



Aalborg Universitet

AALBORG UNIVERSITY  
DENMARK

## Quantification of Biologically and Chemically Bound Phosphorus in Activated Sludge from Full-Scale Plants with Biological P-Removal

Petriglieri, Francesca; Petersen, Jette F.; Peces, Miriam; Nierychlo, Marta; Hansen, Kamilla; Baastrand, Cecilie E.; Nielsen, Ulla Gro; Reitzel, Kasper; Nielsen, Per Halkjær

*Published in:*  
Environmental Science and Technology

*DOI (link to publication from Publisher):*  
[10.1021/acs.est.1c02642](https://doi.org/10.1021/acs.est.1c02642)

*Creative Commons License*  
CC BY-NC-ND 4.0

*Publication date:*  
2022

*Document Version*  
Publisher's PDF, also known as Version of record

[Link to publication from Aalborg University](#)

*Citation for published version (APA):*

Petriglieri, F., Petersen, J. F., Peces, M., Nierychlo, M., Hansen, K., Baastrand, C. E., Nielsen, U. G., Reitzel, K., & Nielsen, P. H. (2022). Quantification of Biologically and Chemically Bound Phosphorus in Activated Sludge from Full-Scale Plants with Biological P-Removal. *Environmental Science and Technology*, 56(8), 5132-5140. <https://doi.org/10.1021/acs.est.1c02642>

### General rights

Copyright and moral rights for the publications made accessible in the public portal are retained by the authors and/or other copyright owners and it is a condition of accessing publications that users recognise and abide by the legal requirements associated with these rights.

- Users may download and print one copy of any publication from the public portal for the purpose of private study or research.
- You may not further distribute the material or use it for any profit-making activity or commercial gain
- You may freely distribute the URL identifying the publication in the public portal -

### Take down policy

If you believe that this document breaches copyright please contact us at [vbn@aub.aau.dk](mailto:vbn@aub.aau.dk) providing details, and we will remove access to the work immediately and investigate your claim.

# Quantification of Biologically and Chemically Bound Phosphorus in Activated Sludge from Full-Scale Plants with Biological P-Removal

Francesca Petriglieri, Jette F. Petersen, Miriam Peces, Marta Nierychlo, Kamilla Hansen, Cecilie E. Baastrand, Ulla Gro Nielsen, Kasper Reitzel, and Per Halkjær Nielsen\*



Cite This: *Environ. Sci. Technol.* 2022, 56, 5132–5140



Read Online

ACCESS |

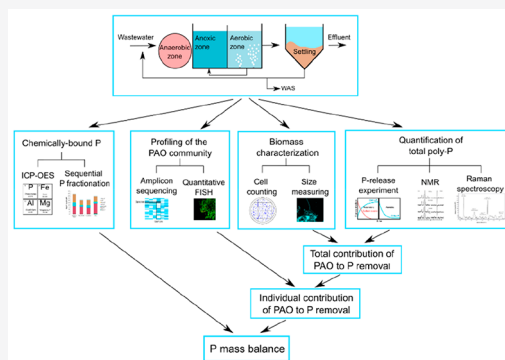
Metrics & More

Article Recommendations

Supporting Information

**ABSTRACT:** Phosphorus (P) is present in activated sludge from wastewater treatment plants in the form of metal salt precipitates, extracellular polymeric substances, or bound into the biomass, for example, as intracellular polyphosphate (poly-P). Several methods for a reliable quantification of the different P-fractions have recently been developed, and this study combines them to obtain a comprehensive P mass-balance of activated sludge from four enhanced biological phosphate removal (EBPR) plants. Chemical characterization by ICP-OES and sequential P fractionation showed that chemically bound P constituted 38–69% of total P, most likely in the form of Fe, Mg, or Al minerals. Raman microspectroscopy, solution state  $^{31}\text{P}$  NMR, and  $^{31}\text{P}$  MAS NMR spectroscopy applied before and after anaerobic P-release experiments, were used to quantify poly-P, which constituted 22–54% of total P and was found in approximately 25% of all bacterial cells. Raman microspectroscopy in combination with fluorescence in situ hybridization was used to quantify poly-P in known polyphosphate-accumulating organisms (PAO) (*Tetrasphaera*, *Candidatus Accumulibacter*, and *Dechloromonas*) and other microorganisms known to possess high level of poly-P, such as the filamentous *Ca. Microthrix*. Interestingly, only 1–13% of total P was stored by unidentified PAO, highlighting that most PAOs in the full-scale EBPR plants investigated are known.

**KEYWORDS:** biological enhanced phosphorus removal (EBPR), P mass-balance, polyphosphate-accumulating organisms (PAO), Raman microspectroscopy, NMR spectroscopy



## INTRODUCTION

Phosphorus (P) is a vital nutrient for all living organisms, but its cycle has been largely altered by human activities, resulting in increasing concerns about its supplies.<sup>1</sup> Moreover, an uncontrolled P discharge into water bodies may cause eutrophication, damaging for animals and humans. This can be partially prevented with efficient removal of P from wastewater,<sup>2</sup> nowadays often achieved by introduction of the enhanced biological phosphorus removal (EBPR) process.<sup>3</sup> The EBPR process offers a sustainable method to recover P, so it can be applied as fertilizer.<sup>4</sup> Worldwide, around 1.3 Mt P are treated every year in municipal wastewater treatment plants (WWTPs), which therefore represent an important source for P recovery and may theoretically substitute 40–50% of phosphate fertilizer applied in agriculture.<sup>5,6</sup>

Activated sludge typically contains 5–15 mgP/gSS for non-EBPR and 30–50 mg P/gSS for EBPR sludge,<sup>7–9</sup> but the P content can vary substantially among different treatment plants. In general, it comprises two main fractions: chemically bound and biogenic P.<sup>10</sup> In most of the cases, the majority of P is present as inorganic P in the form of insoluble metal salts of calcium, iron, or aluminum, sometimes in large amounts, for

example, as vivianite.<sup>10,11</sup> This fraction can vary from 55% to 85% of the total P.<sup>12,13</sup>

Although inorganic P may be predominant in some activated sludge systems, biogenic P also plays a key role as part of the biomass in the form of DNA or other nucleic acids, in cell membranes, in the extracellular polymeric substances (EPS), or in the form of orthophosphate, pyrophosphate, and polyphosphate (poly-P), which, for a long time, has been considered to account for 6.6–10.5% of total P.<sup>10,14</sup> In particular, in EBPR sludge, a large fraction of biogenic P is constituted by poly-P, an intracellular storage compound typical of polyphosphate-accumulating organisms (PAO),<sup>2,3</sup> specifically selected by alternating anaerobic–aerobic conditions. Only recently, the poly-P fraction in some EBPR sludges have been quantified and reported to span between 20 and 60% of total P.<sup>15,16</sup>

**Received:** November 4, 2021

**Revised:** February 8, 2022

**Accepted:** February 24, 2022

**Published:** March 31, 2022



Activated sludge in the EBPR process comprises thousands of bacterial species, but only few genera have been recognized as PAOs. Among these, *Ca. Accumulibacter* and *Tetrasphaera* are the most known.<sup>3</sup> More recently, novel PAOs from the genus *Dechloromonas* have been investigated in situ, showing a similar metabolism to *Ca. Accumulibacter*.<sup>17</sup> Moreover, several putative PAOs, such as *Tessaracoccus* and *Ca. Obscuribacter* are often found in lower abundance in WWTPs,<sup>18</sup> but their importance remains undescribed.<sup>3</sup> Besides typical PAOs, other microorganisms, for example, *Ca. Microthrix*, are known to store poly-P without cycling it in alternating anaerobic/aerobic conditions, but still contributing to P removal.<sup>19–21</sup> Therefore, the identification of novel PAOs and the optimization of their P uptake and release in EBPR systems is of utmost interest for process development and benchmarking.

P recovery strategies rely on the specific types of P actually present in activated sludge. Recently, a new method was developed by Staal et al.<sup>15</sup> for the characterization of different P pools in activated sludge, applying sequential P extractions, which classifies P compounds by their chemical reactivity in specific inorganic P-pools, such as Fe–P or Ca–P. The subsequent application of solution <sup>31</sup>P nuclear magnetic resonance (NMR) spectroscopy, used to identify and estimate P compounds based on the chemical bond type, can then provide information on organic P forms as well as condensed inorganic phosphates (pyro-P and poly-P) in the sample.<sup>15,22</sup> Raman microspectroscopy in combination with fluorescence in situ hybridization (FISH) further provides a reliable single-cell level identification and quantification of intracellular poly-P in activated sludge.<sup>16,17</sup>

In this study, we combined a range of independent techniques to establish a robust and comprehensive P mass-balance covering all main inorganic chemical and biogenic P-species in activated sludge from four full-scale EBPR plants, which were selected based on their large size and long and stable operation. Total P was determined in activated sludge by chemical analysis (ICP-OES), and the inorganic P pools were characterized by P fractionation. The biomass was quantified by fluorescence microscopy, obtaining important information on average cell number and size, and total intracellular poly-P was estimated by Raman microspectroscopy, whereas poly-P in the bulk sludge sample was identified by solid-state <sup>31</sup>P MAS NMR, and solution state <sup>31</sup>P NMR. These three methods, recently developed for a reliable quantification of the different P pools, can be used singularly for poly-P quantification, also in other types of environments, or complementary to each other for a better overview of inorganic and biogenic P-species. Furthermore, the application of FISH-Raman allowed us to determine the contribution of individual PAO genera to P removal, by measuring the levels of intracellular poly-P in probe-defined cells. This has significant implications for the study of EBPR communities, as these microorganisms are recognized as important contributors to P cycling in activated sludge and, therefore, deserve consideration in regard to circular economy and resource recovery from these systems.

## MATERIALS AND METHODS

**Description of the WWTPs Involved.** Four full-scale EBPR WWTPs (Aalborg West, Ejby-Mølle, Lynetten, Viby) were investigated and details about their design, operation, and performance are given in Supporting Information STable 1 and SI Note 1. Activated sludge samples were collected from the aeration tank (at the end of aeration phase for plants with

alternating operation), and transported on ice to the laboratory within few hours. Total suspended solids (TSS) were measured in accordance with Standard Methods.<sup>23</sup> Volatile suspended solids (VSS) information was provided by the plants.

**Sequential P Fractionation.** The modified Psenner fractionation was carried out as previously described by Reitzel,<sup>24</sup> with a fractionation scheme that includes P associated with humic acids.<sup>24</sup> Centrifuged activated sludge (1 g wet-weight) was used for fractionation, and the scheme consisted of six sequential extraction steps: H<sub>2</sub>O, 0.11 M bicarbonate-dithionite (BD), 0.1 M NaOH and 0.5 M HCl, filtration and combustion of the NaOH fraction to obtain Humic-P, and a final combustion step to obtain the residual P (Res-P) as summarized in SI STable 2. This scheme, originally developed for lake sediments, has been previously used for investigation of P pools in activated sludge.<sup>25,26</sup> Total P (TP) in the extracts was measured after conversion of all P in the sample to ortho-P by wet oxidation (5 mL of the fraction mixed with 1 mL potassium peroxodisulfate (K<sub>2</sub>S<sub>2</sub>O<sub>8</sub>, 0.2 M solution) at 120 °C for 1 h). The difference between TP and ortho-P was called nonreactive P (nrP). The P fractions were normalized to the measured TP of the sludge sample, since these were associated with fewer handling steps and potential errors.

**Phosphorus Release Experiments from Activated Sludge and Chemical Analysis.** Batch experiments were conducted in triplicates on fresh activated sludge collected from the aerobic tanks to analyze total P and poly-P-content per cell of FISH-defined cells under anaerobic and aerobic conditions. Fresh activated sludge samples (approximately 4 gSS/L) were aerated for 30 min to reabsorb any phosphate that could have been released during sludge transport. Then, the biomass was used to measure poly-P-full cells. After aeration, sludge was transferred to 200 mL serum bottles and sealed with a rubber stopper and aluminum cap. Pure nitrogen was used to flush the headspace in each bottle to ensure anaerobic conditions. A carbon source containing a mixture of acetate, glucose, and casamino acids was added to provide substrates for as many species of PAO as possible, with a final concentration of the three components of 500, 250, and 250 mg/L, respectively. The serum bottles were kept at room temperature (~22 °C) with shaking for 3 h (150 rpm). Samples for ortho-P analysis were collected every 20 min for the first hour of the experiment, and every 30 min during the remaining 2 h. Biomass from the beginning (0 h) and the end of the anaerobic phase (3 h) were used to quantify poly-P and other P fractions in the sludge by solution NMR (see below). Additionally, samples from the beginning (0 h) and the end of the experiment (3 h) were flash-frozen in liquid nitrogen and subsequently freeze-dried for <sup>31</sup>P MAS NMR or fixed for FISH and Raman analyses (see below). The ortho-P released into the liquid phase was analyzed in accordance with ISO 6878:2004 using the ammonium molybdate-based colorimetric method. Total P, Fe, Al, Ca, Mg, K, and Na were measured in the fresh activated sludge samples. Sludge was homogenized for 10 s with a tissue homogenizer (Heidolph, Schwabach, Germany) and stored at –20 °C until further analysis. Nitric acid (67%) was used to dissolve 0.5 mL of each sludge sample, and the samples were microwave heated, according to U.S. EPA 2007. The total amount of P and other elements in the samples were analyzed in triplicate by ICP-OES, according to Jørgensen et al.<sup>27</sup>

**Poly-P Quantification by Solution State  $^{31}\text{P}$  NMR Spectroscopy and Solid-State  $^{31}\text{P}$  MAS NMR Spectroscopy.** To identify and quantify poly-P in the sludge, solid state (see below), and solution state  $^{31}\text{P}$  NMR spectroscopy was carried out as recently described in Staal et al.<sup>15</sup> Briefly, 30 mL of activated sludge was centrifuged for 10 min at 2000 rpm, and the sludge pellet was used for a two-step extraction, with a 1 h pre-extraction in 40 mL of a 0.05 M EDTA solution followed by a main extraction in 40 mL of 0.25 M NaOH for 16 h. Quantitative  $^{31}\text{P}$  solution NMR spectra were recorded on a JEOL ECZ 500R 500 MHz spectrometer, using a  $90^\circ$  pulse (12  $\mu\text{s}$ ), 2.16 s acquisition time, 25 s relaxation delay, 512 scans, and proton decoupling. Spectra were processed with the MestReNova software using a 5 Hz line broadening with an exponential window function and with zero-filling to 64 K points (32 K points were recorded).

Quantitative solid-state  $^{31}\text{P}$  MAS NMR spectra were recorded on a 500 MHz (11.7 T) NMR spectrometer with a JEOL resonance ECZ 500R console and 11.7 T Oxford magnet using 3.2 mm triple resonance MAS NMR probe, single pulse excitation ( $45^\circ$  pulse), 2–3 min relaxation delay (optimized on sample), 150 scans, and 15 kHz spinning. A synthetic sample of struvite,  $\text{NH}_4\text{MgPO}_4 \cdot 6\text{H}_2\text{O}$ , was used for quantitative determination of the diamagnetic P content in the samples and 85% phosphoric acid was used as a chemical shift reference ( $\delta_{\text{iso}}(^{31}\text{P}) = 0$  ppm). Vnmrj and MestrNova were used for data analyses. A detailed description of the protocol for data analyses was recently reported.<sup>15</sup>

**Biomass Fixation.** Fresh biomass samples from batch reactors and full-scale activated sludge WWTP were either stored at  $-80^\circ\text{C}$  for sequencing workflows or fixed for FISH with 50% ethanol (final concentration) or 4% PFA (final concentration), as previously described<sup>28</sup> and stored at  $-20^\circ\text{C}$  until analyses.

**Cell Counting and Size Measurement.** Total number of bacterial cells, calculated in triplicate, was determined using an Axioskop epifluorescence microscope (Carl Zeiss, Oberkochen, Germany) after staining for 30 min with 50  $\mu\text{g}/\text{mL}$  of DAPI (4,6-diamino-2-phenylindoldihydrochlorid-dilactate). Areas of 1500 cells for Raman microspectroscopy analysis (see below) was measured using ImageJ.<sup>29</sup> The biovolume of the cells was estimated by categorizing cells into perfect geometric shapes. The biovolume of filamentous bacteria was calculated considering filaments as perfect cylinders assuming an average cell length of 1  $\mu\text{m}$ . The biovolume of rod-shaped cells was calculated following Lee and Fuhrman et al.<sup>30</sup> and Frølund et al.<sup>31</sup> methodology. Briefly, the biovolume of rod-shape cells was estimated averaging the volume calculated considering rods as perfect cylinders with half-sphere ends, and the volume calculated considering cocci as perfect spheroids. The average width of filaments and rod-shaped cells was obtained by 2D image analysis using ImageJ<sup>29</sup> for 1000 cells.

**16S rRNA Gene Amplicon Sequencing.** Composition of the microbial community was quantified using FISH and amplicon sequencing. DNA extraction of activated sludge samples from the MiDAS collection<sup>32</sup> was performed as described by Stokholm-Bjerregaard et al.<sup>18</sup> Amplicon sequence variant analysis (ASV) were performed as described elsewhere.<sup>33</sup> Data was analyzed using R (version 3.5.2) and RStudio software (R Development Core Team, 2008).

**Fluorescence In Situ Hybridization and Quantitative FISH.** FISH was performed as described by Daims et al.<sup>34</sup> using a set of specific probes: PAO651<sup>35</sup> targeting *Ca.*

*Accumulibacter*, MCX840<sup>36</sup> targeting the genus *Ca. Microthrix*, Bet135,<sup>37</sup> and Dech443<sup>38</sup> targeting the most abundant species of *Dechloromonas*, and Actino658,<sup>39</sup> Tetra183, and Tetra617<sup>40</sup> to target the genus *Tetrasphaera*. Details about the optimal formamide concentration and use of competitors or helper probes can be found in SI STable 3. Quantitative FISH (qFISH) biovolume fractions of individual taxa were calculated as a percentage area of the total biovolume, hybridizing the EUBmix probes,<sup>41,42</sup> that also hybridizes with the specific probe. Microscopic analysis was performed with a white light laser confocal microscope (Leica TCS SP8 X). qFISH analyses were based on 30 fields of view taken at  $630\times$  magnification using the Daim image analysis software.<sup>43</sup>

**Raman Microspectroscopy and Determination of Total Poly-P and Other Storage Polymers in All Bacteria and in PAOs.** Raman microspectroscopy was applied in combination with FISH as previously described by Fernando et al.,<sup>16</sup> to measure intracellular content of poly-P and other storage polymers in the cells from the beginning (0 h) and the end (3 h) of anaerobic P-release experiments. A detailed description of the method can be found in SI Notes 2. The method assumes that the intensity of the Raman signal is directly dependent on the amount of the analyte in a determined area. Using the same settings as applied in the cited paper and knowing the area of the analyzed cells, it was possible to calculate the amount of poly-P and other storage polymers in chosen cells. The same principle<sup>16</sup> has been applied in this study to calculate the intracellular poly-P content of the total biomass. It was measured by Raman microspectroscopy as an average of 1500 cells randomly selected during the analysis in samples collected from the aeration tanks at the end of the aerobic phase (0 h) and after lab-scale release during anaerobic conditions (3 h) (see SI Notes 3). An average amount of poly-P per cell was calculated as a factor of a constant determined during calibration for poly-P,<sup>16</sup> the average charge-coupled device (CCD) counts determined during the experiment, and the average area of total bacterial cells measured by image analysis (see “Cell Counting and Size Measuring”). This average value of poly-P per cell was multiplied by the total number of all bacterial cells, counted after DAPI staining (see “Cell Counting and Size Measuring”). Additionally, the amount of poly-P in each PAO species was determined using the same procedure, but cells were targeted by their specific FISH probes. As species in *Ca. Microthrix* are known to possess inclusions of storage polymers including poly-P,<sup>19,21,36</sup> they were included in the analysis. As for many filaments, it is very difficult to determine the area of single cells inside filaments with common microscopic techniques, an arbitrary area was determined by multiplying the width of each filament measured with image analysis by 1  $\mu\text{m}$ , obtaining in this way the amount of poly-P in an average segment of 1  $\mu\text{m}$  length. Distribution analysis of the poly-P content and cell size was performed using R (version 3.5.2) (R Development Core Team, 2008) and RStudio software.

## RESULTS AND DISCUSSION

**Overall Chemical Composition of the Four Activated Sludge.** Activated sludge samples from all four EBPR plants had a high P content of 36–50 mgP/gSS. The main inorganic element besides P of the four activated sludge samples were Ca, Na, Fe, and K (SI STable 4). Mg, Al, and Mn were also present in lower amounts. All these elements are normally present in wastewater and may form insoluble salts with P, in



Table 1. Total P, Total Poly-P, and Anaerobic P-Release in Activated Sludge Measured by Independent Methods

WWTP	Total P [mgP/gSS]	Total poly-P			P-release			
		Raman microspectroscopy [mgP/gSS]	<sup>31</sup> P solution NMR [mg/gSS]	<sup>31</sup> P MAS NMR [mg/gSS]	P release experiment [mgP/gSS]	Raman microspectroscopy [mgP/gSS]	<sup>31</sup> P solution NMR [mg/gSS]	<sup>31</sup> P MAS NMR [mg/gSS]
Aalborg West	49.68 ± 1.89	27.11 ± 0.31	22.20	14.00 ± 3.00	27.38 ± 0.89	24.39 ± 0.31	20.10	8.00 ± 3.00
Ejby Mølle	35.85 ± 2.87	13.03 ± 0.52	12.60	3.90 ± 3.00	10.85 ± 0.36	11.58 ± 0.52	11.60	1.90 ± 3.00
Lynetten	37.10 ± 1.74	15.69 ± 0.22	12.40	5.90 ± 3.00	13.75 ± 0.14	13.08 ± 0.22	11.60	3.70 ± 3.00
Viby	45.18 ± 2.26	10.21 ± 0.85	8.80	6.50 ± 3.00	8.47 ± 0.10	8.49 ± 0.85	7.70	0.50 ± 3.00

particular Fe<sup>3+</sup> and Al<sup>3+</sup>. P can be found in complexes with iron oxides or as iron phosphate minerals, such as vivianite or strengite.<sup>44,45</sup> Ca<sup>2+</sup> or Mg<sup>2+</sup> can also form stable precipitates with P, and calcium phosphate precipitation in the form of apatite often takes place in EBPR systems.<sup>46,47</sup> Moreover, these cations are also involved in the PAO metabolism and intervene in stabilizing the intracellular poly-P chains.<sup>47,48</sup> Struvite (NH<sub>4</sub>MgPO<sub>4</sub>·6H<sub>2</sub>O) is also a potential precipitate of P, and its accumulation in the pipes can create operational problems in the plants, causing an increase of the maintenance costs.<sup>49</sup> However, its use for P recovery has been exploited, with the development of struvite precipitation from sludge or anaerobic digesters and its direct application as fertilizer.<sup>50</sup>

**Characterization of P-Pools by Sequential P Fractionation.** To obtain an overview of the major P-pools in the four sludge samples, a sequential P fractionation was carried out (SI SFigure 1, STable 5). Total P in the solid fraction was in the range of 36–50 mgP/gSS, and was dominated by inorganic P, extracted mainly in the BD-P and NaOH-P pools, which constituted 13–22 and 3–7 mgP/gSS, respectively, accounting together for 57–77% of the total P. The BD-P fraction is usually constituted by reducible species of Fe and Mn.<sup>26</sup> The molar Fe:P ratio, calculated using the results obtained with ICP-OES (Table 1), was in the range of 0.31–1.15, much lower than the expected for P bound to Fe hydroxides (8.4 molar ratio)<sup>51</sup> in all plants, and even lower than in common precipitates such as strengite (FePO<sub>4</sub>·2H<sub>2</sub>O) and vivianite (Fe<sub>3</sub>(PO<sub>4</sub>)<sub>2</sub>·8H<sub>2</sub>O) of 1:1 and 1.5:1, respectively,<sup>44,45</sup> with the only exception of Aalborg West. Thus, the BD extract was not specific for extracting P bound to amorphous Fe hydroxides solely. One likely reason for this could be release of bacterial poly-P under the reducing conditions in the BD extract, as previously reported.<sup>25</sup>

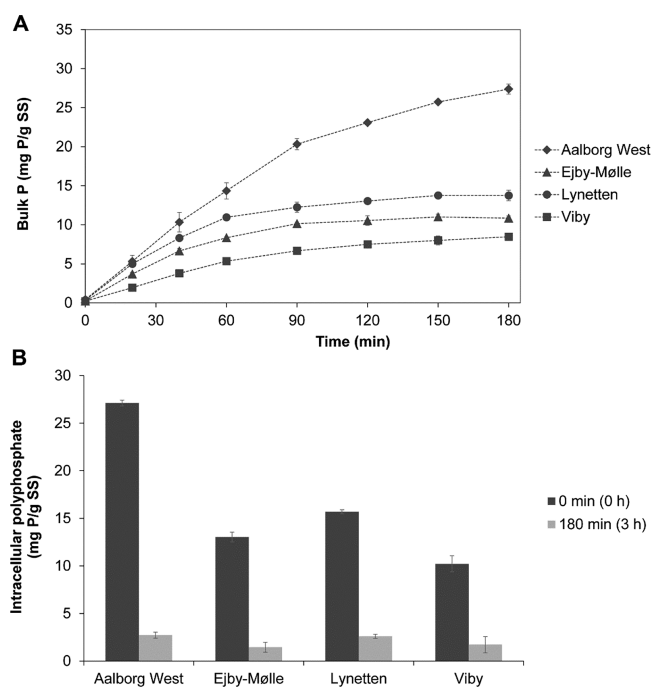
Biogenic P (organic P and poly-P) was mainly extracted in the nrP pool, which accounted for 8–16 mgP/gSS (30–38% of total P). Such high nrP fraction was expected due to the abundance of microbial biomass responsible for the EBPR process, which contains poly-P, in addition to DNA-P, RNA-P, phospholipids, and pyro-P. The presence of large amounts of poly-P was confirmed by solution <sup>31</sup>P NMR (see below), with similar results to previous findings.<sup>25,26</sup> Generally, the nrP quantified by <sup>31</sup>P NMR spectroscopy (the sum of organic P species, pyro-P, and poly-P) was slightly higher than the nrP pool from the fractionation (SI SFigure 1, Table 1, and SI STable 5), most likely due to extraction bias or degradation of poly-P during BD-P extraction, as previously mentioned. As the sequential P fractionation appeared to be imprecise in the determination of the different P pools, we discarded its results from the subsequent analysis and we discourage its application in future studies.

**Characterization of the Bacterial Biomass.** The bacterial biomass was characterized by microscopy, and included number of cells in the activated sludge, their sizes, and their content of poly-P (see next section). In our study, the average number of cells obtained by DAPI staining and epifluorescence microscopy provided results in the same range of 3.78–6.46 × 10<sup>11</sup> cells/gVSS, very similar for all four EBPR plants (SI STable 6) and in accordance with other microscopy-based studies, reporting cell numbers in the range of 2.0 × 10<sup>11</sup> – 26.3 × 10<sup>11</sup> cells/gVSS (SI STable 6).<sup>31,52–56</sup> More recently, flow cytometry has been used for rapid and direct quantification of bacterial cells in activated sludge, with similar results, in the range of 2.7 × 10<sup>11</sup>–11.0 × 10<sup>11</sup> cells/gVSS.<sup>57,58</sup> Fluorescence microscopy<sup>31</sup> and flow-cytometry<sup>58</sup> provided information about the biovolume of bacteria, in the range of 0.16–0.43 μm<sup>3</sup>. However, these estimates do not take into consideration filamentous bacteria which typically constitute 10–20% of the biomass.<sup>59</sup> Therefore, we measured separately biovolumes for spherical/rod-shaped cells and filamentous microorganisms. In accordance with previous findings, the average biovolume of spheroidal/rod-shaped cells for the four plants was 0.363 ± 0.240 μm<sup>3</sup>, while the filaments biovolume was approximated to 0.608 ± 0.245 μm<sup>3</sup> with no difference between the plants.

**Quantification of Total Poly-P by Different Methods.** The intracellular poly-P content is regarded as difficult to analyze reliably, so we applied several independent analytical tools for its absolute quantification. During anaerobic P-release experiment, active PAOs release most of their poly-P as soluble phosphate in the presence of a substrate. Hence, the amount of P released during this phase was compared to the change in intracellular poly-P content obtained by Raman microspectroscopy, <sup>31</sup>P MAS NMR for total poly-P in the sludge, and <sup>31</sup>P solution state NMR after extraction (Table 1).

**P-Release Test.** Similar P-release patterns were observed in all the four plants, with maximum P release after 3 h anaerobic conditions in the range of 8–13 mg P/gSS (Figure 1A, Table 1) in Lynetten, Ejby-Mølle, and Viby (Figure 1A). These values were similar to those previously obtained in Danish EBPR plants of 8–15 mg P/gSS.<sup>60</sup> The highest P release of 27.4 ± 0.9 mg/gSS was observed in Aalborg West.

**Raman Microspectroscopy.** The total amount of intracellular poly-P measured by Raman microspectroscopy varied between the plants, with lowest content observed in Viby (10.2 mg/gSS) and highest in Aalborg West (27.1 mg/gSS) (Figure 1B, Table 1, SI STable 7). A drastic decrease of intracellular poly-P was observed after the 3 h anaerobic conditions in sludge from all plants, reaching values of 1.72–2.72 mg/gSS, corresponding to 10–16% of the poly-P released (Figure 1B, SI STable 7). The difference in total poly-P content measured by Raman corroborated well with the P-released during



**Figure 1.** (A) Bulk ortho-P concentration during the anaerobic P-release experiments with activated sludge from the four WWTPs. (B) Total intracellular poly-P measured as an average of 1500 randomly selected microbial cells by Raman microspectroscopy in initial samples (0 h) and after anaerobic P-release (3 h).

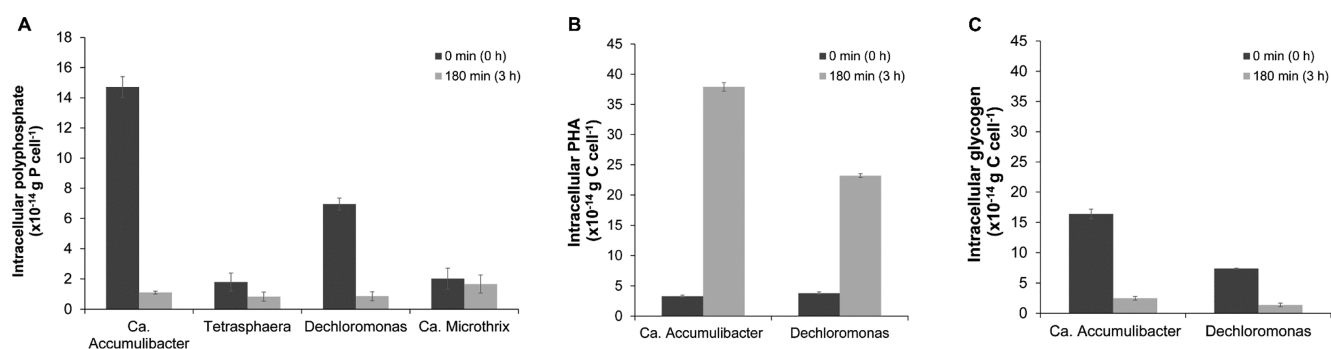
anaerobic batch experiment (Figure 1A and Table 1), and with quantifications by  $^{31}\text{P}$  solution state NMR and  $^{31}\text{P}$  MAS NMR (Table 1, see below). Interestingly, 25–32% of the bacterial cells analyzed contained high amounts of poly-P (SI SFigures 2 and 3). Among these, a fraction (5–8%) also contained high amounts of poly-P after the anaerobic incubation, thus not behaving as canonical PAOs. These may utilize other substrates or they did not show a dynamic P-cycling as observed in canonical PAOs. These could be microorganisms such as the filamentous *Ca. Microthrix* that can store large amount of P, but do not show a typical P uptake-release dynamics.<sup>19</sup> Interestingly, comparison of these fractions to qFISH or amplicon sequencing results (SI STable 8), where the measured fraction of known PAO comprised 12–19% of the total number of bacteria, indicates that there is still up to 10% of unknown PAOs in the activated sludge samples analyzed. Additionally, the lack of poly-P release in 5–10% of the cells corroborates well with the abundance of *Ca. Microthrix*, which was measured by qFISH to comprise 1.5–7.3% of the total number of bacteria. This shows that not only well-recognized PAOs such as *Ca. Accumulibacter* are important for P-removal, but also other unconventional “PAOs” such as *Ca. Microthrix* may be of key importance.

**Poly-P Pools Measured by  $^{31}\text{P}$  Solution State NMR and  $^{31}\text{P}$  MAS NMR.** Two different, but complementary, NMR-based approaches were used to calculate the total amount of poly-P in the four activated sludge samples. While  $^{31}\text{P}$  MAS NMR is used to quantify all poly-P middle groups, solution state  $^{31}\text{P}$  NMR is also showing other organic-bound P forms, as well as poly-P end groups.<sup>15</sup> Their combined application ensures a higher accuracy in the quantification and allows the retrieval of information regarding important organic P species, such as DNA and phospholipids.

According to solution state  $^{31}\text{P}$  NMR (Table 1), poly-P constituted 8.8–22.2 mgP/gSS, which is in the similar range as previous findings in activated sludge of approximately 13 mgP/gSS,<sup>15</sup> and in accordance to the amounts measured by P-release tests (8–27 mgP/gSS) and Raman microspectroscopy (10–27 mgP/gSS). In all plants, the solution state  $^{31}\text{P}$  NMR spectra of the initial samples (time = 0 h, aerobic phase) showed large peaks located at ( $\delta(^{31}\text{P}) \approx -25$  ppm) originating from poly-P middle groups. There were also signals at ( $\delta(^{31}\text{P}) \approx -5$  ppm) originating from poly-P end groups or pyrophosphate (SI SFigures 4–7), which would not be shown with the solely use of  $^{31}\text{P}$  MAS NMR, due to line broadening. These large peaks indicate the presence of large amounts of poly-P in the microbial biomass. In accordance with the results from the P-release experiment and the Raman analysis, the poly-P peaks were not present in the spectra of samples analyzed after anaerobic release (time = 3 h) (SI SFigures 4–7), confirming the sensitivity of the methods and the dynamic levels of this poly-P in the PAO community.

The utilization of solution state  $^{31}\text{P}$  NMR did also provide valuable information about other biologically bound P forms present in bacterial cells, such as DNA-P and P-lipids, where phosphorus is a structural element of their genomes and membranes. DNA-P was found in the biomass from all four WWTP in the range of 1.2–1.4 mg DNA-P/gSS (SI STable 9), which is similar to the DNA-P content measured for non-PAO pure cultures (i.e., *Bacillus subtilis* and *Pseudomonas putida*), ranging from 0.39–2.78 mg DNA-P/SS, analyzed by  $^{31}\text{P}$  NMR.<sup>81</sup> The same study also reports a total P content of 15–18 mgP/g SS for non-PAO pure cultures, which is consistent with common P content in non-EBPR sludge of 5–15 mgP/g SS.<sup>60</sup> Similarly, lipid-P values were approximately 0.5–0.6 mg DNA-P/gSS (SI STable 9), in the range of previously reported measurements of 0.26–9.32 for pure bacterial cultures obtained by NMR or other methods.<sup>61–65</sup> These results suggest that P bound to structural cell components has a fixed stoichiometry, indicating that a small fraction of the P in activated sludge is removed by incorporation during biomass growth, but not readily available for P recovery (e.g., as struvite) before further degradation.

The  $^{31}\text{P}$  MAS NMR spectra (SI SFigure 8) of the four initial samples (time = 0 h, aerobic phase) confirmed the results obtained by other methods, showing the spinning sideband manifold of two broad resonances located ( $\delta(^{31}\text{P}) \approx -2$  and  $-25$  ppm), which originate from ortho- and poly-P, respectively. After 3 h, the poly-P concentration was below the detection limit for Ejby-Mølle, Viby, and Lynetten, implying full degradation of poly-P, whereas for Aalborg West ca. 9% of the total intensity was from pyro-P (SI STable 10). The Aalborg West sample was different from the other three plants as it had the highest concentration of P (14 mg/gSS). We note that  $^{31}\text{P}$  present in magnetic phases such as iron phosphate minerals formed, for example, by addition of Fe(III) salts to the plant, will not be observed under the experimental conditions used, as recently discussed.<sup>15</sup> There was a small (2–4 ppm) shift in the center of gravity of the ortho-P resonance, which contains contributions from both inorganic and organic species after 3 h, implying that the speciation of ortho-P in the sludge changed during the release experiment. At 0 h this resonance was a mixture of the different inorganic and organic phosphate, whereas inorganic P-phases such as magnesium and calcium P prevailed at 3 h, due to the release of biological P.



**Figure 2.** Dynamics of the storage polymers poly-P, PHA, and glycogen in important PAOs in activated sludge. Data from Ejby-Mølle WWTP is shown as example. (A) Poly-P levels during aerobic phase (0 h) and after anaerobic P-release (3 h) in known PAOs and in the unconventional PAO *Ca. Microthrix*. (B) PHA levels in *Ca. Accumulibacter* and *Dechloromonas* cells. No PHA was detected in *Tetrasphaera* and *Ca. Microthrix*. (C) Glycogen levels in *Ca. Accumulibacter* and *Dechloromonas* cells. No glycogen was detected in *Tetrasphaera* and *Ca. Microthrix*.

**PAO Community Profiling.** According to the 16S rRNA gene sequencing results, activated sludge in all four plants comprised a large community of PAOs, with *Tetrasphaera* being the most abundant. However, FISH showed that *Ca. Accumulibacter* had a larger relative biovolume (1.0–5.7% of total biomass) in all plants than indicated by amplicon sequencing (SI SFigure 9, STable 8), showing the difference in quantifying biomass by the two methods due to differences in extraction efficiency, primer bias, 16S rRNA gene copy number, and cell size.<sup>66</sup> This relation seem species-specific, also exemplified by the non-conventional PAO *Ca. Microthrix*, where both amplicon and FISH showed a large relative biomass fraction (1.5–7.3%) in all plants, contributing significantly to the P-removal. Thus, it is still difficult to translate an amplicon community profiling to a poly-P accumulation potential as it very much depends on the exact species composition.

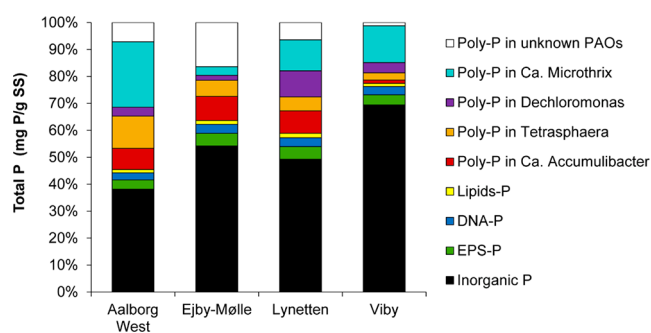
**Quantification of the Main Storage Polymers in PAOs in Full-Scale EBPR Plants.** FISH-Raman was used to determine the level and dynamics of storage polymers accumulated by the known probe-defined PAOs and in the filamentous *Ca. Microthrix*, the latter known to accumulate poly-P, but not releasing it rapidly during anaerobic conditions. The intracellular content of poly-P, PHA, and glycogen was very similar in the same probe-defined species of PAOs across different plants (SI STables 11 and 12). No glycogen and PHA were detected in *Tetrasphaera* and *Ca. Microthrix*, in accordance with their current metabolic models and previous findings.<sup>16,67</sup>

The dynamics and levels of storage polymers in PAOs in Ejby-Mølle plant are shown as an example in Figure 2 (other plants, see SI STables 11 and 12). The amount of poly-P (g/cell) (Figure 2A) was in the range of  $10\text{--}15 \times 10^{-14}$ ,  $6.2\text{--}6.9 \times 10^{-14}$ ,  $1.0\text{--}1.8 \times 10^{-14}$ ,  $1.1\text{--}2.2 \times 10^{-14}$  for *Ca. Accumulibacter*, *Dechloromonas*, *Tetrasphaera*, and *Ca. Microthrix*, respectively. The amount calculated in this study for the well-known PAOs *Ca. Accumulibacter* and *Tetrasphaera* is lower than the amount measured in a previous study applying similar approach.<sup>16</sup> This difference is caused by an over-estimation of the cell area by Fernando et al.,<sup>16</sup> as cell sizes from pure cultures or enrichments were extrapolated to full-scale plants. In this study, the area of the cells measured in situ were smaller, resulting in a lower amount of poly-P per cell. In all three conventional PAOs, poly-P was hydrolyzed and ortho-P released into the bulk liquid during the anaerobic incubation. Considering only the poly-P amount accumulated intra-

cellularly per cell, *Ca. Accumulibacter* had the largest amount due to its larger size (SI STable 13). However, when looking at the average amount of poly-P in the cells ( $\text{g}/\mu\text{m}^3$ ), the three conventional PAOs had almost similar content, in average  $4.5 \times 10^{-14}$ ,  $3.9 \times 10^{-14}$  and  $2.8 \times 10^{-14}$  for *Ca. Accumulibacter*, *Tetrasphaera*, and *Dechloromonas*, respectively. Moreover, all PAOs were highly abundant and, therefore, had a significant contribution to P-removal. As expected, *Ca. Microthrix* released only a small amount of poly-P during the 3 h anaerobic conditions. Although the cell width is relatively small, and thus also the P-content per  $\mu\text{m}$ , they can be significant contributors to P removal with filaments length exceeding  $100 \mu\text{m}$ .<sup>68</sup>

*Ca. Accumulibacter* and *Dechloromonas* cells possessed a dynamic behavior of intracellular PHA and glycogen (Figure 2B,C, SI STable 12), as previously described.<sup>16,17</sup> In both organisms, the C/P and C/C ratio were within the range of 0.3–0.4, in accordance with the accepted metabolic model for conventional PAOs and previous studies.<sup>69,70</sup>

**A Comprehensive P Mass-Balance.** The combination of the data from ICP-OES, Raman analysis, solution state and solid state <sup>31</sup>P MAS NMR, allowed us to obtain a very comprehensive and robust mass-balance of P-fractions in activated sludge from the four EBPR plants (Figure 3). Inorganic P, calculated by subtracting all the other measured fractions from the total P, was the biggest fraction in all plants except Aalborg West, constituting 38–69% of the total P. In Viby more than 70% of the total P was bound as inorganic P, most likely linked to iron or aluminum. A closer inspection of the operation in Viby revealed substantial addition of Fe(III)



**Figure 3.** Comprehensive P-mass balance in the activated sludge. Distribution (in percentage) of P pools in activated sludge in the aeration tanks of four full-scale EBPR plants.



salts in the period before sampling, which could explain its poor EBPR performance at the time of sampling. According to the sequential P fractionations, the 57–77% of the P was in the inorganic fraction (mainly BD-P and NaOH-P fractions). The difference of these values, compared to those that can be extrapolated by solution  $^{31}\text{P}$  NMR,  $^{31}\text{P}$  MAS NMR spectroscopy, and Raman microspectroscopy measurements, were most likely due to extraction bias or degradation of poly-P during BD-P extraction.

In all the plants, a large part of total P was present as biogenic P, and a natural part of the microbial biomass, such as DNA-P or membrane lipids, or included into the EPS.<sup>14</sup> Solution state  $^{31}\text{P}$  NMR provided valuable quantification of these distinct fractions, with DNA-P and lipid-P of approximately 1.7–1.9 mg P/gSS, constituting together 3–4% of total P (Figure 3). Previous studies on P speciation in the EPS have reported EPS-P values in the range of 0.9–2.1 mg P/gSS,<sup>14,71,72</sup> which would account for 3–4% of the total P (Figure 3).

The second largest fraction in all plants but Viby was poly-P, constituting 22–54% of total P (Figure 3). Even though there were some variations between the independent methods applied for total poly-P quantification (3.8 mgP/SS average standard deviation), all of them provided a comparable estimation of the poly-P content in the different samples (Table 1) and could be independently used in future studies on EBPR communities or PAO organisms present in other environments.

Interestingly, approximately 25% (and up to 32%) of all bacterial cells in the activated sludge accumulated large amounts of poly-P, being either PAOs or nonconventional PAOs. In all four plants, the three PAO genera investigated (*Ca. Accumulibacter*, *Tetrasphaera*, and *Dechloromonas*) were important for P-removal, and qFISH demonstrated that the known PAOs accounted for 5–12% of the total biomass, corresponding to approximately 50–65% of all cells containing poly-P. This fraction included the unconventional PAO *Ca. Microthrix*, leaving around 35–50% of the cells accumulating poly-P as undescribed. The contribution of the known PAO genera varied from plant to plant, depending on their biovolumes and relative abundances. The contribution of *Ca. Microthrix* exceeded the other known PAOs in three plants (Aalborg West, Lynetten, and Viby) because of its biovolume and high abundance. *Ca. Microthrix* is considered unwanted because it causes settling (bulking) or foaming problems in full-scale WWTPs,<sup>59</sup> but this study also shows that they should be regarded as important contributors to P removal in EBPR systems.<sup>36</sup> The undescribed cells containing poly-P accounted for a small amount of the total P (e.g., in Viby), most likely because many were very small cells and, therefore, accumulating low amounts of poly-P. This indicates that future efforts in the study of EBPR communities are still needed, but also that most of the important PAOs in the plants investigated are now known.

## ■ ASSOCIATED CONTENT

### SI Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/acs.est.1c02642>.

Additional figures (SFigures 1–9), additional tables (STables 1–13), and additional notes for support and clarification (PDF)

## ■ AUTHOR INFORMATION

### Corresponding Author

Per Halkjær Nielsen – Center for Microbial Communities, Department of Chemistry and Bioscience, Aalborg University, DK-9220 Aalborg, Denmark; Phone: +45 9940 8503; Email: [phn@bio.aau.dk](mailto:phn@bio.aau.dk)

### Authors

Francesca Petriglieri – Center for Microbial Communities, Department of Chemistry and Bioscience, Aalborg University, DK-9220 Aalborg, Denmark; [orcid.org/0000-0002-1676-7690](https://orcid.org/0000-0002-1676-7690)

Jette F. Petersen – Center for Microbial Communities, Department of Chemistry and Bioscience, Aalborg University, DK-9220 Aalborg, Denmark

Miriam Peces – Center for Microbial Communities, Department of Chemistry and Bioscience, Aalborg University, DK-9220 Aalborg, Denmark

Marta Nierychlo – Center for Microbial Communities, Department of Chemistry and Bioscience, Aalborg University, DK-9220 Aalborg, Denmark

Kamilla Hansen – Center for Microbial Communities, Department of Chemistry and Bioscience, Aalborg University, DK-9220 Aalborg, Denmark

Cecilie E. Baastrand – Department of Physics, Chemistry and Pharmacy, University of Southern Denmark, DK-S230 Odense M, Denmark

Ulla Gro Nielsen – Department of Physics, Chemistry and Pharmacy, University of Southern Denmark, DK-S230 Odense M, Denmark; [orcid.org/0000-0002-2336-3061](https://orcid.org/0000-0002-2336-3061)

Kasper Reitzel – Department of Biology, University of Southern Denmark, DK-S230 Odense M, Denmark

Complete contact information is available at:

<https://pubs.acs.org/10.1021/acs.est.1c02642>

### Notes

The authors declare no competing financial interest.

## ■ ACKNOWLEDGMENTS

We thank Susanne Bielidt for assistance with cell counting and microscopical analysis. The project was funded by the Villum Foundation (Dark Matter, grant 13351) and Innovation Fund Denmark (ReCoverP, grant 4106-00014B).

## ■ REFERENCES

- (1) Liu, X.; Sheng, H.; Jiang, S.; Yuan, Z.; Zhang, C.; Elser, J. J. Intensification of phosphorus cycling in China since the 1600s. *Proc. Natl. Acad. Sci. U.S.A.* **2016**, *113*, 2609–2614.
- (2) Oehmen, A.; Lemos, P. C.; Carvalho, G.; Yuan, Z.; Keller, J.; Blackall, L. L.; Reis, M. A. M. Advances in enhanced biological phosphorus removal: From micro to macro scale. *Water Res.* **2007**, *41*, 2271–2300.
- (3) Nielsen, P. H.; McIlroy, S. J.; Albertsen, M. Re-evaluating the microbiology of the enhanced biological phosphorus removal process. *Curr. Opin. Biotechnol.* **2019**, *57*, 111–118.
- (4) Ek, M.; Bergstrom, R.; Bjorhem, J.-E.; Bjorlenius, B.; Hellstrom, D. Concentration of nutrients from urine and reject water from anaerobically digested sludge. *Water Sci. Technol.* **2006**, *54*, 437–444.
- (5) Van Vuuren, D.P.; Bouwman, A.F.; Beusen, A.H.W. Phosphorus demand for the 1970–2100 period: a scenario analysis of resource depletion. *Glob. Environ. Chang.* **2010**, *20*, 428–439.
- (6) Egle, L.; Rechberger, H.; Krampe, J.; Zessner, M. Phosphorus recovery from municipal wastewater: an integrated comparative



- technological, environmental and economic assessment of P recovery technologies. *Sci. Total Environ.* **2016**, *571*, 522–542.
- (7) Jensen, M. D.; Tychsen, P.; Thomsen, M.; Martinsen, L.; Hasler, B. Bæredygtig udnyttelse af fosfor fra spildevand. In *Miljøprojekt nr. 1661*; Miljøstyrelsen: Miljøministeriet, Denmark, 2015.
- (8) Metcalf & Eddy Inc., Abu-Orf, M.; Bowden, G.; Burton, F. L.; Pfrang, W.; Stensel, H. D.; Tchobanoglous, G.; Tsuchihashi, R. *Wastewater Engineering: Treatment and Resource Recovery*, 6th ed.; McGraw Hill Education, 2014.
- (9) Menar, A. B.; Jenkins, D. Fate of phosphorus in waste treatment processes: enhanced removal of phosphate by activated sludge. *Environ. Sci. Technol.* **1970**, *4*, 1115–1121.
- (10) Mino, T.; Kawakami, T.; Matsuo, T. Location of phosphorus in activated sludge and function of intracellular polyphosphates in biological phosphorus removal process. *Water Sci. Technol.* **1985**, *17*, 93–106.
- (11) Wilfert, P.; Mandalidis, A.; Dugulan, A. I.; Goubitz, K.; Korving, L.; Temmink, H. Vivianite as an important iron phosphate precipitate in sewage treatment plants. *Water Res.* **2016**, *104*, 449–460.
- (12) Xie, C.; Zhao, J.; Tang, J.; Xu, J.; Lin, X.; Xu, X. The phosphorus fractions and alkaline phosphatase activities in sludge. *Bioresour. Technol.* **2011**, *102*, 2455–2461.
- (13) González, M. J. J.; Pérez, B. C.; Fernández, G. Analytical phosphorus fractionation in sewage sludge and sediment samples. *Anal Bioanal Chem.* **2005**, *381*, 873–878.
- (14) Zhang, H.; Fang, W.; Wang, Y.; Sheng, G.; Xia, C.; Zeng, R. J.; Yu, H. Species of phosphorus in the extracellular polymeric substances of EBPR sludge. *Bioresour. Technol.* **2013**, *142*, 714–718.
- (15) Staal, L. B.; Petersen, A. B.; Jørgensen, C. A.; Gro, U.; Nielsen, P. H.; Reitzel, K. Extraction and quantification of polyphosphates in activated sludge from waste water treatment plants by <sup>31</sup>P NMR spectroscopy. *Water Res.* **2019**, *157*, 346–355.
- (16) Fernando, E. Y.; McIlroy, S. J.; Nierychlo, M.; Herbst, F.-A.; Petriglieri, F.; Schmid, M. C.; Wagner, M.; Nielsen, J. L.; Nielsen, P. H. Resolving the individual contribution of key microbial populations to enhanced biological phosphorus removal with Raman-FISH. *ISME J.* **2019**, *13*, 1933–1946.
- (17) Petriglieri, F.; Singleton, C.; Peces, M.; Petersen, J. F.; Nierychlo, M.; Nielsen, P. H. *Candidatus* Dechloromonas phosphorotropha and “Ca D. phosphorivorans”, novel polyphosphate accumulating organisms abundant in wastewater treatment systems. *ISME J.* **2021**, *15*, 3605–3614.
- (18) Stokholm-Bjerregaard, M.; McIlroy, S. J.; Nierychlo, M.; Karst, S. M.; Albertsen, M.; Nielsen, P. H. A critical assessment of the microorganisms proposed to be important to enhanced biological phosphorus removal in full-scale wastewater treatment systems. *Front Microbiol.* **2017**, *8*, 718.
- (19) Wang, J.; Qi, R.; Liu, M.; Li, Q.; Bao, H.; Li, Y.; Wang, S.; Tandoi, V.; Yang, M. The potential role of ‘*Candidatus* Microthrix parvicella’ in phosphorus removal during sludge bulking in two full-scale enhanced biological phosphorus removal plants. *Water Sci. Technol.* **2014**, *70*, 367–375.
- (20) Wang, J.; Liu, Q.; Qi, R.; Tandoi, V.; Yang, M. Sludge bulking impact on relevant bacterial populations in a full-scale municipal wastewater treatment plant. *Process Biochem.* **2014**, *49*, 2258–2265.
- (21) Nielsen, P. H.; Roslev, P.; Dueholm, T. E.; Nielsen, J. L. *Microthrix parvicella* a specialized lipid consumer in anaerobic - aerobic activated sludge plants. *Water Sci. Technol.* **2002**, *46*, 73–80.
- (22) Hupfer, M.; Gloess, S.; Schmieder, P.; Grossart, H. P. Methods for detection and quantification of polyphosphate and polyphosphate accumulating microorganisms in aquatic sediments. *Internat. Rev. Hydrobiol.* **2008**, *93*, 1–30.
- (23) APHA, AWWA WEF. *Standard Methods for the Examination of Water and Wastewater*; American Public Health Association, American Water, 2005.
- (24) Reitzel, K. Separation of aluminum bound phosphate from iron bound phosphate in freshwater sediments by a sequential extraction procedure. In *Phosphates in Sediments*, **2005**; pp 109–117.
- (25) Uhlmann, D.; Roske, I.; Hupfer, M.; Ohms, G. A simple method to distinguish between polyphosphate and other phosphate fractions of activated sludge. *Water Res.* **1990**, *24*, 1355–1360.
- (26) Röske, I.; Schonborn, C. Interactions between chemical and advanced biological phosphorus elimination. *Water Res.* **1994**, *28*, 1103–1109.
- (27) Jørgensen, M. K.; Nierychlo, M.; Nielsen, A. H.; Larsen, P.; Christensen, M. L.; Nielsen, P. H. Unified understanding of physico-chemical properties of activated sludge and fouling propensity. *Water Res.* **2017**, *120*, 117–132.
- (28) Nielsen, J. L. Protocol for fluorescence *in situ* hybridization (FISH) with rRNA-targeted oligonucleotides. In *FISH Handbook for Biological Wastewater Treatment*, 2009; pp 73–84.
- (29) Schneider, C. A.; Rasband, W. S.; Elceiri, K. W. NIH Image to ImageJ: 25 years of image analysis. *Nat. Methods* **2012**, *9*, 671–675.
- (30) Lee, S.; Fuhrman, J. A. Relationships between biovolume and biomass of naturally derived marine bacterioplankton. *Appl. Environ. Microbiol.* **1987**, *53*, 1298–1303.
- (31) Frølund, B.; Palmgren, R.; Keiding, K.; Nielsen, P. H. Extraction of extracellular polymers from activated sludge using a cation exchange resin. *Water Res.* **1996**, *30*, 1749–1758.
- (32) McIlroy, S. J.; Kirkegaard, R. H.; McIlroy, B.; Nierychlo, M.; Kristensen, J. M.; Karst, S. M.; Albertsen, M.; Nielsen, P. H. MiDAS 2.0: An ecosystem-specific taxonomy and online database for the organisms of wastewater treatment systems expanded for anaerobic digester groups. *Database* **2017**, *2017*, 1–9.
- (33) Dueholm, M. S.; Andersen, K. S.; McIlroy, S. J.; Kristensen, J. M.; Yashiro, E.; Karst, S. M.; Albertsen, M.; Nielsen, P. H. Generation of comprehensive ecosystem-specific reference databases with species-level resolution by high-throughput full-length 16S rRNA gene sequencing and Automated Taxonomy assignment (AutoTax). *MBio* **2020**, *11*, No. e01557–20.
- (34) Daims, H.; Stoecker, K.; Wagner, M. Fluorescence *in situ* hybridization for the detection of prokaryotes. In *Molecular Microbial Ecology*; Osborn, A. M., Smith, C. J., Eds.; Taylor & Francis: New York, 2005; pp 213–239.
- (35) Crocetti, G. R.; Hugenholtz, P.; Bond, P. L.; Schuler, A. J.; Keller, J.; Jenkins, D.; Blackall, L. L. Identification of polyphosphate-accumulating organisms and design of 16SrRNA-directed probes for their detection and quantitation. *Appl. Environ. Microbiol.* **2000**, *66*, 1175–1182.
- (36) Nierychlo, M.; Singleton, C. M.; Petriglieri, F.; Thomsen, L.; Petersen, J. F.; Peces, M.; Kondrotaitė, Z.; Dueholm, M. S.; Nielsen, P. H. Low global diversity of *Candidatus* Microthrix, a troublesome filamentous organism in full-scale WWTPs. *Front Microbiol.* **2021**, *12*, 690251.
- (37) Kong, Y.; Xia, Y.; Nielsen, J. L.; Nielsen, P. H. Structure and function of the microbial community in a full-scale enhanced biological phosphorus removal plant. *Microbiology* **2007**, *153*, 4061–4073.
- (38) McIlroy, S. J.; Starnawska, A.; Starnawski, P.; Saunders, A. M.; Nierychlo, M.; Nielsen, P. H.; Nielsen, J. L. Identification of active denitrifiers in full-scale nutrient removal wastewater treatment systems. *Environ. Microbiol.* **2016**, *18*, 50–64.
- (39) Kong, Y.; Nielsen, J. L.; Nielsen, P. H. Identity and ecophysiology of uncultured actinobacterial polyphosphate-accumulating organisms in full-scale enhanced biological phosphorus removal plants. *Appl. Environ. Microbiol.* **2005**, *71*, 4076–4085.
- (40) Dueholm, M. S.; Andersen, K. S.; Petriglieri, F.; McIlroy, S. J.; Nierychlo, M.; Petersen, J. F.; Kristensen, J. M.; Yashiro, E.; Karst, S. M.; Albertsen, M.; Nielsen, P. H. Comprehensive ecosystem-specific 16S rRNA gene databases with automated taxonomy assignment (AutoTax) provide species-level resolution in microbial ecology. *bioRxiv* **2019**, 672873.
- (41) Daims, H.; Brühl, A.; Amann, R.; Schleifer, K. H.; Wagner, M. The domain-specific probe EUB338 is insufficient for the detection of all Bacteria: development and evaluation of a more comprehensive probe set. *Syst. Appl. Microbiol.* **1999**, *22*, 434–444.

- (42) Amann, R. I.; Binder, B. J.; Olson, R. J.; Chisolm, S. W.; Devereux, R.; Stahl, D. A. Combination of 16S rRNA-targeted oligonucleotide probes with flow cytometry for analyzing mixed microbial populations. *Appl. Environ. Microbiol.* **1990**, *56*, 1919–1925.
- (43) Daims, H.; Lückner, S.; Wagner, M. Daime, a novel image analysis program for microbial ecology and biofilm research. *Environ. Microbiol.* **2006**, *8*, 200–213.
- (44) Wilfert, P.; Kumar, P. S.; Korving, L.; Witkamp, G.-J.; van Loosdrecht, M. C. M. The relevance of phosphorus and iron chemistry to the recovery of phosphorus from wastewater: a review. *Environ. Sci. Technol.* **2015**, *49*, 9400–9414.
- (45) Wilfert, P.; Dugulan, A.I.; Goubitz, K.; Korving, L.; Witkamp, G.J.; Van Loosdrecht, M.C.M. Vivianite as the main phosphate mineral in digested sewage sludge and its role for phosphate recovery. *Water Res.* **2018**, *144*, 312–321.
- (46) Manas, A.; Biscans, B.; Sperandio, M. Biologically induced phosphorus precipitation in aerobic granular sludge process. *Water Res.* **2011**, *45*, 3776–3786.
- (47) Zhang, H.; Sheng, G.; Fang, W.; Wang, Y.; Fang, C. Calcium effect on the metabolic pathway of phosphorus accumulating organisms in enhanced biological phosphorus removal systems. *Water Res.* **2015**, *84*, 171–180.
- (48) Schonborn, C.; Bauer, H.-D.; Röske, I. Stability of enhanced biological phosphorus removal and composition of polyphosphate granules. *Water Res.* **2001**, *35*, 3190–3196.
- (49) Charles, W.; Ho, G.; Costa, M.; Spencer, P. Solutions to a combined problem of excessive hydrogen sulfide in biogas and struvite scaling. *Water Sci. Technol.* **2006**, *53*, 203–210.
- (50) Yuan, Z.; Pratt, S.; Batstone, D. J. Phosphorus recovery from wastewater through microbial processes. *Curr. Opin. Biotechnol.* **2012**, *23*, 878–83.
- (51) Jensen, H. S.; Kristensen, P.; Jeppesen, E.; Skytthe, A. Iron:phosphorus ratio in surface sediment as an indicator of phosphate release from aerobic sediments in shallow lakes. *Hydrobiologia* **1992**, *235*, 731–743.
- (52) Rasmussen, H.; Bruus, J. H.; Keiding, K.; Nielsen, P. H. Observations on dewaterability and physical, chemical and microbiological changes in anaerobically stored activated sludge from a nutrient removal plant. *Water Res.* **1994**, *28*, 417–425.
- (53) Urban, V.; Block, J. C.; Manem, J. Bioflocculation in activated sludge: an analytic approach. *Water Res.* **1993**, *27*, 829–838.
- (54) Cloete, T. E.; Africa, S. A combined membrane filter-immunofluorescent technique for the *in situ* identification and enumeration of *Acinetobacter* in activated sludge. *Water Res.* **1988**, *22*, 961–969.
- (55) Vollertsen, J. E. S.; Jahn, A.; Nielsen, J. L.; Hvitved-Jacobsen, T.; Nielsen, P. H. Comparison of methods for determination of microbial biomass in wastewater. *Water Res.* **2001**, *35*, 1649–1658.
- (56) Nielsen, J. L.; Nielsen, P. H. Enumeration of acetate-consuming bacteria by microautoradiography under oxygen and nitrate respiring conditions in activated sludge. *Water Res.* **2002**, *36*, 421–428.
- (57) Ma, L.; Mao, G.; Liu, J.; Yu, H.; Gao, G.; Wang, Y. Rapid quantification of bacteria and viruses in influent, settled water, activated sludge and effluent from a wastewater treatment plant using flow cytometry. *Water Sci. Technol.* **2013**, *68*, 1763–1769.
- (58) Foladori, P.; Bruni, L.; Tamburini, S.; Zigliio, G. Direct quantification of bacterial biomass in influent, effluent and activated sludge of wastewater treatment plants by using flow cytometry. *Water Res.* **2010**, *44*, 3807–3818.
- (59) Nierychlo, M.; McIlroy, S. J.; Kucheryavskiy, S. V.; Jiang, C.; Ziegler, A. S.; Kondrotaitė, Z.; Stokholm-Bjerregaard, M.; Nielsen, P. H. *Candidatus* Amarolinea and *Candidatus* Microthrix are mainly responsible for filamentous bulking in municipal Danish wastewater treatment plants. *Front. Microbiol.* **2020**, *11*, 1214.
- (60) Mielczarek, A. T.; Thi, H.; Nguyen, T.; Nielsen, J. L.; Nielsen, P. H. Population dynamics of bacteria involved in enhanced biological phosphorus removal in Danish wastewater treatment plants. *Water Res.* **2013**, *47*, 1529–1544.
- (61) Makarov, M. I.; Haumaier, L.; Zech, W. Nature of soil organic phosphorus: an assessment of peak assignments in the diester region of  $^{31}\text{P}$  NMR spectra. *Soil Biol. Biochem.* **2002**, *34*, 1467–1477.
- (62) Imhoff, J. F.; Kushner, D. J.; Kushwaha, S. C.; Kates, M. Polar lipids in phototrophic bacteria of the *Rhodospirillaceae* and *Chromatiaceae* families. *J. Bacteriol.* **1982**, *150*, 1192–1201.
- (63) Makula, R. A. Phospholipid composition of methane-utilizing bacteria. *J. Bacteriol.* **1978**, *134*, 771–777.
- (64) Pinkart, H. C.; White, D. C. Phospholipid biosynthesis and solvent tolerance in *Pseudomonas putida* strains. *J. Bacteriol.* **1997**, *179*, 4219–4226.
- (65) den Kamp, J. A. F. O.; Redai, I.; van Deenen, L. L. M. Phospholipid composition of *Bacillus subtilis*. *J. Bacteriol.* **1969**, *99*, 298–303.
- (66) Albertsen, M.; Karst, S. M.; Ziegler, A. S.; Kirkegaard, R. H.; Nielsen, P. H. Back to basics - The influence of DNA extraction and primer choice on phylogenetic analysis of activated sludge communities. *PLoS One* **2015**, *10*, 1–15.
- (67) McIlroy, S. J.; Kristiansen, R.; Albertsen, M.; Karst, S. M.; Rossetti, S.; Nielsen, J. L.; Tandoi, V.; Seviour, R. J. Metabolic model for the filamentous ‘*Candidatus* Microthrix parvicella’ based on genomic and metagenomic analyses. *ISME J.* **2013**, *7*, 1161–1172.
- (68) Blackall, L. L.; Stratton, H.; Bradford, D.; Dot, T. D.; Sjørup, C.; Seviour, E. M.; Seviour, R. J. *Candidatus* Microthrix parvicella”, a filamentous bacterium from activated sludge sewage treatment plants. *Int. J. Syst. Evol. Microbiol.* **1996**, *46*, 344–346.
- (69) Qiu, G.; Zuniga-Montanez, R.; Law, Y.; Swa, S. Polyphosphate-accumulating organisms in full-scale tropical wastewater treatment plants use diverse carbon sources. *Water Res.* **2019**, *149*, 469–510.
- (70) Marques, R.; Santos, J.; Nguyen, H.; Carvalho, G.; Noronha, J. P.; Nielsen, P. H.; Reis, M. A. M.; Oehmen, A. Metabolism and ecological niche of *Tetrasphaera* and *Ca* Accumulibacter in enhanced biological phosphorus removal. *Water Res.* **2017**, *122*, 159–171.
- (71) Zhang, H.; Fang, W.; Wang, Y.; Sheng, G.; Zeng, R. J.; Li, W.; Yu, H. Phosphorus removal in an enhanced biological phosphorus removal process: roles of extracellular polymeric substances. *Environ. Sci. Technol.* **2013**, *47*, 11482–11489.
- (72) Li, N.; Ren, N.; Wang, X.; Kang, H. Effect of temperature on intracellular phosphorus absorption and extra-cellular phosphorus removal in EBPR process. *Bioresour. Technol.* **2010**, *101*, 6265–6268.