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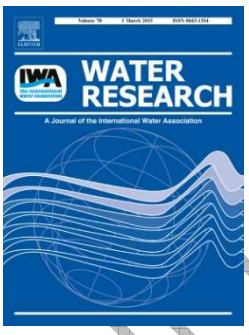

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Denitrifying capabilities of *Tetrasphaera* and their contribution towards nitrous oxide production in enhanced biological phosphorus removal processes

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Abstract

Denitrifying enhanced biological phosphorus removal (EBPR) systems can be an efficient means of removing phosphate and nitrate with low carbon source and oxygen requirements. *Tetrasphaera* is one of the most abundant polyphosphate accumulating organisms present in EBPR systems, but their capacity to achieve denitrifying EBPR has not previously been determined. An enriched *Tetrasphaera* culture, comprising over 80% of the bacterial biovolume was obtained in this work. Despite the denitrification capacity of *Tetrasphaera*, this culture achieved only low levels of anoxic P-uptake. Batch tests with different combinations of nitrate (NO_3^-), nitrite (NO_2^-) and nitrous oxide (N_2O) revealed N_2O accumulation when this electron acceptor was not added externally. Electron competition was observed during the addition of multiple nitrogen electron acceptors species, where P uptake appeared to be slightly favoured over glycogen

production in these situations. This study increases our understanding of the role of *Tetrasphaera*-related organisms in denitrifying EBPR systems.

Keywords: *Tetrasphaera*-related bacteria, Polyphosphate accumulating organisms (PAO), enhanced biological phosphorus removal (EBPR), Denitrification, Nitrous oxide (N_2O), Electron competition

1. Introduction

Phosphorus (P) and nitrogen (N) are known key elements causing eutrophication of water bodies. Combining denitrification with enhanced biological phosphorus removal (EBPR) can reduce both carbon source and aeration requirements of wastewater treatment plants (WWTPs). *Candidatus* Accumulibacter (hereafter Accumulibacter) is the most widely known polyphosphate accumulating organism (PAO), able to store large amounts of polyphosphate (poly-P) anoxically and/or aerobically after taking up organic substrates (e.g., acetate and propionate) anaerobically, unlike ordinary heterotrophic organisms (Oehmen et al., 2007). During the anoxic phase, these organisms can reduce nitrate (NO_3^-) or nitrite (NO_2^-), and oxidize poly-β-hydroxyalkanoates (PHA) to obtain energy to replenish glycogen reserves, take up P and recover their intracellular poly-P level (Carvalho et al., 2007; Kuba et al., 1996).

Another group of organisms present in EBPR systems compete for the same organic carbon sources as the *Accumulibacter* PAOs, which are known as glycogen accumulating organisms (GAOs) without contributing to P removal (Oehmen et al., 2007). Literature studies have enriched mixed cultures of dPAOs and dGAOs, achieving partial or total

denitrification (Carvalho et al., 2007; Ribera-Guardia et al., 2016; Tsuneda et al., 2006; Wang et al., 2008; Zeng et al., 2003a, 2003b).

Tetrasphaera are also present in full-scale EBPR systems, reaching higher abundance than *Accumulibacter*, up to 30% of the total biomass (Kong et al., 2005; Lanham et al., 2013a; Nguyen et al., 2011; Stokholm-Bjerregaard et al., 2017). These organisms can assimilate a wider range of carbon sources (amino acids, sugars, volatile fatty acids (VFAs)) during anaerobic conditions (Kong et al., 2008; Kristiansen et al., 2013; Nguyen et al., 2011). *Tetrasphaera* are capable of fermenting amino acids and sugars, storing either amino acids or glycogen anaerobically, and using it as an energy source for aerobic P uptake (Kristiansen et al., 2013; Marques et al., 2017; Nguyen et al., 2015), and are less competitive for VFA uptake than *Accumulibacter* (Nguyen et al., 2015). With a *Tetrasphaera* enriched culture fed only with casein hydrolysate as carbon source, *Tetrasphaera* were responsible for amino acid consumption and performed the majority of the aerobic P removal observed in this culture (Marques et al., 2017).

Metagenomic results led to the observation that all four existing *Tetrasphaera* isolates (T. *elongate* (member of clade I), T. *australiensis* (clade II), T. *jenkinsii* (clade II) and T. *japonica* (outgroup not covered by clades)) have the genomic capabilities to encode for enzymes to reduce NO_3^- to nitric oxide (NO), while only two of them (T. *australiensis*, T. *japonica*) have the capability to reduce NO to N_2O (Kristiansen et al., 2013). Nevertheless, the capacity of *Tetrasphaera* to couple denitrification with P uptake has never been established, nor the kinetics of denitrification in the presence of different nitrogen electron acceptors.

Complete denitrification involves four consecutive reduction steps, starting with NO_3^-, leading to the sequential production of NO_2^-, NO, and N_2O as three obligatory intermediates, before producing N_2. N_2O is known as a potent greenhouse gas with 300-

fold stronger radiative forcing than carbon dioxide, and is the primary ozone-depleting substance of the 21st century (IPCC , 2013). Emissions from WWTPs have been found to contribute to over 80% of the total greenhouse gases emitted from some plants (Daelman et al., 2013a; Daelman et al., 2013b; Ye et al., 2014) and the need to minimise N_2O emissions is well recognised. The denitrification reduction process is mediated by four different denitrification reductases, NO_3^- reductase (Nar), NO_2^- reductase (Nir), NO reductase (Nor) and N_2O reductase (Nos) (Zumft, 1997). Unbalanced denitrification rates leads to the accumulation of intermediates in the denitrification process. This disturbance can be linked with the competition for electron demand between the four reduction steps when the electron supply rate is the limiting step. This was observed by Pan et al. (2013) for ordinary heterotrophic denitrifiers using only methanol as carbon source, where the reduction rate of NO_2^- was prioritized over the other denitrification steps, consequently leading to N_2O accumulation. Ribera-Guardia et al., (2014) also observed electron competition on N_2O reduction rates in ordinary heterotrophic denitrifiers with multiple external electron donors (acetate, ethanol, and methanol). N_2O has been observed to be emitted from EBPR systems with enriched dPAO and dGAO cultures (Lemaire et al., 2006; Ribera-Guardia et al., 2016; Zeng et al., 2003a, 2003b). The consumption of PHAs as electron donor during the denitrification process has been associated with an increase in the production of N_2O in some cases (Li et al., 2013; Wang et al., 2011; Zhou et al., 2012). *Tetrasphaera* do not synthesise PHAs, and possibly use amino acids or glycogen as internal storage products (Kristiansen et al., 2013; Marques et al., 2017; Nguyen et al., 2015, 2011). The consumption of these internal products might lead to a different behaviour in the formation/consumption of N_2O within these bacteria.

This study focuses on the enrichment of a *Tetrasphaera*-EBPR culture under anaerobic-anoxic-aerobic conditions to evaluate and characterise their denitrifying capabilities and

contribution towards anoxic P uptake. Anoxic batch tests with single or multiple electron acceptors were performed to investigate electron distribution and N_2O production without the presence of external carbon sources. This study contributes to clarify the potential role of *Tetrasphaera,* which are highly abundant organisms in biological nutrient removal plants, on N_2O accumulation during denitrification, as well as their impact on P removal. Increased understanding of the metabolism of *Tetrasphaera*-related PAOs may improve the removal efficiency of P and N in wastewaters with different compositions of organic carbon in EBPR WWTPs.

2. Material and Methods

2.1. Sequencing batch reactor operation

A sequencing batch reactor (SBR), with 2L working volume, was operated for 196 days to enrich a denitrifying *Tetrasphaera* culture. The inoculum was obtained from the study described in Marques et al., (2017). The SBR was fed with sodium casein hydrolysate (Fluka, USA) (hereafter refer as Cas aa) as only carbon source, and operated with an 8-h cycle, including: anaerobic phase (3h), anoxic phase (2h), aerobic phase (2h) and settling/decant phase (1h). Three solutions were used to feed the SBR: A - Mineral media and carbon source (400 mL) was fed continuously during the first 2h of the anaerobic phase; B - Phosphate medium (600 mL) was fed at the start of the anaerobic phase during 3 min; C – Nitrate medium was fed (50 mL) during 5 min in the start of the anoxic phase. The SBR was operated with a hydraulic retention time (HRT) and sludge retention time (SRT) of 16 h and 20 days, respectively. Anaerobic/anoxic or aerobic conditions were obtained by bubbling argon or air, respectively. pH was controlled at 7.1 ± 0.1 by automatic addition of 0.1 M HCl, while temperature was controlled at 20 ± 1ºC with a water bath. The SBR was stirred via an overhead mixer at 300 rpm during the

anaerobic/anoxic and aerobic phases. Aerobic/anoxic and anaerobic conditions were achieved by bubbling argon and air, respectively. The performance and steady state of the SBR was assessed by biological and chemical analyses performed in samples taken during the weekly cycle studies.

2.2. Culture Media

The SBR culture media composition was similar as that used in Marques et al., (2017), briefly: solution (A), mineral media with carbon source contained per litre: 0.79 g Cas aa (150 mg/L in the SBR), 0.37 g NH_4Cl, 0.59 g $MgCl_2.7H_2O$ g, 0.28 g $CaCl_2.2H_2O$, 0.07 g N-Allylthiourea (ATU), 0.2 g ethylene-diaminetetraacetic (EDTA) and 1.98 mL micronutrient solution. The micronutrient solution was prepared based on Smolders et al., 1994; solution (B), Phosphate medium (30 mg-P/L in the SBR) contained 0.32 g K_2HPO_4 and 0.19 g KH_2PO_4 per litre; solution (C), Nitrate medium was increased during the first 20 days of operation until reaching a final concentration of 25 mg-N/L in the SBR (i.e. 6.07 g $NaNO_3$ per litre). The pH of solution A was set to 7.4 ± 0.1, with addition of 1.0 M NaOH, before autoclaving.

2.3. Batch reactor setup and operation

The experimental procedure used for the batch tests was based on Ribera-Guardia et al., (2014) with minor modifications. To assess the denitrifying capabilities of the culture and evaluate the hypothesis of electron competition, seven batch tests with different combinations of nitrogen electron acceptors were performed (Table 1).

A sealable reactor with a volume capacity of 330 mL was used for all batch tests. A 10 mL reservoir filled with the same mixed liquor concentration was connected to the lid to

avoid the entrance of air into the vessel when samples were taken during each batch test. Online N_2O monitoring was performed with an N_2O liquid microsensor connected to an amplifier system (Unisense Environment A/S, Denmark). The microsensor was calibrated before and after each test using a saturated solution obtained by bubbling pure N_2O gas during 5 min, at a flow rate of 5 L/min. A three-point calibration curve was performed by adding twice 0.1 mL of the saturated N_2O solution to 100 mL water free of N_2O. pH was manually controlled at 7.1±0.1 with addition of 0.5 M of NaOH and HCl. All tests were carried out in a temperature controlled lab with minor temperature variations (21-22ºC). The experiments were performed under anoxic conditions with no exchange of N_2O between the liquid and gas phase due to the absence of head space in the vessel.

All batch tests were performed in duplicate between days 139 and 164 of SBR operation. An additional batch test was also performed where external carbon was added (Cas aa at the same concentration fed to the parent SBR, 150 mg/L, but added as a pulse instead of continuous feeding) and with NO_3^- added as electron acceptor (Table 1). The tests were performed using sludge withdrawn from the parent SBR at the end of the anaerobic phase. Sludge was washed twice with mineral media to remove any external carbon source present. The sludge was resuspended with mineral media to a final volume of 450 mL, equally divided between both replicate batch tests. Argon was bubbled to ensure all dissolved oxygen present was removed, prior to starting the experiment. A concentration of 20 mg-N/L of each nitrogen electron acceptor (NO_3^-, NO_2^- and N_2O depending on the test, see Table 1) was added initially as a pulse. Samples were taken along the batch tests to analyse NO_3^-, NO_2^-, NH_4^+ and phosphate. Biomass samples for PHA and glycogen were taken at the beginning and end of each test. Biomass concentration was assessed by volatile suspended solids (VSS) and total suspended solid (TSS) at the end of each cycle.

2.4. Contribution of *Tetrasphaera* and *Competibacter* to nitrogen electron acceptors reduction

The contribution of both *Tetrasphaera* and *Competibacter* GAOs to the reduction of the nitrogen electron acceptors was evaluated according to the following methodology: by calculating the ratio of PHA utilisation to N reduction during the SBR and batch test operation. The model developed by Oehmen et al., (2010) was used to describe the GAOs PHA utilization to serve as electron donor for nitrogen electron acceptors reduction. The remaining nitrogen electron acceptors reduction was then linked with *Tetrasphaera* activity (Table S1, Supplemental Information).

2.5. Calculation of the reduction rates

The maximum measured consumption rates of NO_3^-, NO_2^- and N_2O were determined by applying linear regression to the profiles of NO_3^-, NO_2^- and N_2O, respectively, which were obtained in each test. The observed specific degradation rates of nitrate (mNO_3^-), nitrite (mNO_2^-), and nitrous oxide (mN_2O) were calculated by dividing the rate data determined above by the VSS concentration present in each batch test. The specific degradation rate of (rNO) was assumed to be equal to the specific degradation rate of nitrite. Intracellular concentrations of NO are maintained in low concentration by synchronized regulation of Nir and Nor. NO reduction rate is prioritized at a non-rate-limiting step of denitrification due to the molecule cytotoxicity, causing bacterial decay (Boer et al., 1996; Goretski et al., 1990).

The true reduction rate of each nitrogen electron acceptor (mg-N/(g VSS.h)) was calculated as follows:

$$r_{NO_3^-} = mNO_3^- \quad (1)$$

$$r_{NO_2^-} = r_{NO_3^-} - mNO_2^- \quad (2)$$

$$r_{NO} = r_{NO_2^-} \quad (3)$$

$$r_{N_2O} = r_{NO} - mN_2O \quad (4)$$

where, $r_{NO_3^-}$, $r_{NO_2^-}$, r_{NO}, r_{N_2O} are expressed in (mg-N/(g VSS.h)).

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

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

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

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

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

where, $r_{Nar,e}$, $r_{Nir,e}$, $r_{Nor,e}$, $r_{Nos,e}$ are expressed in (mmol e^-/(g-VSS.h)).

Electron distribution was calculated through the ratio of electron consumption rate by each individual enzyme per total electron consumption rate, expressed as a percentage:

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

2.6. Chemical analyses

Segmented flow analysis (Skalar 5100, Skalar Analytical, The Netherlands) was used for P, poly-P, ammonia, nitrate and nitrite analyses. Poly-P analysis was performed as described in Carvalheira et al., (2014). VFAs were analysed via high-performance liquid chromatography (HPLC) using a Metacarb 87 H (Varian) column and a refractive index detector (RI-71, Merck) as described by Carvalheira et al., (2014). Glycogen was determined as described by Lanham et al., (2012) (conditions: 2 mg biomass, HCl 0.9M and 3h of digestion time). PHA was determined by gas chromatography (GC) according to the methodology described by Lanham et al., (2013b), using a Bruker 430-GC gas chromatograph equipped with a FID detector and a BR-SWax column (60m, 0.53 mm internal diameter, 1 mm film thickness, Bruker, USA). The Cas aa consumption was assessed through the analysis of total organic carbon (TOC) by a Shimadzu TOC-VCSH (Shimadzu, Japan). TSS and VSS were assessed by standard methods (APHA, 2005).

2.7. Microbial characterisation

The microbial composition of the SBR was assessed by fluorescence *in situ* hybridisation (FISH) according to Amann (1995). FISH quantification was performed by image analysis taken with a Zeiss LSM 710 confocal laser scanning microscope. The biomass quantification was performed as described in Marques et al., (2017).

3. Results and Discussion

3.1. SBR performance and microbial composition

To evaluate the reactor performance, cycle studies were made regularly during reactor operation. Pseudo steady-state conditions were achieved in the SBR after 55 days of

operation, and the reactor was operated under these conditions for 115 days prior to executing the batch tests. Identification using FISH analysis detected *Tetrasphaera* as the main PAO present, comprising a volume fraction of 80% of the total bacterial community. *Accumulibacter* PAOs were present in very low abundance (< 2%), *Competibacter* GAOs had an abundance of 12% and *Defluviicoccus* GAO were not detected (Table 3). Two typical profiles of the reactor operation are displayed in Figure 1. During the typical reactor operation on average, 86% of the carbon, 30% of P and 91% of NO_3^- was removed.

The main parameters analysed were compared with those obtained in an enriched *Accumulibacter* SBR and an SBR with a mixture of *Tetrasphaera* and *Accumulibacter* working under similar conditions (Marques et al., 2017; Ribera-Guardia et al., 2016). Table 2 presents a comparison among these three reactors. While the efficiency of carbon removal and NO_3^- reduced agree very well with the removals obtained for an *Accumulibacter* enriched culture operated under similar conditions (Ribera-Guardia et al., 2016), the capacity of the *Tetrasphaera* enriched SBR to perform P-uptake was substantially lower as compared with the P-uptake obtained in the *Accumulibacter* enrichment. When comparing in more detail the P release/substrate ratios, the *Tetrasphaera* SBR displayed a lower ratio (0.11±0.02 P-mmol/C-mmol) as compared with the *Accumulibacter* SBR (0.35±0.15 P-mmol/C-mmol). Furthermore, the P-uptake under anoxic and aerobic conditions was less effective in the *Tetrasphaera* SBR as compared to the *Accumulibacter* SBR. Also, this *Tetrasphaera* culture developed under anaerobic/anoxic/aerobic conditions displayed less than half of the P uptake achieved by a *Tetrasphaera-Accumulibacter* culture operated with an anaerobic/aerobic cycle. Consequently, the intracellular P content displayed by this culture was also very low (Table 2).

The culture mainly consisted of *Tetrasphaera*-related organisms, where the four clades of *Tetrasphaera* comprised over 80% of the total microbial community. Contrary to the anaerobic/aerobic SBR study (Marques et al., 2017), the Tet2-892 clade was the most abundant in this culture and clade Tet2-174 was also present, while the sum of Tet1-266 and Tet3-654 decreased slightly from 60% to 40% between the two studies (Table 3). Various morphologies were observed (short and branched rods, small cocci, cocci in tetrads, filaments, and thin filaments), which is consistent with the morphologies detected in the culture obtained under anaerobic/aerobic conditions discussed in Marques et al., (2017).

The very low fraction of *Accumulibacter* PAOs and the presence of a small fraction of *Competibacter* GAOs likely contributed to the lower P uptake observed in this study as compared to the reactor previously operated under anaerobic/aerobic conditions described in Marques et al., (2017). Since *Tetrasphaera* are not capable of PHA production (Kristiansen et al., 2013), the PHA produced under anaerobic conditions can be assumed to be stored by *Competibacter* through the uptake of mainly fermentation products. The slightly higher anaerobic glycogen consumption and PHA production yields per C uptake and higher PHV fraction are consistent with GAO metabolism (Filipe et al. 2001) as opposed to PAO metabolism (Table 2).

Accumulation of NO_2^- in the anoxic phase was also observed occasionally (Figure 1b), arriving into the aerobic phase. Nitrite accumulation (more specifically in the form of free nitrous acid) has been found to be inhibitory to anoxic and aerobic P uptake in PAOs, and is known to be toxic at different threshold levels to many organisms (Zhou et al., 2011). Nevertheless, nitrite accumulated only rarely, and at low levels (<7 mg-N/L), where cycles without nitrite accumulation (Figure 1a) revealed a similar anoxic and aerobic P uptake level as compared to those with nitrite accumulation (Figure 1b). Thus, it is

unlikely that nitrite was present at levels (0.04±0.07 N mmol/L) that would lead to lower P-uptake in this anaerobic/anoxic/aerobic configuration as compared with the anaerobic/aerobic SBR (Marques et al., 2017).

It should also be noted that the energy obtained by PAOs under anoxic conditions,46% lower as compared to aerobic conditions, leads to lower P-uptake rates (Kuba et al., 1996). A reduction in energy generated anoxically by *Tetrasphaera* would both lower the P taken up under anoxic conditions, and may also deplete their storage compounds that would otherwise have been available for aerobic P uptake. This could also explain the lower P removal efficiency achieved by the *Tetrasphaera* enriched culture under anaerobic/anoxic/aerobic conditions as compared to anaerobic/aerobic conditions (Marques et al., 2017).

3.2. Contribution of *Tetrasphaera* and *Competibacter* to nitrogen electron acceptors reduction

With both *Tetrasphaera* and *Competibacter* present in the culture, it was necessary to assess the contribution of each group to the reduction of nitrogen electron acceptors. To accomplish this, metabolic model predictions of denitrifying GAOs regarding the utilisation of PHA per nitrogen electron acceptor reduction were used. PHA is a differentiating factor between *Tetrasphaera* and *Competibacter*, since previous studies showed *Tetrasphaera*-related organisms are not able to produce PHAs (Kristiansen et al., 2013; Nguyen et al., 2011). Since very low *Accumulibacter* PAOs were detected in this culture, it was assumed that all PHA consumption for nitrogen electron acceptors reduction was associated with denitrification performed by GAOs.

During the anoxic phase, an average of 0.77±0.21 C-mmol/L of PHA was consumed during SBR operation. The ratio obtained of PHA utilisation to nitrogen electron

acceptors reduction was 2.80C-mmol/N-mmol, calculated according to the data shown in Table S1, Supplemental Information. Assuming all PHA is utilised by GAOs to perform nitrogen electron acceptors reduction, an average value of 0.28±0.08 N-mmol/L can be linked with these bacteria. An average of 1.67±0.21 N-mmol/L NO_3^- was reduced in the SBR and NO_2^- accumulation was considered negligible, thus it was assumed that nitrate was fully reduced to N_2O and N_2 gas. This led to 1.39 N-mmol/L reduction linked with *Tetrasphaera* (~83% of the total nitrogen electron acceptors) and 0.28 N-mmol/L (~17%) reduction to *Competibacter* GAOs. This result agrees very well with GAOs abundance in this culture (12.4±5.1%) quantified by FISH (Table 3). These results show that *Tetrasphaera*-related organisms were the main bacteria responsible for the N removal within this culture.

3.3. Denitrification capabilities of *Tetrasphaera* culture

3.3.1. Individual electron acceptors

Batch tests with different electron acceptors were performed to study the denitrifying capacities of the *Tetrasphaera* enriched culture. In tests A, B and C electron acceptors NO_3^-, NO_2^- and N_2O were added individually. Similar reduction rates were obtained for NO_3^- (20.97 ± 2.31 mg-N/g-VSS.h) and NO_2^- (20.30 ± 3.10 mg-N/g-VSS.h), while the N_2O reduction rate (8.53 ± 0.22 mg-N/g-VSS.h) was slower (Figure 2, Table S2 Supplemental Information). This clearly shows higher affinity of this culture for NO_3^- and NO_2^- reduction, while N_2O reduction had the lowest reduction rate of denitrification when fed individually. N_2O accumulation was also observed in both test A and B, although the N_2O reduction rates were higher as compared to the case when only N_2O was added. N_2O accumulation has also been observed in denitrifying PAO and GAO cultures with PHA as the electron donor (Lemaire et al., 2006; Ribera-Guardia et al., 2016; Wei et al., 2014;

Zeng et al., 2003b), with either NO_3^- or NO_2^- as the electron acceptor. However, the increase of the N_2O reduction rate in the presence of NO_2^- vs NO_3^- addition, (20.20 ± 0.19 mg-N/g-VSS.h and 12.80 ± 0.76 mg-N/g-VSS.h, respectively) rules out any inhibition by NO_2^- concentration (Figure 2, Table S2 Supplemental Information).

Higher reduction rates were obtained for NO_3^- (25.78 mg-N/g-VSS.h) in the external carbon source batch test. This result suggests the culture was carbon limited by the amount of internal metabolites (Figure S1 and Table S2 Supplemental Information). Reduction rates of NO_2^- and N_2O were also higher (19.41 and 17.63 mg-N/g-VSS.h, respectively) as compared with test A. A decrease of 42% in N_2O accumulation was observed at the end of the external batch test when compared with the average accumulation obtained for test A. This result was further supported by a higher increase of N_2O reduction rate as compared with NO_2^-, in the external carbon source test (Table S2 Supplemental Information). Limitation of intracellular carbon source during denitrification could potentially contribute to N_2O accumulation in this culture.

When fed alone, the N_2O reduction rate was significantly lower than when in presence of other nitrogen electron acceptors (Figures 2 and 3), which is in contrast to previous studies with ordinary heterotrophic denitrifiers fed with external carbon sources, or dPAOs (Pan et al., 2013; Ribera-Guardia et al., 2016, 2014). One possible explanation for this lower N_2O reduction rate could be less efficient bioenergetics within the cell when metabolising this nitrogen electron acceptors. N_2O reduction creates a lower amount of proton-motive force across the membrane to generate ATP. While reduction of NO_3^- to N_2 requires 10 electrons, the reduction to N_2O requires only 8. These 10 electrons are associated with translocation of 30 protons across the cytoplasmic membrane to drive ATP synthesis (~3.3 proton/ATP). The N_2O reduction can be associated with only 20% of the energy generated by full denitrification, which limits the bioenergetic advantage for a cell to

perform this reduction (Richardson et al., 2009). This may explain why addition of N_2O as the only electron acceptor may lead to lower reduction rates as compared to situations where NO_3^- or NO_2^- are added. When the N_2O is inside the cell it is more readily reduced and generates more energy. If transportation is needed, prior to reduction of N_2O, less energy is generated and does not compensate the transport step as readily.

3.3.2. Combination of electron acceptors

The highest reduction rate of NO_3^- was observed in test A, while it decreased in tests D, F and G when other electron acceptors were added in combination (Figure 3, Table S2 Supplemental Information). A similar pattern was observed for NO_2^- reduction rates, where the highest reduction rate of NO_2^- was observed in test B, while it decreased when other electron acceptors were also added (Figure 3, Supplemental Information Table S2). This indicates that *Tetrasphaera* has no preference for either NO_3^- or NO_2^-, reduction, while when both electron acceptors are present simultaneously the rates decrease, suggesting that electron competition could have an important role in these situations.

The slowest N_2O reduction rate was obtained in test C, however, the rate increased in test D, E and G, respectively (Figure 3, Table S2 Supplemental Information). This higher N_2O reduction rate with increased presence of NO_2^- and/or NO_3^- could be linked to an increased synthesis of enzymes responsible for N_2O reduction (Nos), likely caused by the increased available energy created by NO_2^- and/or NO_3^- reduction as explained above.

This hypothesis is further supported by the accumulation of N_2O produced per nitrogen reduced. Higher accumulation in test A, B and F was observed, whereas in tests D, E and G no accumulation was detected (Table 4). When N_2O was added simultaneously with another electron acceptor, no accumulation was observed. The rate of reduction was similar or higher as compared with the N_2O production rate, although N_2O accumulated

when NO_3^- and NO_2^- were added individually or in combination with no N_2O addition. This higher availability of energy for Nos synthesis combined with a higher availability of N_2O could trigger a higher reduction rate, due to a higher overall energy potential for the cells. Also, less accumulation of N_2O was observed when an external carbon source was added. This higher accumulation in test A, without carbon source, supports the energetic limitation of this culture to reach the full potential of denitrification. When comparing the accumulation of N_2O with the study of Ribera-Guardia et al., (2016), generally the *Tetrasphaera* led to less accumulation of this intermediate when compared with dPAOs and dGAOs. Only in test A, it was observed a higher N_2O accumulation as compared with these two enriched cultures. Similar accumulation of N_2O was obtained for tests B and D as compared with dPAOs, while dGAOs showed much higher accumulation. In the other tests, the *Tetrasphaera* culture achieved lower N_2O accumulation.

Interestingly, P-uptake had a similar rate in all tests performed with different combinations of electron acceptors (0.09±0.01 mmol-P/g-VSS.h) (Table 5). When observing the ratio of P-uptake per nitrogen electron acceptors consumed, the ratio increased as a function of the number or nitrogen electron acceptors provided (Table 5).

3.4. Electron competition and distribution

The lower NO_3^- and NO_2^- reduction rates observed when multiple nitrogen electron acceptors were added as compared to the case where only one was added suggests that electron competition occurred within the culture. Previous studies have shown that electron competition occurs during ordinary heterotrophic denitrification either in conditions of limited or excess carbon substrates (Pan et al., 2013; Von Schulthess et al.,

1994). This is the first study examining electron competition for an enriched *Tetrasphaera*-related PAO culture.

The total average electron consumption rate in the presence of two or more electron acceptors added simultaneously (tests D to G) was very similar (average of 5.27±0.55 mmol e^-/g-VSS.h) (Figure 4). This value was also very similar to the total electron consumption rate obtained in test A with NO_3^- (6.09 mmol e^-/g-VSS.h) (Figure 4). This indicates that these electron consumption rates were limited by the upstream electron supply from the carbon oxidation process of the internal metabolites, suggesting that the denitrification enzymes were competing for electron donors from a limited electron supply system originated from the same internal metabolites. In fact, a higher electron consumption rate (7.72 mmol e^-/g-VSS.h) was obtained in a test performed with external carbon source, at the same concentration as added to the main SBR, with NO_3^- added as sole electron acceptor (Figure 4). This result further supports the idea that cells were unable to supply sufficient electrons from internally stored sources and meet the energy demand to perform denitrification at their maximum rate.

Another interesting observation is that the combination of NO_3^- and N_2O (test D) showed a higher electron consumption rate as compared to NO_2^- and N_2O (test E). It has been observed that Nar receives electrons directly from the ubiquinone/ubiquinol pool (UQ/UQH_2), while Nir, Nor and Nos receive their electrons from the cytochrome c550/pseudoazurin pool (Cyt c550/Ps az) by way of the UQ/UQH_2 pool. Due to this difference in electron flow, it is expected that the electron competition between Nar and Nos would be smaller as compared with Nir and Nos (Pan et al., 2013; Richardson et al., 2009). This hypothesis agrees very well with the results obtained in this study.

Table 6 shows the electron distribution between Nar, Nir, Nor and Nos within each batch test performed. A decrease in Nar activity was confirmed by the electron distribution

between tests where NO_3^- was fed alone or in combination with other nitrogen electron acceptors, decreasing from 49.1% to 43.4%, to 37.1% to 31.5% in tests, A, D, F and G, respectively. Similarly, both NO_3^- and N_2O had a similar impact on Nir activity as can be observed from the electron distribution (Table 6), being highest when NO_2^- was fed individually. This supports the hypothesis that both Nar and Nir activity were affected by electron competition. In the case of N_2O reduction, the total electron consumption rate obtained in test C was only about 1/10 of the value obtained in the other batch tests (Figure 4). This supports the fact that N_2O was energetically unfavourable for the culture when fed in isolation, which can be explained by the fact that N_2O reduction only comprises around 20% of the bioenergetic potential as compared to full denitrification.

When comparing the P-uptake/electron consumption ratio, higher P-uptake was obtained in tests D, E, F and G. This shows that the culture channelled more energy obtained from the reduction of the nitrogen electron acceptors present to perform P-uptake, despite the similar total electron consumption rate (Figure 5). This could be due to higher availability of electron acceptors availability, specially NO3 and NO2, that would lead to higher energy obtained by *Tetraphaera* to be channelled for anoxic P uptake.

In this study, it was not possible to link the internal carbon consumption of the *Tetrasphaera*-related bacteria with the electron consumption. The intracellular storage compound has not been entirely revealed, with glycogen, amino acids or other macromolecules being observed as storage compounds (Kristiansen et al., 2013; Marques et al., 2017; Nguyen et al., 2015). The clarification of this issue would allow improvement in the understanding of the denitrification metabolism by these bacteria.

3.5. Implications of this study

The capabilities of *Tetrasphaera*-related organisms to perform denitrification and a low level of simultaneous P removal have been shown in this study under anaerobic-anoxic-aerobic conditions. According to the results obtained, *Tetrasphaera* performed the majority of the N removal (>80%), in this mixed culture. This result further validates the importance of *Tetrasphaera* in wastewater treatment plants, not only for P removal (Marques et al., 2017), but also for denitrification. This culture achieved anoxic P-uptake with nitrate as the electron acceptor, but at levels far lower than typically observed by *Accumulibacter* enrichments (Table 7). This suggests that *Tetrasphaera* contribute relatively little to anoxic P removal. A comparison of the anoxic and aerobic P uptake kinetics between enriched cultures of *Tetrasphaera* and *Accumlibacter* is shown in Table 7. A higher anoxic P uptake rate of *Accumulibacter* was consistently observed as compared to *Tetrasphaera*, while the denitrification rates were similar for both cultures, leading to an anoxic P uptake per N removed (P/N) ratio for *Tetrasphaera* that was approximately 6 times lower as compared with *Accumulibacter*.

A lower P removal under anoxic conditions as compared to aerobic conditions by *Accumulibacter* is well reported in the literature. Kuba et al., (1996) showed that the energy obtained by PAOs under anoxic conditions was typically 46% lower as compared to aerobic conditions, leading to lower P-uptake rates. This is in good agreement with the average anoxic/aerobic P uptake ratio (0.59 mmol-P/mmol-P) of the *Accumulibacter* studies presented in Table 7. However, the anoxic/aerobic P uptake ratio of 0.08 mmol-P/mmol-P was indeed far lower for *Tetrasphaera* (Table 7). In contrast, the *Tetrasphaera* enriched culture operated under an anaerobic-aerobic SBR cycle (Marques et al., 2017), achieved a similar P uptake rate as compared with *Accumulibacter* studies (0.86±0.29 P-mmol/g-VSS.h) (Table 7).

It is noteworthy that despite this low anoxic P removal, *Tetrasphaera* are active anoxically for denitrification, suggesting an alternate route to achieve denitrification, even in non-EBPR systems, by opening up the possibility of dosing a wider range of supplemental carbon sources. Typical wastewater is composed of a wide range of carbon sources, including proteins (25-35%), polysaccharides (15-25%) and other compounds (Nielsen et al., 2010). These carbon sources could be fermented in the anaerobic tank and amino acids, sugars and VFAs could be obtained as products. This wide variety of carbon sources can be then taken up by *Tetrasphaera* (amino acids, sugars) and *Accumulibacter* (VFAs), which would result in P and N removal in EBPR plants. If carbon supplementation is needed, wastewaters could potentially be supplemented with wastewater rich in proteins instead of more costly pure substrates. Supplementation of carbon sources to augment denitrification is an important issue in WWTPs, whereby amino acids are not typically added for this purpose. Addition of low-cost sources of amino acids, such as from residual streams, could represent an alternative means of achieving both N and P removal in WWTPs. One such example could be fish processing wastes (Ghaly, 2013), which have a high content of amino acids. The potential effectiveness of such a strategy in WWTPs requires further research.

In the batch tests performed with individual electron acceptors, NO_3^- and NO_2^- achieved higher reduction rates, as compared with N_2O. When added with multiple electron acceptors, NO_3^- and NO_2^- achieved lower reduction rates, whereas the N_2O reduction rates increased, especially in combination with NO_2^-. This suggests that electron competition could have an important role in lowering the reduction rates of NO_3^- and NO_2^-. This was further confirmed, by similarity of the total average electron consumption rate in the presence of two or more electron acceptors. This electron consumption rate was identical to the maximum electron consumption rate obtained in test A, confirming the limitation

by the upstream electron supply from the carbon oxidation process of the internal metabolites. The higher electron consumption rate obtained when external carbon was added (batch test Ext A), validates this observation. Electron competition was previously observed with ordinary heterotrophic denitrifiers (Pan et al., 2013; Von Schulthess et al., 1994), while this is the first study confirming electron competition for an enriched *Tetrasphaera*-related PAO culture.

4. Conclusions

Tetrasphaera was enriched in an EBPR system through an anaerobic/anoxic/aerobic cycle operation fed with amino acids. *Tetrasphaera* showed a good capacity for denitrification, being responsible for >80% of the denitrification in the SBR, although only little anoxic P uptake was observed, unlike *Accumulibacter*. The results suggested that the organic carbon taken up anaerobically by *Tetrasphaera* appears to provide sufficient energy to achieve either anoxic denitrification or aerobic P removal rather than both denitrification and P removal simultaneously. Batch tests with different combinations of electron acceptors revealed N_2O accumulation under certain conditions. When two or more electron acceptors were present simultaneously, electron competition occurred, lowering the reduction rates of nitrate and nitrite. The limitation of internal carbon source and the electron distribution within the electron carriers might affect and limit the enzyme activities. A slightly higher anoxic P-uptake was linked with higher electron competition, suggesting a shift in the metabolism when multiple nitrogen electron acceptors were present. The increased understanding of the metabolism of *Tetrasphaera*-related organisms may improve the efficiency of phosphorus and nitrogen removal in EBPR WWTPs.

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Figures and Tables:

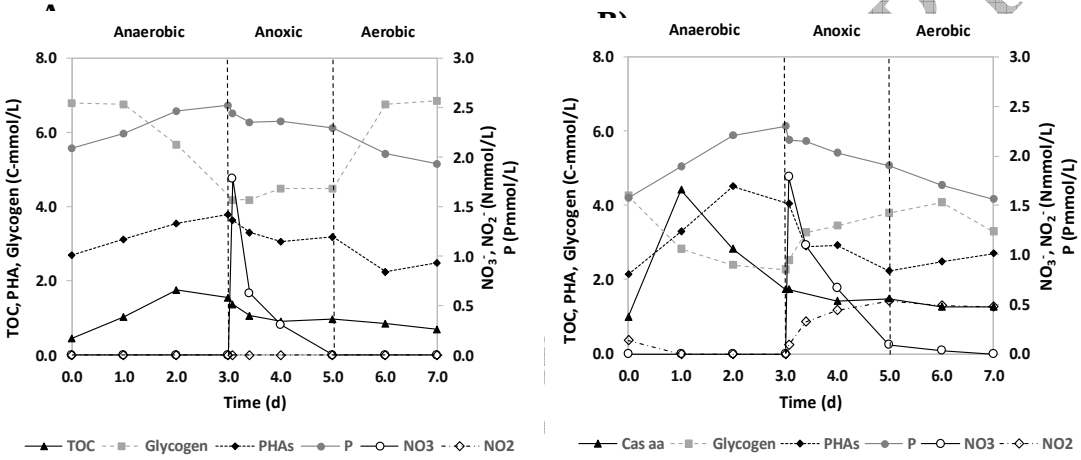

Figure 1 – Typical SBR cycle profile performed on days 82 (A) and 196 (B). Profiles of Cas aa (TOC), Phosphorus (P), Glycogen, PHAs, NO_3^- and NO_2^- are shown.

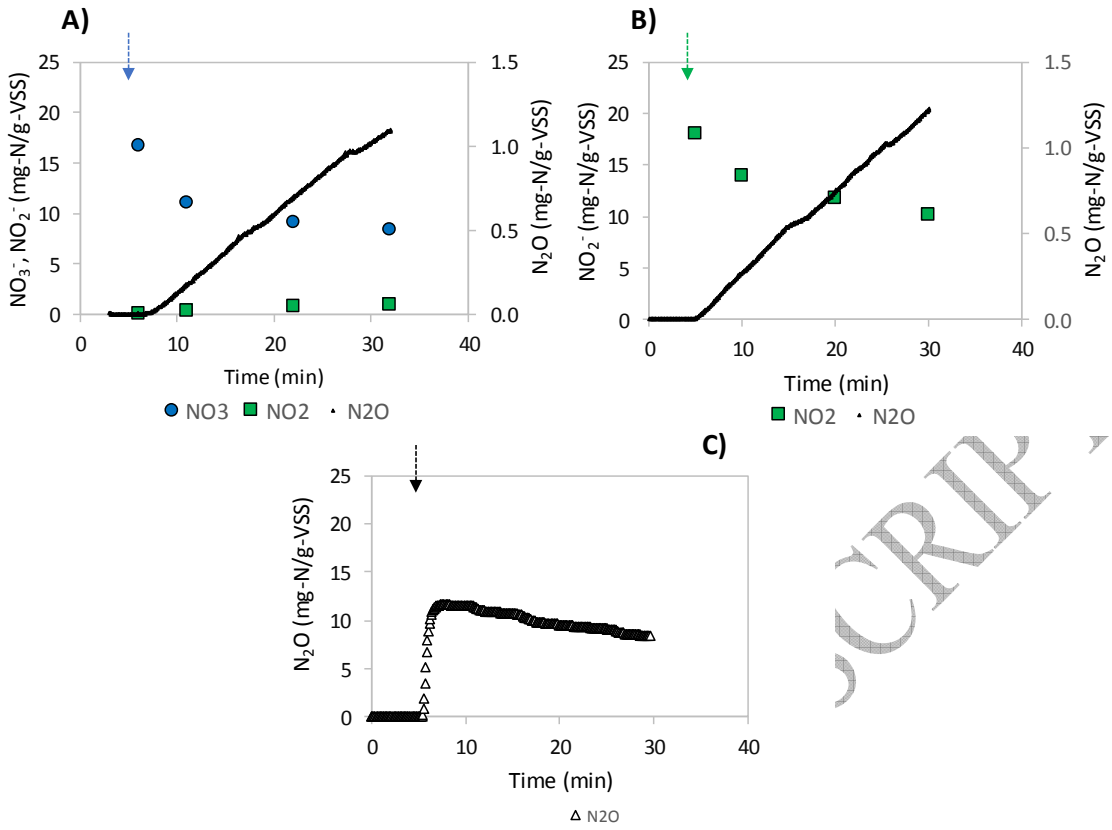

Figure 2 – Batch test profiles performed with different electron acceptors: Nitrate (A), Nitrite (B) and Nitrous oxide (C). The arroz represent the time when nitous oxides were added.

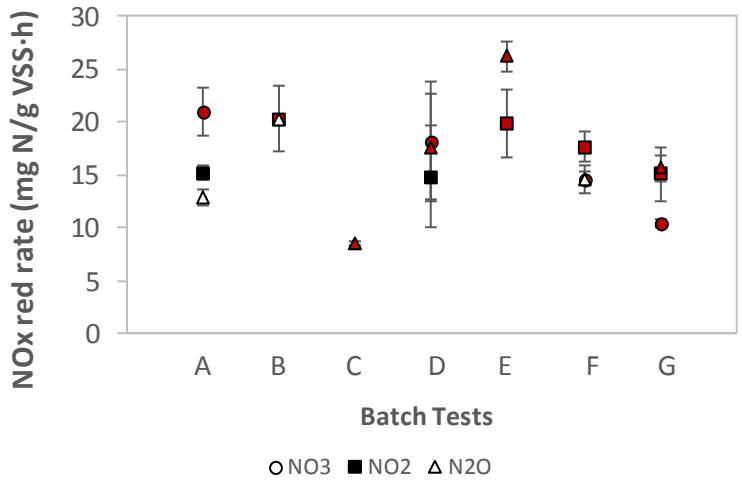

Figure 3 – Nitrous oxide reduction rates for the enriched *Tetrasphaera* culture. Error bars represent standard deviation of duplicate assays. Red filled symbols represent the nitrogen electron acceptors sources fed in each of the batch tests.

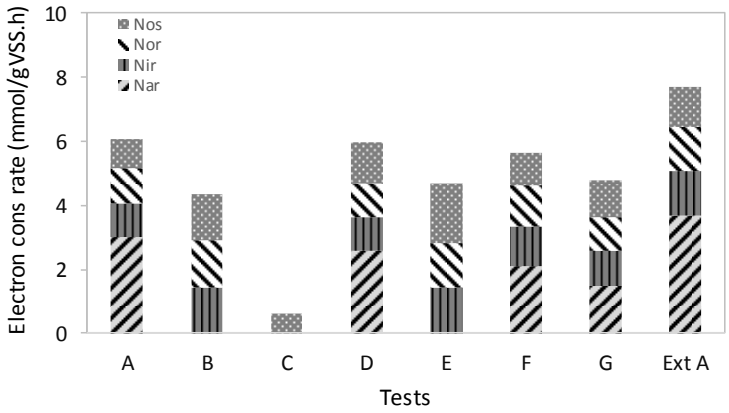

Figure 4 - Electron consumption rates for nitrate reductase (Nar), nitrite reductase (Nir), nitric oxide reductase (Nor) and nitrous oxide reductase (Nos) with the *Tetrasphaera* culture.

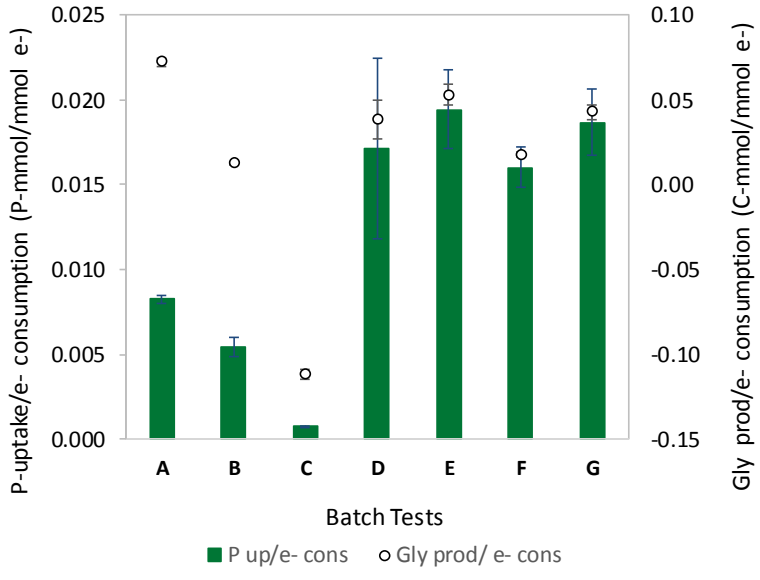

Figure 5 – Ratios of P uptake and Glycogen production per electron consumption obtained for the batch tests performed with different electron acceptors.

Table 1 – Batch tests performed with different combinations of electron acceptors

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

		N_2O	N_2O	NO_2^- NO_2^- N_2O

Table 2 - Typical cycle study (Anaerobic/anoxic/aerobic) obtained during SBR operation and comparison with *Accumulibacter* under similar operational conditions (Ribera-Guardia et al., 2016) and with *Tetrasphaera+ Accumulibacter* under anaerobic/aerobic conditions (Marques et al., 2017).

Anaerobic results			
Dominant organisms	***Tetrasphaera* (this study)**	***Accumulibacter***	***Tetrasphaera+ Accumulibacter***
Carbon source	**Casein hydrolysate**	**Propionate + Acetate**	**Casein hydrolysate**
P release/substrate cons (P-mol/C-mol)	0.11±0.02	0.62±0.25[b]	0.35±0.08 [a]
Glycogen cons/substrate cons (C-mol/C-mol)	0.44±0.19	0.36±0.27[b]	0.38±0.12 [a]
PHB prod/substrate cons (C-mol/C-mol)	0.07±0.05	0.40±0.17[b]	0.03±0.01 [a]
PHV prod/substrate cons (C-mol/C-mol)	0.28±0.08	0.27±0.11[b]	0.09±0.02 [a]
PH2MV prod/substrate cons (C-mol/C-mol)	0.00±0.00	0.11±0.09[b]	0.03±0.01 [a]
PHA prod/substrate cons	0.35±0.13	0.78±0.28[b]	0.15±0.04 [a]

(C-mol/C-mol)			
Anaerobic pH	6.8±0.1	7.0±0.1[b]	6.7±0.1 [a]
Anoxic results			
P uptake (P-mmol/L)	0.28 ±0.08	0.99 ±0.07[b]	---
NOx reduction (N-mmol/L)	1.63±0.22	1.66±0.06[b]	---
Glycogen Production (C-mmol/L)	1.37±0.17	-0.11±0.04[b]	---
PHA Consumption (C-mmoL/L)	0.77±0.21	1.86±0.27[b]	---
Aerobic results			
P uptake (P-mmol/L)	0.37±0.07	1.79±0.39[b]	1.76±0.25 [a]
NOx reduction (N-mmol/L)	0.12±0.11	0.02±0.01[b]	---
% P in TSS	0.6-2.2	5-7[b]	8-19 [a]
Glycogen Production (C-mmol)	0.74±0.23	1.94±0.01[b]	1.38±0.70 [a]
PHA Consumption (C-mmol/L)	0.59±0.22	1.82±0.04[b]	0.75±0.24 [a]
Anoxic/Aerobic results			
P uptake (P-mmol/L)	0.65 ±0.06	2.79±0.43[b]	1.76 ±0.25 [a]
NOx reduction (N-mmol/L)	1.75±0.22	1.69±0.06[b]	---
% P in TSS	0.6-2.2	5-7[b]	8-19 [a]
Glycogen Production (C-mmol/L)	2.11±0.06	1.82±0.03[b]	1.38±0.70 [a]
PHA Consumption (C-mmol/L)	1.36±0.06	3.69±0.30[b]	0.75±0.24 [a]

[a](Marques et al., 2017) [b](Ribera-Guardia et al., 2016)

Table 3 – Morphologies present in the SBR sludge and % volume fraction of *Competibacter* and each *Tetrasphaera*-related clade. Results obtained are an average of 3 samples taken during the experimental period.

Probe	Morphology	% vol. fraction
Tet1-266	Thin filaments, branched rods and cocci in tetrads	21.1±7.1
Tet2-892	Branched rods and filaments	9.1±3.8
Tet2-174	Filaments, tetrads and short rods/branched rods	32.6±8.8

Tet3-654	Branched rods and filament	19.3±6.9
GAOMIX	Rods and short rods in clumps	12.4±5.1
PAOMIX	cocci-bacilli and cocci	1.4±1.4
DFImix		<1
Others		<4

Table 4 – Accumulation of N_2O per N-reduced for this culture and for the dPAO and dGAO enriched cultures used in Ribera-Guardia et al., (2016).

Batch test	**N_2O accumulation per N-reduced (%)**		
	dTET	**dPAOs**	**dGAOs**
A	16.7±0.8	8.7±0.2	7.1±2.2
B	15.5±0.8	17.4±5.9	84.0±4.8
D	0.0	0.0	13.7±5.8
E	0.0	20.1±1.9	56.9±4.9
F	17.7±0.9	31.2±2.7	45.4±0.9
G	0.0	11.3±3.1	48.4±5.9
A (external C-source)	9.6	--	--

Table 5 - Consumption rate of each nitrogen electron acceptor, glycogen production rate, P-uptake rate and ratio P-uptake/electron acceptor consumed obtained during batch tests A, B, D, E, F and G with the *Tetrasphaera* enrichment.

Batch test type	**N electr. acceptors (mmol-N/g VSS.h)**	**Gly Prod (C-mmol/g VSS.h)**	**P-uptake (P-mmol/ g-VSS.h)**	**P-uptake/N electr. acceptors (P-mmol/mmol-N)**
A	0.57±0.00	0.45±0.18	0.05±0.01	0.09±0.02
B	0.64±0.04	0.05±0.02	0.03±0.03	0.05±0.04
C	0.38±0.04	-0.07±0.05	0.01±0.00	0.03±0.01
D	0.86±0.24	0.19±0.13	0.10±0.01	0.12±0.04
E	1.14±0.01	0.25±0.01	0.09±0.01	0.08±0.01

F	0.57±0.10	0.10±0.02	0.09±0.03	0.16±0.08
G	0.71±0.10	0.20±0.04	0.09±0.01	0.13±0.03
Ext A	0.99	0.07	-0.04	-0.04

Table 6 – Electron distribution for anoxic batch tests with the *Tetrasphaera* enriched culture.

	Electron distribution dTET (%)			
Batch Test	**Nar**	**Nir**	**Nor**	**Nos**
A	49.1±3.9	17.9±1.3	17.9±1.3	15.1±1.3
B	0.0	33.2±1.6	33.2±1.6	33.6±3.2
C	0.0	0.0	0.0	100.0
D	43.3±0.3	17.7±0.0	17.7±0.0	21.2±0.4
E	0.0	30.0±1.2	30.0±1.2	40.0±2.5
F	37.1±0.8	22.3±0.2	22.3±0.2	18.4±0.2
G	31.5±2.3	22.5±1.5	22.5±1.5	23.5±0.6
Ext. A	47.7	18.0	18.0	16.3

Table 7 - Comparison of N and P uptake rates and P/N ratio under anoxic and aerobic conditions with values reported in the literature.

Sludge			Electron		Anoxic rates		Ratio	
GAOmix	PAOmix	TET	acceptor	donor	N uptake	P uptake	P/N	Reference
	%				mmol-N /g-VSS.h	mmol-P/g-VSS.h	mmol-P/mmol-N	
5	37	-	NO_3^-	HAc	0.45	0.27	0.60	Carvalho et al., 2007
1	76	-	NO_3^-	HPr	0.24	0.13	0.54	Carvalho et al., 2007
0	90	-	NO_3^-	HPr	0.29	0.31	1.07	Lanham et al., 2011
-	[a]	-	NO_3^-	HAc	0.47	0.39	0.83	Freitas et al., 2005
-	[a]	-	NO_3^-	HAc	0.86	0.97	1.13	Jiang et al., 2006
-	38	-	NO_3^-	HAc	1.08	0.58	0.54	Zeng et al., 2003a
23	42	-	NO_3^-	Hac/HPr	0.32	0.24	0.77	Ribera-Guardia et al., 2016
-	[a]	-	NO_3^-	Hac	1.30	1.19	0.91	Kuba et al., 1993
12	1	82	NO_3^-	Cas aa	0.72	0.09	0.12	This study

Sludge			Electron	Aerobic rates	
GAOmix	PAOmix	TET	donor	P uptake	Reference
	%			mmol-P/g-VSS.h	
17	85	-	Hac/HPr	0.48	Carvalheira et al., 2014
-	41	-	HAc	0.56	Zeng et al., 2003a
-	[a]	-	HAc	1.19	Kuba et al., 1993
24	65	-	HAc	1.22	Oehmen et al., 2005
1	55	-	HPr	0.73	Pijuan et al., 2004
1	55	-	Hac	0.99	Pijuan et al., 2004
-	20	70	Cas aa	1.13	Marques et al., 2017

[a] – majority of the sludge composition was *Accumibacter*, claimed by the author.