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**DOI**
10.1016/j.watres.2020.115531

**Publication date**
2020

**Document Version**
Final published version

**Published in**
Water Research

Water Research 172 (2020) 115531

Contents lists available at ScienceDirect

Water Research

journal homepage: www.elsevier.com/locate/watres

# Biological phosphorus removal in seawater-adapted aerobic granular sludge

Danny R. de Graaff [a, *], Mark C.M. van Loosdrecht [a], Mario Pronk [a, b]

[a] Department of Biotechnology, Delft University of Technology, van der Maasweg 9, 2629, HZ, Delft, the Netherlands
[b] Royal HaskoningDHV, Laan1914 35, Amersfoort, 3800, AL, the Netherlands

ARTICLE INFO

*Article history:*
Received 28 October 2019
Received in revised form
18 January 2020
Accepted 20 January 2020
Available online 21 January 2020

*Keywords:*
Aerobic granular sludge
Seawater
Salinity
Accumulibacter phosphatis
Biological phosphorus removal

ABSTRACT

Seawater can be introduced or intrude in sewer systems and can thereby negatively influence biological wastewater treatment processes. Here we studied the impact of artificial seawater on the enhanced biological phosphate removal (EBPR) process performance by aerobic granular sludge (AGS) with synthetic wastewater. Process performance, granule stability and characteristics as well as microbial community of a seawater-adapted AGS system were observed. In seawater conditions strong and stable granules formed with an $SVI_5$ of 20 mL/g and a lower abrasion coefficient than freshwater-adapted granules. Complete anaerobic uptake of acetate, anaerobic phosphate release of 59.5 ± 4.0 mg/L $PO_4^{3-}$-P (0.35 mg P/mg HAc), and an aerobic P-uptake rate of 3.1 ± 0.2 mg P/g VSS/h were achieved. The dominant phosphate accumulating organisms (PAO) were the same as for freshwater-based aerobic granular sludge systems with a very high enrichment of *Ca.* Accumulibacter phosphatis clade I, and complete absence of glycogen accumulating organisms. The effect of osmotic downshocks was tested by replacing influent seawater-based medium by demineralized water-based medium. A temporary decrease of the salinity in the reactor led to a decreased phosphate removal activity, while it also induced a rapid release of COD by the sludge, up to 45.5 ± 1.7 mg COD/g VSS. This is most likely attributed to the release of osmolytes by the cells. Recovery of activity was immediately after restoring the seawater feeding. This work shows that functioning of aerobic granular sludge in seawater conditions is as stable as in freshwater conditions, while past research has shown a negative effect on operation of AGS processes with NaCl-based wastewater at the same salinity as seawater.



## 1. Introduction

Aerobic granular sludge (AGS) is a technology for wastewater treatment in which chemical oxygen demand (COD), nitrogen, and phosphate can be removed in a single process step (Liu and Tay, 2004; Arrojo et al., 2004; De Kreuk et al., 2005). This technology has successfully been introduced at full-scale for domestic wastewater, which predominantly contains low levels of salinity (freshwater) (Pronk et al., 2015a). Municipal wastewater can also contain high fractions of seawater due to intrusion of seawater or saline groundwater in the sewer system, industrial activity or use of seawater for toilet flushing (Lefebvre and Moletta, 2006; Sefelnasr and Sherif, 2014; Liu et al., 2016). Knowledge about the impact of seawater on the AGS process is therefore essential for maintaining good process performance.

Some crucial aspects for a maintaining a stable effluent quality from the AGS process are nitrification and enhanced biological phosphate removal (EBPR). There is already a substantial body of literature on the effect of salinity on nitrogen conversions (Dinçer and Kargi, 1999; Figueroa et al., 2008; Corsino et al., 2016; Gonzalez-Silva et al., 2016; Li et al., 2017). However, literature on the effect of seawater on EBPR is lacking. The effect of saline wastewater has been studied in either NaCl-supplemented wastewater processes or in NaCl-based enrichment cultures of *Ca.* Accumulibacter phosphatis (Intrasungkha et al., 1999; Welles et al., 2015; Wang et al., 2018). Saline conditions due to NaCl in AGS systems leads to nitrite accumulation which leads to toxicity to polyphosphate accumulating organisms (PAO) (Bassin et al., 2011). Suppression of nitrification decreased the effect of NaCl salinity but still led to a decrease in PAO activity at concentrations of 33 g/L NaCl, which is a similar salinity to seawater (Pronk et al., 2014). Studies on *Ca.* Accumulibacter phosphatis enrichments observed an

* Corresponding author.
*E-mail address:* D.R.deGraaff@tudelft.nl (D.R. de Graaff).

https://doi.org/10.1016/j.watres.2020.115531

increase in maintenance requirements and a decrease in anaerobic COD uptake and aerobic phosphate uptake (Welles et al., 2014, 2015).

EBPR in seawater was unsuccessful in a moving bed biofilm reactor (MBBR), and also shown to be problematic for AGS in high-saline domestic wastewater (Vallet et al., 2009; Thwaites et al., 2018). The reasons for failure were not reported. Successful EBPR in an AGS system in seawater conditions has not been reported yet. Therefore, we focused on the stability and effect of seawater-based biological phosphorus removal in aerobic granular sludge.

Salinity levels in full-scale wastewater treatment plants are prone to fluctuations, due to e.g. rainfall situations. Rainwater has low concentrations of chlorides (<1.0 mg/L) and low conductivity (20–30 μS/cm), which is even in the range of demineralized water (Thomas and Greene, 1993; Valsecchi et al., 1997). The effect of sudden exposure of seawater-adapted aerobic granules to salinity variations is therefore of great importance for maintaining a stable process operation.

In this study, we characterized the EBPR process performance, stability, granule characteristics, and microbial community of a seawater-adapted AGS system. The goal of the study is to assess the impact of seawater on *Ca.* Accumulibacter phosphatis in a seawater-based AGS system. We tested the effect of salinity variations on the biological removal of phosphorus.

## 2. Materials & methods

### 2.1. Reactor operation

A 3.0 L bubble column (5.6 cm diameter) was operated as a sequencing batch reactor (SBR). The inoculation source was Nereda® sludge from wastewater treatment plant Utrecht, the Netherlands, which was fed with municipal wastewater. The granules were physically crushed prior to inoculation. The temperature was controlled at 20 °C. pH was controlled at 7.0 ± 0.1 by dosing either 1 M NaOH or 1 M HCl. Dissolved oxygen (DO) was controlled at 3.7 mg/L $O_2$ (50% saturation). The average sludge retention time (SRT) was 20 days, which was controlled by non-selective sludge removal. The reactor was operated over a period of 700 days.

Reactor cycles had a length of 240 min, consisting of 60 min anaerobic plug-flow feeding, 170 min aeration, 5 min settling, and 5 min effluent withdrawal. The feed of 1500 mL consisted of 1200 mL artificial seawater (Instant Ocean®, final concentration 35 g/L. An overview of its concentrations is given in Atkinson and Bingman, 1997), 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 similar to Vishniac and Santer (1957), but using 2.2 mg/L $ZnSO_4.7H_2O$ instead of 22 mg/L (Pronk et al., 2015b, 2017). 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. Electrical conductivity of the influent was equal to 40 mS/cm.

### 2.2. Osmotic shocks in reactor

Osmotic shocks were introduced into the reactor by replacing the 1200 mL of influent artificial seawater by demineralized water. After one cycle, the influent demineralized water was replaced again by artificial seawater. The 150 mL of medium A and medium B remained the same during each cycle, giving the same concentrations of nutrients in the influent.

Due to the anaerobic plug-flow feeding, followed by aerobic mixing, there was a difference in the salinity that was experienced by the aerobic granules. This led to the following cycles of conductivity, as experienced by the granules (Table 1). The differences in conductivity are rather high, which is due to the batch-wise feeding of the reactor system.

### 2.3. Acetate uptake rate batch test

Granules were taken from the reactor at the end of the aeration phase (day 675 and 676 of operation). Equal amounts of granules were divided over flasks with 200 mL working volume, filled with either filtered effluent or demineralized water. All of the flasks were buffered at pH 7.0 ± 0.1 with a 4.0 mM HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) buffer, and sparged with $N_2$ gas prior to adding the granular sludge. A spike of acetate was added up to a final concentration of 200 mg/L, after which samples were taken for a period of 60 min. All samples were filtered through a 0.45 μm PVDF (polyvinylidene fluoride) filter. Their respective masses were registered to compensate for mass decrease in calculations. The amount of biomass was determined by filtering the granules at the end of the test, washing with demineralized water to remove salts, drying for 24 h at 105 °C, and incinerating for 2 h at 550 °C. All tests were done in duplicate.

### 2.4. COD release batch test

Granules were taken from the reactor at the start of the aeration phase (day 680 of operation). Equal amounts of granules were divided over four flasks with 100 mL working volume, filled with either filtered effluent or demineralized water. All of these flasks were sparged with compressed air prior to adding the granular sludge. Samples were taken for a period of 60 min, and filtered through a 0.45 μm PVDF filter. Their respective masses were registered to compensate for mass decrease in calculations. The amount of biomass was determined by filtering the granules at the end of the test, washing with demineralized water to remove salts, drying for 24 h at 105 °C, and incinerating for 2 h at 550 °C. All tests were done in duplicate.

### 2.5. Analytical methods

Concentrations of phosphate were measured on a Thermo Fisher Gallery Discrete Analyzer (Thermo Fisher Scientific, Waltham, USA). Chemical oxygen demand (COD) was measured using Hach Lange COD 1414 kits on a DR2800 spectrophotometer. Samples were diluted accordingly prior to measurement, to prevent chlorine interference. Acetate was measured by high-performance liquid chromatography (HPLC) with an Aminex HPX-87H column from Biorad, coupled to an RI and UV detector, using 0.01M phosphoric acid as eluent. Strength characterization was carried out as described in de Graaff et al., 2018).

### 2.6. 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.

**Table 1**
Conductivity (mS/cm) as experienced by the aerobic granular sludge during anaerobic feeding and aerobic mixing in a regular cycle, osmotic downshock, and recovery cycle.

| Phase | Regular cycle | Osmotic downshock | Recovery cycle |
|---|---|---|---|
| Anaerobic feeding | 40 | 1.1 | 40 |
| Aerobic mixing | 40 | ~20 | ~30 |

### 2.7. 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) was used for visualizing polyphosphate accumulating organisms (PAO) (Crocetti et al., 2000). A mixture of GAOQ431 and GAOQ989 probes (GAOmix) was used for visualizing glycogen accumulating organisms (GAO) (Crocetti et al., 2002). *Ca.* Accumulibacter clade I was visualized by Acc-I-444, and *Ca.* Accumulibacter clade II was visualized by Acc-II-444 (Flowers et al., 2009). A mixture of EUB338, EUB338-II and EUB338-III probes was 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.

## 3. Results

### 3.1. Reactor operation

Seawater-adapted aerobic granular sludge was cultivated in a 3.0 L lab-scale sequencing batch operated bubble column reactor at 20 days SRT and 20 °C, under the same conditions as usually applied in our laboratory (Pronk et al., 2014). Complete anaerobic acetate consumption was achieved, along with an average anaerobic release of 59.5 ± 4.0 mg/L $PO_4^{3-}$-P (0.35 mg P/mg HAc), and effluent concentrations of 5.9 ± 3.0 mg/L $PO_4^{3-}$-P (Fig. 1) over a period of 12 months. This equals a net removal of 36.5% from the influent, and 91.8% removal of phosphate after anaerobic release. A typical reactor cycle is shown in Fig. 2, from which an average biomass-specific phosphorus removal rate of 3.1 ± 0.2 mg P/g VSS/h was measured. An average concentration of 11.5 ± 1.3 g TSS/L or 8.1 ± 0.8 g VSS/L was present in the reactor during stable operation (i.e. 28% ash content). The effluent TSS was equal to 20.3 mg/L. Neither nitrite nor nitrate production was detected in the reactor. Ammonium was consumed for biomass production with a low consumption rate of 0.6 ± 0.1 mg N/g VSS/h.

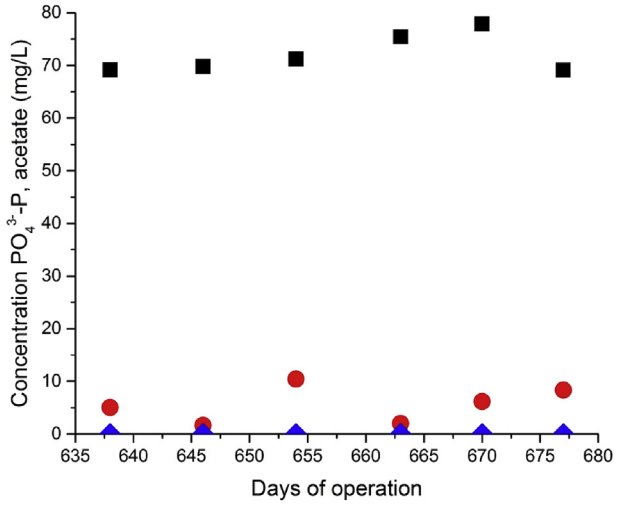


**Fig. 1.** Phosphate concentrations at the start of aeration (black squares ■), end of aeration (red circles ●), and acetate concentrations at the end of anaerobic feeding (blue diamonds ◆) during several weeks of consecutive operation of a seawater-adapted aerobic granular sludge reactor. The reactor was effectively operated from day 525 in stable mode.

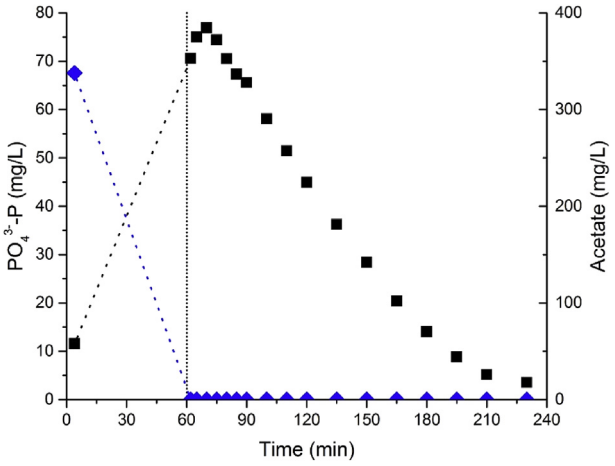


**Fig. 2.** Typical reactor cycle of a seawater-adapted aerobic granular sludge reactor, showing concentrations of phosphate (black squares ■), and acetate (blue diamonds ◆), with 60 min of anaerobic plug-flow feeding, 170 min of aeration, 5 min of settling, and 5 min of effluent withdrawal.

### 3.2. Granule characteristics

Successful granulation from non-granular inoculating sludge was achieved in seawater conditions (Fig. 3). No filamentous outgrowth was observed, and dense well-settling granules were formed. The average sludge volume index after 5 min ($SVI_5$) equalled 20 mL/g for these seawater-adapted granules (between days 635–680 of operation). The average granule size was 1.4 mm. An abrasion coefficient of 1.17 ± 0.01 · $10^{-5}$ $s^{-1}$ was measured for these granules, compared to a value of 1.78 ± 0.20 · $10^{-5}$ $s^{-1}$ for granules grown in the same system on freshwater (de Graaff et al., 2018).

### 3.3. Microbial community

Fluorescence in situ hybridization (FISH) analysis showed the presence of polyphosphate accumulating organisms (PAO), and the absence of glycogen accumulating organisms (GAO) in seawater-adapted aerobic granular sludge (Fig. 4a and b). A high fraction of PAO was observed from all bacteria that were stained with the general eubacteria probe (EUB338). *Ca.* Accumulibacter phosphatis clade I was observed in the seawater-adapted granules, whereas clade II was not observed (Fig. 4c and d).

### 3.4. Osmotic shock

The effect of an osmotic downshock on the reactor performance was assessed by replacing the influent seawater by demineralized water. This resulted in a salinity decrease from 40 mS/cm to 1.1 mS/cm for the granular sludge during plug-flow feeding and a conductivity of approximately 20 mS/cm during the aerated mixed reactor period, due to the 50% volume exchange ratio. Recovery after an osmotic shock was studied by reconnecting influent seawater after the cycle at low saline content. This resulted in a salinity of 40 mS/cm for the granular sludge during plug-flow feeding and approximately 30 mS/cm during the mixed reactor period. Concentrations of acetate and phosphate were measured during a regular cycle, the osmotic downshock cycle, and the subsequent recovery cycle. These cycles of salinity shocks were performed in duplicate; a representative graph is shown in Fig. 5.

During plug-flow feeding in a regular cycle at 100% salinity (40 mS/cm), acetate was completely consumed anaerobically. After

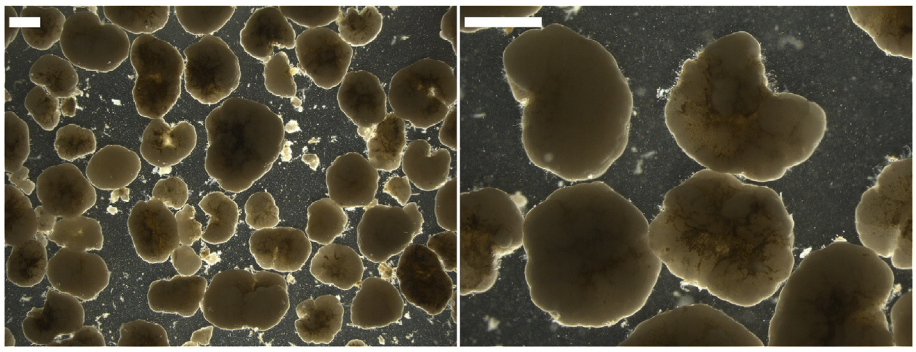

**Fig. 3.** Image analyser pictures of seawater-adapted aerobic granular sludge. Scale bar equals 1 mm.

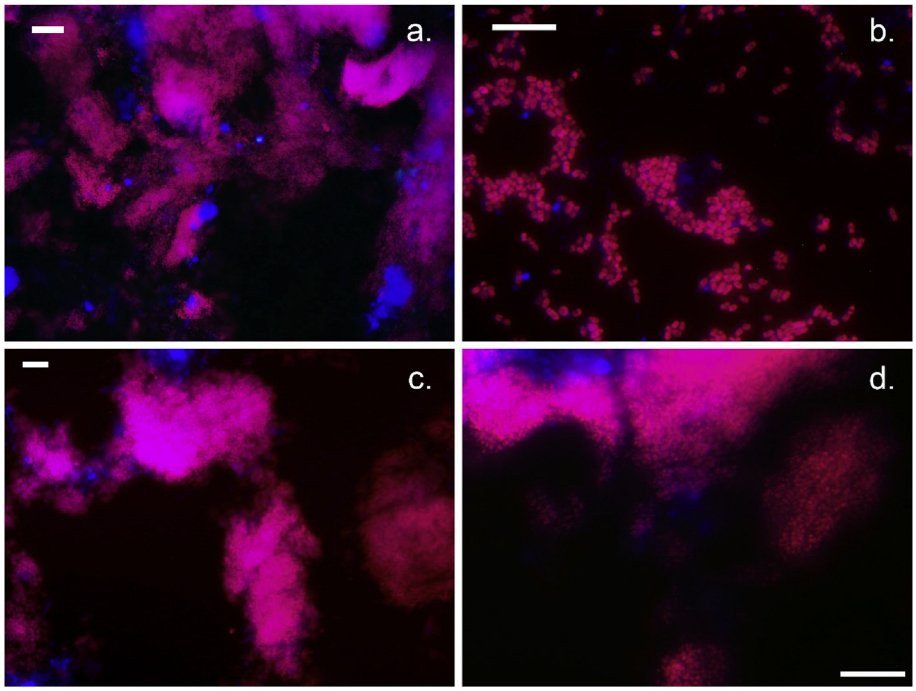


**Fig. 4.** Fluorescence in situ hybridization images of seawater-adapted sludge stained with (a,b) PAOmix, cy3/red, GAOmix, fluos/green, EUB338, cy5/blue. Magenta colour is an overlap between eubacteria (blue) and PAO bacteria (red). (c,d) *Ca.* Accumulibacter I, cy3/red, *Ca.* Accumulibacter II, fluos/green, EUB338, cy5/blue. Magenta colour is an overlap between eubacteria (blue) and *Ca.* Accumulibacter I bacteria (red). Scale bar equals 20 μm.

an osmotic downshock (1.1 mS/cm in feed), anaerobic acetate consumption was incomplete, and acetate became available at the start of the aeration phase. During the mixed reactor phase (approx. 50% salinity, approx. 20 mS/cm), the remaining acetate was completely consumed aerobically while simultaneously a limited amount of phosphate was released during the exact same time. The maximal aerobic phosphate uptake decreased to 72.0 ± 1.2% of the preceding regular cycle. During the subsequent recovery cycle (100% salinity during feeding, 40 mS/cm), acetate was completely anaerobically consumed again. During the mixed reactor phase (approx. 75% salinity, approx. 30 mS/cm), the maximal aerobic phosphate uptake rate increased to 114.1 ± 9.4% of the preceding regular cycle.

### 3.5. Uncoupling biological effect from hydrodynamic effect during anaerobic plug-flow feeding

During feeding of freshwater in a seawater-based reactor, there are two effects occurring simultaneously: a biological effect and a hydrodynamic effect. Due to introduction of influent with a lower density channelling occurred, resulting in a fraction of the influent bypassing the sludge blanket. This was visually observed by streams of influent water appearing on top of the sludge bed (Fig. 6). In order to separate the biological effect from this hydrodynamic effect, batch tests were performed to specifically measure the change in biological performance.

#### 3.5.1. Acetate uptake rate after osmotic downshock

Granules were taken from the reactor at the end of aeration, and transferred to an anaerobic flask containing acetate in either saline reactor effluent or demineralized water. Samples were taken during 60 min and analysed for acetate and phosphate concentrations over time. Results are shown in Fig. 7.

The biomass-specific acetate uptake rates in reactor effluent and demineralized water were similar (18.9 ± 2.3 and 17.7 ± 1.8 mg acetate/g VSS/h, respectively). The anaerobic phosphate release rate increased by 12.3 ± 0.6% in demineralized water. The total release of phosphate after 60 min increased by 32.1 ± 0.8% in

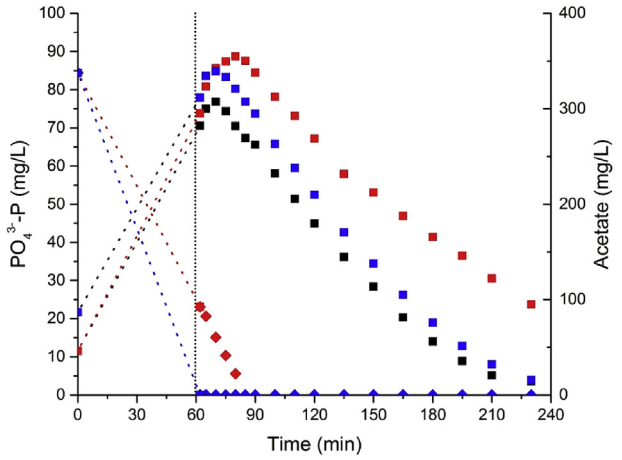


**Fig. 5.** Concentrations of phosphate (squares ■) and acetate (diamonds ◆) during a regular cycle (black, 40 mS/cm during anaerobic feeding, 40 mS/cm during aerobic mixing), downshock cycle (red, 1.1 mS/cm during feeding, approx. 20 mS/cm during mixing), and recovery cycle (blue, approx. 40 mS/cm during feeding, approx. 30 mS/cm during mixing). The first 60 min are anaerobic feeding phase, followed by 170 min of aeration, 5 min of settling, and 5 min of effluent withdrawal.

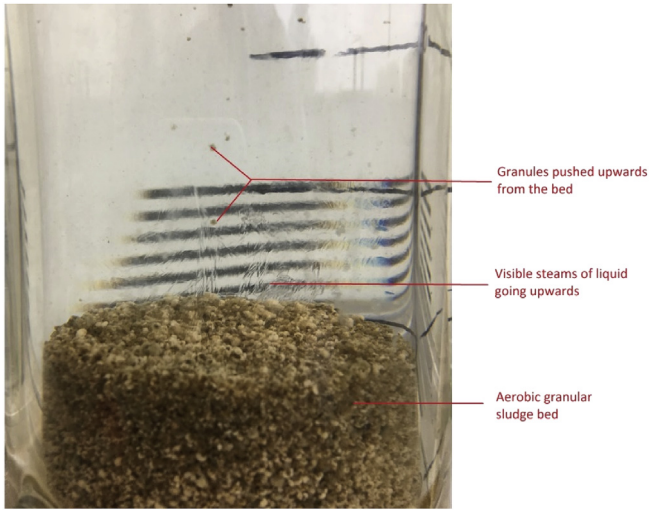


**Fig. 6.** Visualization of hydrodynamic effect of introducing a medium with a much lower density in the granular sludge reactor during upflow mode feeding.

freshwater compared to seawater-based effluent. This equals a P-mol/C-mol ratio of 0.47 ± 0.01 in saline reactor effluent and a P-mol/C-mol ratio of 0.72 ± 0.02 after an osmotic downshock in demineralized water.

### 3.6. COD release during osmotic downshock

Anaerobic incubation of seawater-adapted granules in demineralized water, without addition of acetate, led to release of COD (Fig. 8). Concentration increase of 45.5 ± 1.7 mg COD/g VSS was measured after only 5 min in demineralized water, and this decreased slightly over time down to 34.3 ± 4.4 mg COD/g VSS after 60 min. Incubation in reactor effluent led to much lower release up to a low concentration of 12.1 ± 9.4 mg COD/g VSS after 60 min. No release of suspended solids was observed during this batch test.

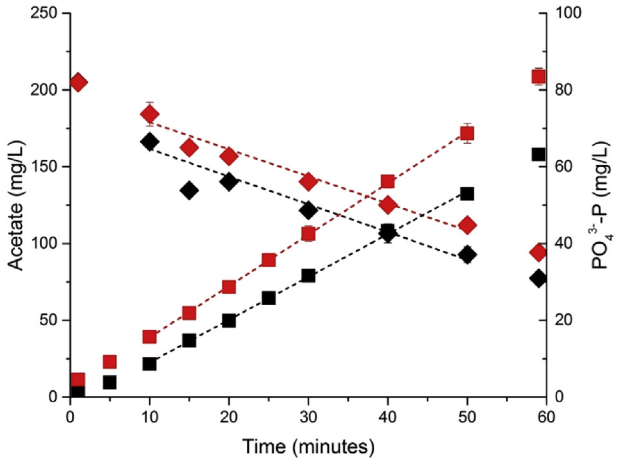


**Fig. 7.** Concentrations of acetate (diamonds ◆) and phosphate (squares ■) during an anaerobic batch test in either seawater-based reactor effluent (black) or demineralized water (red). Lines indicate the points with which the biomass-specific rates have been determined.

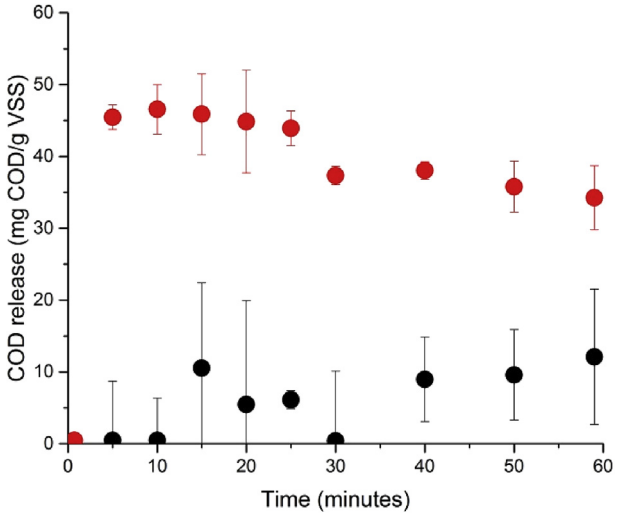


**Fig. 8.** Concentrations of COD (circles ●) during incubation of seawater-adapted aerobic granular sludge in reactor effluent (black) or demineralized water (red).

## 4. Discussion

### 4.1. Successful biological phosphorus removal in seawater

This study shows that biological phosphorus removal and granulation can occur successfully under seawater conditions. Neither granular sludge nor conventional activated sludge have previously been reported to give stable biological removal of phosphorus from seawater-based wastewater streams. The results from this study demonstrate the remarkable flexibility of *Ca.* Accumulibacter phosphatis to adapt to a wide range of salinities. It shows its exceptional resistance to osmotic shocks, and thereby also greatly contributes to increasing its range of full-scale applications.

Stable phosphate removal was measured with an aerobic removal rate of 3.1 ± 0.2 mg P/g VSS/h and a phosphate release of 59.5 ± 4.0 mg P/L. These values are similar to freshwater-based AGS systems (De Kreuk et al., 2005). The amount of phosphate release is higher than AGS in an NaCl-based influent, in which a deterioration

of P-release was observed at 20 g/L $Cl^-$ with the same *Ca.* Accumulibacter phosphatis clade I as present in this study (Pronk et al., 2014). Similarly, in a *Ca.* Accumulibacter enrichment, NaCl caused a decrease in phosphate uptake at 1.8 g/L salinity until a complete inhibition at already 3.5 g/L (Welles et al., 2015).

Complete anaerobic acetate uptake was achieved in our system, with the stable presence of a PAO culture in absence of GAO as determined by FISH. In AGS with pure NaCl, acetate was still completely consumed up to 33 g/L NaCl, but this occurred simultaneously with a shift in microbial community from PAO to GAO (Bassin et al., 2011). PAO enrichment studies have also shown that PAO continues to anaerobically consume acetate while having a near-zero P-release at 2% salinity (Welles et al., 2014). One of the differences between the latter study and our study is the choice of organic substrate (acetate/propionate mixture and only acetate, respectively). The choice for acetate instead of a mixture with propionate is commonly shown to lead to a shift towards GAO (Lopez-Vazquez et al., 2009). These results indicate that *Ca.* Accumulibacter phosphatis is more adaptable to seawater salinity than NaCl-based salinity.

### 4.2. Physical properties of seawater-adapted granules

Stable granulation was achieved in seawater-based conditions, resulting in low $SVI_5$ of 20 mL/g and the absence of filamentous outgrowth (Fig. 3). An essential factor was the complete anaerobic uptake of acetate. The cause of filamentous outgrowth has frequently been discussed in literature (Moy et al., 2002; McSwain et al., 2004; Liu and Liu, 2006). A major reason for their proliferation is the availability of easily degradable COD under aerobic conditions (De Kreuk et al., 2004; Val de Río et al., 2012). Our system does not contain these aerobic feast conditions, due to complete anaerobic consumption of acetate, which is the reason that filamentous outgrowth is absent.

Strength characterization indicated that seawater-adapted granules have a lower abrasion coefficient than freshwater-adapted granules ($1.17 \pm 0.01 \cdot 10^{-5}$ $s^{-1}$ and $1.78 \pm 0.20 \cdot 10^{-5}$ $s^{-1}$, respectively) (de Graaff et al., 2018). These results are supported by Li et al. (2017), who observed that a high-shear cohesion test led to less abrasion in granules that were grown in seawater-based wastewater than those that were grown in less saline wastewater. Moreover, they reported an increased concentration of $Ca^{2+}$ and $Mg^{2+}$ cations in the sludge, which could help bridging of negatively charged functional groups in the polysaccharide fraction of the biofilm matrix.

Interestingly, changes in the composition of extracellular polymeric substances (EPS) can also be the result of adaptation to seawater. Increases in protein content, hydrophobicity, and EPS concentration have been reported when AGS was cultivated in higher salinity wastewater, which could be linked to the increase in biofilm strength (Wang et al., 2015; Corsino et al., 2017; Campo et al., 2018). Adaptation of sugar residues in the EPS was also reported in response to higher salinity, in both anaerobic and aerobic granular sludge (Gagliano et al., 2018; de Graaff et al., 2019). Using improved EPS analysis techniques, the interaction between salinity and EPS composition could be clarified in the future (Seviour at al. 2019).

### 4.3. Difference between NaCl and seawater

The results from this study signify the importance of distinguishing salinity from NaCl and from seawater. This distinction is often not clearly made in literature discussions. Activated sludge studies on EBPR yielded decreased phosphate uptake rates when NaCl was added to the medium (Abu-ghararah and Sherrard, 1993; Intrasungkha et al., 1999; Welles et al., 2015). Decrease in phosphate removal was described in aerobic granular sludge at an NaCl concentration of 33 g/L as well (Bassin et al., 2011; Pronk et al., 2014). The observed shift in microbial community from PAO to GAO in NaCl salinity was not observed in the seawater-based reactor. It is clear that the effect of NaCl salinity should not be used as predictive for seawater salinity.

A major reason for good phosphorus removal in seawater systems could be due to the higher $Na^+/K^+$ ratio in seawater (Millero et al., 2008). Presence of sufficient amounts of potassium cations is required for the functioning of $Na^+/K^+$ pumps required for osmotic stabilization of the cytoplasm (Armstrong, 2003). Moreover, availability of potassium plays a crucial role in the metabolism of PAOs (Brdjanovic et al., 1996).

### 4.4. Release of osmolytes during osmotic downshock

During osmotic downshock of seawater-adapted granules, a remarkably high COD concentration ($45.5 \pm 1.7$ mg COD/g VSS) was measured in the liquid phase (Fig. 8). This COD is likely to be ascribed to the release of osmolytes (Tsapis and Kepes, 1977; Fischel et al., 1993; Roberts, 2005). Osmolytes are accumulated intracellularly to overcome high osmotic pressure, and protect DNA and proteins from denaturation (da Costa et al., 1998; Roeβler and Müller, 2001). During a hypo-osmotic shock, these organic molecules can be released, which has been used as a bioprocess for the production of ectoine (Sauer and Galinski, 1998).

Interestingly, *Ca.* Accumulibacter phosphatis is mainly reported in nature in estuaries which led to the suggestion that they contain osmolytes (Kunin et al., 2008; Peterson et al., 2008). These environments have a natural change from saline to brackish water due to tidal variations, along with a variation in anaerobic nutrient supply and aerobic starvation periods (Watson et al., 2019). However, the link between adaptation to variable salinity and type of osmolytes has not yet been made in neither environmental science literature nor wastewater engineering literature. The advantages of osmolytes to alleviate osmotic stress have frequently been studied for other technologies such as anaerobic digestion. For example, addition of glycine betaine, α-glutamate, and β-glutamate to anaerobic biomass decreases inhibition of methanogenesis by salinity (Vyrides and Stuckey, 2017). It was also found that addition of glutamic acid, aspartic acid, gelatine, and tryptone decreases osmotic stress in anaerobic granular sludge (Sudmalis et al., 2018).

However, due to the wide variety of osmolytes, the positive effect of specific osmolyte dosing in anaerobic sludge cannot directly be copied to EBPR sludge. Understanding the role of osmolytes in the EBPR process is of greatest importance for improving this technology. Basis has been laid in this study, but further research is required to completely comprehend the fascinating flexibility of *Ca.* Accumulibacter phosphatis metabolism to variations in salinity.

## 5. Conclusions

- Stable granulation and biological phosphorus removal can occur in seawater-based aerobic granular sludge with an aerobic P-uptake rate of $3.1 \pm 0.2$ mg P/g VSS/h
- *Ca.* Accumulibacter clade I is adaptable to both freshwater and seawater
- Seawater-adapted AGS releases $45.5 \pm 1.7$ mg COD/g VSS during a salinity downshock, which is likely ascribed to release of osmolytes
- Salinity impact due to NaCl or seawater is very different on EBPR processes

## 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.

## Acknowledgements

This research is financed by the Netherlands Organisation for Scientific Research (NWO), which is partly funded by the Ministry of Economic Affairs and Climate Policy, and co-financed by the Netherlands Ministry of Infrastructure and Water Management and partners of the Dutch Water Nexus consortium. We would like to thank Nick Ivens with help with reactor operation, and Roel van de Wijgaart for his help with FISH analysis.

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