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Denitrification of nitrate and nitrite by '*Candidatus* Accumulibacter phosphatis' clade IC

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ABSTRACT

Phosphate accumulating organisms (PAO) are assumed to use nitrate as external electron acceptor, allowing an efficient integration of simultaneous nitrogen and phosphate removal with minimal organic carbon (COD) requirements. However, contradicting findings appear in literature regarding the denitrification capacities of PAO due to the lack of clade specific highly enriched PAO cultures. Whereas some studies suggest that only PAO clade I may be capable of using nitrate as external electron acceptor for anoxic P-uptake, other studies indicate that PAO clade II may be responsible for anoxic P-removal. In the present study, a highly enriched PAO clade IC culture (>99% according to FISH) was cultivated in an SBR operated under Anaerobic/Oxic conditions and subsequently exposed to Anaerobic/Anoxic/Oxic conditions using nitrate as electron acceptor. Before and after acclimatization to the presence of nitrate, the aerobic and anoxic (nitrate and nitrite) activities of the PAO I culture were assessed through the execution of batch tests using either acetate or propionate as electron donor. In the presence of nitrate, significant P-uptake by PAO I was not observed before or after acclimatization. Using nitrite as electron acceptor, limited nitrite removal rates were observed before acclimatization with lower rates in the acetate fed reactor without P-uptake and slightly higher in the propionate fed reactor with a marginal anoxic P-uptake. Only after acclimatization to nitrate, simultaneous P and nitrite removal was observed. This study suggests that PAO clade IC is not capable of using nitrate as external electron acceptor for anoxic P-removal. The elucidation of the metabolic capacities for individual PAO clades helps in better understanding and optimization of the relation between microbial ecology and process performance in enhanced biological phosphate removal processes.

1. Introduction

In the enhanced biological phosphorus removal (EBPR) process, sludge is cycled through anaerobic and aerobic/anoxic zones (Mino et al., 1998; Oehmen et al., 2007). The group of microorganisms responsible for EBPR performance is broadly known as Polyphosphate Accumulating Organisms (PAO). These organisms are able to anaerobically take up volatile fatty acids (VFA) and store them as poly-β-hydroxyalkanoates (PHA) generating energy from the degradation of intracellular polyphosphate, which leads to anaerobic *ortho*-phosphate release into the bulk liquid. In the following aerobic phase, PAO grow and take-up *ortho*-phosphate to recover their poly-P pools, leading to P removal from the bulk liquid via PAO cell removal by wastage of activated sludge (Mino et al., 1987, Mino et al., 1998).

Several studies have demonstrated that PAO can also grow and take up phosphate under anoxic conditions. The so-called denitrifying phosphate accumulating organisms (DPAO) are thought to use both nitrate and oxygen as electron acceptors instead of solely oxygen (Kerrn-Jespersen and Henze, 1993; Kuba et al., 1993; Hu et al., 2002), which promoted the development of combined nitrogen and phosphate removal processes with lower COD

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consumption such as the Modified University of Cape Town (MUCT) and Dephanox (or A^2/N) process (Bortone et al., 1996; Kuba et al., 1996; Henze, 2008).

The denitrification capacity of PAO is still a matter of debate in the scientific literature. In some studies, anoxic P-uptake gradually decreased and disappeared while nitrate was still available. In these studies, P-uptake continued in the subsequent aerobic phase which was thought to be related to the depletion of the PHA pools of DPAO under anoxic conditions (Parco et al., 2007), while conventional PAO (not capable of using nitrate) still contained PHA and was therefore active under aerobic conditions (Kerrn-Jespersen and Henze, 1993; Meinhold et al., 1999). This observation led to the distinction of two PAO phenotypes with different denitrification capabilities namely PAO that were only capable of using oxygen as external electron acceptor and DPAO that were able to use nitrate and nitrite as external electron acceptor. In the first studies that focused on the differentiation of PAO and DPAO, it was shown that both PAO and DPAO were closely related genotypes (Ahn et al., 2002; Kong et al., 2002; Zeng et al., 2003b). However, on the basis of the 16SrRNA gene and the poly-phosphate kinase gene (ppk1) as a genetic marker, it was revealed that '*Candidatus* Accumulibacter phosphatis' was organized into two main types, type I (PAO I) and type II (PAO II) each subdivided in several distinct clades (He et al., 2007; Peterson et al., 2008), which led to the speculation that these types or clades may have diverse functionality and morphology. In two more studies, PAO and DPAO were distinguished by morphology. Rod type morphology was observed to be dominant in cultures that succeeded to denitrify from nitrate, while cocci type was more abundant in the ones that failed (Carvalho et al., 2007; Guisasola et al., 2009). Flowers et al. (2009) developed FISH probes for PAO I and II and demonstrated by FISH analysis that a PAO I dominated system showed better anoxic P-removal than a PAO II dominated system and concluded that PAO I was capable of using nitrate whereas PAO II was not. These findings were supported by the study of Oehmen et al. (2010a) that demonstrated by FISH analysis that the organisms with the rod type and cocci type morphology, observed in the study of Carvalho et al. (2007), were PAO I and PAO II, respectively. Thus, in these and several other studies, the full denitrification capacity (starting from nitrate) of PAO was, based on FISH analysis, attributed to PAO I species (DPAO), while PAO II was thought to be only capable of using oxygen and nitrite (Carvalho et al., 2007; Flowers et al., 2009; Oehmen et al., 2010a; Lanham, et al., 2011). On the contrary, Kim et al. (2013) demonstrated with FISH-MAR, that clade IA, IIA, IIC and IIF were not capable of using nitrate (Kim et al., 2013). In addition, several metagenomic studies revealed that only the metagenome of PAO clade IIF encodes the nitrate reductase and nitrite reductase genes, whereas the metagenomes of the other clades (IA, IB, IC, IIA, IIF) only encoded genes for periplasmic nitrate reductase. The role of this reductase in denitrification remains unclear (Moreno-Vivián et al., 1999; Skennerton et al., 2015).

Overall, the results of previous studies are contradictory and inconclusive as many studies lack information on the prevailing microbial populations or were not conducted with cultures that were highly enriched with specific PAO clades. The significant presence of microbial populations other than PAO in past studies and the lack of cultures highly enriched with only one specific PAO clade have hampered the elucidation of functional differences among PAO clades including their denitrification capabilities. For instance, Flowers et al. (2009) conducted a study with two cultures containing different ratios of PAO clades (culture 1 enriched with 67% PAO I, 5% PAO II and 28% by other organisms; and, culture 2 containing 32% PAOI, 50% PAO II, and 18% by other organisms). Whereas the authors suggest that in both cultures PAO I were mostly responsible for the initial denitrification rates, the determination of the biomass kinetic rates for the specific PAO clades (taking into account their relative abundance in each culture) does not show any considerable difference between the two clades (estimated 0.154, 0.16 mmol NO_3-N/gVSS.hr, for culture 1 and 2, respectively).

Despite the inconsistent and inconclusive findings of past studies, it seems from the literature that a general perception appears to be present that PAO I can use both nitrate and nitrite, while PAO II can only use nitrite (Carvalho et al., 2007; Flowers et al., 2009; Oehmen et al., 2010a,b; Lanham et al., 2011; Bassin et al., 2011; Tayà et al., 2013; Tian et al., 2013; Wang et al., 2014). Moreover, it has been suggested that the presence of an anoxic zone with nitrate may serve as a selective pressure leading to the enrichment of PAO I dominated cultures (Lanham et al., 2011; Tayà et al., 2013).

The objective of this study was to assess the anoxic (NO_2^- and NO_3^-) activity of a highly enriched and well characterized PAO IC culture in comparison to the aerobic activity before and after acclimatization to nitrate after feeding with either acetate or propionate. The acclimatization period was selected to expose the organisms long enough to nitrate to allow induction of potential denitrification capacity, but short enough to avoid a significant change in the microbial community. Different carbon sources were used to verify if the carbon source also affects the ability of PAO to denitrify.

2. Materials and methods

2.1. Enrichment of PAO

2.1.1. Operation of parent SBR

PAO were enriched and cultivated during two different experimental phases. The reactor was operated under conditions similar to those applied in many past EBPR studies, except for the higher pH, the influent composition of the carbon source (including propionate), influent phosphorus to carbon ratio and DO limitation that were more distinctive and may have led to this specific enrichment culture. Furthermore, the ratio of propionate over acetate applied in this study is in the range of the ratio observed in many domestic wastewaters. In phase I, a PAO culture was enriched and cultivated under Anaerobic/Oxic conditions in a double-jacketed laboratory sequencing batch reactor (SBR), while in phase II, PAO were cultivated under Anaerobic/Anoxic/Oxic conditions. The SBR was operated and controlled automatically by an Applikon ADI controller. Online operating data (e.g. pH and O_2) was stored using BioXpert software (Applikon, Delft, The Netherlands). The reactor had a working volume of 2.5 L. Activated sludge from Harnaschpolder WWTP (Den Horn, The Netherlands), was used as inoculum for the enrichment of the PAO culture.

2.1.1.1. Operation in (Anaerobic/Oxic) mode. In the first operation mode, the SBR was operated in cycles of 6 h (135 min anaerobic, 135 min aerobic and 90 min settling and decanting phase) following similar operating conditions used in previous studies (Smolders et al., 1994; Brdjanovic et al., 1997). pH was maintained at 7.6 ± 0.05 by dosing 0.4 M HCl and 0.4 M NaOH. Temperature was controlled at 20 ± 1 °C. The mixed liquor was mixed at 500 rpm, except during settling and decant phases when mixing was switched off. In the aerobic phase, dissolved oxygen was controlled not to exceed 20% of saturation (around 1.8 mg/L) by an on-off valve controlling the flow of compressed air into the reactor, as lower dissolved oxygen concentrations seem to favor the growth of PAO over GAO (Carvalheira et al., 2014) and many full-scale WWTP's have DO control at around 20% to optimize the efficiency of oxygen transfer without compromising the sludge specific activity.

The HRT was controlled at 12 h while the SRT at 8 days, without