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Recurrent multi-stressor floc treatments with sulphide and free ammonia enabled mainstream partial nitritation/anammox

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Abstract

Selective suppression of nitrite-oxidising bacteria (NOB) over aerobic and anoxic ammonium-oxidising bacteria (AerAOB and AnAOB) remains a major challenge for mainstream partial nitritation/anammox implementation, a resource-efficient nitrogen removal pathway. A unique multi-stressor floc treatment was therefore designed and validated for the first time under lab-scale conditions while staying true to full-scale design principles. Two hybrid (suspended + biofilm growth) reactors were operated continuously at 20.2 ± 0.6 °C. Recurrent multi-stressor floc treatments were applied, consisting of a sulphide-spiked deoxygenated starvation followed by a free ammonia shock. A good microbial activity balance with high AnAOB (71 ± 21 mg N L^{-1} d^{-1}) and low NOB (4 ± 17% of AerAOB) activity was achieved by combining multiple operational strategies: recurrent multi-stressor floc treatments, hybrid sludge (flocs & biofilm), short floc age control, intermittent aeration, and residual ammonium control. The multi-stressor treatment was shown to be the most important control tool and should be continuously applied to maintain this balance. Excessive NOB growth on the biofilm was avoided despite only treating the flocs to safeguard the AnAOB activity on the biofilm. Additionally, no signs of NOB adaptation were observed over 142 days. Elevated effluent ammonium concentrations (25 ± 6 mg N L^{-1}) limited the TN removal efficiency to 39 ± 9%, complicating a future full-scale implementation. Operating at higher sludge concentrations or reducing the volumetric loading rate could overcome this issue. The obtained results ease the implementation of mainstream PN/A by providing and additional control tool to steer the microbial activity with the multi-stressor treatment, thus advancing the concept of energy neutrality in sewage treatment plants.

Keywords

Deammonification; Biological nitrogen removal; Nitrite-oxidizing bacteria; Nitrification; Nitrospira; Brocadia

1. Introduction

Partial nitritation/anammox (PN/A) is a carbon- and energy-efficient nitrogen removal process due to its 100% lower chemical oxygen demand (COD) (~carbon) and 60% lower oxygen (~energy) demand compared to conventionally applied nitrification/denitritation (Jetten et al., 1997). Its implementation in the main (water) stream of a sewage treatment plant (STP) is pursued to allow improved carbon utilisation by the combination of an upstream carbon-capture stage and anaerobic sludge digestion in the downstream side (sludge) stream. Consequently, energy-positive sewage treatment can be achieved once these concepts are combined (Gao et al., 2014; Liu et al., 2018). This can result in considerable energy savings, given sewage treatment's average energy consumption of about 37 kWh IE^{-1} y^{-1} for Western European countries (Maktabifard et al., 2018).

Successful implementation of mainstream PN/A is challenging as operational conditions typically allow the growth of unwanted nitrite-oxidising bacteria (NOB), competing for, respectively, oxygen and nitrite with the essential aerobic and anoxic ammonium-oxidising bacteria (AerAOB and AnAOB). The latter, AnAOB, are also often referred to as anammox bacteria. A combination of multiple dedicated control strategies is essential to avoid NOB activity as they reduce both the nitrogen removal as well as the energy efficiency (Agrawal et al. (2018); Laureni et al. (2019); Van Tendeloo et al. (2021a)). The most effective strategies described so far include a (partial) combination of: i) oxygen availability control by either continuous aeration at low dissolved oxygen (DO) setpoints (Laureni et al., 2016; Seuntjens et al., 2020; Van Tendeloo et al., 2021b; Zheng et al., 2023) or intermittent control at a relative higher DO setpoint (Miao et al., 2016; Pedrouso et al., 2019), ii) maintaining residual ammonium as substrate for AerAOB and AnAOB (Laureni et al., 2016; Liu et al., 2019; Zheng et al., 2023), iii) differential sludge retention time (SRT) control in combination with hybrid sludge (flocs + biofilm) to pressure the NOB in the aerobic flocs with a low SRT_{floc} while the AnAOB in the partially-anoxic biofilm (either granules or attached to carriers) are additionally retained to overcome their low growth rate (Laureni et al., 2016; Liu et al., 2019; Van Winckel et al., 2019), and iv) applying inhibitory agents or stressors to selectively suppress NOB activity over the other essential bacteria, preferentially as a temporary treatment in the more concentrated return-sludge line to limit the total chemical addition. Successful examples of such return-sludge treatments include recurrent exposure to *in-situ* generated free ammonia (FA, NH_3) (Duan et al., 2019a; Wang et al., 2021) or free nitrous acid (FNA, HNO_2) (Duan et al., 2019a; Peng et al., 2020; Wang et al., 2018; Zheng et al., 2023), substrate starvation (Ye et al., 2019), and exposure to other compounds such as sulphide (Erguder et al., 2008), either *in-situ* generated by sulphate reduction or externally dosed.

Despite promising results achieved in multiple lab studies (Laureni et al., 2019; Pedrouso et al., 2019; Seuntjens et al., 2020), long-term and large-scale successes remain scarce (Agrawal et al., 2018; Cao et al., 2017; Zheng et al., 2023). The ability of NOB to adapt to certain operational strategies over time threatens the development. For example, Wang et al. (2021) observed NOB adaptation to low DO control and afterwards to regular FA sludge treatments. Adaptation to single-stressor treatments such as FA and FNA were also frequently observed (Duan et al., 2019a; Li et al., 2020; Ma et al., 2017; Peng et al., 2020; Wang et al., 2021), compelling authors to either alternate between multiple treatments (Duan et al., 2019a; Peng et al., 2020) or apply additional strategies such as bioaugmentation (Wang et al., 2021). Additionally, the continuous intake of NOB via the influent challenges the long-term stability (Duan et al., 2019b; Yu et al., 2020). Another issue is that many studies either exclude the AnAOB-rich biofilm from treatment to safeguard the AnAOB activity (Peng et al., 2020; Wang et al., 2018; Wang et al., 2021), or even exclude AnAOB completely from their study (Li et al., 2020; Ma et al., 2017), potentially causing the NOB to grow uncontrolled in the biofilm if not dealt with care. Limiting nitrite accumulation could however alleviate this threat (Gu et al., 2019; Gu et al., 2021; Laureni et al., 2019). Finally, certain strategies are difficult to extrapolate to full-scale applications such as strict or low DO control (Wang et al., 2021), thus reducing its NOB suppression effectivity, or due to extensive cost or logistical challenges and being system dependant.

The development of a multi-stressor treatment could overcome these challenges as there are indications that the combination of multiple stressors is less susceptible to NOB adaptation (Seuntjens et al., 2018; Torà et al., 2010). Additionally, this technology would provide an extra control tool to limit NOB as a combination of multiple strategies is essential for mainstream PN/A implementation (Agrawal et al., 2018). Seuntjens et al. (2018) successfully tested the combination of a 2-day deoxygenated starvation with sulphide exposure and a subsequent 1-hour exposure to FA, after screening for the most effective combination of known NOB inhibitors.

In this study, the unique multi-stressor treatment concept formulated by Seuntjens et al. (2018) was validated under lab-scale conditions while staying true to full-scale design principles: continuous process with influent COD present, including AnAOB activity in a hybrid sludge system, and testing the combination with other control tools. Additional attention was given to avoiding uncontrolled NOB growth in the biofilm, while safeguarding the AnAOB activity, and any potential indications for adaptation. The overall aim was to achieve mainstream nitrogen removal by AnAOB with minimal NOB contribution. A combination of multiple strategies was tested: recurrent multi-stressor floc treatments, aeration strategy (continuous and intermittent), hybrid sludge consisting of flocs and attached (carriers) or suspended (granules) biofilm growth, short floccular SRT (8.5 days) control, and residual ammonium (≥ 5 mg N L^{-1}) control. Multiple combinations and variations on control setpoints were tested in two separate reactors to identify crucial control tools and optimal settings. The return-sludge treatment could act as an additional NOB activity control tool, providing the additional flexibility needed for mainstream PN/A implementation and thus pave the way towards energy autonomous sewage treatment.

2. Materials and Methods

2.1. Reactor and experimental design

Two identical integrated fixed-film activated sludge (IFAS) reactors with hybrid sludge (flocs and biofilm) were operated in parallel under mainstream conditions at 20.2 ± 0.6 °C (Figure 1). Each reactor consisted of a reaction vessel (4.7L) coupled to a cylindrical settler with a flat bottom (1L). A return activated sludge (RAS) pump was installed to cycle the sludge to the reaction vessel. Influent was continuously added at 11.3-16.1 L d^{-1} from a shared influent vessel and consisted of tap water spike with 50 mg NH_4^+-N L^{-1} (as $(NH_4)_2SO_4$), 7.5 mg PO_4^{3-}-P L^{-1} (as KH_2PO_4), 30 mg Mg^{2+} L^{-1} (as $MgCl_2 \cdot 6H_2O$), 50 mg Ca^{2+} L^{-1} (as $CaCl_2 \cdot 2H_2O$), 300-500 mg HCO_3^- L^{-1} (as $NaHCO_3$), and 1 mL L^{-1} of trace elements A and B (according to van de Graaf et al. (1996)). COD was mostly dosed separately from a 2000-2250 mg COD L^{-1} stock solution consisting of demineralised water spiked with 20% acetate (as $NaC_2H_3O_2$), 15% yeast extract, and 65% sucrose (as $C_{12}H_{22}O_{11}$), to achieve a final influent COD/N ratio of 1.5. Exceptionally, COD was directly added to the main influent (75 mg COD L^{-1}) when one of the COD dosing pumps was out of order (see supplemental information, Table S1). Both pumps were continuously operated at a RAS:influent ratio of 0.95 ± 0.03 and 0.74 ± 0.04 for reactor 1 and 2 (R1 and R2), respectively. pH (7.2-7.4) and DO were controlled by a multichannel controller (Liquiline CM44X), coupled to a pH (Memosens CPS11E) and DO probe (Oxymax COS61D), all from Endress+Hauser (Switzerland), and as actioner an acid (0.1 M HCl) or base (0.05 M NaOH) dosage pump and an air pump (TEC APS 150, Tetra, Germany) connected to a cylindrical aeration stone. All liquid pumps were supplied by Watson-Marlow (United Kingdom). Oxygen availability was controlled by an on/off feedback control system that powers the airflow pump with the DO probe's input. A rotameter was installed after the air pump to manually adjust the airflow rate to finetune the control. The oxygen availability control was conducted through either continuous aeration at DO setpoint 0.3 mg O_2 L^{-1} (R2, Phase I) or intermittent aeration (4/8 min on/off aeration) at DO setpoint 0.9 mg O_2 L^{-1} for all other phases. Continuous mixing (300 rpm) was provided by an overhead mixer with propeller blades (ES Overhead Stirrer, Velp Scientifica, Italy). Floccular sludge was wasted thrice a week and separated from the biofilm using a 200 µm sieve to obtain the target SRT_{floc} of 8.5 days. Biofilm was not actively wasted.

Figure 1. Schematic overview of the reactor and experimental design. Multi-stressor floc treatments were conducted twice a week, treating about 33% of the total flocs, resulting in an overall frequency of 0.095 d^{-1}. Biofilm (both attached and suspended) were excluded from the treatment.

The two reactors were operated independent of each other. Operational conditions were frequently changed to derive the optimal combination of control tools and settings by comparing the change in performance between subsequent phases within the same reactor. A summary of the operational conditions per reactor and phase is given in **Table 1**.

Table 1. Summary of the operational conditions per reactor and per operational phases. Values in bold indicate the main change between subsequent phases.

2.2. Sludge origin and reactivation

Sludge used for inoculation and bioaugmentation originated from three different sources (Table 2):

- DEMON® sludge: Full-scale sidestream PN/A reactor applying DEMON technology (Wett, 2006) at STP Nieuwveer (Breda, Netherlands), consisting of flocs and granules (> 200 µm).
- Long-term stored carriers: AnoxKaldnes K1 carriers with PN/A biofilm, operated in an IFAS (4.7L) under mainstream conditions at 20°C (Peng et al., 2020). Stored for 2 years with nitrate (≤ 250 mg N L^{-1}) at 4°C.
- RBC biofilm: lab-scale rotating biological contactor (RBC) with a thick PN/A biofilm, operated under mainstream conditions at 20 ± 2°C (Van Tendeloo et al., 2021b).

The initial biofilm inoculum for each reactor was (re)activated by first operating both IFAS reactors in sequencing batch reactor (SBR) mode under anoxic conditions for 18 days (53 days for R1) to promote AnAOB growth. R1 was inoculated with 1.3L (28% filling ratio) of the long-term stored carriers while R2 was inoculated with empty K1 carriers, manually filled with RBC biofilm to promote attachment. Each SBR cycle consisted of 15 min pulse feeding with mixing, 210 min reaction, 10 min extraction, and 5 min idle time. No settling phase was foreseen to select for attached growth. The volume exchange ratio was 31%. The influent was similar to the main operation (section 3.1.) except that 27 mg NH_4^+-N L^{-1} (as $(NH_4)_2SO_4$), 35 mg NO_2^--N L^{-1} (as $NaNO_2$), and 15 mg NO_3^--N L^{-1} (as $NaNO_3$) instead of solely ammonium were dosed, and a COD/N ratio of 0.75 instead of 1.5, solely consisting of acetate. Once the actual experiment was started, the reactor operation switched to the conditions described in section 2.1. On day 35, DEMON® sludge (flocs + granules) were added to R1 and on day 12, RBC flocs (mixed biofilm) to R2. Additionally, an extra SBR reactivation reactor, applying identical reactivation conditions, was operated to further reactivate the long-term stored carriers until they were added to R1 on day 95, replacing 0.75L of the original carriers.

Occasionally, additional bioaugmentations were conducted with mainly DEMON® sludge (flocs and/or granules, Table 2) to boost the maximum AnAOB activity and/or the overall biomass concentration and conversion rates. R1 was considered to have solely attached biofilm growth (the granules were afterwards shown to have low activity, Section 3.4) and flocs while R2 was considered as suspended growth (no biofilm was observed on the empty carriers over the total 113 days they were in use, and extra granules were added) and flocs. On day 95, the inactive granules and empty carriers were removed from R1 and R2, respectively. In the second last phase (IIIb for R1 and V for R2), 57% of the carriers from R1 (0.74L) were inoculated to R2 to test if limited AnAOB and/or high NOB activity in the biofilm could explain the limited performance of R2 (see Section 3.1.). Simultaneously, all the granules present in R2 were removed to better compare suspended (granular) versus fixed (on carriers) biofilm growth.

Table 2. Inoculation and bioaugmentation overview per reactor.

2.3. Multi-stressor floc treatment

Over the full reactor operation (excluding the final phase for each reactor), the floccular sludge was regularly treated at a frequency of 0.095 d^{-1} by exposing 33% of the flocs twice a week to the multi-stressor treatment. Each time, a pre-determined mixed liquid volume was harvested and sieved (200 μm) to separate the flocs and obtain about 33% of the total floc mass. Each multi-stressor treatment consisted of three subsequent steps (Figure 1): pre-treatment, sulphide-spiked deoxygenated starvation, and an FA shock. During the pre-treatment, the separated flocs were concentrated to 2.5 ± 2.0 g VSS L^{-1} by discarding the supernatants after settling. They were then transferred to a 500 mL Erlenmeyer, sealed, and deoxygenated by sparging the headspace with N_2 gas for 10 minutes to starve the biomass from substrates like oxygen as previous experiments indicated that this increased the inhibitor's efficiency (Seuntjens et al., 2018). Sulphide (157 ± 16 or 305 ± 12 mg S^{2-}-S L^{-1} as $Na_2S.xH_2O$ (35% H_2O)) was dosed using a syringe and needle to maintain deoxygenated conditions. pH was corrected to either 7.1 ± 0.5 or 6.2 ± 0.7 by injecting a predetermined volume of 1 M HCl. The flocs were then stirred (250 rpm) for 2 days at 21 ± 1°C. The starvation was ended by transferring the sludge to an open 500 mL Erlenmeyer. A one-hour FA shock was conducted by adding 640 ± 13 mg NH_4^+-N L^{-1} at pH 8.1, resulting in an FA concentration of 34 ± 2 mg NH_3-N L^{-1}. Stirring at 250 rpm was applied during the FA shock while the pH was maintained at 8.1 using 1 M NaOH. The treatment was concluded by correcting the pH to 7.2 and returning the sludge to their respective reactor, resulting in a 10- to 50-fold dilution to lift chemical stress conditions. Additionally, the presence of oxygen caused all the sulphide to reactor to harmless sulphate. Exact treatment conditions are listed in the supplementary information (SI, Table S3).

2.4. Physical and chemical analyses

Influent and effluent samples were taken five times per week from each reactor to determine its performance. All samples were filtered (0.22 μm) and stored for maximally two weeks at 4°C prior to analysis. Ammonium concentration was photometrically determined according to the Nessler method (APHA, 1995) while nitrite and nitrate were measured with an anion exchange chromatograph (EcoIC, Metrohm, Switzerland). Total nitrogen (TN) was calculated as the sum of these measurements. COD was occasionally determined with measuring kits (Nanocolor, Macherey-Nagel, Germany).

Total and volatile suspended solids (TSS and VSS) were determined according to APHA (1995). The suspended VSS concentration (sum flocs and suspended biofilm) of the reactors was measured thrice a week. Biomass content on the carriers was determine twice in quadruple by scrubbing the biofilm of five randomly selected carriers, then suspending it in tap water to 5 mL, and afterwards measuring the VSS concentration. Additionally, fractionation tests were conducted twice a week on the harvested sludge for the floc treatment. Two samples were therefore taken from the sludge and from the retentate (>200 µm). VSS concentrations were measured for each sample and corrected for any dilution step. The floccular VSS concentration was calculated as the difference between the total and the biofilm VSS. A similar test was executed every week on the effluent.

2.5. Molecular analysis

Biomass was sampled from each IFAS reactor over time for successive bacterial community analysis to gain insights in community changes over time. Regular samples consisted of suspended sludge: flocs + granules. Attached biofilm on carriers were occasionally sampled separately, separating the biofilm with a needle from 5 random carriers. Samples were stored at -20°C after centrifugation and prior to DNA extraction. Powerfecal kits (Qiagen, Germany) were used to extract total DNA content, in accordance with the manufacturers protocol (incubation steps excluded). Dedicated dual-index paired-end sequencing primers (Kozich et al., 2013) were used to amplify the V4 region of the 16S rRNA gene. Paired-end sequencing was performed at the Medical genetics research group, University of Antwerp, on a Miseq Desktop sequencer (M00984, Illumina) using 2x250 cycle chemistry. Analysis was performed as described in Peng et al. (2020). In short: raw sequencing reads were processed with DADA2 (Callahan et al., 2016) and Rstudio (v 3.6.3), using an in-house developed package (www.github.com/SWittouck/tidyamplicons).

2.6. Batch maximum activity tests

Batch maximum activity tests were conducted twice to gain more insights in the dynamics in AerAOB, NOB, and AnAOB activity in both the floc and biofilm fraction as well as between R1 and R2. A 200 µm sieve was used to separate the floccular and biofilm fraction. No COD was added to avoid nitrogen removal via denitrification. Two types of tests were performed: *in-situ* aerobic tests applying the reactor's intermittent aeration control and ex-situ anoxic tests to determine the maximum AnAOB activity. For the *in-situ* tests, both reactors were operated as batch (no influent nor RAS flow) and the settlers were emptied. Ammonium was added at 50 mg N L^{-1} and 6-8 liquid samples were taken over time. The activity in the flocs was measured first (20-22 h) and afterwards the biofilm (20-22 h). After each test was concluded, a sludge sample was taken to determine the biomass concentration. Simultaneously, the maximum AnAOB activity was measured *ex situ* in closed Erlenmeyers in triplicate for each reactor. Ten min sparging with N_2 gas and 50 mg NH_4^+-N L^{-1} and 50 mg NO_2^--N L^{-1} was spiked to guarantee optimal conditions for AnAOB. The sampling frequency was identical to the *in-situ* tests. Floccular activity was not tested *ex situ* and were temporarily stored at 20°C with 15 mg NO_3^--N L^{-1} instead.

2.7. Calculations

A microbial activity balance (or nitrite balance) was made by estimating how much nitrite was produced by AerAOB in the reactor, and how much of this nitrite was consumed by AnAOB, NOB, or remained residual. All TN removal was hereby assumed to originate from AnAOB activity, thus neglecting any potential denitrification activity. The following stoichiometry was used (Lotti et al., 2014; Strous et al., 1998):

$$AerAOB: NH_4^+ + 1.382\ O_2 + 0.091\ HCO_3^- \\ \rightarrow 0.982\ NO_2^- + 1.891\ H^+ + 1.036\ H_2O + 0.091\ CH_{1.4}O_{0.4}N_{0.2}$$

$$NOB: NO_2^- + 0.488\ O_2 + 0.003\ NH_4^+ + 0.01\ H^+ + 0.013\ HCO_3^- \\ \rightarrow\ NO_3^- + 0.008\ H_2O + 0.013\ CH_{1.4}O_{0.4}N_{0.2}$$

$$AnAOB: NH_4^+ + 1.32\ NO_2^- + 0.066\ HCO_3^- + 0.13\ H^+$$
$$\rightarrow 1.02\ N_2 + 0.26\ NO_3^- + 2.03\ H_2O + 0.066\ CH_2O_{0.5}N_{0.15}$$

The SRT_{floc} was calculated using the reactor and effluent VSS concentrations, reactor mixed liquor sampling volumes, and the wasted floc volume.

3. Results and Discussion

Two identical IFAS reactors were continuously operated to study the effectivity of the multi-stressor treatment and other operational control tools (oxygen availability control, residual ammonium, and differential SRT control with hybrid sludge) on the nitrogen-converting community. The main goal was to achieve mainstream nitrogen removal by AnAOB with minimal NOB contribution, also referred to as a good microbial activity balance. Multiple variations in control tools were tested over time to observe the most optimal combination (**Table 1**): Continuous versus intermittent aeration (R2, Phase I versus II), doubled sulphide dose during the multi-stressor treatment tested at neutral starvation pH (R1, Phase I versus II-III and R2, Phase IV-V) and at slightly acidic pH (R2, Phase I-II versus IIII), and attached (R1) versus suspended (R2, Phase I-IV) biofilm growth. In the final phase of each reactor, the recurrent multi-stressor floc treatment was concluded to study the necessity of such treatment.

3.1. Overall reactor nitrite balance

R1 was first operated for an additional 34 days in anoxic mode to improve biofilm acclimatisation to mainstream conditions and promote AnAOB growth (Section 2.2). Afterwards, flocs were added and intermittent aeration was initiated. R1 started (day 35-45) with an initially balanced system as all the nitrite produced by AerAOB was consumed by AnAOB or remained residual (Figure 2 R1). The combination of a PN/A inoculum (reactivated long-term stored PN/A carriers, and DEMON® flocs and granules, Section 2.2) with intermittent aeration and recurrent multi-stressor floc treatments at standard sulphide dose (151 ± 15 mg S^{2-} L^{-1}) and neutral pH (7.1 ± 0.5), resulted in full NOB suppression during days 35-45. This was also reflected in the microbial community composition, with an initial low relative NOB abundance (≤ 0.2%, Figure 3).

Afterwards, NOB activity could consistently be observed at an average relative nitrite consumption of about 30% while nitrite accumulation was no longer observed. Consequently, these initial conditions failed to maintain stable and long-term NOB suppression. Insufficient AnAOB compared to AerAOB activity resulting in nitrite accumulation, potentially caused by the switch from sidestream (inoculum) to mainstream (reactor) conditions, was believed to be the main cause for the occurrence of NOB activity as high nitrite levels promote NOB activity (Gu et al., 2021).

From Phase IIIa onwards, the good microbial activity balance was restored with low nitrite uptake by NOB (4 ± 17%), high uptake by AnAOB (93 ± 20%) and neglectable nitrite accumulation (

Table 3). This was achieved by doubling the sulphide dose to 307 ± 15 mg S^{2-} L^{-1} (Phase IIa onwards), a temporary reduction in DO levels due to technical difficulties (Phase IIb and IIc) and the addition of additional reactivated long-term stored carriers in combination with fixing the aeration control (Phase IIIa). Once again, a reduced relative NOB abundance was observed at the start of Phase IIIa (0.2-0.5%), although it recovered to about 1% throughout the rest of Phase IIIa (Figure 3). In the final two phases, the balance deteriorated after purposedly removing 57% of the carriers (Phase IIIb), resulting in nitrite accumulation and overall lower rates, and concluding the recurrent floc treatment (Phase IV), resulting in a rapid revival of NOB activity (Figure 2 R1). This corresponded with the highest relative NOB abundance detected throughout R1's operation, namely 2% at day 164.

Figure 2. Microbial activity balance (or nitrite balance) of reactor 1 (R1, top) and 2 (R2, bottom). The nitrite production by AerAOB is visualised as well as the fate of this nitrite: consumption by AnAOB, NOB, or remains residual. Alternating grey and white zones correspond to different phases. The exact conditions per phase are summarised in Table 1.

The imposed reactor conditions on R2 together with the different inoculum (RBC suspended biofilm, DEMON® flocs and granules (section 2.2)) failed to achieve a good microbial activity balance with minimal NOB contribution during most phases (I-IV). Phase I started with continuous aeration at an average DO of 0.35 ± 0.23 mg O_2 L^{-1} in combination with recurrent multi-stressor floc treatments with a sulphide dose of 159 ± 17 mg S^{2-} L^{-1} and starvation pH of 6.2 ± 0.7. From Phase IIa onwards, the DO control was switched to intermittent aeration. Starting from Phase IIIa, the sulphide dose in the multi-stressor treatment was doubled to 303 ± 9 mg S^{2-} L^{-1}. And in Phase IV, the starvation pH was increased to 7.2 ± 0.5. Nevertheless, none of these changes could succeed in completely suppressing the NOB activity. This was also reflected in the relative NOB abundance which remained high throughout these phases (2-6%, Figure 4). Only after

exchanging all the suspended biofilm in R2 with 57% of the attached biofilm in R1 (day 138), to guarantee the presence of sufficient AnAOB activity, a good microbial activity balance was achieved with AnAOB consuming 76 ± 5% of the produced nitrite and NOB only 19 ± 4% (

Table 3, Phase V). Similar to R1, this balance was severely disturbed after the recurrent floc treatments were concluded (Phase VI).

The contribution of denitrification (DN) and denitritation (DNit) in TN removal and thus in the nitrite balance cannot be excluded since COD was added to the influent. Other IFAS studies observed that 80-100% of the added COD was removed aerobically, even at low DO setpoints between 0.05 and 0.30 mg O_2 L^{-1} (Laureni et al., 2016; Seuntjens et al., 2020). Since the DO in this study was on average 81% of the time above 0.05 mg O_2 L^{-1} (88.6% for the best performing Phase R1-IIIa) and continuously fed, it can be assumed that most COD was also removed aerobically. TN removal by DN is desired as it can improve the overall reactor performance by removing nitrate and COD without the use of aeration energy, but consequently it could influence the calculated nitrite balance, resulting in an overestimation of the NOB suppression and AnAOB activity. DNit is less preferred as it competes with AnAOB although it is still more resource-efficient compared to DN. Nevertheless, COD will also be present in the full-scale implementation at similar or even higher influent COD/N ratios (typically ≤ 2, Verstraete and Vlaeminck (2011)), thus the obtained results were not compromised. To further boost the DN potential, and thus TN removal, anoxic feeding rather than continuous could be implemented.

Table 3. Summary of the main performance indicators: TN removal rate (NRR) and efficiency, effluent nitrite concentration, and estimated NOB activity.

3.2. Dynamics in microbial community composition

The microbial community composition of the suspended sludge (flocs and granules) was frequently determined through the experiments to study any dynamics, possibly linked to the imposed control tools. Results therefore solely focus on nitrogen-converting species (AerAOB, NOB, and AnAOB), excluding heterotrophic bacteria. Flocs, granules, and carriers were occasionally measured separately as well.

The dominant genera in both reactors were *Nitrosomonas* (AerAOB), *Nitrospira* (NOB), and Ca. *Brocadia* (AnAOB) (Figure 3 and Figure 4). Additionally, *Nitrotoga* (NOB) was frequently observed while Ca. *Anammoxoglobus* (AnAOB) occasionally got detected in low relative abundance (≤ 0.2%). Ca. *Kuenenia* (AnAOB) was sometimes detected in relative high abundance, especially towards the end of the experiment (day 93+). Its presence was linked to the carriers: they occurred in high relative abundance in the seeded carrier's biofilm (Figure S1), detected after an additional 60-day reactivation in an anoxic SBR reactor (Section 2.2), seeded to R1 on day 0, and afterwards used again as bioaugmentation in R1 on day 95, and finally partially transferred to R2 on day 138 from R1. Moreover, Ca. *Kuenenia* was not detected in the other seeded sludges (Van Tendeloo et al., 2021a; Van Tendeloo et al., 2021b).

By comparing the microbial community composition of R1 and R2, it can be noted that R2 had a consistent and considerable elevated relative NOB abundance (0.4-8% versus 0.2-2%). This is in line with the performance data, where the lowest relative NOB activity was achieved in R1 (

Table 3). Similarly, a higher relative AerAOB abundance was detected (3-9% versus 2-5%). No consistent differences were observed for AnAOB.

Figure 3. Microbial community evolution of R1. Relative abundance of all identified NOB (red), AerAOB (green) and AnAOB (blue) amplicon sequence variants in the suspended sludge (flocs + granules), expressed relatively over the total community. "Days since inoculation" shows the number of days since the latest inoculation (**Table 2**).

Microbial community analyses of each sludge fraction (flocs, granules, and total suspended sludge) were conducted twice for each reactor (Figure S1). Carriers were also separately analysed towards the end of the experiment. The relative abundance of NOB was generally higher in the granules than in the flocs. This could be linked to the multi-stressor floc treatment, pressuring the NOB population in the flocs. No consistent difference in species could be detected. Unexpectedly, a high relative abundance of AnAOB on the flocs was detected (4-17%), which was unlikely as the short SRT_{floc} (3-17 days) would prevent AnAOB growth in the floccular fraction. A possible explanation could be the disintegration of granules or detachment of biofilm from the carriers. This could explain the high relative AnAOB abundance in R1 after day 97 when most of the biofilm was assumed to be on carriers, despite carriers not being included in the suspended sludge microbial community analyses (Figure 3).

Figure 4. Microbial community evolution of R2. Relative abundance of all identified NOB (red), AerAOB (green) and AnAOB (blue) amplicon sequence variants in the suspended sludge (flocs + granules), expressed relatively over the total community. “Days since inoculation” shows the number of days since the latest inoculation (Table 2).

3.3. Importance of the recurrent multi-stressor floc treatments

Necessity of the treatment

The application of recurrent multi-stressor floc treatments was essential for both achieving and maintaining a well-balanced nitrogen system. This was shown by the quick deterioration in performance after the recurrent treatment was concluded. For R1, already 6 days after the final treatment was concluded (day 147), nitrite consumption by NOB increased at the expense of AnAOB activity (Figure 2 R1). On average, 59 ± 35% of all produced nitrite was consumed by NOB in Phase VI, compared to only 11 ± 9% when the recurrent floc treatment was still applied in Phase IIIb (

Table 3). However, the relocation of 57% of R1’s carriers to R2 might have sped up this process, as insufficient potential AnAOB activity remained and consequently nitrite accumulation was observed (Figure 2 R1, Phase IIIb). As previously stated, the presence of elevated nitrite concentrations can boost NOB activity and was most likely responsible for this fast deterioration in performance in R1 (Section 3.1). For R2, a consistent elevated nitrite consumption by NOB was only observed 28 days after the final treatment, with a relative consumption of 41-75% (day 167-187) compared to 15-25% in Phase V. However, already 4 days after concluding the last treatment, a small increase in relative nitrite consumption by NOB (15-25% to 33-41%) was observed (Figure 2 R2). This could not be linked to concluding the recurrent floc treatment given the short time frame. The underlying trigger remains unknown, but after 5 days low NOB activity was observed again.

Relative abundance of NOB fluctuated over time, although a clear increase could be observed in R2 from day 115 onwards, from 0.4% to 8%, mainly due to an increase of *Nitrospira* (Figure 4). This could be linked to concluding the recurrent multi-stressor floc treatments at day 143, although this trend already started between day 115 and 129 for an unknown reason (no changes in operation) (Figure 4). The domination of *Nitrospira* could indicate that this NOB genus was the least susceptible to the imposed reactor conditions and multi-stressor treatments, followed by *Nitrotoga*. This was in accordance with other studies, in which the above-mentioned genera were also the most resilient towards FNA stress (Duan et al., 2019a; Ma et al., 2017).

Additional batch tests were performed to obtain more insights in the dynamics in AerAOB, NOB, and AnAOB activity in both the floc and biofilm faction (Section 2.6). By comparing the activity before (day 131) and 17 days after the final treatment was conducted (day 156), the effect of the recurrent floc treatment could be derived (

Table 4). A decrease in the floccular AerAOB/NOB activity ratio of both reactors could be observed from 3.0 and 1.6 to 1.2 and 1.2 for R1 and R2, respectively. Similarly, this ratio also decreased in the biofilm from 3.9 (both R1 and R2 shared the same attached biofilm before concluding the treatments) to 2.5 and 2.1, respectively. This showed that concluding the recurrent multi-stressor floc treatment resulted in a selective increase in NOB activity over AerAOB and AnAOB. Overall, the NOB activity measured in the batch test (sum flocs and biofilm) increased with 70% and 81% for R1 and R2, respectively, assuming that the initial NOB activity in the biofilm was respectively 43% and 57% of the activity measured in R1 on day 132 (as 57% of R1's carriers were relocated to R2 on day 137). AnAOB activity consistently decreased with respectively 47% and 31% while for AerAOB no (R1) or only a 22% increase could be observed (R2). The *in-situ* tested AnAOB activity was only 23% and 26% of the maximum AnAOB activity (*ex-situ* batch test) for R1 and R2, respectively, while this was 62% for the carriers from R1 before the recurrent treatment was concluded. This additionally highlights the importance of the floc treatment to limit NOB activity and consequently reduce the competition with AnAOB for nitrite.

Table 4. Summary of the batch maximum activity test results on days 131 and 156.

Beneficial effects of the treatment

Next to the importance of the floc treatment on maintaining a balanced microbial system, the batch test results also showed that excessive NOB growth on the biofilm could be avoided despite only treating the flocs. Contrary to the expected threat of uncontrolled NOB growth in the flocs, a positive (>1) AerAOB/NOB activity ratio could be achieved in the biofilms of both R1 and R2 (

Table 4). Additionally, this ratio was similar or even slightly higher for the biofilm compared to the flocs, while only the latter were treated. Consequently, the recurrent treatment of the flocs had an indirect, positive effect on the biofilm. Uncontrolled NOB growth in the biofilm can thus be avoided by treating solely the flocs with the multi-stressor treatment, in combination with the other applied operational strategies, in contrary to the issues encountered/concerns raised in other studies (Peng et al., 2020). A possible explanation could be the occurrence of sufficient AnAOB activity in the biofilm, competing with NOB for nitrite and thus avoiding uncontrolled NOB growth as described in the source-sink concept (Laureni et al., 2019; Seuntjens et al., 2020; Wang et al., 2021). This can be explained by the difference in nitrite affinity, which is 1-2 orders higher for AnAOB compared to NOB, allowing the active biofilm to serve as a sink and therefore impede the NOB activity (Lotti et al., 2014; Park and Noguera, 2007).

No signs of adaptation were observed over the 108 and 142 days of reactor operation with recurrent floc treatments for R1 and R2, respectively. Almost complete NOB suppression could still be achieved through the means of these treatments, until they were concluded in the final phase of each reactor as previously discussed. This was in contrary to Wang et al. (2021) who observed an increase in NOB activity up to 100% of the AerAOB activity after 60 days of recurrent exposure to FA treatments, caused by a shift from *Nitrospira* to *Nitrotoga* as main NOB genera.

Most-effective treatment conditions

Throughout the multiple phases in both reactors, some multi-stressor treatment conditions were varied, allowing to determine the most-effective conditions. Overall, the highest AnAOB and lowest NOB activity were obtained in combination with a high sulphide dose (307 ± 15 mg S^{2-} L^{-1}) and neutral pH (7.1 ± 0.5) during the multi-stressor treatment (

Table 3; R1-IIIa, R2-IV, and R2-V). The isolated effect of varying one treatment condition was however difficult to observe as it often coincided with other variations such as inoculation of new sludge and unforeseen changes in aeration control. Overall, the effect of concluding the multi-stressor treatment was considerably more severe than these small variations in treatment conditions (Figure 2). The higher effectivity of the multi-stressor treatment at doubled sulphide dose was in line with previous, unpublished own research, for which a 112% increase in nitrite accumulation (thus selective suppression of NOB over AerAOB) was observed compared to the same treatment with a lower sulphide dose and similar reactor conditions and inoculum. The optimal starvation pH according to that study on the other hand was at reduced pH (6.1 ± 0.1), yielding a 48% increase in nitrite accumulation compared to neutral pH (7.2 ± 0.1) while in this research the neutral pH seemed to be the most promising. A possible explanation could be the reduced AerAOB activity of -32% that was linked to the pH reduction, limiting the overall performance.

3.4. Importance of the other control strategies

Aeration control: continuous versus intermittent

The application of intermittent aeration (4/8 min on/off aeration, DO setpoint 0.9 mg O_2 L^{-1} and 0.60 ± 0.44 mg O_2 L^{-1} on average) yielded better results compared to continuous aeration (0.35 ± 0.23 mg O_2 L^{-1}) under the tested reactor conditions as shown by the transition from continuous to intermittent aeration in R2 (Figure 2 R2, Phase I to IIa). An increase in AnAOB activity was observed (14 ± 11 to 23 ± 4 mg TN-N L^{-1} d^{-1}) at the expense of less relative nitrite production by NOB (77 ± 38% to 65 ± 12%) and reduced nitrite accumulation (17 ± 62% to 0 ± 5%). The AerAOB activity remained almost identical (61 ± 29 and 62 ± 9 mg NO_2^--N L^{-1} d^{-1}). This observation was in line with Miao et al. (2016)

for whom NOB suppression was only achieved after switching from continuous (0.17 ± 0.08 mg O_2 L^{-1}) to intermittent aeration (7/21 min on/off at 0.5 mg O_2 L^{-1}).

The reduced airflow rate for R1 in Phases IIb and especially IIc compared to Ia-IIa resulted in a decreased average DO concentration of respectively 0.60 ± 0.32, 0.20 ± 0.24 and 0.72 ± 0.42 mg O_2 L^{-1} at a similar intermittent aeration pattern (

Table 3). With all other operational conditions constant, except for the inoculation of DEMON® flocs on day 82 (Phase IIc), a clear reduction in AerAOB activity could be observed from 55 ± 13 to 29 ± 7 and 28 ± 15 mg NO_2^--N L^{-1} d^{-1} for Phase IIa, IIb and IIc, respectively. Moreover, the TN removal rate decreased, although the relative nitrite consumption by AnAOB remained similar or even increased (58 ± 14%, 54 ± 16% and 68 ± 32%). In contrast, the relative nitrite consumption by NOB considerably decreased from 45 ± 17% (Phase IIa) to 33 ± 15% (IIb) and 10 ± 21% (IIc), resulting in relatively more nitrite accumulation (up to 22 ± 13%). This could not be completely linked to the inoculation of additional flocs, as they were only added at the start of Phase IIc and thus cannot explain the increase in Phase IIb. The reduced average DO concentration thus resulted in overall lower aerobic rates, boosted AnAOB activity and resulted in a relative reduction in NOB activity and nitrite accumulation. The full-scale application of these reduced DO setpoints would however not be feasible as it would results in too low conversion rates, making it a space-intensive technology. The originally targeted DO setpoint was therefore restored from Phase IIIa onwards, despite potentially being slightly less effective. Restoring these settings resulted in a further decrease in NOB activity (to 4 ± 17% in Phase IIIa), in combination with the addition of extra DEMON® flocs and reactivated long-term stored carriers to supply more potential AnAOB activity which successfully consumed all residual nitrite.

Biofilm growth mode: attached or suspended

One of the main differences between R1 and R2 was the biofilm type that was used as inoculum, being mainly attached (carriers) for R1 and suspended (granules) for R2. The occurrence of granules in R1 during the first 61 days can be neglected since: i) their biomass concentration was always limited (< 0.09 g VSS L^{-1} and < 19% of the full suspended VSS, Table S2), ii) no additional granular sludge was added over time (solely flocs), and iii) even after the granular biomass concentration dropped below

0.02 g VSS L^{-1} or 4% of the total suspended solids, no differences in performance could be observed (Figure 2 R1). For R2, no biofilm growth was observed on the empty carriers during the 113 days they were present in the reactor, thus AnAOB were only present in suspended biofilm.

Attached biofilm appeared to be the most optimal growth mode under the tested conditions as it achieved the best microbial activity balance (R1-IIIa,

Table 3). This could also be derived from the batch test results, with a AerAOB/NOB and AnAOB/NOB activity ratio of 3.9 and 3.0, respectively, for the attached growth in R1 and only 1.7 and 0.3, respectively, for the suspended growth in R2 (

Table 4). Replacing the granules in R2 with 57% of R1's carriers considerably improved its performance, reaching a relative nitrite consumption by NOB similarly to R1 (19 ± 4% and 4 ± 17%). A potential explanation for the supremacy of attached growth could be that the carriers are retained in the reactor vessel (HRT = 3.5 - 5.7 h) and do not cycle through the settler (HRT = 0.8 - 1.2) unlike the granules. The anoxic conditions in the settler create additional stress, possibly resulting in the disintegration of granular sludge due to the anoxic starvation (Yamamoto et al., 2011), and limits the growth potential. Other studies also showed that carriers might be advantaged over granules growth as it is easier to retain biofilm on carriers compared to granules (O'Shaughnessy, 2016). Carriers do not have these disadvantages, which could potentially explain the higher AnAOB activity retention on the carriers. Additionally, the use of carriers in a full-scale application would improve the robustness as they are easier to retain in the system.

The presence of sufficient AnAOB activity in general, regardless of the growth mode, was additionally essential in achieving and maintaining a good microbial activity balance. For R1, the addition of reactivated long-term stored carriers with high AnAOB activity (Section 2.2) at the start of Phase IIIa, in combination with the applied control strategies, improved the microbial activity balance. Wang et al. (2021) made a similar observation for which the addition of AnAOB activity improved the selective NOB suppression. This was linked to the source-sink concept and limiting nitrite availability to avoid NOB activity, as explained in Section 3.1. Moreover, after 57% of the carriers, and thus AnAOB activity, was removed in Phase IIIb, nitrite accumulation was observed which boosted NOB growth and resulted in a

faster deterioration of the microbial activity balance compared to R2, where almost no nitrite accumulated (Figure 2;

Table 3).

Bioaugmentation

The occasional addition of PN/A sludge (Table 2) also influenced the reactor performance and can therefore be seen as a control tool. Sidestream PN/A sludge was added to the reactors if either the AnAOB activity was too low (adding granules, mainly to R2) or if the conversion rates or floccular VSS concentration was too low (adding flocs, to both R1 and R2). These bioaugmentations most likely helped to achieve the good microbial activity balance (R1 Phase IIIa and R2 Phase V) but were not sufficient on themselves to maintain this. Firstly, inoculations typically have an immediate but short-lasting effect and are mainly beneficial if the operational conditions favour PN/A. Bioaugmentations are therefore often successful for start-up (Bartroli et al., 2011; Zhang et al., 2012) but not necessary as process control (Kamp et al., 2019; Mannucci et al., 2015). Secondly, no more sidestream PN/A flocs were added to R1 after day 95 and yet the microbial balance was maintained for 95 days until the experiment was concluded (about 10 times SRT_{floc}). Finally, it was clearly shown in the final phase of each reactor that the multi-stressor treatments were essential to avoid NOB to dominate the community while this was not the case for the bioaugmentation. For future applications, bioaugmentation is therefore not proposed as a control strategy but was rather used in these lab experiments to overcome process hiccups like unwanted AnAOB activity loss due to suboptimal process conditions.

3.5. Application potential

Due to the combination of all the applied operational strategies (intermittent aeration, hybrid sludge growth, residual ammonia, and short SRT_{floc} control) but especially due to the application of the recurrent multi-stressor floc treatment, a well-balanced lab-scale PN/A system was established at mainstream conditions with low nitrite consumption by unwanted NOB. The best-performing phase

was R1-IIIa in which an average volumetric TN removal rate of 71 ± 21 mg N L^{-1} d^{-1} was achieved with NOB activity equal to only 4 ± 17% of the AerAOB activity (

Table 3). This obtained rate is similar to what is currently achieved in a full-scale sewage treatment plant (100 mg N L^{-1} d^{-1}, Tchobanoglous et al. (2013)), although the latter includes other processes such as organic matter and phosphorus removal. However, the TN removal efficiency (39 ± 9%), and consequently the effluent quality, was insufficient to be applicable full-scale. The main reason was an elevated effluent ammonium concentration (25 ± 6 mg N L^{-1}), limiting the TN removal efficiency to the ammonium conversion efficiency of 50 ± 10%. This was due to the relatively low suspended biomass levels of on average 0.3 ± 0.1 g VSS L^{-1} in that phase. In a future application, higher biomass levels should be applied to achieve higher conversion rates and consequently avoid overloading of the system. This however affects the floccular SRT, possibly affecting the NOB suppression efficiency and should thus be tested out first under experimental settings. Additionally, further optimisation of the technology could be considered, preferably on pilot scale, like the SRT_{floc} control and aeration settings (minor changes) to boost the rates without compromising the NOB suppression. In a full-scale application, ammonium-sensors would be linked to the aeration control to guarantee desired ammonium levels, with gradually lowered setpoints towards the final basins to avoid high ammonium concentration in the final effluent. Additionally, continuous aeration could be applied at the final basin to oxidise any remaining ammonium into nitrate to improve the effluent environmental quality. Finally, post-treatment technologies like post-denitrification with methanol dosing could be considered to reach (future) strict discharge limits (e.g., 3 mg TN L^{-1}), similar to conventional treatment plants, or reducing the overall volumetric TN loading rate.

The recurrent multi-stressor floc treatment would be applied continuously in a full-scale application. Flocs from the return-sludge line, separated from the biofilm by, for example, a sieve or hydro cyclone and concentrated in the secondary settler, would be continuously subjected to the multi-stressor treatment. The application of a belt press could be considered to further concentrate the return-sludge and consequently reduce the total costs (Peng et al., 2020). Sulphide should be added from an external source but is globally available in large quantities as it is a recovered resource from fossil fuel purification. The acid and base for respectively the pH correction after the sulphide dose and the FA shock should also be provided externally. The FA shock on the other hand can be generated *in situ*, utilising the ammonium-rich reject water after anaerobic digestion of the sludge, which was calculated to be sufficient at similar conditions by Peng et al. (2020). Other manipulations such as sparging with N_2 gas are redundant for the full-scale application since return sludge is treated, originating from a settler and thus already be deprived of oxygen. The other control strategies can easily be implemented as they are currently already applied in STP, in either the main or side stream. The treatment's stress is lifted by reintroducing the sludge into the main stream due to dilution effects, reduces pH (less FA speciation), and presence of oxygen (converting sulphide into harmless sulphate) and other substrate (lifting the starvation). A flash aeration basin at the end of the treatment line could be foreseen to avoid the introduction of any sulphide into the main stream.

The subsequent steps in the development of the multi-stressor treatment and achieving mainstream PN/A in general would be to first further fine-tune the control strategies as mentioned before. Secondly, validation with real pre-treatment sewage including fluctuations in both influent concentration and load should be tested to increase the technology readiness level, as well as verifying the effectivity of the control tool at lower temperatures (<20°C). Additionally, a life cycle and economic assessment should be carried out to determine whether the proposal is sustainable and economically viable.

4. Conclusions

A good microbial activity balance with high AnAOB (71 ± 21 mg N L^{-1} d^{-1}) and low NOB activity (4 ± 17% of AerAOB) could be achieved under mainstream conditions by combining multiple operational strategies: recurrent multi-stressor floc treatments (307 ± 15 mg S^{2-} L^{-1} spiked 2-day starvation at pH 7.1 ± 0.5, followed by a 1-hour 34 ± 2 mg FA-N L^{-1} shock), hybrid sludge (flocs and attached biofilm), short SRT_{floc} control (8.5 days), intermittent aeration (4/8 min on/off at DO setpoint 0.9 mg O_2 L^{-1}), and residual ammonium control (≥ 5 mg N L^{-1}). The multi-stressor floc treatment was the most important control tool and should be continuously applied to maintain a good microbial activity balance. No signs of NOB adaptation were observed, and uncontrolled NOB growth on the biofilm could successfully be avoided by solely treating the flocs to safeguard the AnAOB activity. The presence of sufficient AnAOB activity to limit residual nitrite levels was hereby also essential to avoid NOB activity.

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6. Author contributions

M. Van Tendeloo: Conceptualization, Methodology, Investigation, Visualization, Writing – original draft, Funding acquisition. M. C. Baptista: Conceptualization, Methodology, Investigation, Visualization. T. Van Winckel: Conceptualization, Methodology, Writing – review & editing. Siegfried E. Vlaeminck: Conceptualization, Writing – review & editing, Methodology, Visualization, Supervision, Project administration, Funding acquisition.

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