Challenges for simultaneous nitrification, denitrification, and phosphorus removal in microbial aggregates: mass transfer limitation and nitrous oxide production

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Abstract

The microbial community composition and activity was investigated in aggregates from a lab-scale bioreactor, in which nitrification, denitrification and phosphorus removal occurred simultaneously. The biomass was highly enriched for polyphosphate accumulating organisms facilitating complete removal of phosphorus from the bulk liquid; however, some inorganic nitrogen still remained at the end of the reactor cycle. This was ascribed to incomplete coupling of nitrification and denitrification causing NO$_3^-$ accumulation. After 2 h of aeration, denitrification was dependent on the activity of nitrifying bacteria facilitating the formation of anoxic zones in the aggregates; hence, denitrification could not occur without simultaneous nitrification towards the end of the reactor cycle. Nitrous oxide was identified as a product of denitrification, when based on stored PHA as carbon source. This observation is of critical importance to the outlook of applying PHA-driven denitrification in activated sludge processes.

Keywords: Simultaneous nitrification denitrification; Microsensors; Aggregates; Nitrous oxide

1. Introduction

Removal of nitrogen (N) and phosphorus (P) through biological treatment of wastewater is enforced in most developed countries to protect local waterways from eutrophication. In biological nutrient removal, inorganic nitrogen in the form of ammonium (NH$_4^+$) is removed through aerobic, autotrophic nitrification followed by anoxic, heterotrophic denitrification. The process is normally split into two stages as the environmental conditions required for each process are very different.

Phosphorus can be removed biologically through enhanced biological phosphorus removal (EBPR), exploiting the ability of polyphosphate-accumulating organisms (PAOs) to take up P in excess of metabolic requirements and accumulate it intracellularly as polyphosphate [1]. This metabolic phenotype is facilitated by a continuing cycle of provision of organic carbon largely in the form of volatile fatty acids (VFAs) to the microorganisms, and then exposure of the organisms to first anaerobic, then aerobic conditions. It is essential that NO$_3^-$ and carbon are not present simultaneously, and for clarification we will use the term “anoxic” for the absence of O$_2$ but presence of NO$_3^-$, and the term “anaerobic” for the absence of both O$_2$ and NO$_3^-$. PAOs take up and store VFAs as polyhydroxyalkanoates (PHA) under anaerobic conditions using energy...
obtained by hydrolysis of stored polyphosphate and glycogen. Stored PHA is then oxidized in the following aerobic period, providing energy for growth and replenishment of the polyphosphate storage by P uptake. The environmental conditions required to obtain P removal are thus different from those required for nitrification and/or denitrification.

Several studies have pointed out that nitrification and denitrification can occur simultaneously at low oxygen levels [2–4], offering a possibility for designing a one-stage treatment process for N removal. Furthermore, EBPR has been demonstrated under alternating anaerobic-anoxic conditions, where PAOs carry out denitrification of their stored PHA in the anoxic phase, rather than aerobic metabolism [5]. It was recently demonstrated that EBPR can be incorporated in a simultaneous nitrification-denitrification (SND) process by encouraging denitrification to be mediated by PAOs [6]. In this simultaneous nitrification-denitrification-phosphorus removal (SNDPR) system, and in the reactor of the present study, carbon is supplied in an initial anaerobic period and can therefore selectively be taken up by PAOs and stored as PHA. In the following aerobic period, SNDPR is facilitated by the presence of adjacent aerobic and anoxic microzones in microbial aggregates caused by mass transport limitation of oxygen, providing conditions for both nitrification and denitrification. Theoretically, denitrification with simultaneous P uptake is carried out by PAOs using PHA stored in the previous anaerobic period as the carbon source.

Organic carbon is often the limiting substrate for both denitrification and P removal, and many wastewater treatment plants add extra carbon for denitrification to balance the processes [7]. A combination of denitrification and EBPR in one process could offer substantial savings on carbon for the overall nutrient removal process, which makes this approach highly attractive. However, balancing three different processes (nitrification, denitrification and EBPR) simultaneously in a single-sludge system requires skilful management of the bacterial populations. Successful enrichment of PAOs can fail due to the proliferation of glycogen-accumulating organisms (GAOs) that have a similar metabolism to PAOs, however without contributing to P removal[1]. Nitrifying organisms must be able to compete successfully for oxygen with aerobic heterotrophs in the system, and denitrifying organisms require the presence of anaerobic conditions as well as organic carbon to remove nitrate and nitrite produced by nitrification.

This study addresses some of the challenges to the SNDPR process imposed by the complex interplay between highly different microbial processes situated in a three-dimensional aggregate, which plays an important role for obtaining the precise conditions for all processes to occur simultaneously. We specifically focus on nitrification and denitrification as these processes need to be carefully balanced and closely coupled to obtain complete conversion of ammonium to dinitrogen gas. The production of nitrous oxide (N2O) from denitrification in this system is also investigated, as this highly potent greenhouse gas is an unwanted bi-product which could preclude the application of SNDPR in full-scale wastewater treatment processes.

2. Materials and methods

2.1. Reactor set-up and operation

Biomass was enriched in a 5 l lab-scale sequencing batch reactor (SBR) seeded with sludge from the Caboolture Sewage Treatment Plant, Qld, Australia. The SBR was operated with a cycle time of 6 h consisting of a 117 min non-aerated and a 190 min aerated period, followed by 43 min settling and 10 min decant. The oxygen concentration during the aerobic period was measured with an on-line dissolved oxygen probe (YSI 5739, Yellow Springs Instruments, USA) and kept between 11 and 16 μmol l⁻¹ by on/off control of air sparging at 300 ml min⁻¹. Three litres of synthetic wastewater containing 3.84 mmol l⁻¹ (230 mg l⁻¹) acetic acid, 1.64 mmol l⁻¹ NH₄⁺ (23 mg l⁻¹), 0.58 mmol l⁻¹ PO₄³⁻ (18 mg/l P), and 0.525 ml micronutrient solution [8] were pumped into the reactor in the first 7 min of the anaerobic period, resulting in concentrations in the reactor at the start of the cycle of approximately 2.3 mmol l⁻¹ acetate, 0.98 mmol NH₄⁺, and 0.35 mmol PO₄³⁻. After the settling period, 31 supernatant was removed, resulting in a hydraulic retention time (HRT) of 10 h. The solids retention time (SRT) was kept at about 15 days. The pH in the system was recorded but not controlled, and fluctuated between 7.0 and 7.5. Biomass concentration, biomass content of glycogen and PHA, influent nitrogen and phosphorus, as well as phosphorus release at the end of the anaerobic period were monitored twice weekly. Cycle studies were carried out weekly by taking the samples every 20–30 min within a 6-h cycle. Ammonium, nitrate, nitrite, orthophosphate, glycogen, PHA, mixed liquor suspended solid (MLSS), and mixed liquor volatile suspended solids (MLVSS), were analysed as described in Zeng et al. [8]. The on-line oxygen data were used to calculate the oxygen uptake rate (OUR) during the aerobic period. In the first 1.5 h of the aerobic period (from 2 to 3.5 h of the cycle), air was sparged continuously because the concentration did not reach the upper set point due the high oxygen uptake. The OUR during this period was calculated as OUR = K₅a * (C*–C), where K₅a is the oxygen transfer coefficient (estimated 3 h⁻¹, R. Zeng, unpublished data), C* is the oxygen concentration at saturation (270 μmol l⁻¹ in this study) and C is measured oxygen concentration. During the rest of the aerobic period (from 3.5 to 5 h of the cycle), oxygen
sparging was turned off every time the concentration reached the upper set-point, and the OUR was calculated from the oxygen consumption rate measured when oxygen sparging was off: \( \text{OUR} = \frac{C_I - C_O}{(2 - t)} \), where \( C_I \) is the oxygen concentration at the time \((t)\).

2.2. Anoxic batch test

1.2 l mixed liquor was removed from the 5 l reactor at the end of the anaerobic period and transferred to a separate reactor vessel and continuously sparged with helium gas. Nitrate was added from a stock KNO\(_3\) solution to achieve an initial nitrate concentration of 4.64 mmol l\(^{-1}\) NO\(_3^-\), and off-line samples for nitrite, NO\(_2^-\), orthophosphate and ammonium were taken every 15 min after the addition of nitrate. Nitrous oxide was monitored on-line in the bulk liquid with a N\(_2\)O microsensor (described below).

2.3. Microsensors and microsensor analysis

Oxygen microsensors (tip size 10 \(\mu\)m, 90% response time 2 s) were constructed as described by Revsbech [9] and microscale biosensors for NO\(_3^-\) + NO\(_2^-\) (NO\(_3^-\) and NO\(_2^-\)) (tip size 30 \(\mu\)m, 90% response time 40 s) were constructed according to Larsen et al. [10] and Nielsen et al. [11]. Microsensors for N\(_2\)O (tip size 30 \(\mu\)m, response time 25 s) were purchased from Unisense A/S (Aarhus, Denmark).

Oxygen profiles measured in aggregates with a diameter of approximately 500 \(\mu\)m or larger, were performed by transferring aggregates from the 5 l reactor and placing them in a flow-cell with an upward vertical flow of medium recycled through a 1 l reservoir [12]. The sensor was moved stepwise into the aggregates from above. Movement of the sensor and data acquisition was obtained with the software Profix (Unisense A/S, Aarhus, Denmark). Aggregates were sampled from the reactor at the end of the anaerobic period for measurements of oxygen profiles under “nitrifying conditions” (in the presence of ammonium). These measurement were carried out over a 2 h period from when the aggregates were removed from the reactor. The medium in the flow cell was adjusted to have approximately the same pH and nutrient composition in the parent reactor at the beginning of the aerobic period of the reactor cycle, i.e. pH 7, no acetate, 1 mmol l\(^{-1}\) NH\(_4^+\), and 5 mmol l\(^{-1}\) PO\(_4^{3-}\). Measurements were repeated at different oxygen concentrations, and the oxygen level of the flow-cell was adjusted by sparging with air and/or N\(_2\). It is important to note that the nutrient level in flow-cell would not change substantially during the measurements, as the amount of biomass (a few aggregates) contained in the flow cell was very small compared to the volume of media cycling from the reservoir through the flow cell.

2.4. Analysis of biomass composition and structure

Approximately 20 aggregates were sampled from the 5 l reactor at the end of the anaerobic period and fixed with 4% paraformaldehyde [13] and embedded in OCT for cryosectioning [12]. Sections (20 \(\mu\)m) were transferred to microscope slides and dried by sequential immersion for 3 min in 50%, 80%, and 98% ethanol and air dried. Fluorescence in situ hybridization (FISH) was performed on at least 10 different granules [13] targeting ‘*Candidatus Competibacter phosphatis*’ (henceforth referred to as *Competibacter*) using a mix of the probes GAOQ989, GAOQ431 [14], and GB_G2 [15] all labeled with Cy3. ‘*Candidatus Accumulibacter phosphatis*’ (henceforth referred to as *Accumulibacter*) was targeted with Cy5.
labeled PAOMIX probes (PAO462, PAO651 and PAO846) [16], and FITC labeled EUBMIX probes [17] targeted all bacteria. FISH was also used to study nitrifying bacteria, with probes NSO1225 and NSO190 [18] targeting ammonia-oxidising bacteria, and NIT3 [19] and Ntspa662 [20] targeting nitrite-oxidizing bacteria. Sixteen images were taken of FISH targeting Competibacter and Accumulibacter, and 11 images targeting ammonia- and nitrite-oxidising bacteria. Through image analysis, the abundance of Competibacter, Accumulibacter, and nitrifying bacteria was determined in the outer 100 µm of the aggregates and compared with their abundance in the central part (deeper than 100 µm) of the aggregates. Samples were analysed using a BioRad Radiance 2000 Confocal Laser Scanning Microscope and collecting eight bit images for each fluorochrome sequentially. Post FISH staining of PHA accumulated intracellularly was performed with Nile blue A [21] and visualized on the CLSM using Cy3 settings. The same field as analysed by FISH was located manually.

The aggregate size distribution was measured spectrophotometrically using a Malvern laser light scattering instrument, Mastersizer 2000 series (Malvern Instruments, Worcestershire, UK: www.malvern.co.uk). Three replicate samples of 30 ml were analysed weekly.

3. Results

3.1. Process performance of the bioreactor

Weekly cycle studies (e.g. Fig. 1), stable effluent nutrient levels, and MLSS and MLVSS (data not shown) confirmed that the reactor performance was in steady state. The reactor biomass was able to completely transform carbon and phosphorus, but not nitrogen. An example of a cycle study is shown in Fig. 1. Acetate was fully uptaken within the first half hour of the 2 h anaerobic period with a simultaneous release of PO₄³⁻ from the biomass to the bulk liquid. The phosphorus release to carbon uptake ratio was 0.58 (P mol:C mol), which according to the stoichiometry of the metabolic model with acetate at pH 7 [22], indicates that all acetate was taken up by PAOs. The intracellular concentrations of PHA and glycogen at the end of the anaerobic period were 1.61 and 5.7 C mmol l⁻¹, respectively. In the subsequent 3.5 h aerobic period, PO₄³⁻ was taken up by the biomass and depleted from the bulk liquid (Fig. 1). PHA was also largely depleted (0.098 C mmol l⁻¹), and glycogen increased to 8.11 C mmol l⁻¹. During the aerobic period, ammonium was oxidized through nitrification and the resulting nitrite and nitrate were partly removed through denitrification. From the point when NH₄⁺ was depleted, the NOₓ⁻ concentration remained virtually the same for the rest of the cycle (Fig. 1). At this point the oxygen uptake rate also decreased dramatically. It should be noted some of the NOₓ⁻ was reduced to N₂O rather than N₂ giving rise to a transient accumulation of N₂O during the aerobic period. It was not quantified how much of the reduced nitrogen appears as N₂O as opposed to N₂.

Specific investigations of the denitrification process through anoxic batch tests (Fig. 2) showed that N₂O was produced from denitrification. The N₂O production was highest in the first hour of the test, at which time the highest NOₓ⁻ reduction rate could be found, and a small accumulation of NO₂⁻ was detected.

3.2. Biomass composition and structure

The biomass consisted of a mix of aggregates that appeared very different in density and size. Some had a
loose, floc-like structure and others a round granular shape with a more defined surface. The average size of the aggregates was 290 µm (SE = 49, n = 3) in diameter. Cryosectioning and FISH analysis of the granule shaped aggregates revealed a very loose structure with large void spaces inside the biomass (Fig. 3(a)). *Accumulibacter* were highly abundant and clearly exceeded *Competibacter* (*t* test, *p* < 0.05), which is in accordance with the excellent phosphorus removal characteristics of the biomass and the high carbon uptake to phosphorus release ratio. Small clusters of ammonia oxidizing *Nitrosomonas* and nitrite oxidizing *Nitrospira* were also identified, however, *Nitrobacter* could not be detected (images not shown). The aggregates did not appear to have any consistent three-dimensional arrangement of the different members (*Accumulibacter*, *Competibacter*, and

Fig. 2. Anoxic batch test following a 1 h anaerobic period in which acetate was fed and depleted. Time 0 indicates the time of addition of NO$_3$–.

Fig. 3. Confocal laser scanning micrographs showing FISH of a sectioned aggregate (a, b) and PHA-specific staining (c) of the same field as shown in (b). *Competibacter* (GAOMIX) are shown in orange, *Accumulibacter* (PAOMIX) in blue, and other Bacteria in green. (d) shows a grey-scale version of (b) overlaid with (c).
nitrifying bacteria) of the microbial community (outer 100 μm of the granules quantified against the inner part, t test, p < 0.05).

3.3. Microbial processes and the gradient environment

Post FISH staining of stored PHA of a sample taken at the end of the anaerobic period (Fig. 3(b)–(d)) confirms that primarily *Accumulibacter* accumulated PHA under anaerobic conditions. The distribution of PHA did not appear to be restricted to any particular part of the aggregate. Oxygen limitation in the central part of the larger aggregates was demonstrated by oxygen microsensor profiles measured at different bulk liquid oxygen concentrations (Fig. 4(a)). Oxygen penetrated 50–100 μm into the aggregates at the oxygen level of the reactor (10–15 μmol l⁻¹), and most aggregates would thus have aerobic and anoxic zones, given the average aggregate diameter of 290 μm. Increasing the bulk liquid oxygen concentration led to further penetration of oxygen into the aggregates. At 30 μmol l⁻¹ bulk liquid oxygen, the penetration was approximately 200 μm, and at 80 μmol l⁻¹, oxygen fully penetrated even large aggregates with a diameter of 700 μm.

The profiles in Fig. 4(a) were measured under nitrifying conditions (in the presence of NH₄⁺) and therefore represent the oxygen distribution in the aggregates in the first 2 h of the aerobic period. However, if aggregates were sampled from the reactor at the time of ammonium depletion (2 h into the aerobic period) and analysed with microsensors under non-nitrifying conditions, oxygen fully penetrated the aggregates even at bulk liquid concentrations of 12 μmol l⁻¹ (Fig. 4(b)). The oxygen uptake rate of the aggregates under non-nitrifying conditions, as calculated from the slope of the oxygen profile at the aggregate surface, was only 18% of the rate observed when nitrifying bacteria were active.

Nitrogen transformations were studied with microsensors in the artificial biofilm setting. The concentration profiles show a net production of NO₃⁻ in the aerobic (upper) part of the biofilm and diffusion of NO₃⁻ to the overlying water phase and to the anoxic zone below where it was consumed, as indicated by the concave shape of the concentration profile (Fig. 5). Nitrous oxide was produced in the bottom part of the biofilm under anoxic conditions and diffused out of the medium above the biofilm made from 2:1 volumes of homogenized mixed liquor and 1% agarose. The medium above the biofilm contains no carbon source, all other nutrient concentrations are otherwise identical to what is found in the reactor at the beginning of the cycle after the feeding period. All profiles are single profiles at different time points, T = 0 being when the biofilm was cast and incubated in the medium.
4. Discussion

4.1. Coupling between nitrification and denitrification

Successful enrichment of *Accumulibacter* led to efficient phosphorus uptake, and the phosphorus concentration in the effluent of the reactor was constantly below 6 µmol l⁻¹. Complete conversion of nitrogen was, however, never obtained (Fig. 1). In SND, efficient nitrogen removal requires a tight coupling between nitrification and denitrification so that no NO₃⁻ accumulates in the bulk liquid. To obtain this, the nitrifying and denitrifying bacteria must be in close proximity. The relatively-loose structure of the aggregates (e.g., Fig. 3(a)) could offer one explanation as to why some NO₃⁻ escapes from the biomass to the bulk liquid. Another cause for NO₃⁻ accumulation in the bulk could be linked to the size distribution of the aggregates. Oxygen penetrated at least 50 µm into the aggregates at the oxygen level present in the reactor, and aggregates with a diameter of 100 µm or less would therefore be fully penetrated by oxygen providing conditions for nitrification but not for denitrification. When the oxygen concentration was increased to just 30 µmol l⁻¹, oxygen penetration increased to 200 µm, which would leave most aggregates fully aerobic. Accurate control of the bulk liquid oxygen concentration is therefore extremely important.

Another contribution to incomplete transformation of ammonium to N₂, was the fact that no NO₃⁻ reduction occurred after the time of NH₄⁺ depletion in the aerobic period (Fig. 1), hence denitrification activity ceased when nitrification was not occurring. Oxygen microsensor profiles measured in the aggregates revealed that the oxygen uptake without the activity of nitrifying bacteria was too small to facilitate development of anoxic zones in the aggregate centre. Any NO₃⁻ diffusing from the biomass to the bulk liquid during SND would therefore not be removed. The importance of oxygen consumption by nitrifying bacteria for the formation of anoxic microsites in the aggregates was further illustrated by an abrupt decrease in the oxygen uptake rate at the time of NH₄⁺ depletion (Fig. 1).

It is possible that denitrification ceased partly due to lack of carbon, as intracellular PHA would be consumed gradually during the aerobic period of the reactor cycle. We did however, observe PO₄³⁻ uptake for at least 40 min after the depletion of NH₄⁺, which indicates the presence of stored carbon at that time as carbon oxidation is required to provide energy for PO₄³⁻ uptake.

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Fig. 6. Oxygen, NO₃⁻, NO₂⁻, and N₂O profiles into biofilm (as shown in Fig. 5) with 100 µmol l⁻¹ NO₃⁻ added to the overlying water (a) and 100 µmol l⁻¹ NO₃⁻ plus 1.55 mmol l⁻¹ acetate (b). Profiles in are averages of replicate profiles measured at least 30 min after addition of nitrate (a) or acetate and nitrate (b), error bars = SE (n = 3–4).
Increased PHA accumulation in the biomass could be one strategy for stimulating denitrification towards the end of the reactor cycle, but perhaps a more simple approach would be introduction of a post-nitrification anoxic period, which allows denitrification to proceed if PHA has not been depleted.

4.2. Using PHA-driven denitrification for SNDPR

In the present study, carbon was fed to the bioreactor under anaerobic conditions, and dissolved carbon was not present in the bulk liquid at any time during the aerobic period. This strategy offers two advantages to SNDPR. Firstly, carbon uptake is restricted to PAOs (and GAOs) as these organisms have the unusual ability to take up carbon and convert it to intracellular PHA under conditions where no electron acceptor is available. As biological phosphorus removal relies on the abundance of PAOs, this is highly desirable.

Secondly, carbon for heterotrophic respiration during the aerobic period is supplied from within the aggregates and is restricted to the cells that were able to take up and store carbon in the previous anaerobic period. If oxygen and carbon is supplied simultaneously from the bulk liquid, both substrates have to diffuse into the aggregate and most of the oxygen would likely be used by heterotrophic bacteria for aerobic carbon oxidation. Nitrifying bacteria compete very poorly against aerobic heterotrophic bacteria as they generally have a higher $K_m$ for oxygen [23–25], and nitrification is usually undetectable in nitrifying/denitrifying systems in the presence of dissolved organic carbon [26]. When carbon is supplied from stored PHA, heterotrophic respiration may dominate in the first part of the aerobic period, but as PHA is gradually depleted from the cells in the aerobic zone near the surface of the aggregate, nitrifying bacteria would have access to oxygen. The curvature of the $\text{NH}_4^+$ graph in Fig. 1 indicates a slower $\text{NH}_4^+$ uptake in the first hour of the aerobic period, after which it increased to an almost constant rate (linear decrease in the concentration) until $\text{NH}_4^+$ was depleted. The low ammonium oxidation rate in the first hour of the aerobic period can most likely be attributed to competition for oxygen with aerobic PHA-oxidizing bacteria (PAOs and GAOs), and such a delay in the onset of nitrification has also been observed for nitrifying bacteria in a phosphorus-removing biofilm by Gieseke et al. [27]. The oxygen profiles in Fig. 4(b) illustrate the difference in the oxygen uptake of the aggregates during the first 2 h of the aerobic period, and during the last 1.5 h when ammonium is depleted.

The use of PHA rather than an external carbon source such as acetate may further slow down the carbon oxidation rate, which allows not only for nitrifying bacteria to better compete for available oxygen; it also prolongs the time in which carbon is available for denitrification [26], which is important to maintain good coupling between nitrification and denitrification until ammonium is depleted.

4.3. Production of $\text{N}_2\text{O}$ from denitrification of PHA

Bulk liquid measurements in the bioreactor showed accumulation of $\text{N}_2\text{O}$ from approximately 0.5 h to approximately 2.5 h into the aerobic period. As $\text{N}_2\text{O}$ was stripped from the liquid by air sparging, $\text{N}_2\text{O}$ measurements could not be evaluated quantitatively. Production of $\text{N}_2\text{O}$ can occur from either nitrification [28] or denitrification [29]. Investigation of nitrification and denitrification in the biomass through $\text{O}_2$, $\text{NO}_x$ and $\text{N}_2\text{O}$ microsensor measurements in a biofilm set-up showed that $\text{N}_2\text{O}$ was accumulated under anoxic conditions in the bottom of the biofilm, and thus occurred as a result of denitrification. This was further confirmed by anoxic batch tests (Fig. 2).

A number of factors have been suggested in the literature to affect $\text{N}_2\text{O}$ production from denitrification. Otte et al. [30] showed in pure culture studies of *Alcaligenes faecalis* that the $\text{NO}_x$ reductase is more sensitive to oxygen inhibition than the nitrite reductase, and this can cause $\text{N}_2\text{O}$ accumulation when denitrification occurs under microaerobic conditions. The gradient of oxygen into the aggregates in the present study certainly offers a possibility for denitrification to occur under such conditions, however, $\text{N}_2\text{O}$ production also occurred from denitrification in batch tests under strictly anoxic conditions (Fig. 2) and oxygen therefore does not appear to be the causative factor.

Incomplete denitrification caused by a low C/N ratio of the substrate has also been shown to cause accumulation of $\text{N}_2\text{O}$ [31]. Hanaki et al. [32] demonstrated in lab-scale reactors that low chemical oxygen demand (COD)/N ratio (3.5), short SRT (0.5 d), and low pH (6.5) favoured $\text{N}_2\text{O}$ production from denitrification. With a SRT of 15 days and a COD/N ratio of 10, the present reactor does not fall into this category. However, the COD/N ratio of the substrate cannot be directly compared to the COD/N ratio present during denitrification as carbon is used both for aerobic respiration and for denitrification at the same time, and $\text{NO}_x$ for denitrification is being produced by nitrification within the aggregates at the same time as it is consumed in denitrification. The amount of $\text{NO}_x$ and carbon available for denitrification at any given time is therefore not directly measurable. Accumulation of $\text{NO}_x$ in the bulk liquid testifies to impairment of denitrification and it could be argued that the COD/N ratio is relatively low at least toward the end of the aerobic period. However, there is no indication that the $\text{N}_2\text{O}$ production rate increases at this time.

Some studies suggest that $\text{N}_2\text{O}$ production is highly affected by accumulation of nitrite [33], and this has spe-
cifically been suggested to be the cause of N₂O production in a previous study of the SNDP process [6]. Zeng et al. [6] observed accumulation of N₂O during anoxic batch tests, when the bulk liquid NO₃⁻ concentration exceeded 35 μmol l⁻¹. The NO₃⁻ concentration in the aggregates would be significantly less due to mass transport limitation, and indeed N₂O production was also observed during the normal operation of the reactor where nitrite produced inside the aggregates by nitrification did not accumulate in the bulk liquid. Even though nitrite was not measured directly in the aggregates, the Zeng et al. [6] study suggests that even very low levels of nitrite could cause N₂O accumulation in this system.

In the present study, nitrite was not measured in the aggregates under in situ conditions. However, bulk liquid measurements showed accumulation of nitrite to approximately 100 μmol l⁻¹ and microsensor profiles measured in the biofilm set-up (Fig. 6(b)) identified that the accumulation occurred as a result of nitrification in the aerobic part of the biomass. Nitrite was present only at 10 μmol l⁻¹ or less in the anoxic part of the biofilm where N₂O production occurred. If NO₂ triggers N₂O accumulation in the present study, it happens at concentrations much lower than what was observed by von Schulthess [33] in denitrifying activated sludge (250 μmol l⁻¹; 3.5 mg l⁻¹). However, it is consistent with similar studies of an enriched culture of denitrifying GAOs [34] which found N₂O accumulation in anoxic batch tests when NO₂ produced by NO₃⁻ reduction in the biomass caused the bulk liquid concentration to exceed approximately 0.1 mg l⁻¹ NO₂⁻N (7 μmol l⁻¹).

Production of N₂O in the biofilm set-up was stimulated by the addition of excess NO₃⁻ to the water phase above the biofilm (Fig. 6(a)). Addition of acetate together with nitrate dramatically increased the denitrification rate as reflected by the depletion of NO₃⁻ in the biofilm (Fig. 6(b)). However, no N₂O production could be detected under these conditions. The addition of an external carbon source significantly changed three parameters, all of which could influence the N₂O production. Firstly, the C/N ratio was increased and denitrification was occurring under N limiting conditions. Secondly, the carbon source for denitrification was changed from intracellular PHA to external acetate, and thirdly, denitrification activity was no longer restricted to PAOs and GAOs as carbon was available to any bacteria capable of utilizing acetate. Production of N₂O in the present study can thus be linked to one or more of the following: (1) the C/N ratio, (2) the use of stored versus external carbon, or (3) the organisms (PAOs and GAOs) capable of PHA storage. We could not demonstrate any effect of the C/N ratio, and as N₂ production has been shown to occur in bioreactors enriched for the same organisms, it does not seem reasonable to conclude that Accumulibacter and Competibacter are incapable of reducing N₂O at all. We therefore point to the use of PHA as carbon source as being the key to N₂O production in combination with the presence of even very low levels of NO₃⁻.

Itokawa et al. [35] studied N₂O production in denitrifying sludge enriched in lab-scale bioreactors and found that N₂O only accumulated during denitrification of endogenous carbon and in the presence of nitrite. Interestingly, the potential N₂O reduction rate measured in the presence of excess acetate in their study was more than twice as high as the potential NO₂⁻ or NO₃⁻ reduction rates, indicating that N₂O accumulation is highly unlikely in the presence of excess external carbon. However, when denitrification was based on stored carbon, the picture is clearly very different. These data strongly support our findings and clearly identify denitrification of stored carbon together with the presence of nitrite as an important issue with respect to production of N₂O in wastewater treatment processes.

Further research into N₂O production from PHA driven denitrification is necessary to clarify whether N₂O production is generally associated with denitrification based on stored carbon, or whether it is associated with the denitrifying bacteria enriched in this reactor (Accumulibacter and Competibacter). Few studies have measured N₂O production from activated sludge, as it is generally assumed that N₂ is the sole product from denitrification. However, this study clearly identifies N₂O as a product from denitrification. Understanding the mechanisms behind N₂O production in these systems is therefore essential to develop a strategy to diminish production of this bi-product from SNDP or any other wastewater treatment process relying on PHA-driven denitrification, before these processes can be implemented as commercial wastewater treatment processes.

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