



# 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 (P) and nitrate (NO<sub>3</sub><sup>-</sup>) 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 NO<sub>3</sub><sup>-</sup>, nitrite (NO<sub>2</sub><sup>-</sup>) and nitrous oxide (N<sub>2</sub>O) revealed lower N<sub>2</sub>O accumulation by *Tetrasphaera* as compared to *Accumulibacter* and *Competibacter* when multiple electron acceptors were added. 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.

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## 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 NO<sub>3</sub><sup>-</sup> or NO<sub>2</sub><sup>-</sup>, 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 (Lanham et al., 2013a; Muszyński and Mitobędzka, 2015; Nguyen et al., 2015, 2011; Qiu et al., 2017; Stokholm-Bjerregaard et al., 2017; Tooker et al., 2016). 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

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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 available for four *Tetrasphaera* isolates (*T. elongata* (member of clade I), *T. australiensis* (clade II), *T. jenkinsii* (clade II) and *T. japonica* (not targeted by the probes developed for *Tetrasphaera*-PAOs)) have the genomic capabilities to encode for enzymes to reduce  $\text{NO}_3^-$  to nitric oxide (NO), while only two of them (*T. australiensis*, *T. japonica*) have the capability to reduce NO to  $\text{N}_2\text{O}$  (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  $\text{NO}_3^-$ , leading to the sequential production of  $\text{NO}_2^-$ , NO, and  $\text{N}_2\text{O}$  as three obligatory intermediates, before producing  $\text{N}_2$ .  $\text{N}_2\text{O}$  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  $\text{N}_2\text{O}$  emissions is well recognised. The denitrification reduction process is mediated by four different denitrification reductases,  $\text{NO}_3^-$  reductase (Nar),  $\text{NO}_2^-$  reductase (Nir), NO reductase (Nor) and  $\text{N}_2\text{O}$  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  $\text{NO}_2^-$  was prioritized over the other denitrification steps, consequently leading to  $\text{N}_2\text{O}$  accumulation. Ribera-Guardia et al. (2014) also observed electron competition on  $\text{N}_2\text{O}$  reduction rates in ordinary heterotrophic denitrifiers with multiple external electron donors (acetate, ethanol, and methanol).  $\text{N}_2\text{O}$  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  $\text{N}_2\text{O}$  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  $\text{N}_2\text{O}$  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  $\text{N}_2\text{O}$  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  $\text{N}_2\text{O}$  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 2 L 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 (3 h), anoxic phase (2 h), aerobic phase (2 h) and settling/decant phase (1 h). Three solutions were used to feed the SBR: A - Mineral media and carbon source (400 mL) was fed continuously during the first 2 h 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  $7.1 \pm 0.1$  by automatic addition of 0.1 M HCl, while temperature was controlled at  $20 \pm 1^\circ\text{C}$  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  $\text{NH}_4\text{Cl}$ , 0.59 g  $\text{MgCl}_2 \cdot 7\text{H}_2\text{O}$  g, 0.28 g  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ , 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  $\text{K}_2\text{HPO}_4$  and 0.19 g  $\text{KH}_2\text{PO}_4$  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  $\text{NaNO}_3$  per litre). The pH of solution A was set to  $7.4 \pm 0.1$ , 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

**Table 1**  
Batch tests performed with different combinations of electron acceptors.

Batch test	A	B	C	D	E	F	G	Ext A
Electron acceptors	$\text{NO}_3^-$	$\text{NO}_2^-$	$\text{N}_2\text{O}$	$\text{NO}_3^-$ $\text{N}_2\text{O}$	$\text{NO}_2^-$ $\text{N}_2\text{O}$	$\text{NO}_3^-$ $\text{NO}_2^-$	$\text{NO}_3^-$ $\text{NO}_2^-$ $\text{NO}_2$	$\text{NO}_3^-$

test. Online  $\text{N}_2\text{O}$  monitoring was performed with an  $\text{N}_2\text{O}$  liquid microsensor connected to an amplifier system (Unisense Environment A/S, Denmark) (Figure S1 Supplemental Information). The microsensor was calibrated before and after each test using a saturated solution obtained by bubbling pure  $\text{N}_2\text{O}$  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  $\text{N}_2\text{O}$  solution to 100 mL water free of  $\text{N}_2\text{O}$ . pH was manually controlled at  $7.1 \pm 0.1$  with addition of 0.5 M of NaOH and HCl. All tests were carried out in a temperature controlled lab with minor temperature variations ( $21\text{--}22^\circ\text{C}$ ). The experiments were performed under anoxic conditions with no exchange of  $\text{N}_2\text{O}$  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  $\text{NO}_3^-$  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 ( $\text{NO}_3^-$ ,  $\text{NO}_2^-$  and  $\text{N}_2\text{O}$  depending on the test, see Table 1) was added initially as a pulse. Samples were taken along the batch tests to analyse  $\text{NO}_3^-$ ,  $\text{NO}_2^-$ ,  $\text{NH}_4^+$  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 utilisation 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  $\text{NO}_3^-$ ,  $\text{NO}_2^-$  and  $\text{N}_2\text{O}$  were determined by applying linear regression to the profiles of  $\text{NO}_3^-$ ,  $\text{NO}_2^-$  and  $\text{N}_2\text{O}$ , respectively, which were obtained in each test. The observed specific degradation rates of nitrate ( $m\text{NO}_3^-$ ), nitrite ( $m\text{NO}_2^-$ ), and nitrous oxide ( $m\text{N}_2\text{O}$ ) were calculated by dividing the rate data determined above by the VSS concentration present in each batch test. The specific degradation rate of ( $r\text{NO}$ ) was assumed to be equal to the specific degradation rate of  $\text{NO}_2^-$ . 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 glucose 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 (Pan et al., 2013; Ribera-Guardia et al., 2016):

$$r\text{NO}_3^- = m\text{NO}_3^- \quad (1)$$

$$r\text{NO}_2^- = r\text{NO}_3^- - m\text{NO}_2^- \quad (2)$$

$$r\text{NO} = r\text{NO}_2^- \quad (3)$$

$$r\text{N}_2\text{O} = r\text{NO} - m\text{N}_2\text{O} \quad (4)$$

where,  $r\text{NO}_3^-$ ,  $r\text{NO}_2^-$ ,  $r\text{NO}$ ,  $r\text{N}_2\text{O}$  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 (Pan et al., 2013; Ribera-Guardia et al., 2016):

$$r_{\text{Nar},e} = \frac{r_{\text{NO}_3^-}}{14} \cdot 2 \quad (5)$$

$$r_{\text{Nir},e} = \frac{r_{\text{NO}_2^-}}{14} \cdot 1 \quad (6)$$

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

$$r_{\text{Nos},e} = \frac{r_{\text{N}_2\text{O}}}{14} \cdot 1 \quad (8)$$

where,  $r_{\text{Nar},e}$ ,  $r_{\text{Nir},e}$ ,  $r_{\text{Nor},e}$ ,  $r_{\text{Nos},e}$  are expressed in (mmol  $\text{e}^-$ /(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 (Pan et al., 2013; Ribera-Guardia et al., 2016):

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

#### 2.6. Chemical analyses

Segmented flow analysis (Skalar 5100, Skalar Analytical, The Netherlands) was used for P, poly-P, ammonia,  $\text{NO}_3^-$  and  $\text{NO}_2^-$  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 0.9 M and 3 h 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 (60 m, 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 2). Two typical profiles of the reactor operation are displayed in Fig. 1. During the typical reactor operation on average, 86% of the carbon, 30% of P and 91% of  $\text{NO}_3^-$  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 3 presents a comparison among these three reactors. While the efficiency of carbon removal and  $\text{NO}_3^-$  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 ( $0.11 \pm 0.02$  P-mmol/C-mmol) as compared with the *Accumulibacter* SBR ( $0.35 \pm 0.15$  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 3).

The culture mainly consisted of *Tetrasphaera*-related organisms, where the four clades of *Tetrasphaera* comprised over 80% of the total microbial community. This included about 20% or more of each of clades 1–3, showing a highly diverse and even *Tetrasphaera* community that possibly comprised multiple metabolic activities previously detected in studies with *Tetrasphaera* isolates belonging to different clades (Kristiansen et al., 2013). 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 2). 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 3).

Accumulation of  $\text{NO}_2^-$  in the anoxic phase was also observed occasionally (Fig. 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,  $\text{NO}_2^-$  accumulated only rarely, and at low levels (<7 mg-N/L), where cycles without  $\text{NO}_2^-$  accumulation (Fig. 1a) revealed a similar anoxic and aerobic P uptake level as compared to those with  $\text{NO}_2^-$  accumulation (Fig. 1b). Thus, it is unlikely that  $\text{NO}_2^-$  was present at levels ( $0.04 \pm 0.07$  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 (amino acids by *Tetrasphaera*, as opposed to the PHA stored by *Accumulibacter* and *Competibacter*) 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  $0.77 \pm 0.21$  C-mmol/L of PHA was consumed during SBR operation. The ratio obtained of PHA utilisation to nitrogen electron acceptors reduction was 2.80 C-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  $0.28 \pm 0.08$  N-mmol/L can be linked with these bacteria. An average of  $1.67 \pm 0.21$  N-mmol/L  $\text{NO}_3^-$  was reduced in the SBR and  $\text{NO}_2^-$  accumulation was considered negligible, thus it was assumed that  $\text{NO}_3^-$  was fully reduced to  $\text{N}_2\text{O}$  and  $\text{N}_2$  gas. This led to 1.39 N-mmol/L reduction linked with *Tetrasphaera* (~83% of the total nitrogen

**Table 2**

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	$21.1 \pm 7.1$
Tet2-892	Branched rods and filaments	$9.1 \pm 3.8$
Tet2-174	Filaments, tetrads and short rods/branched rods	$32.6 \pm 8.8$
Tet3-654	Branched rods and filament	$19.3 \pm 6.9$
GAOMIX	Rods and short rods in clumps	$12.4 \pm 5.1$
PAOMIX	cocci-bacilli and cocci	$1.4 \pm 1.4$
DFImix		<1
Others		<4



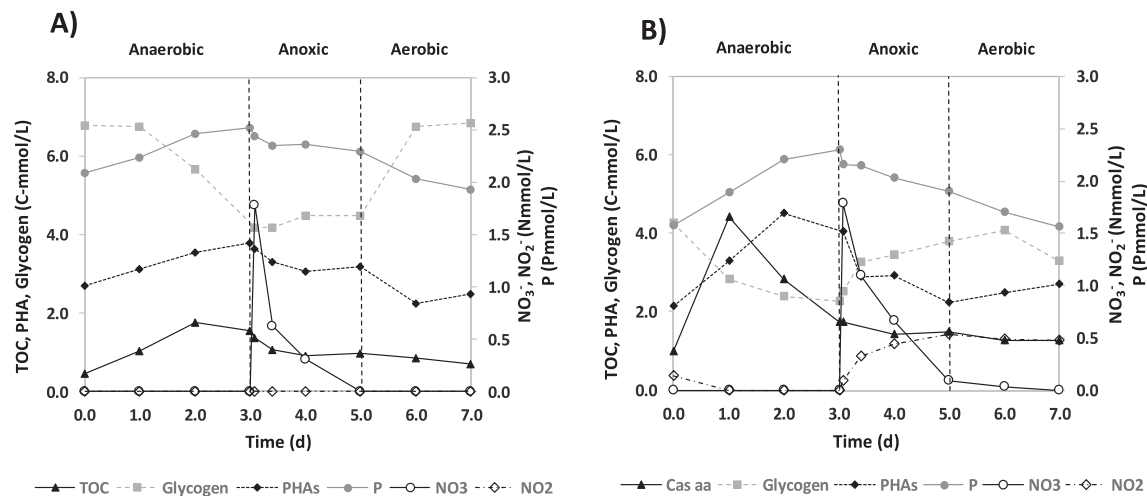


Fig. 1. Typical SBR cycle profile performed on days 82 (A) and 196 (B). Profiles of Cas aa (TOC), Phosphorus (P), Glycogen, PHAs,  $\text{NO}_3^-$  and  $\text{NO}_2^-$  are shown.

electron acceptors) and 0.28 N-mmol/L (~17%) reduction to *Competition* GAOs. This result agrees very well with GAOs abundance in this culture ( $12.4 \pm 5.1\%$ ) quantified by FISH (Table 2). 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  $\text{NO}_3^-$ ,  $\text{NO}_2^-$  and  $\text{N}_2\text{O}$  were added individually. Similar reduction rates were obtained for  $\text{NO}_3^-$  ( $20.97 \pm 2.31$  mg-N/g-VSS.h) and  $\text{NO}_2^-$  ( $20.30 \pm 3.10$  mg-N/g-VSS.h), while the  $\text{N}_2\text{O}$  reduction rate ( $8.53 \pm 0.22$  mg-N/g-VSS.h) was slower (Fig. 2, Table S2 Supplemental Information). This clearly shows higher affinity of this culture for  $\text{NO}_3^-$  and  $\text{NO}_2^-$  reduction, while  $\text{N}_2\text{O}$  reduction had the lowest reduction rate of denitrification when fed individually.  $\text{N}_2\text{O}$  accumulation was also observed in both test A and B, although the  $\text{N}_2\text{O}$  reduction rates were higher as compared to the case when only  $\text{N}_2\text{O}$  was added.  $\text{N}_2\text{O}$  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  $\text{NO}_3^-$  or  $\text{NO}_2^-$  as the electron acceptor. However, the increase of the  $\text{N}_2\text{O}$  reduction rate in the presence of  $\text{NO}_2^-$  vs  $\text{NO}_3^-$  addition, ( $20.20 \pm 0.19$  mg-N/g-VSS.h and  $12.80 \pm 0.76$  mg-N/g-VSS.h, respectively) rules out any inhibition by  $\text{NO}_2^-$  concentration (Fig. 2, Table S2 Supplemental Information).

Higher reduction rates were obtained for  $\text{NO}_3^-$  (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 (Fig. S2 and Table S2 Supplemental Information). Reduction rates of  $\text{NO}_2^-$  and  $\text{N}_2\text{O}$  were also higher (19.41 and 17.63 mg-N/g-VSS.h, respectively) as compared with test A. A decrease of 42% in  $\text{N}_2\text{O}$  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  $\text{N}_2\text{O}$  reduction rate as compared with  $\text{NO}_2^-$ , in the external carbon source test (Table S2 Supplemental Information). Limitation of intracellular carbon source during denitrification could potentially contribute to  $\text{N}_2\text{O}$  accumulation in this culture.

When fed alone, the  $\text{N}_2\text{O}$  reduction rate was significantly lower

than when in presence of other nitrogen electron acceptors (Figs. 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  $\text{N}_2\text{O}$  reduction rate could be less efficient bioenergetics within the cell when metabolising this nitrogen electron acceptor.  $\text{N}_2\text{O}$  reduction creates a lower amount of proton-motive force across the membrane to generate ATP. While reduction of  $\text{NO}_3^-$  to  $\text{N}_2$  requires 10 electrons, the reduction to  $\text{N}_2\text{O}$  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  $\text{N}_2\text{O}$  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  $\text{N}_2\text{O}$  as the only electron acceptor may lead to lower reduction rates as compared to situations where  $\text{NO}_3^-$  or  $\text{NO}_2^-$  are added. When the  $\text{N}_2\text{O}$  is inside the cell it is more readily reduced and generates more energy. If transportation is needed, prior to reduction of  $\text{N}_2\text{O}$ , 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  $\text{NO}_3^-$  was observed in test A, while it decreased in tests D, F and G when other electron acceptors were added in combination (Fig. 3, Table S2 Supplemental Information). A similar pattern was observed for  $\text{NO}_2^-$  reduction rates, where the highest reduction rate of  $\text{NO}_2^-$  was observed in test B, while it decreased when other electron acceptors were also added (Fig. 3, Supplemental Information Table S2). This indicates that *Tetrasphaera* has no preference for either  $\text{NO}_3^-$  or  $\text{NO}_2^-$  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  $\text{N}_2\text{O}$  reduction rate was obtained in test C, however, the rate increased in test D, E and G, respectively (Fig. 3, Table S2 Supplemental Information). This higher  $\text{N}_2\text{O}$  reduction rate with increased presence of  $\text{NO}_2^-$  and/or  $\text{NO}_3^-$  could be linked to an increased synthesis of enzymes responsible for  $\text{N}_2\text{O}$  reduction (Nos), likely caused by the increased available energy created by  $\text{NO}_2^-$  and/or  $\text{NO}_3^-$  reduction as explained above.

This hypothesis is further supported by the accumulation of  $\text{N}_2\text{O}$  produced per nitrogen reduced. Higher accumulation in test A, B

**Table 3**

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 (%)	<i>Tetrasphaera</i> (this study)	<i>Accumulibacter</i>	<i>Tetrasphaera</i> + <i>Accumulibacter</i>
<b><i>Tetrasphaera</i></b>	82.1 ± 2.1	N/A	69.5 ± 2.8 <sup>a</sup>
<b><i>Accumulibacter</i></b>	1.4 ± 1.4	42.4 ± 8.3 <sup>b</sup>	21.7 ± 8.8 <sup>a</sup>
<b><i>Competibacter</i></b>	12.4 ± 5.1	22.9 ± 4.4 <sup>b</sup>	<1 <sup>a</sup>
Carbon source	Casein hydrolysate	Propionate + Acetate	Casein hydrolysate
P release/substrate cons (P-mol/C-mol)	0.11 ± 0.02	0.62 ± 0.25 <sup>b</sup>	0.35 ± 0.08 <sup>a</sup>
Glycogen cons/substrate cons (C-mol/C-mol)	0.44 ± 0.19	0.36 ± 0.27 <sup>b</sup>	0.38 ± 0.12 <sup>a</sup>
PHB prod/substrate cons (C-mol/C-mol)	0.07 ± 0.05	0.40 ± 0.17 <sup>b</sup>	0.03 ± 0.01 <sup>a</sup>
PHV prod/substrate cons (C-mol/C-mol)	0.28 ± 0.08	0.27 ± 0.11 <sup>b</sup>	0.09 ± 0.02 <sup>a</sup>
PH2MV prod/substrate cons (C-mol/C-mol)	0.00 ± 0.00	0.11 ± 0.09 <sup>b</sup>	0.03 ± 0.01 <sup>a</sup>
PHA prod/substrate cons (C-mol/C-mol)	0.35 ± 0.13	0.78 ± 0.28 <sup>b</sup>	0.15 ± 0.04 <sup>a</sup>
Anaerobic pH	6.8 ± 0.1	7.0 ± 0.1 <sup>b</sup>	6.7 ± 0.1 <sup>a</sup>
Anoxic results			
P uptake (P-mmol/L)	0.28 ± 0.08	0.99 ± 0.07 <sup>b</sup>	—
NOx reduction (N-mmol/L)	1.63 ± 0.22	1.66 ± 0.06 <sup>b</sup>	—
Glycogen Production (C-mmol/L)	1.37 ± 0.17	−0.11 ± 0.04 <sup>b</sup>	—
PHA Consumption (C-mmol/L)	0.77 ± 0.21	1.86 ± 0.27 <sup>b</sup>	—
Aerobic results			
P uptake (P-mmol/L)	0.37 ± 0.07	1.79 ± 0.39 <sup>b</sup>	1.76 ± 0.25 <sup>a</sup>
NOx reduction (N-mmol/L)	0.12 ± 0.11	0.02 ± 0.01 <sup>b</sup>	—
% P in TSS	0.6–2.2	5–7 <sup>b</sup>	8–19 <sup>a</sup>
Glycogen Production (C-mmol)	0.74 ± 0.23	1.94 ± 0.01 <sup>b</sup>	1.38 ± 0.70 <sup>a</sup>
PHA Consumption (C-mmol/L)	0.59 ± 0.22	1.82 ± 0.04 <sup>b</sup>	0.75 ± 0.24 <sup>a</sup>
Anoxic/Aerobic results			
P uptake (P-mmol/L)	0.65 ± 0.06	2.79 ± 0.43 <sup>b</sup>	1.76 ± 0.25 <sup>a</sup>
NOx reduction (N-mmol/L)	1.75 ± 0.22	1.69 ± 0.06 <sup>b</sup>	—
% P in TSS	0.6–2.2	5–7 <sup>b</sup>	8–19 <sup>a</sup>
Glycogen Production (C-mmol/L)	2.11 ± 0.06	1.82 ± 0.03 <sup>b</sup>	1.38 ± 0.70 <sup>a</sup>
PHA Consumption (C-mmol/L)	1.36 ± 0.06	3.69 ± 0.30 <sup>b</sup>	0.75 ± 0.24 <sup>a</sup>

<sup>a</sup> (Marques et al., 2017).

<sup>b</sup> (Ribera-Guardia et al., 2016).

and F was observed, whereas in tests D, E and G no accumulation was detected (Table 4). When N<sub>2</sub>O was added simultaneously with another electron acceptor, no accumulation was observed. The rate of reduction was similar or higher as compared with the N<sub>2</sub>O production rate, although N<sub>2</sub>O accumulated when NO<sub>3</sub><sup>−</sup> and NO<sub>2</sub><sup>−</sup> were added individually or in combination with no N<sub>2</sub>O addition. This higher availability of energy for Nos synthesis combined with a higher availability of N<sub>2</sub>O could trigger a higher reduction rate, due to a higher overall energy potential for the cells. Also, less accumulation of N<sub>2</sub>O 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<sub>2</sub>O 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<sub>2</sub>O accumulation as compared with these two enriched cultures. Similar accumulation of N<sub>2</sub>O 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<sub>2</sub>O 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<sub>3</sub><sup>−</sup> and NO<sub>2</sub><sup>−</sup> 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

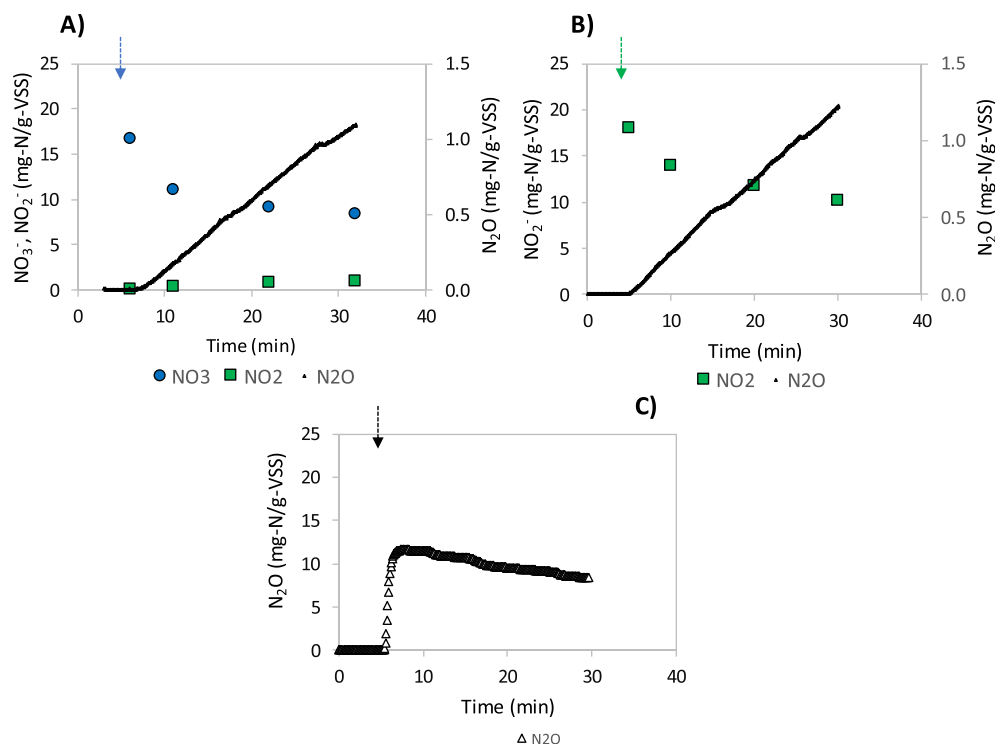


Fig. 2. Batch test profiles performed with different electron acceptors: Nitrate (A), Nitrite (B) and Nitrous oxide (C). The arrows represent the time when nitrous oxides were added.

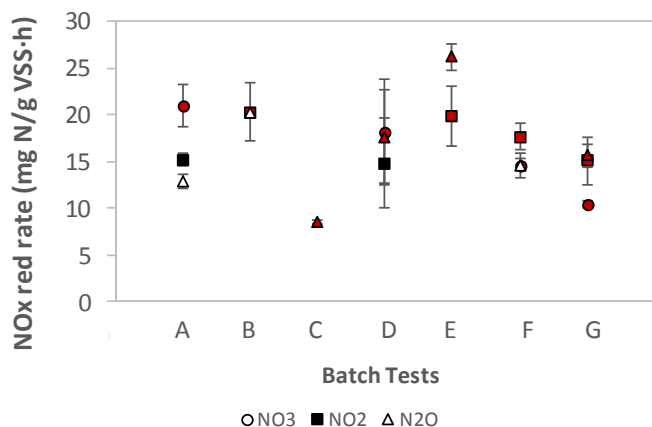


Fig. 3. Nitrous oxide reduction rates for the enriched *Tetrasphaera* culture. Error bars represent standard deviation of duplicate assays. Red filled symbols represent the nitrogen electron acceptors sources fed in each of the batch tests. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Table 4

Accumulation of  $\text{N}_2\text{O}$  per  $\text{N}$ -reduced for this culture and for the dPAO and dGAO enriched cultures used in Ribera-Guardia et al. (2016).

Batch test	$\text{N}_2\text{O}$ accumulation per $\text{N}$ -reduced (%)		
	dTET	dPAOs	dGAOs
A ( $\text{NO}_3^-$ )	16.7 $\pm$ 0.8	8.7 $\pm$ 0.2	7.1 $\pm$ 2.2
B ( $\text{NO}_2^-$ )	15.5 $\pm$ 0.8	17.4 $\pm$ 5.9	84.0 $\pm$ 4.8
D ( $\text{NO}_3^- + \text{N}_2\text{O}$ )	0.0	0.0	13.7 $\pm$ 5.8
E ( $\text{NO}_2^- + \text{N}_2\text{O}$ )	0.0	20.1 $\pm$ 1.9	56.9 $\pm$ 4.9
F ( $\text{NO}_3^- + \text{NO}_2^-$ )	17.7 $\pm$ 0.9	31.2 $\pm$ 2.7	45.4 $\pm$ 0.9
G ( $\text{NO}_3^- + \text{NO}_2^- + \text{N}_2\text{O}$ )	0.0	11.3 $\pm$ 3.1	48.4 $\pm$ 5.9
A ( $\text{NO}_3^-$ ) (external C-source)	9.6	—	—

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, using the methodology developed by Pan et al. (2013).

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  $5.27 \pm 0.55 \text{ mmol e}^-/\text{g-VSS.h}$ ) (Fig. 4). This value was also very similar to the total electron consumption rate obtained in test A with  $\text{NO}_3^-$  ( $6.09 \text{ mmol e}^-/\text{g-VSS.h}$ ) (Fig. 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 ( $7.72 \text{ mmol e}^-/\text{g-VSS.h}$ ) was obtained in a test performed with external carbon source, at the same concentration as added to the main SBR, with  $\text{NO}_3^-$  added as sole electron acceptor (Fig. 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.

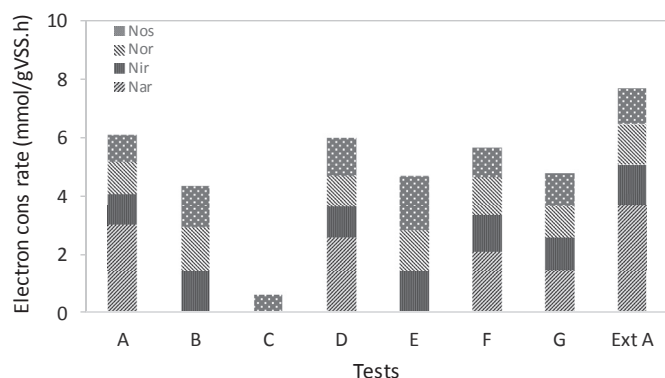
Another interesting observation is that the combination of  $\text{NO}_3^-$  and  $\text{N}_2\text{O}$  (test D) showed a higher electron consumption rate as compared to  $\text{NO}_2^-$  and  $\text{N}_2\text{O}$  (test E). It has been observed that Nar receives electrons directly from the ubiquinone/ubiquinol pool ( $\text{UQ/UQH}_2$ ), while Nir, Nor and Nos receive their electrons from the cytochrome c550/pseudoazurin pool (Cyt c550/Ps az) by way of the  $\text{UQ/UQH}_2$  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

**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 (NO <sub>3</sub> -)	0.57 ± 0.00	0.45 ± 0.18	0.05 ± 0.01	0.09 ± 0.02
B (NO <sub>2</sub> -)	0.64 ± 0.04	0.05 ± 0.02	0.03 ± 0.03	0.05 ± 0.04
C (N <sub>2</sub> O)	0.38 ± 0.04	-0.07 ± 0.05	0.01 ± 0.00	0.03 ± 0.01
D (NO <sub>3</sub> - + N <sub>2</sub> O)	0.86 ± 0.24	0.19 ± 0.13	0.10 ± 0.01	0.12 ± 0.04
E (NO <sub>2</sub> - + N <sub>2</sub> O)	1.14 ± 0.01	0.25 ± 0.01	0.09 ± 0.01	0.08 ± 0.01
F (NO <sub>3</sub> - + NO <sub>2</sub> -)	0.57 ± 0.10	0.10 ± 0.02	0.09 ± 0.03	0.16 ± 0.08
G (NO <sub>3</sub> - + NO <sub>2</sub> - + N <sub>2</sub> O)	0.71 ± 0.10	0.20 ± 0.04	0.09 ± 0.01	0.13 ± 0.03
A (NO <sub>3</sub> -) (external C-source)	0.99	0.07	-0.04	-0.04



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

**Table 6**

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

Batch Test	Electron distribution dTET (%)			
	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

where NO<sub>3</sub><sup>-</sup> was fed alone or in combination with other nitrogen electron acceptors, decreasing from 49.1% to 43.4%, to 37.1%–31.5% in tests, A, D, F and G, respectively. Similarly, both NO<sub>3</sub><sup>-</sup> and N<sub>2</sub>O had a similar impact on Nir activity as can be observed from the electron distribution (Table 6), being highest when NO<sub>2</sub><sup>-</sup> was fed individually. This supports the hypothesis that both Nar and Nir activity were affected by electron competition. In the case of N<sub>2</sub>O reduction, the total electron consumption rate obtained in test C was only about 1/10 of the value obtained in the other batch tests (Fig. 4). This supports the fact that N<sub>2</sub>O was energetically unfavourable for the culture when fed in isolation, which can be explained by the fact that N<sub>2</sub>O reduction only comprises around 20% of the bio-energetic potential as compared to full denitrification.

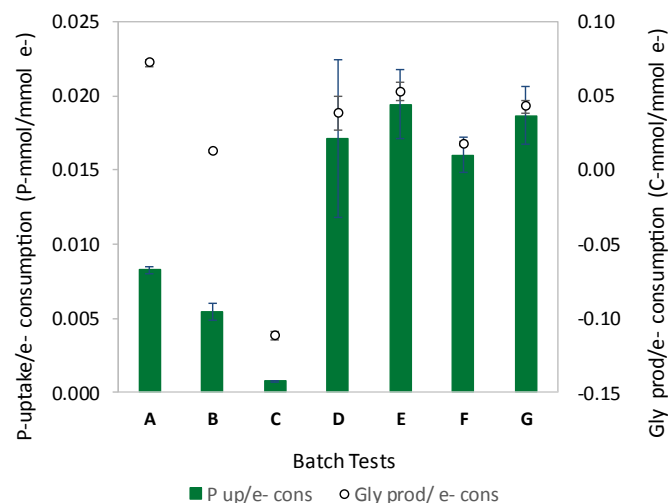
When comparing the P-uptake/electron consumption ratio, higher P-uptake was obtained in tests D, E, F and G. This shows that the culture channelled more energy obtained from the reduction of the nitrogen electron acceptors present to perform P-uptake, despite the similar total electron consumption rate (Fig. 5). This

could be due to higher availability of electron acceptors availability, especially NO<sub>3</sub><sup>-</sup> and NO<sub>2</sub><sup>-</sup>, that would lead to higher energy obtained by *Tetrasphaera* 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 NO<sub>3</sub><sup>-</sup> 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 *Accumulibacter* is shown in Table 7. A higher anoxic P uptake rate of *Accumulibacter* was



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



**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	Reference
GAOmiox	PAOmiox	TET	acceptor	donor	N uptake	P uptake	P/N	
%					mmol-N/g-VSS.h	mmol-P/g-VSS.h	mmol-P/mmol-N	
5	37	—	NO <sub>3</sub> <sup>-</sup>	HAc	0.45	0.27	0.60	Carvalho et al., 2007
1	76	—	NO <sub>3</sub> <sup>-</sup>	HPr	0.24	0.13	0.54	Carvalho et al., 2007
0	90	—	NO <sub>3</sub> <sup>-</sup>	HPr	0.29	0.31	1.07	Lanham et al., 2011
—	<sup>a</sup>	—	NO <sub>3</sub> <sup>-</sup>	HAc	0.47	0.39	0.83	Freitas et al., 2005
—	<sup>a</sup>	—	NO <sub>3</sub> <sup>-</sup>	HAc	0.86	0.97	1.13	Jiang et al., 2006
—	38	—	NO <sub>3</sub> <sup>-</sup>	HAc	1.08	0.58	0.54	Zeng et al., 2003a
23	42	—	NO <sub>3</sub> <sup>-</sup>	HAc/HPr	0.32	0.24	0.77	Ribera-Guardia et al., 2016
—	<sup>a</sup>	—	NO <sub>3</sub> <sup>-</sup>	HAc	1.30	1.19	0.91	Kuba et al., 1993
12	1	82	NO <sub>3</sub> <sup>-</sup>	Cas aa	0.72	0.09	0.12	This study
Sludge	GAOmiox	PAOmiox	TET	Electron donor	Aerobic rates		Reference	
%					P uptake			
					mmol-P/g-VSS.h			
17	85	—	HAc/HPr		0.48		Carvalho et al., 2014	
—	41	—	HAc		0.56		Zeng et al., 2003a	
—	<sup>a</sup>	—	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	

<sup>a</sup>—sludge composition was qualitatively reported as dominated by *Accumulibacter*.

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 (Kuba et al., 1996). 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 ( $0.86 \pm 0.29$  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 then be taken up by *Tetrasphaera* (amino acids, sugars) and *Accumulibacter* (VFAs), which would result in P and N removal in EBPR plants. The different ecological niche of *Tetrasphaera* and *Accumulibacter* could lead to an increase in efficiency of P and N removal as compared to systems without *Tetrasphaera*. The ratios of C/N and C/P due to a more efficient carbon utilisation can also contribute to augment denitrification in WWTPs.

In the batch tests performed with individual electron acceptors, NO<sub>3</sub><sup>-</sup> and NO<sub>2</sub><sup>-</sup> achieved higher reduction rates, as compared with N<sub>2</sub>O. When added with multiple electron acceptors, NO<sub>3</sub><sup>-</sup> and NO<sub>2</sub><sup>-</sup>

achieved lower reduction rates, whereas the N<sub>2</sub>O reduction rates increased, especially in combination with NO<sub>2</sub><sup>-</sup>. This suggests that electron competition could have an important role in lowering the reduction rates of NO<sub>3</sub><sup>-</sup> and NO<sub>2</sub><sup>-</sup>. 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<sub>2</sub>O accumulation by *Tetrasphaera* that was lower than *Accumulibacter* or *Competibacter* when multiple electron acceptors were provided simultaneously. When two or more electron acceptors were present simultaneously, electron competition occurred, lowering the reduction rates of NO<sub>3</sub><sup>-</sup> and NO<sub>2</sub><sup>-</sup>. 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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## Appendix A. Supplementary data

Supplementary data related to this article can be found at <https://doi.org/10.1016/j.watres.2018.03.010>.

## References

- Amann, R., 1995. In situ identification of micro-organisms by whole cell hybridization with rRNA-targeted nucleic acid probes. *Mol. Microb. Ecol.* 4, 331–345.
- APHA, 2005. Standard Methods for the Examination of Water and Wastewater, twenty-first ed. ed. Washington, D.C.
- Boer, A.P.N.D.E., Oost, J.V.A.N.D.E.R., Reijnders, W.N.M., Westerhow, H.V., 1996. Mutational analysis of the nor gene cluster which encodes nitric-oxide reductase from *Paracoccus denitrificans*. *Eur. J. Biochem.* 242, 592–600.
- Carvalho, M., Oehmen, A., Carvalho, G., Reis, M.A.M., 2014. The effect of substrate competition on the metabolism of polyphosphate accumulating organisms (PAOs). *Water Res.* 64, 149–159.
- Carvalho, G., Lemos, P.C., Oehmen, A., Reis, M. a M., 2007. Denitrifying phosphorus removal: linking the process performance with the microbial community structure. *Water Res.* 41, 4383–4396.
- Daelman, M.R.J., De Baets, B., van Loosdrecht, M.C.M., Volcke, E.I.P., 2013a. Influence of sampling strategies on the estimated nitrous oxide emission from wastewater treatment plants. *Water Res.* 47, 3120–3130.
- Daelman, M.R.J., van Voorthuizen, E.M., van Dongen, L.G.J.M., Volcke, E.I.P., van Loosdrecht, M.C.M., 2013b. Methane and nitrous oxide emissions from municipal wastewater treatment - results from a long-term study. *Water Sci. Technol.* 67, 2350–2355.
- Filipe, C.D.M., Daigler, G.T., Grady, C.P.L., 2001. Stoichiometry and kinetics of acetate uptake under anaerobic conditions by an enriched culture of phosphorus-accumulating organisms at different pHs. *Biotechnol. Bioeng.* 76, 32–43.
- Freitas, F., Temudo, M., Reis, M. a M., 2005. Microbial population response to changes of the operating conditions in a dynamic nutrient-removal sequencing batch reactor. *Bioproc. Biosyst. Eng.* 28, 199–209.
- Goretski, J., Zafiriou, O.C., Hollocher, T.C., 1990. Steady-state nitric-oxide concentrations during denitrification. *J. Biol. Chem.* 265, 11535–11538.
- IPCC, 2013. In: Stocker, T.F., Qin, D., Plattner, G.-K., Tignor, M., Allen, S.K., Boschung, J., Nauels, A., Xia, Y., Bex, V., Midgley, P.M. (Eds.), *Climate Change 2013: The Physical Science Basis. Contribution of Working Group I to the Fifth Assessment Report of the Intergovernmental Panel on Climate Change*. Cambridge University Press, Cambridge, United Kingdom and New York, NY, USA. <https://doi.org/10.1017/CBO9781107415324>, 1535 pp.
- Jiang, Y., Wang, B., Wang, L., Chen, J., He, S., 2006. Dynamic Response of Denitrifying Poly-p Accumulating Organisms Batch Culture to Increased Nitrite Concentration as Electron Acceptor 4529, pp. 2557–2570.
- Kong, Y., Xia, Y., Nielsen, P.H., 2008. Activity and identity of fermenting microorganisms in full-scale biological nutrient removing wastewater treatment plants. *Environ. Microbiol.* 10, 2008–2019.
- Kristiansen, R., Nguyen, H.T.T., Saunders, A.M., Nielsen, J.L., Wimmer, R., Le, V.Q., McIlroy, S.J., Petrovski, S., Seviour, R.J., Calteau, A., Nielsen, K.L., Nielsen, P.H.H., 2013. A metabolic model for members of the genus *Tetrasphaera* involved in enhanced biological phosphorus removal. *International Soc. Microb. Ecol.* 7, 543–554.
- Kuba, T., Murnleitner, E., Loosdrecht, M.C.M. Van, Heijnen, J.J., 1996. A metabolic model for biological phosphorus removal by denitrifying organisms. *Biotechnol. Bioeng.* 52, 685–695.
- Kuba, T., Smolders, G., Van Loosdrecht, M.C.M., Heijnen, J.J., 1993. Biological phosphorus removal from wastewater by anaerobic-anoxic sequencing batch reactor. *Water Sci. Technol.* 27, 241–252.
- Lanham, A.A.B., Oehmen, A.A., Saunders, A.M.A., Carvalho, G., Nielsen, P.H., Reis, M. a M., 2013a. Metabolic versatility in full-scale wastewater treatment plants performing enhanced biological phosphorus removal. *Water Res.* 47, 7032–7041.
- Lanham, A.B., Moita, R., Lemos, P.C., Reis, M.A.M., 2011. Long-term Operation of a Reactor Enriched in Accumulibacter Clade I DPAOs : Performance with Nitrate, Nitrite and Oxygen, pp. 352–360.
- Lanham, A.B., Ricardo, A.R., Albuquerque, M.G.E., Paredelha, F., Carvalho, M., Coma, M., Fradinho, J., Carvalho, G., Oehmen, A., Reis, M. a M., 2013b. Determination of the extraction kinetics for the quantification of polyhydroxyalkanoate monomers in mixed microbial systems. *Process Biochem.* 48, 1626–1634.
- Lanham, A.B., Ricardo, A.R., Coma, M., Fradinho, J., Carvalho, M., Oehmen, A., Carvalho, G., Reis, M. a M., 2012. Optimisation of glycogen quantification in mixed microbial cultures. *Bioresour. Technol.* 118, 518–525.
- Lemaire, R., Meyer, R., Taske, A., Crocetti, G.R., Keller, J., Yuan, Z., 2006. Identifying causes for N<sub>2</sub>O accumulation in a lab-scale sequencing batch reactor performing simultaneous nitrification, denitrification and phosphorus removal. *J. Biotechnol.* 122, 62–72.
- Li, C., Zhang, J., Liang, S., Ngo, H.H., Guo, W., Zhang, Y., Zou, Y., 2013. Nitrous oxide generation in denitrifying phosphorus removal process: main causes and control measures. *Environ. Sci. Pollut. Res.* 20, 5353–5360.
- Marques, R., Santos, J., Nguyen, H., Carvalho, G., Noronha, J.P.P., Nielsen, P.H., Reis, M.A.M.M., Oehmen, A., 2017. Metabolism and ecological niche of *Tetrasphaera* and *Ca. Accumulibacter* in enhanced biological phosphorus removal. *Water Res.* <https://doi.org/10.1016/j.watres.2017.04.072>.
- Muszyński, A., Miobędzka, A., 2015. The effects of carbon/phosphorus ratio on polyphosphate- and glycogen-accumulating organisms in aerobic granular sludge. *Int. J. Environ. Sci. Technol.* 12, 3053–3060.
- Nguyen, H.T.T., Kristiansen, R., Vestergaard, M., Wimmer, R., Nielsen, P.H., 2015. Intracellular accumulation of Glycine in polyphosphate-accumulating organisms in activated sludge, a novel storage mechanism under dynamic anaerobic-aerobic conditions. *Appl. Environ. Microbiol.* 81, 4809–4818.
- Nguyen, H.T.T., Le, V.Q., Hansen, A.A., Nielsen, J.L., Nielsen, P.H.H., 2011. High diversity and abundance of putative polyphosphate-accumulating *Tetrasphaera*-related bacteria in activated sludge systems. *FEMS Microbiol. Ecol.* 76, 256–267.
- Nielsen, P.H.H., Mielczarek, A.T., Kragelund, C., Nielsen, J.L., Saunders, A.M., Kong, Y., Hansen, A.A., Vollertsen, J., 2010. A conceptual ecosystem model of microbial communities in enhanced biological phosphorus removal plants. *Water Res.* 44, 5070–5088.
- Oehmen, A., Lemos, P.C., Carvalho, G., Yuan, Z., Blackall, L.L., Reis, M.A.M., Keller, J.J., Blackall, L.L., Reis, M.A.M., 2007. Advances in enhanced biological phosphorus removal: from micro to macro scale. *Water Res.* 41, 2271–2300.
- Oehmen, A., Lopez-Vazquez, C.M.M., Carvalho, G., Reis, M.A.M.A.M., van Loosdrecht, M.C.M.C.M., 2010. Modelling the population dynamics and metabolic diversity of organisms relevant in anaerobic/anoxic/aerobic enhanced biological phosphorus removal processes. *Water Res.* 44, 4473–4486.
- Oehmen, A., Teresa Vives, M., Lu, H., Yuan, Z., Keller, J.J., Vives, M.T., Lu, H., Yuan, Z., Keller, J.J., Teresa Vives, M., Lu, H., Yuan, Z., Keller, J.J., 2005. The effect of pH on the competition between polyphosphate-accumulating organisms and glycogen-accumulating organisms. *Water Res.* 39, 3727–3737.
- Pan, Y., Ni, B.J., Bond, P.L., Ye, L., Yuan, Z., 2013. Electron competition among nitrogen oxides reduction during methanol-utilizing denitrification in wastewater treatment. *Water Res.* 47, 3273–3281.
- Pijuan, M., Saunders, A.M., Guisasola, A., Baeza, J.A., Casas, C., Blackall, L.L., 2004. Enhanced Biological Phosphorus Removal in a Sequencing Batch Reactor Using Propionate as the Sole Carbon Source.
- Qiu, G., Montanez, R.E.Z., Law, Y., Williams, R.B.H., Thi, S.S., Nguyen, T.Q.N., Kaliyamoorthy, E., Nielsen, P.H., Wuertz, S., 2017. Relating carbon source usage profiles to polyphosphate-accumulating organisms (PAO) communities in full-scale enhanced biological phosphorus removal (EBPR) plants in Singapore. In: Qiu, Guangle, Montanez, Rogelio Ernesto Zuniga, Law, Yingyu, et al. (Eds.), *The IWA 2017 Conference on Sustainable Wastewater Treatment and Resource Recovery: Research, Planning, Design and Operation*. Chongqing, China. Nanyang Technol.
- Ribera-Guardia, A., Kassotaki, E., Gutierrez, O., Pijuan, M., 2014. Effect of carbon source and competition for electrons on nitrous oxide reduction in a mixed denitrifying microbial community. *Process Biochem.* 49, 2228–2234.
- Ribera-Guardia, A., Marques, R., Arangio, C., Carvalho, M., Oehmen, A., Pijuan, M., 2016. Distinctive denitrifying capabilities lead to different N<sub>2</sub>O production in dPAO and dGAO. *Bioresour. Technol.* 219, 106–113.
- Richardson, D., Felgate, H., Watmough, N., Thomson, A., Baggs, E., 2009. Mitigating release of the potent greenhouse gas N<sub>2</sub>O from the nitrogen cycle - could enzymic regulation hold the key? *Trends Biotechnol.* 27, 388–397.
- Smolders, G.J.F., Meij, J. Van Der, Loosdrecht, M.C.M. Van, Heijnen, J.J., van der Meij, J., van Loosdrecht, M.C., Heijnen, J.J., 1994. Model of the anaerobic metabolism of the biological phosphorus removal process: stoichiometry and pH influence. *Biotechnol. Bioeng.* 43, 461–470.
- Stokholm-Bjerregaard, M., McIlroy, S.J., Nierychlo, M., Karst, S.M., Albertsen, M.,

- Nielsen, P.H., 2017. A critical assessment of the microorganisms proposed to be important to enhanced biological phosphorus removal in full-scale wastewater treatment systems. *Front. Microbiol.* 8, 718.
- Tooker, N.B., Barnard, J.L., Bott, C., Carson, K., Dombrowski, P., Dunlap, P., Martin, K., McQuarrie, J., Menniti, A., Phillips, H., Schauer, P., Shaw, A., Stevens, G., Takács, I., Onnis-Hayden, A., Gu, A.Z., 2016. Side-stream enhanced biological phosphorus removal as a sustainable and stable approach for removing phosphorus from wastewater. In: *Proc. WEF/IWA Nutr. Remov. Recover.* 2016.
- Tsuneda, S., Ohno, T., Soejima, K., Hirata, A., 2006. Simultaneous nitrogen and phosphorus removal using denitrifying phosphate-accumulating organisms in a sequencing batch reactor. *Biochem. Eng. J.* 27, 191–196.
- Von Schulthess, R., Wild, D., Gujer, W., 1994. Nitric and nitrous oxides from denitrifying activated sludge at low oxygen concentration. *Water Sci. Technol.* 30, 123–132.
- Wang, X., Zeng, R.J., Dai, Y., Peng, Y., Yuan, Z., 2008. The denitrification capability of cluster 1 *Dechlorococcus* vanus-related glycogen-accumulating organisms. *Biotechnol. Bioeng.* 99, 1329–1336.
- Wang, Y., Geng, J., Ren, Z., He, W., Xing, M., Wu, M., Chen, S., 2011. Effect of anaerobic reaction time on denitrifying phosphorus removal and N<sub>2</sub>O production. *Bioresour. Technol.* 102, 5674–5684.
- Wei, Y., Wang, S., Ma, B., Li, X., Yuan, Z., He, Y., Peng, Y., 2014. The effect of poly-B-hydroxyalkanoates degradation rate on nitrous oxide production in a denitrifying phosphorus removal system. *Bioresour. Technol.* 170, 175–182.
- Ye, L., Ni, B.J., Law, Y., Byers, C., Yuan, Z., 2014. A novel methodology to quantify nitrous oxide emissions from full-scale wastewater treatment systems with surface aerators. *Water Res.* 48, 257–268.
- Zeng, R.J., Lemaire, R., Yuan, Z., Keller, J.J., 2003a. Simultaneous nitrification, denitrification, and phosphorus removal in a lab-scale sequencing batch reactor. *Biotechnol. Bioeng.* 84, 170–178.
- Zeng, R.J., Yuan, Z., Keller, J.J., 2003b. Enrichment of denitrifying glycogen-accumulating organisms in anaerobic/anoxic activated sludge system. *Biotechnol. Bioeng.* 81, 397–404.
- Zhou, Y., Lim, M., Harjono, S., Ng, W.J., 2012. Nitrous oxide emission by denitrifying phosphorus removal culture using polyhydroxyalkanoates as carbon source. *J. Environ. Sci.* 24, 1616–1623.
- Zhou, Y., Oehmen, A., Lim, M., Vadivelu, V., Ng, W.J., 2011. The role of nitrite and free nitrous acid (FNA) in wastewater treatment plants. *Water Res.* 45, 4672–4682.
- Zumft, W.G., 1997. Cell biology and molecular basis of denitrification. *Microbiol. Mol. Biol. Rev.* 61, 533–616.