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ABSTRACT

Enhanced Biological phosphorous removal (EBPR) processes, often operated at low temperatures, are utilised world-wide, but currently little is known regarding enrichment cultures and the characteristics of active organisms ("*Candidatus* Accumulibacter phosphatis" (*Accumulibacter*)) under psychrophilic conditions. This study assesses the long-term performance, metabolic activity, microbial community characteristics and sludge morphology in an EBPR community enriched from activated sludge at 10 °C. Long solid retention times (SRT) and low temperatures resulted in the dominance of Accumulibacter type II over type I. Despite changes in the microbial community, P-removal efficiencies did not show obvious differences and although no specific measures were implemented, the enriched *Accumulibacter*-PAO culture formed stable dense granules. A high level of Alginate-like exopolysaccharides (ALE) were observed, with a large number of Guluronic acid-Guluronic acid (GG) blocks derived from the biomass at 10 °C. This characteristic favors sludge granulation, increasing the mechanical strength of granules formed, which encourages solid-liquid separation and consequently, contributes to the stable operation of EBPR systems.

1. Introduction

Basic activated sludge can achieve enhanced biological phosphorus removal (EBPR) by changing dissolved oxygen concentration (Barnard, 1975; Shi and Lee, 2006). Such a selective environment promotes the proliferation of polyphosphate-accumulating organisms (PAO's), which are primarily to remove phosphorus in EBPR systems (Liu and Li, 2015; Semerci and Hasilci, 2016). Anaerobically, PAO's can effectively utilize volatile fatty acids (VFA) and accumulate polyhydroxyalkanoates (PHA), whereas under aerobic conditions, these accumulative PHA are applied as a carbon and energy source to support the orthophosphate uptake by PAO's (Kuba et al., 1996). This mechanism offers a selective advantage over many heterotrophs, allowing survival in both aerobic and anaerobic conditions.

By investigating the associations between EBPR performance and microbial community dynamics, the phenomenon of PAO metabolism was found from the subclass 2 Betaproteobacteria, which was related to *Rhodocyclus,* within EBPR systems (Hesselmann et al., 1999; Crocetti et al., 2000). Poly-phosphate kinase gene (ppk1) used as genetic marker and the16S rRNA gene were applied to analyse the bacterial group, named "*Candidatus* Accumulibacter phosphatis". It was found that *Accumulibacter* were mainly classified into PAO I and II, with both clades formed of distinct types (He et al., 2007).

Several studies have found significant functional differences among varying PAO clades, affecting not only EBPR performances, but also play a fundamental and important role in the development of syntrophic relations between specific microbial communities. In this regard, two main PAO clades in terms of their denitrifying

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capabilities were classified as: *Accumulibacter* Type I (PAO I), taking advantage of NO_3^- and/or NO_2^- as electron acceptor; and *Accumulibacter* Type II (PAO II), which only can utilize NO_2^- for anoxic phosphorus removal (Flowers et al., 2009). Slater et al. (2010) monitored the relationship of sub-clade variations, community structure and ecosystem dynamics from an EBPR activated sludge system by terminal-restriction fragment length polymorphism (T-RFLP). This study found a variation occurred between PAO I and PAO II dominancy, with PAO I groups dominating EBPR systems in times of high phosphorus removal efficiency; while PAO II groups dominated in EBPR systems associated with poor phosphorus removal efficiency. Under limiting phosphate conditions, PAO I and II groups had varying abilities to transform to GAO (glycogen accumulating organisms) metabolism, with PAO II apparently more capable of utilising volatile fatty acids (Welles et al., 2015).

The functional differences between closely related PAO clades are relevant for effective process control and the relationship between PAO and GAO are greatly complicated by the ability of some PAO clades to exhibit a GAO phenotype, which can also bring new potential for the exploitation of the behaviour and metabolic characteristics of different PAO clades. However, to get deep comprehension of the functional differences between PAO clades and the relevance of clade specific traits on PAO-GAO interactions, comprehensive investigations are required in highly enriched cultures. Unfortunately, PAO cultures enriched with specific clades have rarely been reported and the conditions required for the selection of specific PAO clades, remains unclear (Lopez-Vazquez et al., 2009a,b). Previous researches showed that PAO clades could be selected at low temperatures (<15 °C) (Lopez-Vazquez et al., 2009a,b; Tian et al., 2013), while other studies indicated that higher temperatures (>20 °C) are in favour of GAO clades (Brdjanovic et al., 1998; Lopez-Vazquez et al., 2009a,b; Amorim et al., 2016), although it has been established that PAO can function effectively at higher temperatures of 30–35 °C (Winkler et al., 2011; Ong et al., 2014; Law et al., 2016). For the application of EBPR in colder climates, to the best knowledge of the authors, the population dynamics of specific PAO clades, the long-term performance and biomass characteristics of EBPR have only been marginally reported (Tian et al., 2013). Additionally, the characterization of alginate-like exopolysaccharides in flocs and granules (Lin et al., 2013b) and the mechanical strength of anaerobic granule sludge (Lin et al., 2013a) have recently been on researcher's attention.

The main objectives of this study were therefore to study the long-term performance, metabolic activity, microbial community characteristics, alginate-like EPS and their mechanical properties in an EBPR community with long SRT(32days) at 10 °C.

2. Materials and methods

2.1. Operation and set up of the sequencing batch reactor

A lab-scale sequencing batch reactor (SBR) with an effective volume of 2 L (Tian et al., 2013) was inoculated with activated sludge collected from WWTP Harnaschpolder (the Netherlands). The cycle time was conducted as 6 h (anaerobic, aerobic and settling phase were 2.0 h, 3.0 h and 1.0 h, respectively), hydraulic retention time (HRT) and SRT were 12 h 32 days, respectively. The temperature was controlled at 10 ± 0.5 °C with pH of 7.0 ± 0.1. In anaerobic and aerobic phases, the SBR had a constant mixing speed of 500 rpm. Further details related to the operating parameters can be found elsewhere (Tian et al., 2013).

2.2. Synthetic media

To avoid the growth of microorganisms in the feed tank, the synthetic feed media was prepared as separate solutions A and B. Solution A contained the carbon source and solution B contained minerals and trace elements. Solution A and B with a volume ratio of 1:1 were simultaneously supplied to the SBR reactor as influent, where following mixing, the synthetic feed contained per litre: 300 mg/L COD ($NaAc \cdot 3H_2O$); 28 mg N/L (NH_4Cl); 15 mg P/L ($NaH_2PO_4 \cdot 2H_2O$). Other nutrient components were prepared according to Tian et al. (2013).

2.3. Analyses

Orthophosphate (PO_4^{3-}-P); acetate (HAc); mixed liquid suspended solids (MLSS); and mixed liquor volatile suspended solids (MLVSS) were according to Standard Methods (APHA, 1998) to evaluate the SBR performance. Poly-hydroxy-butyrate (PHB), poly-hydroxy-valerate (PHV) were extracted according to Johnson et al. (2009) and glycogen concentrations were determined according to Lanham et al. (2012) in the typical cycle measurements.

2.4. Microbial community structure and biomass morphology

Biomass samples were collected at different stages of enrichment and analysed using Fluorescence *in situ* Hybridization (FISH) microscopy. EUBMIX (including EUB 338, EUB338-II and EUB338-III) were used to target all bacteria; PAOMIX (mixture of probes PAO462, PAO651, and PAO 846) and GAOMIX probe (mixture of probes GAOQ431 and GAOQ989) were used to target '*Candidatus* Accumulibacter phosphatis' and'*Candidatus* Competibacter phosphatis', respectively; PAO I (clade IA and other type I clades) and PAO II (clade IIA, IIC and IID) were targeted by the probes Acc-1-444 and Acc-2-444, respectively (Welles et al., 2015). FISH slides were analysed using a Zeiss Axioplan 2 microscope, as reported by Lopez-Vazquez et al. (2007). The morphology of the flocs/granule biomass was examined by a Leica DFC420 imaging system.

2.5. Anaerobic stoichiometry and kinetics

During the anaerobic phase, the kinetic rates were investigated based on the HAc uptake and P release respectively (Welles et al., 2015). PHV/HAc, PHV/PHB, P/HAc, Gly/HAc and PHB/HAc were calculated to characterize the stoichiometric ratios of interest. During the aerobic phase, the aerobic PO_4-uptake rate was evaluated. The specific acetate uptake rates (C-mol/C-mol/h) were calculated as reported previously (Lopez-Vazquez et al., 2007).

2.6. Characterization of ALE

The extraction, fractionation process and the partial hydrolysis method of ALE were in accordance with Lin et al. (2013a,b).

Biomass usually display viscous and elastic characteristics if deformed by external forces, which is named viscoelasticity (Wineman and Rajagopal, 2000). The biomass viscoelasticity was detected using the low load compression tester (LLCT) at 10 °C (Sharma et al., 2011; Lin et al., 2013a,b) with 15 randomly selected granule samples and the data was analyzed as described below.

The strain was calculated as:

$$strain_t = \frac{(h_0 - h_t)}{h_0} \quad (1)$$

while h_0 is the size of the granule, and h_t is the position of the top plate at any time t.

The relaxation modulus G(t) was calculated as:

$$G(t) = F(t)/0.2 \quad (2)$$

where F(t) is the compressive force, and the applied strain ($\varepsilon = 0.2$).

$$G(t) = G_1 e^{-t/\tau_1} + G_2 e^{-t/\tau_2} + G_3 e^{-t/\tau_3} + G_4 e^{-t/\tau_4} \ldots\ldots \quad (3)$$

where $\tau_i = \eta_i/G_i$, G_i is the spring constant and η_i is the viscosity of the ith element and i have the value 1, 2, 3 ….

3. Results

3.1. Enrichment of psychrophilic Accumulibacter-PAO culture at 10 °C

The laboratory scale SBR system was continuously operated for 150 days, with a steady-state performance achieved following 90 cycles of operation. Fig. 1a illustrates long-term phosphorus removal performance, under steady-state conditions at 10 °C. Throughout the period of enrichment, phosphate concentrations gradually climbed from 50 mg P/L, to 60 mg P/L before the anaerobic phase ended. The effluent phosphate concentration decreased to 0.5 mg P/L (98% removal rate), when the aerobic phase almost finished. The P/HAc ratios were within 0.31–0.37 Pmol/Cmol (Fig. 1a), with VSS and TSS content increasing from 2.6 g/L to 4.5 g/L and 3.6 g/L to 7.2 g/L respectively, and a decreasing VSS/TSS ratio, from 0.73 to 0.61 g/g (Fig. 1b).

3.2. Microbial community structure based on FISH

The microbial community was analyzed eight times at different periods during reactor operation, as shown in Fig. 2. Following 90 cycles of operation, the microbial community was highly enriched with PAO I, with dominance by the clade I group '*Candidatus* Accumulibacter phosphatis'. FISH analysis of the community present in the 350th cycle (Fig. 3a) showed that the fractions of β-*Proteobacteria* occupied 96 ± 2% (relative to EUBmix) of the total microbial community and that γ-*Proteobacteria* were not detected. The fractions of *Accumulibacter*, which belong to β-*Proteobacteria,* represented 94 ± 4% (relative to EUBmix) of bacteria present, with no '*Candidatus* Competibacter phosphatis', which belong to γ-*Proteobacteria*, detected by FISH probes (Fig. 3b). Other microorganisms occupied 2 ± 1% of the remaining bacterial community. These results indicate that complete phosphorus removal in EBPR systems, is associated with communities dominated by *Accumulibacter* PAOs and that the presence of *Competibacter*-GAO was effectively suppressed at 10 °C.

During cycles 90–510 of operation, '*Candidatus* Accumulibacter phosphatis' remained the dominant organism within the biomass, but a gradual shift was observed from clade I to clade II. Fig. 2 shows P/HAc in the anaerobic phase slightly increased with the population shift between PAO I and II. However, phosphorus removal performance was not influenced regardless of dominance of PAO clade I or II within the EBPR community (Figs. 1a and 4).

3.3. Metabolic conversions

Fig. 4 illustrates the behavior of the EBPR reactor in the 350th cycle, where the stoichiometric and kinetic parameters (the anaerobic conversions) were determined. During the anaerobic phase, complete HAc uptake occurred with a specific rate of 0.041 C-mmol HAc/C-mol active biomass/hr, with acetate uptake associated with PHA production and glycogen consumption. In the following aerobic phase, PHA was degraded, coupled to glycogen and poly-P pool replenishment with a phosphate uptake rate of

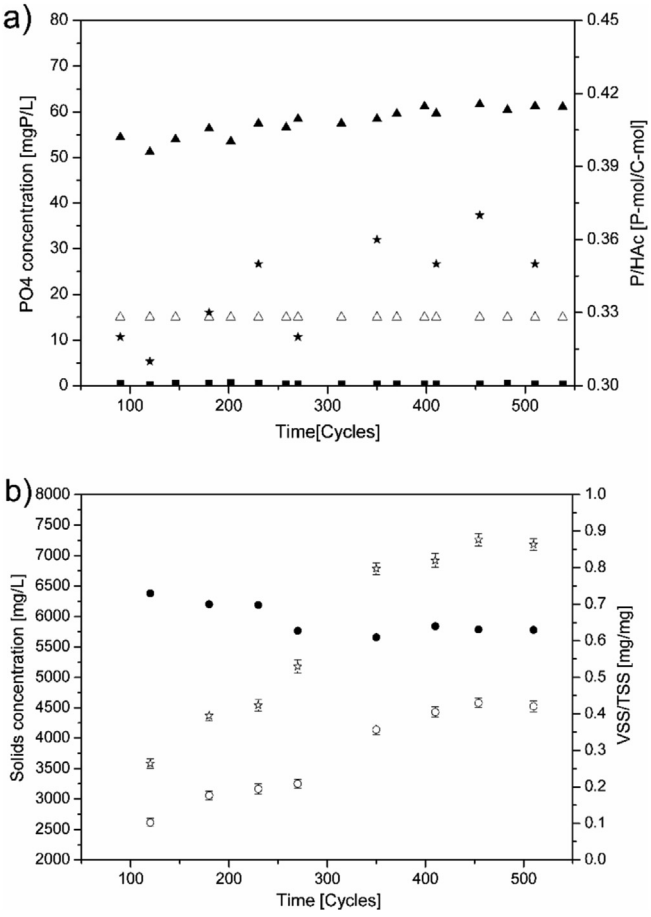

Fig. 1. Long-term performance of the EBPR-SBR system at 10 °C. Phosphate concentrations (a): at the end of the anaerobic phase (▲), influent (△), at the end of the aerobic phase (■); P/HAc (★). Solids concentrations (b): TSS (☆), VSS (○), VSS/TSS (●).

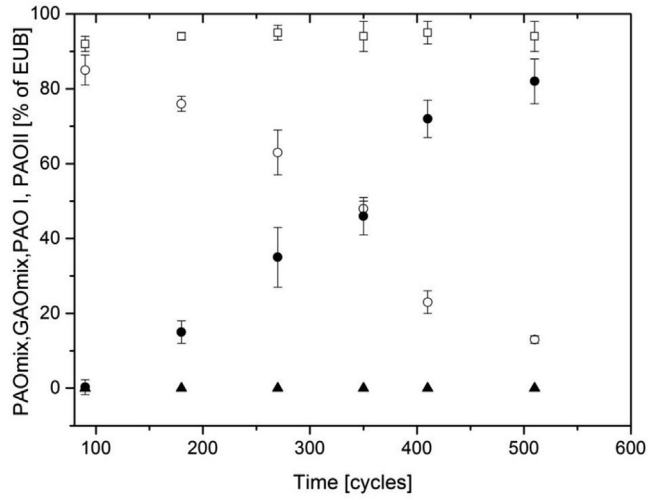

Fig. 2. Microbial composition as observed by FISH during the long term cultivation of an EBPR-SBR system at 10 °C. FISH probes: PAO mix (□), GAO mix (▲), PAO I (○), PAO II (●) (Three replicates were analyzed at each time).

0.011 P-mol/(C-mol active biomass/hr). This resulted in complete P-removal occurring within the liquid phase.

Table 1 shows a comparison of kinetic and stoichiometric

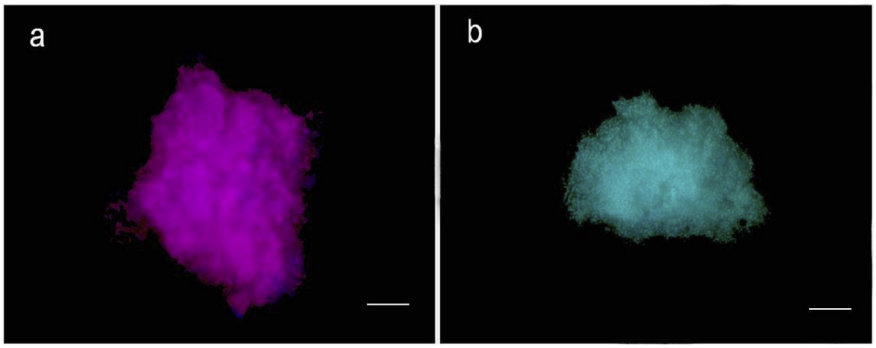

Fig. 3. Microbial community structure observed in the 350th cycle of operation of the SBR at 10 °C, by applying Fluorescence *in situ* Hybridization. a) γ-Proteobacteria (FLUOS) in green; β-Proteobacteria (Cy3) in red (or light violet due to the superposition of EUBmix); b) Accumulibacter (FLUOS) in green, and Competibacter (CY3) in red (or light violet due to the superposition of EUBmix). EUBacteria (Cy5) appear in blue. In all Figs, the bar indicates 20 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

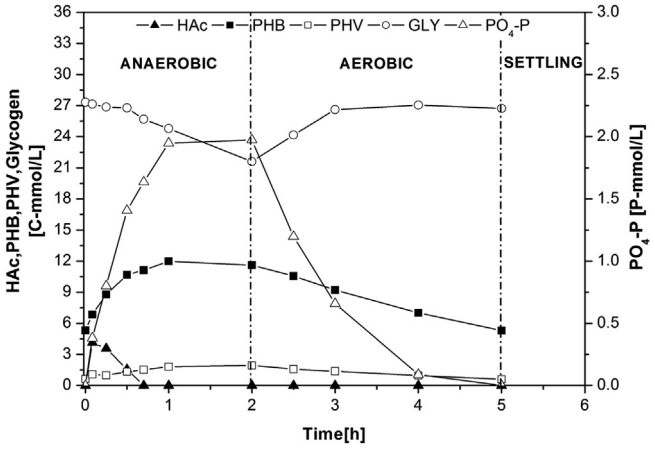

Fig. 4. Cycle profiles observed in the 350th cycle of SBR operation of enriched with *Accumulibacter*-PAO cultures at 10 °C.

parameters determined within the present study at 10°Cand reported in the literature. It is of note, that during the anaerobic phase, a low anaerobic P/HAc ratio (0.36 P-mol/C-mol) and a relatively high Gly/HAc and PHV/PHB ratio were observed, while a low poly-P content was obtained when the system initially dominated by PAO I culture (Fig. 1b). Due to PAO metabolism can shift to GAO metabolism (by PAO) dependent on their poly-P content (Acevedo et al., 2012; Welles et al., 2015), therefore, the stoichiometry information in this study indicate that HAc was consumed via a partial GAO-like metabolism.

3.4. Characteristics of psychrophilic Accumulibacter-PAO enriched biomass. Morphology of the biomass of psychrophilic Accumulibacter-PAO

The biomass formed within the EBPR system was highly stable, with compact granules, following stirring of the SBR enriched culture with *Accumulibacter*-PAO at 10 °C. The size distribution of the granules and a figure showing the biomass morphology is given in Fig. 5, showing that the compact, well-formed granular sludge to be around 1 mm in diameter. From an engineering perspective, this morphological characteristic could assist the ability for sludge to settle, as well as improving nutrient removal performance,

Table 1
Anaerobic stoichiometry parameters observed in this study, compared to those reported in literatures.

Temperature [°C]	Stage	Organisms	SRT [d]	P/HAc P-mol/C-mol	glycogen/HAc C-mol/C-mol	PHA/HAc	PHB/HAc	PHV/HAc	PHV/PHB	Reference
10	Long-term	PAO I (48 ± 2%) and PAO II (46 ± 4%)	32	0.36 ± 0.02	1.09 ± 0.01	1.62 ± 0.05	1.34 ± 0.04	0.28 ± 0.03	0.21 ± 0.02	This study
10	Long-term	PAO I (81 ± 2%) *Competibacter* (18 ± 1%)	16	0.56 ± 0.02	0.55 ± 0.05	1.44 ± 0.07	1.31 ± 0.05	0.13 ± 0.02	0.10 ± 0.02	(Lopez-Vazquez et al., 2009a,b; Tian et al., 2013)
10	Short-term test	PAO	8	0.48 ± 0.03	N/A	0.94 ± 0.29	N/A	N/A	N/A	(Brdjanovic et al., 1997)
20	Long-term	PAO	8	0.50	0.50	1.33	1.33	0.00	0.00	(Smolders et al., 1994)
20	Long-term	GAO	6.6	0.00	1.12	1.86	1.40	0.47	0.34	(Zeng et al., 2003a,b)
20	Long-term[a]	PAOIand PAO II	10	0.16–0.52	0.50–0.69	1.10–1.44	0.76–0.96	0.14–0.36	N/A	(Carvalho et al., 2007; Oehmen et al., 2010)
20	Short-term test	[b] PAO I (66 ± 7%) PAO II(8 ± 3%)	8	0.73	0.35	1.36	1.30	0.06	0.05	(Acevedo et al., 2012)
20	Short-term test	[c] PAO I (23 ± 5%) PAO II(36 ± 7%)	8	0.08	1.08	2.02	1.74	0.28	0.16	(Acevedo et al., 2012)
20	Long-term	DPAO	8	0.44	0.48	1.20	1.20	N/A	N/A	(Kuba et al., 1996)
20	Long-term	DPAO	14	0.42	0.80	1.52	1.52	N/A	N/A	(Kuba et al., 1996)
20	Long-term	DPAO/DGAO	15	0.35	0.64	1.48	1.35	0.13	0.10	(Zeng et al., 2003a,b)
20	Long-term	DPAO/DGAO	8	0.32	0.76	1.52	1.34	0.18	0.13	(Zeng et al., 2003a,b)

Note: N/A = not applicable.
[a] pH varied between 7.0 and 8.2.
[b] Poly-P/VSS = 0.30; pH ranged from 7.0 to 8.9.
[c] Poly-P/VSS = 0.01; pH ranged from 7.0 to 8.9.

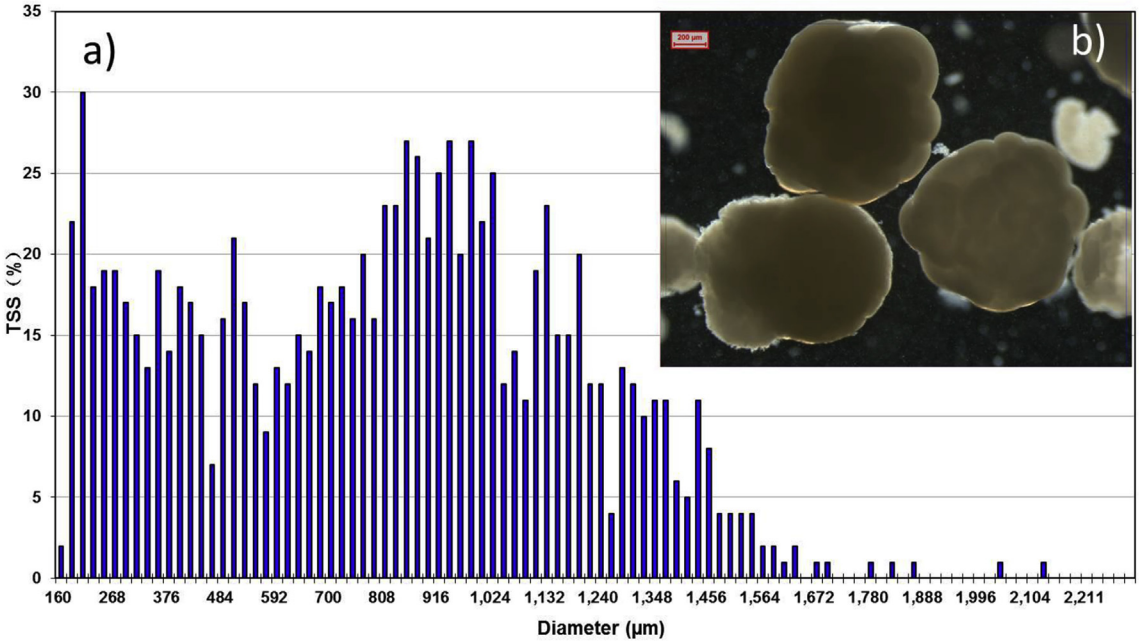

Fig. 5. Morphology of biomass in a stable *Accumulibacte*r dominated SBR formed at 10 °C. a) Particle size distribution; b) Microscope image with 50× amplification, the bar indicates 200 μ.

encouraging the stabilization of EBPR systems (Van Loosdrecht et al., 1998; De Kreuk et al., 2005; Wang et al., 2015).

3.5. ALE isolation and fractionation

ALE have been reported to take significant effect in aerobic granular sludge formation (Lin et al., 2013a,b). To identify the presence of this kind of exopolysaccharides within biomass, an ALE extraction was performed, using alginate as a block co-polymer, with three kinds of blocks formed from two monomers (GG, MG and MM blocks are formed from mannuronic acid (M) and guluronic acid (G) monomers, respectively). The measured yield of ALE was very high (52 ± 5%, VSS/VSS), with a high level of GG blocks (73 ± 7%) and comparatively low levels of MM (1.4 ± 0.9%) and MG (6.5 ± 3.1%) blocks.

3.6. Mechanical properties of biomass in Accumulibacter-PAO EBPR at 10 °C

Alginate with higher content of GG blocks normally presents with a stronger mechanical strength (Martinsen et al., 1989; Kuo and Ma, 2001). The mechanical strength of the biomass granules were quantitatively measured by a stress relaxation test, the experimental details of which have been described by Lin et al. (2013a,b).

The force-time profiles are provided in Fig. 6. During the first 0.5 s, the biomass was compressed by 90% compared to the original volume (strain = 0.1), with the strain then kept stable at 0.1 for 100 s to enable force relaxation. The force-strain profile is presented in Fig. 6b, showing the force-strain curve for biomass to be a straight line, with a slope of 116 ± 20 mN. The degree of slope is a measure for material stiffness, showing that the stiffness of the biomass in EBPR systems under colder conditions, is higher than that of the Ca-ALE beads produced from aerobic granular sludge (Lin et al., 2013a,b).

A generalized Maxwell model was illustrated in Fig. 6c, and the fitting parameters for which are summarized in Table 2. Four Maxwell elements were needed for an effective fit with experimental data, with these elements designating that the first element (the relaxation time $\tau \leq 1$ s) relaxed very fast; the second element ($1 < \tau \leq 10$ s) relaxed slower; the third element ($10 < \tau \leq 100$ s) was even slower and the fourth element ($\tau > 100$ s) was the slowest. The element with $\tau \leq 1$s means free water, $1 < \tau \leq 10$ s represents bound water (Turco et al., 2011), $10 < \tau \leq 100$ s represents the organic matrix (Lin et al., 2013a,b), and $100 < \tau \leq 1000$ s represents minerals.

4. Discussion

This study investigated the performance, metabolic conversions, microbial characteristics and sludge morphology within a 'Candidatus Accumulibacter phosphatis' culture, operated under long SRT (32 days) and low temperature (10 °C) conditions, showing these operating conditions to provide a competitive edge for Candidatus Accumulibacter phosphatis' clade II over I. Although the change in Accumulibacter-PAO clades was accompanied with a change in the anaerobic stoichiometry, from a practical perspective the change in the prevailing Accumulibacter-PAO population did not affect the overall P-removal performance. The biomass appeared morphologically as dense granules, which may be attributed to the high concentration of ALE in the sludge, potentially induced by the lower temperature operating conditions.

The bacterial distribution of *Accumulibacter* clades was regularly monitored by applying FISH analysis during the long-term cultivation (Fig. 2), where after a relatively short period of enrichment (120^{th} cycles), PAO I was the dominant group in the reactor, comprising 85 ± 4% of the microbial community. Moreover, *Competibacter* were not present after the 120^{th} cycle in this study, in comparison with an 18 ± 1% proportion in a previous study at 10 °C (Lopez-Vazquez et al., 2009a,b; Tian et al., 2013). Lopez-Vazquez et al. (2009a,b) and Oehmen et al. (2010) found using mathematical modeling that the competitive differences between PAO and GAO (and potentially also within PAO clades) are small, resulting in the longer time required to shift a culture towards the optimally

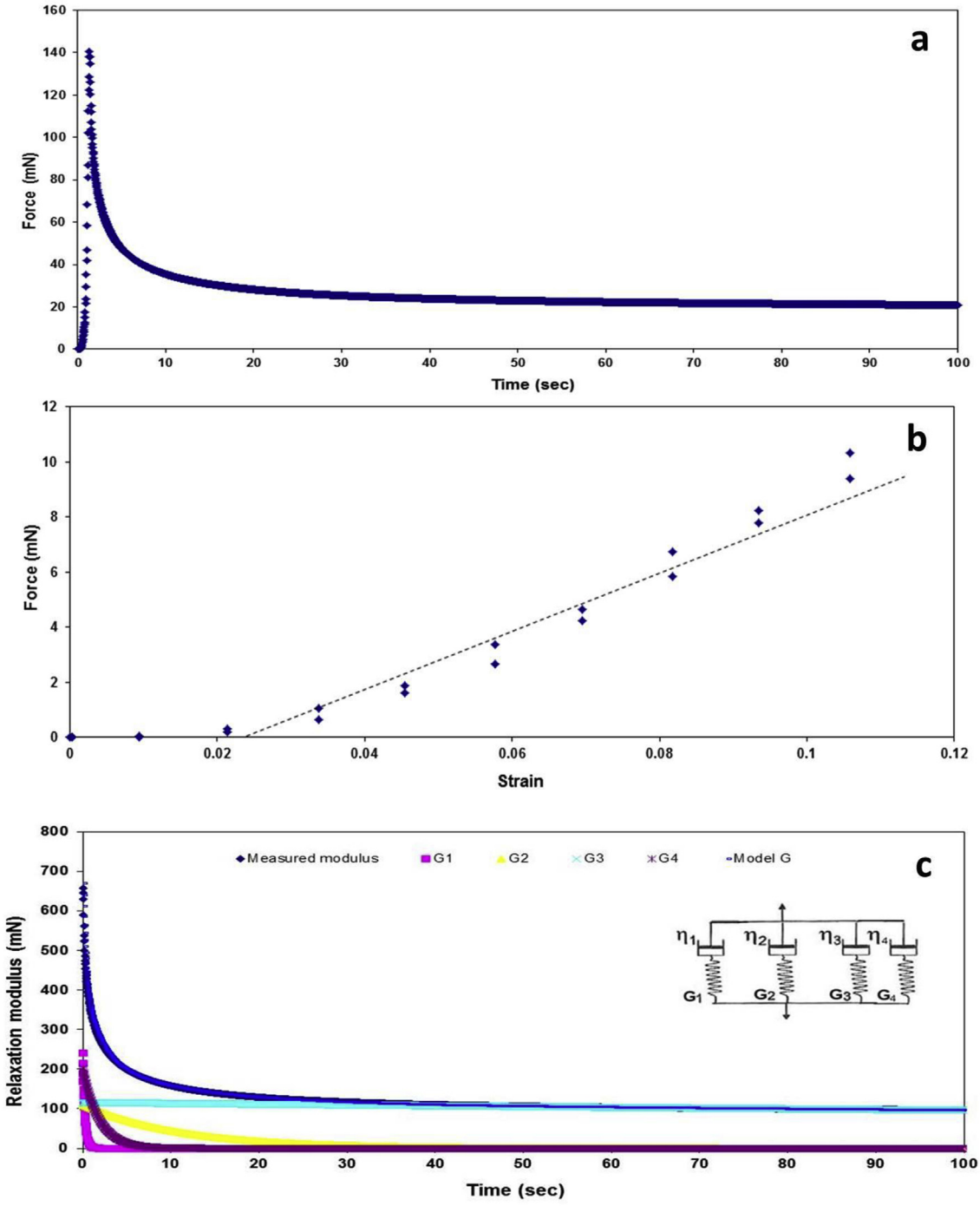

Fig. 6. Data acquisition and analysis from the low load compression tester. a. Raw output of force and strain as a function of time; b. Derivation of stiffness from the linear region where deformation is imposed on the biomass; c. Derivation of the viscoelastic parameters from the relaxation phase of the part of force-time curve by fitting a generalized Maxwell model.

Table 2
Generalized Maxwell parameters for biomass, represented with G as spring constant and τ as relaxation time constant.

Maxwell Element	1 ($\tau < 1$)	2 ($1 < \tau < 10$)	3 ($10 < \tau < 100$)	4 ($\tau > 100$)
G (mN)	240.8 ± 12.5	203.2 ± 10.1	110.4 ± 18.9	115.6 ± 9.7
τ (s)	0.25 ± 0.07	1.85 ± 0.18	10 ± 1.25	541 ± 15

adapted microbial community. Initially the community will continue to resemble the inoculum, which in the present study showed a dominance of PAO I in the initial culture. Following a period of stable operation, a gradual shift occurred from a PAO I

dominated culture to a PAO II dominated culture, with the fractions of PAO II, finally comprising 82 ± 6% of the total bacterial biomass, whereas PAO I comprised only 13± 1% in the 510th cycle. This reveals that the operational conditions utilized in this study were selective for PAO II, with the observation of this high PAO enrichment, with no detectable presence of GAO, consistent with the findings of other studies conducted under similar incubation temperatures of 10 °C (Brdjanovic et al., 1997; Tian et al., 2013). The study by Tian et al. (2013) found that cultures with an SRT period of 16 days had PAO I as the prevailing PAO clade, while PAO II dominated the culture following an SRT period of 32 days, suggesting that a longer SRT period provides a competitive edge for *Candidatus* Accumulibacter phosphatis' clade IIA over I.

The two periods dominated by either PAO I or PAO II, allow a comparison of the behavior of both clades at low temperature. Slater et al. (2010) suggested that specific PAO clades show different effectiveness in EBPR systems, with poor performance associated with PAO clade IIC, whereas good EBPR performance was associated with PAO clade I. In the present study, the overall phosphate removal performance did not change dramatically, despite the shift in PAO community from clade I to II, and therefore from an engineering perspective, this shift is not directly relevant to the phosphate removal performance, as both PAO clades are active. However, to understand the behavior of the system under adverse conditions or in combined nutrient removal and recovery systems, it is necessary to further understand the differences in behavior of the different PAO clades.

The anaerobic P/HAc ratio slightly increased following the transfer from PAO I to PAO II domination (Figs. 1b and 2), while the VSS/TSS ratio significantly increased. This may be explained by the findings of Acevedo et al. (2012) and Welles et al. (2015), showing that both PAO clades can shift the metabolism from a PAO to GAO system, depending on their poly-P content, however the intrinsic anaerobic P-release/HAc-uptake stoichiometry of a PAO II culture is in general lower than that of PAO I, at a defined poly-P content. In the present study the biomass was initially dominated by PAO I, but had a low poly-P content (as shown by the VSS/TSS ratio), triggering partial GAO metabolism, as shown by the low P/HAc ratio of 0.36 P-mol/C-mol. When the poly-P content gradually increased, a metabolic shift happened, from mixed PAO-GAO metabolism to a PAO-dominant metabolism, likely due to the higher poly-P fraction present within the sludge.

When comparing these findings with similar studies conducted at low temperature (10 °C) operating conditions, Brdjanovic et al. (1998) found that the anaerobic stoichiometry (0.5 P-mol/C-mol) and ash-content resulted in a PAO I dominated culture when using a SRT of 16 days (Welles et al., 2015). Furthermore, Tian et al. (2013) reported a well enriched PAO I culture under the same SRT and temperature conditions. The present study also established that the dominant culture shifted from PAO clade I to IIA with a longer SRT of 32 days at 10 °C, indicating that the shorter SRT periods favor the enrichment of PAO I, while long SRT periods favor enrichment with PAO II communities at low temperatures (10 °C).

PAO and GAO organisms are usually reported to grow in dense clusters within the sludge, enhancing the stabilization of granular sludge (De Kreuk et al., 2005; Wang et al., 2015). The biomass formed highly stable, with compact granules at 10 °C in this study may be related to the psychrophilic environment, as it has been suggested that higher temperature conditions can reduce the stability of granular sludge (Nor-Anuar et al., 2012). In general, we have observed well settled sludge within our EBPR-SBR systems, consisting of small compact aggregates or granules (<0.1 mm), possibly due to the induction of extracellular polymeric substances (EPS) at lower temperatures (Smidsrod and Draget, 1996; Seviour et al., 2012), or intrinsic granulation behavior of PAOs, which has been frequently reported in dense sludge microcolonies. Now, a lab-scale EBPR-SBR system enriched with *Accumulibacter*-PAO resulted in dense granular sludge like aggregates, which may be attributed to the higher concentration of Alginate-Like Exopolysaccharides (ALE) formed at low temperatures. The yield of ALE from biomass at lower temperatures was greater than 50% of the VSS, which is about 3-fold higher than the reported yield from aerobic granular sludge enriched with municipal wastewater (Lin et al., 2010).

The mechanical strength of these small granules is comparatively strong, due to the extremely high percentage of GG blocks within ALE. ALE contains an extremely high ratio of GG blocks, and low ratio of MG and MM blocks, with the estimation of G/M ratio found to be around 16, which is 2-fold higher than the G/M ratio reported for aerobic granular sludge (Lin et al., 2010). Which further reinforced the findings that PAO (and potentially GAO) bacteria are at the basis of a stable aerobic granular sludge process. In general, the EPS of PAO has received limited attention, as well as alginate-like polymer production by activated sludge organisms in general.

The genetics of bacterial alginate synthesis have been reported by numerous studies, such as the *Pseudomonadaceae* (Tatnell et al., 1994; Remminghorst and Rehm, 2006) and the *Azotobacteriaceae* (Campos et al., 1996; Rehm and Valla, 1997; Remminghorst and Rehm, 2006). Fialho et al. (1990) found alginate genes in the *Pseudomonas* RNA homeotic group 1, while other *Pseudomonas* strains are able to produce alginate with the presence of mutagens or special antibiotics (DeVault et al., 1990). P-mannose dehydrogenase (Martins and Sá-Correia, 1991) is a crucial enzyme for alginate biosynthesis, existing in several bacteria including Candidatus "*Accumulibacter* phosphatis" clade IIA str. UW-1 (Martín et al., 2006); *Azoarcus aromaticum* EBN1; *Thauera* sp. MZ1T; and *Alcanivorax borkumensis* SK2. For PAOI this capability still has to be evaluated (Skennerton et al., 2015; Barr et al., 2016) and as a large number of bacteria probably contain the genetic machinery for alginate synthesis, a better approach would be the exploration of alginate synthesis induction mechanisms, which are still poorly understood (Seviour et al., 2012; Skennerton et al., 2015).

Considering that within the EBPR-SBR system there is no selection for granular sludge, using long settling periods, it appears that the formation of alginate-like material and growth in granular form, are intrinsic properties of PAOs. It is of note, that under low temperature conditions, alginate with a high GG content and therefore mechanical strength, was obtained. In the EBPR-SBR system only small aggregates/granules were observed, which is either caused by the absence of a settling velocity based selection force or the presence of relatively high shear (with the presence of an impeller). Future research on the regulation of EPS formation and the effect of environmental parameters on the mechanical quality of the EPS, is required to better understand these aspects, which is essential in the optimisation of wastewater treatment plant designs with stable and compact sludge separation properties.

5. Conclusions

This study investigated the performance, metabolic conversions, microbial characteristics and sludge morphology of a 'Candidatus Accumulibacter phosphatis' culture operated at long SRT (32 days) and low temperature (10 °C). The following conclusions can be drawn: The long SRT and low temperature provided a competitive advantage for Candidatus Accumulibacter phosphatis' clade II over I. Although the change in Accumulibacter-PAO clades was accompanied with a change in the anaerobic stoichiometry, from a practical perspective the change in the prevailing Accumulibacter-PAO population did not affect the P-removal performance. The

morphology of biomass appeared as dense granule. This should be attributed to a high fraction of Alginate-Like Exopolysaccharides (ALE) in the sludge, potentially induced by the lower temperature.

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