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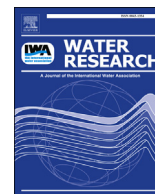
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Stress-induced assays for polyphosphate quantification by uncoupling acetic acid uptake and anaerobic phosphorus release

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ABSTRACT

Phosphorus has been successfully eliminated from wastewater by biological techniques of enhanced biological phosphorus removal (EBPR) process, which relies on a specific microbiota of polyphosphate accumulating organisms (PAOs) that accumulate phosphate as polyphosphates (poly-P). Most methods for quantification of poly-P pools suffer from low accuracy and specificity. More powerful and implementable P-analysis tools are required for poly-P quantification, which will help in improved evaluation of processes in laboratory and full-scale EBPR systems. This study developed two methods to quantify poly-P pools by releasing the poly-P from the cell. During experimental optimization, it was observed that two different methods resulted in the highest phosphate release: acetate addition at a pH of 4.8 and exposure to EDTA solution with a concentration of 1% (w/v). Treatment with EDTA resulted in a higher amount of phosphate release from all sludge samples. This was characterized by P-release of 1.5–2.5 times higher than the control tests. In contrast, treatments with acetate addition at a low pH exhibited that P-release depended upon the types of the sludge samples. The highest P-release amount and rate were found in highly-enriched PAO sludge samples, but with fewer influences on the sludge collected from WWTP, which may be attributed to the lower fraction of PAOs in the sludge. Overall, the proposed approaches to quantify the poly-P concentration can be applied in simple, user-friendly, and cost-effective ways.

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1. Introduction

Phosphorus (P) is one of the most abundant elements in the Earth's crust and is present in a large variety of forms, either in organic or inorganic forms, and as a monomer (phosphate) or as a constituent part of macromolecules (polyphosphates, referred to poly-P). Enhanced biological phosphorus removal (EBPR) has been recognized as the most sustainable and economical process to remove P from wastewater (Oehmen et al., 2007; Van Loosdrecht

et al., 1997). EBPR relies on the enrichment of phosphate accumulating organisms (PAOs) by alternating anaerobic (feast) and aerobic or anoxic (famine) conditions.

The EBPR process imposes a selective pressure favoring the growth of PAOs. Under anaerobic conditions, PAOs are capable of taking up carbon substrates, primarily volatile fatty acids (VFAs), and storing them in the form of polyhydroxyalkanoate (PHA) (Comeau et al., 1986; Martin et al., 2006; Mino et al., 1998; Oehmen et al., 2007; Wentzel et al., 1985) (Fig. 1a). The energy required for this metabolic process is primarily gained from the degradation of intracellular poly-P, which releases ortho-P to the bulk liquid. The reduction equivalents for the conversion of VFA into PHA are generated through glycolysis of intracellularly stored glycogen. In the subsequent aerobic phase, PAOs take up ortho-P to recover the poly-P level using the stored PHA as the energy source. Glycogen is a key component in PAO metabolism. Poly-P is composed of three to several hundred phosphate units (Fig. 1b). As indicated above,

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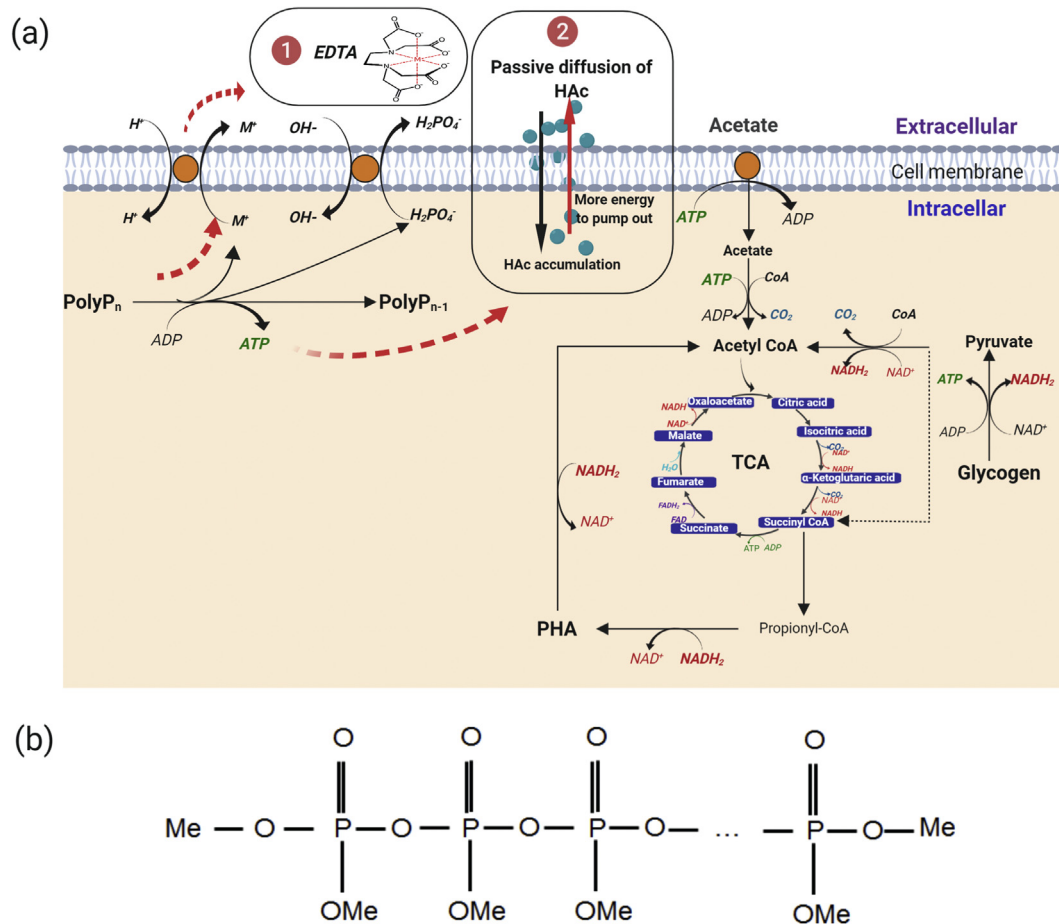


Fig. 1. (a) Schematic diagram of the anaerobic metabolism of PAOs with acetate as the carbon source (Acevedo et al., 2012; Hesselmann et al., 2000). The inserted figures marked with numbers display the potential mechanisms involvement of EDTA treatment and acetic acid presence at low pH values. (b) Structure of linear condensed phosphate (poly-P), where Me is monovalent (NH_4^+ , K^+ , Na^+) or divalent (Mg^{2+} , Ca^{2+} etc.) cations. Poly-P: polyphosphate, VFA: volatile fatty acid, ATP: adenosine triphosphate, NADH: nicotinamide adenine dinucleotide, PHA: polyhydroxyalkanoate, TCA: tricarboxylic acid.

poly-P plays a significant role in P-storage reservoirs (one constituent of nucleic acids) and in energy storage (for ATP formation) (Kulakovskaya et al., 2012; Serafim et al., 2002).

The poly-P content in sludge is an important parameter to study at a fundamental level including its role in the metabolism of specific PAO and the niche differentiation of different PAO clades. Moreover, reliable quantification of the poly-P content is of great importance to understand process performance and nutrient recovery potential of wastewater treatment systems. To investigate the effect of poly-P on the metabolism of different types of PAO clades in laboratory studies and modelling approaches (Saia et al., 2017), reliable methods for the determination of poly-P are needed. During the application of EBPR in full-scale wastewater treatment systems or nutrient reclamation facilities, the P-removal capacity of the process can be indicated by the level of poly-P content. Poly-P also shows the potential for P-recovery using biological and/or chemical, physical methods. To facilitate future studies and optimization of full-scale processes, developing simple and user-friendly methods are required for quantification of intracellular poly-P.

For a comprehensive evaluation of a technique, several elements in terms of reliability, cost, complexity, and pre-treatment steps should be considered. The development of fast, simple, and feasible poly-P analytical and quantitative tools is impending. For this, researchers need to elucidate the nature of the biochemistry, and the roles and dynamics of various phosphorus-containing molecules

in vitro and in vivo. Table 1 summarizes the principle, advantages, and disadvantages of techniques related to poly-P quantification. The reported techniques have their limitations for poly-P quantification. In general, conventional P-analysis methods involve fastidious extraction and pre-treatment procedures (Hupfer et al., 2008), includes conversion of poly-P to ortho-P through chemical digestion, followed by ortho-P analysis. On the other hand, due to the strong acid (perchloric acid, and nitric-sulfuric acid etc.) used during the digestion process, all forms of P can be converted to ortho-P, resulting in overestimation poly-P amount. Most chemical fractionation methods are laborious and not efficient at separation of poly-P, organic P and precipitated P. Glycogen plays a role in maximal P-release in biological methods (Brdjanovic et al. 1997, 1998; Welles et al., 2015), and for this complicated experimental systems are needed (Acevedo et al., 2012). Biological methods have the potential of releasing poly-P from the cells but there is a need for methods that uncouple the energy consumption from the consumption of reduction equivalents. Biological methods can be achieved by creating conditions where cells will use the poly-P for maintenance properties by inducing ATP consuming processes. Anaerobic metabolism of PAOs relies on the hydrolysis of internally stored poly-P for ATP generation. Microorganisms exposed to adverse environments, consume more energy in order to resist the environmental stress conditions, thus inducing greater P-release from EBPR cultures. Fig. 1a depicts the mechanism for two potential methods (i.e. EDTA treatment and acetic acid (HAC) presence at low

Table 1

Summary of poly-P analysis techniques. The contents were modified based on Tarayre et al. (2016) and Majed et al. (2012b).

	Techniques	Advantages	Disadvantages	Cost	Reference
Chemical analysis	Conventional analysis	Quantitative	Non-specific Fastidious pre-treatment procedures	Low	APHA (2005)
	Extraction - ortho-P analysis	Quantitative Possible to characterize different poly-P fractions	Possible poly-P loss and composition changes Fastidious extraction and pre-treatment procedures	Low	(Aravind et al., 2015; Majed et al., 2012b)
	Extraction-purification methods	Quantitative Applicable for unicellular organisms	Cell damage Unknown if it is applicable for mixed cultures	Low	Bru et al. (2017)
	Extraction-chloroform/ isoamyl alcohol addition-staining	Quantitative Sodium phosphate glass type 45 as the specific standard Specific dye of Toluidine blue Less time consuming (~3.5 h)	Fastidious procedures Cell damage (boiling at 100 °C for 2 h) Lack of proper protocols for EBPR experiments Expensive staining reagent	High	(Mukherjee and Ray, 2015)
	High-performance liquid chromatography	Sensitive separation and determination of linear poly-P up to a chain length of 35 Analysing up to 80 samples once with a low relative standard deviation of below 1.5%	Non-quantitative	Low	Baba et al. (1985)
	Electron ionization mass spectrometry	Highly selective/sensitive (1–10 ng/ml) No preparation steps Possibly to characterize different poly-P fractions	Destructive Standard required Lack of proper protocols for EBPR experiments or pure cultures of PAOs	High	(Choi et al., 2000; Rao et al., 2009)
	Raman spectromicroscopy	Quantitative Simple preparation Applicable for microbial consortia study	Weak Raman signal which requires an adapted device Interferences from impurities and fluorescence	High	(Majed et al. 2009, 2012a)
	Nuclear magnetic resonance spectroscopy	Non-destructive Non-invasive Possible to study different forms and species of P Applicable for liquid/solid samples	Tagged substrates required Frequency of interferences Non-specific to the poly-P measurement but phosphodiester linkages	High	Günther (2011)
Microscopy/ Staining	X-ray	Quantitative; powerful combination with EM Minimal preparation of samples Possible to combine with X-ray fluorescence spectromicroscopy to study the poly-P repartition inside the cells	Destructive Excessive preparation Possible loss of poly-P during preparation	High	(Baxter and Jensen, 1980; Diaz et al. 2008, 2009; Hupfer et al., 2008)
	DAPI ^a	Highly sensitive	Non-quantitative Destructive Expensive staining reagent Non-specific to poly-P Possible to stain other polymeric substances e.g., DNA and lipids	High	(Aschar-Sobbi et al., 2008; Serafim et al., 2002)
	EM ^b	Possible to locate poly-P inside the cells Possible to observe poly-P granules inferior to 100 nm	Non-quantitative Complicated sample preparation	High	Gu et al. (2008)
Biochemical analysis	Gel electrophoresis	Semi-quantitative Effective to separate poly-P with different chain lengths Possible to measure the poly-P size	Destructive Poly-P extraction required Poly-P must be solubilized	Low	(Clark and Wood, 1987)
	Enzyme assays	Quantitative Sensitive Applicable to assess the metabolism of poly-P extracts	Destructive Complex samples preparation Complex enzymatic reaction Less simple than with simple chemical reagents	Low	(Ault-Riché et al., 1998; Clark et al., 1986; Ohtomo et al., 2008)
	Proteic affinity	Quantitative Possible to locate poly-P by an immunocytochemical method High resolution when combining with EM	Complex sample preparation Specific antibodies for poly-P	Low	Saito et al. (2005)
Biological analysis	Poly-P depletion	Quantitative Non-destructive Non-invasive.	Time-consuming Laborious due to many cycles Less accurate	Low	(Acevedo et al., 2012; Brdjanovic et al., 1998; Welles et al., 2015; Zhou et al., 2008)

^a DAPI: 4',6'-Diamidino-2-Phenylindole.^b EM: Electron microscopy.

pH) of stress-induced energy consumption for poly-P quantification by uncoupling HAc uptake and P release. Previous studies (Liu et al., 1996; Mino et al., 1998) have shown that VFA uptake at low pH causes an uncoupling effect, which can uncouple the P-release from the VFA uptake and thereby allow rapid release of all biologically

available poly-P. A low pH leads to an accumulation of HAc in the cells and subsequent pH decrease, which the cell activity tries to counteract by producing extra energy in the form of ATP. An alternative method is the use of a compound that affects membrane integrity (such as ethylenediaminetetraacetate, EDTA) and thereby

disrupts the proton motive force, which the cells try to restore by ATP usage derived from poly-P.

The current study focusses on two different methods for poly-P quantification: (i) HAC addition; (ii) EDTA presence. The principle of poly-P quantification relies on uncoupling P release and HAC uptake, which arise from environmental stresses. The objectives of this study were 3-fold, (1) to develop easily-performable, non-destructive, and cost-effective methods to quantify poly-P; (2) to optimize the experimental procedure, including EDTA concentration, reaction time and pH; (3) to define and validate the protocol for poly-P quantitation using different sludge types.

2. Materials and methods

2.1. Sludge sources and characterization

To compare the reliability and availability of the proposed methods, flocculent sludge (FS) and granular sludge (GS) samples were harvested from lab-scale and full-scale reactors treating domestic sewage (4 different types of samples). The samples included GS (Sample 1) and FS (Sample 2) collected from PAO-enriched lab-scale EBPR system; Sample 3 was aerobic GS collected from a full-scale Nereda plant (Epe, NL), and Sample 4 was FS collected from a local Waste Water Treatment Plant (WWTP) Harnaspolder in the Netherlands. After collection of the fresh granules for GS samples from the lab-scale SBR reactors or WWTP, the granules were instantly and gently rinsed for 2–3 times using tap water (for 2–3 min each time) on a fine sieve with a mesh aperture of 0.1 mm. Afterwards, a piece of quantitative filter paper was put beneath the fine sieve for 5 min to absorb excessive moisture. Following this, the wet granules were harvested. Fresh sludge for the flocculent samples was firstly centrifuged at 3000 rpm and the supernatant was decanted. Then, the pellets were rinsed using the same amount of tap water as decanted supernatant for 2–3 min. Finally, we obtained the mixture of biomass pellets and tap water, which was sparged with air for mixing.

2.2. Procedures for poly-P determination

The procedures for detecting and quantifying poly-P are shown in Fig. 2. The whole process consists of three stages: (I) Optimization of pre-treatment conditions, (II) Protocol optimization and improvement, (III) Demonstration and validation using WWTP sludge. Stage I includes three steps: Step (1) assessed the sensitivity of granular biomass to stress conditions. The physical pre-treatment resulted in destabilizing the compact structure, thereby making the biomass more sensitive to environmental stress. Step (2) involved the optimization of the concentration of EDTA and verification of its biodegradability. EDTA is as an ideal aminopolycarboxylic acid chelating agent for this test, because it binds with cations (calcium, magnesium) and therefore it avoids chemical precipitation of released ortho-P. The function of EDTA in P-release in this study was not only to stress the bacteria, but also to avoid complete disintegration of the bacteria. Hence, three different levels of EDTA (from low to high concentrations of 0.1% [3 mM], 1% [30 mM] and 1.5% [50 mM]) were examined to determine the suitable EDTA concentration for the experiments. Previous studies have reported that EDTA can be utilized as a carbon source specially by enriched bacterial cultures (Nörtemann, 1999; Thomas et al., 1998). Details of the procedures are listed under Section 2.3. To demonstrate its biodegradability in the present study, we also compared P-release with the EDTA addition under anaerobic and aerobic conditions using GS. Finally, Step 3) involved the assessment of the effects of undissociated HAC concentrations on P-release by adjusting pH.

In stage II, P-release was investigated by the two methods developed from stage I (with optimized EDTA concentration and pH), using two types of PAO-enriched sludge (GS and FS). A general concern of these tests was potential cell lysis of the microorganisms exposed to the extreme conditions, e.g., presence of VFA at low pH, EDTA or other adverse environments. Cell lysis could disrupt the stress-induced P-release response and thereby result in underestimated poly-P levels. To prevent poly-P underestimation as a consequence of cell lysis, it was important to assess whether the cells were still active. However, the extent of cell lysis during batch tests is difficult to evaluate. To demonstrate the bacteria were still alive, an aerobic P uptake test was performed after the anaerobic test (Stage II). In the final stage (Stage III), the sludge collected from full-scale installations treating domestic sewage was used to further demonstrate the feasibility of the protocol.

2.3. Optimization of physical and chemical pre-treatment and EDTA treatment

Due to the compact matrices of GS, the granules were first treated using combined physical methods (i.e. mechanical grinding and sonication) to evaluate the effect of the pre-treatment as described next. For each test, 0.15 g fresh wet granules were added to a 50 ml-Falcon tube containing 20 ml tap water. Then the sludge-water mixture was homogenized for 10 s at 800 W (Labgen tissue homogenizer, Cole-Parmer, USA) and then subjected to pulsed sonication on ice for 1 min at 36 W. EDTA was then added to the mixture with final concentrations of 0.1%, 1% and 1.5% (w/v). Finally, air or N₂ were sparged to maintain aerobic or anaerobic conditions, respectively for 3 h. Solution pH was kept constant at pH of 8.0 using 0.1 M NaOH. Samples were taken at 0 and 3 h, respectively for ortho-P measurement. For the control tests, the same procedures were carried out without EDTA addition.

2.4. Anaerobic P-release batch test by EDTA treatment or acetate addition under various low pH conditions

After physical and chemical treatment, the optimum concentration of EDTA for the following anaerobic phosphate release test was chosen. Given that the prevailing pH of anaerobic phase affects anaerobic metabolism (Mulkerrins et al., 2004), it is important to understand the behaviour of P release at various pH values during acetate addition. For each test 2 g of wet GS was transferred into a bottle which was filled with 80 ml of tap water; or 80 ml of FS was transferred after rinsing it (as described in Section 2.1). Then the mixture was continually sparged with N₂ to maintain the strictly anaerobic conditions. The carbon source (CH₃COONa·3H₂O) was quickly added to obtain a final concentration of 860 mg/L (400 mg/L COD, 6.3 mM) (Welles et al., 2015). For the EDTA test, the final concentration of EDTA was 1% (w/v, 30 mM) and the pH was maintained at 8.0. For acetate addition tests, the pH was manually controlled at various low pH conditions (4.8, 5.5, 6.0, 6.5, and 7.0) using 1M HCl during stage II. For Stage III, the pH values were 4.8, and 7.0. Samples for ortho-P measurement were collected at 0, 1.5, 3, 6, 9, 12, 21, 24, and 30 h.

2.5. Aerobic P uptake batch test for bioactivity study

At the end of anaerobic P-release, GS or FS was immediately washed for three times with a buffer containing 120 mg P/L phosphate without carbon source under anaerobic conditions. Liquid pH was controlled at 7.0 (Zhou et al., 2008). Subsequently, all the groups were instantly sparged with air for 2 h. Initial and final samples were collected for ortho-P concentration detection.

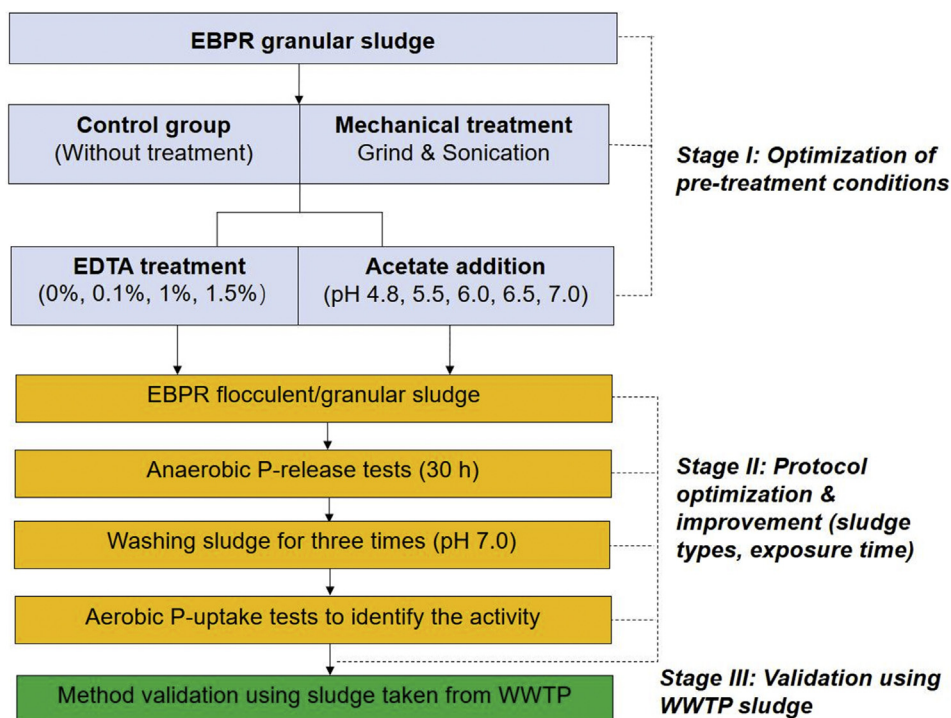


Fig. 2. Flow chart of experimental design for anaerobic phosphate release test by using granular and flocculent sludge.

2.6. Chemical analysis

Samples for the determination of soluble components were immediately filtered using membrane filters with a pore size of 0.45 μm . The sludge samples were collected for the analysis of total suspended solids (TSS) and volatile suspended solids (VSS). The measurements of TSS, VSS, total phosphorus (TP), and ortho-P (PO_4^{3-}P) were carried out according to the standard ammonium molybdate spectrophotometric methods (APHA, 2005). Samples were digested using Potassium Peroxodisulfate to convert various forms of phosphorus among biomass and bulk liquid to ortho-P for TP measurements. TSS and VSS were determined by triplicate to calculate the phosphate release per g TSS/VSS. Cations including Ca, Mg, Al, and Fe were detected by ICP-MS (XSeries II, Thermo Scientific, Germany). Acetate was measured by the gas chromatograph (Agilent 6890, USA). Three replicates were performed for each analysis.

2.7. PHREEQC program modelling theory

Phosphorus in sludge is composed of poly-P, assimilation, and chemical precipitant. pH is a key factor that influences the P formation. At low pH, precipitated forms in sludge matrix (in particular for GS) could be solubilized under the conditions of the tests; while at high pH, released ortho-P could precipitate. Besides, EDTA addition may release chemically precipitated P (e.g., ferric or aluminium phosphates) due to the chelating function of EDTA on cations (Zou et al., 2017). To clarify whether the chemical precipitation formation or re-dissolution would occur in batch tests, model predictions or simulations were performed using PHREEQC program (version 3.1.4) (Parkhurst and Appelo, 1999), which can serve as a theoretical guide for the effects of reaction conditions by examining saturation-index (SI). SI is defined as:

$$SI = \log \frac{IAP}{K_{SP}}$$

Where IAP is the free ionic activities product and K_{SP} is the thermodynamic solubility product. SI can be selected as an indicator of equilibrium and the thermodynamic basis for crystallization reaction. The solution is in equilibrium ($SI = 0$), undersaturated ($SI < 0$), or supersaturated ($SI > 0$) (Parkhurst and Appelo, 1999; Song et al., 2002).

3. Results and discussion

3.1. Effect of EDTA presence on P release under anaerobic and aerobic conditions using PAO - enriched granular sludge

The presence of EDTA causes P release regardless of aerobic or anaerobic conditions (as shown in Fig. 3a) thus supporting the hypothesis that EDTA has a stressful effect on PAOs. The initial ortho-P concentration was 0.9 ± 0.1 mg P/g TSS. Under aerobic conditions, substantial P release occurred in the EDTA addition tests, where the P release amount rose in the tests with increased EDTA addition, ranging from 1.3 to 10.4 mg P/g TSS. The P-release amounts under anaerobic conditions were slightly higher than those observed under aerobic conditions, ranging from 5.5 to 12.0 mg P/g TSS. The results indicated that in general anaerobic conditions were the most favorable for increased P release. However, under the most favorable conditions (anaerobic with EDTA), the soluble P concentration at the end of the tests accounted for only 7%–20% of TP, and thus we did not observe a substantial P-release in the duration of the test, suggesting that the EDTA could not be utilized by PAOs as a substrate. Furthermore, more phosphate was released from mechanically pre-treated PAO sludge (i.e. grind and sonication) under aerobic conditions, indicating that under these conditions, EDTA penetrated the cell wall of bacteria

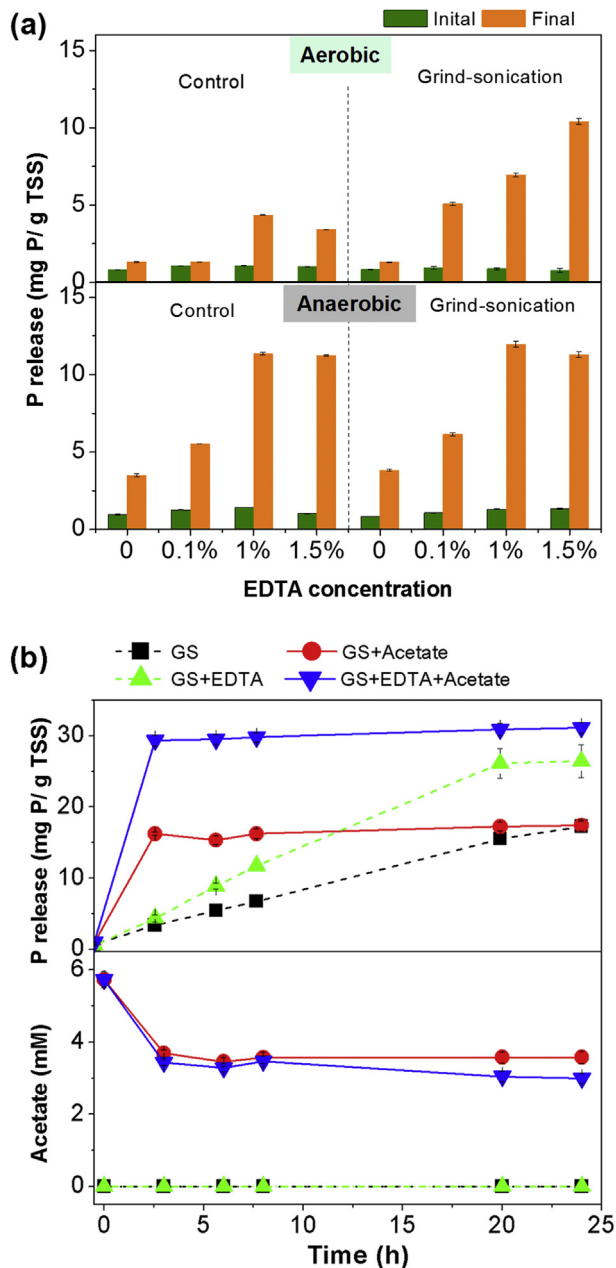


Fig. 3. (a) Effects of the presence of EDTA and sludge physical pre-treatment on the phosphate release using EBPR granular sludge under aerobic and anaerobic conditions. Tests were carried out right after dosing EDTA (Initial: 0 h) and ended after 3 h (Final) without the addition of acetate. Solution pH was controlled at 8.0. (b) EDTA addition effects on the P-release and acetate concentration during anaerobic phosphorus release tests, 1% (w/v, 30 mM) EDTA concentration.

and resulted in P-release. Differences arising from mechanical treatment also imply that the robust and compact matrix of aerobic granules plays significant roles in protecting the cells against the stressful conditions due to the extracellular polymeric substances, particularly exopolysaccharides (Lin et al., 2010). However, under anaerobic conditions for the mechanical treatment group, pre-treatment showed no substantial influences on P release. More specifically, final P concentrations (at 3 h) were 12 mg P/g TSS with both 1% and 1.5% EDTA tests. Given that there were no significant differences in P-release with the high level of EDTA under anaerobic conditions with or without pre-treatment, 1% EDTA without pre-treatment was selected as the optimal condition to carry out the

subsequent batch tests.

In agreement with previous studies (Brdjanovic et al., 1998; Hesselmann et al., 2000), typical curves of P release and acetate consumption were obtained under anaerobic conditions in our study (Fig. 3b). Generally, EDTA treatment significantly boosted the anaerobic P-release. It was observed that GS with the EDTA treatment (GS + EDTA + Acetate) showed a higher P-release capacity, characterized by the final P-release amount of 31.1 ± 0.8 mg P/g TSS (70.0 ± 0.2 mg/L) within 24 h, which was 1.8 fold of the amount released in the no EDTA test (GS + Acetate). Also, acetate uptake was incomplete and stopped within 3 h with the presence of EDTA. This led to a higher ratio of P-release and acetate uptake (P/HAC) up to 0.93 P-mol/C-mol for the EDTA addition than the control test (up to 0.57 P-mol/C-mol) and typical P/HAC ratios as high as 0.75 P-mol/C-mol (Schuler and Jenkins, 2003) of highly enriched PAO cultures. The increased P/HAC ratio demonstrated a significant contribution of poly-P in the anaerobic conversions, attributing to stressful effects of EDTA on anaerobic P-release of PAOs. In addition, the results indicated that the EDTA treatment successfully uncoupled P depletion from the acetate uptake. According to the anaerobic metabolism of PAOs, internally-stored poly-P were hydrolyzed for ATP generation (Fig. 1). Thus, the cultures resisting environmentally stressful conditions require more energy, thus inducing more P release from EBPR cultures. In this regard, the released P using 1% EDTA is considered as a fraction of poly-P.

3.2. P release from PAO enriched granular sludge with acetate addition at different pH values

In order to investigate the influence of un-dissociated acetic acid on the anaerobic metabolism of P-release of PAOs, the sludge was exposed to HAC at different pH values ranging from 4.8 to 7.0. According to pKa value of HAC ($pK_a = 4.8$), the ratios of $[HAC]/([HAC] + [A^-])$ were 0.50, 0.16, 0.06, 0.02 and 0.01 under pH values of 4.8, 5.5, 6.0, 6.5 and 7.0, respectively. Seemingly phosphate concentration increased linearly during the first 3 h for the tests with pH conducted at all pH values (Fig. 4a). Subsequently, the concentration gradually rose to different maximum levels in individual tests. Previous literature has reported that pH presents substantial influences on the amount and specific rate of P release (Boswell et al., 1999; Liu et al., 1996). HAC of approximately 4.2–6.1 mM was still detected after 30 h, indicating that in these tests VFA did not become limiting. Fig. 4b shows the sludge specific P-release amount under anaerobic conditions at the different pH values as well as the release ortho-P to total P fraction. An increase in phosphate concentration was observed under low pH conditions. In particular, the highest P concentration of around 48.5 ± 2.4 mg P/g TSS (84.5 ± 4.2 mg P/L, 98% of TP) was detected at a pH of 4.8. The corresponding fractions at pH 5.5, 6.0, 6.5 and 7.0 represented approximately 86%, 78%, 71% and 66% of TP in PAO-enriched granular sludge (w/w), respectively. As reported previously (Boswell et al., 1999; Liu et al., 1996; Smolders et al., 1994), acidic conditions (pH lower than 6.5) inactivate the acetate metabolism thereby inhibiting P release when exposure time is shorter than 1.5 h. Similarly, Boswell et al. (1999) found that anaerobic P-release (at 30 °C for 30 h) continuously increased with pH incrementally increasing from 5.0 to 8.0. However, our study indicated that low pH enhanced the P release even at pH 4.8. These differences are attributed to toxification arising from undissociated HAC at low pH, which allowed undissociated HAC to penetrate the cell membrane freely at such a high rate that it became toxic (Smolders et al., 1994). In this case, PAOs were forced to pump out the excess HAC, which could consume more energy, finally resulting in massive P-release at low pH.

After the anaerobic release test, the sludge was washed under

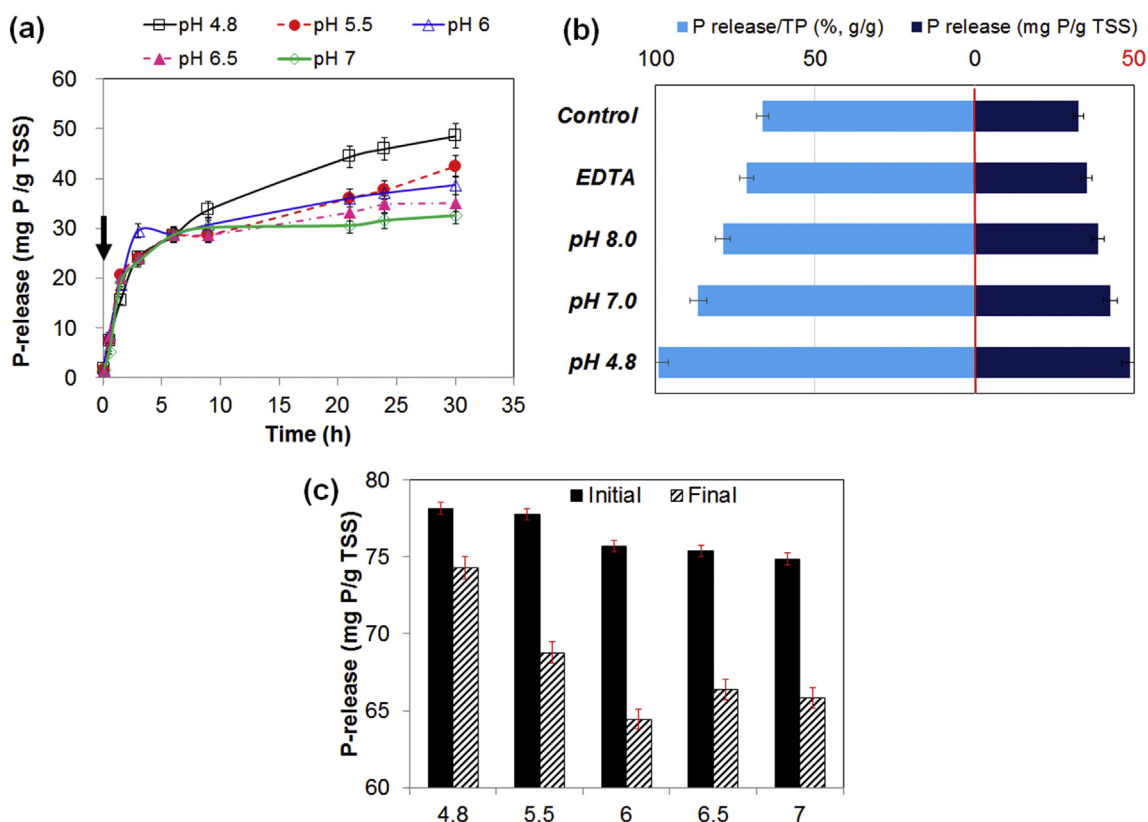


Fig. 4. (a) Effects of pH on anaerobic phosphate release. The arrows indicate the acetate addition point. (b) Maximum P-release amount at different pH values. (c) Initial and final phosphate concentration under aerobic conditions for bioactivity verification. Error bars show standard deviations ($n = 3$).

anaerobic conditions in a phosphate buffer (containing 120 mg P/L and no VFA or COD) and subjected to aerobic conditions (for 2 h) to test the aerobic uptake activity and thereby the cell integrity. As shown in Fig. 4c and 5% of phosphate was taken in at pH of 4.8, while the corresponding proportion accounted for 12%–16% in other tests. The uptake of phosphate suggested that the metabolic activity of PAOs was still present and that cells were not disintegrated. On the basis of the results, pH of 4.8 and 7.0 were chosen to conduct the subsequent tests.

3.3. Comparison of two strategies to uncouple P release and HAC uptake: acetate addition at low pH and EDTA treatment

To further demonstrate the feasibility of the two methods, i.e., acetate addition at low pH and the presence of EDTA, P-release tests were carried out using granular and flocculent EBPR sludge without mechanical pre-treatment. As demonstrated above, treatments with pH 4.8, pH 7.0, pH 8.0 (as a positive control for aerobic uptake activity), with addition of 1% EDTA (pH 8.0) versus control (without adjusting pH), were used to evaluate the level of P release. Fig. 5a and b shows the batch tests of PAO enriched granular and flocculent EBPR sludge. Under stressful conditions (both pH 4.8 and EDTA addition), spherical granules were largely intact after 30 h, further indicating the robust structure of aerobic granular sludge. Fig. 5c and e depict the anaerobic phosphate release into the solution in the five groups using granular sludge during 30 h. After sparging with N_2 gas, phosphate was immediately released into the solution by the PAO-enriched cultures. In Fig. 5c, the released phosphate initially presented a linear increase for 3 h. Afterwards, the phosphate concentration rose gradually to a stable level. After 9 h the phosphate profiles in all the tests became stable, except for the

phosphate profile from the test conducted at pH 4.8. The EDTA treatment presented the highest initial specific P-release rate, reaching 21.9 mg P/(g TSS·h), presumably underlying the stronger stressful effects of EDTA presence on the anaerobic P release behaviour of PAOs. With respect to the phosphate release amount (Fig. 5e), at pH 4.8, the phosphate concentration in the solution accounted for 96% of TP after 30 h, indicating that the amorphous phosphate precipitate dissolved into the solution. The highest released P concentration was 68.4 ± 2.7 mg P/g TSS (90 ± 1.8 mg/L), which was 2.2 folds of that in the control test. Final P concentrations resulted from EDTA treatment were the second highest, representing 54.5 ± 2.1 mg P/g TSS (69.5 ± 3.5 mg/L, 78% of TP). In agreement with P-release results of GS, phosphate was released from cells instantly by flocculent sludge, particularly EDTA tests witnessed the highest P release rate of 16 mg P/g TSS·h within 3 h (Fig. 5d and f). EDTA treatment resulted in the highest phosphate release, but it took a longer time to release phosphate (over 9 h) in comparison with GS. To further identify the negative impact on sludge activity, a P-uptake test under aerobic conditions was performed after the anaerobic release tests (Fig. 5g and h). The results illustrate that PAOs were still active after an EDTA-exposure of 30 h, because partial phosphate for EDTA group was taken up by PAOs in granular sludge, in line with disrupted HAC uptake after 3 h in Fig. 3b. A quite small fraction of phosphate (0.653 mg P/g TSS) was taken in with pH at 4.8, which inhibited the PAOs' activity at low pH. Overall, P-release and uptake tests demonstrated that the EDTA treatment was superior and can be an effective way of uncoupling acetate uptake and P-release.

Fig. 6 illustrates the simulated and calculated SI values which could be used to simulate the possibility of chemical precipitation formation of ortho-P at high pH and the dissolution of intrinsic

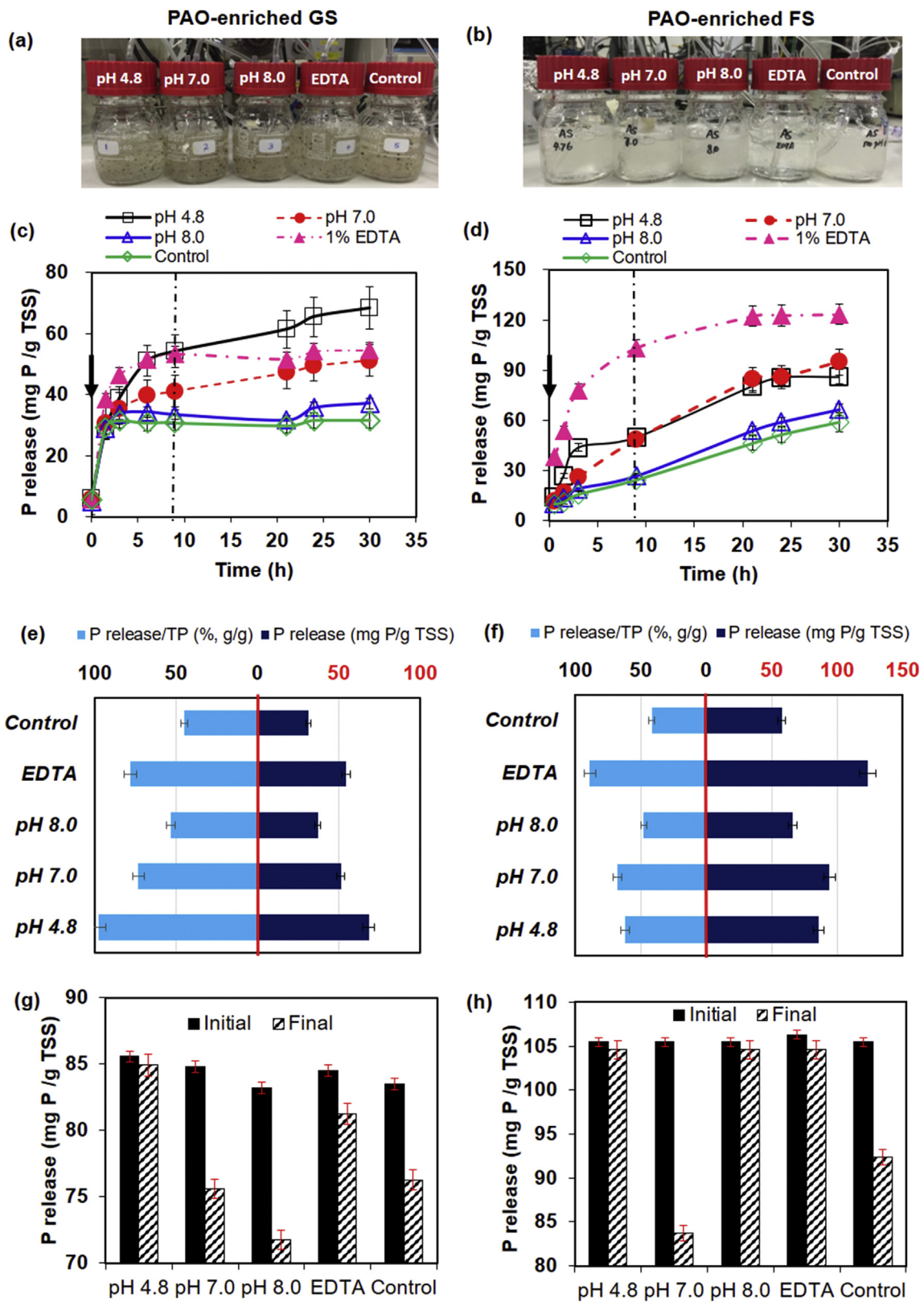


Fig. 5. (a, b) Anaerobic phosphate release tests using PAO enriched granular sludge (GS) or flocculent sludge (FS). (c, d) Anaerobic phosphate release concentration during 30 h at different pH with acetate addition or EDTA presence. The arrows indicate the acetate addition point. (e, f) Maximum phosphate released and the fraction in total phosphate in five groups. (g, h) The initial and final phosphate concentration under aerobic conditions. Error bars show standard deviations ($n = 3$).

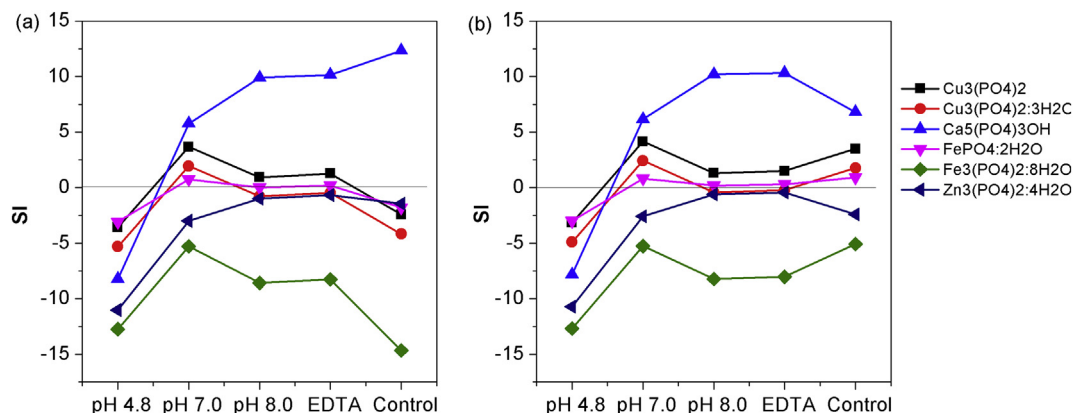


Fig. 6. Saturation index (SI) calculation and simulation for the phosphate release tests at variable pH with acetate addition or the EDTA presence: (a) granular sludge, (b) flocculent sludge.

chemical precipitation in the sludge at low pH. At pH 4.8, the SI values of phosphate precipitants were lower than zero, ranging from -3.6 to -11 , indicating the aqueous phase was undersaturated and the chemical precipitant can dissolve into the aqueous phase. At low pH, PO_4^{3-} could be released along with cations such as Mg^{2+} and Ca^{2+} , which are responsible for sludge P precipitation (Latif et al., 2017). Regarding the EDTA treatment, the SI value of $\text{FePO}_4 \cdot 2\text{H}_2\text{O}$ was -8.3 . SI values for other chemical precipitants were above and near zero, indicating that the chemical precipitants may occur under these reaction conditions. However, the phenomenon of dissolution or chemical precipitation largely depends on the ion concentration in the supernatant and the composition of chemical precipitants in the sludge. Although sludge matrix composition is unknown, the relevant counter ions (Ca, Mg, Al and Fe) were observed at a very low level in the initial and final samples for acetate or EDTA addition for two kinds of sludge (Table A1 in Supplementary Information). Therefore, it was speculated that the influence of chemical precipitants was relatively small on P-release determination. Meanwhile, it should be noticed that the EDTA method has limitations to study the P-release behaviour of sludge samples which were subjected to chemical precipitation. Because of the chelating effects of EDTA on chemical P precipitation (Zou et al., 2017), poly-P values may be overestimated. In this regard, the sludge samples were recommended to be taken from EBPR plants without chemical addition to precipitate P.

3.4. Application of the proposed methods using real sludge samples from WWTPs

To further demonstrate the capability of the proposed methods, two kinds of sludge samples were taken from WWTPs, i.e. GS-WWTP and FS-WWTP. Fig. 7a and b presents results for the P-release under anaerobic conditions during a period of 30 h. In line with the aforementioned results, a higher P-release rate for both sludge samples was witnessed with EDTA treatment compared with other groups. Particularly, P-release rate for EDTA groups was up to $13.4 \text{ mg P}/(\text{g TSS} \cdot \text{h})$ within 3 h; while the corresponding data in other groups were $2.3\text{--}10.8 \text{ mg P}/(\text{g TSS} \cdot \text{h})$. The final P-release amounts after 30 h are displayed in Fig. 7c and d. As the figures indicate, the presence of EDTA resulted in the maximum P release for both granular sludge and floc sludge, representing 29 and $31 \text{ mg P}/\text{g TSS}$, which were 1.8–2.5 fold the values from control tests. Notably, FS is more sensitive than GS, given that the P-release amount and rate at pH 4.8 were lower than other tests, which is likely associated with toxic effects of over intracellular acid accumulation.

3.5. Feasibility of two methods and suggestions for future experiment

Comparison of the two methods indicates that the observed P-release in the tests with 9 h exposure to EDTA (1% w/v, 30 mM) at pH 8 is considered to be the biological available poly-P. The EDTA treatment resulted in the highest P-release amount and highest release rate less than 9 h in most sludge types with an exception for GS samples from a lab-scale SBR system for which the highest P-release was observed with extremely-high fraction of 98% TP. In accordance with the previous study (Zhang et al., 2013), this present study showed that the TP contents in GS or FS from EBPR systems took a fraction of 4%–14% of the dry solids. Poly-P represented the main form with a content ranging from 77% to 89% of TP by the EDTA treatment, leaving 8–16 mg P/g TSS, which is considered as a minimal organic bound P content associated with active biomass. Finally, aerobic activity tests confirmed that the bacteria were still active and that the P-release is not caused by cell disintegration.

Regarding the method of pH treatment, it seems very effective for GS from the lab-scale SBR with a high P-release amount, accounting for 98% of TP (Fig. 5e), which was even higher than that of the EDTA treatment. However, this finding suggests that other phosphate forms have contributed to this number. Considering that various other forms of P, including ortho-P, inorganic phosphates (pyro, meta, and poly-P), organic phosphates also exist in the sludge. For instance, a recent study reported that the P-content in extracted extracellular polymeric substances (EPS) of EBPR sludge took an unneglectable fraction of $6.7 \pm 1.1\%$ of TP (Zhang et al., 2013). Especially, the P-release amount was observed to approach to that of EDTA test at 9 h, reaching $54 \pm 2 \text{ mg P}/\text{g TSS}$. However, the P-release amount gradually increased with a very low release rate of $<1.4 \text{ mg P}/(\text{g TSS} \cdot \text{h})$ after 9 h, which could be attributed to dissolution of inert precipitates (as supported by the SI) and cell lysis during the long-time exposure to low-pH treatment, thus leading to the hydrolysis of long-chain poly-P released from cells. Overall, EDTA treatment was found effective to quantify the poly-P concentration based on the kinetic analysis and P release amount. The method of acetate addition at low pH values depended upon the sludge types, in which GS due to a robust structure was more applicable than FS, but the experimental time should be less than 9 h. For further use of the pH treatment method for granular sludge, it is required to validate if it is applicable to different types of aerobic GS.

For confirmation of the results, the poly-P content in the cells was also estimated using the equation below

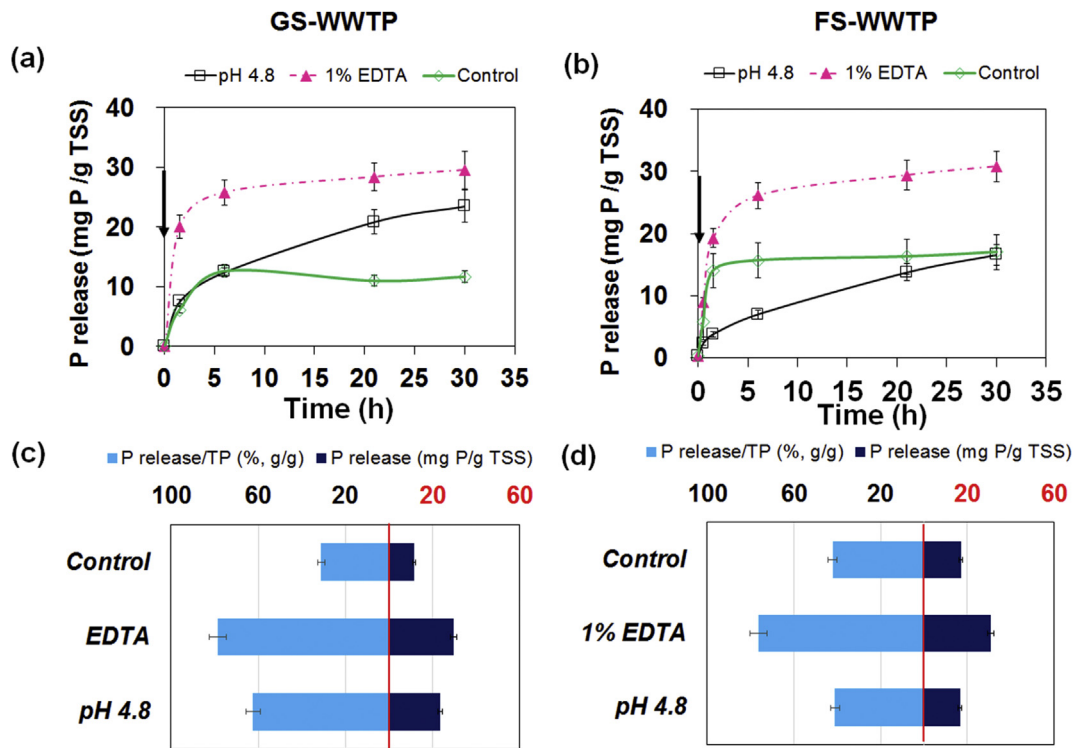


Fig. 7. Anaerobic phosphate release with the presence of acetate at different pH values and with EDTA treatment using samples from WWTPs (a, c) granular sludge (GS); and (b, d) Flocculent sludge (FS). All the tests were conducted in triplicate. The arrows indicate the acetate addition point.

$$P \text{ cell content (g P/g TSS)} = (\text{TSS-VSS})/\text{TSS} \cdot 1/3.23 \quad (1)$$

Assuming that the ash content is mainly poly-P ($\text{Mg}_{0.33} \cdot \text{K}_{0.33} \cdot \text{PO}_3$) (Brdjanovic et al., 1996; Marcelino et al., 2009; Tayà et al., 2013). As shown in Table A2, the measured poly-P contents (0.030–0.123 g P/g TSS) by EDTA treatment for all samples were much lower than the calculated data (0.070–0.204 g P/g TSS). In particular, calculated poly-P was even unreasonably higher than TP, indicating a substantial part of the ash is not poly-P. Moreover, the results indicate that the method of ash-content dependent calculation exists limitations. Many factors including operational conditions (HRT, SRT), sludge types, sludge compositions (e.g. EPS), and microbial communities may influence the poly-P concentration, which should be taken into account for the poly-P calculations (Welles et al., 2015). However, the EDTA treatment developed in this study showed robustness which can achieve excess anaerobic P-release with limited influences of other factors.

4. Conclusions

In this study, practical implementable strategies to quantify the poly-P content in the PAOs were investigated. The strategies were based on uncoupling P-release from VFA uptake under extreme environmental conditions, including: (i) acetate addition at low pH; and (ii) the presence of EDTA in the aqueous solution. A protocol associated with poly-P quantification was developed. The proposed strategies demonstrated improvement in degradation of poly-P and subsequent release of ortho-P into the bulk liquid.

- Under stressful conditions, poly-P consumption is not stoichiometrically limited by the VFA-uptake and therefore full hydrolysis of poly-P to ortho-phosphate under anaerobic conditions can be achieved.

- EDTA treatment is a feasible method to quantify the poly-P concentration in both flocculent and granular sludge. Exposure to 1% (w/v) of EDTA at pH 8.0 is optimal for maximized anaerobic P-release.
- The recommended experimental time for anaerobic P-release treatment should not be over 9 h, given that longer exposure at stressful conditions inhibits the bioactivity of PAOs.
- TP in GS or FS samples of biological phosphate removal system takes a fraction of 4%–14% of dry solids, and mainly represents in the forms of poly-P ranging from 77% to 89% of TP as determined by the EDTA treatment test.

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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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.watres.2019.115228>.

Acknowledgments.

Nomenclature

poly-P Polyphosphates
GS Granular sludge

FS	Flocculent sludge
PAO	Phosphate accumulating organism
GAO	Glycogen accumulating organism
TSS	Total suspended solids
VSS	Volatile suspended solids
EBPR	Enhanced biological phosphorus removal
ATP	Adenosine triphosphate
VFA	Volatile fatty acid
TCA	Tricarboxylic acid
PHA	Polyhydroxyalkanoate
HAc	Acetic acid
NADH	Nicotinamide adenine dinucleotide
Acetyl-CoA	Acetyl coenzyme A
SI	Saturation index
EPS	Extracellular polymeric substances

References

- Acevedo, B., Oehmen, A., Carvalho, G., Seco, A., Borras, L., Barat, R., 2012. Metabolic shift of polyphosphate-accumulating organisms with different levels of polyphosphate storage. *Water Res.* 46 (6), 1889–1900.
- APHA, 2005. Standard Methods for the Examination of Water and Wastewater. American Water Works Association and Water Environmental Federation, Washington, DC, USA.
- Aravind, J., Saranya, T., Kanmani, P., 2015. Optimizing the production of polyphosphate from acinetobacter townieri. *Glob. J. Environ. Sci. Manag.* 1 (1), 63.
- Aschar-Sobbi, R., Abramov, A.Y., Diao, C., Kargacin, M.E., Kargacin, G.J., French, R.J., Pavlov, E., 2008. High sensitivity, quantitative measurements of polyphosphate using a new DAPI-based approach. *J. Fluoresc.* 18 (5), 859–866.
- Ault-Riché, D., Fraley, C.D., Tzeng, C.-M., Kornberg, A., 1998. Novel assay reveals multiple pathways regulating stress-induced accumulations of inorganic polyphosphate in *Escherichia coli*. *J. Bacteriol.* 180 (7), 1841–1847.
- Baba, Y., Yoza, N., Ohashi, S., 1985. Simultaneous determination of phosphate and phosphonate by flow injection analysis and high-performance liquid chromatography with a series detection system. *J. Chromatogr. A* 318, 319–324.
- Baxter, M., Jensen, T., 1980. A study of methods for in situ X-ray energy dispersive analysis of polyphosphate bodies in *Plectonema boryanum*. *Arch. Microbiol.* 126 (3), 213–215.
- Boswell, C.D., Dick, R.E., Macaskie, L.E., 1999. The effect of heavy metals and other environmental conditions on the anaerobic phosphate metabolism of *Acinetobacter johnsonii*. *Microbiology* 145 (7), 1711–1720.
- Brdjanovic, D., Hooijmans, C., Van Loosdrecht, M., Alaerts, G., Heijnen, J., 1996. The dynamic effects of potassium limitation on biological phosphorus removal. *Water Res.* 30 (10), 2323–2328.
- Brdjanovic, D., Loosdrecht, M.C.v., Hooijmans, C.M., Alaerts, G.J., Heijnen, J.J., 1997. Temperature effects on physiology of biological phosphorus removal. *J. Environ. Eng.* 123 (2), 144–153.
- Brdjanovic, D., Van Loosdrecht, M., Hooijmans, C., Mino, T., Alaerts, G., Heijnen, J., 1998. Effect of polyphosphate limitation on the anaerobic metabolism of phosphorus-accumulating microorganisms. *Appl. Microbiol. Biotechnol.* 50 (2), 273–276.
- Bru, S., Jiménez, J., Canadell, D., Ariño, J., Clotet, J., 2017. Improvement of biochemical methods of polyP quantification. *Microb. Cell* 4 (1), 6.
- Choi, B.K., Hercules, D.M., Houalla, M., 2000. Characterization of polyphosphates by electrospray mass spectrometry. *Anal. Chem.* 72 (20), 5087–5091.
- Clark, J.E., Beegen, H., Wood, H.G., 1986. Isolation of intact chains of polyphosphate from "*Propionibacterium shermanii*" grown on glucose or lactate. *J. Bacteriol.* 168 (3), 1212–1219.
- Clark, J.E., Wood, H.G., 1987. Preparation of standards and determination of sizes of long-chain polyphosphates by gel electrophoresis. *Anal. Biochem.* 161 (2), 280–290.
- Comeau, Y., Hall, K., Hancock, R., Oldham, W., 1986. Biochemical model for enhanced biological phosphorus removal. *Water Res.* 20 (12), 1511–1521.
- Diaz, J., Ingall, E., Benitez-Nelson, C., Paterson, D., de Jonge, M.D., McNulty, I., Brandes, J.A., 2008. Marine polyphosphate: a key player in geologic phosphorus sequestration. *Science* 320 (5876), 652–655.
- Diaz, J., Ingall, E., Vogt, S., de Jonge, M.D., Paterson, D., Rau, C., Brandes, J.A., 2009. Characterization of phosphorus, calcium, iron, and other elements in organisms at sub-micron resolution using X-ray fluorescence spectromicroscopy. *Limnol. Oceanogr. Methods* 7 (1), 42–51.
- Gu, A.Z., Saunders, A., Neethling, J., Stensel, H., Blackall, L., 2008. Functionally relevant microorganisms to enhanced biological phosphorus removal performance at full-scale wastewater treatment plants in the United States. *Water Environ. Res.* 80 (8), 688–698.
- Günther, S., 2011. Population Structure and Dynamics of Polyphosphate Accumulating Organisms in a Communal Wastewater Treatment Plant.
- Hesselmann, R., Von Rummell, R., Resnick, S.M., Hany, R., Zehnder, A., 2000. Anaerobic metabolism of bacteria performing enhanced biological phosphate removal. *Water Res.* 34 (14), 3487–3494.
- Hupfer, M., Gloss, S., Schmieder, P., Grossart, H.-P., 2008. Methods for detection and quantification of polyphosphate and polyphosphate accumulating microorganisms in aquatic sediments. *Int. Rev. Hydrobiol.* 93 (1), 1.
- Kulakovskaya, T.V., Vagabov, V.M., Kulaev, I.S., 2012. Inorganic polyphosphate in industry, agriculture and medicine: modern state and outlook. *Process Biochem.* 47 (1), 1–10.
- Latif, M.A., Mehta, C.M., Batstone, D.J., 2017. Influence of low pH on continuous anaerobic digestion of waste activated sludge. *Water Res.* 113, 42–49.
- Lin, Y., de Kreuk, M., Van Loosdrecht, M., Adin, A., 2010. Characterization of alginate-like exopolysaccharides isolated from aerobic granular sludge in pilot-plant. *Water Res.* 44 (11), 3355–3364.
- Liu, W.-T., Mino, T., Matsuo, T., Nakamura, K., 1996. Biological phosphorus removal processes-effect of pH on anaerobic substrate metabolism. *Water Sci. Technol.* 34 (1–2), 25–32.
- Majed, N., Chernenko, T., Diem, M., Gu, A.Z., 2012a. Identification of functionally relevant populations in enhanced biological phosphorus removal processes based on intracellular polymers profiles and insights into the metabolic diversity and heterogeneity. *Environ. Sci. Technol.* 46 (9), 5010–5017.
- Majed, N., Li, Y., Gu, A.Z., 2012b. Advances in techniques for phosphorus analysis in biological sources. *Curr. Opin. Biotechnol.* 23 (6), 852–859.
- Majed, N., Matthäus, C., Diem, M., Gu, A.Z., 2009. Evaluation of intracellular polyphosphate dynamics in enhanced biological phosphorus removal process using Raman microscopy. *Environ. Sci. Technol.* 43 (14), 5436–5442.
- Marcelino, M., Guisasola, A., Baeza, J.A., 2009. Experimental assessment and modelling of the proton production linked to phosphorus release and uptake in EBPR systems. *Water Res.* 43 (9), 2431–2440.
- Martin, H.G., Ivanova, N., Kunin, V., Warnecke, F., Barry, K.W., McHardy, A.C., Yeates, C., He, S., Salamov, A.A., Szeto, E., 2006. Metagenomic analysis of two enhanced biological phosphorus removal (EBPR) sludge communities. *Nat. Biotechnol.* 24 (10), 1263.
- Mino, T., Van Loosdrecht, M., Heijnen, J., 1998. Microbiology and biochemistry of the enhanced biological phosphate removal process. *Water Res.* 32 (11), 3193–3207.
- Mukherjee, C., Ray, K., 2015. An improved method for extraction and quantification of polyphosphate granules from microbial cells. *Protoc. Exch.* <https://doi.org/10.1038/protex.2015.06710>.
- Mulkerrins, D., Dobson, A., Colleran, E., 2004. Parameters affecting biological phosphate removal from wastewaters. *Environ. Int.* 30 (2), 249–259.
- Nörtemann, B., 1999. Biodegradation of EDTA. *Appl. Microbiol. Biotechnol.* 51 (6), 751–759.
- Oehmen, A., Lemos, P.C., Carvalho, G., Yuan, Z., Keller, J., Blackall, L.L., Reis, M.A., 2007. Advances in enhanced biological phosphorus removal: from micro to macro scale. *Water Res.* 41 (11), 2271–2300.
- Ohtomo, R., Sekiguchi, Y., Kojima, T., Saito, M., 2008. Different chain length specificity among three polyphosphate quantification methods. *Anal. Biochem.* 383 (2), 210–216.
- Parkhurst, D.L., Appelo, C., 1999. User's Guide to PHREEQC (Version 2): A Computer Program for Speciation, Batch-Reaction, One-Dimensional Transport, and Inverse Geochemical Calculations.
- Rao, N.N., Gómez-García, M.R., Kornberg, A., 2009. Inorganic polyphosphate: essential for growth and survival. *Annu. Rev. Biochem.* 78, 605–647.
- Saia, S.M., Sullivan, P.J., Regan, J.M., Carrick, H.J., Buda, A.R., Locke, N.A., Walter, M.T., 2017. Evidence for polyphosphate accumulating organism (PAO)-mediated phosphorus cycling in stream biofilms under alternating aerobic/anaerobic conditions. *Freshw. Sci.* 36 (2), 284–296.
- Saito, K., Ohtomo, R., Kuga-Uetake, Y., Aono, T., Saito, M., 2005. Direct labeling of polyphosphate at the ultrastructural level in *Saccharomyces cerevisiae* by using the affinity of the polyphosphate binding domain of *Escherichia coli* exopolysphatase. *Appl. Environ. Microbiol.* 71 (10), 5692–5701.
- Schuler, A.J., Jenkins, D., 2003. Enhanced biological phosphorus removal from wastewater by biomass with different phosphorus contents, part I: experimental results and comparison with metabolic models. *Water Environ. Res.* 75 (6), 485–498.
- Serafim, L.S., Lemos, P.C., Levantesi, C., Tandoi, V., Santos, H., Reis, M.A., 2002. Methods for detection and visualization of intracellular polymers stored by polyphosphate-accumulating microorganisms. *J. Microbiol. Methods* 51 (1), 1–18.
- Smolders, G., Van der Meij, J., Van Loosdrecht, M., Heijnen, J., 1994. Model of the anaerobic metabolism of the biological phosphorus removal process: stoichiometry and pH influence. *Biotechnol. Bioeng.* 43 (6), 461–470.
- Song, Y., Hahn, H.H., Hoffmann, E., 2002. Effects of solution conditions on the precipitation of phosphate for recovery: a thermodynamic evaluation. *Chemosphere* 48 (10), 1029–1034.
- Tarayre, C., Nguyen, H.-T., Brognaux, A., Delepierre, A., De Clercq, L., Charlier, R., Michels, E., Meers, E., Delvigne, F., 2016. Characterisation of phosphate accumulating organisms and techniques for polyphosphate detection: a review. *Sensors* 16 (6), 797.
- Tayá, C., Guerrero, J., Vanneste, G., Guisasola, A., Baeza, J.A., 2013. Methanol-driven enhanced biological phosphorus removal with a syntrophic consortium. *Biotechnol. Bioeng.* 110 (2), 391–400.
- Thomas, R.A., Lawlor, K., Bailey, M., Macaskie, L.E., 1998. Biodegradation of metal-EDTA complexes by an enriched microbial population. *Appl. Environ. Microbiol.* 64 (4), 1319–1322.
- Van Loosdrecht, M., Hooijmans, C., Brdjanovic, D., Heijnen, J., 1997. Biological phosphate removal processes. *Appl. Microbiol. Biotechnol.* 48 (3), 289–296.

- Welles, L., Tian, W., Saad, S., Abbas, B., Lopez-Vazquez, C., Hooijmans, C., van Loosdrecht, M., Brdjanovic, D., 2015. Accumulibacter clades Type I and II performing kinetically different glycogen-accumulating organisms metabolisms for anaerobic substrate uptake. *Water Res.* 83, 354–366.
- Wentzel, M., Dold, P., Ekama, G., Marais, G.v.R., 1985. Kinetics of biological phosphorus release. *Water Sci. Technol.* 17 (11–12), 57–71.
- Zhang, H.-L., Fang, W., Wang, Y.-P., Sheng, G.-P., Zeng, R.J., Li, W.-W., Yu, H.-Q., 2013. Phosphorus removal in an enhanced biological phosphorus removal process: roles of extracellular polymeric substances. *Environ. Sci. Technol.* 47 (20), 11482–11489.
- Zhou, Y., Pijuan, M., Zeng, R.J., Lu, H., Yuan, Z., 2008. Could polyphosphate-accumulating organisms (PAOs) be glycogen-accumulating organisms (GAOs)? *Water Res.* 42 (10), 2361–2368.
- Zou, J., Zhang, L., Wang, L., Li, Y., 2017. Enhancing phosphorus release from waste activated sludge containing ferric or aluminum phosphates by EDTA addition during anaerobic fermentation process. *Chemosphere* 171, 601–608.