample prior to further analysis. Taxonomic assignment of metagenomics reads was done following the method described by Ilott et al. (2016). High quality reads were aligned against the NCBI non-redundant (NR) protein database (March 2016) using DIAMOND v.0.7.10.59 (Buchfink et al. 2015) with default parameters. The lowest common ancestor approach implemented in MEGAN Community Edition v.6.5.5 (Huson et al. 2016) was used to assign taxonomy to the NCBI-NR aligned reads with the following parameters (maxMatches=25, minScore=50, minSupport=20, paired=true). On average, 36.8% of the high-quality reads were assigned to cellular organisms, of which 98.4% were assigned to the bacterial domain. Adequacy of sequencing depth was corroborated with rarefaction curves at the genus taxonomic level.

3. Results

3.1. Dynamics in ecosystem function

During the acclimation phase, the F:M and C:N values were maintained at around 0.21 (mg COD mg TSS^{-1} d^{-1}) and 3.5 (mg COD mg TKN^{-1}), respectively (Table 1). Ammonium concentrations in the effluent decreased gradually while nitrate concentrations increased (Fig. 1). Sludge related parameters like settleability (SVI) and biomass fraction (VSS:TSS) varied during this acclimation period (Supplementary Fig. S1). Most of these variations decreased after 30 d and trends were stable after 45 d.

During the perturbation phase of the study (d54 onwards), more sludge was wasted so as to better control the F:M (Supplementary Fig. S1), which is the reason why the SRT for the low F:M-C:N reactors is lower than during the acclimation phase. The average F:M and C:N values for the low F:M-C:N reactors were similar to those during the acclimation phase (0.19 and 3.5), while the ones for the high F:M-C:N reactors were controlled to be almost double (0.36 and 6.3). As expected, controlling for a higher F:M resulted in a lower SRT in reactors subjected to this treatment (Table 1). This period showed a clear distinction between high and low F:M-C:N reactors in terms of nitrification and organic carbon removal, with the high F:M-C:N reactors displaying partial nitrification with high NO_2-N and COD effluent concentrations. To ensure that the partial nitrification was due to different F:M-C:N values and not a lack of available dissolved oxygen, we increased the aeration rate from 1 to 4 L min^{-1} from d97 onwards without observing significant changes in effluent compounds. The last two weeks of the study involved shifting operational parameters in the high F:M-C:N reactors to match those of the low F:M-C:N ones (Table 1). During this period a transition towards recovery of the nitrification function was observed, with high variability of effluent values for NO_2-N, NO_3-N and COD across reactors (Fig. 1).

Among the high F:M-C:N replicates, reactor three (R3) had an air diffuser blockage on d64 that impeded proper aeration, affecting the performance of this reactor compared to others. Nitrification was completely inhibited and the COD and NH_3-N residuals were high (Supplementary Fig. S2). The diffuser problem was solved when the aeration rate was increased on d97. R3 effluent variables evolved

to approach those of the other high F:M-C:N reactors, matching the consumption/generation pattern of N compounds during the aerobic phase, although still showing a diminished COD degradation ability during the anoxic phase, as observed in the cycle study on d110 (Supplementary Fig. S3). For this reason we did not include R3 in the reported average parameters for high F:M-C:N reactors, but the recovery of the nitrification function in this reactor after d97 is discussed.

3.2. Dynamics in nitrification and nitrifiers

Nitrite accumulation was found in the effluent of high F:M-C:N reactors, together with a higher residual COD (Fig. 1). The acclimation phase (d1-d53) displayed negligible nitrite accumulation at 0.3% (±1.5%). Low F:M-C:N reactors had zero percent nitrite accumulation after d61 and only 1.1% (±2.0%) during the first week (d54-d60). However, high F:M-C:N reactors showed an initial transient nitrite accumulation of 18% (±21%) on d54-d60, which subsequently increased and stabilized at 77% (±6.0%) during the d61-d113 period. Finally, after shifting from high to low F:M-C:N conditions, nitrite accumulation decreased to 55% (±29%) in the first week (d114-d120) and to 24% (±35%) in the second week (d121-d127).

Among nitrifiers, the three most abundant bacterial genera detected through DNA sequencing were *Nitrospira*, *Nitrosomonas* and *Nitrobacter* (Fig. 2). Similar patterns of nitrifier abundance across low and high F:M-C:N replicates were observed for both metagenomics and 16S rRNA gene amplicon sequencing datasets. After the acclimation phase *Nitrospira* was suppressed in high F:M-C:N reactors, while *Nitrosomonas* remained at around half the abundance levels observed for low F:M-C:N reactors (Fig. 2). This coincided with the accumulation of nitrite in high F:M-C:N reactors during the d54-d113 period of the study (Fig. 1). The fourth and fifth most abundant nitrifying genera, *Nitrosospira* and *Nitrolancea*, were only detected through metagenomics sequencing at very low (~0.01%) relative abundances (Supplementary Fig. S4). *Nitrosospira* abundance decreased during the course of the study, but similar to *Nitrosomonas* abundance was higher for the low F:M-C:N reactors. On the other hand, *Nitrolancea* abundance increased throughout the experiment, being higher for the low F:M-C:N reactors from d96 onwards. Interestingly, after the aeration problem in R3 was fixed, the relative abundances of *Nitrosomonas*, *Nitrosospira* and *Nitrolancea* returned to the same level as that of the other high F:M-

C:N reactors, which is commensurate with the observed partial recovery of the nitrification function for this reactor (Supplementary Fig. S2).

Following the shift from high to low F:M-C:N it was *Nitrobacter* that rose to be the dominant NOB instead of *Nitrospira*, but only in two of four reactors (Fig. 2). Variations in performance among replicate reactors were also evident from cycle study profiles before (d110) and after (d124) the shift in feeding regime for the high F:M-C:N reactors (Fig. 3). Two weeks after the change, only two high to low F:M-C:N reactors displayed functional profiles similar to those of the low F:M-C:N reactors. The reactors which recovered functionality were the same as those that registered around 1% of *Nitrobacter* abundance (Fig. 2).

The higher resolution of 16S rRNA gene amplicon sequencing allowed us to taxonomically identify four ASVs as *Nitrospira*, twenty-one as *Nitrosomonas*, and one as *Nitrobacter* (Fig. 4). From these, only two ASVs were identified at the species level. The genus *Nitrospira* was dominated by the *N. defluvii* species, with the other three ASVs detected only at low abundances across the low F:M-C:N reactors after d97, the day the aeration rate was increased. The dominant *Nitrosomonas* ASVs during the perturbation phase were different from the initial ones. Furthermore, *N. europaea* and *Nitrosomonas* ASV-70 saw their relative abundances increasing with time across high F:M-C:N reactors (Fig. 4).

4. Discussion

4.1. Function stabilization and nitrifier dynamics during acclimation phase

Sludge acclimation served to stabilize important functions like nitrification, organic carbon removal, and sludge settling capacity across reactors (Fig. 1, Supplementary Fig. S1). The only initially abundant nitrifier that prevailed after the acclimation stage was *Nitrospira*. The next most abundant nitrifying genus, *Nitrosomonas*, increased sixfold (Fig. 2) and the changes in abundance of different *Nitrosomonas* ASVs suggested a succession of organisms within this genus (Fig. 4). Additionally, through the use of exact sequence variants (Callahan et al. 2017) we could observe that *Nitrosomonas* was the most diverse nitrifying genus with twenty-one different ASVs being detected. The C:N used during the acclimation phase (3.5 mg COD mg TKN^{-1}) was lower in comparison to that of the full-scale plant from where the inoculum was collected (~6.5 mg COD mg TKN^{-1}), which meant that more N was available for *Nitrosomonas* and *Nitrospira* to grow under similar F:M conditions as in the full-scale plant (0.21 mg COD mg TSS^{-1} d^{-1}).

4.2. Disturbance leads to stable partial nitrification unveiling system's resistance

Ecosystem function in terms of COD removal, ammonia removal, and complete nitrification was optimal and stable for the low F:M-C:N reactors, particularly towards the end of the study (Fig. 1). On the other hand, press disturbed reactors (high F:M-C:N) experienced a reduction in nitrification and COD removal in comparison. The shift in function was not immediate as the first seven days registered transient nitrite accumulation, which was variable among replicates, before stabilizing with little within-treatment variability for the rest of the perturbation phase. The increase in residual nitrite likely had a negative effect on COD removal due to the known toxicity of NO_2^- to bacteria (Philips et al. 2002).

Nitrification was not completely inhibited because 23% of the NO_X-N products still consisted of nitrate during the d61-d113 period. This percentage constitutes a quantitative measure of resistance for the nitrification function, as it shows the degree to which this particular function was insensitive to disturbance (Pimm 1984). The higher variability in function observed during the first seven-day transition period is an example of the stochastic effects that a disturbance initially triggers in ecosystems

(Zhou and Ning 2017). Since the disturbance was sustained in this case, selective pressure likely promoted deterministic mechanisms resulting in a less variable function over time (Fig. 1). This is similar to what has been previously reported for studies on sludge bioreactors under sustained 3-chloroaniline perturbation (Santillan et al. 2019a).

4.3 Dynamics of nitrifiers during the perturbation phase

Understanding community and activity dynamics of nitrifying bacteria is essential for improving design and operation of wastewater treatment biological processes (Nowka et al. 2015). The suppression of *Nitrospira* in the high F:M-C:N reactors (Fig. 2) was likely due to competition for DO with heterotrophs and *Nitrosomonas* that possess a higher affinity for DO (Garrido et al. 1997, Okabe et al. 2011). The same competition for DO occurs between *Nitrosomonas* and heterotrophs, which explains the observed lower abundance of this genus in high F:M-C:N reactors. It is known that AOB have a higher oxygen affinity than NOB (Blackburne et al. 2008). Still, the increase in aeration rates from d97 onward did not prevent nitrite accumulation, indicating that a low DO in the system was not the main reason behind our observations. DO concentrations higher than 1 mg L^{-1} are enough to achieve optimal nitrification performance (Gonzalez et al. 2009), which was the case for the reactors in this study (Supplementary Fig. S3). However, nitrifying communities grow in stratified biofilms where AOB are located closer to the water interface and NOB are in the interior zone (Lydmark et al. 2006, Okabe et al. 1999). The concentration of oxygen deep inside a biofilm or floc is lower than in the mixed liquor. Moreover, stratification in AOB biofilms due to an increase in C:N has been reported (Okabe et al. 2011), highlighting that increases in biofilm thickness due to heterotrophic growth further reduce oxygen diffusion inside, which is detrimental to the growth of NOB. In our study, the period during the aerobic phase of a cycle when almost all ammonia had been removed and COD concentrations were either low or remained constant (around 400-500 min, Fig. 3) implies that heterotrophs and AOB were not competing with NOB for oxygen anymore. Although this should have provided plenty oxygen to NOB to be active during the rest of the aerobic phase, an increase in nitrate production was not observed. A possible reason for this could be nitrite accumulation, which was reported to be toxic to NOB at high concentrations (Anthonisen et al. 1976). In summary, the observed NOB suppression at high F:M-C:N

could have been due to a combination of competition for DO with heterotrophs and *Nitrosomonas*, reduced oxygen diffusion into the nitrifier biofilm due to heterotrophic growth, and nitrite accumulation due to AOB activity.

AOB growth rates are normally higher than those of NOB at 30 °C, which implies that the SRT can be reduced to achieve partial nitrification (Lackner et al. 2014). In our study, increasing F:M while keeping TSS constant implied a SRT reduction of 35% in the high F:M-C:N reactors compared to the low F:M-C:N ones. It is conceivable that part of the observed reduction in nitrifiers was due to washout given their low growth rates. In a mathematical model, Wu et al. (2016) suggested that reducing the SRT has a stronger effect on NOB than on AOB. However, the SRT of 8.2 d used to operate the high F:M reactors is common in activated sludge processes performing complete nitrification (Tchobanoglous et al. 2003), and just above the operating SRT of 5-6 d at the full-scale plant that provided the sludge inoculum for this study. Thus, our observed changes in nitrifiers and nitrification function are more likely driven by changes in F:M and C:N values than by washout due to low SRT.

4.4 Importance of assessing F:M, C:N and SRT simultaneously for nitrification studies

We showed that a combined high F:M-C:N approach led to stable and reproducible nitrite accumulation (77%) after seven days of transition. This finding holds promise as a new partial nitrification strategy to achieve biological nitrogen removal from wastewater via partial nitritation/anammox (Ge et al. 2015). Similarly to our results, conditions at high F:M resulted in higher nitrite accumulation compared to low F:M in studies on full-scale sludge systems that focused on the effect of varying F:M directly (Wu et al. 2013) or indirectly through changes in SRT (Meerburg et al. 2016, Vuono et al. 2015). However, contrary to our results low influent C:N values (1 to 3 mg COD mg NH_4-N^{-1}) in high-strength industrial wastewaters were reported to yield partial nitrification in a review of full-scale anammox processes (Lackner et al. 2014). Likewise, Tan et al. (2012) reported better nitrite accumulation at low C:N (~2 mg COD mg TN^{-1}) on aerobic SBRs. On the other hand, there was complete nitrification without nitrite accumulation at low influent C:N (3 ± 1 mg COD mg TN^{-1}) in pilot-scale membrane batch reactors (Ma et al. 2013), which is similar to our results. Furthermore, increasing C:N molar ratios from 2 to 5 was shown to significantly reduce nitrification

rates in a laboratory denitrification-nitrification system (Ballinger et al. 2002), although the study did not mention whether this caused nitrite accumulation. These opposing findings in the literature imply that other factors in addition to C:N affected nitrification. As neither of the aforementioned studies manipulating C:N controlled the covariations in F:M, its role cannot be disentangled. Hence, we emphasize the need to evaluate these interconnected parameters simultaneously.

In our study we showed that doubling the amount of COD in the influent almost doubled both F:M and C:N values, as operational TSS and influent TKN were controlled to remain close to constant. Double influent COD also doubled the OLR. More COD also resulted in higher cell growth, thus more sludge had to be wasted to keep the TSS constant, reducing the SRT as a consequence. This illustrates how important operational parameters like SRT, C:N and OLR also covary with F:M (Tchobanoglous et al. 2003), reason why they have to be evaluated simultaneously during experimentation. However, this does not seem to be the case in practice. A recent study on the effect of reducing the SRT from 30 to 3 days in a full-scale plant (Vuono et al. 2015) reported a washout of *Nitrospira* and an associated decrease in nitrogen removal efficiency and nitrite accumulation, but this happened at a expense of a ten-fold increase in F:M (0.03 to 0.33 d^{-1}). Additionally, the C:N values for such study are unknown since influent COD and TKN concentrations were not reported. We warn that manipulations of SRT, without controlling for covariations in F:M and C:N, can lead to questionable results about nitrifiers and nitrification dynamics due to uncontrolled confounding factors.

4.5 Resilience of ecosystem function and nitrifiers

Since perturbations often occur in biotechnological systems, it is desirable to understand the mechanisms of recovery after disturbance (Cabrol et al. 2016), for which experimental replication is paramount to ensure reproducibility and capture fluctuations in process instability (Wells et al. 2017). Hence, we tested the effect of returning the high F:M-C:N to their previous low levels on the functional resilience of the perturbed reactors. The switch back had noticeable effects on nitrifier communities (Fig. 2, Fig. 4, and Supplementary Fig. S4), as well as on the nitrification function (Figs. 1 and 3). Relative abundances of *Nitrosomonas*, *Nitrosospira*, and *Nitrolancea* genera recovered quickly after returning to pre-perturbation conditions. Although there is little information about *Nitrolancea* (Sorokin

et al. 2012), its rapid recovery after disturbance suggests that it could be categorized as an r-strategist, along with the known r-strategists *Nitrosomonas* and *Nitrosospira* (Dytczak et al. 2008). High F:M-C:N reactors exhibited variable functional resilience because after ten days of returning to pre-perturbation conditions only two of three reactors completely recovered the nitrification function. This inconsistency among independent replicate reactors could be due to the stochastic growth after disturbance typically associated with r-strategists (Pianka 1970, Santillan et al. 2019b). Hence, nitrification recovery after removing the high F:M-C:N perturbation seemed to be due to stochastic colonization by r-strategist NOB (*Nitrobacter*), replacing the niche initially occupied by K-strategist NOB (*Nitrospira*) before perturbation. Besides having a fast growth rate (Blackburne et al. 2007) and prevailing at alternating conditions (Dytczak et al. 2008), *Nitrobacter* has been shown to thrive at higher DO (Huang et al. 2010) and nitrite (Wu et al. 2013) concentrations, which explains why its abundance increased in the previously high F:M-C:N reactors after the shift to low F:M-C:N conditions. However, as part of a secondary succession process after disturbance, it is possible that *Nitrospira* would have recovered as a dominant NOB if more time had been allowed in the study. Another example of resilience was the reactor (R3) that recovered the nitrification function and nitrifier community when aeration was restored and mimicked that of the remaining high F:M-C:N reactors (Figs. 3 and 4). *Nitrobacter* abundance did not increase in R3 during the period we assessed, which is consistent with the higher susceptibility to disturbance associated with NOB (Graham et al. 2007).

Our reactors constituted a closed system, which implies that the *Nitrobacter* colonizers came from low-abundance seed-bank populations. It was proposed that disturbance opens niches for bacterial colonization in open sludge systems (Vuono et al. 2016), but we showed that recruitment of organisms from the seed-bank is also possible. Moreover, as immigration of nitrifiers into sludge systems has been shown to alter the local community composition and function (Jauffur et al. 2014, Yu et al. 2018), studies of nitrification resilience under SRT changes in open sludge systems (Vuono et al. 2015) cannot disentangle the effect of immigrating populations from their observations.

Here we showed that functional resilience greatly differed from nitrifying community resilience. Reactors with recovered function after returning to low F:M-C:N conditions (Fig. 3) remained distinct

from the control reactors in terms of NOB composition (Fig. 2). The fact that an altered community can perform the same functions as the original one supports the idea of functional redundancy (Allison and Martiny 2008) and the insurance hypothesis (Yachi and Loreau 1999). This finding is similar to that of Vuono et al. (2015) in an unreplicated full-scale sludge system after a press disturbance, but contrary to what was reported for lake microbial systems after a pulse disturbance (Shade et al. 2012b). These contrasting reports highlight the complexity of assessing disturbance-diversity-function relationships (Santillan 2018), as they depend not only on the system assessed but also on the disturbance frequency (i.e., pulse or press). Finally, the variability in the recovery of the nitrification function and fluctuations in nitrifier populations could only be captured thanks to the replication employed in the design of our study. As we move towards stable operation for microbial resource management (Cabrol et al. 2016), future perturbation studies on sludge bioreactors should be robustly designed to ensure process reproducibility and highlight operational ranges where functional variability can be encountered.

5. Conclusions

- A combined high F:M and C:N approach for NOB suppression can be used to achieve stable and reproducible nitrite accumulation (around 77%) in activated sludge at tropical temperatures. This approach is relevant to nitritation-denitritation systems, particularly for industrial wastewater treatment applications.
- Variations in interconnected operational parameters in bioreactors (such as F:M, C:N, OLR and SRT) should be assessed simultaneously to better understand their effects on nitrification and nitrifiers, as well as on microbial community function and structure.
- Functional resistance and resilience can be variable across identically operated reactors, highlighting the importance of replication for perturbation studies.
- Resilience of the nitrification function can differ from nitrifying community structure resilience, as reactors with recovered function after returning from high to low F:M and C:N conditions remained distinct from the low F:M and C:N reactors in terms of the predominant nitrite oxidizers present.
- The partial return of complete nitrification after removing the high F:M and C:N perturbation seemed to be due to stochastic colonization by seed-bank r-strategist NOB (*Nitrobacter*), replacing the niche initially occupied by K-strategist NOB (*Nitrospira*) before perturbation. Hence, immigration of new taxa was not required.
- Controlled perturbation studies on sludge communities using parameters within the range of plant operation can lead to not only ecologically but also practically meaningful insights.

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Author Contributions

ES and SW conceived the study. ES designed the experiment. SW obtained the funding for the study. ES and WXP performed the experiments and conducted laboratory and molecular analyses (except library preparation and sequencing). ES performed the 16S rRNA gene bioinformatics analyses. FC performed the metagenomics bioinformatics analyses. ES interpreted the data, generated the results, and elaborated the main arguments in the introduction and discussion. ES and SW wrote the manuscript. FC and WXP critically reviewed the manuscript.

Data availability

DNA sequencing data will be available at NCBI BioProjects upon publication. Supplementary information related to this article can be found in the online version, including details about sludge inoculum and acclimation phase, complex synthetic wastewater preparation, chemical analysis, DNA extraction and purification, and 16S rRNA gene and metagenomics library preparation and sequencing.

Competing interests

The authors declare no competing interests.

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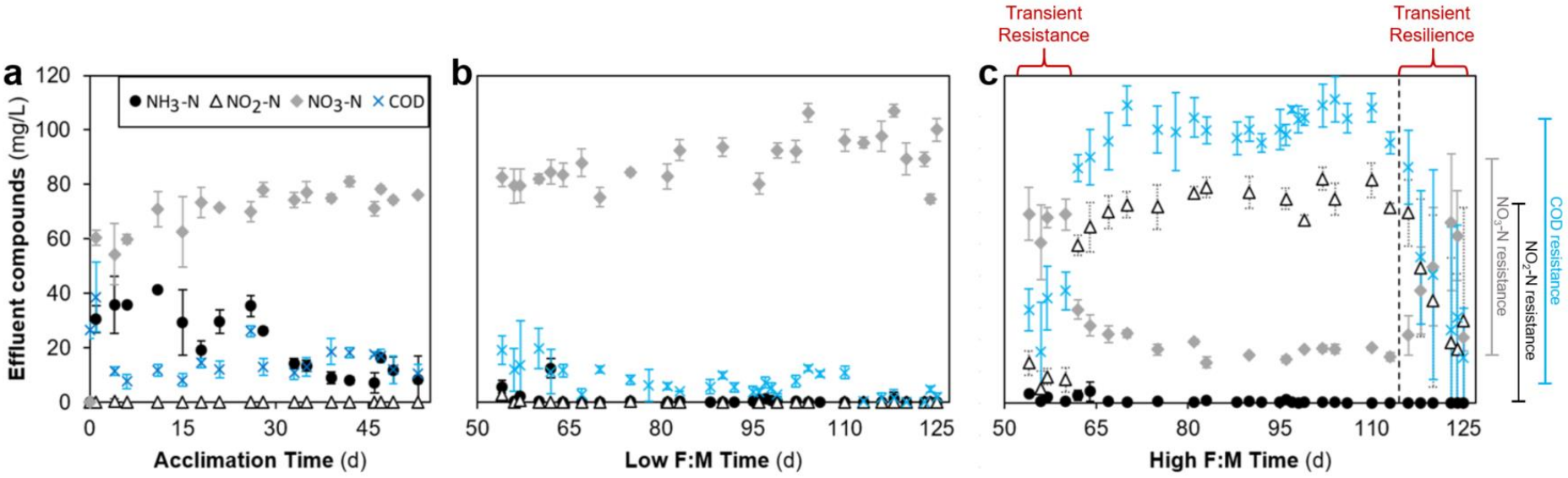

Fig. 1. Temporal average effluent concentrations of NH_3-N, NO_2-N, NO_3-N and soluble COD. (**a**) Acclimation phase (n = 4), (**b**) low F:M-C:N phase (n = 4), (**c**) high F:M-C:N phase (n = 3) (R3 not included). Error bars represent one s.d.m. Vertical dashed line indicates the shift from high to low F:M-C:N. Periods of transient functional resistance and resilience are indicated in red in (**c**), where longer error bars denote higher variability among replicates. Average resistance levels for affected functions are indicated to the right side of panel (**c**).

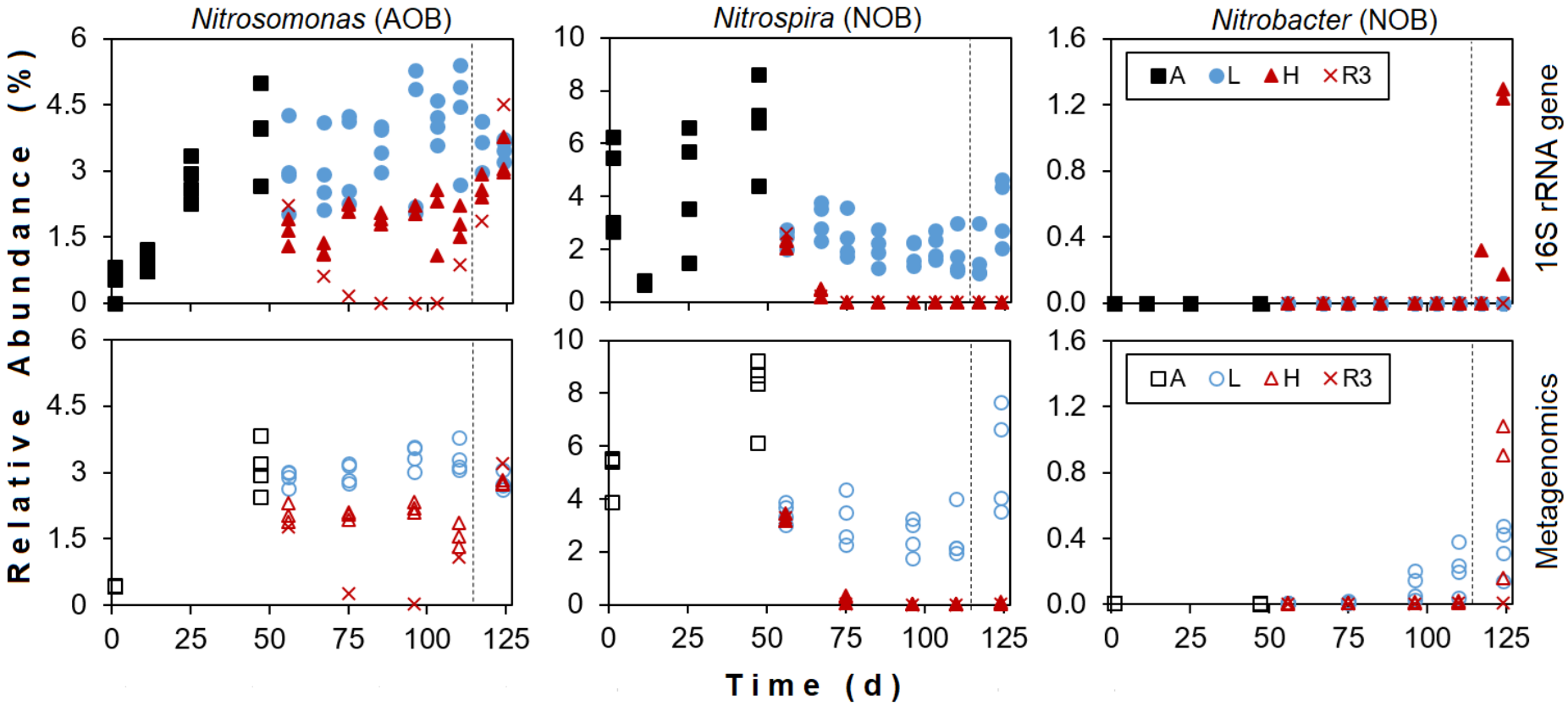

Fig. 2. Temporal relative abundance of main nitrifier genera in each reactor. Phases: A, acclimation; L, low F:M-C:N; H, high F:M-C:N; and R3, reactor three. Vertical dashed line indicates the shift from high to low F:M-C:N. Upper panels display 16S rRNA gene amplicon ASV data and lower panels shotgun metagenomics data. Left, *Nitrosomonas*; centre, *Nitrospira*; right, *Nitrobacter*. AOB (*Nitrosomonas*) display more resistance and resilience than NOB. Variable NOB resilience is due to the increase in *Nitrobacter* replacing previously predominant *Nitrospira* in some high F:M-C:N reactors.

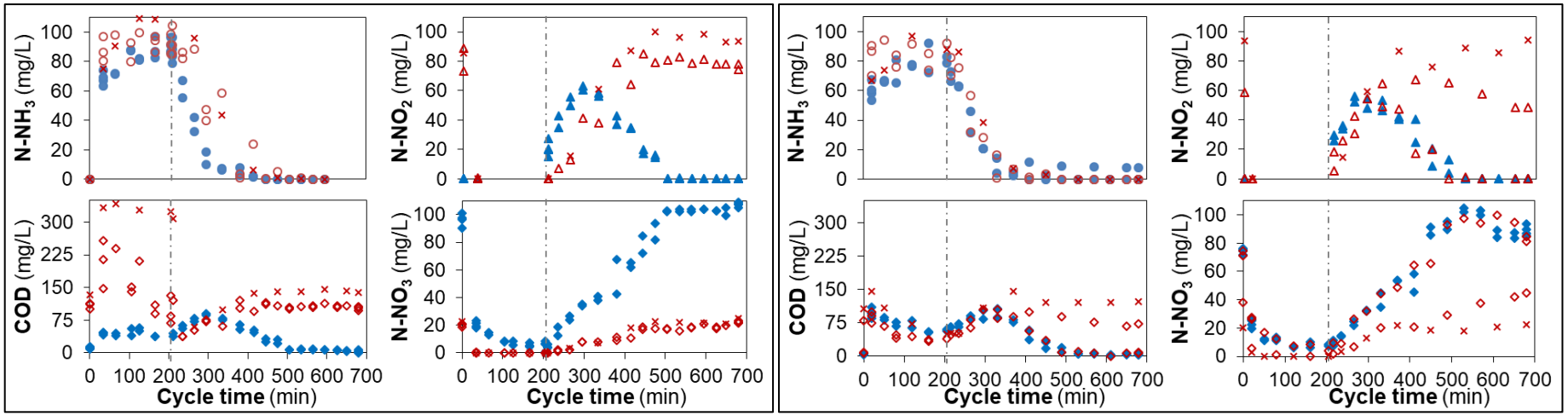

Fig. 3. Chemical profiles during a full SBR cycle lasting 12 hours, before and after high to low F:M-C:N changes. Concentrations are shown for all eight reactors on day 110 (left panel group) and day 124 (right panel group). Low F:M-C:N reactors (n = 4) have blue closed symbols, high F:M-C:N reactors (n = 3) red open symbols, and R3 has red X symbols. Vertical dotted dashed line indicates the start of the aerobic stage in each cycle. Right panel shows that only two high F:M-C:N reactors recovered the nitrite oxidation function on d124.

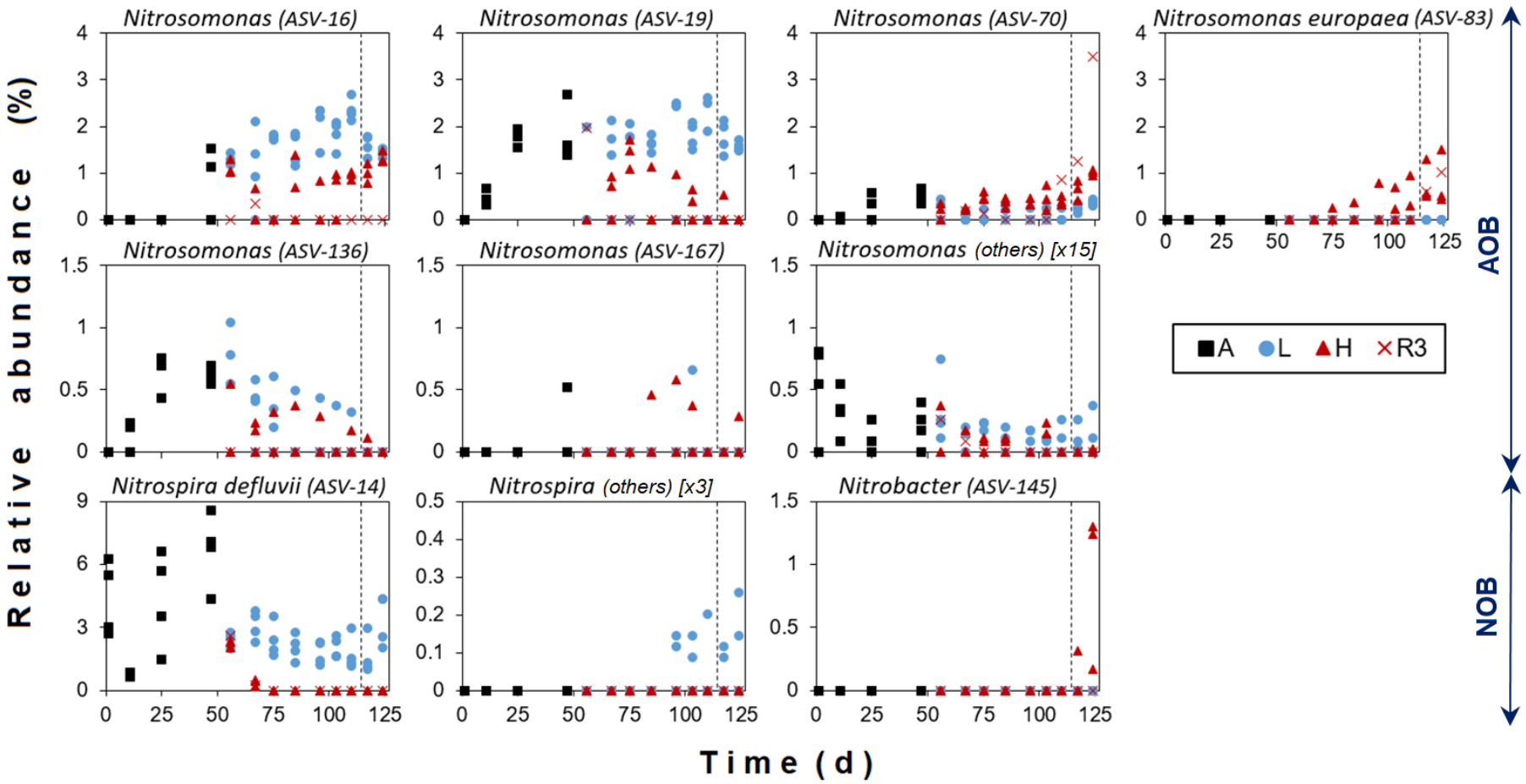

Fig. 4. Temporal relative abundance of ASVs assigned to nitrifier genera in each reactor. Phases: A, acclimation; L, low F:M-C:N; H, high F:M-C:N; and R3, reactor three. Vertical dashed line indicates the shift from high to low F:M-C:N. The abundance rank number (among all 1646 ASVs detected) is shown in parentheses. Brackets indicate the number of other additional lower abundance ASVs summed as 'others' within a panel. Higher number of ASVs suggest that AOB populations were more diverse than NOB populations.

Table 1. Influent synthetic wastewater characteristics and reactor operational parameters per phase.

Day	Phase†	COD* [mg L^{-1}]	TKN [mg L^{-1}]	C:N [mg COD mgTKN^{-1}]	TSS [mg L^{-1}]	F:M [mg COD mg TSS^{-1} d^{-1}]	SRT [d]	VSS:TSS [%]
1-53	Acclimation	374 (106)	105 (27)	3.5 (0.7)	1934 (502)	0.21 (0.08)	18.7 (0.7)	92.0 (2.3)
54-127	Low F:M-C:N	323 (24)	92 (3.6)	3.5 (0.3)	1727 (251)	0.19 (0.05)	12.8 (0.4)	96.1 (1.6)
54-113	High F:M-C:N‡	629 (67)	100 (19)	6.3 (0.9)	1943 (476)	0.36 (0.11)	8.2 (1.4)	95.6 (1.4)
114-127	High to low F:M-C:N‡	326 (19)	90 (2.2)	3.6 (0.3)	1774 (256)	0.19 (0.06)	12.4 (1.0)	95.4 (1.8)

*Average values, including standard deviation of the mean (s.d.m.) in parentheses.

†Each phase operated four independent 5-L reactors. Samples were generated 2-3 times per week.

‡These two phases involved the same four reactors, where the F:M-C:N was changed from high to low on d114. Reactor three (R3) was not included.