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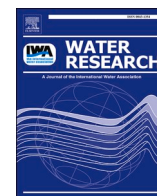
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Phosphorous speciation in EPS extracted from Aerobic Granular Sludge

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ABSTRACT

Wastewater treatment technologies opened the door for recovery of extracellular polymeric substances (EPS), presenting novel opportunities for use across diverse industrial sectors. Earlier studies showed that a significant amount of phosphorus (P) is recovered within extracted EPS. P recovered within the extracted EPS is an intrinsic part of the recovered material that potentially influences its properties. Understanding the P speciation in extracted EPS lays the foundation for leveraging the incorporated P in EPS to manipulate its properties and industrial applications. This study evaluated P speciation in EPS extracted from aerobic granular sludge (AGS). A fractionation lab protocol was established to consistently distinguish P species in extracted EPS liquid phase and polymer chains. ³¹P nuclear magnetic resonance (NMR) spectroscopy was used as a complementary technique to provide additional information on P speciation and track changes in P species during the EPS extraction process. Findings showed the dominance of organic phosphorus and orthophosphates within EPS, besides other minor fractions. On average, 25% orthophosphates in the polymer liquid phase, 52% organic phosphorus (equal ratio of mono and diesters) covalently bound to the polymer chains, 16% non-apatite inorganic phosphorus (NAIP) precipitates mainly FeP and AlP, and 7% pyrophosphates (6% in the liquid phase and 1% attached to the polymer chains) were identified. Polyphosphates were detected in initial AGS but hydrolyzed to orthophosphates, pyrophosphates, and possibly organic P (forming new esters) during the EPS extraction process. The knowledge created in this study is a step towards the goal of EPS engineering, manipulating P chemistry along the extraction process and enriching certain P species in EPS based on target properties and industrial applications.

1. Introduction

Utilization of microbial-produced phosphorus compounds could lead to more circular and economic phosphorus usage (Blank, 2023). Microorganisms can make very defined products, such as poly-P or organic P esters, out of a complex matrix, such as wastewater. Besides many other P₄ "so-called elemental phosphorus or white phosphorus," derivatives, poly-P, and organic P compounds are often synthesized from P₄, through a cost and energy-intensive thermal route from phosphate rock. These compounds are used to produce flame retardants, lubricants, or organophosphorus chemicals (ESPP webinar, 2020; Horstink et al., 2021; Jupp et al., 2021). The use of P products made by microorganisms has the potential to create a higher value of P recovered from wastewater via its use in high-value industrial niches and, in this way, reduce dependence on cost and energy-intensive processes to produce similar products from P₄ and boost the P recovery market.

Extracellular Polymeric Substances (EPS) extracted from waste sludge contain significant amounts of P, as reported by Huang et al., 2015; Zhang et al., 2013; Zeng et al., 2019; Bahgat et al., 2023. These polymeric substances have potential applications across sectors such as agriculture, construction, textiles, and paper industry since it has demonstrated distinctive properties, serving as composite materials, bio-stimulants, or flame retardants (van der Knaap et al., 2019; Pronk et al., 2020; Feng et al., 2019; Kim et al., 2020). P is already intensively used in various contexts to modify polymers to improve or endow many properties. For instance, chemical phosphorylation of polyesters and cotton in textiles and polyvinyl chloride in construction materials make these materials flame-resistant (Liang et al., 2013; Salmeia et al., 2016; ESPP webinar, 2020). Similarly, protein phosphorylation is used in food industries to change the properties of substances such as egg white, potatoes, and rice to enhance wettability, dispersibility, and water absorption capacity (Hu et al., 2023; Hu et al., 2019; Li et al., 2020;

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Miedzianka & Pęksa, 2013). Understanding the role of P in EPS will assist in making high-value P products from WWTPs and potentially replace some of the P_4 derivatives. Still, it will also assist in modifying the recovered EPS with certain processing characteristics or even engineering EPS to target specific industrial applications.

The Netherlands has established the world's first two large-scale demonstration facilities for extracting EPS from sludge. These two facilities are in Epe municipal and Zutphen industrial WWTPs, where EPS is extracted from aerobic granular sludge (AGS) (Bahgat et al., 2023; van der Knaap et al., 2019). The EPS extraction process consists of two main steps. The first step is to combine high pH (9–11) and temperature (80°C) to solubilize EPS, and the second step is acidification, pH (2–4), to precipitate the EPS (Bahgat et al., 2023; van der Knaap et al., 2019; Felz et al., 2019). Bahgat et al. (2023) reported that approximately 20% of the total P within AGS ultimately becomes incorporated into the recovered EPS. No research has been reported on P speciation in EPS extracted using the extraction conditions used for extracting EPS from sewage sludge on a large scale (i.e., for application). Furthermore, no information is available on how P speciation in AGS changes under these conditions and whether they select for a certain P species compared to other EPS extraction conditions. Addressing these knowledge gaps leads to the opportunity to make high-value use of P in WWTPs to promote EPS properties and industrial applications.

In this study, the phosphorus speciation was determined by 1) establishing a P-fractionation protocol that can distinguish between different P species in the liquid phase and polymer chains in the EPS gel-forming polymer and 2) using ^{31}P nuclear magnetic resonance (NMR) spectroscopy as a complementary method to reveal additional information about different organic and inorganic bound P species. ^{31}P NMR was also used to show how P-speciation changes along the EPS extraction process by analyzing P in all different stages of the extraction process.

The knowledge of P chemistry during the extraction process and how much and in what manner P ends up incorporated in EPS serves as a basis to produce EPS with desired or optimized properties (analogue to phosphorylation of synthetic polymers). Based on the results obtained in this study, P distribution in EPS was better elucidated, which opens more opportunities for combined P and EPS recovery from WWTPs and sets the foundation for future research into leveraging the incorporated P in EPS to manipulate its properties and industrial applications.

2. Methodology

2.1. Sludge samples

Laboratory extractions were performed using AGS surplus sludge samples collected after belt thickening from Epe AGS WWTP. Epe WWTP was selected as it is a municipal WWTP, and the majority of AGS WWTPs worldwide are municipal, so it is a good representative of the majority of AGS plants. When sampling, the Nereda® reactors had an 8 g MLSS/L sludge concentration. They were operated with process cycles of approximately 6 h: 3 h of aeration, 1 h of settling, and 2 h of anaerobic feeding/simultaneous effluent withdrawal. The so-called "selection spill" is the sludge removed after every cycle and stored in a buffer tank for 4 hours before it partially goes to the EPS extraction installation. The sludge was collected after the belt thickener and had the following average composition: 5.1 %TS, 4.4 %VS, 33 g P/kg TS, and pH 5.9–6.2. The sludge samples were stored in the fridge for 2–3 days at 4°C until all EPS extractions were performed. AGS sample from Zutphen WWTP, dairy industrial wastewater from FrieslandCampina, was also collected in this study, as shown in detail in the supplementary material. ^{31}P NMR was performed on Zutphen EPS as a quick method to confirm P speciation in EPS from another different source.

2.2. EPS extraction

Lab extractions were performed based on the lab protocol adapted from demonstration-scale practice (Bahgat et al., 2023). The sludge samples were first heated to 80 Celsius in a water bath; 25%(w/v) KOH was then added to the heated sludge until it reached pH 10 ± 0.05 ; the mixture was stirred for two hours at 400 rpm and 80°C and then cooled down for centrifugation at $4,000 \times g$ and 30°C for 20 min. 9.5 M HCl acidified the alkaline supernatant until it reached a final pH of 2.2 ± 0.05 , centrifuged at $4,000 \times g$ and 30°C for 20 min, then the acidified gel-like EPS pellet precipitates. Samples along the extraction process were collected for analysis.

2.3. P-fractionation protocol

The acidic EPS (obtained by the end of the extraction process) was used for P fractionation and analysis. EPS consists of a polymer chain in a liquid phase, and each of these two fractions has P species, as shown in Fig. 1. Total phosphorous in EPS (TP) can be either part of the polymer chain via covalent bonds (organic P), it can also be slightly bound/adsorbed to the polymer chain via charge (exchangeable O-P) or just floating around in the liquid phase as free orthophosphate ions (O-P) or as bound inorganic and/or organic P in the liquid phase. P could also be present as a precipitate mineral as a non-apatite mineral (NAIP) (iron phosphate, aluminum phosphate, or magnesium phosphate) or an apatite mineral (AIP) (calcium phosphate). Since the EPS analyzed is acidic EPS at a pH of 2.2, it is not expected to find AIP because of its solubility at this pH, and therefore, only NAIP is expected to be found (predicted by visual Minteq as shown in supplementary material Table S1 and Fig. S1).

The P fractions determined with the protocol are summarized as follows:

- TP (liquid+ chain): Total phosphorus in the liquid phase and polymer chains in the EPS.
- Bound inorganic and/or organic P (liquid): The bound phosphorus is either organic or inorganic in the liquid phase of EPS.
- O-P (Liquid): The free orthophosphates in the liquid fraction of EPS
- NAIP: The non-apatite inorganic fractions of phosphorus present in the EPS matrix as a precipitate. FeP, AlP, and MnP were presumed to be possibly present due to the supersaturation conditions of these ions as predicted by Visual Minteq (shown in supplementary material table S1 and fig. S1).
- Exchangeable O-P (chain): The exchangeable orthophosphates are adsorbed to the EPS chains by electrostatic interactions and can be displaced by changes in pH or ionic strength.
- Organic-P (chain): The organic phosphorus fraction is covalently bound to the EPS chain and not present in the liquid fraction.

Based on these expected P groups, the P-fractionation protocol was developed to measure and validate the measurements of these fractions in four steps, as described in Sections 2.3.1, 2.3.2, 2.3.3, 2.3.4, summarized in Table 1 and Fig. 2. Each step in the protocol was performed in triplets, and average values and standard deviations were obtained.

2.3.1. Characterization (crude samples)

This step was included in the protocol to measure TP (chain+ liquid) in the acidic EPS and O-P (liquid) and bound inorganic and/or organic P (liquid) in the acidic supernatant. So, the precipitated EPS and the acidic supernatant at the end of the EPS extraction process were collected and analyzed: TS/VS, pH, conductivity, elemental analysis by IC, ICP, and liquid state ^{31}P NMR.

2.3.2. Highly- spun EPS

This step was undertaken to obtain the full liquid phase from the interior of the EPS matrix to evaluate its similarity in composition, O-P

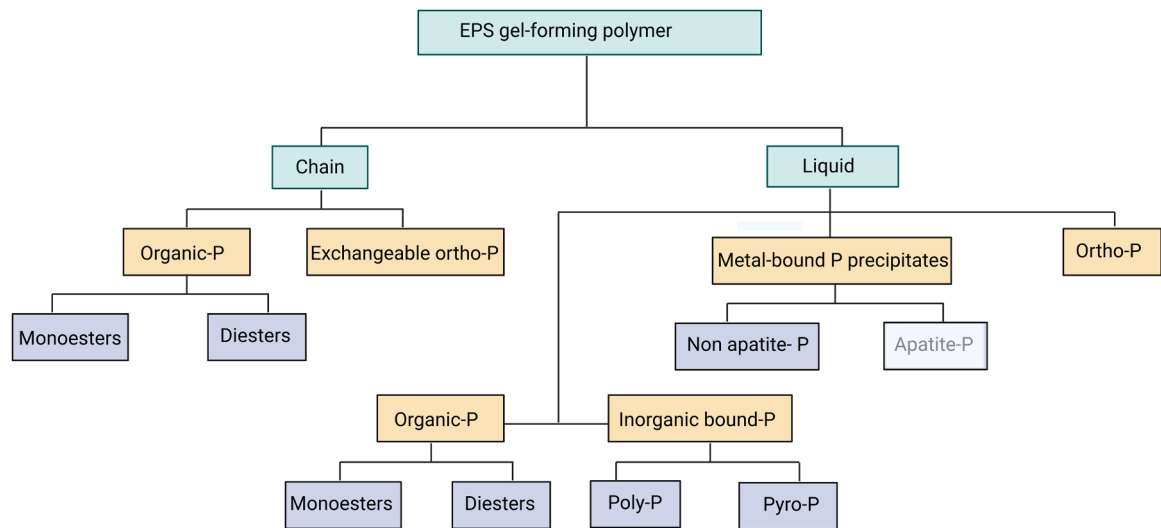


Fig. 1. Summary of possible P-species present in both chain and liquid phases of the EPS gel-forming polymer. In this study, P fractions in yellow are measured with the P-fractionation protocol, and P fractions in grey are measured with ³¹P NMR. Apatite-P is shaded as it is thermodynamically not expected in the EPS analyzed in this study.

Table 1
Summary of the measurements and calculations of P-fractions measured with the P-fractionation protocol. The numbers in blue are related to the numbers shown in Fig. 2 on the top left of measured P fractions.

Numbers in Fig. 2	Measurements
(1)	TP (chain+ liquid)
(2)	O-P (liquid)
(3)	Exchangeable O-P (chain)
(4)	O-P (liquid) + bound inorganic and/or organic P (liquid)
(5)	Organic (chain)+ Exchangeable O-P (chain)+ NAIP precipitates
(6)	O-P (liquid) + Exchangeable O-P (chain) + NAIP precipitates
P fractions	Measured or calculated
TP (liquid +chain)	(1) measured
O-P (liquid)	(2) measured
bound inorganic and/or organic P (liquid)	(7) calculated= (4) measured – (2) measured
NAIP precipitates	(8) calculated= (6) measured – (2) measured – (3) estimated
Exchangeable O-P (chain)	(3) estimated from the EPS washing curve
Organic (chain)	(9) calculated= (5) measured- (3) estimated - (8) calculated

(liquid) and bound inorganic and/or organic P (liquid) fractions, with the acidic supernatant and assess if any pocket effects are present in the polymer or not. The pocket effect phenomenon occurs when some regions within the polymer can have different compositions/concentrations than the bulk matrix, as reported in polymer systems containing different monomers or blocks (Henderson and Clarke, 2004). EPS was encapsulated in 5 mL Eppendorf tubes and subjected to high-speed centrifugation at 21,000 g for 20 minutes at 20°C. After centrifugation, the liquid was collected and analyzed using ICP, IC, and COD kits. The gel compression was validated visually and by TS% change from ≈ 7% to ≈12%.

2.3.3. EPS washing

EPS washing was done to remove the P fractions present in the liquid phase, O-P (liquid), bound inorganic and/or organic P (liquid), and keep organic-P (chain), exchangeable O-P (chain), and NAIP precipitates in the pellet. The concept was to wash EPS with a solution that has similar pH, ionic strength, and temperature as polymers are most prone to changes in solubility, swelling, dissolution, hydrophobicity, or others by those conditions due to changes in intermolecular forces which we

wanted to avoid in our P-fractionation protocol (Lv et al., 2022; Hong et al., 2017). An HCl/KCl solution was used for EPS washing (purification) with a pH of 2.2, and a conductivity level of 19 mS/cm was made by adding 12 g of KCl (chem-lab 99%) and 12.5 ml of 0.1 M HCl in a 500 ml volumetric flask. Washings are carried out by adding 25 mL of solvent to 25 g EPS tubes with a 1:1 ratio. The tubes are then shaken vigorously until homogeneous. Subsequently, the tubes were placed in a centrifuge and subjected to centrifugation at 4000 g for 20 minutes at a temperature of 20°C. After each wash cycle, the supernatant was collected from the tubes for analysis, and the pellet went through the next washing step. The supernatants were analyzed by IC and ICP, and the pellet was analyzed by ICP and liquid state ³¹P NMR.

2.3.4. EPS at pH=0

This step aimed to estimate non-apatite phosphorous (NAIP) fraction in EPS due to its dissolution at extremely low pH conditions. This step was adopted from the SMT (Standards in Measurements and Testing) protocol to determine non-apatite inorganic phosphorus as FeP, AlP, and MnP (Pardo et al., 2004; Zeng et al., 2019b). Additionally, according to the pKa values of phosphate species, at that pH, phosphates are non-ionized, and H₃PO₄ species dominate, so if there are exchangeable orthophosphates adsorbed to the polymer chains, they were also expected to be released into the solution. 3.5 M HCl was added to EPS samples to lower the pH to close to zero, while the pH meter was immersed inside the EPS samples. The EPS samples were shaken vigorously until homogeneous and centrifuged at 21,000 xg for 20 minutes to obtain the supernatant and the pellet separately for analysis. Two confirmation steps were performed at this step. The first is to confirm with calculations that the added amount of acid was enough to lower pH to zero as pH meters are sensitive at this pH range (calculations are shown in the supplementary material table S2). The second step is to confirm that lowering the pH did not cause polymer disintegration. For this purpose, the COD of the liquid phase of EPS at pH=0 was measured and compared to the original COD value of the liquid phase of EPS at pH=2.2 and the COD of the total EPS (chain and liquid fractions) as explained in the results Section 3.1.4. Liquid samples were analyzed by IC and ICP.

2.4. Analysis

2.4.1. Microwave digestion

All solid samples were destructured by microwave digestion to convert

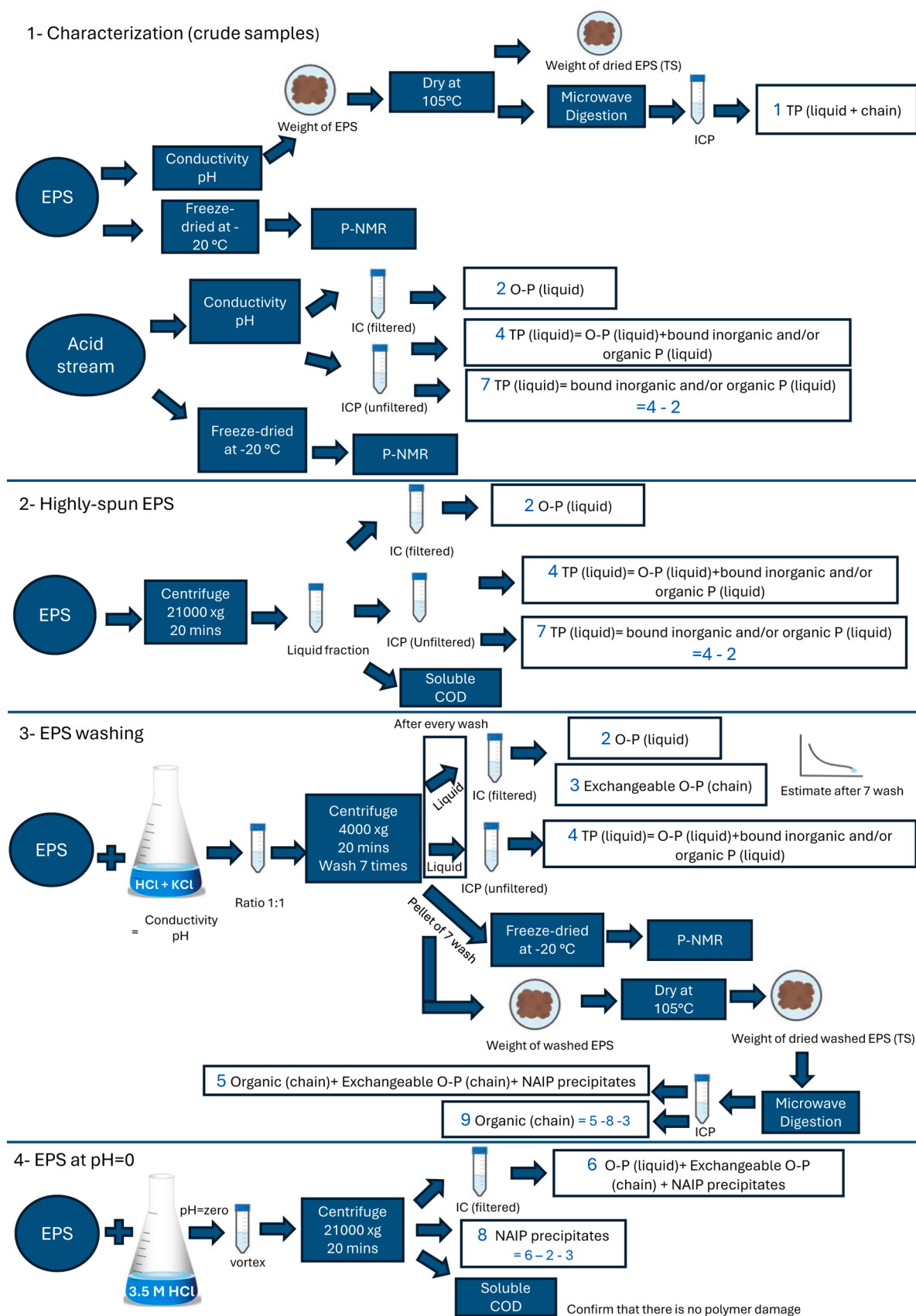


Fig. 2. Schematic diagrams of the four steps in the P- fractionation lab protocol- analysis performed.

them to liquid analyses. Samples were digested in an Ethos Easy from Milestone with an SK-15 High-Pressure Rotor. Around 50 mg of solids were put in a Teflon vessel in which 10 mL of ultrapure HNO₃ (64.5–70.5% from VWR Chemicals) was poured. The digester is set to reach 200 Celsius in 15 minutes, run at this temperature for 15 minutes, and cool down for 1 hour.

2.4.2. ICP-OES (Inductively Coupled Plasma with optical emission spectrometer)

The elemental inorganic composition was measured via Inductively Coupled Plasma (Perkin Elmer, type Optima 5300 DV) with an Optical Emission Spectroscopy as a detector (ICP-OES). The device was equipped with an Autosampler, Perkin Elmer, type ESI-SC-4 DX fast, and the data were processed with the software Perkin Elmer WinLab32. The rinse and standard internal solutions were 2% HNO₃ and 10 mg/L of Yttrium.

2.4.3. IC (Ion chromatography)

Liquid samples were pre-treated first by filtering the samples through 0.45 µm followed by 0.22 µm membrane filters before analysis. Anions and cations (free dissolved ions) were measured by Metrohm Compact ion chromatograph Flex 930.

2.4.4. Chemical oxygen demand (COD)

Total COD was measured using a Hach Lange test kit (LCK514 Hach Lange, UK), heated in a thermostat (HT200S, Hach Lange) to 170 Celsius, and then analyzed using a spectrophotometer (DR. 3900 VIS spectral photometer, wavelength range 320–750 nm).

2.4.5. TS/VS

TS and VS were analyzed according to the Standard Methods for the Examination of Water and Wastewater.

2.5. Liquid ³¹P NMR

The presence of phosphor-containing compounds in the sludge, alkaline supernatant, alkaline pellet, acidic supernatant, crude EPS, and washed EPS pellet (described in section 2.3.3) was evaluated using ³¹P NMR as a complementary method to the P-fractionation protocol. Samples preparation included EDTA and NaOH extractions. The dose of EDTA and NaOH solutions was to avoid the interference of divalent cations and to adjust the pH above 12 to ensure consistent chemical shifts and optimal spectral resolution during the NMR measurement (Turner et al., 2003). After adjusting the pH, alkaline, acidic supernatants, crude EPS, and washed EPS samples were completely dissolved. The sludge and alkaline pellet samples had insoluble residual material, which was removed by centrifuging the sample (3900 rpm, 22 Celsius, 20 min); the P extraction efficiency was ≈ 97% and 92% for sludge and alkaline pellet, respectively (calculations are shown in fig. S5 in the supplementary material). The samples were prepared as follows:

- Alkaline supernatant, alkaline pellet (including the alkaline gel layer described by Bahgat et al., 2023), acidic supernatant, crude EPS, and washed EPS samples: phosphorus was extracted using one-step extraction with 0.1M EDTA and 1M NaOH solution. This method represents the most commonly used extraction protocol for environmental samples (Cade-Menun & Liu, 2014; Turner et al., 2003) and EPS samples (Zeng et al., 2019; Zhang et al., 2013). The samples were prepared one hour before acquiring data.
- Sludge sample: the phosphorus was extracted with a two-step extraction with EDTA pre-extraction followed by NaOH extraction. Staal et al., 2019 reported that a two-step EDTA and NaOH extraction protocol achieved complete poly-P from activated sludge extraction compared to a single-step EDTA-NaOH extraction protocol. So, freeze-dried AGS was pre-extracted with 0.1M EDTA solution for one hour, followed by centrifugation at 3900 rpm for 10 mins and

decanting the EDTA extract. The resulting pellet was extracted with 1M NaOH for 16 hours. The sample was being prepared for almost 18 hours before acquiring data.

All liquid samples were placed in a 10 mm NMR tube (Norel). The spectra were obtained using a Bruker Ascend- 600 MHz spectrometer operating at 162.00 MHz for ³¹P. The acquisition parameters were as follows: acquisition time, 0.8 s; relaxation delay, 50 s (to ensure full relaxation); 90° pulse width, number of scans 1500, and the total measurement period per sample was 23 hours. The spectrum was acquired twice for the sludge sample, one after four hours (Staal et al., 2019) and another after 23 hours, to confirm that the measurement length did not affect polyphosphate content. The ratio between orthophosphate and polyphosphate peaks was the same in the two spectra (shown in the supplementary material Fig. S2). The NMR data processing software determined individual signals' chemical shift and area (MestReNova, version 12.0.4-22023, Mestrelab Research S.L., Spain).

3. Results & Discussion

3.1. P-fractionation protocol

The results of the phosphorus species measured with the P-fractionation protocol are summarized in Fig. 3. It shows that EPS consists of, on average, 24% of O-P (liquid), 18% of NAIP precipitates with iron and aluminum, < 1% of exchangeable O-P (chain), 6% of bound inorganic and/or organic P (liquid), and 51% of organic (chain) phosphorus integrated into the polymer chains. Detailed calculations and validation steps for each step of the fractionation protocol are described in the below sections.

3.1.1. Characterization (crude samples)

The EPS acidic gel samples were characterized as summarized in Tables 2 and 3. Table 2 summarizes the EPS samples' TS, VS, water content, pH, conductivity, and COD. Table 3 shows the TP in EPS polymer (liquid+ chain) as ≈ 1.4gP/kg EPS, the O-P (liquid), and the bound inorganic and/or organic P (liquid) in the acidic supernatant as ≈ 350 and 97 mgP/L, respectively. The measurements are in line with measurements reported in Bahgat et al. (2023) for the general characterization of EPS and acidic by-product stream from EPS extraction installation in Epe WWTP.

3.1.2. Highly-spun EPS

The EPS was centrifuged at high g force to observe if there was a different O-P (liquid) concentration in the acidic supernatant and the liquid trapped by the EPS gel-forming polymer (pocket effect). The liquid fraction of EPS squeezed via high centrifugal spinning had O-P (liquid) and the bound inorganic and/or organic P (liquid) concentrations ≈ of 340 and 110 mgP/L, respectively, as shown in Table 3. These concentrations are similar to the concentrations of the acidic supernatant obtained after EPS precipitation at pH 2.2 measured in step 3.1.1, indicating phosphate in the liquid phase was distributed equally between the acid supernatant and EPS and it was not influenced by any pocket effects. The liquid phase retrieval from the EPS matrix interior was confirmed as the TS% increased from ≈ 7% to ≈ 12%, as shown in Table 2. This step is unnecessary in future EPS P speciation measurements since no pocket effect on phosphate concentration was observed, and the results are similar to those of the previous step.

3.1.3. EPS Washing

EPS washing was performed with a synthetic solution to evaluate the O-P (liquid) and the exchangeable O-P (chain) fractions. The free orthophosphates and the total phosphorus concentrations in the washing solution after each washing step are shown in Fig. S4 in the supplementary material. Free orthophosphates were observed to be approximately halving each time, indicating no "binding" interactions till the 6th

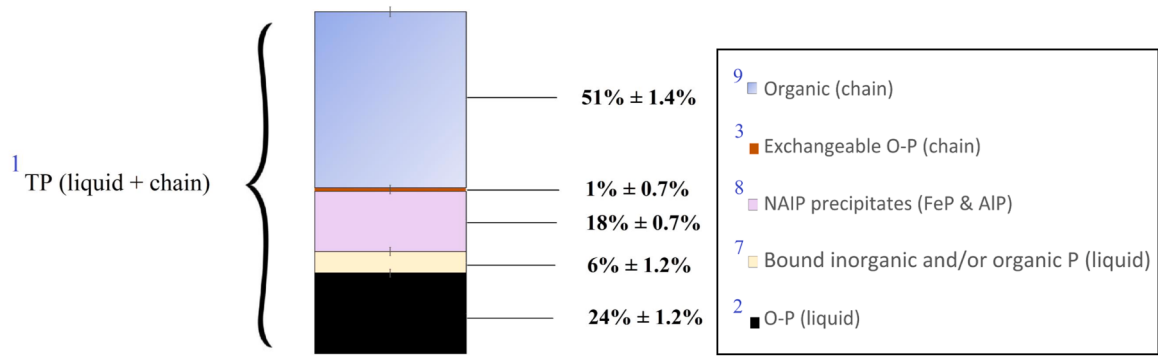


Fig. 3. % of phosphorus fractions in crude EPS extracted from AGS-Epe WWTP measured by established P-fractionation protocol. Extractions were performed in triplets, average values are shown, standard deviation of all fractions was approximately 1%. Blue numbers on the top left are related to numbers in fig. (2) and Table (1) in the methodology section

Table 2
Measurements of pH, conductivity, COD, and TS% performed in each step of the P-fractionation protocol- EPS extracted from Epe WWTP.

(1)- characterization (crude samples)	EPS (liquid+chain)	TS%	7.0±0.4
		VS%	5.9± 0.3
		pH	2
		Conductivity (mS/cm)	19
		COD ¹ (mg/L)	99,000 ± 7,000
(2)- highly spun EPS	EPS (liquid+chain)	TS% of the centrifuged pellet	12.3 ± 0.1
	EPS (liquid)	COD ² (mg/L)	14,000± 300
	EPS (chain)	COD ³ (mg/L) = COD ¹ - COD ²	85,000 ±6,000
(3)- EPS washing	EPS pellet after washing	TS% of the washed pellet	17.4 ±0.4
		TS% of EPS (corrected)	6.0±0.6
(4)-pH=zero EPS	EPS (liquid)	COD ⁴ (mg/L)	21,000 ±700
	EPS (chain)	COD ⁵ = COD ¹ -COD ⁴	78,000 ±6,000

washing step. If there had been a binding interaction, the phosphate concentration of the washing solution would not have followed the halving trend, as the polymer would have interfered with the washing process. Total phosphorus measurements followed a similar trend but did not decrease linearly, which can be explained by the bound phosphorus in the liquid fraction of EPS washed out. The O-P (liquid) washed from EPS was calculated as ≈ 350 mgP/L and bound inorganic and/or organic P (liquid) as the subtraction of washed total phosphorus and O-P (liquid) ≈ 90 mgP/L. Starting from the 6th washing step, the concentration of O-P was stable, then slightly increased in the 8th washing step, and decreased again, not following the earlier halving trend anymore, indicating binding interactions taking place starting from the 6th washing step. The concentration of exchangeable O-P (chain) was estimated to leave the polymer at the point of these binding interactions taking place after the 6th washing step as ≈ 16 mg P/L. The exchangeable O-P (chain) is negligible as it is less than 1% of the total phosphorus in EPS, so measuring this fraction is not needed in future measurements of EPS. The O-P (liquid), bound inorganic and/or organic P (liquid) fractions are in line with measurements in the previous two steps, as shown in Table 3.

3.1.4. EPS at pH=0

The pH of EPS was adjusted to zero to evaluate the metal-bound (NAIP) fraction of phosphorus. This step resulted in an increase in the

Table 3
Concentration of different phosphorus fractions (gP/kg EPS) measured by the P-fractionation protocol in EPS extracted from Epe WWTP.

Phosphorus fraction	Concentrations	
	mg/L	g P/kg EPS
(1)- Characterization		
TP (liquid+ chain)	-	1.41±0.01
O-P (liquid)	353± 6	0.33 ± 0.01
bound inorganic and/or organic P (liquid)	97±12	0.09±0.01
(2)-Highly Spun EPS		
O-P (liquid)	340±10	0.32± 0.01
bound inorganic and/or organic P (liquid)	110± 10	0.10± 0.01
(3)-EPS washing		
O-P (liquid)	360± 10	0.35± 0.01
bound inorganic and/or organic P (liquid)	95± 10	0.07 ± 0.01
Exchangeable O-P (chain)	15±5	0.01 ± 0.00
Organic (chain)+ Exchangeable O-P (chain)+ NAIP precipitates	-	0.99± 0.02
Organic (chain)		0.73± 0.02
(4)- EPS at pH=0		
O-P (liquid) + Exchangeable O-P (chain) + NAIP precipitates	640 ±10	0.60 ± 0.01
NAIP precipitates	-	0.25± 0.01

free orthophosphate concentrations O-P(liquid) up to 640 mgP/L (20720 μ mole P/L) due to the dissolution of P metal-bound precipitates (NAIP, e.g., FeP, AlP, and MnP), and the release of exchangeable O-P (chain) to the liquid. Although CaPO₄ and MgNH₄PO₄ are commonly present in sludge, they are not expected to be found in acidic EPS as they will be dissolved during the acid precipitation step of EPS extraction. Two validation steps were performed in this experiment:

- 1) The first one was to confirm our assumption that the release of NAIP (Fe, Al, Mn) to the liquid is equivalent to P release, considering the small fraction of exchangeable O-P estimated in Section 3.1.3. Fe release from FeOOH was also assumed not to occur or occur to a minimum extent as it would have already been dissolved at pH 2.2 during the acid precipitation step of EPS. The P-precipitates, as predicted by visual Minteq, are Strengite (FePO₄·2H₂O), Variscite (AlPO₄·2H₂O), and MnHPO₄ (fig. S1 in supplementary material), all have a ratio of 1:1 to phosphate. Table S3 in the supplementary material shows the measurements of the P, Fe, Al, and Mn moles released upon decreasing the pH from 2.2 to zero. Fe and Al are the

main fractions of the NAIP present in the EPS sample, as the contribution of Mn is almost negligible. Subtracting the initial O-P (liquid) measured in 3.1.1 and 3.1.2 as 10970 $\mu\text{mole P/L}$, exchangeable O-P fraction which was estimated as 16 mgP/L (520 $\mu\text{mole P/L}$) from the total P released, the moles of P released upon NAIP dissolution are calculated as 9230 $\mu\text{mole P/L}$. The total Fe and Al moles released are measured to be 9460 $\mu\text{moles/L}$, with a 1.02 ratio to P released moles. This ratio agrees with our assumptions and VisualMinteq predictions.

- 2) The second validation step was to confirm that lowering the pH to zero did not cause polymer damage and that no P attached to polymer chains dissolved to the liquid fractions, causing an over-estimation of the phosphorous measured in the liquid. At pH= zero, the COD of EPS (chain) decreased by ≈ 7 g COD/L as calculated by subtracting the initial COD of EPS (chain) at pH=2.2 ≈ 85 g/L and the COD of EPS (chain) at pH=0 ≈ 78 g/L. The COD decrease of EPS (chain) is probably due to some minor hydrolysis of organics. Since the ratio between TCOD and TP in EPS at pH 2.2 is calculated as ≈ 0.00015 g P/ g COD, we could correlate the COD of EPS (chain) change with the change of P concentrations to assess its influence on the measured phosphorus species. The change in P concentrations due to the minor hydrolysis organics was estimated to be 0.007%, which is negligible. Calculations are shown in detail in Table S4 in the supplementary material.

3.2. Liquid ^{31}P NMR

3.2.1. ^{31}P NMR peaks assignment

^{31}P NMR measurements were performed on initial sludge, alkaline supernatant, alkaline pellet, acidic supernatant, acidic EPS, and washed EPS. The primary goal of these measurements was to confirm the EPS phosphorus speciation obtained by the fractionation protocol and to provide additional information about the organic phosphorus fraction.

The secondary goal was to gain more insights regarding the changes in phosphorus species along the extraction process. The results are shown in Figs. 4 and 5. The peaks in the ^{31}P NMR spectrum were assigned as previously reported (Cade-Menun & Liu, 2014; McDowell et al., 2006; Turner et al., 2003). The pH of the extracts (12.6) was close enough to the pH in those studies that the chemical shifts can be compared (Grouse et al., 2000). Detected inorganic P compounds included orthophosphate (fixed at 6.00 ppm to compare between different spectra easily), pyrophosphate at -5 ppm, polyphosphate middle groups between -19 and -24 ppm, and terminal groups between -4 and -4.5 ppm, organic P included orthophosphate monoesters between 5.9 and 3.7 ppm, and orthophosphate diesters between 2 and -1 ppm. Peaks in the monoester region could possibly be derived from monoribonucleotides due to RNA hydrolysis under alkaline conditions (Lemire et al., 2016), inositol phosphates, metabolic phosphate compounds such as nicotinamide adenine dinucleotide (NAD), sugar phosphates (e.g., glucose 6-P), phosphoproteins (e.g., 3-phosphoserine), or other compounds present due to the degradation of orthophosphate diesters as reported in the literature (Cade-Menun, 2015). Additionally, the peaks in the diester region could possibly be derived from DNA, but also from phospholipids (e.g., phosphatidylcholine, phosphatidylglycerol). Spiking was recommended as the only way to specifically identify phosphorus forms in complex matrices (Koopmans et al., 2007). Therefore, future spiking experiments could be performed on EPS with standard purchased phosphorus compounds and their degradation products if the intention is to identify a specific phosphorus compound in EPS.

3.2.2. P species in EPS- complementary to the P-fractionation protocol

From spectrum E) in Fig. 5, of total phosphorus present in crude EPS, total orthophosphate (includes free O-P(liquid), and dissolved NAIP) was calculated as 40%, organic phosphorus as 50% (25% as monoesters, and 25% as diesters), and pyrophosphates as 10% (includes pyrophosphate in liquid or attached to the polymer chains). From spectrum F) in

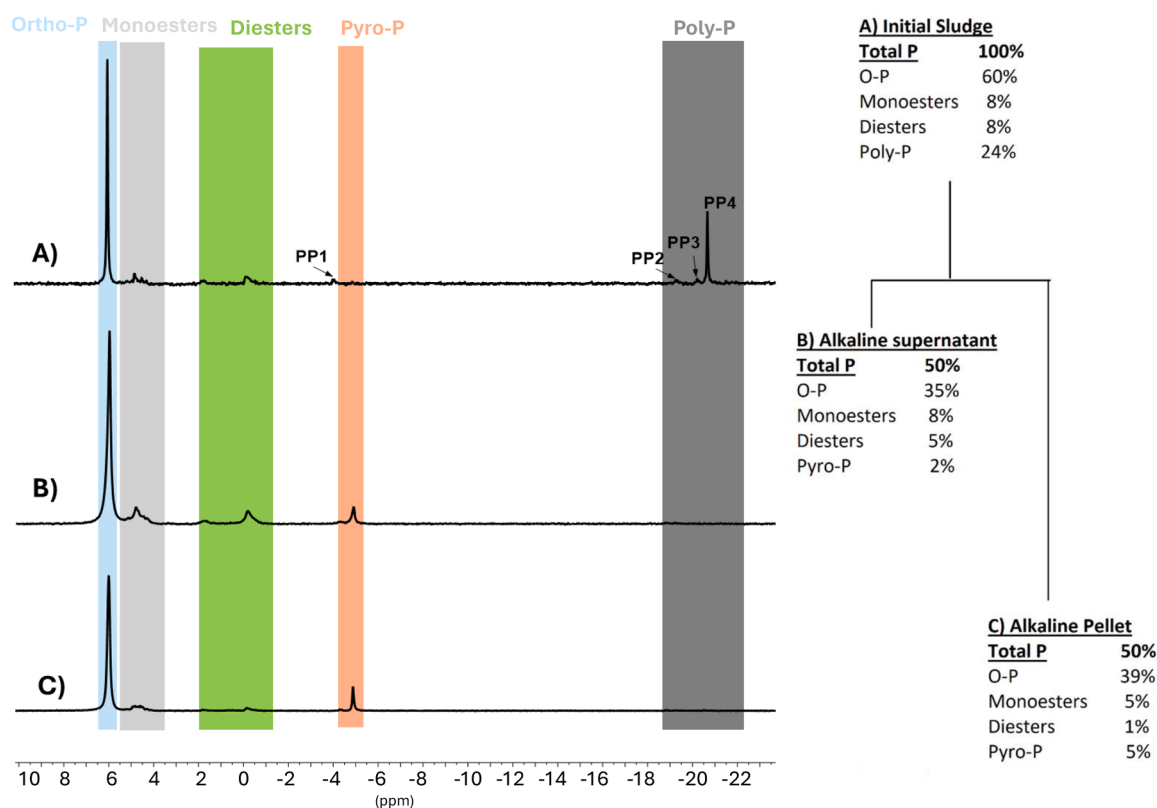


Fig. 4. ^{31}P NMR spectra of the first step of EPS extraction step: A) initial AGS, B) alkaline supernatant, C) alkaline pellet, - Epe municipal WWTP- All % are related to the initial total P in initial sludge approximated to the first whole numbers or first decimal point.

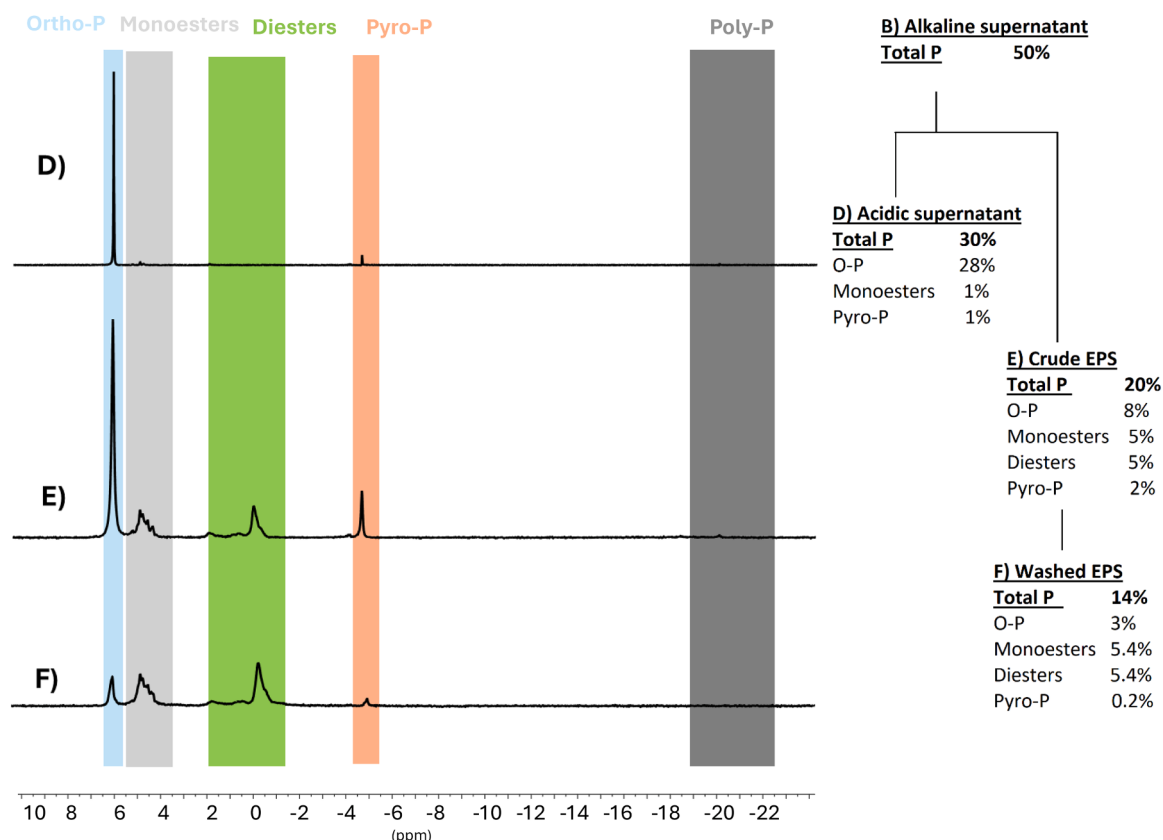


Fig. 5. ^{31}P NMR spectra of the second step of the EPS extraction process: D) acidic supernatant, E) crude EPS, F) washed EPS- Epe municipal WWTP- All % are related to the initial total P in initial sludge approximated to the first whole numbers or first decimal point.

Fig. 5, the washed EPS had a less intense peak of orthophosphate compared to graph E) as expected, showing that O-P (liquid) was removed upon washing, and the remaining phosphates represented the NAIP, which will remain with the washed EPS pellet after washing and centrifugation. The orthophosphates (representing dissolved NAIP due to the high pH <12 of ^{31}P NMR sample) in washed EPS were calculated as 15% of the total phosphorus in crude EPS. Comparing the orthophosphate peaks in spectrum F) and spectrum E), the removed orthophosphates (O-P (liquid)) were calculated as 25% of the total phosphorus present in crude EPS. The organic phosphorus in spectrum F) was estimated as 53 % (27% as monoesters, 27% as diesters) of total phosphorus present in crude EPS, confirming the organic P fraction calculated in spectrum E). Comparing the pyrophosphate peaks in spectrum F) and E), the removed pyrophosphate (free in the liquid) was calculated as 9%, and the remaining attached to the organics in washed EPS was 1%. The ^{31}P NMR results were in line with the P-fractionation protocol developed. In comparison to the protocol, ^{31}P NMR showed a 1% difference in O-P (liquid), a 3% difference in NAIP, < 1% difference in organic P (chain), and a 4% difference in the bound inorganic and/or organic P (liquid) which was revealed to be only pyrophosphates and no organics.

To understand how variant the P speciation could be in EPS samples from different sources, EPS was extracted from Zutphen (dairy industrial AGS) WWTP, and its ^{31}P NMR was compared to Epe (municipal AGS) as described in Fig. S3 in the supplementary material. Of total phosphorus present in crude Zutphen EPS, 47% orthophosphates (O-P(liquid) and NAIP), 46% organic phosphorus (28% diesters, and 18% monoesters), 1% pyrophosphates, and 6% polyphosphates were present. Compared to Epe, P speciation of Zutphen EPS showed a similar trend despite the sludge being from municipal or industrial sources. Orthophosphates and organic phosphorus are still the dominant species, with 7% and 6% differences from Epe, respectively. The diesters and monoesters do not

have an equal ratio as Epe; Zutphen had a higher content of diesters compared to monoesters. A minor fraction of polyphosphate was also detected, unlike Epe. Still, these differences are minor, not affecting the major trend of the dominant P-species and the expected % range of these species.

3.2.3. Change of P species along the extraction process

Fig. 4 shows the ^{31}P NMR spectra of the initial AGS, alkaline supernatant, and alkaline pellet streams produced along the first step (EPS alkaline solubilization) of the extraction process, and all % are related to total P in the initial sludge. Fig. 5 shows the ^{31}P NMR spectra of the acidic supernatant, crude EPS, and washed EPS produced along the second step of the extraction process, and all % are related to total P in the initial sludge. Spectrum A) shows the excess AGS used for EPS extraction contained $\approx 24\%$ polyphosphates, 60% orthophosphates, and 16% organic phosphorus (8% monoesters and 8% diesters). It was anticipated that polyphosphate would be the dominant fraction, as reported by Huang et al. (2015). However, our findings showed that orthophosphate was the predominant form. This discrepancy can be attributed to poly-P hydrolysis to orthophosphate during storage in the buffer tank (with an average SRT of approximately 4 hours, slightly anaerobic conditions, and a pH of around 6) in the WWTP. Storage sludge samples in the fridge for 2-3 days before laboratory extractions could have further contributed to this transformation. Furthermore, most of the phosphorus could have been initially present as inorganic phosphorus precipitates, such as apatite (AIP) and non-apatite (NAIP) in the core of granules not as poly-P. There is a slaughterhouse discharge to Epe WWTP, which contains most likely high levels of Fe. Mañas et al., 2011 reported that biologically induced precipitation of hydroxyapatite was responsible for 45% of the overall P removal in AGS, and Cai et al., 2018 also reported vivianite and siderite precipitation in the core of the granules with iron dosing to the reactor.

Comparing spectrum A (initial AGS) to B (alkaline supernatant) and C (alkaline pellet) in Fig. 4 and the mass balances of P species over the alkaline extraction step, it is observed that polyphosphate was completely hydrolyzed as it only appeared in the initial sludge. Pyro-P peaks appeared in the alkaline supernatant (spectrum B) and pellet (spectrum C). Polyphosphates ($\approx 24\%$ in initial sludge) hydrolyzed into pyrophosphates and orthophosphates. Orthophosphates increased by $\approx 14\%$ and pyrophosphate by $\approx 7\%$, but $\approx 3\%$ of polyphosphates remained. Similarly, the total organic-P content increased compared to the initial sludge by $\approx 3\%$. We hypothesize that this could indicate that phosphorylation is taking place, and that part of the polyphosphates was used to form ester bonds. Compared to chemical phosphorylation conditions reported in literature (Cen et al., 2022; Kaewruang et al., 2014; Xiong & Ma, 2017; Miedzianka & Peksa, 2013), the EPS extraction conditions are promising to induce phosphorylation because of the presence of high energy P bonds (P-O-P) in polyphosphates, high temperature, alkaline conditions, and long residence times in the alkaline reactor. However, confirming the hypothesis from a 3% increase of peak integration in the NMR spectra is challenging because it is a very small percentage to be trusted. Consequently, we recommend performing experiments that specifically target phosphorylation effects to substantiate or refute our hypothesis. In these experiments, different parameters that could influence phosphorylation have to be assessed e.g., the type of P salt used, P concentration, pH, temperature, residence time, molecular configuration, and available phosphorylation sites.

Comparing the P speciation in our study with other EPS extracted under different conditions, reported in the literature, showed that extraction conditions used in our study are selecting for organic phosphorus. For example, Huang et al., 2015 reported no organic phosphorus in EPS extracted by EDTA-ultrasound from AGS, Zhang et al., 2013 also reported no organic phosphorus in EPS extracted by cation exchange resin (CER) from WAS, and Zeng et al., 2019 reported poly-P and ortho-P were the dominant forms with negligible organic P in EPS extracted by formaldehyde-NaOH from WAS. Then, the extraction conditions we use in our study are hypothesized to produce EPS with high organic-P content, as around 51%-54% of total phosphorus in EPS is organic phosphorus as measured by the P-fractionation protocol and ^{31}P NMR. This hypothesis was further confirmed since EPS extracted from Zutphen under the same conditions also had organic-P as a dominant species in EPS, similar to Epe, despite the difference in the type of wastewater and sludge in the two WWTPs.

Spectrum D) in Fig. 5, confirmed Bahgat et al., 2024 findings that around 30% of TP as ortho-P in sludge ends in the acidic by-product stream, and it is a relatively pure stream as O-P represents 95% of its total phosphorus composition, so it is suitable for phosphate recovery by precipitation.

4. Recommendations & outlook

This section provides recommendations for future studies on P species in EPS using the P-fractionation protocol established in this study. Also, it offers insights into the ultimate objective of EPS engineering and the goal to leverage the incorporated P in EPS to manipulate its properties and enhance its industrial applications.

4.1. Regarding the P-fraction protocol

1) The P-fractionation protocol to measure EPS samples extracted under similar conditions: The protocol involved four steps, but future studies could be more concise. For example, the "highly spun EPS" step may be omitted in future speciation evaluations as it showed no pocket effects or concentration difference compared to the acidic by-product. Similarly, the "EPS washing" step can be skipped since the exchangeable O-P (chain) is negligible ($<1\%$). The steps "Characterization of crude samples" and "EPS at pH=0" are essential for assessing the main P fractions. "Characterization of crude samples"

identifies O-P (liquid) (25% of TP in EPS) and bound inorganic and/or organic P (liquid) (7% of TP in EPS). "EPS at pH=0" identifies organic P covalently bound to the polymer chains (52% of TP in EPS) and non-apatite inorganic phosphorus NAIP (15% of TP in EPS).

2) The P-fractionation protocol to measure EPS samples extracted under different conditions: It's crucial to note that the EPS analyzed in this study was acidic, with a pH of 2.2, so no apatite inorganic phosphorus (AIP) was anticipated. However, caution is needed for EPS at higher pH values, as when EPS is brought to pH=0, combined NAIP and AIP will be released, and a method to differentiate them must be incorporated into the protocol. One approach could involve adjusting the pH to high alkaline conditions to dissolve NAIP and precipitate AIP and then bringing the EPS to zero to dissolve AIP. Additionally, the exchangeable O-P fraction may not be negligible for higher pH EPS, as P is more ionized (with a higher charge of -2 or -3), potentially having a greater affinity for polymer chains.

4.2. Regarding the ultimate goal of EPS engineering

The results reveal the P species incorporated in EPS and how P changes throughout the extraction process. These results highlight opportunities to tailor the P species in EPS according to industrial needs. Comparing EPS extraction conditions to the chemical phosphorylation work done on biopolymers in the literature and considering the resultant phosphorus content (wt%) and its influence on properties as shown in Table 4 demonstrates the potential for follow-up research focused on EPS engineering and tailoring its properties to meet industry needs. For example, since P esters are commonly added to macromolecules to make them flame retardants, and approximately 50% of the P in EPS was found to be organic phosphorus, EPS could create an effective flame retardant (Kim et al., 2020, 2022). Further increasing the organic P content in EPS could be a way to make an EPS super flame retardant that competes with commercial flame retardants. Also, further research into phosphorylation under extraction conditions, as pinpointed in this study, could be a way to influence the P chemistry to form higher organic P content to maximize EPS thermal stability property and its application as a flame retardant. Another way to change the speciation could be introducing excess Fe or Al cations in the wastewater treatment reactors or during the EPS extraction process to increase non-apatite inorganic phosphorus (NAIP) in EPS and reduce O-P (liquid), potentially useful for creating slow-release fertilizers from EPS to prevent rapid O-P (liquid) leaching from the soil. Other possibilities will appear with further research on how different P species/content influence different EPS properties.

5. Conclusions

The P-fractionation protocol established in this study and ^{31}P NMR showed that P species in acidic EPS extracted from aerobic granular sludge are free orthophosphate in the liquid phase as 25%, organic P (covalently bound to polymer chains) as 52%, NAIP metal bound-precipitates as 15%, free pyrophosphates in the liquid phase as 7%, and $<1\%$ of exchangeable orthophosphate adsorbed to the polymer chains. ^{31}P NMR also showed that polyphosphates detected in the initial AGS completely/partially transformed along the extraction process to pyrophosphates, orthophosphates, and possibly to new P-esters (phosphorylation) as hypothesized in this study, which is to be further confirmed in future studies. The knowledge about P-speciation in EPS will set the foundation for future work on utilizing P to engineer EPS properties for industrial applications.

CRedit authorship contribution statement

Nouran T. Bahgat: Writing – original draft, Writing – review & editing, Methodology, Formal analysis, Data curation, Conceptualization. **Philipp Wilfert:** Writing – review & editing, Supervision,

Table 4

Phosphorylation conditions, %wt P content and the effect on the properties of some biopolymers from literature in comparison to EPS extraction conditions from AGS and EPS %P content. TSPP stands for tetrasodium pyrophosphate salt, STPP stands for sodium tripolyphosphate, and STMP stands for sodium trimetaphosphate.

Polymer	P-salt added	Temp	Time	Phosphorus content (wt %)	conditions	References	Effect of phosphorylation
Gelatin	TSPP	50°C	0.5 h	3.2% (calculated based on data set)	Aqueous solution pH7	Cen et al., 2022	Enhanced rheological behavior, emulsifying properties, and hydrophobicity
Gelatin	STPP	65°C	1-3 h	0.25%-1%	Aqueous solution pH5-11	Kaewruang et al., 2014	Enhanced rheological behavior
Chitin or chitosan	TSPP	60°C	3 h	6-7%	-	Yalpani, 1992	Creating metal-chelating agents and enhanced thermal stability
Gelatin	STMP	50°C	05-3 h	0.07% (calculated based on the data set)	Aqueous solution pH9	Yang et al., 2022	Enhanced solubility, gel structure, and emulsifying properties
Cellulose paper	STMP	Room temperature 50°C	4 weeks 2 weeks	0.17% 0.15%	Aqueous solution pH12	Inoue et al., 1995	Creating metal-chelating agents
*EPS from AGS	NA	80°C	2 h	2-2.5%	Aqueous solution pH9-11	Bahgat et al., 2023	*Follow-up studies to determine P effect on EPS properties

Methodology, Formal analysis, Conceptualization. **Stephen J. Eustace:** Writing – review & editing, Methodology, Data curation. **Leon Korving:** Writing – review & editing, Supervision, Funding acquisition, Formal analysis, Conceptualization. **Mark C.M. van Loosdrecht:** Writing – review & editing, Supervision, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at [doi:10.1016/j.watres.2024.122077](https://doi.org/10.1016/j.watres.2024.122077).

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