Understanding the role of *Tetrasphaera* in enhanced biological phosphorus removal

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Abstract
The enrichment of enhanced biological phosphorus removal (EBPR) sludge with *Tetrasphaera* was obtained for the first time using glutamate as sole carbon source. Two configurations were tested, an SBR with anaerobic/aerobic sequence and a continuous anaerobic/anoxic/aerobic (A^2/O) pilot plant. Better results and improved stability was obtained in the continuous A^2/O operation. *Accumulibacter* were also detected in both systems, as anaerobic conditions allow fermentation of glutamate producing volatile fatty acids, which are its preferred carbon source. This blocks the possibility to obtain a pure culture of *Tetrasphaera* using only glutamate.

Results with SBR cycle studies indicate that the recovery of PHA and glycogen during the anaerobic phase only accounts around 20% of the carbon source consumed, indicating that other storage routes should be studied to identify the fate of the carbon source for *Tetrasphaera*.

Keywords
Accumulibacter, EBPR, glutamate, PAO, *Tetrasphaera*

INTRODUCTION

Enhanced biological phosphorus removal (EBPR) is implemented in many wastewater treatment plants (WWTPs) to achieve high phosphorus removal efficiency. EBPR is based on the ability of polyphosphate-accumulating organisms (PAO) to take up phosphorus and accumulate it intracellularly as polyphosphate when they are exposed to alternating anaerobic and aerobic conditions. *Candidatus Accumulibacter phosphatis* (referred to as *Accumulibacter* hereafter) are the most frequently found species of PAO in WWTPs performing EBPR (Crocetti *et al.*, 2000). However, other putative PAO from the genus *Tetrasphaera* have also been abundantly found in real WWTP (Nguyen *et al.*, 2012). *Tetrasphaera* present important differences in comparison to *Accumulibacter: i)* they cannot take up short-chain fatty acids under anaerobic conditions, ii) they do not store polyhydroxyalkanoates (PHA) and iii) they can take up some amino acids and glucose (Nguyen *et al.*, 2011).

Although *Tetrasphera* PAO could play an important role in many full-scale EBPR WWTPs, there is a lack of research about them mainly because most lab studies are conducted with synthetic VFA-rich wastewaters. Hence, our work aims to gain knowledge on this new PAO genus and reveal its role in real WWTP. With this purpose, two reactor configurations were used: a sequencing batch reactor (SBR) and a continuous pilot plant system with an anaerobic/anoxic/oxic (A^2/O) configuration.

MATERIALS AND METHODS

An anaerobic/aerobic SBR with a working volume of 10 L and a volume exchange ratio of 50% was inoculated with sludge from an EBPR WWTP (Igualada, Barcelona) and fed with a mixture of aspartate and glutamate as carbon source for 88 days. The proportion of aspartate and glutamate varied along the operation but a concentration of 44-113 mg COD/L was maintained in the influent.

The phosphate concentration in the inlet was maintained at 10 mg P/L. Daily wastage was performed to maintain a sludge retention time (SRT) of 10-15 days.

The A^2/O pilot plant consisted of three continuous stirred tank reactors with a total volume of 146 L and a 50 L settler. This reactor operated at room temperature during 383 days treating a synthetic wastewater containing 10 mg $P\text{-}PO_4^{-3}$/L, 50 mg $N\text{-}NH_4^{+}$/L and 400 mg COD/L of glutamate as a sole carbon source. Apart from the periodic plant monitoring, fluorescence in situ hybridization (FISH) coupled with confocal laser scanning microscopy (CSLM) was used to follow the relative abundance of *Accumulibacter* PAO, GAO and *Tetrasphaera* PAO in both systems.

RESULTS AND DISCUSSION

Enrichment in SBR

The SBR start-up was fast after day 20 (Fig 1A), when proper anaerobic conditions during the anaerobic phase were ensured by nitrogen gas sparging. The system was successfully operated for one month with glutamate and aspartate as sole carbon sources. The P-uptake versus P-release ratio was 1.22 ± 0.10 and the P-release versus carbon uptake ratio was 0.282 ± 0.030 mol P/mol C. These ratios were lower than those obtained with volatile fatty acids (VFA) as propionate or acetate but correlate well with other less common carbon sources tested, as for example methanol (Tayà *et al*., 2012). P-removal was not complete, but around 3.4 ± 1.2 mg P/L were removed. Figure 1B shows a complete cycle monitoring at this point of successful operation (day 46). Glutamate and aspartate were completely consumed during the anaerobic phase, linked to P-release, but very low PHA increase was observed, only 0.69 mmol C/g VSS were accumulated during this phase. In fact, PHA concentration during all the cycle was much lower than in conventional EBPR studies with VFA as sole carbon source where the sludge was enriched in *Accumulibacter*. The average PHA concentration at the end of the anaerobic phase in this cycle was 1.49 ± 0.09 mmolC/gVSS, whereas in a conventional *Accumulibacter*-enriched SBR was 6.87 ± 0.07 mmol C/g VSS (Tayà *et al*. 2013). On the other hand, glycogen was slightly accumulated during the anaerobic phase (an increase of 0.41 mmol C/g VSS), instead of the glycogen consumption always detected in *Accumulibacter*-enriched sludge. However, glycogen concentration at the end of the anaerobic phase in this work was 3.9 ± 0.2 mmol C/g VSS, very similar to the concentration around 4 mmol C/g VSS for the *Accumulibacter*-enriched sludge (Tayà *et al*. 2013). Considering that the initial carbon source concentration used in the experiment of Figure 1B was 5.0 mmol C/g VSS and that only 0.69 mmol C/g VSS of PHA and 0.41 mmol C/g VSS of glycogen were accumulated during the anaerobic phase, these results would indicate that other non-quantified carbon storage process is probably used by *Tetrasphaera*.

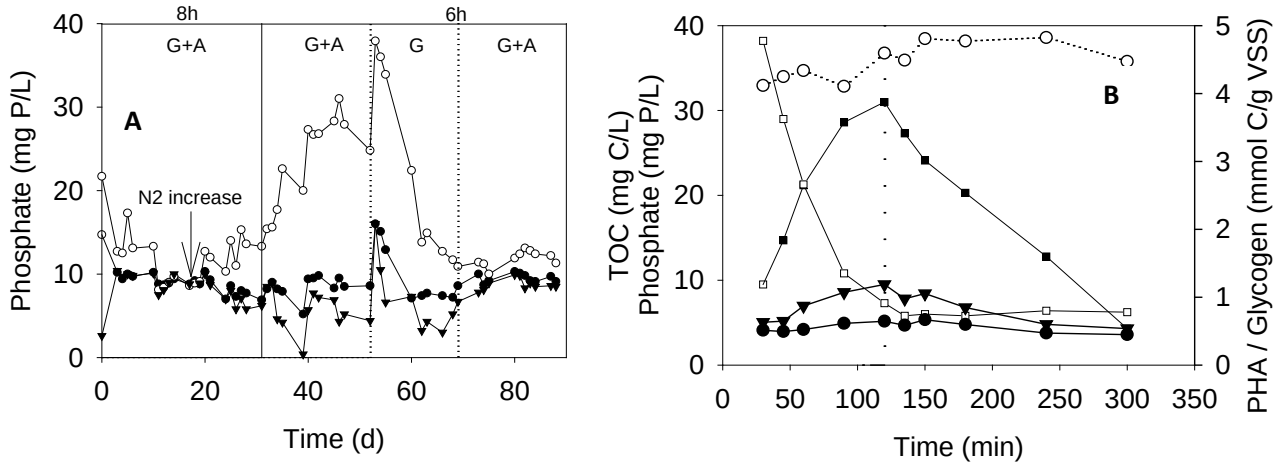

Figure 1. A) P concentration at initial time (●) and at the end of anaerobic (○) and aerobic phase (▼) during

all the SBR operation time. **B)** P concentration (■), TOC concentration (□), PHB concentration (●), PHV concentration (▼) and glycogen concentration (○) along a complete SBR cycle at day 46 of operation.

On day 52 of operation, when the carbon source was changed to only glutamate and COD concentration was increased from 77 to 113 mg/L, the system started to fail (Figure 1A). SBR became unstable and system recovery was not possible, even returning to the previous conditions, probably because of GAO growth.

Long-term A^2/O continuous operation

The experimental results in the A^2/O pilot plant showed high P-removal efficiency (>95%) until day 380 of operation (Figure 2A). Between days 106 and 140 EBPR activity was lost due to a batch of deteriorated glutamate source used for the synthetic wastewater preparation. Once the deteriorated feed was replaced by the original glutamate source, high P-removal efficiencies were reached again. Between days 217 and 256, EBPR activity failed again due to excessive nitrate input to the anaerobic reactor. The internal recycle was increased to a ratio of 4 with respect the influent flowrate and the external recycle was decreased to a ratio of 0.5 with respect to the influent, in order to improve denitrification and to reduce the input of nitrate to the anaerobic reactor. Complete net P-removal around 10 mgP/L and stable performance was systematically observed after these changes.

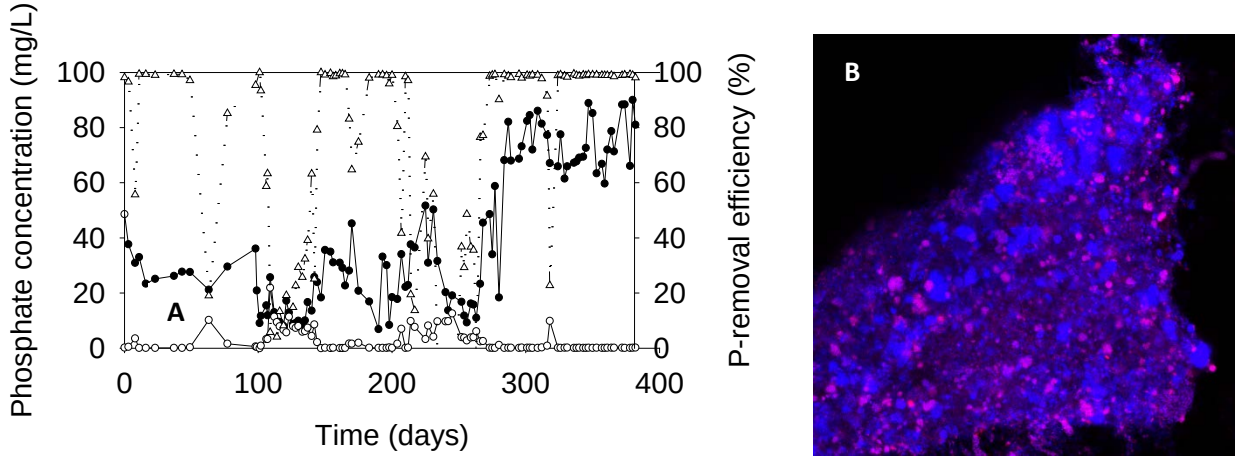

Figure 2. A) Continuous operation of the A^2/O plant. P concentration in the anaerobic chamber (●) and in the effluent (○) and P-removal efficiency (Δ). **B)** FISH image show bacteria hybridization with the bacterial probes EUBmix (blue) and probe Tet3-654 (pink).

A batch experiment to study the role of intracellular storage polymers in the *Tetrasphaera*-enriched biomass was designed in a 10 L vessel (Figure 3). This vessel was operated under anaerobic/aerobic conditions. Similar PHA and glycogen concentrations than in the SBR were achieved: PHA concentration at the end of the anaerobic phase in this experiment was very low (1.06 ± 0.04 mmol C/gVSS) in comparison with values reported by other authors for PAO enriched systems, 6.87 ± 0.07 mmol C/gVSS (Tayà *et al*., 2013), as already observed in the previous SBR study. The increase of PHA and glycogen during the anaerobic phase were very low when compared to the amount of carbon source consumed during this cycle, as only around 20% of the COD consumed during the anaerobic phase was quantified as increase of PHA and glycogen. Previous studies with pure cultures of *Tetrasphaera* have identified glycogen as the most probable intracellular storage

polymer. However, our results indicate that other storage routes should be studied to identify the fate of the carbon source stored under anaerobic conditions.

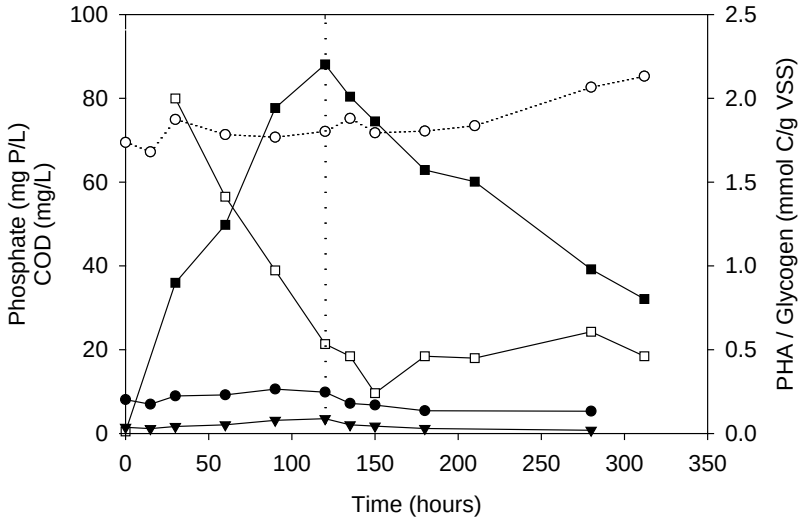

Figure 3. P concentration (■), COD concentration (□), PHB concentration (●), PHV concentration (▼) and glycogen concentration (○) along a batch experiment at day 308 of operation in the A^2/O pilot plant.

On the other hand, it should be noted obtained a highly-enriched *Tetrasphaera* sludge with minimum *Accumulibacter* presence is not a straightforward issue since the fermentation of aminoacids (in this case, glutamate) cannot be avoided and *Accumulibacter* can live off fermentation products, generally VFA. Ongoing research includes the study of the effect of different carbon sources and different electron acceptors in EBPR activity with this microbial community enriched in *Tetrasphaera*.

Bacterial community assessment

FISH-CSLM technique confirmed the enrichment in *Tetrasphaera* for both systems. In the SBR, hybridization of PAOMix, GAOMix and 4 probes of *Tetrasphaera* was observed. In the A^2/O plant, hybridization of PAOMix and *Tetrasphaera* was observed, while hybridization of GAOMix was very weak. Next generation MiSeq sequencing of bacterial 16S rDNA is being conducted and will be presented to show a broader assessment of the bacterial community.

Table 1. Results of FISH quantification for both systems

	PAO Mix	GAO Mix	*Tetrasphaera*
SBR	36.2 ± 1.2%	21.2 ± 0.8%	42.5 ± 9.3%
A^2/O plant	26 ± 4%	0.7 ± 0.3%	66 ± 5%

CONCLUSIONS

This work shows for the first time a successful enrichment of bio-P sludge with *Tetrasphaera* using glutamate as sole carbon source. Moreover, this enrichment was achieved not only using an SBR configuration but also, for the first time, in a continuous pilot plant (A^2/O) with better results and improved stability with respect to SBR operation. Nevertheless, *Accumulibacter* are able to live from fermentation products of glutamate, which avoids the possibility to obtain a pure culture of *Tetrasphaera* using glutamate.

Results with SBR cycle studies indicate that the increase of PHA and glycogen during the anaerobic phase only accounts around 20% of the carbon source consumed. Although previous studies with pure cultures of *Tetrasphaera* have identified glycogen as the most probable intracellular storage polymer, our results indicate that other storage routes should be studied to identify the fate of the carbon source stored under anaerobic conditions.

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