The Effect of Bioinduced Increased pH on the Enrichment of Calcium Phosphate in Granules during Anaerobic Treatment of Black Water

Jorge Ricardo Cunha,† Taina Tervahauta,‡ Renata D. van der Weijden,*†‡ Hardy Temmink,*‡ Lucia Hernández Leal,† Grietje Zeeman,§ and Cees J.N. Buismann

ABSTRACT: Simultaneous recovery of calcium phosphate granules (CaP granules) and methane in anaerobic treatment of source separated black water (BW) has been previously demonstrated. The exact mechanism behind the accumulation of calcium phosphate (Ca₅(PO₄)₃) in CaP granules during black water treatment was investigated in this study by examination of the interface between the outer anaerobic biofilm and the core of CaP granules. A key factor in this process is the pH profile in CaP granules, which increases from the edge (7.4) to the center (7.9). The pH increase enhances supersaturation for Ca₅(PO₄)₃ phases, creating internal conditions preferable for Ca₅(PO₄)₃ precipitation. The pH profile can be explained by measured bioconversion of acetate and H₂, HCO₃⁻ and H⁺ into CH₄ in the outer biofilm and eventual stripping of CO₂ and CH₄ (biogas) from the granule. Phosphorus content and Ca₅(PO₄)₃ crystal mass quantity in the granules positively correlated with the granule size, in the reactor without Ca²⁺ addition, indicating that the phosphorus rich core matures with the granule growth. Adding Ca²⁺ increased the overall phosphorus content in granules >0.4 mm diameter, but not in fine particles (<0.4 mm). Additionally, H⁺ released from aqueous phosphate species during Ca₅(PO₄)₃ crystallization were buffered by internal hydrogenotrophic methanogenesis and stripping of biogas from the granule. These insights into the formation and growth of CaP granules are important for process optimization, enabling simultaneous Ca₅(PO₄)₃ and CH₄ recovery in a single reactor. Moreover, the biological induction of Ca₅(PO₄)₃ crystallization resulting from biological increase of pH is relevant for stimulation and control of (bio)crystallization and (bio)mineralization in real environmental conditions.

INTRODUCTION

Phosphorus (P) is an essential, irreplaceable, and scarce element for humanity. Therefore, phosphate (PO₄³⁻) recovery from waste streams is an important measure to reduce P scarcity and to minimize eutrophication, which is associated with P discharge to natural water bodies.

Current technologies for P recovery focus on precipitation/(bio)crystallization and (bio)mineralization in real environmental conditions. An increase of soluble calcium (Ca²⁺) and a decrease of bicarbonate (HCO₃⁻) in BW enhanced the accumulation of P in the UASB reactor (PO₄³⁻ removal). By supplementing at least 250 mgCa²⁺ L⁻¹ BW to the UASB reactor, the P sampled as CaP granules (>0.4 mm diameter) was enhanced from 2 to 31% and the P accumulation from 51 to 89% of the total incoming P. However, a significant fraction of P (58%) was still present as fine particles (<0.4 mm diameter), which are unfeasible to recover. Therefore, more understanding of the mechanism behind formation and growth of CaP granules is essential to stimulate granule formation, enabling simultaneous recovery of Ca₅(PO₄)₃ and methane (CH₄) during anaerobic treatment of BW. Additionally, it can give insights into the application of this process to other wastewater streams, such as manure.

Supporting Information

Received: June 26, 2018
Revised: September 14, 2018
Accepted: October 17, 2018
Published: October 17, 2018

DOI: 10.1021/acs.est.8b03502
Environ. Sci. Technol. 2018, 52, 13144−13154
Formation of Ca₅(PO₄)₃ in the granules does not necessarily start with the most stable phase thermodynamically, but with phases which require less energy of formation. Formation of hydroxyapatite (HAP), which is the thermodynamic most stable Ca₅(PO₄)₃ phase, occurs gradually via precursor phases, such as amorphous calcium phosphate (ACP) and octacalcium phosphate (OCP). ACP and OCP are less stable and, therefore, precipitate first. Parameters, such as pH and Ca²⁺ and PO₄³⁻ionic activities, influence the saturation state of Ca₅(PO₄)₃ phases. Since the pH and Ca²⁺ and PO₄³⁻ concentrations in BW are dictated by the dilution factor during BW collection and by the treatment conditions, the chemical speciation in the interphase between bulk liquid of the reactor and CaP granule is crucial to understand the enrichment of Ca₅(PO₄)₃ in the granules. For instance, syntrophic production and consumption of hydrogen (H₂) by acidifiers, acetogens, and hydrogenotrophic methanogens in the biofilm, which surrounds the Ca₅(PO₄)₃ core, could induce a local increase in pH, due to the conversion of H₂ to H⁺ and HCO₃⁻ into CH₄. This would favor Ca₅(PO₄)₃ enrichment of the granules over bulk precipitation.

In this study, pH as a function of granule depth, biological activity, chemical composition, and structural and crystal properties of CaP granules are experimentally assessed and correlated with the granule size. The goal is to describe the mechanism behind the desired formation of Ca₅(PO₄)₃ in CaP granules over bulk precipitation. Additionally, the biological impact of the outer biofilm on crystallization of HAP is modeled by correlating the biological production and consumption of HCO₃⁻ and H⁺ with crystallization of HAP from ACP and OCP phases in CaP granules.

### EXPERIMENTAL SECTION

#### pH Microelectrode Measurements

The internal pH profile of 11 CaP granules (2.0 mm diameter) was analyzed with a 25 μm microelectrode (Unisense, Denmark) coupled with a reference electrode model ref-RM (Unisense, Denmark). The source of granules for the pH measurements was the laboratory scale 50 L UASB reactor previously described in Tervahauta et al. (2014b), which set the motion of the sensor and logged the data simultaneously. The motion of the sensor was controlled in three axis using a two-dimensional stage MT-65 (Phytron Inc., Williston, VT) and a motorized micromanipulator MM3M (Unisense, Denmark), which controlled the vertical motion. The calibration was performed using buffers at pH 7 and 9 (VWR International S.A.S., France) at 25 °C.

#### Modeling of Saturation State of Ca₅(PO₄)₃ Phases and Calcite

The saturation states of HAP, precursors ACP and OCP, and calcite in function of the local pH in the granules were calculated using the software Visual MINTEQ version 3.1 (KTH, Sweden). This software uses the Davies equation to calculate activity coefficients, while taking into account the measured chemical composition of BW (g L⁻¹): 0.87 NH₄⁺, 0.01 Mg²⁺, 0.35 Na⁺, 0.23 K⁺, 0.5 × 10⁻³ Fe²⁺, 0.05 Ca²⁺, 2.36 HCO₃⁻, 0.46 Cl⁻, 0.04 SO₄²⁻, 0.7 × 10⁻³ NO₃⁻, and 0.16 PO₄³⁻. Methods used for the chemical measurements were as described in Cunha et al. (2017). Humic substances, which are known to interact with Ca²⁺ and PO₄³⁻, were included using the complexation model available in the software used; the entering parameters were total dissolved organic carbon (determined by Shimadzu TOC analyzer, 1.3 gC L⁻¹) and dissolved humic acids (136 mgC L⁻¹). The latter were measured with liquid chromatography—organic carbon detection (LC-OCD, Model 8 with a NDIR-detector Siemens Ultramat 6E and UV and OND detectors Agilent 1260 Infinity). The temperature was set at 25 °C and pH was defined according to the obtained pH depth profile. Then, the saturation state (SI) of each specie (y) was defined by eq 1.

\[
\text{SI} = \frac{\text{IAP}_y}{K_{sp,y}},
\]

where IAPₘₙₙₖ is the ion activity product of the elements in y and Kₚₛₙₜₜₜ is the solubility product constant of y. For SI > 0 y is supersaturated, for SI < 0 y is undersaturated and for SI = 0 y is in apparent equilibrium.

#### Specific Methanogenic Activity Tests of CaP Granules

CaP granules used for the specific methanogenic activity tests were also harvested from the previously mentioned 50 L UASB reactor. Granules separated by fluidization in the upflow column above-described were used for the specific methanogenic activity tests, which were performed at 25 °C in triplicate according to Angelidaki et al. (2007). Then, Acetate (1 g L⁻¹) and H₂/CO₂ (1 bar overpressure 80/20) were separately used as substrate to determine the activity of acetoclastic and hydrogenotrophic microorganisms, respectively. Serum bottles (250 mL) were used with a working liquid volume of approximately 125 mL (weight corrections were done for each bottle). The concentration of inoculum was 2.3 ± 0.5 gVSS L⁻¹. Control (without substrate) and blank (only substrate) tests were performed in duplicate. Macronutrients and micronutrients stock solutions were prepared according to Angelidaki et al. (2007). Sørensen buffer was used to control the operational pH at 7.4. CH₄ production was measured by means of pressure using a manometer GMH 3150—EX (Greisinger electronic, Germany). The composition of headspace gas was determined using gas chromatography (Varian CP4900 Micro GC with two separate column models controlled at 25 ± 0.2 °C and 7.5 ± 0.02, respectively. The data was acquired using a pH meter PHM210 (Radiometer analytical SAS, France), an A/D converter ADC-216 (Unisense, Denmark) and the software Profi 3.10 (Unisense, Denmark), which set the motion of the sensor and logged the data simultaneously. The motion of the sensor was controlled in three axis using a two-dimensional stage MT-65 (Phytron Inc., Williston, VT) and a motorized micromanipulator MM3M (Unisense, Denmark), which controlled the vertical motion. The calibration was performed using buffers at pH 7 and 9 (VWR International S.A.S., France) at 25 °C.
Mol sieve 5 Å (MSS) and Poraplot U (PPU) and the acetate concentration using ion chromatography (Metrohm 761 Compact). Specific methanogenic activity (SMA) rates were calculated for all the tests according to Angelidaki et al. (2007). For the tests with H2/CO2 the partial pressure of CH4 ($P_{CH_4}$) was quantified according to eq 2.

$$P_{CH_4} = \frac{\Delta P_{\text{total}}}{nCH_4 - 4nH_2 - nCO_2}$$ (2)

where $\Delta P_{\text{total}}$ is the pressure variation measured with manometer, and $n$ is the partial proportions for anaerobic CH4 production from H2/CO2, which is assumed 1 for standard temperature and pressure conditions.

**Particle Size Distribution (PSD), Elemental Composition and X-ray Diffraction Analysis (XRD).** PSD, elemental analysis and XRD measurements were performed on CaP granules from two 5 L UASB reactors treating BW. The vacuum collected black water used as influent for all UASB reactors was collected in the same community in Sneek (The Netherlands), but at different periods. Reactor 1 was a control reactor with the similar operation as the 50 L UASB reactor used for the micro pH measurements and methanogenic activity tests. Reactor 2 was used to study the effect of Ca2+ addition on CaP granulation. Both 5 L reactors, without and with Ca2+ addition, operated for 460 days at 25 °C and at an OLR of 1.2 ± 0.3 and 1.4 ± 0.4 kgCOD m⁻³ d⁻¹ and HRT of 8 ± 1 and 7 ± 1 days, respectively. These conditions resulted in an upflow velocity of 0.4 cm h⁻¹ for both reactors and SRT of 163 and 186 days for reactor without and with Ca2+ addition, respectively. Sludge samples (55 mL) were sampled on operation days 350, 415, 436, and 460 at three different heights from top to bottom (20, 10, and 5 cm), using a syringe connected to a hose with 0.5 cm diameter. Results are presented as an average of the four sampling occasions.

PSD was analyzed with mesh sieves, dividing particles in the sludge samples as <0.4, 0.4−0.9, 0.9−1.4, 1.4−2.0, 2.0−2.5, and >2.5 mm diameter. A sludge sample (50 mL) was sieved sequentially using first the mesh sieve with the larger pore size (2.5 mm), followed by 2.0, 1.4, 0.9, and finally 0.4 mm pore size. Then, each size fraction was used for elemental and XRD analysis and total and volatile suspended solids (TSS and VSS, respectively) quantification. Elemental composition (P, Ca and Mg) was determined by inductively coupled plasma−optical emission spectroscopy (PerkinElmer Optima 5300 DV ICP-OES) after an HNO3 digestion at 148 °C for 45 min, using a microwave (MWD Milestone). TSS and VSS were determined by gravimetric standard method.

XRD analyses were performed with a Bruker D8 Advance diffractometer 280 mm measurement radius using Cu radiation with Linear PSD 3° detector opening, divergence slit at 0.58° and a soller slit at 2.5°. The samples were dried at 105 °C for 12h and ground before the measurements. The software TOPAS (Bruker) based on Rietveld method was used for pattern fitting. Amorphous content was deduced by the degree of crystallinity obtained in the model. The R-weighted pattern was kept below 7 for all spectra model, fitting only the Lorentzian and Gaussian component convolutions of the identified phase structures of hydroxyapatite (COD 9002216 P_63/m) and calcite (COD 9016706 R_3_c). The morphology and the elemental distribution of CaP granules were assessed with a scanning electron microscope (SEM) JEOL JSM-6480LV in backscattered detection at 15 kV.

**Figure 1.** Measured pH profile of a representative CaP granule (~3.0 mm diameter) and saturation state of amorphous calcium phosphate, octacalcium phosphate and calcite in function of the pH measured. For SI > 0 precipitation can occur (supersaturated), for SI < 0 precipitation hardly occurs (undersaturated) and for SI = 0 each specie is in apparent equilibrium.
coupled with NORAN Systems SIX EDX (Thermo Scientific, Waltham, MA). The EDX system is factory calibrated using pure minerals. Then the compositional quantification is done via peak integration considering the known spectrum of each element and the peaks intensity. After sieve separation, a set of 10 CaP granules from the reactor without Ca\textsuperscript{2+} addition were dried at room temperature and sectioned with a scalp for cross-sectional line scan analysis and only dried for visualization of the outer biofilm and elemental mapping.

**Calculation of Mass Flows of HCO\textsuperscript{3−}, H\textsuperscript{+} and H\textsubscript{2} during Anaerobic Digestion of BW and HAP Crystalization from Less Stable Ca\textsubscript{x}(PO\textsubscript{4})\textsubscript{y} Phases.** Anaerobic digestion of BW was previously modeled by Feng et al. (2006).\textsuperscript{20} The model and adopted parameters were then used in this study to calculate the mass flow of HCO\textsuperscript{3−}, H\textsuperscript{+}, and H\textsubscript{2} between the bulk and granule. In the model, acidification of products from hydrolysis (monosaccharides (MS), amino acids (AA), and long-chain fatty acids (LCFA)) are assumed to occur near the granule edge. Though acetogenic activity was not determined, acetogens were assumed in the granule biofilm as syntrophy between acetogens and hydrogenotrophic and acetoclastic methanogens prevails.\textsuperscript{15} Acetate in BW (influent) is converted into CH\textsubscript{4} and HCO\textsuperscript{3−} in the bulk of the reactor and propionate and butyrate are only consumed in the outer biofilm, along with consumption of produced H\textsubscript{2} by hydrogenotrophic methanogens. Equations and parameters used are extensively described in the Supporting Information (S3). The calculation was based on a volumetric unit (L) of BW. COD input parameters were taken from previous experiments. These values were 6.9 g L\textsuperscript{−1} of particulate and colloidal COD and 1.6 g L\textsuperscript{−1} of soluble COD. Soluble COD was assumed to be a mixture of acetate, propionate, and butyrate (66%, 32%, and 2%, respectively), which corresponded with the measured VFA concentration in BW.\textsuperscript{7}

Crystallization of HAP at pH between 7 and 7.5 and room temperature is occurring via Ca deficient ACP precursors.\textsuperscript{12} OCP is the intermediate phase, which consists of an agglomeration of ACP complexes mediated by Ca\textsuperscript{2+} assimilation.\textsuperscript{12} A complete conversion of ACP into HAP was assumed to estimate the complete release of H\textsuperscript{+}. The fact that HAP is the thermodynamically most stable Ca\textsubscript{x}(PO\textsubscript{4})\textsubscript{y} phase, and therefore, it is the prevailing crystal phase when the time is not limited is the basis for this assumption. Moreover, only the accumulated Ca\textsuperscript{2+} and PO\textsubscript{4}\textsuperscript{3−}, which was further divided into H\textsubscript{2}PO\textsubscript{4}\textsuperscript{−} and HPO\textsubscript{4}\textsubscript{2−} according to bulk pH (7.5), were considered as input for the calculation. Solid Ca and P were not accounted.

## RESULTS AND DISCUSSION

**Internal pH Profile and Saturation State of Ca\textsubscript{x}(PO\textsubscript{4})\textsubscript{y} phases and calcite.** The average internal pH peak of the measured CaP granules (n = 11) was 7.93 ± 0.08 while the mobile phase was kept at 7.5 ± 0.03, which corresponded with the effluent pH (7.48 ± 0.19) in the 50 and 5 L UASB reactors used in this study.

As a representative example, the pH profile of a complete cross-section of a granule is given in Figure 1. Considering the soluble chemical composition of raw BW, both precursors ACP and OCP are supersaturated at the higher pH inside the granule as shown in Figure 1. Yet, in the granule core, the chemical speciation will differ as the diffusion of substances will change with the physical structure and thickness of the biofilm. Near the edge and outside of the granule ACP and OCP are undersaturated, indicating that precipitation will not

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**Figure 2.** Elemental (P and Ca) line scan (a) of a granule cross section from the 5 L reactor without Ca\textsuperscript{2+} addition (b) measured with energy dispersive X-ray (EDX) coupled with scanning electron microscope (SEM). SEM representation of a CaP granule partially detached from the outer biofilm (c) taken from the 5 L reactor with Ca\textsuperscript{2+} addition, and its respective elemental distribution measured with EDX (d).
In the reactor is in between in plug-fi coprecipitation of CaCO3 within the granule is possible. Yet, rated only within the boundaries of the granule, suggesting that dispersed sludge, is reduced. Model results in Figure 1 are granule cross-section as shown further in Figure 2a, using validated by measuring the Ca and P concentrations in a Figure 3. Correlation between size (diameter) and P, Ca, Mg, and VSS (as the ratio VSS/TSS) contents and the Ca/P molar ratio for each size fraction of sludge taken from the 5 L reactors, without Ca2+ addition (a) and with Ca2+ addition (b). Values are averages of four samples taken on operation days 350, 415, 436, and 460.

Figure 4. SEM-EDX line scan. The pH of influent and effluent of the UASB reactors used in this study was measured, but not bulk pH. The pH in the influent BW was 7.9 ± 0.3, with a few peaks up to 8.7, which could induce Ca and P precipitation as CaCO3, ACP, OCP, and struvite. Effluent pH was always below 7.7. Bulk pH in the reactor is in between influent and effluent pH, due to the plug-flow mode of the liquid phase in the UASB reactor (low mixing). Considering the effluent pH, CaCO3, ACP, and OCP are undersaturated. Thus, fine particles observed in the bulk solution most likely were already formed prior the entry in the UASB reactor. If bulk pH rose above 7.8, bulk precipitation of ACP and OCP would be triggered, reducing the favored enrichment of Ca₈(PO₄)₂ in granules over bulk precipitation.

HAP is supersaturated for the entire pH range measured (SI > 6), and because of its low solubility (Ksp = 4.7 × 10⁻⁶⁵), hydroxyapatite is thermodynamically the dominant crystal phase for the BW chemical matrix. However, at this phase no kinetic predictions about the formation time can be made. This is because HAP nucleation and mineralization time can be influenced by several uncontrolled factors, such as presence organic compounds (proteins and collagen fibers), existing surface and ionic activity of inhibitors, such as Mg and carbonate (CO3²⁻).10,21–23 CaCO3 (calcite) was supersaturated only within the boundaries of the granule, suggesting that coprecipitation of CaCO3 within the granule is possible. Yet, over time Ca₈(PO₄)₂ phases prevail due to the thermodynamic equilibrium (lower Ksp), leading to recrystallization of CaCO3 into Ca₈(PO₄)₂.14,15 Note that the retention time for solids in the UASB reactor treating BW varied from 163 to >365 days as previously demonstrated.7,26

The supersaturation in the granule core, which is induced by the internal higher pH, is crucial for formation of Ca₈(PO₄)₂ within CaP granules. Consequently, the formation of Ca₈(PO₄)₂ fines, which are difficult to separate from the dispersed sludge, is reduced. Model results in Figure 1 are validated by measuring the Ca and P concentrations in a granule cross-section as shown further in Figure 2a, using SEM-EDX line scan.

Methanogenic Activity and Outer Biofilm of CaP Granules. Methanogenic activity tests on CaP granules showed that acetate and H2/CO2 were metabolized into CH4 at 0.245 ± 0.042 and 0.088 ± 0.026 gCOD-CH4 g⁻¹VSS d⁻¹, respectively. The obtained activity rates are within the range found in existing literature, but lower than the average.27 The lower methanogenic activity for H2 compared to acetate might be partly due to the low temperature (25 °C) applied in the activity tests and in the UASB reactor or the spatial distribution of microorganisms in the biofilm of CaP granules. For instance, acetoclastic methanogens might be located on the outer part of the biofilm, due to the relatively high concentration of acetate in the influent black water (1.1 ± 0.5 g L⁻¹), whereas hydrogenotrophic methanogens might be located deeper in the biofilm with lower access to substrate from the bulk solution.

The characteristic outer biofilm of CaP granules is determined with SEM in backscattering mode in Figure 2b. This allows distinguishing an outer biofilm (darkened) from an inorganic core (lightened). SEM images of other granule samples are shown in Supporting Information (S4) and show a similar observation. The SEM-EDX line scan (white line in Figure 2b) shows that the P and Ca contents (wt %) are higher in the core than in the outer biofilm of the granule (Figure 2a). Data points are recorded each µm during the line scan (812 measurements in total) to measure the elemental composition in the background, outer biofilm, and granule core. The average P content for the background (from 0 to 150 µm) was 0.3 wt %, which is below the detection limit of 1 wt %. The average P content for the organic outer layer (from 150 to 260 µm and from 360 to 420 µm) was 2.1 wt % and for the core (from 420 to 800 µm) increased to 10.9 wt %. The P and Ca peak between 260 and 360 µm in the line scan corresponds to an isolated Ca₈(PO₄)₂ particle within the outer biofilm. The P content of the outer biofilm is higher than the P content commonly observed for nonlimiting P anaerobic biomass (1.2 wt %), due to the presence of fine Ca₈(PO₄)₂ particles.28,29 In the core, the Ca/P molar ratio is 1.74 ± 0.63, which is higher than the theoretical Ca/P molar ratio in HAP of 1.67,12 caused by the presence of CaCO3 as it will be explained later on Figure S5. Both microbial groups were found in cross-sectioned CaP granules using fluorescence in situ.
the P content of particles >0.4 mm diameter increased from HCO₃⁻ (Figure 3). Moreover, the degree of crystallinity in particles <0.4 mm diameter did not increase by adding Ca²⁺ (Figure 5).

The absence of bulk precipitation can be explained by saturation for Ca²⁺, which is in line with the calculated supersaturation for Ca₃(PO₄)₂. However, results in Figure 1 strongly indicate that CaP granules do not precipitate directly in the bulk of the reactor is created. Without the addition of Ca²⁺, but the granule formation rate and granule size as demonstrated in Cunha et al. (2018). This confirms that Ca₃(PO₄)₂ enrichment of granules is favored over bulk precipitation, which is in line with the calculated supersaturation for Ca₃(PO₄)₂ phases in the center of the granule (Figure 1).

By adding extra Ca²⁺, supersaturation for Ca₃(PO₄)₂ phases in the bulk of the reactor is created. However, results in Figure 3 show that Ca₃(PO₄)₂ bulk precipitation did not occur, because the P content in fine particles (<0.4 mm diameter) with Ca²⁺ addition was lower than without Ca²⁺ addition (Figure 3). Moreover, the degree of crystallinity in particles <0.4 mm diameter did not increase by adding Ca²⁺ (Figure 5). The absence of bulk precipitation can be explained by complexation of added Ca²⁺ with negatively charged extracellular polymeric substances (EPS) and microbial cell surfaces in the outer biofilm, stimulating Ca₃(PO₄)₂ precipitation in the granule over bulk precipitation. Without the complexation of Ca²⁺ with existing EPS and microorganisms and the consequent local increase of Ca²⁺ and pH, the P content in fine particles would have increased due to Ca₃(PO₄)₂ bulk precipitation.

There are two possible mechanisms for formation of CaP granules (Figure 4): (1) attachment of biomass (EPS and microorganisms) to an existing inorganic seed particle, which further grows developing a CaP granule, consisting of an outer biofilm and an inorganic core containing mainly Ca₃(PO₄)₂, and (2) complexation of Ca²⁺ with biomass (EPS and microorganisms) creating local conditions for Ca₃(PO₄)₂ precipitation and subsequent formation of a CaP granule by agglomeration of biomass and formed Ca₃(PO₄)₂; the formed Ca₃(PO₄)₂ remains in the core along with the internal decay of biomass. The growth of CaP granules can occur via two pathways (Figure 4): (1) diffusion of Ca²⁺ and PO₄³⁻ through the biofilm and precipitation of Ca₃(PO₄)₂ directly in the granule core, and (2) precipitation of Ca₃(PO₄)₂ in the outer biofilm and eventual transport of Ca₃(PO₄)₂ precipitates to the core along with the internal decay of the outer biofilm. The latter is supported by isolated inorganic particles (white particles) within the outer biofilm as shown in Figures 2b and 4. The agglomeration of Ca₃(PO₄)₂ assemblies was previously observed, and it occurs via an amorphous interface. Over time, precursor phases (Ca₃(PO₄)₂) formed in the outer biofilm or core edge recrystallize to HAP as the core grows. The decrease in VSS content along the granule growth (Figure 1) is due to the internal decay of the outer biofilm. Although individual granule development could not be followed in time, the results of the elemental composition and crystallinity of different particle sizes from 16 representative sludge samples taken at different operation times and reactor heights (Figures 3 and S, respectively) strongly indicate that CaP granules do mature in time.

Figure 4. Proposed mechanism for CaP granulation supported by SEM and SEM-EDX images of CaP granules. The numbers (1) and (2) refer to the possible mechanisms for formation (initiation) and growth of CaP granules.
With Ca\(^{2+}\) addition, a maximum P content was observed for CaP granules with a size between 1.4 and 2.5 mm diameter (Figure 3b). Moreover, the VSS content was higher for granules >2.5 mm diameter than the latter size range, suggesting that granule maturation was not completely in line with the CaP granule growth (size) when Ca\(^{2+}\) was added. This indicates the existence of two populations of CaP granules with similar size, but different Ca\(_x\)(PO\(_4\))\(_y\) content or maturation stages. The reduction in granule size during maturation is most probably related to a decrease in the surface available for attachment of organic material along with the growth of the inorganic core or increase in core density.

Crystal Properties of CaP Granules. Crystal phase identification showed that HAP and calcite were the most prominent phases in all particle sizes for both conditions, with and without Ca\(^{2+}\) addition (Figure 3b). Moreover, the VSS content was higher for granules >2.5 mm diameter than the latter size range, suggesting that granule maturation was not completely in line with the CaP granule growth (size) when Ca\(^{2+}\) was added. This indicates the existence of two populations of CaP granules with similar size, but different Ca\(_x\)(PO\(_4\))\(_y\) content or maturation stages. The reduction in granule size during maturation is most probably related to a decrease in the surface available for attachment of organic material along with the growth of the inorganic core or increase in core density.

The formation, growth, and maturation of CaP granules are stimulated by adding Ca\(^{2+}\). When Ca\(^{2+}\) is added, the Ca\(_x\)(PO\(_4\))\(_y\) content in mature CaP granules (with a diameter between 0.9 and 2.5 mm) increases, while the VSS content decreases (Figure 3b). This was not observed for CaP granules formed without Ca\(^{2+}\) addition, where a linear relationship between VSS and P contents and size was obtained (Figure 3a).

Crystal Properties of CaP Granules. Crystal phase identification showed that HAP and calcite were the most prominent phases in all particle sizes for both conditions, with and without Ca\(^{2+}\) addition (Figure 5). Additional XRD spectra are in Supporting Information (SS) and show the same observation. The broadening and overlap of the HAP peaks between positions (2\(^\theta\)) 30 and 35 are most likely due to the presence of HAP nanocrystals.\(^{31,32}\) The percentage of HAP was always higher than calcite, and a positive correlation between the particle size and HAP content was observed (Figure 5). By adding Ca\(^{2+}\) the crystal percentage of HAP in CaP granules (>0.4 mm diameter) increased from 8.4 ± 2.0 to 21.5 ± 5.4%, while the crystal percentage of calcite remained relatively constant (2.0 ± 0.3% without Ca\(^{2+}\) addition and 2.3 ± 0.6% with Ca\(^{2+}\) addition). Moreover, the amorphous content of CaP granules decreased with the Ca\(^{2+}\) addition from 89.6 ± 2.1 to 76.2 ± 5.9%. Additionally, pattern fitting results showed that crystallite size for HAP and calcite were on average 5.5 ± 0.5 and 31.4 ± 11.3 nm for all granule size fractions and those are similar to the crystallite sizes reported in previous studies.\(^{12,33-35}\) The increase in crystallite size of HAP in CaP granules was minor by adding Ca\(^{2+}\) (from 5.1 ± 0.4 to 5.8 ± 0.2 nm). Thus, Ca\(^{2+}\) addition increased the Ca\(_x\)(PO\(_4\))\(_y\) content in CaP granules by enhancing the formation and agglomeration of nanocrystals. According to existing literature, the crystallization of HAP nanocrystals is mediated by ACP complexes, which facilitate the \(\text{H}^+\) release from H\(_2\)PO\(_4\)\(^−\) and HPO\(_4\)\(^{2−}\) by binding Ca\(^{2+}\) (Ca\(^{2+}\)-HPO\(_4\)\(^{2−}\)), evolving to the intermediate phase OCP.\(^{8,10}\) Then, the growth of the HAP nanocrystal is occurring via OCP dissolution and recrystallization of OCP lattice ions into the prenanocrystal until the maximum size, which is based on the ordered structure of the surface (P\(_{63/m}\)) for the solution conditions, being.

![Figure 5. XRD scans of particles <0.4 mm diameter and CaP granules with diameter between 2 and 2.5 mm from sludge samples taken from the reactor without Ca\(^{2+}\) addition (1 and 2) and reactor with Ca\(^{2+}\) addition (3 and 4), and XRD scans of Hydroxyapatite (HAP) and Calcite (5 and 6). The weight percentage of amorphous (a), crystal HAP (b), and crystal calcite (c) contents, according to Rietveld pattern fitting.](image)
reached. 9,10,32 Then, the HAP nanocrystals (∼5 nm) agglomerate through the formation of a hydration layer.8,32 The hydration layer dictates the overall particle size of the HAP conglomerate and the reactivity with cationic (e.g., Ca2+ and Mg2+), inorganic (e.g., CaCO3) and organic compounds (e.g., collagen fibers).12,22,36,37 Moreover, the exact composition and structural properties of the hydration layer are dependent on the water-pore composition of the granules.32,37 Therefore, the internal conditions in CaP granules play a crucial role not only in the Ca\textsubscript{x}(PO\textsubscript{4})\textsubscript{y} formation but also on the agglomeration mechanism.

**Effect of HCO\textsubscript{3}−, H\textsuperscript{+} and H\textsubscript{2} Flows on CaP Granulation.** Conversion of H\textsuperscript{+}, HCO\textsubscript{3}− and H\textsubscript{2} into CH\textsubscript{4} in the outer biofilm, mass transfer limitations created by the outer biofilm and eventual stripping of CO\textsubscript{2} (H\textsubscript{2}CO\textsubscript{3}) and CH\textsubscript{4} gases from the granule induce an internal environment in CaP granules with reduced H\textsuperscript{+} ionic activity (higher pH) and lower CO\textsubscript{3}\textsuperscript{2−} concentration. This is crucial for Ca\textsubscript{x}(PO\textsubscript{4})\textsubscript{y} enrichment of the granules, because H\textsuperscript{+} released from the deprotonation of H\textsubscript{2}PO\textsubscript{4}− and HPO\textsubscript{4}\textsubscript{2−} and from the recrystallization of ACP and OCP into HAP need to be locally buffered, in order to maintain the favorable conditions for Ca\textsubscript{x}(PO\textsubscript{4})\textsubscript{y} formation along with the granule maturation.8,11,12

In Figure 6 the estimated formation and consumption of HCO\textsubscript{3}−, H\textsuperscript{+} and H\textsubscript{2} throughout the anaerobic digestion of BW is shown. Acetate in BW is readily converted into CH\textsubscript{4} and HCO\textsubscript{3}− by disperse sludge in the bulk at the bottom of the reactor. The calculated production of HCO\textsubscript{3}− from acetate degradation in the bulk (16 mM) is similar to the experimentally measured increase of HCO\textsubscript{3}− during the digestion of the sludge in the anaerobic reactor. The mass balance between the biological degradation of solid and soluble organic compounds in BW is shown in Figure 6.
treatment (13 ± 5 mM) in the reactor without Ca²⁺ addition. In the reactor with Ca²⁺ addition, the measured increase of HCO₃⁻ was only 6 ± 2 mM, most likely because of higher CaCO₃ formation in the bulk and granules.

Disintegration/hydrolysis of biodegradable organic solids and colloids yield MS, AA, and LCFA which are further acidified in the interface between bulk and granule. This is because H₂ produced during acidogenesis is rapidly consumed by H₂ consuming organisms during hydrogenotrophic methanogenesis in the outer edge of the granular structure to enable further degradation of propionate and butyrate, as previously proposed by Batstone et al. (2004) for anaerobic granules treating brewery wastewater.¹⁵ The anaerobic degradation of propionate and butyrate is only exergonic when H₂ partial pressure is lower than 10⁻³ atm, and since H₂ is a byproduct of propionate and butyrate acetogenic reactions, hydrogenotrophic methanogenesis must be locally associated with acetogenesis.¹⁴,³³ Thus, degradation of propionate and butyrate, which are products of acidogenesis, was assumed to undergo within the outer biofilm of CaP granules. Yet, hydrogenotrophic methanogenesis is also possible with dispersed sludge due to the low up-flow velocity applied (<1 cm h⁻¹), enabling conditions for exchange of substrates. H₂ was never detected in the biogas produced in the two 5 L reactors and the 50 L reactor used in this study. Sludge bed analysis indicated that the percentage of VSS (organic matter) in the granules (particles >0.4 mm diameter) represented 72% and 81% of the total VSS in the sludge bed without and with Ca²⁺ addition, respectively, after 460 days of operation. Therefore, biological activity is expected to occur mainly in the granules. In the model, products from acidogenesis were assumed to be metabolized by acetogens and methanogens in the granule to estimate the internal conversion of H₂/H⁺, and HCO₃⁻ into CH₄, explaining the internal higher pH.

The calculated concentration of H⁺ during acidogenesis of MS, AA and LCFA was 57.1 mM (Figure 6). The consumption of H⁺ produced during acidogenesis was assumed to take place within the outer biofilm of the granule. Yet, part of the H⁺ may be responsible for the decrease in bulk pH from 7.94 ± 0.33 (n = 58) in the influent BW to 7.44 ± 0.04 (n = 70). Nevertheless, the uptake of H⁺ during hydrogenotrophic methanogenesis and the buffering capacity by the HCO₃⁻ produced during internal acetoclastic methanogenesis is sufficient to neutralize the total produced H⁺, including the H⁺ released during the formation and recrystallization of Ca₅(PO₄)₃(OH) phases.

Increased local pH in anaerobic granules measured with micro pH sensors was previously reported by Lens et al. (1993) and Yamaguchi et al. (2001).⁵⁹,⁶⁰ Lens et al. (1993) clearly demonstrated that methanogenic activity was responsible for internal pH buffering in anaerobic granules, and when methanogens are inhibited an internal pH drop was observed instead of increase in aged anaerobic granules.⁵⁹ Yamaguchi et al. (2001) demonstrated that internal methanogenic activity in anaerobic granules increases the internal pH by acid (H⁺) consumption.⁶⁰ Moreover, Garcia-Robledo et al. (2016) by supplying H₂ in an anaerobic membrane bioreactor treating sieved (2 mm) cattle manure observed an increase in pH from 7 to 9.5 at 0.5 μm depth in the biofilm. The calculated concentration of H⁺ during acidogenesis of MS, AA and LCFA volatile fatty acids (VFA), and H₂ along the biofilm depth in an anaerobic granule and also obtained an increasing pH gradient from the edge toward the center. This is in line with the internal pH gradient measured in CaP granules presented in this study (Figure 1). Mañas et al. (2012) also observed internal precipitation of calcium phosphate in anaerobic granules.⁶² It was proposed that the increase in internal pH is because of biological consumption of H⁺ during methanogenesis in anaerobic granules and contributed to the internal precipitation.⁶² Therefore, the favorable internal conditions for Ca₅(PO₄)₃(OH) formation are most likely biologically induced.

For the formation of HAP only the soluble P (Total Ortho-PO₄³⁻) retained in the reactors without (0.3 mM) and with (1.2 mM) Ca²⁺ addition was considered (Figure 6). Solid P, which represented 68% of the total P in BW, was mostly accumulated in the reactor in both situations, with and without Ca²⁺ addition. Solid P was in the form of organically bound P, struvite, and Ca₅(PO₄)₃(OH).⁶³ Organically bound P is hydrolyzed and solubilized as PO₄³⁻, which leaves the reactor in the effluent or is used for Ca₅(PO₄)₃(OH) formation within the granules, depending on the Ca²⁺ concentration.⁶⁴ Because of the HCO₃⁻ surplus, CaCO₃ is likely formed in the outer biofilm (up to 11 wt %, derived from the Ca/P molar ratio presented in Figure 3). However, CaCO₃ dissolves over time due to the thermodynamic advantage of HAP formation.⁶⁴ Traces of struvite were also observed in the outer biofilm, representing up to 12 wt % of the granules without Ca²⁺ addition, but they dissolve when Ca²⁺ is added, representing less than 2.5 wt % (Figure 3). Struvite was detected in the XRD spectrum of solids from the influent black water, but not in flocculent sludge nor CaP granules.

The time evolution of a CaP granule is a complex process involving three main aspects: (1) recrystallization and maturation of crystals and amorphous complexes from different species (Ca₅(PO₄)₃(OH), CaCO₃, and struvite); (2) microbial growth and decay in the outer biofilm and (3) anaerobic degradation of organics in BW. In this study, these three aspects were correlated based on the anaerobic treatment of a volumetric unit (L) of BW, without a kinetic reference (Figures 3 and 4), larger granules are not necessarily fully mature. Therefore, size separation in the reactor is not ideal. The density of HAP varies from 2 to 6 g/cm³, depending on the formation conditions. Because a much lower density was observed for dispersed sludge (1.4 g/cm³), internal liquid or gas upflow mixing could be used to enhance
the concentration of denser granules at the bottom part. Simultaneously, lifted granules and fine inorganic particles would have higher contact with biomass, PO$_4^{3-}$ and Ca$^{2+}$, stimulating the further growth of younger granules and the formation of the initial nuclei; note that with the UASB configuration used in this study the liquid flows in a vertical plug-flow mode. Additionally, attention should be given to the bulk pH, which should be kept below 7.8 to avoid unwanted Ca$_3$(PO$_4$)$_2$ precipitation in the bulk of the reactor.

**ASSOCIATED CONTENT**

2 Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.est.8b03502.

Description of apparatus for separation of granules used for micro pH measurements and methanogenic activity tests and results obtained for chemical composition of each size fraction. Half-cross section of the pH gradient analyzed in 11 granules and respective statistical analysis. Modeling calculation used for determination of HCO$_3^-$, H$^+$ and H$_2$S flows in the interphase between bulk and granule. SEM-EDX images of other CaP granules. XRD profile for each particle size fraction of each reactor, with and without Ca$^{2+}$ addition (PDF).

**AUTHOR INFORMATION**

Corresponding Author

*E-mail: renata.vanderweijden@wur.nl. ORCID

Jorge Ricardo Cunha: 0000-0002-6791-0119

Notes

The authors declare no competing financial interest.

**ACKNOWLEDGMENTS**

We thank Tanya Georgieva and Jelmer Dijkstra for their contribution in the experimental work. The fruitful discussions with Leon Korving and Filip Kurte are much appreciated. This work was performed in the cooperation framework of Wetsus, European Centre of Excellence for Sustainable Water Technology (www.wetsus.eu). Wetsus is cofunded by the Dutch Ministry of Economic Affairs and Ministry of Infrastructure and Environment, The Province of Fryslân and the Northern Netherlands Provinces. We also thank the members of the research theme Source Separated Sanitation and Environment, The Province of Fryslân.

**REFERENCES**

(1) U.S. Geological Survey. Mineral Commodity Summaries 2015. U.S. Geol. Surv. 2015, 196.

(2) Schröder, J. J.; Cordell, D.; Smit, A. L.; Rosemarin, A. Sustai...
(24) Song, Y.; Weidler, P. G.; Berg, U.; Nüesch, R.; Donnert, D. Calcite-Seedmed Crystallization of Calcium Phosphate for Phosphorus Recovery. *Chemosphere* 2006, 63 (21), 236–243.

(25) Rokidi, S.; Combes, C.; Koutsookus, P. G. The Calcium Phosphate – Calcium Carbonate System: Growth of Octacalcium Phosphate on Calcium Carbonates. *Cryst. Growth Des.* 2011, 11 (5), 1683–1688.

(26) Graaff, M. S. De; Temmink, H.; Zeeman, G.; Buismam, C. J. N. Anaerobic Treatment of Concentrated Black Water in a UASB Reactor at a Short HRT. *Water* 2010, 2 (1), 101–119.

(27) Schmidt, J. E.; Ahring, B. K. Granular Sludge Formation in Uplow Anaerobic Sludge Blanket (UASB) Reactors. *Biotechnol. Bioeng.* 1996, 49 (3), 229–246.

(28) Langerak, E. P. A. Van; Gonzalez-Gil, G.; Aelst, A. Van; Lier, J. B. Van; Hamelers, H. V. M.; Lettinga, G. Effects of High Calcium Concentrations on the Development of Methanogenic Sludge in Uplow Anaerobic Sludge Bed (UASB) Reactors. *Water Res.* 1998, 32 (4), 1255–1263.

(29) Arne Alphenaar, P.; Sleyrster, R.; De Reuver, P.; Ligthart, G. J.; Lettinga, G. Phosphorus Requirement in High-Rate Anaerobic Wastewater Treatment. *Water Res.* 1993, 27 (5), 749–756.

(30) Tung, J.; Pan, H.; Zeng, Y.; Xu, R.; Tang, R. Roles of Amorphous Calcium Phosphate and Biological Additives in the Assembly of Hydroxyapatite Nanoparticles. *J. Phys. Chem. B* 2007, 111 (47), 13410–13418.

(31) Scardi, P.; Leoni, M.; Beyerlein, K. R. On the Modelling of the Powder Pattern from a Nanocrystalline Material. *Zeitschrift fur Krist.* 2011, 226 (12), 924–933.

(32) Bian, S.; Du, L. W.; Gao, Y. X.; Huang, J.; Gou, B. Di; Li, X.; Liu, Y.; Zhang, T. L.; Wang, K. Crystallization in Aggregates of Calcium Phosphate Nanocrystals: A Logistic Model for Kinetics of Fractal Structure Development. *Cryst. Growth Des.* 2012, 12 (7), 3481–3488.

(33) Wang, L.; Ruiz-Agudo, E.; Putnis, C. V.; Menneken, M.; Putnis, A. Kinetics of Calcium Phosphate Nucleation and Growth on Calcite: Implications for Predicting the Fate of Dissolved Phosphate Species in Alkaline Soils. *Environ. Sci. Technol.* 2012, 46 (2), 834–842.

(34) Feng, Q. L.; Pu, G.; Pei, Y.; Cui, F. Z.; Li, H. D.; Kim, T. N. Polymorph and Morphology of Calcium Carbonate Crystals Induced by Proteins Extracted from Mollusk Shell. *J. Cryst. Growth* 2000, 216 (1), 459–465.

(35) Cölfer, H.; Antonietti, M. Crystal Design of Calcium Carbonate Microparticles Using Double-Hydrophilic Block Copolymers. *Langmuir* 1998, 14 (3), 582–589.

(36) Bar-Yosef Ofer, P.; Govrin-Lippman, R.; Garti, N.; Füredi-Milhofer, H. The Influence of Polyelectrolytes on the Formation and Phase Transformation of Amorphous Calcium Phosphate. *Cryst. Growth Des.* 2004, 4 (1), 177–183.

(37) Bertinetti, L.; Drouet, C.; Combes, C.; Rey, C.; Tampieri, A.; Coluccia, S.; Mastra, G. Surface Characteristics of Nanocrystalline Apatites: Effect of Mg Surface Enrichment on Morphology, Surface Hydration Species, and Cationic Environments. *Langmuir* 2009, 25 (10), 5647–5654.

(38) Hori, T.; Haruta, S.; Ueno, Y.; Ishii, M.; Igarashi, Y. Dynamic Transition of a Methanogenic Population in Response to the Concentration of Volatile Fatty Acids in a Thermophilic Anaerobic Digester Dynamic Transition of a Methanogenic Population in Response to the Concentration of Volatile Fatty Acids in a The. *Appl. Environ. Microbiol.* 2006, 72 (2), 1623–1630.

(39) Lens, P. N. L.; Beer, D. D. E.; Cronenberg, C. C. H.; Houwen, F. P.; Engraf, S. P. P. O.; Verstraelen, W. H. Heterogeneous distribution of microbial activity in methanogenic aggregates: PH and glucose microprofiles. *Appl. Environ. Microbiol.* 1993, 59 (11), 3803–3815.

(40) Yamaguchi, T.; Yamazaki, S.; Uemura, S.; Tseng, L. C.; Ohashi, A.; Harada, H. Microbial-Ecological Significance of Sulfide Precipitation within Anaerobic Granular Sludge Revealed by Micro-Electrodes Study. *Water Res.* 2001, 35 (14), 3411–3417.