



Occurrence of PAOI in a low temperature EBPR system



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HIGHLIGHTS

- At 10 °C and 6 d net aerobic SRT, an enriched GAO culture shifted to PAO I.
- PAO I did not switch to a GAO-like metabolism under P-limiting conditions at 10 °C.
- Low temperature can be used to enrich PAO I cultures for their metabolic research.

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ABSTRACT

The occurrence of *Accumulibacter* Type I (a known phosphorus-accumulating organism, PAO) has received increased attention due to the potential operating benefits associated with their denitrifying activity in enhanced biological phosphorus removal (EBPR) wastewater treatment plants. In this study, after a shift from an enriched glycogen-accumulating organism (GAO) culture (competitors of PAO) to a PAO-enriched system, *Accumulibacter* Type I (PAO I) became dominant in an anaerobic–aerobic EBPR system fed with acetate and operated at 10 °C with a net aerobic solids retention time (SRT) of 6 d. Since *Accumulibacter* Type II (PAO II) were not detected, the low temperature in combination with the net aerobic SRT applied appeared to have suppressed their growth as well. The stoichiometry of PAO I was in agreement with previous metabolic models, suggesting that it was the main PAO organisms present in previous studies operated under similar conditions. Moreover, under poly-P limiting conditions, PAO I were unable to switch to a GAO-like metabolism at low temperatures. These results contribute to increase the understanding of the physiology, microbial metabolism and microbial ecology of PAO I.

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1. Introduction

The enhanced biological phosphorus removal (EBPR) process in activated sludge systems has been implemented as an economical and environmentally friendly technology in wastewater treatment plants worldwide. Denitrifying dephosphatation carried out by denitrifying phosphorus accumulating organisms (DPAO) is considered to be a highly desirable process for wastewater treatment since DPAO are capable of using nitrate instead of oxygen as electron acceptor to simultaneously achieve anoxic phosphorus uptake and denitrification (Kuba et al., 1993; Zeng et al., 2004; Kong et al., 2004). This process requires less carbon for P-uptake (Kuba et al., 1996), has lower aeration requirements (Kuba et al., 1996; Tian et al., 2011), and leads to lesser sludge production (Kuba et al., 1994; Murnleitner et al., 1997).

The existence of DPAOs had been first observed in anaerobic–anoxic–aerobic batch tests when alternating or switching the final electron acceptor (nitrate or oxygen) (Kern-Jespersen and Henze, 1993). Kuba et al. (1993) showed that DPAOs can utilise either nitrate or oxygen as electron acceptor for biological phosphorus removal in HAC-fed anaerobic–anoxic (A₂) or anaerobic–aerobic (A/O) systems. Until a few years ago, it was assumed that DPAOs and PAOs were seemingly the same microorganisms when comparing their stoichiometry and kinetics in A/O and A₂ systems (Ahn et al., 2002; Zeng et al., 2003a). However, those observations were still in conflict with the results of Kern-Jespersen and Henze (1993) and Meinhold et al. (1999), who suggested the existence of two types of PAOs (DPAOs and non-DPAOs) according to the analysis of chemical transformations.

A recent study concerning the lineage of these organisms revealed the existence of two main Types of *Accumulibacter* (I and II) each one comprising several clades (He et al., 2007; Peterson et al., 2008) with different denitrification capabilities (He et al., 2007; Flowers et al., 2009). *Accumulibacter* Type I (hereafter referred to as PAO I) are able to use nitrate and/or nitrite as final

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electron acceptor whereas *Accumulibacter* Type II (hereafter referred to as PAO II) are capable of using only nitrite for anoxic P removal (Flowers et al., 2009; Oehmen et al., 2010). The use of nitrate as electron acceptor seems to favour the growth of PAO I over PAO II, but in most of the cases they tend to co-exist in EBPR systems (Carvalho et al., 2007; He et al., 2010; Acevedo et al., 2012).

Though Kuba et al. (1993) and Zeng et al. (2003a) were able to cultivate a DPAO culture (presumably PAO I) on acetate using nitrate as electron acceptor, Carvalho et al. (2007) found that HPr instead of HAc was more suitable to sustain the anoxic P-removal process after switching from an A/O to an A₂ system, apparently due to the presence of PAO I (Oehmen et al., 2010). Lanham et al. (2011), using nitrate as electron acceptor in a HPr-fed reactor operated under anaerobic/anoxic/aerobic conditions, confirmed the observations of Carvalho et al. (2007) by achieving a relatively high enrichment of PAO I (approx 90%), with practically no PAO II present. In a recent study (Acevedo et al., 2012), PAO I was enriched in a HAc-fed A/O system but eventually switched over to PAO II when poly-P decreased drastically in short-term experiments. This resulted in low intracellular poly-P levels, reduced anaerobic P-release/HAc ratios and higher anaerobic glycogen requirements for HAc uptake associated to a GAO-like metabolism performed by PAO. However, it was unclear whether the GAO-like metabolism occurred due to a population shift from PAO I to PAO II, or because PAO II were able to adjust their metabolism under low intracellular Poly-P levels. The aforementioned information seemingly suggests that propionate, nitrate as final electron acceptor, and higher influent COD/P ratios provide competitive advantages to PAO I over PAO II. However, to the best of our knowledge, the effects of other factors influencing the occurrence of PAO I and PAO II have not been documented yet (such as pH, temperature, dissolved oxygen concentration and solids retention time (SRT), among others). The main objectives of this study were therefore: (1) to explore the population distribution of *Accumulibacter* sub-clades (namely, PAO I and PAO II) in a sequencing batch reactor (SBR) where the dominant microbial populations shifted from a highly enriched GAO to an enriched PAO culture after reducing the temperature from 20 to 10 °C under P-limiting conditions and adjusting the total SRT to 16 d, 26 d and then back to 16 d, that led to net aerobic SRT of 6 d, 13.5 d and 6 d, respectively (Lopez-Vazquez et al., 2009); and, (2) to evaluate the biomass metabolism of the SBR enriched with PAO with particular emphasis on the anaerobic and aerobic stoichiometry of the dominant *Accumulibacter* clade(s). The results of this study will contribute to get a better understanding about the environmental factors affecting the occurrence of *Accumulibacter* sub-clades, potential metabolic and microbial population shifts in EBPR systems and ultimately about the performance of EBPR processes.

2. Materials and methods

2.1. Operation of the SBR

A double-jacketed lab-scale SBR was inoculated with sludge from a highly enriched GAO reactor operated at 20 °C (parent SBR) (Lopez-Vazquez et al., 2009). After the sludge transfer from the parent SBR to the second SBR, the temperature in the second SBR was reduced to 10 °C. The second SBR had a working volume of 2.5 L, it was operated at 10 ± 0.5 °C and pH was maintained at 7.0 ± 0.1. In order to avoid temperature fluctuations, prior to filling up the reactor, the temperature of the synthetic influent was adjusted to 10 °C in another double-jacketed batch reactor equipped with a water bath. The SBR cycle consisted of 2.25 h anaerobic, 2.25 h aerobic and 1.5 h settling. The SBR was operated with a SRT of 16 d in phase I and phase III, and 36 d in phase II. Table 1

shows the operating parameters of interest during the three different experimental phases. The net aerobic SRT was determined as a fraction of the total time that the biomass spends under aerobic conditions in relation to the total applied SRT. Further details regarding the operation of the SBR can be found elsewhere (Lopez-Vazquez et al., 2009).

2.2. Synthetic media

The synthetic media fed to the SBR (both parent and the second SBR) contained in mg L⁻¹: 850 NaAc·3H₂O (12.5 C-mmol, approximately 400 mg COD L⁻¹) and 107 NH₄Cl (2 mmol). 2 allylthiourea were added to inhibit nitrification. In phases I and II of the operation of the second SBR, the phosphorus concentrations in the synthetic media were limited to 2.2 mg PO₄³⁻-P L⁻¹ (0.07 mM P) (Liu et al., 1997). However, in phase III the influent phosphorus concentration was increased to 15 mg PO₄³⁻-P L⁻¹ (0.48 mM P) in order to favour the growth of PAO (Smolders et al., 1994a,b). Other minerals and trace metals present in the media were prepared as described by Smolders et al. (1994b). Prior to use, the media were autoclaved for 1 h at 110 °C.

2.3. Chemical analyses

On a regular basis, the performance of the SBR was monitored through the determination of mixed liquor suspended solids (MLSS), mixed liquor volatile suspended solids (MLVSS) and orthophosphate. For MLSS and MLVSS determination, grab samples were collected by triplicate in the end of the aerobic stage, whereas for PO₄³⁻-P analyses samples were taken in the end of the anaerobic and end of the aerobic phases. When the SBR exhibited steady-state conditions (e.g. phase III), cycle measurements were executed in consecutive days and the average values were reported (Table 2). During the execution of the cycle measurements the parameters of interest were PO₄³⁻-P, acetate, poly-hydroxy-butyrate (PHB), poly-hydroxy-valerate (PHV), glycogen and ammonium. They were measured by duplicate in addition to MLSS and MLVSS that were measured by triplicate. All off-line analyses were performed in accordance with Standard Methods (APHA, 1995). The polyhydroxyalkanoates (PHA, concluding PHB and PHV) and glycogen contents were determined as described elsewhere (Lopez-Vazquez et al., 2007).

2.4. Microbial characterisation

Fluorescence *in situ* Hybridization (FISH) was performed as described in Amann (1995). In order to assess the evolution of microbial population from a highly enriched GAO culture to a PAO culture at 10 °C, the rRNA oligonucleotide probes used for FISH in this study were EUBMIX (including EUB 338, EUB338-II and EUB338-III) to target the entire bacterial population (Daims et al., 1999), and *Accumulibacter* Type I and Type II probes to target PAOI and PAOII, respectively (Flowers et al., 2009). Other probes such as PAOMIX and GAOMIX to target the PAO and GAO populations, respectively, are described elsewhere (Lopez-Vazquez et al., 2009). The quantification of the population distribution was carried out as described in Lopez-Vazquez et al. (2008b).

3. Results and discussion

3.1. Shift from a GAO- to a PAO I-enriched system

Fig. 1a illustrates the first cycle of phase I (after reducing the temperature from 20 to 10 °C at an SRT of 16 d). It showed the typical phenotype of an enriched GAO culture: full anaerobic

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