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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 (P) and nitrate (NO_3^-) 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_3^-, nitrite (NO_2^-) and nitrous oxide (N_2O) revealed lower N_2O 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.

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_3^- or 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 (Lanham et al., 2013a; Muszyński and Miłobędzka, 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 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 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 ± 0.1 by automatic addition of 0.1 M HCl, while temperature was controlled at 20 ± 1 °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 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

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^- N_2O	NO_2^- N_2O	NO_3^- NO_2^-	NO_3^- NO_2^- NO_2^-	NO_3^-