Sustained organic loading perturbation favors nitrite accumulation in bioreactors with variable resistance and resilience of nitrification and nitrifiers

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Abstract

Sustained perturbations are relevant for 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 nitrification and nitrifiers. Two sets of replicate 5-liter sequencing batch reactors were operated at two different, low (n=4) and high (n=3), F:M (0.19-0.36 mgCOD/mgTSS/d) and C:N (3.5-6.3 mgCOD/mgTKN) conditions for a period of 74 days, following 53 days of sludge acclimation. The inoculum was taken from a full-scale treatment plant. Resilience was tested during the last 14 days by operating all seven 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 three 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 are central tenets of environmental engineering and biotechnology, to help achieve the sustainable development goal of guaranteeing availability and sustainable management of water and sanitation for everyone [1]. In ecology, perturbations or disturbances are believed to have direct effects on the stability of ecosystems by altering community structure and function (Table 1) [2]. For engineered systems like activated sludge bioreactors, it is important to identify the effect of different disturbances on the microbial community structure so as to relate them to changes in process performance [3]. However, disturbance is also deemed to affect underlying mechanisms of community assembly [4] which, if predominantly stochastic, could drive microbial communities to divergent trajectories in terms of composition and function [5]. Therefore, robust replication is required to properly assess the effect of perturbations in the stability of sludge bioreactors.

Sustained perturbations or press disturbances that impose a long-term continuous change of species densities through an alteration of the environment [6, 7], are relevant since they can lead a system to alternative stable states that may or may not be reversible in terms of both community composition and function [8]. Press disturbance studies on wastewater treatment systems often employ harsh perturbations like toxic aromatic pollutants [9] or washout of organisms through sludge discharge [10, 11]. However, 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 [12]. In bioreactors, a switch in the substrate feeding scheme employed could then elicit changes in community structure and function. Such perturbations can occur in the form of organic shocks within activated sludge [13-15] and anaerobic reactor systems [16, 17]. In wastewater treatment, the food-to-biomass ratio (F:M) or sludge loading rate is an important parameter as it determines the growth type and settleability of sludge microorganisms [18]. 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 [19, 20], despite the fact that

C:N variations are known to affect nitrification [21]. This is also overlooked in studies where F:M values change through modifications in solids retention time (SRT) [10, 11] and organic carbon loading rate (OLR) [22, 23].

Partial nitrification is an important stage in biological nitrogen removal from wastewater via nitritation/anammox [24], for which the accumulation of nitrite is desired by promoting the growth of ammonia oxidizing bacteria (AOB) while suppressing nitrite oxidizing bacteria (NOB) [25]. Different strategies have been employed towards this goal, like varying temperature, dissolved oxygen (DO), pH, SRT, and substrate concentrations [26]. Influent manipulation in particular has been reported to yield contrasting results in terms of C:N [27]. Low influent C:N values (2-3 mg COD/mg TN) have been shown to prevent [28], but also promote [29-31] nitrite accumulation in different sludge systems. Other studies have reported nitrite accumulation at high (10 mg COD/mg NH_4^+-N) [32] and also fluctuating influent C:N values (2.5-8 mg COD/mg NH_4^+-N) [33, 34]. Additionally, although adjustments in C:N also impact F:M values by altering the available carbon for heterotrophic growth, none of these studies accounted for covariations in F:M, and hence there is a knowledge gap about system performance when both factors are controlled simultaneously. For better nitrite accumulation strategies, more research is needed to understand the effect of variations in influent C:N together with controlled variations of other important operational parameters like F:M and SRT.

The aim of this work was to test the effect of a sustained perturbation (press disturbance) of doubling F:M and C:N values in a set of replicated lab-scale bioreactors after an acclimation period, with controlled parameters to avoid loose covariations. The focus was on the nitrification function and the nitrifying microbial community. 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. Nitrite accumulation was also tracked due to its relevance for practical applications. Changes in composition of nitrifying organisms were assessed by metagenomics and 16S rRNA gene amplicon sequencing. The resistance of relevant functions and specific nitrifier abundance was evaluated by monitoring their transition to a different steady state after the

perturbation. We further tested resilience by shifting the F:M and C:N back to the original pre-perturbation state.

2. Materials and Methods

2.1. Experimental design

The experiment was conducted using seven 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 with intermittent aeration. Initially, four reactors were acclimated to lab conditions and fed with complex synthetic wastewater for 53 days. The complex synthetic feed was adapted from Hesselmann [35]. At the start of the experiment (d54), the sludge of the acclimation reactors was thoroughly mixed and redistributed across seven reactors. From these, three 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 2). For the sake of simplicity, we will refer to both F:M and C:N as F:M-C:N. A schematic representation of the experimental design (Fig. S1) and details about sludge inoculum, acclimation phase and complex synthetic wastewater preparation are available as supplementary information.

2.2. Operational parameters

The reactor temperature was maintained at 30°C and sludge was continuously mixed with a magnetic stirrer. In each cycle, SBR phases were: 5 min feed, 200 min anoxic/anaerobic react, 445 min aerobic react, 50 min sludge settle, and 20 min supernatant drain. The DO concentration was controlled at 2 – 6 mg/L during the aerobic phase. The pH ranged from 6 – 9, owing to alkalinity provided in the feed. Two cycles per day corresponded to a hydraulic retention time (HRT) of 24 h. Effluent and influent compositions were measured 2-3 times per week in accordance with Standard Methods [36]. 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. Detailed equations and explanations for F:M, C:N and SRT calculations, as well as analytical methods, are available as supplementary information.

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 [37]. 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 [38] 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 [39]. 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) [40]. Adequacy of sequencing depth after reads processing was corroborated with rarefaction curves at the ASV level (Fig. S2).

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* [41]. 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 [42]. High quality reads were aligned against the NCBI non-redundant (NR) protein database (March 2016) using DIAMOND v.0.7.10.59 [43] with default parameters. The lowest common ancestor approach implemented in MEGAN Community Edition v.6.5.5 [44] 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 (Fig. S2).

3. Results

3.1. Dynamics in bioreactor performance

During the acclimation phase, the F:M-C:N values were maintained at 0.21 (mg COD/mg TSS/d) and 3.5 (mg COD/mg TKN), respectively (Table 2). 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 (Fig. S3). Most of these variations decreased after 30 d and trends were stable after 45 d.

During the perturbation phase of the study (d54 onwards), sludge was wasted more often to better control the TSS and thus the F:M (Fig. S2), 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 (details in supplementary information), controlling for a higher F:M resulted in a lower SRT in reactors subjected to this treatment (Table 2). 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 nitrite accumulation with high NO_2^--N and COD effluent concentrations (Fig. 1). 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 2). 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).

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). Conversely, 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 all the way to zero in the second week (d121-d127) for two of the three reactors (Fig. 1).

Among nitrifiers, the three most abundant bacterial genera detected through DNA sequencing were *Nitrospira*, *Nitrosomonas* and *Nitrobacter*. During the perturbation 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). Similar patterns of nitrifier abundance across low and high F:M-C:N replicates were observed for metagenomics and 16S rRNA gene amplicon sequencing datasets (Fig. 2).

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 three 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, Fig. S3). The most abundant nitrifying genus was *Nitrospira*, which increased around 50% after the acclimation stage. The next most abundant nitrifier, *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 [39] we could observe that *Nitrosomonas* was the most diverse nitrifying genus with twenty-one different ASVs being detected (Fig. 4).

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 nitrate production and COD removal. 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 [45].

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 [46]. The higher variability in function among replicates during the first seven-day transition period is an example of the stochastic effects that a disturbance initially triggers in ecosystems [4]. 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 [5].

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 [47]. 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 [48, 49]. 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 [50]. 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 are enough to achieve optimal nitrification performance [51], which was the case for the reactors in this study (Fig. 3). However, nitrifying communities grow in stratified biofilms where AOB are located closer to the water interface and NOB are in the interior zone [52, 53]. 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 [48], 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 sufficient oxygen to NOB to be active during the remainder 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 [54]. 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 [55]. In our study, increasing F:M while keeping

TSS constant implied an SRT reduction of 35% in the high F:M-C:N reactors compared to the low F:M-C:N reactors. It is conceivable that part of the observed reduction in nitrifiers was due to washout given their low growth rates. It was suggested based on mathematical modelling that a reduction in SRT has a stronger effect on NOB than on AOB [56]. However, the SRT of 8.2 d (aerobic SRT of 5.1 d) used to operate the high F:M reactors is common in activated sludge processes performing complete nitrification [18, 57], and well above the operating SRT of 5-6 d at the full-scale plant that provided the sludge inoculum for this study. SRT values of 4-8 d have been suggested as optimum for nitrification in practice [57, 58], while complete nitrification has been reported for SRT values as low as 2 d [59]. Thus, our observed changes in nitrifiers and nitrification function were driven by controlled changes in F:M and C:N values and not by washout of NOB due to a 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 [27]. A comparison with earlier studies where either F:M or C:N was the parameter of interest reveals conflicting and inconclusive outcomes. For example, similar 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 [60] or indirectly through changes in SRT [10, 23]. Likewise, nitrite accumulation was found at high influent C:N (10 mg COD/mg NH_4^+-N) in a pilot-scale study using a CSTR [32]. However, contrary to our results, low influent C:N values (1-3 mg COD/mg NH_4^+-N) in high-strength industrial wastewaters were reported to yield partial nitrification in a review of full-scale anammox processes [55]. Also, nitrite accumulation was reported at low C:N values in studies using a 35-L SBR (~2 mg COD/mg TN) [29], a lab-scale 6.3-L SBR (3.33 mg COD/mg NH_4^+-N) [31], and a pilot-scale continuous-flow A/O/A reactor (3.19 mg COD/mg NH_4^+-N) [30]. On the other hand, there was complete nitrification without nitrite accumulation at low influent C:N (3 ± 1 mg COD/mg TN) in pilot-scale membrane batch reactors [28], similar to our results. Increasing C:N molar ratios from 2 to 5 was shown to

significantly reduce nitrification rates in a laboratory denitrification-nitrification system [61], although the study did not mention whether this caused nitrite accumulation. Moreover, nitrite accumulation was even reported for fluctuating low and high influent C:N values (2.5-8 mg COD/mg NH_4^+-N) in two different pilot-scale studies using SBRs [33, 34]. These multiple opposing findings in the literature imply that other factors in addition to C:N affect nitrification, which need to be accounted for. Changes in C:N also affect F:M values by altering the available carbon for heterotrophic growth. As neither of the aforementioned studies controlled the covariations in F:M when changing the C:N, their respective roles cannot be disentangled. Hence, we emphasize the need to evaluate these interconnected parameters simultaneously.

In practice, control of TSS is critical to operational control strategies based on either F:M or SRT [57, 58]. In our study design, 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. Doubling the influent COD also doubled the OLR, and higher COD concentrations also increased the biomass produced per unit time; thus more sludge had to be wasted to keep the TSS constant, reducing the SRT as a consequence (details in supplementary information). This point serves to illustrate that important operational parameters like SRT, C:N and OLR also co-vary with F:M [57], which is precisely why they have to be evaluated simultaneously during experimentation. Our literature review suggests that this interrelationship has not received the attention it deserves. For example, a recent study on the effect of reducing the SRT from 30 to 3 days in a full-scale plant [10] reported a washout of *Nitrospira* and an associated decrease in nitrogen removal efficiency and nitrite accumulation, but this happened at the expense of a ten-fold increase in F:M (0.03-0.33 mg COD/mg TSS/d). The C:N values in this study were not controlled. This seems to be common practice as several recent studies, using sludge bioreactors to test the effect of varying SRT on nitrification and nitrifiers, neither controlled covariations in F:M nor accounted for changing C:N values [59, 62-64]. One of the studies, however, highlighted the importance of F:M covariations with SRT for assessing P removal [62]. We posit that experimental changes in SRT, without also controlling for covariations in

F:M and C:N, can lead to erroneous conclusions about dynamics of nitrifying communities and the nitrification process due to 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 [65], for which experimental replication is paramount to ensure reproducibility [66] and capture fluctuations in process instability [67]. Several recent studies on variations of C:N and SRT in sludge bioreactors could not make such an assessment because their designs did not include replication [10, 30-34, 56, 59, 68, 69]. Hence, we tested the effect of returning the high F:M-C:N to their previous low levels on the functional resilience of the replicated perturbed reactors. The switch back had noticeable effects on nitrifier communities (Figs. 2 and 4) as well as on the nitrification function (Figs. 1 and 3). Relative abundances of *Nitrosomonas* genera recovered quickly after returning to pre-perturbation conditions. 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 [70] and ruderal organisms [71]. According to the r/K ecological framework (Table 1), early niche colonization stages should favour r-strategists, whereas K-strategies should prevail at a later stage when many organisms attempt to colonize [72]. In our study, the variable 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 [73] and prevailing at alternating conditions [74], *Nitrobacter* has been shown to thrive at higher DO [75] and nitrite [60] 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.

Our reactors constituted a closed system, which implies that the *Nitrobacter* colonizers came from low-abundance seed-bank populations (Table 1). It was proposed that disturbance opens niches for bacterial colonization in open sludge systems [76], 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 [77, 78], studies of nitrification resilience in open sludge systems [10] 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 [79] and the insurance hypothesis [80]. This finding is similar to what was found in a full-scale sludge system after a press disturbance [10], but contrary to what was reported for lake microbial systems after a pulse disturbance [81]. These contrasting reports highlight the complexity of assessing disturbance-diversity-function relationships [82], 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 [65], 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 general 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 recovery of nitrite oxidation 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 manuscript. ES and SW wrote the manuscript.

Data availability

DNA sequencing data are available at NCBI BioProjects PRJNA559245. See supplementary information for details about sludge inoculum and acclimation phase, complex synthetic wastewater preparation, chemical analysis, calculation of parameters (F:M, C:N, SRT), 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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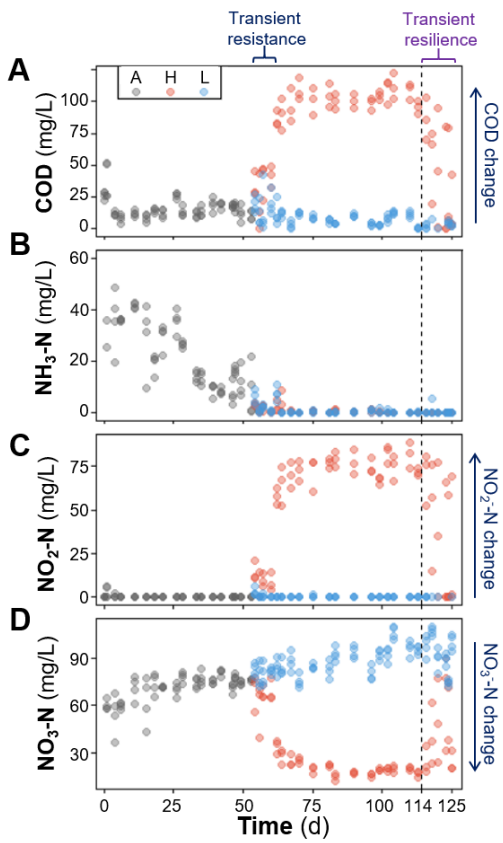

Fig. 1. Temporal average effluent concentrations in mg/L of (**A**) soluble COD, (**B**) NH_3-N, (**C**) NO_2^--N and (**D**) NO_3^--N. Phases: A, acclimation (n = 4); L, low F:M-C:N (n = 4); H, high F:M-C:N (n = 3). Vertical dashed line indicates the shift from high to low F:M-C:N. Functional resistance (i.e., degree of insensitivity to disturbance) is indicated on the right-hand side of panels. Periods of transient functional resilience (i.e., capacity to return to the pre-disturbance condition) and resistance are indicated by curly brackets.

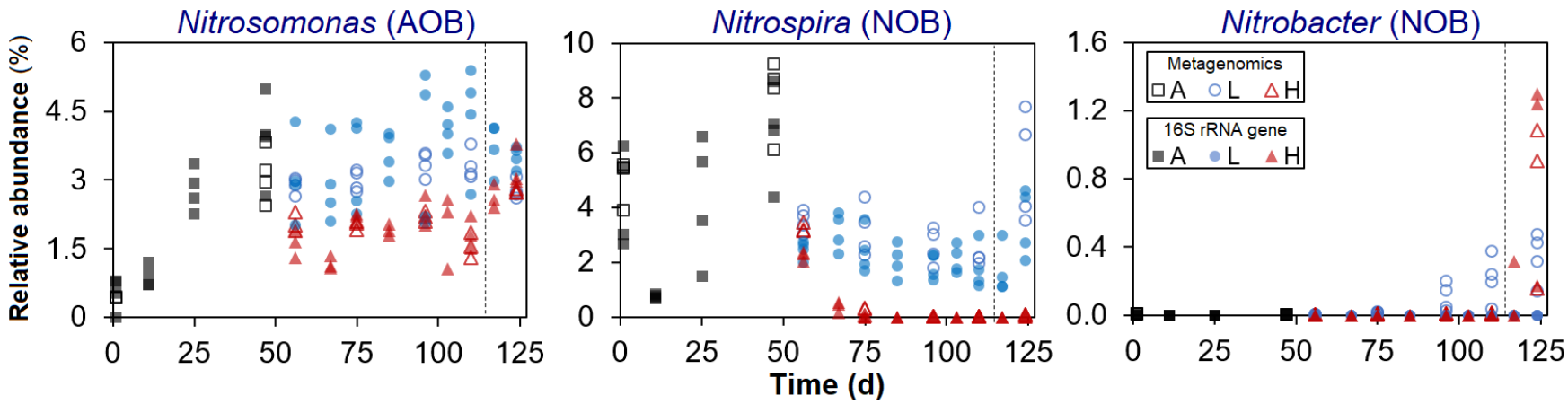

Fig. 2. Temporal relative abundance of main nitrifier genera in each reactor. Phases: A, acclimation (n = 4); L, low F:M-C:N (n = 4); H, high F:M-C:N (n = 3). Vertical dashed line indicates the shift from high to low F:M-C:N. Closed symbols display 16S rRNA gene amplicon ASV data and open symbols shotgun 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 two out of three high (shifted to low) F:M-C:N reactors.

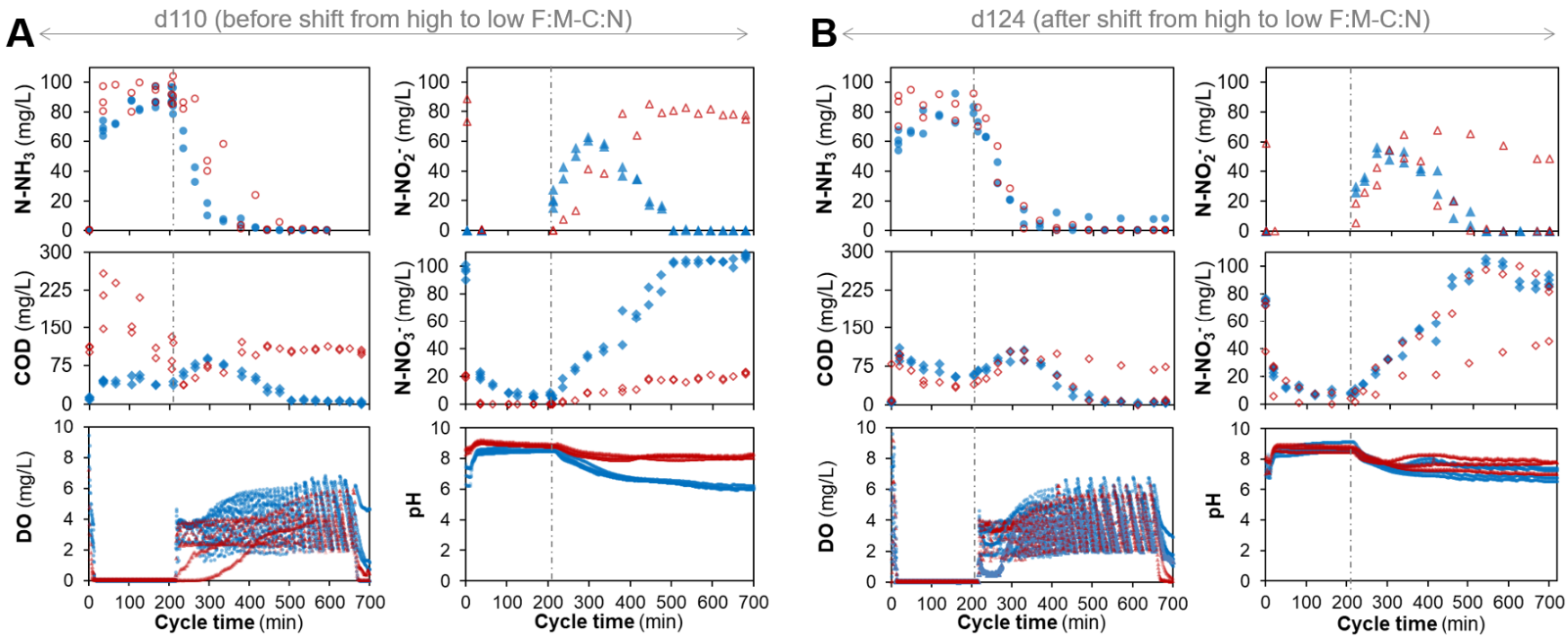

Fig. 3. Chemical profiles during a full intermittently-aerated SBR cycle lasting 12 hours. Concentrations are shown for all reactors on (**A**) day 110 and (**B**) day 124, showing profiles before and after high to low F:M-C:N changes. Low F:M-C:N reactors (n = 4) have blue closed symbols, high F:M-C:N reactors (n = 3) red open symbols. Vertical dotted dashed line indicates the start of the aerobic stage in each cycle. Right panels (**B**) show that only two out of three high (shifted to low) F:M-C:N reactors recovered the nitrite oxidation function and full COD removal on d124.

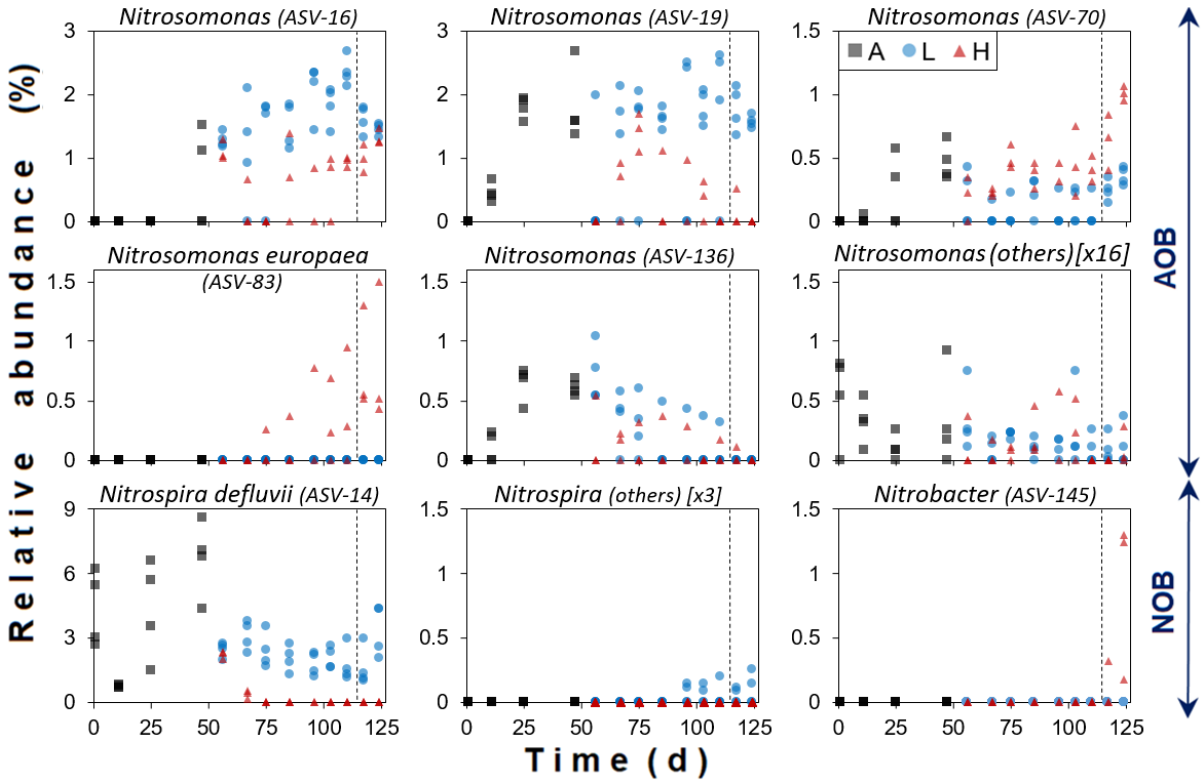

Fig. 4. Temporal relative abundance of ASVs assigned to nitrifier genera in each reactor. Phases: A, acclimation (n = 4); L, low F:M-C:N (n = 4); H, high F:M-C:N (n = 3). Vertical dashed line indicates the shift from high to low F:M-C:N. The abundance rank number (among all 1646 ASVs detected) is shown in parentheses. Brackets indicate the number of other additional lower abundance ASVs summed as 'others' within a panel. Higher number of ASVs detected suggest that AOB populations were more diverse than NOB populations.

658 **Table 1.** Glossary of key microbial ecology concepts

Concept	Definition
Community	A group of populations of two or more different microbial taxa (*e.g.* nitrifiers) occupying the same geographical space (*e.g.* bioreactor) at a particular time [2].
Community structure	The composition and relative abundance of different microbial taxa within a community at a given time [2].
Disturbance	An event that significantly alters the environment of a community, creating opportunities for individuals to grow and reproduce [12] (*e.g.* a change in feeding strategy). Also referred to as *perturbation*.
Ecosystem	A community of living organisms in conjunction with the abiotic components of their environment, interacting as a system [2] (*e.g.* a bioreactor).
Ecosystem function	Outputs of functional processes at a whole ecosystem level [83] (*e.g.* ammonia removal within a bioreactor).
Immigration	Large-scale movement of members of a species to a different environment [84] (*e.g.* bacteria within the feed entering a bioreactor).
Niche	The abiotic and biotic conditions that a microbial taxa need to grow, survive and reproduce [85].
Press disturbance	A long-term or continuous disturbance [7] (*e.g.* a sustained change in organic loading).
r- and K- strategists	Classification given to organisms under the r- vs. K-strategies ecological framework. Among nitrifiers, *Nitrosomonas* and *Nitrobacter* are r-strategists able to grow fast under high substrate concentration conditions, while *Nitrosospira* and *Nitrospira* are K-strategists that grow slowly but are adapted to low substrate concentrations [70, 72].
Resilience	The capacity of a community and/or function to return to a pre-disturbance condition [86].
Resistance	The degree to which a community and/or function is insensitive to a disturbance [86].
Seed-bank population	Refers to the members within a community that are present in low abundances, but are able to grow when given the appropriate conditions [87].
Stability	The response of a community to disturbance [12]. It is comprised of resistance and resilience, which are two quantifiable metrics useful for comparing community disturbance responses [79, 88]. The stability of a community can be studied in terms of functional and/or compositional parameters [7].
Stochastic growth	Refers to growth under conditions that are not deterministic but random in nature [4]. Examples are priority effects (*i.e.* colonizing first, by chance, a recently open niche) and ecological drift (*i.e.* random events of birth and death).

Succession	The process of change in the community structure over time [89].

Table 2. Influent synthetic wastewater characteristics and reactor operational parameters per phase.

Day	Phase†	n	COD* [mg/L]	TKN [mg/L]	C:N [mg COD/mg TKN]	TSS [mg/L]	F:M [mg COD/mg TSS/d]	SRT [d]	VSS:TSS [%]
1-53	Acclimation	4	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	4	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‡	3	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‡	3	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 independent 5-L reactors. Samples were generated 2-3 times per week.

‡These two phases involved the same three reactors, where the F:M-C:N was changed from high to low on d114.