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Published in:
Environmental Science and Technology

DOI:
10.1021/acs.est.1c02642

Publication date:
2022

Document version:
Final published version

Document license:
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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

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Download date: 15. Sep. 2023
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*

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 31P NMR, and 31P 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. 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, nowadays often achieved by introduction of the enhanced biological phosphorus removal (EBPR) process. The EBPR process offers a sustainable method to recover P, so it can be applied as fertilizer.

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 substituted 40–50% of phosphate fertilizer applied in agriculture.

Activated sludge typically contains 5–15 mgP/gSS for non-EBPR and 30–50 mg P/gSS for EBPR sludge, but the P content can vary substantially among different treatment plants. In general, it comprises two main fractions: chemically bound and biogenic P. 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. This fraction can vary from 55% to 85% of the total P.

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. In particular, in EBPR sludge, a large fraction of biogenic P is constituted by poly-P, an intracellular storage compound of polyphosphate-accumulating organisms (PAO), 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.
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. More recently, novel PAOs from the genus Deschloromonas have been investigated in situ, showing a similar metabolism to Ca. Accumulibacter. Moreover, several putative PAOs, such as Tesraroccus and Ca. Obscuribacter are often found in lower abundance in WWTPs, but their importance remains undescribed. 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. 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. 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 31P 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. 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.

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 31P MAS NMR, and solution state 31P 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 Table 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. 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, with a fractionation scheme that includes P associated with humic acids. Centrifuged activated sludge (1 g wet-weight) was used for fractionation, and the scheme consisted of six sequential extraction steps: H2O, 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. 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 peroxydisulfate (K2S2O8, 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 31P 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.
Poly-P Quantification by Solution State $^{31}$P NMR Spectroscopy and Solid-State $^{31}$P MAS NMR Spectroscopy. To identify and quantify poly-P in the sludge, solid state (see below), and solution state $^{31}$P NMR spectroscopy was carried out as recently described in Staal et al. 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 $^{31}$P solution NMR spectra were recorded on a JEOL ECZ 500R 500 MHz spectrometer, using a 90° pulse (12 μs), 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 $^{31}$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° pulse), 2–3 min relaxation delay (optimized on sample), 150 scans, and 15 kHz spinning. A synthetic sample of struvite, NH$_4$MgPO$_4$·6H$_2$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 ($\delta_{iso}(^{31}P) = 0$ ppm). VnmrJ and MestrNova were used for data analyses. A detailed description of the protocol for data analyses was recently reported.

Biomass Fixation. Fresh biomass samples from batch reactors and full-scale activated sludge WWTP were either stored at −80 °C for sequencing workflows or fixed for FISH with 50% ethanol (final concentration) or 4% PFA (final concentration), as previously described and stored at −20 °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, Oberko-chen, Germany) after staining for 30 min with 50 μg/mL of DAPI (4,6-diamino-2-phenylindoldihydrochlorid-dilactate). Areas of 1500 cells for Raman microspectroscopy analysis (see below) were measured using ImageJ. 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 μm. The biovolume of rod-shaped cells was calculated following Lee and Fuhrman et al. 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 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 was performed as described by Stokholm-Bjerregaard et al. Amplicon sequence variant analysis (ASV) was performed as described elsewhere. 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. using a set of specific probes: PAO651 targeting Ca. Accumulibacter, MCX840 targeting the genus Ca. Micro-thrix, Bet135, and Dech443 targeting the most abundant species of Dechloromonas, and Actino658, Tetra183, and Tetra617 to target the genus Tetrasphaera. Details about the optimal formamide concentration and use of competitors or helper probes can be found in SI Table 3. Quantitative FISH (qFISH) biovolume fractions of individual taxa were calculated as a percentage of the total biovolume, hybridizing the EUBmix probes, 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 630X magnification using the Daimie image analysis software.

Raman Microscopy and Determination of Total Poly-P and Other Storage Polymers in All Bacteria and in PAOs. Raman microscopy was applied in combination with FISH as previously described by Fernando et al. 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 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, 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,29,30,36 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 μm, obtaining in this way the amount of poly-P in an average segment of 1 μm 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 Table 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
particular Fe³⁺ and Al³⁺. P can be found in complexes with iron oxides or as iron phosphate minerals, such as vivianite or phospholipids, and pyro-P. The presence of large amounts of the abundance of microbial biomass responsible for the EBPR total P). Such high nrP fraction was expected due to the in stabilizing the intracellular poly-P chains. Struvite fi

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poly-P during BD-P extraction, as previously mentioned. As the sequential P fractionation appeared to be imprecise in the

developed struvite precipitation from sludge or anaerobic digester’s and its direct application as fertilizer.  

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. 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) in all plants, and even lower than in common precipitates such as strengite (Fe₃(PO₄)·2H₂O) and vivianite (Fe₅(PO₄)₂·8H₂O) of 1:1 and 1.5:1, respectively, 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. 

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 ³¹P NMR (see below), with similar results to previous findings. Generally, the nrP quantified by ³¹P 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 × 10¹¹ 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 × 10¹¹ – 26.3 × 10¹¹ cells/gVSS (SI STable 6). 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 × 10¹¹–11.0 × 10¹¹ cells/gVSS. Fluorescence microscopy and flow-cytometry provided information about the biovolume of bacteria, in the range of 0.16–0.43 μm³. However, these estimates do not take into consideration filamentous bacteria which typically constitute 10–20% of the biomass. 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 μm³, while the filaments biovolume was approximated to 0.608 ± 0.245 μm³ 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, ³¹P MAS NMR for total poly-P in the sludge, and ³¹P 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 8–15 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. The highest P release of 27.4 ± 0.9 mg/gSS was observed in Aalborg West.

Raman Microscopy. 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). 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, Table 7). The difference in total poly-P content measured by Raman corroborated well with the P-released during
According to solution state $^{31}$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, 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 $^{31}$P NMR spectra of the initial samples (time = 0 h, aerobic phase) showed large peaks located at $(\delta(^{31}P) \approx -25$ ppm originating from poly-P middle groups. There were also signals at $(\delta(^{31}P) \approx -5$ ppm originating from poly-P end groups or pyrophosphate (SI SFigures 4–7), which would not be shown with the solely use of $^{31}$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 $^{31}$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/gSS, analyzed by $^{31}$P NMR.61 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.60 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.61–63 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 $^{31}$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 $(\delta(^{31}P) \approx -2$ 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 $^{31}$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.15 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.
(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.

**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 Figure 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. This relation seems 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.

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 $10^{-15}$ to $10^{-14}$, 6.2–6.9 $\times 10^{-14}$, 1.0–1.8 $\times 10^{-14}$, 1.1–2.2 $\times 10^{-14}$ 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. This difference is caused by an overestimation of the cell area by Fernando et al., 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 orthophosphate released into the bulk liquid during the anaerobic incubation. Considering only the poly-P amount accumulated intracellularly 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 (g/μm$^3$), 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 μm, they can be significant contributors to P removal with filaments length exceeding 100 μm.

Ca. Accumulibacter and *Dechloromonas* cells possessed a dynamic behavior of intracellular PHA and glycogen (Figure 2B,C, SI STable 12), as previously described. 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.

**A Comprehensive P Mass-Balance.** The combination of the data from ICP-OES, Raman analysis, solution state and solid state $^{31}$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)
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 31P NMR, 31P MAS NMR spectroscopy, and Raman microspectroscopy measurements, were mostly 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.14 Biofilm P, and a natural part of the microbial biomass, such as DNA-P or membrane lipids, or included into the EPS.14 Biofilm P.

Solution state 31P 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 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).

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,59 but this study also shows that they should be regarded as important contributors to P removal in EBPR systems.59 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
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)

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Notes
The authors declare no competing financial interest.

ACKNOWLEDGMENTS
We thank Susanne Bieldt 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).

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