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Stable granulation of seawater-adapted aerobic granular sludge with filamentous *Thiothrix* bacteria

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ABSTRACT

Many sources of wastewater contain sulfides, which can cause excessive growth of filamentous bacteria such as *Thiothrix* sp. resulting in bulking sludge in conventional activated sludge systems. Granular sludge systems could potentially also suffer from the growth of filamentous bacteria. Uptake of easily degradable COD by the relatively slow growing *Ca.* Accumulibacter phosphatis bacteria and the absence of strong diffusion gradients due to plug flow feeding through the settled granular sludge bed are assumed to be the dominant factors for successful granulation. Sulfides will remain after this anaerobic phase and cause growth of sulfide-consuming bacteria such as *Thiothrix* sp. Here we observed the impact of growth of *Thiothrix* sp bacteria in a laboratory aerobic granular sludge reactor by feeding a mixture of acetate and thiosulfate in the influent. *Thiothrix* sp, proliferated when 18% of the influent COD was due to thiosulfate, forming 51.4 ± 8.3% of the total granular biomass. Despite the strong presence of these filamentous bacteria a well settling sludge was maintained (SVI_{10} equal to 13.3 mL/g). These results confirm that sludge morphology is not necessarily a reflection of the cell morphology of the bacteria, but is highly influence by reactor operation. It also reiterates the fact that compact biofilms are formed when the substrate consumption rate is lower than the substrate transport rate.

1. Introduction

Aerobic granular sludge (AGS) is an upcoming technology for simultaneous removal of organic carbon (COD), nitrogen, and phosphorus in a single process step (De Kreuk et al., 2005; Pronk et al., 2015a). Stability of the process depends on anaerobic uptake and intracellular storage of easily biodegradable COD. The stored substrate is used for energy generation and growth during the aeration phase (Dawes and Senior, 1973; Carta et al., 2001; Beun et al., 2002). This results in a low growth rate, which is favorable for obtaining a smooth biofilm (van Loosdrecht et al., 1995; Liu and Tay, 2002). In general, polyphosphate accumulating organisms (PAO) thrive under the applied reactor conditions (Bassin et al., 2011; Winkler et al., 2013). An essential aspect of the aerobic granular sludge process is the excellent settling property, which might deteriorate when filamentous bacteria proliferate. Typically, very low (30–60 mL/g) sludge volume indexes (SVI) are obtained in laboratory and full-scale installations (De Kreuk et al., 2005; Pronk et al., 2015a).

Many sources of wastewater contain sulfides. This can originate from iron sulfate usage in drinking water production, from industrial discharges, or due to seawater intrusion into sewer systems (Sears et al., 2004; Lefebvre and Moletta, 2006; Lu et al., 2012; Pikaar et al., 2014). Commonly, sulfide presence is associated in wastewater treatment with proliferation of filamentous organisms, especially *Thiothrix* species, leading to bulking sludge (Williams and Unz, 1985). Many studies report on prevention and handling of filamentous bulking (Chiesa and Irvine, 1985; Jenkins et al., 2003; Martins et al., 2004). Multiple engineering solutions have been developed for minimizing filamentous microorganisms in activated sludge systems, such as addition of a selector or intermittent feeding (Chudoba et al., 1973; Verachtert et al., 1980; Houtmeyers et al., 1980). There is limited information on the impact of proliferation of filamentous bacteria on granular sludge morphology.

Occurrence of filamentous microorganisms in enhanced biological phosphorus removal (EBPR) systems leading to problems have been reported in literature (Wanner et al., 1987; Gonzalez-Gil and Holliger, 2011). A study by Rubio-Rincón et al. (2017b) reported the presence of *Thiothrix caldifontis* next to *Ca.* Accumulibacter

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phosphatis in an EBPR culture, performing complete removal of both phosphate and acetate. *T. caldifontis* is a typical filamentous organism with a cell length up to 6.5 μm, growing in multicellular filaments that are protected by polysaccharide sheaths. This strain can anaerobically consume acetate and store it as polyhydroxyalkaonates (PHA) (Wanner et al., 1987; Williams et al., 1987; Brigmon et al., 1994, Rubio-Rincon et al., 2017a). *T. caldifontis* also encodes the *ppk2* gene, which is a widely conserved gene that is responsible for the synthesis of poly-P from GTP or ATP (Garcia Martin et al., 2006). Simultaneously, this strain can oxidize both sulfide and thiosulfate. This oxidation of reduced sulfur compounds serves as another energy source next to PHA oxidation, which leads to an increased growth yield on acetate. In this way, *T. caldifontis* can effectively compete with *Ca.* Accumulibacter sp. and other PAOs in the presence of sulfides.

The metabolic similarity between *T. caldifontis* and *Ca.* Accumulibacter phosphatis led to the question whether stable granulation can be achieved when this filamentous microorganism proliferates in sulfide-containing wastewater. Formation of compact and non-filamentous granules is based on diffusion-based selection, which prescribes that compact biofilms are formed when the substrate consumption rate is lower than the substrate transport rate (van Loosdrecht et al., 1995; Picioreanu et al., 1998). Based on this theory, our hypothesis is that granule morphology is determined by reactor operation, rather than by bacterial morphology (van Loosdrecht et al., 1995; Martins et al., 2003; De Kreuk and Van Loosdrecht, 2004).

To verify this hypothesis, a lab-scale reactor has been operated with up to 25% COD in the form of thiosulfate, and the remaining COD from acetate. The choice for thiosulfate over sulfide has been made to purely study the increase in filamentous *Thiothrix*, and to prevent a potential toxicity of sulfide to the microbial community. Granule morphology, changes in microbial community, and phosphate removal rates have been monitored over time to identify the presence of filamentous bacteria and successful stable granulation.

2. Materials & methods

2.1. Reactor operation

Aerobic granular sludge was cultivated in a 3.0 L bubble column (5.6 cm diameter), operated as a sequencing batch reactor (SBR), similar to the setup as described in de Graaff et al. (2020). The reactor was seeded with biomass from a lab-scale seawater-adapted AGS reactor. The temperature was controlled at 20 °C. The pH was controlled at 7.0 ± 0.1 by dosing either 1M NaOH or 1M HCl. During the first month of startup, the pH was not controlled. The dissolved oxygen (DO) concentration was controlled at 3.7 mg/L O_2 (50% saturation). The average sludge retention time (SRT) was 20 days.

The influent was 1.5 L per cycle, consisting of 1200 mL artificial seawater (Instant Ocean®, final concentration 35 g/L), 150 mL of medium A, and 150 mL of medium B. Medium A contained 57.2 mM sodium acetate trihydrate. Medium B contained 42.8 mM NH_4Cl, 4.2 mM K_2HPO_4, 2.1 mM KH_2PO_4, and 10 mL/L trace elements solution (4.99 g/L $FeSO_4 \cdot 7H_2O$, 2.2 g/L $ZnSO_4 \cdot 7H_2O$, 7.33 g/L $CaCl_2 \cdot 2H_2O$, 4.32 g/L $MnSO_4 \cdot H_2O$, 2.18 g/L $Na_2MoO_4 \cdot 2H_2O$, 1.57 g/L $CuSO_4 \cdot 5H_2O$, 1.61 g/L $CoCl_2 \cdot 6H_2O$, 50 g/L EDTA). The combination of these feed streams led to influent concentrations of 366 mg/L COD, 60 mg/L NH_4^+-N and 9.3 mg/L PO_4^{3-}P. The reactor was seeded with only 200 mL of granular biomass. To make sure all the COD was taken up during the anaerobic feeding time the COD influent concentration for the first two weeks was limited to 14.3 mM sodium acetate.

Thiosulfate was added to the influent by addition to the artificial seawater vessel. Its concentration was gradually increased up to 80 mg S-COD/L influent concentration (equal to 18% COD from thiosulfate) over a period of 58 days. Afterwards the thiosulfate concentration was increased to 120 mg S-COD/L (equal to 25% COD from thiosulfate) in a single step, and operated for a period of 53 days. Reactor cycles with 80 mg S-COD/L (18% S-COD) consisted of 60 min of anaerobic feeding, 195 min aeration, 10 min settling and 5 min effluent withdrawal. Reactor cycles under 120 mg S-COD/L (25% S-COD) consisted of 90 min of anaerobic feeding, 200 min aeration, 5 min settling and 5 min effluent withdrawal.

2.2. Analytical methods

Concentrations of phosphate were measured on a Thermo Fisher Gallery Discrete Analyzer (Thermo Fisher Scientific, Waltham, USA). Acetate was measured by HPLC with an Aminex HPX-87H column from Biorad, coupled to an RI and UV detector, using 0.01M phosphoric acid as eluent.

The sludge volume index after 10 min (SVI_{10}) was determined by taking a mixed liquor sample from the reactor during aeration, and allowing this sample to settle in a measuring cylinder for 10 min. This sludge was subsequently used for determination of the biomass concentration. The settled granules in the measuring cylinder were washed with demineralized water to remove soluble salts, dried for 24 h at 105 °C, and used for TSS calculation. The dried granules were subsequently incinerated at 550 °C for determining the ash content and for VSS calculation.

2.3. Granule morphology

Pictures of whole granules were taken with a stereo zoom microscope (Leica Microsystems Ltd, M205 FA, Germany), and processed with Leica Microsystems Qwin (V3.5.1) image analysis software.

2.4. Fluorescent in-situ hybridization (FISH)

The handling, fixation and staining of FISH samples was performed as described in Bassin et al. (2011). A mixture of PAO462, PAO651, and PAO846 probes (PAOmix) were used for visualizing polyphosphate accumulating organisms (PAO) (Crocetti et al., 2000). A mixture of GAOQ431 and GAOQ989 probes (GAOmix) were used for visualizing glycogen accumulating organisms (GAO) (Crocetti et al., 2002). GAM42A probes were used for staining gammaproteobacteria (Manz et al., 1992). G123T probes were used for staining *Thiothrix* bacteria (Kanagawa et al., 2000). A mixture of EUB338, EUB338-II and EUB338-III probes were used for staining all bacteria (Amann et al., 1990; Daims et al., 1999). Images were taken with a Zeiss Axioplan 2 epifluorescence microscope equipped with filter set 26 (bp 575e625/FT645/bp 660e710), 20 (bp 546/12/FT560/bp 575e640), 17 (bp 485/20/FT 510/bp 5515e565) for Cy5, Cy3 and fluos respectively. Quantification of FISH images was done by counting the relative amounts of pixels per fluorescence signal of cy3, fluos, and cy5 signals, using Leica Microsystems Qwin (V3.5.1) image analysis software.

3. Results

3.1. Reactor operation

An aerobic granular sludge sequencing batch reactor was operated with addition of thiosulfate in the influent up to 80 mg S-COD/L in combination with addition of 366 mg HAc-COD/L, equaling 18% of COD derived from thiosulfate. A representative measurement of a typical cycle in the SBR reactor is shown in Fig. 1;

the development of biomass concentration and phosphorus concentrations during reactor operation are shown in Fig. 2. Acetate was completely removed by the biomass during the 60 min anaerobic feeding phase, resulting in a net release of phosphate up to 65.4 mg/L. Aerobic uptake of phosphate was linear over time, with a rate of 3.2 mgP/gVSS/h. Biomass concentration was stable with a concentration of 7.9 ± 0.7 g VSS/L and a sludge bed density of 77.0 ± 3.2 g TSS/L sludge bed. An identical, but thiosulfate-free, reactor operation is described in de Graaff et al. (2020); this reactor operation was used as the reference reactor.

After increasing the influent concentration to 120 mg S-COD/L (25% COD from thiosulfate, from day 58 onwards), anaerobic acetate uptake and anaerobic phosphate release began to decrease over a period of 36 days. Due to an accidental washout of around 50% of biomass from the reactor at day 104, acetate started leaking into the aeration phase and phosphate removal collapsed. The sludge bed density decreased to 13.4 g TSS/L, and the effluent started containing filaments with a solids concentration of 0.11 g/L (which was near zero before); at this stage reactor operation was stopped.

3.2. Granule morphology

Stable granules were obtained when the reactor was fed with 18% COD from thiosulfate. These thiosulfate-based granules had a similarly dense structure as the thiosulfate-free grown granules used as inoculation source (Fig. 3a and b). Minor amounts of filamentous outgrowth from the granule core were visible, still resulting in proper settling behavior with a sludge volume index after 10 min (SVI_{10}) of 13.3 mL/g. After a sudden drop in SRT after 104 days of operation, the granule morphology changed markedly into a more fibrillous structure (Fig. 3c and d).

3.3. Microbial community

Robust granules were formed with thiosulfate in the feed, despite the presence of filamentous *Thiothrix* bacteria. Both the inoculum and the granules that were grown with 18% S-COD were analyzed using fluorescence in situ hybridization (FISH), using probes that were specific for PAO, GAO, *Thiothrix* sp., and eubacteria.

The inoculum contained a large fraction of PAO, and GAO were not detected (Fig. 4a). No filamentous organisms were detected (Fig. 4b). Granules from the thiosulfate-added reactor showed a high abundance of *Thiothrix* bacteria (Fig. 4c). Based on quantification of FISH images, the fraction of *Thiothrix* was estimated as 51.4 ± 8.3% of the total amount of biomass. The filamentous morphology of *Thiothrix* was clearly visible and these bacteria coexisted with PAO in the granule microbial community (Fig. 4d and e). Besides these two groups, also a smaller amount of GAO was present (Fig. 4f).

FISH analysis on a slice of whole non-crushed granules showed that gamma proteobacteria (to which *Thiothrix* belong) are mainly present on the outer layer of the granules (Fig. 5).

Inspection of the filamentous bacteria with bright field microscopy showed the presence of a large number of intracellular globules. These were morphologically similar to storage polymers which are commonly found in *Thiothrix* bacteria (Fig. 6) (Dahl and Prange, 2006; Rubio-Rincon et al., 2017a).

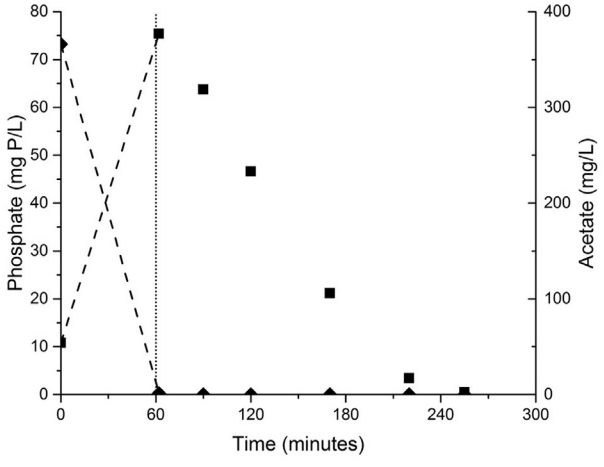

Fig. 1. Concentrations of phosphate (squares ■) and acetate (diamonds ◆), during a reactor cycle with the addition of 80 mg S-COD/L in the influent (18% of total COD). The dotted vertical line indicates the switch between anaerobic feeding and aerobic mixing.

4. Discussion

4.1. Successful granulation with the presence of filamentous bacteria

Stable aerobic granules were obtained despite a dominant presence of filamentous *Thiothrix* bacteria. Roughly half of the microbial biomass in the reactors was formed by *Thiothrix* (determined by FISH analysis; Fig. 4). Nevertheless, this still resulted in the formation of dense and well-settling granules (Fig. 3). The filamentous *Thiothrix* bacteria mainly grew inside the granular structure, rather than deteriorate settling properties and forming bulking sludge (Jenkins et al., 2003; Martins et al., 2004).

The results from this study show that the stability of aerobic granular sludge is not necessarily sensitive for the growth of filamentous bacteria unlike conventional activated sludge systems. The standard theory states that bulking sludge occurs when there is a low substrate concentration in the bulk, giving a competitive advantage to organisms with a low affinity constant (K_S), especially filamentous organisms (Chudoba et al., 1973). The generally higher μ^{max} of floc-forming bacteria as compared to filamentous bacteria is an important factor in their relative proliferation when substrate is present in higher amounts (Chudoba, 1985; Chiesa and Irvine, 1985; Liu and Liu, 2006). This principle has led to the development of aerobic selectors with high spatial substrate gradients (Verachtert et al., 1980; Chiesa et al., 1985), and explains why SBR systems suffer less from filamentous outgrowth than CSTR systems (Martins et al., 2003). In an earlier experiment by Martins et al. (2011) it was shown that proliferation of filamentous *Nostocoida* bacteria can lead to compact flocs or bulking sludge depending on the presence of substrate gradients.

A key factor for stable granulation is the temporal separation of diffusion and consumption of COD and growth. Compact granules are obtained when the biofilm has a higher substrate diffusive transport than substrate consumption rate (Picioreanu et al., 1998). The SBR operation in our study consists of anaerobic plug-flow feeding, during which the high substrate concentration allows for complete diffusion of acetate into the granules (Fig. 1). This acetate is stored as PHA inside the granule, which is consumed for growth only during the subsequent aeration phase. If the acetate were supplied aerobically, the diffusive flux of oxygen or acetate would be rate limiting compared to the metabolic capacity and would lead to less stable granule formation. *Thiothrix*, much like PAO and GAO, have the ability to store acetate as PHA under anaerobic conditions (Rubio-Rincón et al., 2017a). This means that the same granulation principles apply. Stable granule formation (Fig. 5) with the filamentous *Thiothrix* bacteria can be formed under the correct reactor

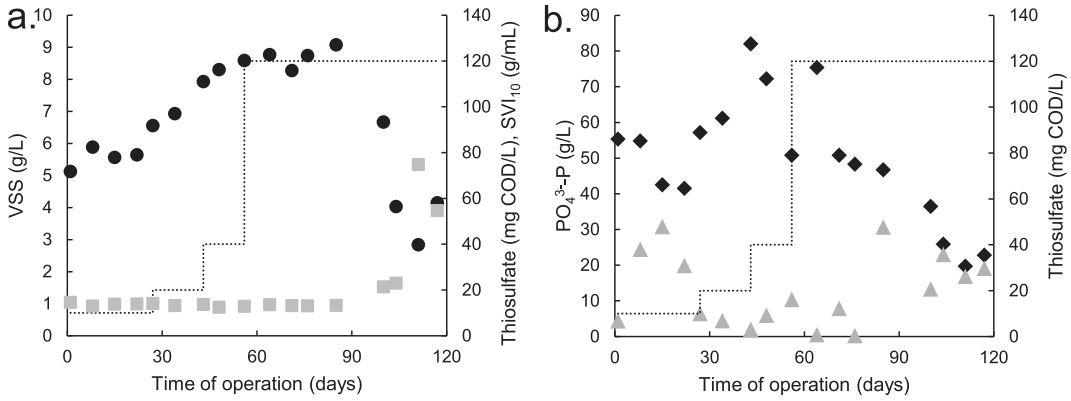

Fig. 2. Reactor performance over time of reactor operation with increasing influent thiosulfate concentration (dotted line). a) Concentration of VSS (black circles) and SVI_{10} (grey squares); b) Phosphate at start (black diamonds) and end (grey triangles) of aeration.

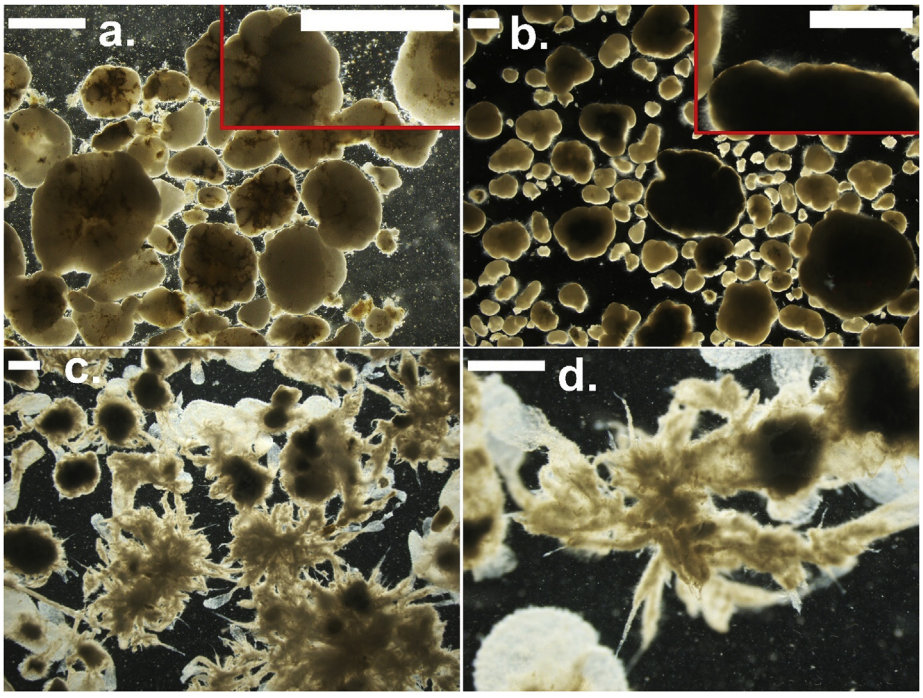

Fig. 3. Morphology of aerobic granular sludge: (a) Granular biomass prior to crushing them for inoculating the thiosulfate-added reactor; (b) Granules grown with 18% S-COD in seawater conditions after 58 days of operation; (c, d) Granules after a sudden drop in SRT after 104 days of operation, with 25% S-COD in the influent. Scale bar indicates 1 mm.

operations.

4.2. Proliferation of Thiothrix in an EBPR system

In our study, with only 18% of the influent COD being reduced sulfur roughly 50% of the biomass consisted of *Thiothrix* with the remainder mainly formed by *Ca.* Accumulibacter phosphatis. An essential factor for the successful proliferation of *Thiothrix* is the capacity of using reduced sulfur compounds as energy source. Reduced sulfur is rapidly sequestered as intracellular sulfur pools. These are oxidized for energy generation during the aerobic phase to sulfate.

Rubio-Rincon et al. (2017a) observed an increase in the yield by 116.7% when compared to typical PAO *Ca.* Accumulibacter, resulting in a higher fraction of *Thiothrix* over *Ca.* Accumulibacter in a suspended EBPR system (Rubio-Rincon et al., 2017a). This was linked to the extra energy that becomes available by oxidation of poly-S pools, allowing them to achieve mixotrophic metabolism (Chernousova et al., 2009).

The choice of thiosulfate instead of sulfide in our study led to a difference in terms of toxicity to *Ca.* Accumulibacter. Sulfide affects its anaerobic metabolism more severely than its aerobic metabolism (Rubio-Rincón et al., 2017b). Complete anaerobic uptake of acetate is essential for stable granulation, so issues with filamentous outgrowth due to acetate overshoot into aeration were prevented by supplying thiosulfate rather than sulfide. Thiosulfate can be disproportionated by *Thiothrix* to sulfide and sulfite or sulfate prior to being taken up by the cells (Hao et al., 1996). This sulfide gets taken up rapidly by *Thiothrix* under aerobic conditions, in order to replenish their poly-S pools (Rubio-Rincon et al., 2017a). The potentially toxic effect of sulfide production from thiosulfate is therefore deemed minor with this small exposure time.

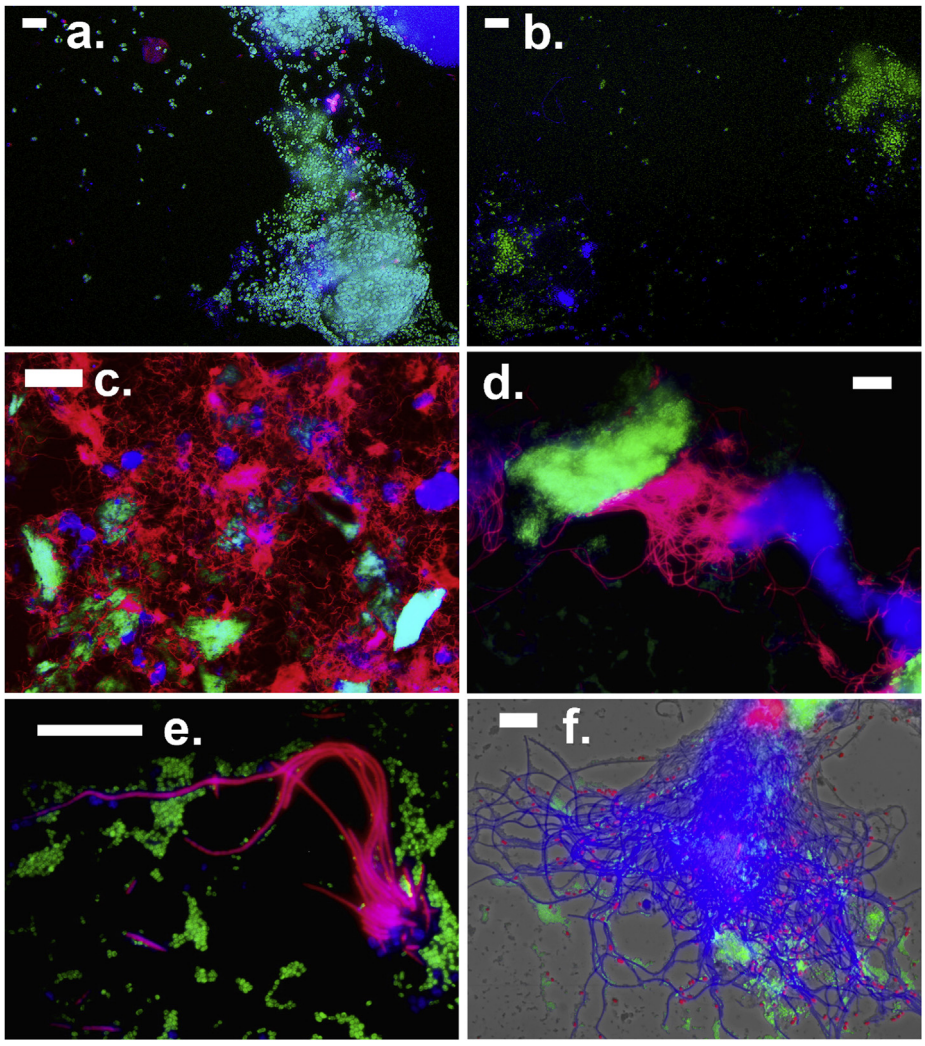

Fig. 4. Fluorescence in-situ hybridization (FISH) analysis (a, b) crushed granules that were used for inoculation of the thiosulfate-based reactor (corresponding to Fig. 3a) with (a) GAOmix (cy3, red), PAOmix (fluos, green), EUB338 (cy5, blue), and (b) gamma proteobacteria (cy3, red), PAOmix (fluos, green), EUB338 (cy5, blue); (c, d, e, f) 18% S-COD seawater-adapted aerobic granular sludge after 58 days of operation (corresponding to Fig. 3b). Probes that were used are (c, d, e) *Thiothrix*-specific G123T (cy3, red), PAOmix (fluos, green), EUB338 (cy5, blue); (f) GAOmix (cy3, red), PAOmix (fluos, green), EUB338 (cy5, blue). Magenta color indicates overlap between blue cy5 and red cy3 signals. Scale bar (a, b, d, e, f) 20 μm, and (c) equals 100 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

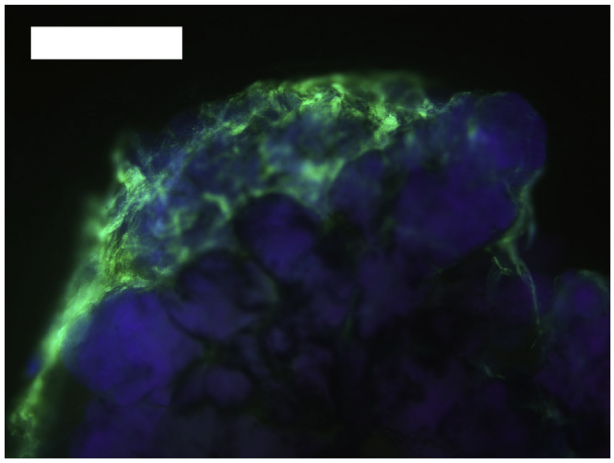

Fig. 5. Fluorescence in-situ hybridization (FISH) analysis on a slice of a whole granule, with probes for gamma proteobacteria (fluos, green), PAO mix (cy3, red), and EUB 338 (cy5, blue). Scale bar equals 200 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

4.3. Biological phosphate removal with Thiothrix bacteria

EBPR activity in our system was high at a remarkably high sulfur content of 18% COD next to acetate (Figs. 1 and 2). This amount of sulfur was higher than usual observed in domestic wastewaters, which is usually up to 10 mg S/L in gravity-driven sewer systems (Nielsen et al., 1992). These results therefore imply that presence of sulfides in wastewater with subsequent growth of filamentous *Thiothrix* bacteria, is not a problem for development of good settling aerobic granular sludge.

Removal of phosphate and anaerobic acetate uptake in our system started deteriorating when the reactor was fed with 120 mg S-COD/L and 366 mg HAc-COD/L (25% COD present as thiosulfate). Rubio-Rincón et al. (2017b) observed steady phosphate removal with the presence of 65 ± 3% filamentous *T. caldifontis* bacteria after feeding with an influent concentration of 100 mg S-COD/L, 295 mg HAc-COD/L, and 100 mg HPr-COD/L (20% COD from sulfide). The VFA uptake rate decreased by 65% compared to sulfide-free influent, but their anaerobic time was long enough for complete consumption.

The remarkable change in granule morphology after an SRT drop

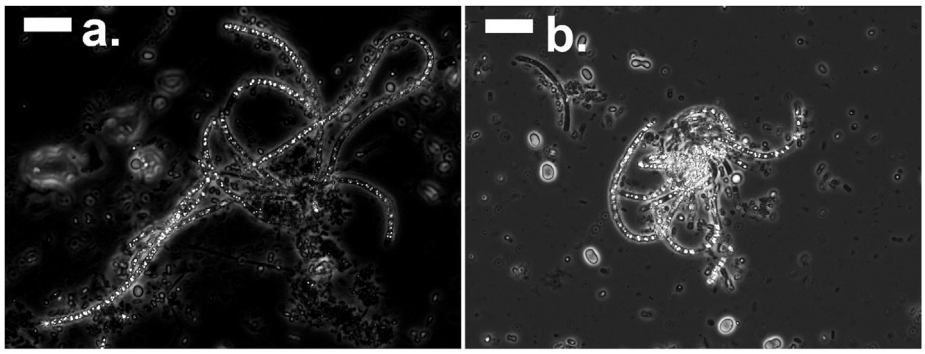

Fig. 6. Brightfield microscopy images of crushed 120 mg S/L thiosulfate-adapted granular sludge after 104 days of operation (corresponding to Fig. 3c and d). Bright storage polymers are visible inside the bacteria. Scale bar equals 10 μm.

at 25% S-COD (Fig. 3c and d) is likely due to the overshoot of acetate into the aeration phase, rather than an effect of the growth of *Thiothrix* sp. (Mosquera-Corral et al., 2005). The granules showed a more fibrous structure. The lower uptake rate of VFA by the *Thiothrix* community, combined with a system more prone to preferential flow in the granular sludge bed with fibrous outgrowth, resulting in acetate presence in the aerated phase. Where limited sulfide in the influent (as in municipal wastewater) seems not to give problems, higher sulfide loadings might need extra attention for the influent distribution and flow rate through the granular sludge bed.

5. Conclusions

- Reduced sulfur compounds select for *Thiothrix* type of phosphate accumulating bacteria.
- Presence of *Thiothrix* bacteria leads to stable aerobic granular sludge, despite their filamentous cell morphology
- Granule morphology is determined by reactor operation, and in a much lower extent by morphology of the individual cells
- When easy degradable COD is still present when aeration starts in an aerobic granular sludge system the sludge morphology and P-removal efficiency can rapidly deteriorate.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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