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Ribera-Guardia Anna, Marques Ricardo, Arangio Corrado, Carvalheira Mónica, Oehmen Adrian, Pijuan Maite

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Distinctive denitrifying capabilities lead to differences in N_2O production by denitrifying polyphosphate accumulating organisms and denitrifying glycogen accumulating organisms

Ribera-Guardia, Anna[a]; Marques, Ricardo[b]; Arangio, Corrado[a,c]; Carvalheira, Mónica[b] ; Oehmen, Adrian[b]; Pijuan, Maite[a*]
[a]Catalan Institute for Water Research (ICRA); Scientific and Technological Park of the University of Girona, 17003 Girona, Spain
aribera@irca.cat, mpijuan@icra.cat
[b]UCIBIO, REQUIMTE, Chemistry Department, Faculdade de Ciências e Tecnologia, Universidade Nova de Lisboa, 2829-516 Caparica, Portugal
rmd.marques@campus.fct.unl.pt; mic16141@campus.fct.unl.pt, a.oehmen@fct.unl.pt
[c]Dipartimento di ingegneria civile e architettura (DICAR), Università di Catania, 95100, Catania, Sicily, Italy
corrauzzo@yahoo.it

*Corresponding author: mpijuan@icra.cat

Abstract

This study aims at investigating the denitrification kinetics in two separate enriched cultures of denitrifying polyphosphate accumulating organisms (dPAO) and denitrifying glycogen accumulating organisms (dGAO) and compare their N_2O accumulation potential under different conditions. Two sequencing batch reactors were inoculated to develop dPAO and dGAO enriched microbial communities separately. Seven batch tests with different combinations of electron acceptors (nitrate, nitrite and/or nitrous oxide) were carried out with the enriched biomass from both reactors. Results indicate that in almost all batch tests, N_2O accumulated for both cultures, however dPAOs showed a higher denitrification capacity compared to dGAOs due to their higher nitrogen oxides reduction rates. Additionally, the effect of the simultaneous presence of several electron acceptors in the reduction rates of the different nitrogen oxides was also assessed in dPAOs and dGAOs.

Keywords: denitrification; dGAO; dPAO; multiple electron acceptors; nitrous oxide.

1. Introduction

Enhanced biological phosphorous removal (EBPR) is a frequently used, economical and sustainable process to remove phosphorus (P) from wastewater as it can help to reduce the carbon requirements for nutrient removal and the energy consumption of wastewater treatment plants (WWTP). EBPR is mainly carried out by a group of bacteria known as polyphosphate accumulating organisms (PAOs), and for the process to result in a net P removal alternate anaerobic and aerobic steps are needed. During anaerobic conditions PAOs utilize an external carbon source to produce poly-β-hydroxyalkanoates (PHA) whilst hydrolyzing their intracellular poly-phosphate to obtain energy and releasing orthophosphates. In the aerobic phase PAOs oxidize their stored PHA to generate the energy needed for orthophosphate uptake and to recover their intracellular poly-phosphate levels. The aerobic step can also be accomplished under anoxic conditions by a specific group of PAOs, namely denitrifying PAOs (dPAOs), which can remove nitrogen and phosphorus simultaneously using nitrate (NO_3^-) or nitrite (NO_2^-) as electron acceptors (Kuba et al., 1996). However, another group of bacteria, known as glycogen accumulating organisms (GAOs), can also be found in this process. The presence of GAOs can lower the EBPR efficiency because they compete with PAOs for the carbon substrates without performing phosphorus removal (Cech and Hartman, 1993; Whang and Park, 1999). GAOs hydrolyze internal glycogen under anaerobic conditions to obtain energy for carbon uptake and storage as PHA. In aerobic conditions, GAOs oxidize their internal PHA for cell growth and glycogen replenishment without phosphorus removal. Under anoxic conditions, the so called denitrifying glycogen accumulating organisms (dGAOs) can perform the same metabolism as in aerobic conditions but also achieving N removal through the denitrification process.

Previous studies have reported the accumulation of nitrous oxide (N_2O), a strong greenhouse gas, in those systems where denitrification was conducted using internal storage polymers (PHA), such as in biological reactors containing dPAO or dGAO (Wang et al., 2011; Zeng et al., 2003a). N_2O is an intermediate of the denitrification process, being formed during the reduction of NO_3^- or NO_2^- to nitrogen gas (N_2). A possible explanation for N_2O accumulation in these systems is the limited electron flow across the different steps of denitrification caused by the slower PHA oxidation rates as compared with the oxidation rates obtained with external substrates. This would affect the reduction rates of the nitrogen oxides, causing electron competition between the different denitrification enzymes (Kampschreur et al., 2009). It has been speculated that another important factor leading to the accumulation of N_2O, nitrite and/or NO is the incomplete denitrification pathway of some of the PAO and/or GAO groups based on the genomes that have been reported for both cultures (McIlroy et al., 2014; Oehmen et al., 2010).

The negative effect of the simultaneous presence of different nitrogen oxides (NO_3^-, NO_2^- and N_2O) on their reduction rates during denitrification was first reported under low COD/N ratios for ordinary heterotrophic denitrifiers that metabolized externally available carbon sources as the electron donor (von Schulthess and Gujer, 1996). This concept, known as electron competition, was also reported when external carbon was available in excess in a denitrifying culture using methanol as the sole carbon source, and also when different COD loadings were applied (Pan et al., 2013). This electron competition effect on the N_2O reduction was corroborated with other substrates such as acetate and ethanol in a mixed denitrifying microbial community (Ribera-Guardia et al., 2014). However, little is known about the effect that the simultaneous presence of

different electron acceptors can have on dPAO and dGAO cultures during PHA-driven denitrification.

This study explores the denitrification kinetics and the N_2O accumulation potential in two separate enriched cultures of dPAO and dGAO performing denitrification with PHA as their only carbon source. Experiments were conducted under anoxic conditions with single and multiple electron acceptors to assess the preferred nitrogen oxide for each culture and the occurrence of electron competition in the different denitrification kinetics from the two cultures.

2. Materials and methods

2.1. Bioreactors set-up and operation

Two lab-scale sequential batch reactors (SBRs) were operated to develop a dPAO and a dGAO enriched culture, respectively. The dPAO reactor consisted of a 2L SBR and it was inoculated with sludge from the WWTP of Beirolas (Portugal). The dGAO reactor had a volume of 6L and it was inoculated with sludge from Girona's WWTP (Spain). Both reactors were operated in a 6h cycle consisting in: 5min feed-1; 102min anaerobic phase, 4min feed-2, 114min anoxic phase, 90min aerobic phase and 45min of settling and decant. The feed used in both reactors was slightly different and is described below. The pH was controlled at 7.5 ± 0.1 with 0.1M HCl. The sludge retention time (SRT) was 10 days in both reactors and was maintained by wasting mixed liquor at the end of the aerobic phase.

Both reactors were fed with synthetic wastewater with the following characteristics:

- dPAO reactor feed: Feed-1 (950mL added) consisted of 0.59 g NH_4Cl/L, 0.95 g $MgSO_4 \cdot 7H_2O$/L, 0.44 g $CaCl_2 \cdot 2H_2O$/L, 0.01 g ATU/L, 0.03 g EDTA/L, 1.91 g $C_2H_3O_2Na \cdot 3H_2O$/L, 0.2mL $C_3H_6O_2$/L (200 mg COD/L in the reactor), 0.25g

K_2HPO_4/L, 0.15 g KH_2PO_4/L (30mg P/L in the reactor) and 3.17mL of trace element stock solution per liter of feed (Carvalheira et al., 2014a). Feed-2 (50mL added) consisted of 6.07 g $NaNO_3$/L (25 mg N-NO_3^-/L in the reactor).

- dGAO reactor feed: Feed-1(900 mL added) contained 0.03 g K_2HPO_4/L (0.7 mg P/L in the reactor), 0.13 g NH_4Cl/L, 0.89 g $MgSO_4 \cdot 7H_2O$/L, 0.41 g $CaCl_2 \cdot 2H_2O$/L, 0.2 g ATU/L, 0.03 g EDTA/L, 11.33 g $C_2H_3O_2Na$/L, 2.57 g $C_3H_5NaO_2$/L (200 mg COD/L in the reactor) and 2.97mL of trace element stock solution per liter of feed. The trace element solution was the same as for the dPAO reactor. Feed-2 (100mL added) consisted of 14.5 g $NaNO_3$/L (30 mg N-NO_3^-/L in the reactor)

Cycle study analyses were performed weekly. Samples were taken during each phase to analyze nitrate, nitrite, phosphate, ammonia and VFAs. Samples were filtered through 0.22µm Millipore filters. At the end of the cycle samples for mixed liquor suspended solids (MLSS) and volatile MLSS (MLVSS) were taken.

2.2. Batch tests experiments

Both reactors were in steady state conditions and displaying typical dPAO and dGAO phenotypes when the batch tests were conducted.

7 different batch tests (A-G, Table 1) with different combinations of electron acceptors (NO_3^-, NO_2^-, N_2O) were carried out in a sealed batch reactor with no head-space (in order not to have N_2O stripping) with enriched dPAO or dGAO sludge withdrawn from the end of the anaerobic phase of the parent SBR. Nitrogen gas was sparged into the reactor to ensure anoxic conditions during the batch tests. In each batch, a concentration of 20 mg N-NOx/L of each nitrogen oxide indicated in Table 1 was initially added as a pulse. Dissolved N_2O concentration was continuously monitored with an online N_2O

microsensor (Unisense A/S, Denmark; Ribera-Guardia et al., 2014; Wang et al., 2015) and samples for the analysis of nitrate, nitrite, phosphate, ammonia and VFAs were taken along the experiment. All the experiments were carried out in duplicates. Biomass concentration was also analyzed at the end of each test to calculate the specific reduction rates. Batch tests for both cultures were conducted over a period of 2 months.

2.3. Chemical and microbial analysis

Nitrate, nitrite, ammonia and phosphate were determined through segmented flux analysis (Skalar 5100, Skalar Analytical, Netherlands) at UNL (Universidade Nova de Lisboa) and via ion chromatography (ICS5000, DIONEX) at ICRA (Catalan Institute for Water Research). Volatile fatty acids were analyzed via liquid chromatography at high resolution using a Biorad Aminex precolumn and an HPX-87H column and a UV detector adjusted to 210nm. Sulfuric acid (0.01M) was used as eluent in a 0.6mL/min flow-rate and 50ºC of operating temperature at UNL. VFAs were analyzed via gas chromatography (Trace GC Ultra ThermoFisher Scientific) at ICRA (Catalan Institute for Water Research). MLSS and MLVSS were determined following the standard methods (American Public Health Association, 1995).

Fluorescence *in situ* hybridization (FISH) was also performed at the end of the anaerobic and aerobic phases using the following oligonucleotide probes: EUB338, EUB338II, and EUB338III were applied together (EUBMIX), for most *Bacteria* (Daims et al., 1999); as well as PAO651, PAO462 and PAO846, (PAOMIX) which refer to most of the members of *Accumulibacter* group , Acc-I-444 which refers to Type I of PAOs (able to denitrify from nitrate and nitrite), Acc-II-444 which refers to Type II of PAOs (able to denitrify from nitrite only) (Flowers et al., 2009), GAOQ989, GAOQ431 and GB_G2 (GAOMIX) which refer to the *Candidatus Competibacter phosphatis* (able

to denitrify from nitrate and nitrite) (Crocetti et al., 2000); TFO_DF218 and TFO_DF618, (DFImix) for Cluster I of *Defluviicoccus*-related GAOs (able to denitrify from nitrate but not from nitrite); DEF988 and DEF1020 with helpers H966 and H1038, (DFIImix) for Cluster II of *Defluviicoccus*-related GAOs (not able to denitrify); DF198 for Clusters III of *Defluviicoccus*-related GAOs (DFIII) and DF181A and DF181B for Cluster IV of *Defluviicoccus*-related GAOs (DFIV). FISH preparations were visualized with a Nikon CS1 confocal laser-scanning microscope (CLSM) using Plan-Apochromat 63 x oil (NA1.4) objective. Thirty images were taken from each sample for quantification. The area containing Cyt-3 labelled specific probe (PAOMIX, PAOI, PAOII, GAOMIX, DEFIMIX, DEFIIMIX, DEFIII and DEFIV, respectively) cells was quantified as percentage of the Cyt-5 labelled bacteria probe (EUBMIX) within each image using the ImageJ and Pixel Counting programs.

An N_2O microsensor was used to monitor continuously the dissolved N_2O in the liquid phase. This type of microsensor is a miniaturized Clark-type sensor with an internal reference and a guard cathode (N_2O-R), it has a detection limit of 0.1µM in water and a response time less than 1 sec (Unisense A/S, Arhus, Denmark).

2.4. Calculations

The measured maximum specific nitrate, nitrite and nitrous oxide reduction rates ($r_{NO3-,m}$, $r_{NO2-,m}$ and $r_{N2O,m}$) were determined through linear regression of the nitrate, nitrite and N_2O profiles, respectively divided by the MLVSS concentration. The true reduction rate of each nitrogen oxide was calculated as follows:

$$r_{NO3-} = r_{NO3-,m} \quad \text{(Eq. 1)}$$

$$r_{NO2-} = r_{NO3-} - r_{NO2-,m} \quad \text{(Eq. 2)}$$

$$r_{NO} = r_{NO2-} \quad \text{(Eq. 3)}$$

$r_{N2O} = r_{NO2^-} - r_{N2O,m}$ (Eq. 4)

N_2O production rate was considered to be equal to the nitrite reduction rate based on the assumption that NO did not accumulate. NO is a potent cytotoxin and its accumulation causes bacterial decay (De Boer et al., 1996). Also in order to prevent accumulation of cytotoxic levels, intracellular concentrations of nitric oxide are typically maintained at low nanomolar levels through synchronized regulation of Nir and Nor (Goretski et al., 1990). Therefore, the NO reduction reaction is prioritized and not the rate-limiting step of denitrification.

The specific electron consumption rates for nitrate (Nar), nitrite (Nir), nitric oxide (Nor) and nitrous oxide (Nos) were calculated as follows:

$$r_{Nar,e} = \frac{r_{NO_3^-}}{14} \cdot 2 \quad \text{(Eq. 5)}$$

$$r_{Nir,e} = \frac{r_{NO_2^-}}{14} \cdot 1 \quad \text{(Eq. 6)}$$

$$r_{Nor,e} = \frac{r_{NO}}{14} \cdot 1 \quad \text{(Eq. 7)}$$

$$r_{Nos,e} = \frac{r_{N_2O}}{14} \cdot 1 \quad \text{(Eq. 8)}$$

Eq. 5- 8 express the electron consumption of Nar, Nir, Nor and Nos respectively, in mmol e-/ gVSS·h. For the case of Nor, the reduction rate of NO was assumed to be equal to the nitrite reduction rate. Electron distribution was calculated as the ratio of electron consumption rate for each of the nitrogen oxide reductases to the total electron consumption rate, expressed as a percentage (Eq. 9):

$$\text{Electron distribution (\%)} = \frac{r_{NOx,e}}{r_{Nar,e}+r_{Nir,e}+r_{Nor,e}+r_{Nos,e}} * 100 \quad \text{(Eq. 9)}$$

3. Results and discussion

3.1. Reactor performance and microbial community characterization

After 5 months of operation, stable nitrogen and phosphorus removal was achieved in the dPAO SBR. The reactor was operating with 100% volatile fatty acids removal, 72% phosphorus removal and 93% nitrate removal, with no nitrite accumulation. During the anaerobic phase acetate and propionate were completely consumed, releasing phosphorus into the liquid phase. During the following anoxic phase the nitrate added was almost completely removed with a simultaneous phosphorus uptake. No nitrite accumulation was detected. Finally, during the aerobic phase, the remaining phosphate was taken up (Figure 1a). The P release/VFA uptake ratio was 0.44 ± 0.07 Pmol/Cmol. This P/C ratio agrees well with the one obtained by (Carvalheira et al., 2014b) using an enriched PAO culture fed with the same combination of acetate-propionate, suggesting that the activity observed in the bioreactor resulted mainly by dPAO rather than dGAO. The dGAO reactor was operated for half a year before the experiments were conducted. Figure 1b shows a typical cycle study profile. All VFAs were consumed during the anaerobic period. During the following anoxic phase, the nitrate added was completely consumed and nitrite accumulated while nitrate was present. Afterwards, nitrite was also consumed. Phosphate concentration did not change and remained very low during the whole cycle (<1 mg P/L).

Microbial analysis were conducted in each SBR at the time when the batch tests were carried out. Table 2 shows the quantification of each microbial community through the FISH technique.

42% of the bacterial community present in the dPAO-SBR was targeted by PAOMIX (comprising the microorganisms belonging to the *Accumulibacter*-PAO group), with 26% being type PAO I (able to denitrify from nitrate and nitrite) and 15% being type PAO II (only able to denitrify from nitrite). Also GAOs were detected in this biomass

with 23% of the bacterial community belonging to the *Competibacter*-GAO group and 4% belonging to the *Defluviicoccus*-GAO group.

For the case of dGAO-SBR, *Competibacter* (targeted by GAOMIX) and *Defluviicoccus-GAO* comprised around 75% of the microbial population while around 14% of the bacterial population belonged to the *Accumulibacter*-PAO group. An example of two images from the FISH quantification of the PAOmix for the dPAO culture and of the GAOmix for the dGAO culture can be found in the supplementary material (Figure S1).

3.2. Distinctive denitrification kinetics of dPAO and dGAO cultures with different electron acceptors

Figure 2 shows the experimental profiles obtained in the batch tests conducted with one electron acceptor (tests A-C, see Table 1) for the dPAO and the dGAO cultures respectively.

In tests A and B, N_2O accumulated in both cultures, since its reduction rate was slower than the nitrite reduction rate. For the case of dGAOs, nitrite also accumulated (batch test A) indicating that dGAO had a preference to consume nitrate against nitrite. That was not the case for dPAOs where nitrite did not accumulate in any of the cases. The nitrite reduction rate in dPAOs was around 2 times higher than that of dGAOs in test B (21.24 ± 3.96 mg N/g VSS·h and 9.96 ± 1.44 mg N/g VSS·h, respectively). Therefore, nitrate reduction can be considered as the rate-limiting step for dPAO.

Nitrite addition caused an important increase on N_2O accumulation in the case of dGAOs (Figure 2B-right). dPAOs, had a higher nitrous oxide reduction rate than dGAOs, especially when nitrite was added as the sole NOx in test B (18.9 ± 4.62 mg N/g VSS·h and 1.63 ± 0.71 mg N/g VSS·h, respectively), suggesting a possible

inhibitory effect of nitrite on the nitrous oxide reductase for dGAOs, which was not observed for dPAOs.

Figure 3 shows the specific reduction rates for each electron acceptor added in each type of batch test conducted. When comparing both cultures, dPAOs had higher reduction rates in general compared with dGAOs, showing higher denitrifying capacity.

In the case of dPAOs, nitrate reduction rate was relatively constant across the different tests. Interestingly, nitrite reduction rate significantly increased in those tests where it was added simultaneously with nitrate (Tests F & G). It was found that dPAOs had a preference for nitrite as electron acceptor, presenting the highest N reduction rates in all the tests where nitrite was added. N_2O reduction rate was slightly lower than nitrite reduction rate in the majority of the tests, resulting in some N_2O accumulation (see Table 3).

On the other hand, the dGAO population presented a preference for nitrate, having higher nitrate reduction rates than those of nitrite. Also, the rate of nitrate reduction was relatively constant in all tests where nitrate was added, independently if it was added alone (test A) or in combination with other electron acceptors (Tests D, F & G). An important reduction on the nitrous oxide reduction rate was observed in those tests where nitrite was added (tests B, E, F & G), suggesting an inhibitory effect of nitrite towards the last step of denitrification in dGAOs.

Denitrification kinetics for both cultures differ depending on the electron acceptors used. When using nitrate, whether as a sole electron acceptor or in combination with nitrite and/or nitrous oxide, NO_3^- reduction rates for dPAO and dGAO cultures are similar (around 15.88 ± 2.40 mg N/g VSS·h and 13.43 ± 1.80 mg N/g VSS·h, respectively) in all the scenarios tested. However nitrite reduction rates are only similar when nitrate is not present (batches B & E; around 13.92 ± 0.22 mg N/g VSS·h for

dPAOs and 12.01 ± 2.94 mg N/g VSS·h for dGAOs). In the cases where nitrate is present (batches A, D, F and G) nitrite reduction rate decreases significantly in the case of dGAOs compared to dPAOs (around 20.73 ± 7.30 mg N/g VSS·h in the dPAO culture and 8.39 ± 1.00 mg N/g VSS·h in the dGAO culture), which might be due to a preference to reduce nitrate over nitrite. In the study of Zeng et al. (2003b) it was reported that when adding nitrate as the electron acceptor there was accumulation of nitrite and N_2O for a dGAO culture which agrees well with the results in this study. They postulated that this accumulation could be due to different dGAO populations mediating the different steps in denitrification. McIlroy et al., (2014) found that subgroup 1 of *Competibacter*-related GAOs called "*Candidatus Competibacter denitrificans*" was able to denitrify from nitrate to nitrite, from nitrate to nitrogen gas and also from nitrite to nitrogen gas whereas another subgroup (subgroup 5) of *Competibacter*-related GAOs called "*Candidatus Contendobacter odensis*" was only able to denitrify from nitrate to nitrite. In our study it was not possible to determine the different sub-groups of *Competibacter* present in the SBR, but the results obtained are consistent with this reasoning behind the preference of dGAOs towards nitrate.
Table 3 shows the percentage of N_2O produced per nitrogen reduced for all the tests conducted.

In almost all cases, dGAOs presented higher N_2O accumulation per N-reduced than dPAOs. The percentage of accumulation was very high for the test where nitrite was added alone, around 80%. In general, the N_2O accumulation levels in dPAOs were lower than those found in dGAOs, with the highest being 31% for test F. These values indicate that high N_2O emissions are very likely to occur in those systems where denitrification is carried out by dGAOs, and/or where nitrite accumulates.

Nitrous oxide reduction rates were lower for dGAOs than dPAOs in all the scenarios tested. Therefore, there was more N_2O accumulation in the dGAO culture. Lemaire and co-workers (2006) reported that the net N_2O production from denitrification was linked to dGAOs, which were responsible for denitrification in a simultaneous nitrification, denitrification and phosphorus removal reactor. Also, Zeng et al. (2003b) and Zhu and Chen (2011) reported that dGAOs were the major contributor to N_2O production in their study.

NO_2^- did not accumulate in any of the batch tests for the dPAO culture, which is in agreement with the results found by Carvalho et al. (2007), who found no accumulation of nitrite in a dPAO reactor fed with propionate as the sole carbon source. However, nitrite accumulated in all the batch tests with the dGAO culture, which can be explained by two possible reasons: i) the microbial population characteristics, with a predominant dGAO group only being able to conduct the first step of denitrification; ii) an inhibition by nitrite/free nitrous acid (FNA) on the reduction step of this compound. The concentration of FNA in the batch tests where nitrite was accumulated ranged from 0.31- 1.92 µg HNO_2-N/L. This concentration is similar to the one reported by Semerci and Hasılcı, (2016) (0.01-2.27 µg HNO_2^-N/L) in a dPAO and dGAO culture, who found an increase of dGAOs over dPAOs under these FNA levels . Also, Ye and co-workers, (2013) studied the effect of FNA on the anaerobic and aerobic metabolism of GAOs and found that PAOs were more affected by FNA than GAOs under the same FNA concentrations. The fact that nitrite reduction was not affected in dPAOs under the same FNA/nitrite concentrations as in the dGAO culture suggests that FNA inhibition did not play an important role in the accumulation of nitrite in dGAOs. We hypothesize that nitrite accumulation was due to the microbial composition within the dGAO culture. Indeed, Tayà et al., (2013) showed that nitrite was more readily utilized by dPAO than

Defluviicoccus GAO when propionate was fed as the C source, which corroborates our results. Overall, the fact that a wider diversity of *Accumulibacter* PAO sub-groups seem to be capable of nitrite reduction as compared to the diversity of GAO sub-groups (Oehmen et al., 2010) could explain why PAOs were more able than GAOs to denitrify the ~20 mg NO_2^--N/L fed during the batch tests.

The highest denitrification rate within the dPAO culture was obtained when nitrite was used as electron acceptor. Also, when using nitrate as the electron acceptor, all the nitrogen oxide reduction rates decreased. This suggests the presence of two different types of dPAO groups, one able to denitrify from nitrate and another able to denitrify from nitrite. This hypothesis is consistent with previous reports from Oehmen et al., (2010) and would imply that the denitrification rates are lower in the case of nitrate since only one of the dPAO groups is able to reduce nitrate, while both are able to reduce nitrite. This hypothesis is corroborated with the quantification of the microbial community, being 26% of the dPAO culture from *Accumulibacter* group Type PAO I and 15% from *Accumulibacter* group Type PAO II. Therefore, as there were more dPAOs able to denitrify nitrite than to denitrify nitrate, nitrite reduction rates were higher than nitrate in all cases for the dPAO culture. This hypothesis would also explain the fact that in the cases where nitrate and nitrite was added (batches F & G), nitrite reduction rate was higher than when adding nitrate (batches A & D). When there was addition of NO_3^-, PAO Type I were responsible for reducing it to NO_2^-, making it the rate limiting step for nitrite reduction by both PAO Types, while in batches F and G both sub-groups of PAOs could reduce NO_3^- and NO_2^- simultaneously at their maximum rates, due to the higher simultaneous abundance of both nitrogen oxides, thereby reducing nitrite faster.

3.3. Electron competition and distribution when using PHA as carbon source for denitrification

A flow of electrons is required during the denitrification process for all the reductive steps, which are provided from the oxidation of the PHA. Electron competition is defined here as the competition of the different electrons between the different denitrifying enzymes (nitrate reductase (Nar), nitrite reductase (Nir), nitric oxide reductase (Nor) and nitrous oxide reductase (Nos)) of the denitrification process.

Tests D-G were carried out in order to see if there was a competition for electrons when several electron acceptors were present simultaneously. Figure 4 shows the electron consumption rates of all the nitrogen oxides reductases for all the batch tests in both dPAO (a) and dGAO (b) cultures and the electron distribution for dPAOs (c) and dGAOs (d) respectively.

The electron consumption rates by Nar, Nir, Nor and Nos were very similar in all the experiments independently of the electron acceptor addition scheme. This suggests that there was not competition for electrons in either dPAOs or dGAOs. Higher electron consumption rates were obtained in the dPAO tests due to the fact that this culture had higher denitrification rates. The maximum electron consumption rate in the dPAO culture was obtained in experiment G, where all the electron acceptors were added simultaneously.

The electrons were distributed depending on the electron acceptors added in each test. For example, in the case of Test A in dPAOs, the expected electron distribution was found, with around 40% of the electrons going to Nar and the remaining 60% almost evenly distributed among the other reductases. This was also the case for Test D, where nitrate was added together with N_2O. Interestingly, in those tests where nitrate was

added together with nitrite, the percentage of electrons distributed to Nar decreased, increasing the fraction diverted to the other reductases. This suggests the activation of another microbial group which denitrifies from nitrite.

In the case of dGAOs, the clear preference for nitrate is highlighted in figure 4d. Between 50 to 60% of electrons were derived to Nar, with the remaining evenly distributed among the other reductases when nitrate was added alone or in combination with nitrous oxide (tests A & D). The addition of nitrite (tests B, F & G) caused a clear decrease on the electrons diverted to Nos.

It is likely that electron competition was not significant due to the different subgroups of PAO and GAO organisms present in the SBRs and their preferences for utilising nitrate or nitrite, which were activated depending on the electron acceptors added. Since there were different groups of microorganisms performing the different steps of the denitrification process, electron competition between Nar and the other reductases was not detected. This is expected since the electron supply system from the different subgroups of dPAOs and dGAOs is independent of each other.
Accumulibacter, Competibacter and Defluviicoccus (Cluster I) have been found to possess different mechanisms for anaerobic acetate uptake (Saunders et al., 2007; Burow et al., 2008). Wei et al., (2014) showed that the electron consumption rate of Nir and Nos descended with the PHA degradation rate in a dPAO culture. Accordingly, electron competition between nitrite reductase and nitrous oxide reductase did not get intensified when carbon was degraded more slowly in denitrification with PHA. These findings are in agreement with our results. Overall, it appears that electron competition during the reduction of different nitrogen oxides is a significant factor in ordinary heterotrophic denitrification processes based on external carbon sources as the electron donor, and not in PHA-driven denitrification processes by PAOs or GAOs.

3.4 Implication of the study

N_2O is an intermediate compound in the denitrification process and its accumulation is strictly linked to the activity of the nitrous oxide reductase (NoS) enzyme. N_2O can accumulate due to two main reasons: i) when the majority of the denitrifying community does not possess the gene encoding for NoS, therefore having nitrous oxide as the end product of denitrification; ii) when nitrous oxide reduction rate is affected by a certain environmental or operational factor becoming lower than the nitrate or nitrite reduction rates. Several environmental factors have been reported to lead to N_2O accumulation during denitrification such as the effect of electron acceptors (oxygen, nitrite/FNA or nitric oxide), pH, electron donors (type of organic carbon used for denitrification or internal storage compounds such as PHA) or the relationship between COD/N in the wastewater (Alinsafi et al., 2008; Du et al., 2016; Lu and Chandran, 2010; Park et al., 2000; Zhou et al., 2008). In this study the N_2O emissions of the denitrification process using PHA as the carbon source has been investigated using a dPAO and a dGAO enriched cultures, respectively. Results showed that generally, higher N_2O accumulation was detected in the tests conducted with dGAOs than those conducted with dPAOs. This accumulation becomes critical when nitrite is present, substantially inhibiting the last step of denitrification in dGAOs. This inhibition does not seem to occur in dPAOs (at least on the concentration range tested in this study). Special attention needs to be paid on those systems were nitrite pathway is promoted since the abundance of dGAOs will not only affect the effectiveness of the P removal process of the plant but also will most likely increase its overall N_2O emissions.

5. Conclusions

(1) N_2O accumulation was higher in dGAOs compared to dPAOs. This accumulation was intensified in dGAOs when nitrite was added due to its inhibitory effect on N_2O reduction. Contrary, this effect was not observed in the dPAO biomass.

(2) No electron competition was detected in either of the two cultures. This was likely due to different sub-groups of PAO and GAO organisms and their preferences for reducing different nitrogen oxides.

(3) Favouring dPAOs over dGAOs can improve P removal efficiency in WWTPs and lead to lower levels of N_2O accumulation, particularly with nitrogen removal via the nitrite pathway.

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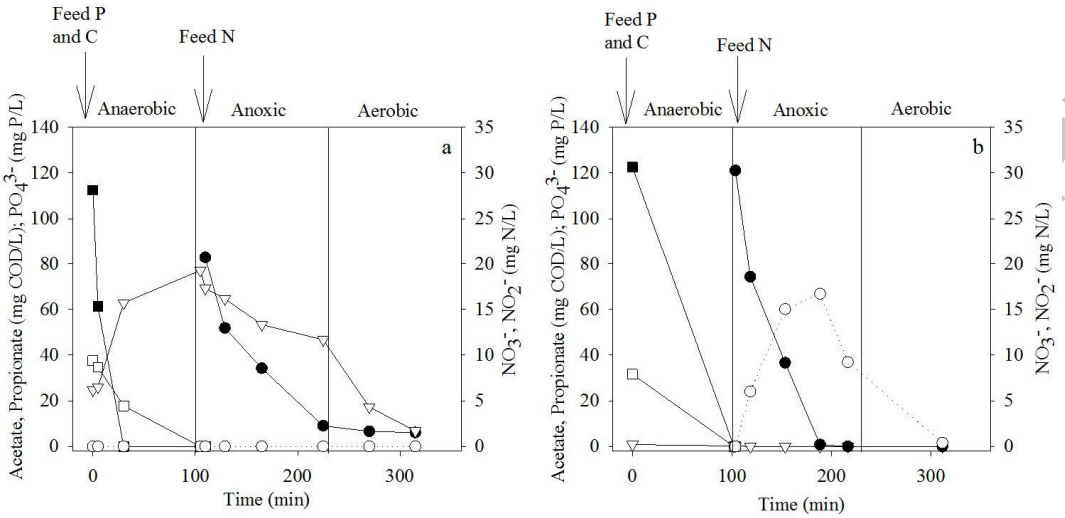


Figure 2. Experimental acetate (■), propionate (□), phosphate (▽), nitrate (●), and nitrite (○) profiles analyzed during a typical cycle study conducted in the dPAO (a) and dGAO (b) reactors.

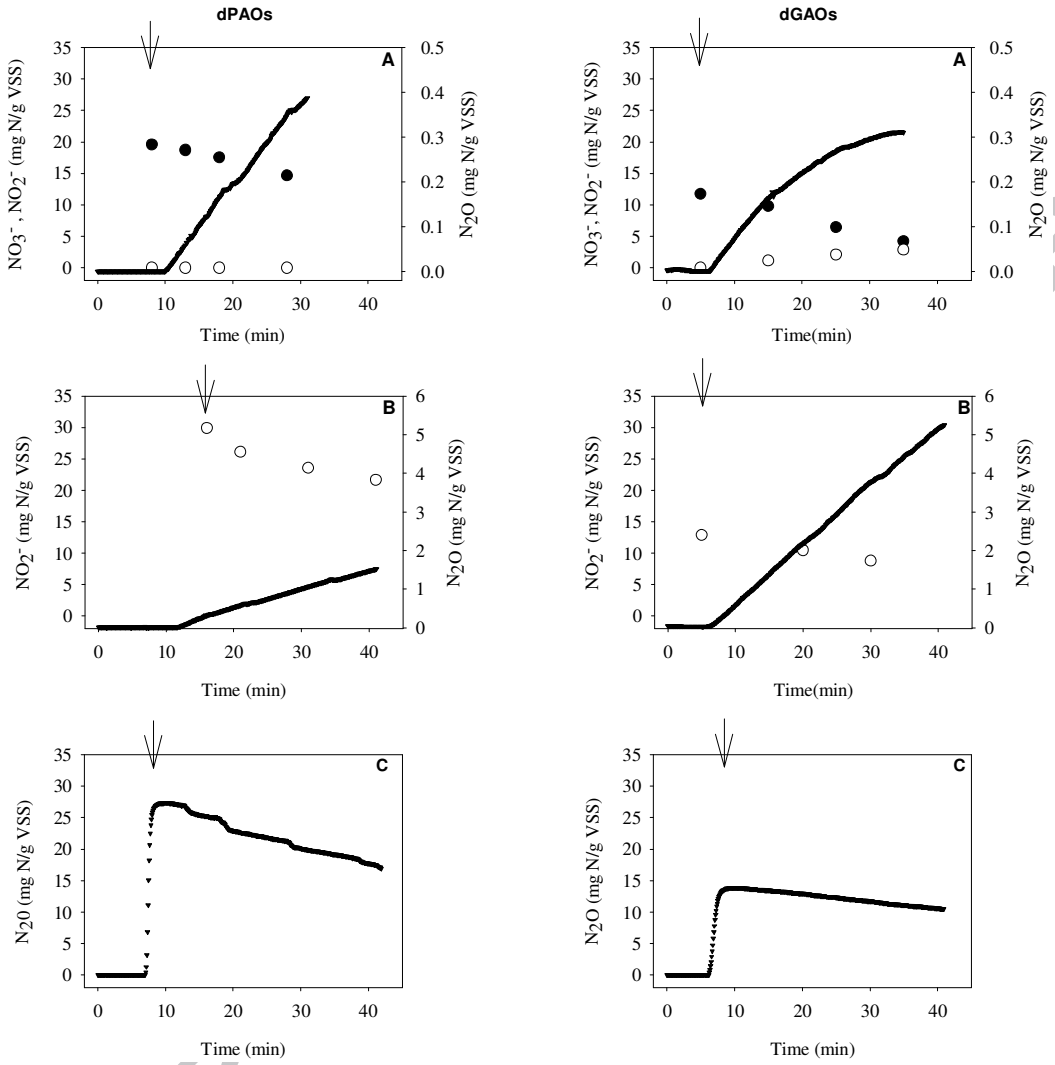


Figure 2. Nitrate (●), nitrite (○), and N_2O (▼) profiles for batch tests A, B and C for dPAO (left) and dGAO (right) cultures. The arrows represent the moment when NOx was added. Notice the different N_2O axis scale in Tests B compared with Tests A.

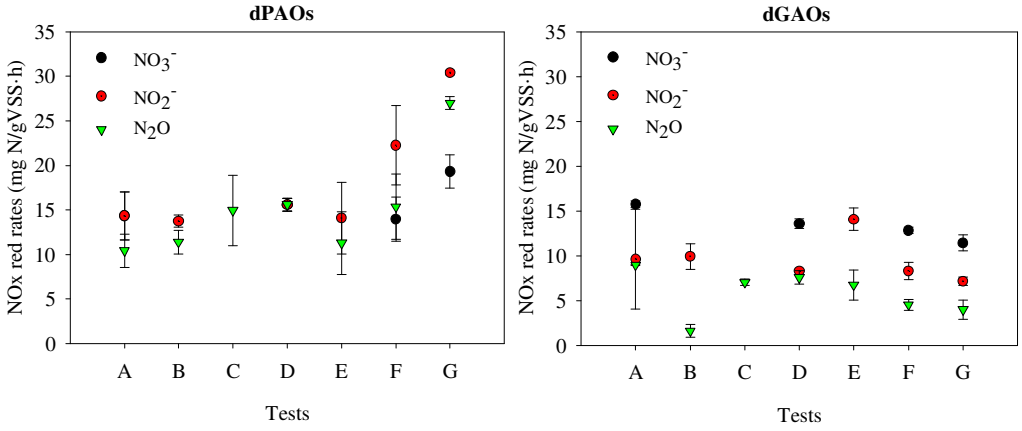


Figure 3. Nitrogen oxides reduction rates for dPAOs (left) and dGAOs (right) cultures.

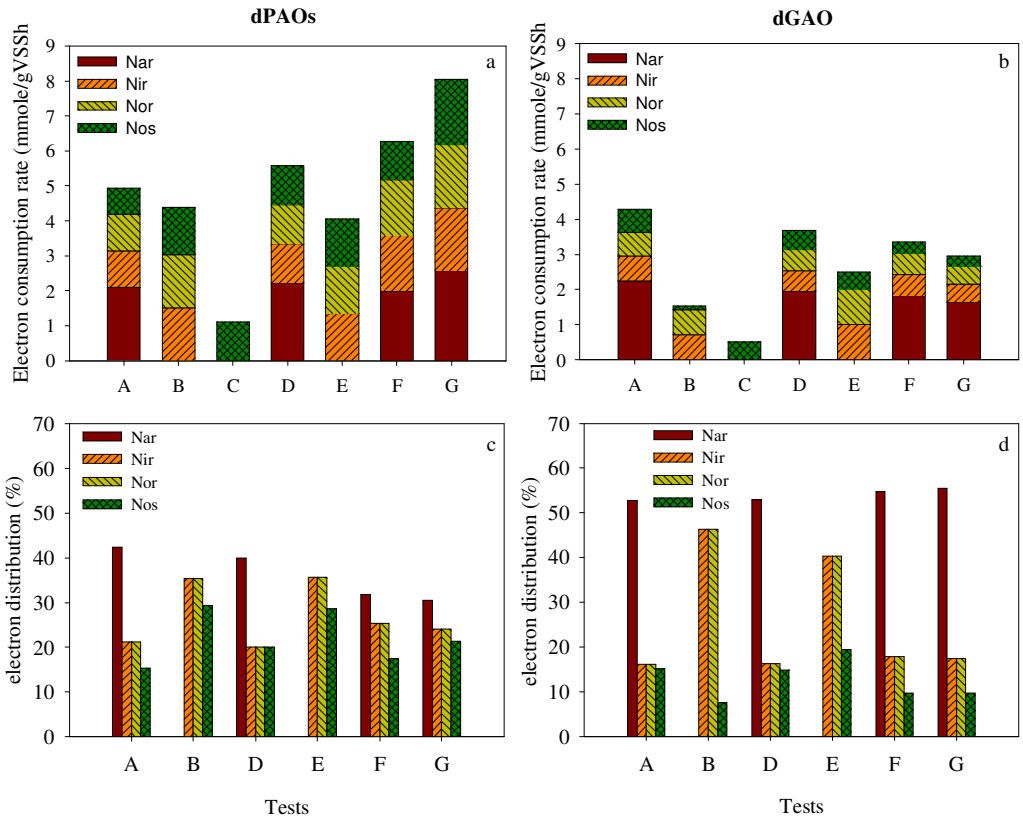


Figure 4. Electron consumption rates (a and b) and electron distribution (c and d) for nitrate reductase (Nar), nitrite reductase (Nir), nitric oxide reductase (Nor) and nitrous oxide reductase (Nos) for dPAO (left) and dGAO (right) cultures.

Table 1. Batch tests conducted with dPAO and dGAO cultures.

Batch test type	*A*	*B*	*C*	*D*	*E*	*F*	*G*
Electron acceptors used	NO_3^-	NO_2^-	N_2O	NO_3^- N_2O	NO_2^- N_2O	NO_3^- NO_2^-	NO_3^- NO_2^- N_2O

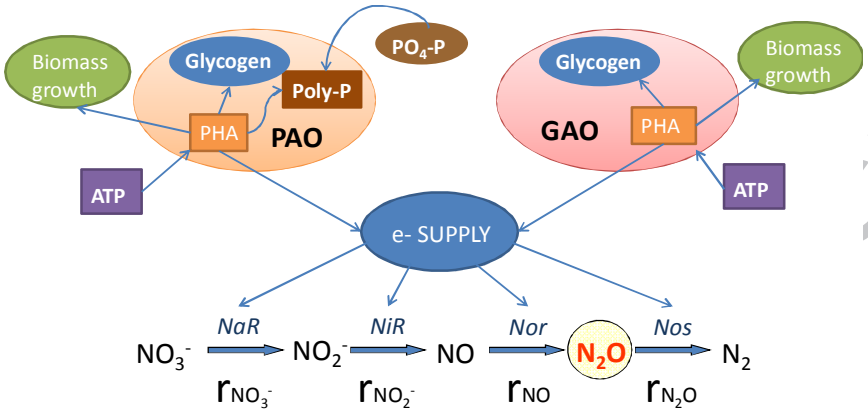
Table 2. FISH quantification of the dPAO and dGAO SBR cultures used in the batch tests.

FISH PROBES	**Relative abundance**
dPAO-SBR	
PAO I	26.03 ± 4.75 %
PAO II	15.42 ± 2.82 %
PAOMIX	42.40 ± 8.32 %
GAOMIX	22.93 ± 4.41 %
DFImix, DFIImix and DFIII	4.17 ± 0.16 %
dGAO-SBR	
GAOMIX	55.60 ± 1.86 %
DFImix, DFIII and DFIV	6.33 ± 0.24 %
DFIImix	13.20 ± 0.88 %
PAOMIX	14.30 ± 1.52 %

Table 3. Percentage of N_2O accumulated per N-reduced for both cultures.

Batch test type*	**N_2O accumulation per N-reduced (%)**	
	dPAOs	**dGAOs**
A	8.72 ± 0.20%	7.12 ± 2.16 %
B	17.40 ± 5.90%	83.95 ± 4.79 %
D	0.00	13.71 ± 5.81 %
E	20.11 ± 1.90%	56.90 ± 4.92 %
F	31.20 ± 2.70%	45.45 ± 0.89 %
G	11.30 ± 3.10 %	48.45 ± 5.94 %

*Test C is not presented since only N_2O was added.



Highlights

- Denitrification kinetics for a dPAO and a dGAO culture are studied separately
- N_2O accumulation is higher in dGAOs than in dPAOs
- Nitrite inhibits the nitrous oxide reduction in dGAOs but not in dPAOs
- There is no electron competition in any of the cultures