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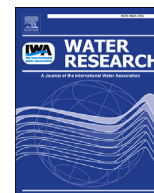

Long-term effects of sulphide on the enhanced biological removal of phosphorus: The symbiotic role of *Thiothrix caldifontis*

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ABSTRACT

Thiothrix caldifontis was the dominant microorganism (with an estimated bio-volume of 65 ± 3%) in a lab-scale enhanced biological phosphorus removal (EBPR) system containing 100 mg of sulphide per litre in the influent. After a gradual exposure to the presence of sulphide, the EBPR system initially dominated by *Candidatus* Accumulibacter phosphatis Clade I (98 ± 3% bio-volume) (a known polyphosphate accumulating organism, PAO) became enriched with *T. caldifontis*. Throughout the different operating conditions studied, practically 100% phosphate removal was always achieved. The gradual increase of the sulphide content in the medium (added to the anaerobic stage of the alternating anaerobic-aerobic sequencing batch reactor) and the adjustment of the aerobic hydraulic retention time played a major role in the enrichment of *T. caldifontis*. *T. caldifontis* exhibited a mixotrophic metabolism by storing carbon anaerobically as poly-β-hydroxy-alkanoates (PHA) and generating the required energy through the hydrolysis of polyphosphate. PHA was used in the aerobic period as carbon and energy source for growth, polyphosphate, and glycogen formation. Apparently, extra energy was obtained by the initial accumulation of sulphide as an intracellular sulphur, followed by its gradual oxidation to sulphate. The culture enriched with *T. caldifontis* was able to store approximately 100 mg P/g VSS. This research suggests that *T. caldifontis* could behave like PAO with a mixotrophic metabolism for phosphorus removal using an intracellular sulphur pool as energy source. These findings can be of major interest for the biological removal of phosphorus from wastewaters with low organic carbon concentrations containing reduced S-compounds like those (pre-)treated in anaerobic systems or from anaerobic sewers.

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1. Introduction

The enhanced biological phosphorus removal (EBPR) process is broadly applied in sewage treatment plants to meet the phosphorus discharge standards of treated wastewater. In this process, phosphorus is removed by polyphosphate accumulating organisms (PAOs) that store phosphorus beyond their growth requirements and are enriched by recirculating the activated sludge mixed liquor through anaerobic and aerobic/anoxic conditions (Barnard, 1975). Under anaerobic conditions, PAOs store volatile fatty acids (VFAs) present in the wastewater as poly-β-hydroxy-alkanoates (PHAs), using the energy generated from the hydrolysis of polyphosphate (Poly-P) and glycogen. In the subsequent aerobic/anoxic phase, PAOs oxidize the stored PHA to restore their Poly-P storage pools (resulting in the biological removal of phosphorus from the water phase) as well as to replenish the intracellular glycogen pools, for biomass synthesis and maintenance purposes (Comeau et al., 1986; Mino et al., 1998). Phosphorus is removed from the system by sludge wasting. A member of the betaproteobacterial family *Rhodocyclaceae* (identified as genus "*Candidatus* Accumulibacter") has been suggested to be one of the main PAOs involved in the EBPR process in wastewater treatment plants (WWTPs) (Hesselmann et al., 1999; Seviour et al., 2003).

In urban environments, wastewaters with sulphate

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concentrations of up to 500 mg SO_4^{2-}/L can be formed due to: (i) discharge of industrial effluents rich in sulphate (Sears et al., 2004), (ii) use of sulphate-based chemicals in drinking water treatment (e.g. aluminium sulphate) (Bratby, 2016), (iii) saline (sea and brackish) water intrusion into the sewage network, and (iv) the direct use of sea and brackish water as secondary quality water (e.g. cooling, toilet flushing) (Lee and Yu, 1997). In the absence of other electron acceptors, sulphate can be reduced to sulphide (Koster et al., 1986). García De Lomas et al. (2006) reported sulphide concentrations as high as 112 mg H_2S-S/L before the grift chamber of a 200,000 PE wastewater treatment plant located in southern Spain. At concentrations as low as 8 mg H_2S-S/L, sulphide has been observed to inhibit the anaerobic and (more severely) the aerobic metabolism of *Ca.* Accumulibacter after a sudden short-term exposure to this compound (Comeau et al., 1986; Rubio-Rincón et al., 2017). Yamamoto et al. (1991) and Baetens et al. (2001) assessed the long-term effects of the sulphate reduction process (resulting in HS^- formation) on the EBPR process, but the proliferation of filamentous bacteria led to the failure of their systems at sulphide concentrations lower than those studied by Rubio-Rincón et al. (2017).

Interestingly, some bacteria have the ability to use sulphide as energy source for the intracellular accumulation of phosphorus (Schulz and Schulz, 2005; Brock and Schulz-Vogt, 2011; Ginestet et al., 2015; Guo et al., 2016). Schulz and Schulz (2005) observed that under anaerobic conditions *Thiomargarita namibiensis* used their intracellularly stored nitrate and Poly-P to oxidize sulphide into sulphur and stored it as polysulphur (Poly-S; or elemental sulphur). While acetate triggered the anaerobic metabolism, PHA inclusions were not observed. Instead, acetate was stored as glycogen. When an electron acceptor was available, *T. namibiensis* generated the required energy from Poly-S and glycogen to replenish their Poly-P storage pools (Schuler, 2005). Also, Brock and Schulz-Vogt (2011) observed that a marine *Beggiatoa* strain was capable to store Poly-P above their growth requirements using intracellularly stored Poly-S as a source of energy. But, contrary to the metabolism of *Ca.* Accumulibacter, the anaerobic phosphorus released was not affected by the addition of VFAs. The phosphorus release observed under anaerobic conditions was only associated to maintenance requirements, which interestingly increased proportionally to the concentration of sulphide.

Ginestet et al. (2015) observed that certain organisms could use intracellularly stored sulphur to generate energy for phosphorus uptake in a full-scale WWTP. However, the microbial communities were not identified and the phosphorus uptake rates and net phosphorus removal were relatively low (2.9 mg PO_4-P/L). Guo et al. (2016) observed that the addition of sulphide improved the biological removal of phosphorus, where sulphur oxidizing bacteria (SOB) were involved in the main EBPR process. However, the SOB culture comprised only 2.6% of the total biomass with a net P-removal of 27 mg P/g VSS, that, arguably, is close to the phosphorus growth requirements of conventional systems (estimated around 20 to 30 mg P/g VSS) (Henze et al., 2008), and arguably cannot be considered an EBPR process. One of the most common sulphur oxidizing bacteria observed in EBPR belong to the genus *Thiothrix* (Wanner et al., 1987; García Martín et al., 2006; Gonzalez-Gil and Holliger, 2011). Certain species from this genus, such as *Thiothrix caldifontis*, have been isolated and studied in the past (Chernousova et al., 2009). In particular, *T. caldifontis* has shown the capability to grow aerobically not only using organic carbon, such as acetate, but also by fixing atmospheric carbon dioxide (exhibiting a mixotrophic growth). Under aerobic conditions, *T. caldifontis* was able to take up and store sulphide or thiosulphate as Poly-S for its later oxidation into sulphate (Chernousova et al., 2009). Interestingly, the genome of *T. caldifontis* (access number FNQP01000046) encode the ppk2 gene, previously reported in cultures of *Ca.* Accumulibacter as in important gene for the metabolism of Poly-P (García Martín et al., 2006). Nevertheless, to the best of our knowledge the capability of these organisms to store VFAs as PHA under anaerobic conditions and store phosphorus beyond their growth requirements has not been documented in literature.

Due to the increasing generation of saline sulphate-rich wastewaters, and the commitment to reduce phosphorus emissions, there is a need to assess the long-term effects of sulphide on the EBPR process. This will allow to address the potential selection or adaptation of sulphide-tolerant PAO species or side populations that can contribute to reduce the deleterious effects of sulphide, while maintaining satisfactory EBPR. For this purpose, long-term studies were conducted in a lab-scale anaerobic-aerobic EBPR sequencing batch reactor (SBR) system, initially enriched with *Ca.* Accumulibacter, by exposing the biomass to different sulphide concentrations added to the substrate in the anaerobic stage.

2. Material and methods

2.1. Reactor operation

Prior to the execution of this study, a 3.0 L double-jacketed reactor of a working volume of 2.5 L was enriched with "*Ca.* Accumulibacter" clade I. The system was operated for more than 200 days showing a stable EBPR performance (Rubio-Rincón et al., 2017). At the start of the present study, 50 mL of activated sludge taken from the WWTP Nieuwe Waterweg (Hoek van Holland, The Netherlands) were added to the SBR and two experimental phases were carried out. In the first phase, a cycle of 8 h was applied (composed of 2 h 15 min anaerobic, 4 h aerobic, 1 h 15 min settling, and 30 min effluent removal stages). Because phosphorus was not fully taken up at sulphide concentration of 20 mg S/L, the aerobic phase was extended to 5 h and the anaerobic phase was reduced to 1 h 15 min in order to keep the same (8 h) cycle length (experimental phase two). At the start of each cycle, nitrogen gas was sparged for 15 min (at a flowrate of 70 L/h) to create anaerobic conditions and 1.25 L of synthetic media was fed during the next 5 min. Thereafter, sulphide was fed at different concentrations that were gradually increased from 10 to 100 mg S/L. In order to control the solids retention time (SRT) at 20 d, 41 mL of mixed liquor were withdrawn at the end of aerobic phase. The pH in the reactor was kept at 7.6 ± 0.1 by the addition of 0.1 M HCl and 0.4 M NaOH. In the aerobic stage, compressed air was supplied at a flowrate of 20 L/h. Temperature in the reactor was controlled at 20 ± 1 °C by recirculating water between a thermostat bath and the double-jacketed reactor. Volatile fatty acids (VFAs), ortho-phosphate (PO_4-P), sulphide, volatile suspended solids (VSS), and total suspended solids (TSS) concentrations were measured twice per week. It was considered that the system had reached pseudo steady-state conditions when no significant changes (about 5% change) in the concentrations of these parameters were observed (approximately after 30 days).

2.2. Media

The synthetic media fed to the reactor contained per litre 637 mg $NaOAc \bullet 3H_2O$ (295 mg COD/L), 66.7 μL propionic acid (100 mg COD/L), 107 mg NH_4Cl, 135 mg $NaH_2PO_4 \cdot H_2O$ (30 mg PO_4-P/L), 90 mg $MgSO_4 \cdot 7H_2O$, 14 mg $CaCl_2 \cdot 2H_2O$, 36 mg KCl, 1 mg yeast extract, and 300 μL of trace element solution prepared according to Smolders et al. (1994). In order to inhibit the nitrification process 20 mg N-allylthiourea (ATU) per litre were fed into the system. Sulphide was fed from a bottle with a stock solution containing 3.2 g S/L dissolved in 0.1 M NaOH. When the sulphide solution was

added to the reactor, a 0.1 M HCl solution was fed simultaneously to keep the pH at the desired set point (7.6 ± 0.1). At pH 7.6 the speciation of sulphide was composed of approximately 20% dihydrogen sulphide (H_2S) and 80% hydrogen sulphide (HS^-).

2.3. Cycle tests

When the activity of the biomass in the parent reactor reached pseudo steady-state conditions, cycle tests were conducted in the parent reactor (3.0 L). Samples for the determination of VFA, orthophosphate, sulphide, and ammonia concentrations were collected with a higher frequency along the cycle, while samples for the determination of PHA, glycogen, VSS, TSS, magnesium, calcium and potassium were only collected at the shifting point between phases.

2.4. Batch activity tests

Once the gradual addition of sulphide reached concentration of 100 mg S/L and the biomass in the parent reactor exhibited a pseudo steady-state activity, additional anaerobic-aerobic (of 1 h and 3 h length, respectively) batch activity tests were conducted to assess the physiology of the enriched culture. Five batch activity tests were carried out with or without VFA (acetate), sulphide and/or biomass (Table 1). Ortho-phosphate, sulphide, sulphate, and ammonia were measured at different times along the batch test. VSS and TSS were measured at the start, and the end of the anaerobic phase, and at the end of the aerobic phase.

2.5. Analytical measurements

Ortho-phosphate (PO_4^{3-}-P), ammonium (NH_4^+-N), sulphide ($H_2S + HS^-$), VSS and TSS were measured as described in APHA et al. (2005). Sulphate was measured using an Ion Chromatography system equipped with a Dionex Ionpack AS4A-SC column (Dreieich, Germany). Potassium (K^+), magnesium (Mg^{2+}) and calcium (Ca^+) were measured in an Inductively Coupled Plasma, Mass Spectroscopy manufactured by Thermo Scientific in Bremen, Germany. Acetate (HAc) and propionate (HPr) were measured in a gas chromatography system G420-C (Nieuwegein, The Netherlands). All analyses were performed within 2 h after the cycle test finished and handled as described elsewhere (Rubio-Rincón et al., 2017).

2.6. Kinetic and stoichiometric parameters of interest

The net P released to VFA consumed (P-mol/C-mol), net PHA stored to VFA consumed (C-mol/C-mol) and the net glycogen utilized to VFA consumed ratio (C-mol/C-mol) were the anaerobic stoichiometric parameters of interest. Also, the potassium to phosphorus (mol-K^+/P-mol), magnesium to phosphorus (mol-Mg^{2+}/P-mol), and calcium to phosphorus (mol-Ca^+/P-mol) conversion ratios in the anaerobic and aerobic stages were calculated. The Poly-P content of the biomass and observed growth were estimated with a mass balance as described by Kuba et al. (1993) and expressed as mass of phosphorus over mass of VSS (g P/g VSS) and as mass of VSS over organic carbon consumed (g VSS/g COD_{VFA}), respectively. The kinetic rates were calculated by linear regression as described in Smolders et al. (1995). The kinetic rates of interest were:

i) q_{VFA}^{MAX}: Maximum specific VFA consumption rate, in mg COD/g VSS.h
ii) $q_{PO_4,AN}^{MAX}$: Maximum specific total phosphorus release rate, in mg PO_4-P/g VSS.h
iii) $q_{PO_4,Ox}$: Specific phosphorus uptake rate, in mg PO_4-P/g VSS.h
iv) $q_{NH_x,Ox}$: Specific ammonia uptake rate, in mg NH_4-N/g VSS.h
v) $q_{H_2S,Ox}$: Specific sulphide oxidation rate, in mg S/g VSS.h
vi) $q_{O_2,Ox}$: Oxygen consumption rate, in mg O_2/g VSS.h

2.7. Microscopic identification of intracellular polymers

To preserve the sludge samples, 6 drops of Formaldehyde were added to 10 mL of sludge samples immediately after collection as described in Marzluf et al. (2007). PHA inclusions in the biomass were detected using Nile Blue A and BODIPY 505/515 applied according to Seviour and Nielsen (2010) and Cooper et al. (2010), respectively. Poly-P was identified using DAPI staining as described in Seviour and Nielsen (2010). Images were collected with an Olympus BX5i microscope equipped with a SC100 and XM10 cameras (Hamburg, Germany).

2.8. Fluorescence in situ Hybridization (FISH)

In order to visualize and identify the most representative microbial communities, Fluorescence *in situ* Hybridization (FISH) analyses were performed according to Amman (1995). PAO were targeted with the PAOMIX probe (composed of probes PAO462, PAO651 and PAO846) (Crocetti et al., 2000). The presence of PAO clade I and clade II was determined through the addition of probes Acc-1-444 (1A) and Acc-2-444 (2A, 2C, 2D) (Flowers et al., 2009). *Candidatus* Competibacter phosphatis, a known glycogen accumulating organism (GAO), was targeted with the GB probe (Kong et al., 2002). *Defluvicoccus* cluster 1 and 2 were identified with the TFO-DF215, TFO-DF618, DF988, and DF1020 probes (Wong et al., 2004; Meyer et al., 2006). In order to target filamentous bacteria from the *Thiothrix* genus, the probe G123T was used as described by Kanagawa et al. (2000). Vectashield containing a DAPI concentration was used to preserve the fluorescent signal and stain all organisms present (Nielsen et al., 2009). The estimation of the relative abundance of the organisms of interest was performed as described by Flowers et al. (2009).

2.9. MAR-FISH (microautoradiography-fluorescence in situ Hybridization)

To address the potential of anaerobic carbon uptake by the biomass, samples were collected from the reactor at the end of the anaerobic and aerobic phase and cooled down at 4 °C. MAR incubations were performed within 24 h after sampling as described previously (Nielsen and Nielsen, 2005). To exhaust the PHA

Table 1
General information of batch activity tests conducted in this study to assess the physiology of the enriched EBPR culture.

Batch test No.	VFA (mg COD/L)	Sulphide (mg S/L)	VSS (mg)	Target activity or purpose
1	100	45	540	Simultaneous PAO and sulphur oxidation activities
2	100	None	595	PAO activity
3	None	45	877	Sulphide oxidation process
4	None	None	823	Blank
5	None	45	None	Control test and potential chemical sulphide oxidation

contents of the cells while maintaining their intracellular sulphur storage pools, biomass sample taken at the end of the anaerobic phase was pre-incubated aerobically at room temperature for 4 h in a 5 mM thiosulfate solution. Samples collected at the end of the aerobic phase (containing neither PHA nor S granules) were pre-incubated anaerobically at room temperature for 1 h for acclimatization purposes and to remove any potential residual concentration of an electron acceptor or donor. Thereafter, both samples were diluted to 1 g MLSS/L concentration in a synthetic media similar to that fed to the parent reactor, but excluding any carbon source. 2 mL of sample were introduced to 10 mL serum bottles and sealed with thick rubber stoppers. To achieve anaerobic conditions, oxygen was removed prior to substrate addition through the repeated evacuation of headspace and injection of oxygen-free nitrogen. Unlabeled and tritium-labeled acetate (PerkinElmer Inc., Waltham, MA, USA) were degassed and added to serum vials under a nitrogen gas flow to reach final concentrations of 6 mM and 30 μCi/mL, respectively. After 3 h incubation at room temperature, cells were immediately fixed with cold 4% [w/v] paraformaldehyde (final concentration), washed three times with sterile filtered tap water and re-suspended in 1 ml of 1:1 PBS/EtOH solution. Aliquots of 30 μL were applied onto coverslips and the FISH procedure using EUBmix targeting most bacteria (Amman, 1995), labeled with 5(6)-carboxyfluorescein-N-hydroxysuccinimide ester (FLUOS) and G123T targeting *Thiothrix* (Kanagawa et al., 2000) labeled with cyanine Cy3 was performed as described previously. Thereafter, microscope slides were coated with Ilford K5D emulsion (Polysciences Inc., Warrington, PA, USA), exposed in the dark for 10 days at 4 °C and developed with Kodak D-19 developer (Artcraft Camera and Digital, Kingston, NY, USA). Microscopic analyses were carried out with an Axioskop epifluorescence microscope (Carl Zeiss, Oberkochen, Germany).

2.10. Community composition analysis using denaturing gradient gel electrophoresis (DGGE) and 16S rRNA amplicon sequencing

In order to confirm the results gathered using the FISH technique, and to further identify the species of microorganism present in the biomass, 16S-rDNA-PCR DGGE analyses were performed. The complete procedure was carried out as described by Bassin et al. (2011). In addition, the community composition in the reactor fed with 100 mg S/L was analysed using 16S rRNA amplicon sequencing. DNA was extracted using the FastDNA® Spin kit for soil (MP Biomedicals, USA), V1-3 variable region of the 16S rRNA gene was amplified and samples were prepared and sequenced as described in Albertsen et al. (2015). The sequences were classified using the MiDAS taxonomy, v. 1.20 (McIlroy et al., 2015). All sequenced sample libraries were subsampled to 10,000 reads and the data analysis was performed in R v. 3.2.3 (R Core Team, 2015) using the ampvis package v. 1.24.0 (Albertsen et al., 2015).

2.11. Molecular analysis of PAO clades by PCR on the ppk1 functional gene

Genomic DNA was extracted using the Ultraclean Microbial DNA extraction kit supplied by MOBIO laboratories Inc. (CA, USA) following the suggested protocol except that the bead-beating was substituted by a combination of 5 min heat at 65 °C and 5 min beat-beating to ensure maximum yields. To check for quality and quantity, the genomic DNA was loaded onto a 1% agarose gel in 1x TAE running buffer. Analysis of the extracted DNA showed a large high molecular weight fraction and well visible DNA yields in comparison to the Smart ladder (Eurogentech Nederland b.v.).

A direct PCR to identify the PAO clade was performed based on the polyphosphate kinase (*ppk1*) functional gene as described by McMahon et al. (2007). The PCR amplicons were sequenced using ACCppk1-254F and ACCppk1-1376R primers. The phylogenetic tree was constructed using the neighbour joining method as described by Saad et al. (2016).

3. Results

3.1. Effect on the process performance of the biological removal of phosphorus by the increasing sulphide concentrations

Prior to the beginning of this study, the parent SBR was operated with a SRT of 8 days for more than 200 days (data not shown). In the anaerobic phase, all VFA were taken up by the biomass at a specific rate of 534 mg COD/g VSS.h, releasing ortho-phosphate at the rate of 377 mg PO_4-P/g VSS.h. Under aerobic conditions, ortho-phosphate was removed at specific rate of 57.9 mg PO_4-P/g VSS.h. According to FISH image analysis, *Ca.* Accumulibacter clade I was the dominant microorganism in the system (reaching a relative abundance of 99% with respect to DAPI) (Rubio-Rincón et al., 2017).

In the first experimental phase, the extension of the SRT to 20 d and the addition of 10 mg S/L slightly decreased the anaerobic and aerobic rates with regard to those observed at the 8 d SRT when no sulphide was added (Table 2). The inhibition of the VFA uptake rate continued when adding up to 20 mg S/L (from 370 to 194 mg COD/g VSS.h at 10 and 20 mg S/L, respectively). In a similar manner, the phosphorus release rate ($q^{MAX}_{PO_4,AN}$) decreased from 257 to 187 mg PO_4-P/g VSS.h. However, at 20 mg S/L not all phosphorus was aerobically taken up. Therefore, the aerobic phase was extended to 5 h (experimental phase 2), while the overall hydraulic retention time (HRT) and the sulphide concentration in the feed were kept at 18 h and at 20 mg S/L, respectively.

Once the EBPR system became stable, the sulphide concentration was further increased. Above 20 mg S/L the VFA uptake rate remained around 202 ± 15 mg COD/g VSS.h. On the contrary, the maximum phosphorus release ($q^{MAX}_{PO_4,AN}$) decreased from 174 to 124 mg PO_4-P/g VSS.h when the sulphide concentration increased from 20 to 100 mg S/L. The P/VFA ratio remained at 0.73 ± 0.02 P-mol/C-mol at sulphide concentrations of up to 50 mg S/L, but decreased to 0.64 P-mol/C-mol at 100 mg S/L. A lower Poly-P content and a higher GLY/VFA ratio were observed as sulphide reached 100 mg S/L (Table 2). However, the PHA/VFA decreased from 1.00 to 0.76 C-mol/C-mol as the sulphide concentrations reached 100 mg S/L. The anaerobic COD balance closed to 99% and to 81% at 10 mg S/L and 100 mg S/L, respectively. In the anaerobic stages, the sulphide and ammonia concentrations remained relatively stable (Supplementary information A).

Regarding the aerobic metabolism, 20 mg S/L inhibited the aerobic phosphorus uptake, not all phosphorus was removed and 5 mg PO_4-P/L remained in the effluent (Supplementary information A) (Table 2). As previously described, this led to the start of the second experimental phase where the anaerobic phase was shortened from 2 h 15 min to 1 h 15 min and the aerobic stage was extended from 4 h to 5 h. Extending the aerobic phase helped to gradually increase the net aerobic phosphorus uptake from 27.6 mg PO_4-P/g VSS.h at 20 mg S/L (when phosphorus was observed in the effluent) to 61.1 mg PO_4-P/g VSS.h at 100 mg S/L, contributing to achieve full P removal (Fig. 1B). At 100 mg S/L the sludge did not settle well resulting in the loss of biomass through the effluent (around 220 mg TSS/cycle), leading to a reduction of the SRT from 20 d to approximately 4.5 d, which remained so til the end of the present study.

The Mg/P and K/P ratios measured in the tests were in line with the theoretical composition of Poly-P ($Mg_{1/3}K_{1/3}PO_3$; Smolders et al., 1994), contributing to discard the potential chemical precipitation of phosphorus. In addition, no considerable changes in

Table 2
Anaerobic and aerobic kinetic rates and stoichiometric ratios observed in the cycle tests at different sulphide feed content (from 10 to 100 mg S/L).

Sulphide concentration (mg S/L)	q_{VFA}^{MAX}	$q_{PO_4,AN}^{MAX}$	P/VFA	PHV/VFA	PHB/VFA	PHA/VFA	Gly/PHA
	(mg COD/gVSS.h)	(mg PO_4-P/gVSS.h)	(P-mmol/C-mmol)	(C-mmol/C-mmol)	(C-mmol/C-mmol)	(C-mmol/C-mmol)	(C-mmol/C-mmol)
Anaerobic kinetic and stoichiometric rates							
10	370	257	0.75	0.31	0.69	1.00	0.12
20	269	187	0.75	N.M[c]	N.M[c]	N.M[c]	N.M[c]
20[a]	194	174	0.74	N.M[c]	N.M[c]	N.M[c]	N.M[c]
30[a]	213	198	0.72	N.M[c]	N.M[c]	N.M[c]	N.M[c]
50[a]	218	175	0.71	N.M[c]	N.M[c]	N.M[c]	N.M[c]
100[a]	187	124	0.64	0.21	0.54	0.76	0.20
Aerobic kinetic and stoichiometric rates							
	$q_{PO_4,Ox}$	$q_{NH_x,Ox}$	$q_{H_2S,Ox}$	$q_{O_2,Ox}$	**Mg/P**	**K/P**	**Poly-P**[b]
	(mg PO_4-P/gVSS.h)	(μg NH_4-N/gVSS.h)	(mg H_2S-S/gVSS.h)	(mg O_2/gVSS.h)	(Mg-mmol/P-mmol)	(K-mmol/P-mmol)	(mg P/mg VSS)
10	43.5	594	13.0	19.3	N.M[c]	N.M[c]	0.41
20	27.6	433	23.1	17.7	N.M[c]	N.M[c]	0.25
20[a]	36.1	179	34.4	15.1	N.M[c]	N.M[c]	0.36
30[a]	56.4	885	131.0	23.7	0.33	0.36	N.C
50[a]	51.0	678	140.0	36.2	0.30	0.28	N.C
100[a]	61.1	1291	333.0	34.9	0.33	0.35	0.19

[a] Experiments performed in experimental phase 2 (with a 5 h aerobic phase).
[b] Estimated as described in Kuba et al. (1993).
[c] N.M. Not measured; N.C. Not calculated.

the concentrations of calcium were detected (data not shown). Nevertheless, a higher consumption of ammonia was observed which increased from 594 up to 1291 μg NH_4-N/g VSS.h at 10 and 100 mg S/L, respectively. Simultaneously, the oxidation of sulphide reached up to 333 mg S/g VSS.h at 100 mg S/L, whereas at 10 mg S/L it was only 13.0 mg S/g VSS.h (Fig. 1). It was observed that the production of sulphate was slower than the oxidation of sulphide (39.4 mg SO_4-S/g VSS.h at 100 mg S/L). Moreover, only around 58% of the sulphide fed to the system was oxidized to sulphate, suggesting that either there were certain intermediate compounds formed or that certain sulphur compounds were stored intracellularly.

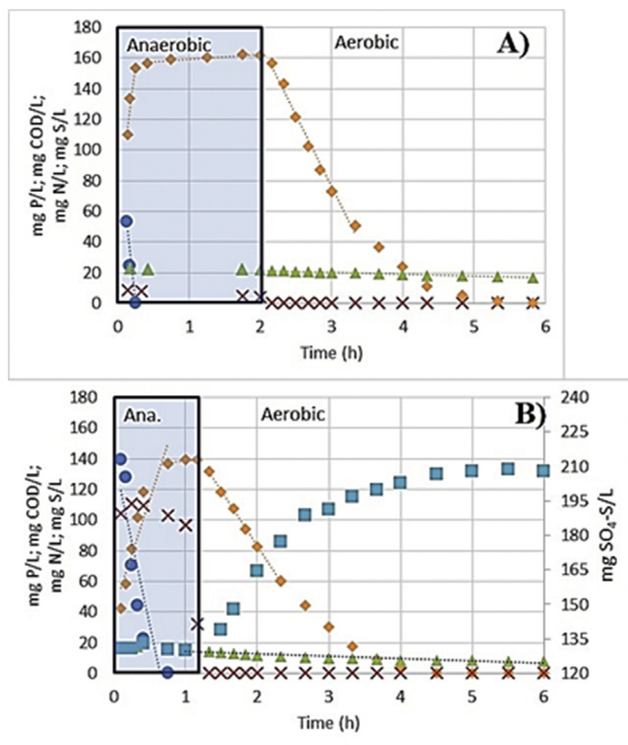


Fig. 1. VFA (circle), phosphorus (diamond), ammonia (triangle), sulphide (cross) and sulphate (square) profiles of the cycle tests performed at (A) 10 mg S/L with an aerobic phase of 4 h and (B) 100 mg S/L with a duration of the aerobic stage of 5 h.

3.2. *Putative role of sulphide in the biological removal of phosphorus*

In order to assess the potential effects of sulphide on EBPR, five batch tests were performed (Table 1) with biomass from the system feed with 100 mg S/L. Fig. 2 shows the phosphorus (Fig. 2A and B), ammonia (Fig. 2C), and sulphide and sulphate (Fig. 2D) profiles observed in the different batch tests. In the batch tests fed with VFA (Fig. 2A; diamond and square markers), the net phosphorus removal values were comparable between each other regardless whether sulphide was or was not added (96 and 92 mg PO_4-P/g VSS, respectively). On the other hand, in the tests where no VFA were added (Fig. 2B; triangle and circle markers), the phosphorus removed was almost twofold higher in the experiment carried out with sulphide than without sulphide (13 and 6.1 mg PO_4-P/g VSS, respectively). Interestingly, the aerobic ammonia consumption was also twofold higher in the experiments conducted with sulphide, regardless whether VFA were dosed or not. Moreover, in none of the batch test neither nitrate nor nitrite was detected during the aerobic stage and no ammonia consumption was observed in the anaerobic stage. Remarkably, the phosphorus removal was but the consumption of ammonia differed between the tests performed with VFA and with or without sulphide. This suggests that the higher phosphorus removal observed in the batch test fed with sulphide compared to the batch test performed without sulphide (both conducted without VFA) was not due to an increase in the observed growth (reflected in a higher ammonia consumption).

In addition, in the aerobic stages, a complete oxidation of sulphide was observed within the first 10 min in the control experiment (with biomass present); whereas in the blank test (performed without biomass), the oxidation of sulphide took 2 h. Moreover, the sulphate production was slower than the sulphide consumption. These observations indicate that sulphide was biologically oxidized

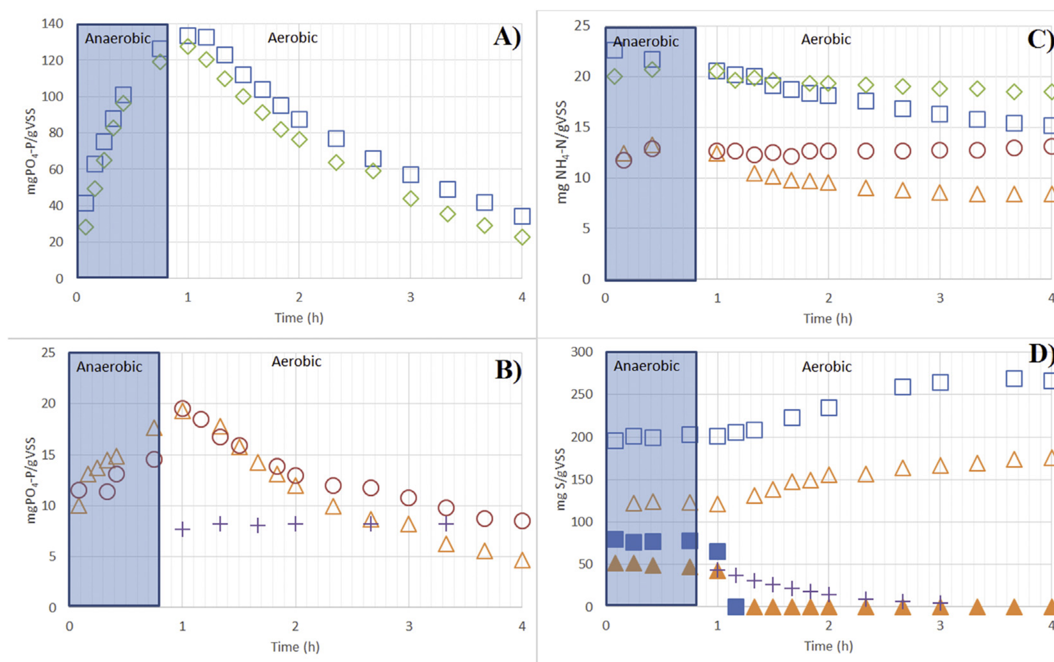


Fig. 2. Profiles of the compounds of interest observed in the batch tests performed with sludge from the parent EBPR reactor illustrating the concentrations of: phosphorus (A and B); ammonia (C); and sulphur compounds (D; sulphide concentrations with closed shape and sulphate with open shape). The batch tests were conducted with the addition of: sulphide and VFA (square); only VFA (diamond); and, only sulphide (triangle). The circle markers show the profiles of the control test performed without VFA and without sulphide, and the cross markers display the profiles of the blank test conducted without biomass.

and presumably stored intracellularly as elemental sulphur in the first minutes of the aerobic stage (likely as Poly-S) and afterwards oxidized and released as sulphate.

3.3. Selection and adaptation of the microbial community

In view of the stable EBPR activity at relatively high sulphide concentrations (100 mg S/L), different microbiological characterization and identification analyses were performed to assess the potential selection or adaptation of sulphide-tolerant PAOs. FISH image analyses indicated that *Ca.* Accumulibacter (PAO clade I) were the dominant microorganisms at 10 mg S/L (Fig. 3A) comprising around 76 ± 2% of the total bacterial population, while filamentous bacteria composed only 4 ± 0.5% of the biomass. In contrast, at 100 mg S/L, the fraction of filamentous organisms increased up to 65 ± 3%, while that of *Ca.* Accumulibacter decreased to 33 ± 2% (Fig. 3; Supplementary information B). Using 16S rRNA amplicon sequencing, a relative abundance of 81% of one OTU belonging to the *Thiothrix* genus was obtained (Fig. 4; Supplementary information C). DGGE analyses supported these observations, indicating an increase in the dominance of *T. caldifontis* (based on 99% similarity) in the reactor when it was fed with 100 mg S/L (Fig. 5, bands 11, and 21). Based on polyphosphate kinase (*ppk*) analysis, the main *Ca.* Accumulibacter clade switched from IC to IA, as the sulphide concentration increased (Fig. 6). This could indicate the selection of a sulphide tolerant *Ca.* Accumulibacter culture.

The increase in the relative abundance of filamentous bacteria, identified as mostly *T. caldifontis*, led to an increase in suspended solids in the effluent. Nevertheless, despite the deterioration of the settleability of the sludge, the system was still able to achieve full phosphorus removal (Fig. 1B). Further sludge characterization indicated the existence of different intracellular polymers in the *Thiothrix*: PHA inclusions at the end of the anaerobic stage (Fig. 7A and C), while at the end of the aerobic phase Poly-S and Poly-P were detected (Fig. 7B and D, respectively). The ability of *T. caldifontis* to take up carbon anaerobically (and possibly store it as PHA) regardless the presence of intracellular Poly-S granules was confirmed by MAR-FISH analyses (Fig. 8). Similar to the metabolism of PAO (Comeau et al., 1986; Smolders et al., 1994, 1995), further microscopic investigations showed that the Poly-P inclusions present in *Thiothrix* decreased at the end of the anaerobic phase and increased at the end of the aerobic phase (Supplementary information D).

4. Discussion

4.1. Selection and enrichment of Thiothrix caldifontis

The gradual increase in the sulphide concentration and to shorted the SRT from 20 d to 4.6 d, resulted in a switch from the *Ca.* Accumulibacter clade IC to IA. At this stage of the research it is not possible to identify if the switch in the *Ca.* Accumulibacter clade was due to a higher sulphide concentration or lower SRT. With the increase in the sulphide concentration in the feed, FISH microscopy showed that the relative abundance of *T. caldifontis* increased while the relative abundance of *Ca.* Accumulibacter decreased. Rubio-Rincón et al. (2017) observed that the anaerobic carbon uptake of *Ca.* Accumulibacter decreased from 221 mg COD/g VSS.h at 48 mg S/L to 112 mg COD/g VSS.h at 86 mg S/L, which is 41% lower than the anaerobic carbon uptake rate observed in this study at 100 mg S/L (187 mg COD/g VSS.h). Most likely, as suggested by Rubio-Rincón et al. (2017), the presence of sulphide inhibited the anaerobic activity of *Ca.* Accumulibacter, hence favouring the anaerobic activity of *T. caldifontis*. Furthermore, *T. caldifontis* was able to store VFA as PHA under anaerobic conditions (Figs. 7 and 8), a process observed in similar cultures (e.g. *Beggiatoa, Thiothrix*) but only under aerobic conditions (Martins et al., 2003; Schwedt et al., 2012).

Under aerobic conditions, *T. caldifontis* rapidly stored intracellularly the sulphide present as Poly-S. Later on, Poly-S was likely

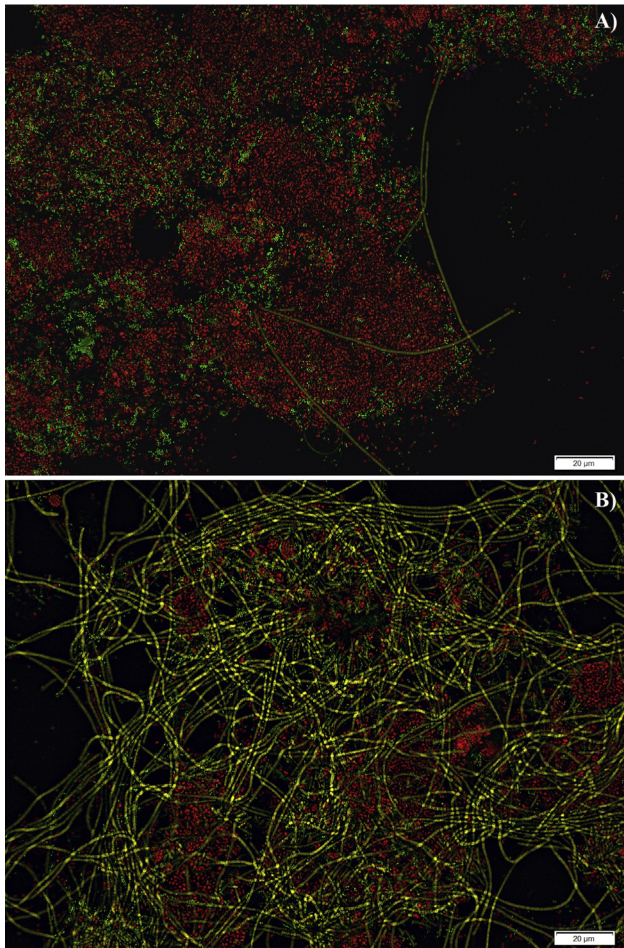


Fig. 3. FISH images performed with EBPR sludge from the parent bioreactor after long-term exposure to (A) 10 mg S/L and (B) 100 mg S/L displaying: all living organism in green (DAPI); GAO in blue (GB and DF215,618,988,1020 FISH probes); in red *Ca.* Accumulibacter (FISH probes PAO 462, 651, 846); and, in yellow *Thiothrix* (FISH probe G123T). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

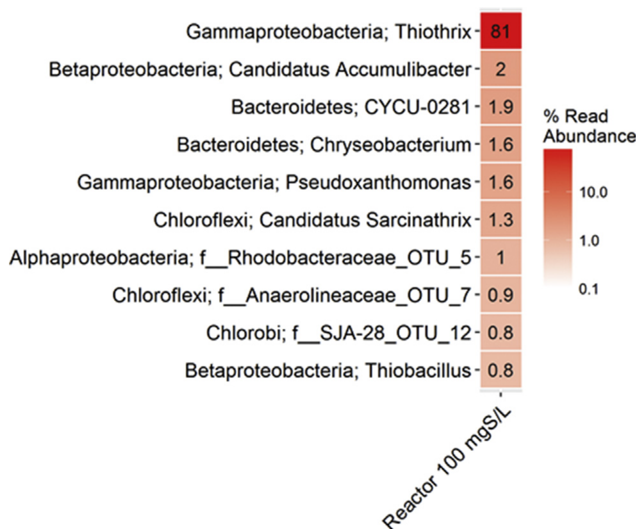
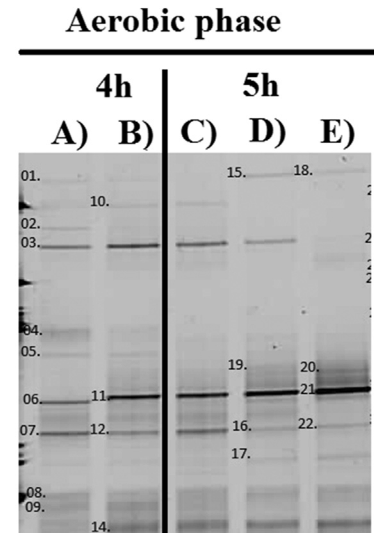


Fig. 4. 16S rRNA amplicon sequencing indicating the relative read abundance of 10 most abundant genera present in the EBPR sludge after long-term exposure to 100 mg S/L.



Band number	Related organism	Access number	similarity
3	Uncultured bacterium	DQ413120.1	99 %
6	Uncultured *Thiobacillus*	FJ439098.1	99 %
7,12,16	Uncultured *Thermomonas*	GQ891782.1	99 %
11 ,21	*Thiothrix caldifontis*	KF926094.1	99 %

Fig. 5. DGGE analyses performed with EBPR sludge from the parent bioreactor after long-term exposure with an aerobic phase of 4 h to: (A) 10 mg S/L and (B) 20 mg S/L; and, after long-term exposure with an aerobic phase of 5 h to: (C) 20 mg S/L; (D) 50 mg S/L; and, (E) 100 mg S/L.

used, together with the anaerobically stored PHA, as energy source to support the aerobic metabolic pathways for growth and formation of Poly-P and glycogen. Due to the simultaneous presence of two energy sources (Poly-S and PHA) the biomass yield of *T. caldifontis* on acetate could be higher. Indeed, a higher growth yield of 0.39 mg VSS/gCOD$_{VFA}$ was observed at 100 mg S/L than at 10 mg S/L (estimated at 0.18 VSS/mg COD$_{VFA}$). The increase in the observed growth yield at 100 mg S/L is in line with an increase in the ammonia consumption, which is associated to biomass growth since nitrification did not take place due to the addition of Allyl-N-thiourea (Fig. 1). The oxidation of the intracellular PHA and Poly-S compounds to generate energy by *T. caldifontis* suggests that *T. caldifontis* grew aerobically following a mixotrophic metabolism as proposed by Chernousova et al. (2009). The ability to grow mixotrophically is well distributed among sulphur oxidizing bacteria, and it is believed to provide an advantage over specialized autotrophic and heterotrophic organisms (such as *Ca.* Accumulibacter) under alternating growth conditions (Kuenen and Beudeker, 1982). Overall, these observations (a higher carbon uptake rate and their increase in biomass production) can explain the proliferation of *T. caldifontis* in the sulphide fed EBPR system, which was initially dominated by *Ca.* Accumulibacter.

4.2. *T. caldifontis contribution to the biological removal of nutrients*

In the aerobic stage, complete phosphorus removal was observed at 100 mg S/L when *T. caldifontis* was the dominant organism in the system. On the contrary, at 20 mg S/L up to 5 mg PO_4-P/L was observed in the effluent, when *Ca.* Accumulibacter was the dominant bacteria in the system. Rubio-Rincón et al. (2017) reported that sulphide inhibited more severely the aerobic metabolism than the anaerobic metabolism of *Ca.* Accumulibacter. Thus, in this study higher sulphide concentrations should have

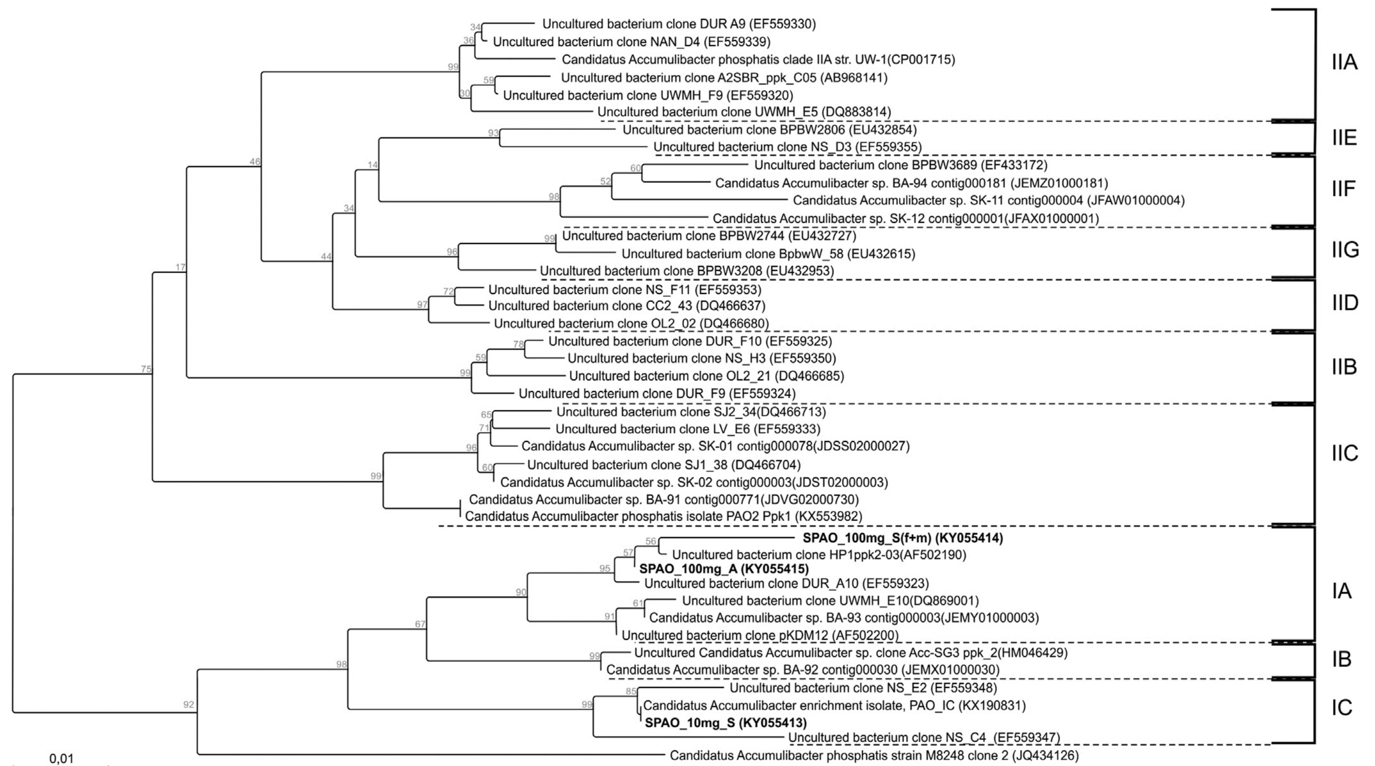


Fig. 6. Phylogenetic tree of genus *Ca.* Accumulibacter based on the *ppk* gene. Samples SPAO_10_mg and SPAO_100_mg, were taken when the system reached pseudo steady-state conditions when dosing 10 and 100 mg S/L, respectively.

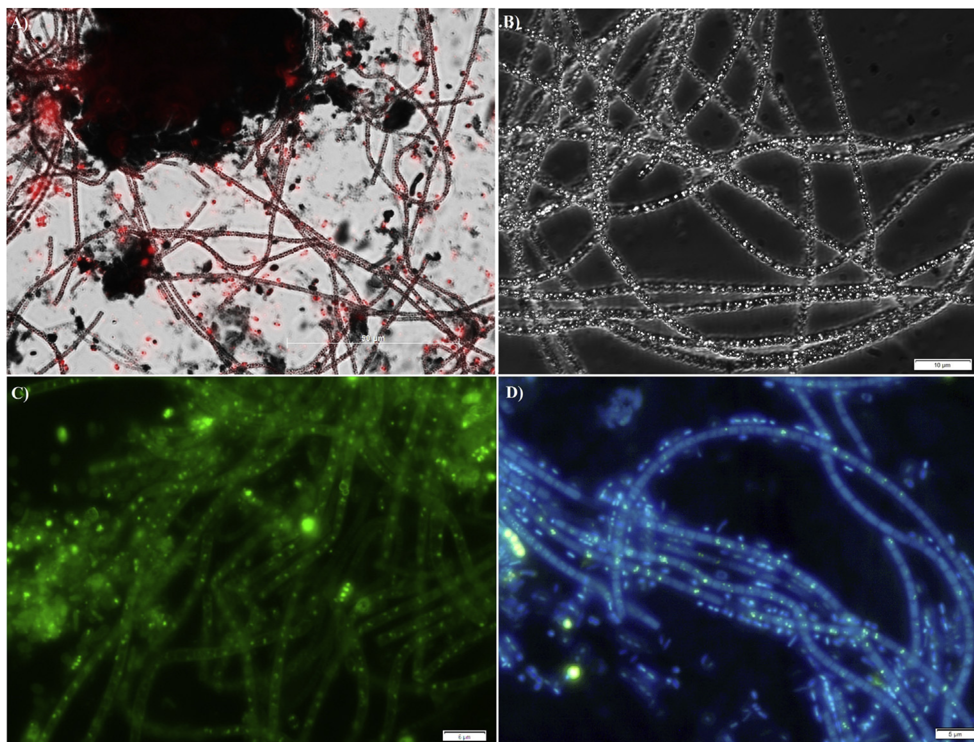


Fig. 7. *Thiothrix* images of the EBPR sludge after the long-term exposure to 100 mg S/L stained with: (A) Nile blue showing in red PHA; (B) Contrast image showing the elemental Sulphur inclusions as bright white spots; (C) BODIPY displaying in bright green PHA; and, (D) DAPI staining showing in yellow the intracellular Poly-P. Poly-P in Fig. 7D looks light green due to the superposition of blue and yellow. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

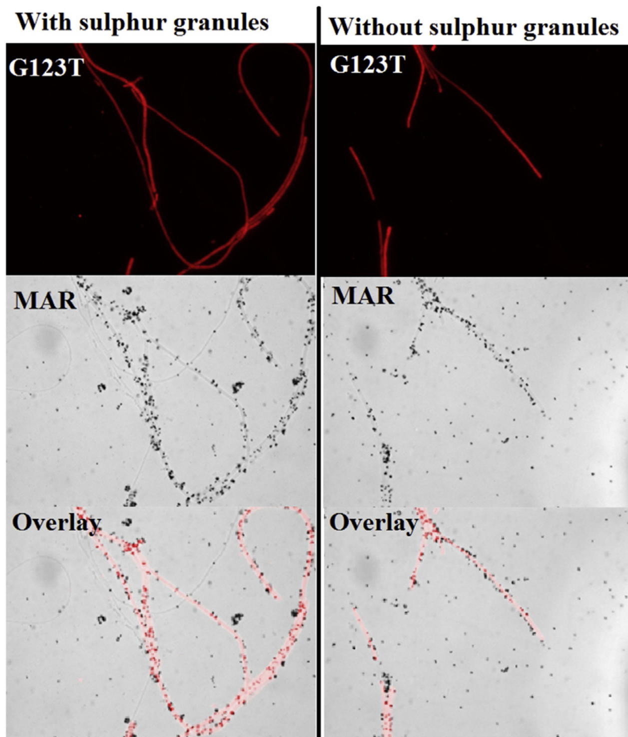


Fig. 8. MAR-FISH micrographs of the *Thiothrix*-enriched sludge fed with 100 mg S/L incubated with labeled acetate under anaerobic conditions. Left and right panels show a positive substrate uptake in *Thiothrix* filaments with and without sulphur granules, respectively. *Thiothrix* cells that hybridized with G123T probe appear red (top panel); middle panel are the corresponding bright-field MAR images; bottom panel shows overlay of MAR and FISH images. Positive MAR signal is represented by the high density of silver grains (observed as black granules) associated with *Thiothrix* filaments. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

deteriorated the aerobic phosphorus uptake, which was not the case. It seems that an increase in the abundance of *T. caldifontis* (from 4% to 65% at 10 and 100 mg S/L, respectively) and the rapid oxidization of sulphide observed in the first 10 min of the aerobic stage decreased the sulphide inhibiting effects on the aerobic metabolism of *Ca.* Accumulibacter, contributing to a stable EBPR. This is in line with the observed increase in the phosphorus uptake rate from 27.6 to 61.1 mg PO_4-P/gVSS.h at 20 and 100 mg S/L in the influent, respectively.

In addition to the previous observations, *T. caldifontis* might have also directly contributed to the EBPR process by storing phosphorus as Poly-P. According to Wentzel et al. (1989), the maximum storage capacity of *Ca.* Accumulibacter lies around 0.38 mg P/mg VSS. Hence, based on the bio-volume quantification (at 100 mg S/L) of *Ca*. Accumulibacter performed through FISH image analysis of 33%, at an SRT of 4.6 d, and the biomass concentration of the reactor of 1.081 g VSS/L, then around 64.5 mg VSS of *Ca.* Accumulibacter were removed in each cycle. Thus, considering the maximum Poly-P storage capacity of *Ca.* Accumulibacter and discarding any potential chemical precipitation (since calcium concentrations and Mg/P and K/P ratios were rather typical to those reported for EBPR systems), it can be estimated that *Ca.* Accumulibacter contributed up to a maximum net removal of 24.4 mg PO_4-P per cycle (65% phosphorus removal; Supplementary information E). Clearly, this is not sufficient to explain the full net removal of phosphorus observed in the system of 37.5 mg PO_4-P per cycle (15 mg PO_4-P/L removed), suggesting that another organism likely contributed to the biological removal of phosphorus. Thus, *T. caldifontis* (the most dominant organism in the EBPR system at 100 mg S/L) likely stored additional phosphorus beyond its biomass growth requirements, reaching up to 0.10 mg P/mg VSS. The DAPI image analysis for Poly-P detection supports these observations showing phosphorus inclusions inside the filamentous bacteria (displayed in yellow or white) (Fig. 7, Supplementary information D). This is in line with the presence of the ppk2 gene found in the genome of *T. caldifontis* (access number FNQP01000046) which can be used to synthesize PolyP (Zhang et al., 2002).

The ability of sulphur oxidizing organisms to perform the biological removal of phosphorus has been reported in literature. Brock and Schulz-Vogt (2011) observed that a *Beggiatoa* culture was capable to intracellularly store phosphorus as Poly-P, using the energy generated by the oxidation of sulphide. However, contrary to this study, Brock and Schulz-Vogt (2011) did not observe any PHA accumulation. Hence, *T. caldifontis* may rely on the oxidation of sulphide and PHA to generate energy for its different metabolic process, among them the replenishment of Poly-P storage pools, which might be used to store PHA in the subsequent anaerobic phase and afterwards grow mixotrophically (Kuenen and Beudeker, 1982). Likely, the extra energy provided by sulphide led to a higher growth yield and thereby to a higher ammonium consumption (7.4 and 1.5 mg NH_4-N/VSS, when sulphide was fed or not, respectively; Fig. 2C), implying that *T. caldifontis* could not solely remove phosphorus and sulphur, but also assimilate a higher amount of ammonia. Nevertheless, further research is needed to assess the maximum nutrient removal capacity of these organisms using a pure or highly enriched culture and assess whether it is practically relevant for wastewater treatment technologies and applications.

4.3. Possible anaerobic and aerobic conversions of *T. caldifontis*

In this study, an estimation of the anaerobic and aerobic conversions of *T. caldifontis* (Tables 3 and 4, respectively) was made based on: (i) the measurements presented in Table 2, (ii) assuming that the *Ca.* Accumulibacter population present in the system was capable to store up to 36 ± 1% of the COD fed to the reactor (as function of the P-released to COD consumption ratio) (Supplementary information E), (iii) considering that all COD was in the form of acetate, and (iv) following the stoichiometry for *Ca.* Accumulibacter clade IC reported by Welles et al. (2015). Therefore, *Thiothrix* might have stored up to 64 ± 1% of the COD fed in the anaerobic phase. However, only 47% of the COD fed was tracked down and identified as an intracellular carbon storage compound (PHB/VFA of 0.12 C-mol/C-mol and PHV/VFA of 0.29 C-mol/C-mol). The rest could not be identified. Interestingly, the ratio PHV/PHB was 2.4 C-mol/C-mol, which is close to the theoretical ratio calculated for the reductive branch of the TCA cycle for GAO (2.5 C-mol/C-mol PHV/PHB; Zeng et al., 2003). Possibly, the missing carbon dosed in the feed was stored as another PHA polymer, such as PH_2MV. But, still, even if it was stored as PH_2MV (assuming a PH_2MV/VFA ratio of 0.17 C-mol/C-mol as reported by Zeng et al., 2003), based on the COD balance only 69% of the COD load assumed to be stored by *T. caldifontis* could be explained. Arguably, this might suggest that part of the carbon fed was oxidized in the anaerobic phase to carbon dioxide using the intracellular Poly-S as energy source which should have been subsequently reduced to sulphide. However, because the sulphide profiles were rather stable in the anaerobic stages, this hypothesis is unlikely. On the other hand, it cannot be discarded that the unaccounted carbon was stored as another carbon polymer not detected with the analytical methods applied in this study, which could explain why the overall COD balance only closed to 81% when feeding 100 mgS/L. This can be supported by the studies of Schulz and Schulz (2005) who observed that, even though *T. namibiensis* was capable of storing

Table 3
Anaerobic conversions estimated for the *Thiothrix* culture in the cycle conducted with 100 mg S/L.

	Ratio	Unit	Measured	Estimated for Ca. Accumulibacter[a]	Estimated for Thiothrix[b]
Anaerobic conversions	PHV/PHB	C-mol/C-mol	0.39	0.07	2.42
	PHV/VFA	C-mol/C-mol	0.21	0.09	0.29
	PHB/VFA	C-mol/C-mol	0.54	1.27	0.12
	PH_2MV/VFA	C-mol/C-mol	N.M.	N.M.	0.17[c]
	GLY/VFA	C-mol/C-mol	0.20	0.29	0.14
	P/VFA	P-mol/C-mol	0.64	0.64	0.64
Anaerobic net conversion	PHV/L	C-mmol/L	1.34	0.20	1.14
	PHB/L	C-mmol/L	3.39	2.92	0.47
	PH_2MV/L	C-mmol/L	N.M	N.M.	0.68[c]
	P/L	P-mmol/L	4.00	1.47	2.53
	GLY/L	C-mmol/L	1.25	0.67	0.58
	VFA/L	C-mmol/L	6.25	2.3	3.95

N.M. Not measured.
[a] Values obtained based on Welles et al. (2015).
[b] Values estimated according to the bio-volume of *Thiothrix* and assuming that acetate was the main carbon source.
[c] Estimated based on the stoichiometric conversions of the reductive branch of the TCA cycle proposed by Zeng et al. (2003).

Table 4
Aerobic net conversions and energy balance estimated for the *Thiothrix* culture in the cycle test fed with 100 mg S/L.

	Ratio	Units	Measured		*Ca.* Accumulibacter	*T. caldifontis*		
			PHA[a]	PHA + Poly-S		PHA[a]	PHA[b]	PHA + Poly-S
Net conversions	PHV	C-mol/L	1.34	1.34	0.2	1.14	1.14	1.14
	PHB	C-mol/L	3.39	3.39	2.92	0.47	2.8	2.8
	PO_4-P	P-mol/L	4	4	1.47	2.5	2.5	2.5
	Glycogen	C-mol/L	1.25	1.25	0.67	0.58	0.58	0.58
	Biomass growth	C-mol/L	3	3	0.99	1.99	1.99	1.99
	Sulphide oxidation	S-mol/L	N.A	2.43	N.A.	N.A	N.A	2.43
Energy balance of the aerobic metabolism	**Source/Use**		**NADH balance**					
	PHA		−[0.74] [c]	−[0.74] [c]	2.18	−[1.69][c]	1.44	1.44
	Biomass growth		1.84	1.84	0.61	1.22	1.22	1.22
	Phosphate transport		−0.44	−0.44	−0.16	−0.27	−0.27	−0.27
	Glycogen		1.25	1.25	0.67	0.58	0.58	0.58
	Sulphide oxidation		N.A	7.29	N.A	N.A	N.A.	7.29
	Balance		**2.69**	**9.94**	**3.3**	**1.53**	**2.97**	**10.26**
	Source/Use		**ATP balance**					
	Oxidative phosphorylation		4.98	18.38	6.10	2.83	5.49	18.98
	PHA		N.A	N.A	0.48	N.A	0.32	0.32
	Biomass growth		−4.50	−4.50	−1.48	−2.98	−2.98	−2.98
	Phosphate transport		−4.00	−4.00	−1.47	−2.50	−2.50	−2.50
	Glycogen		−1.04	−1.04	−0.82	−0.48	−0.48	−0.48
	Balance		**-[4.56]**[d]	**8.84**	**2.81**	**-[3.13]** [d]	**-[0.15]** [d]	**13.34**

[a] Estimations based on the measured PHA concentrations.
[b] Estimations assuming a ratio of 1 C-mol PHA stored per 1 C-mol of VFA consumed.
[c] Negative values indicate insufficient carbon conditions for biomass growth and glycogen formation.
[d] Negative values indicate energy deficient conditions.

acetate under anaerobic conditions, no PHA formation observed. This may imply that, besides the study of a pure or at least highly enriched culture of these organisms, the adaptation and/or development and application of specific or more advanced analytical techniques is possibly needed to identify their intracellular compounds.

The transport and storage of acetate requires energy (ATP) and reducing equivalents (NADH) (Smolders et al., 1994). The anaerobic P-release/VFA-uptake ratio estimated for *Thiothrix* was 0.64 P-mol/C-mol. Thus, the ATP provided by the hydrolysis of Poly-P can be assumed to be higher than that required for the reduction and storage of acetate as PHA at pH 7.6 (Smolders et al., 1994). On the other hand, the reducing equivalents should be partially provided by glycogen because the glycogen consumption to PHA formation ratio was 0.14 C-mol/C-mol. Clearly, this is insufficient to provide all the reducing equivalents necessary for PHA formation (Smolders et al., 1994). The source of NADH for reduction of PHA to PHB/PHV is as yet unclear and deserves further attention to elucidate the metabolic pathways of *Thiothrix* under the conditions applied in this study.

Under aerobic conditions with 50 and 100 mg S/L in the feed when sulphide was oxidized in the first 10 min, it was not possible to observe any phosphorus uptake (Supplementary information A). This suggests that *T. caldifontis* stored sulphide as Poly-S, before the storage of phosphorus as Poly-P. Once Poly-S was stored, both PHA and Poly-S were possibly used to generate energy (Smolders et al., 1994; Brock and Schulz-Vogt, 2011) to cover the different metabolic activities of the bacteria. Table 4 shows the net aerobic conversions and energy balance under aerobic conditions calculated based on the bio-volume quantification (estimating a relative abundance of about 33% *Ca.* Accumulibacter and 65% *T. caldifontis*), considering the stoichiometry for *Ca.* Accumulibacter IC reported by Welles et al. (2015), and assuming that there is not accumulation over time of neither PHA nor glycogen (Supplementary information E3).

While *Ca.* Accumulibacter could cover their carbon and energy requirements from the estimated PHA storage pools, *T. caldifontis* would have severe limitations if PHA was their only carbon and energy source (Table 4, Note a). For instance, carbon would be insufficient for biomass synthesis and/or glycogen formation. Either the required carbon might be provided in the form of an intracellular carbon polymer, not measured in this study, or *T. caldifontis* could use an alternative inorganic carbon source like CO_2. Moreover, even if a ratio of 1 C-mol PHA stored per C-mol of VFA consumed is assumed as an attempt to explain the metabolism of *Thiothrix,* the ATP produced by the oxidation of PHA would be insufficient to cover their different metabolic activities (Table 4, Note b). Alternatively, the energy required could be provided by the oxidation of the intracellularly stored Poly-S into sulphate. The carbon, reducing power and energy balances estimated for the *Thiothrix* culture (Table 4, PHA + Poly-S) strongly suggest that this was the most likely mechanism that took place. This is fully supported by the potential mixotrophic growth that these organisms can perform (Chernousova et al., 2009).

The preference of *Thiothrix* to carry out certain metabolic processes using either PHA or Poly-S can be regulated by the presence or absence of other intracellular polymers (e.g. Poly-P). The batch tests fed with VFA with and without sulphide, exhibit a similar phosphorus profile, whereas only the one fed with sulphide had a higher ammonia consumption (2.0 and 5.4 mg NH_4-N/g VSS, respectively). This indicates, that as Poly-P needs to get restored (due to its anaerobic release), growth is the last metabolic process that takes place with the energy that remains from the oxidation of PHA and/or Poly-S oxidation. Interestingly, in the batch test fed without VFA and with sulphide, twice as much phosphorus was removed and a higher ammonia consumption was observed when compared to the one conducted without VFA and without sulphide (Fig. 2B). In this case, Poly-P was not hydrolysed as much as compared when VFA was added (anaerobic phosphorus release), thus as less phosphorus has to be replenish more energy can be used for growth.

In this study, *Thiothrix caldifontis* exhibited a mixotrophic growth and an enhanced removal of phosphorus using sulphide as a key compound instead of organic carbon. For wastewater treatment applications, this can contribute to increase the potential recovery of carbon for energy generation and therefore deserves to be the focus of further research efforts that may lead to the development of innovative treatment technologies for nutrient removal and recovery.

5. Conclusions

Thiothrix caldifontis, a mixotrophic sulphide oxidizing organism, contributed to the stability and performance of an EBPR system (fed with sulphide concentrations of up to 100 mg S/L) achieving full P-removal. Also, *T. caldifontis* directly contributed to the removal of phosphorus via aerobic P-uptake, accumulating up to 100 mg P/g VSS. *T. caldifontis* was able to perform (i) the anaerobic storage of VFA as PHA, (ii) aerobic storage of sulphide as Poly-S, (iii) mixotrophic growth using Poly-S and PHA, (iv) and aerobic phosphorus uptake. This research suggests that *T. caldifontis* has the potential to be used for the biological removal (and recovery) of phosphate and sulphur.

Acknowledgements

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Appendix A. Supplementary data

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