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Extraction and quantification of polyphosphates in activated sludge from waste water treatment plants by ^{31}P NMR spectroscopy

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ABSTRACT

Polyphosphate (poly-P) is a major constituent in activated sludge from wastewater treatment plants with enhanced biological phosphorus removal due to poly-P synthesis by poly-P accumulating organisms where it plays an important role for recovery of phosphorus from waste water. Our aim was to develop a reliable protocol for poly-P quantification by ^{31}P NMR spectroscopy. This has so far been complicated by the risks of inefficient extraction and poly-P hydrolysis in the extracts. A protocol for complete extraction, identification and quantification of poly-P in activated sludge from a waste water treatment plant was identified based on test and evaluation of existing extraction protocols in combination with poly-P determination and quantification by solution and solid state ^{31}P NMR spectroscopy. The total poly-P middle group content was quantified by solid state NMR for comparison with the poly-P middle groups quantified by solution NMR, which is novel. Three different extraction protocols previously used in literature were compared: 1) a single 0.25 M NaOH-0.05 M EDTA extraction, 2) a 0.05 M EDTA pre-extraction followed by a 0.25 M NaOH main extraction and 3) a 0.05 M EDTA pre-extraction followed by a 0.25 M NaOH-0.05 M EDTA main extraction. The results showed that the extraction protocol 2 was optimal for fresh activated sludge, extracting 10.8 ± 0.4 to 11.4 ± 1.2 mgP/gDW poly-P. Extraction protocols 1 and 3 extracted less than 9.4 ± 0.5 mgP/gDW poly-P. A comparison of the quantification of poly-P by ^{31}P solution NMR and by ^{31}P solid state NMR spectroscopy of lyophilised activated sludge showed 86 ± 9% extraction efficiency of poly-P, which confirms that the extraction protocol recovered most of the poly-P from the samples without pronounced poly-P degradation.

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1. Introduction

Phosphorus (P) recovery from waste water is an alternative P resource that becomes increasingly important as global P reserves are limited (Cordell et al., 2011). Phosphorus recovery from domestic waste water can cover up to 20% of the global phosphorus consumption (Yuan et al., 2012). Phosphorus and nitrogen are removed during the treatment of waste water in order to protect the recipient from excess nutrients. Today, the most common methods of P removal from municipal waste water include enhanced biological P removal (EBPR) (Jing et al., 1992) and precipitation by aluminum(III), Al^{3+}, or iron(III), Fe^{3+} compounds. Enhanced biological P removal relies on aerobic uptake of phosphate and conversion to internal inorganic polyphosphate (poly-P) by poly-P accumulating organisms (PAOs) (Yuan et al., 2012). The use of EBPR is cost-effective, as it saves chemicals and enhances the value of the sludge as a fertilizer (Kahiluoto et al., 2015; O'Connor et al., 2004). Furthermore, poly-P might also be used to recover P, e.g., as struvite if the degradation of poly-P and the subsequent release of orthophosphate from PAOs can be controlled (Yuan et al., 2012). Optimisation of the P uptake in PAOs by EBPR systems and control of the subsequent phosphate release requires correct identification and quantification of the total amount of poly-P in the sludge. In order to better understand and optimise the EBPR process, and retain more P, one should be able to precisely quantify and identify the poly-P formed by the PAOs to, e.g., monitor changes in the poly-P accumulation under different conditions. However, reliable methods for the quantification of the poly-P

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Abbreviations

NMR	nuclear magnetic resonance
EDTA	ethylenediaminetetraacetic acid
EBPR	enhanced biological phosphorus removal
EPS	extracellular polymeric substances
ICP	inductively coupled plasma
poly-P	polyphosphate
ppm	parts per million
$\delta(^{31}P)$	^{31}P chemical shift
PAO	polyphosphate accumulating organism
SSNMR	solid state nuclear magnetic resonance
TP	total phosphorus

species are needed as current methods have several shortcomings such as inefficient extraction and poly-P degradation (Hupfer et al., 2008).

Although several methods exist for poly-P identification and quantification, none of these methods have been proven to reliably quantify the total poly-P content of bulk activated sludge. One of the most common methods for quantification of poly-P in environmental samples is staining followed by fluorometry (Hupfer et al., 2008; Majed et al., 2012), which often includes an alkaline extraction with NaOH (Diaz and Ingall, 2010; Majed et al., 2012) or a permeabilisation step which allows the dye to cross cell membranes (Gomes et al., 2013). Thus, absolute quantification of poly-P by staining techniques may be hindered due to, e.g., insufficient extraction/permeabilisation and the risk of degradation of poly-P in the extract (Majed et al., 2012). Furthermore, many dyes only bind to longer poly-P chains (>10 P_i) (Diaz and Ingall, 2010; Hupfer et al., 2008), which excludes short-chain poly-P from the quantification. Raman micro-spectroscopy allows for identification and quantification of poly-P on a cellular level in activated sludge, but this has so far not been transferred into absolute, bulk quantities (Majed et al., 2009), even though a recent study has successfully quantified species-specific poly-P contents by Raman-fluorescence *in situ* hybridisation (FISH) (Fernando et al. 2019).

Solution state ^{31}P NMR analyses have been used for investigations of poly-P in sludge since 1983 (Cade-Menun, 2005b; Florentz and Granger, 1983). The ^{31}P chemical shift reflects the position of the phosphate group in the poly-P chain: Terminal phosphate at the end of the chain (PP1 group) can be distinguished from penultimate phosphate groups near the end of the chain (PP2 and PP3) and phosphate groups inside the poly-P chain (PP4). These groups can be directly quantified by ^{31}P solution NMR spectroscopy (Hupfer et al., 2008). However, comparisons among studies are hampered by the large differences in sludge preparation, extraction procedures, and preparation of the extracts for the ^{31}P NMR analysis. Hence, previous ^{31}P solution NMR studies of organic P and poly-P from different environmental samples including sludge used a wide range of combinations of pre-treatment (air-drying, freezing/lyophilisation etc.), pre-extractant (ethylenediaminetetraacetic acid (EDTA), trichloroacetic acid, etc.), main extractant (EDTA-NaOH, NaOH, etc.) and post-treatments of the extracts (e.g., lyophilisation or rotary evaporation) (Cade-Menun and Liu, 2013). A list with examples of extraction protocols including references is given in supporting information (Table S1). Often the effects of the different pre- and post-treatments are unknown (Cade-Menun and Liu, 2013; Cade-Menun, 2005a). Lyophilisation of NaOH or EDTA-NaOH extracts of soil followed by dissolution of the lyophilised extract before ^{31}P solution NMR analysis is a very common way to concentrate samples prior to ^{31}P NMR analysis. However, poly-P degradation after lyophilisation of EDTA-NaOH extracts has been observed (Cade-Menun et al., 2006; Reitzel et al., 2009), and neutralization of the extract prior to lyophilisation has been suggested as a way to prevent this, as demonstrated for the short-chain poly-P sodium tripolyphosphate (Cade-Menun et al., 2006). Thus, there is no evidence in the literature for the NMR analysis' ability to accurately quantify the total poly-P content, and the risks of incomplete extraction and/or degradation of poly-P have not been addressed (Hupfer et al., 2008).

Solid state ^{31}P magic angle spinning NMR (^{31}P SSNMR) is a non-destructive characterisation technique that only requires minimum pre-analysis treatment of the sample, but is sparingly used for environmental samples as the resolution is lower than for solution NMR (Turner et al., 2005). SSNMR is a useful tool for sludge P characterisation due to relatively high P concentrations in activated sludge from waste water treatment plants compared to, e.g., soil samples (Frossard et al., 1994; Hinedi et al., 1989; Huang and Tang, 2015). However, analysis by ^{31}P solution NMR is often quicker than by SSNMR and produces spectra with a better resolution that allows identification of specific organic P compounds (Cade-Menun, 2005a). The main limitation for quantification of poly-P by ^{31}P solution NMR spectroscopy is the unknown extraction effeciency of the extraction protocol and the possible degradation (hydrolysis) of poly-P by this (Hupfer and Gachter, 1995; Hupfer et al., 2008). These uncertainties limit the comparability among studies, and to our knowledge, no estimates of the poly-P extraction efficiencies of these protocols have been reported before.

In this study, SSNMR was used to quantify the poly-P middle groups in sludge prior to extraction, and this poly-P content was compared to the poly-P extracted by three different extraction protocols and used as a reference for evaluating potential poly-P degradation in the extracts. The advantage of solution NMR over SSNMR is described above, but in addition to this, solution NMR enables the detection of poly-P terminal groups. Our objective was to identify the best suited extraction protocol for poly-P from activated sludge, i.e., a protocol that ideally ensures full extraction of poly-P with limited degradation. This was obtained through a series of laboratory experiments where SSNMR and solution NMR were used to evaluate three known extraction protocols' ability to extract and preserve poly-P. In addition, effects of pre-concentration of the extracts prior to ^{31}P solution NMR analysis by either rotary evaporation or lyophilisation were tested. These variables were investigated as they are most commonly used for sample preparation for ^{31}P solution NMR studies of poly-P in sludge and sediments. First, the poly-P middle group content of lyophilised sludge quantified directly by ^{31}P SSNMR is presented. Following this, the effect of different combinations of pre-extractants, main extractants, and sample concentration is described. A comparison of the two methods for poly-P quantification provides insight into the poly-P extraction efficiencies of the different protocols. Finally, ^{31}P SSNMR analyses of sludge pellets after extraction are used to elucidate the reason behind poly-P extraction inefficiencies.

2. Materials and methods

Three different extraction protocols for poly-P in activated sludge were tested (Fig. 1):

1) A single-step EDTA-NaOH extraction (EN)
2) A two-step extraction with EDTA pre-extraction followed by a NaOH extraction (E→N)
3) A two-step extraction with EDTA pre-extraction followed by an EDTA-NaOH extraction (E→EN)

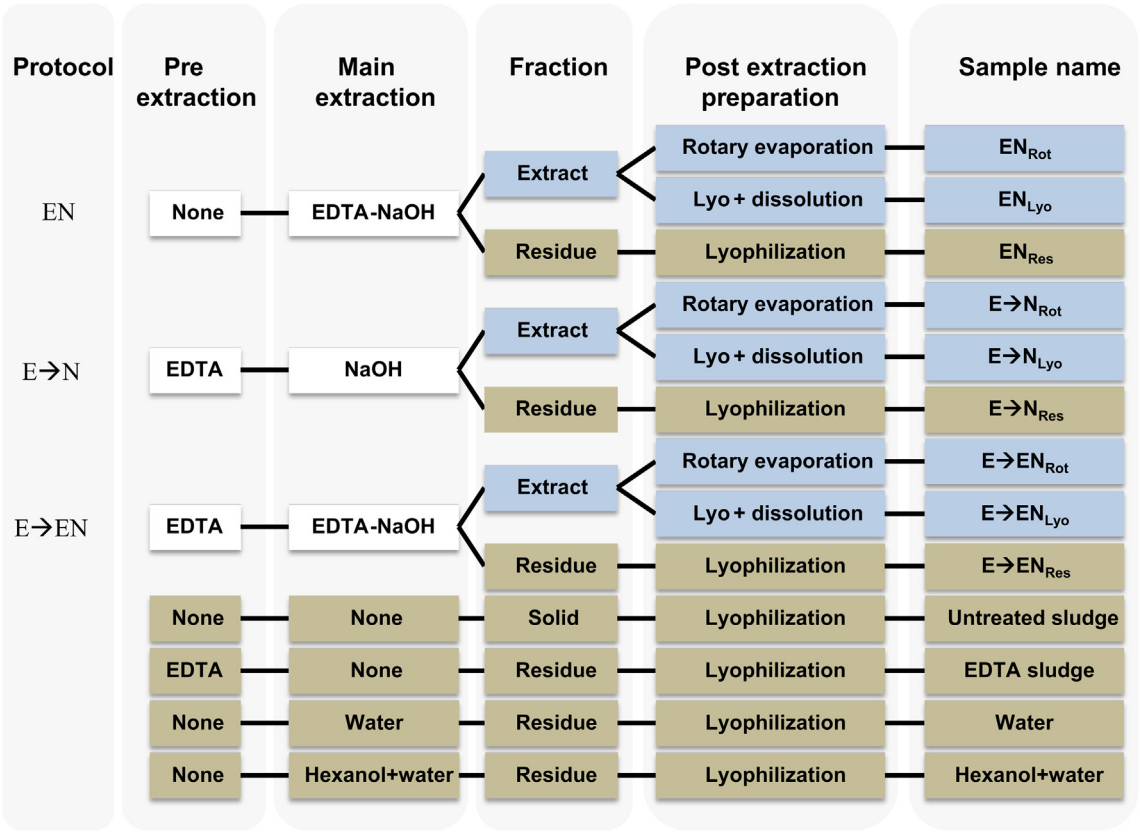

Fig. 1. An overview of the samples. There are six different combinations of extraction protocols and post-extraction sample concentration (blue) and seven samples for SSNMR analysis (brown). Samples marked with light blue or dark brown were studied by ^{31}P solution NMR and ^{31}P SSNMR, respectively. Lyo = lyophilisation. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

The single-step EN extraction represents the most commonly used extraction protocol for environmental samples (Cade-Menun and Liu, 2013; Turner et al., 2005). The E→EN extraction and the E→N extraction protocols were tested, as both have been developed for extraction of P from sediments, with emphasis on organic P (Ahlgren et al. 2006, 2007) and poly-P (Hupfer and Gachter, 1995), respectively. A fourth extraction protocol with a single-step 0.25 M NaOH main extraction was tested but excluded based on preliminary studies, as the poly-P recovery was very low (Fig. S1).

^{31}P solution NMR was used to identify and quantify poly-P in the extracts of the activated sludge, and ^{31}P SSNMR was used to estimate the total poly-P content of the sludge prior to extraction and to examine the sludge residues after extraction to establish whether all the poly-P was extracted. Finally, the poly-P middle group content determined from ^{31}P solution NMR and ^{31}P SSNMR was compared to calculate the poly-P extraction efficiencies of the different extraction protocols.

2.1. Activated sludge sample from Ejby Mølle waste water treatment plant

Activated sludge was sampled from Ejby Mølle waste water treatment plant (WWTP) in Odense, Denmark. The plant (corresponding to ca. 210,000 person equivalents) receives a mixture of domestic and industrial waste water, and P is removed by a combination of precipitation with iron(III) chloride ($FeCl_3$) and biological P removal (Stokholm-Bjerregaard et al., 2017). The activated sludge sample was taken from the aerated activated sludge tank and was kept refrigerated in a 10 L plastic bottle until analysis (maximum four hours after sampling). All sludge samples used for NMR extractions and SSNMR were centrifuged and decanted.

2.2. Protocols for extraction of poly-P from activated sludge

30 mL of activated sludge (5.7 g DW/L) was centrifuged 10 min at 2000 rpm and decanted prior to extraction. The resulting sludge pellet (approx. 0.17 g DW) was used for the NMR extractions. The pellet was resuspended in 40 mL solution (details below) at a shaking table (speed 54–60 rpm). The duration of the pre-extraction step and main extraction was one hour and 16 h, respectively. After extraction, the NMR extract was separated from the sludge by centrifugation (3000 rpm, 10 min). The following three protocols were tested (Fig. 1):

Protocol EN: The activated sludge pellet was extracted using a one-step extraction with 40 mL of an EDTA-NaOH solution (0.25 M NaOH and 0.05 M EDTA) for 16 h.

Protocol E→N. The activated sludge pellet was extracted using a two-step extraction, with a pre-extraction by 40 mL by a 0.05 M EDTA solution for one hour followed by centrifugation at 3000 rpm for 10 min, followed by decanting of the EDTA extract. The resulting pellet was extracted with 40 mL of 0.25 M NaOH for 16 h.

Protocol E→EN. The activated sludge pellet was extracted using a two-step extraction, with a pre-extraction by 40 mL of a 0.05 M EDTA solution for one hour followed by centrifugation at 3000 rpm for 10 min followed by decanting of the EDTA extract. The resulting pellet was extracted with 40 mL of an EDTA-NaOH solution (0.25 M NaOH and 0.05 M EDTA) for 16 h.

Subsamples (5 mL) of the resulting main extracts were used for analysis of total P by inductively coupled plasma optical emission spectroscopy (ICP-OES). The subsample was centrifuged at 10,000×*g* for 5 min and diluted with milliQ water before analysis by ICP.

The preparation of sludge and acquisition of the ^{31}P solution NMR spectrum can be accomplished within 24 h of sludge sampling and has the following steps with the estimated duration of each given in parentheses: Centrifugation of sludge (0.5 h), pre-extraction (1 h), centrifugation and separation of sludge pellet and extract (15 min), main extraction (16 h), centrifugation and separation of sludge pellet and extract (15 min), concentration by rotary evaporation (1–1.5 h), and recording of the ^{31}P solution NMR spectrum (3–5 h per sample).

2.3. Samples for ^{31}P solid state NMR spectroscopy

^{31}P SSNMR spectra were recorded on seven sludge samples from Ejby Mølle WWTP (Fig. 1). One activated sludge sample was frozen, lyophilised and subsequently analysed by ^{31}P SSNMR spectroscopy ("untreated sludge"). Four samples were extracted by a 0.05 M EDTA solution ("EDTA sludge") or extraction protocol 1 to 3 ("EN_{Res}", "$E \rightarrow N_{Res}$", and "$E \rightarrow EN_{Res}$") to evaluate the effect of EDTA pre-extraction on poly-P recovery and investigate if there was a complete extraction of poly-P by the three extraction protocols. Furthermore, two sludge pellets recovered after a water/hexanol (release of microbial P, called "Hexanol + water") (Cheesman et al., 2010) and a water extraction (a reference to water/hexanol solution, called "water") were analysed (experimental details in supporting information page S5, Fig. S2). This was done to establish whether the poly-P resonance in the ^{31}P SSNMR spectra should be ascribed to microbial origin (signal removed after hexanol extraction) or to overlapping Al phosphate resonances (signal present after hexanol extraction).

2.4. Sample concentration for solution NMR spectroscopy

Two different methods used to increase the P concentration in the main extract prior to solution NMR analysis were tested:

1) A 10-fold concentration of the samples by rotary evaporation (samples referred to with a subscript "Rot") (Hupfer and Gachter, 1995).
2) Neutralisation of the extracts followed by lyophilisation and redissolution of the lyophilised extract (samples referred to with a subscript "Lyo") (Cade-Menun et al., 2006).

All NMR extracts for rotary evaporation were kept at −20 °C until the day of the NMR analysis, where the samples were thawed at room temperature and concentrated approximately 10-fold by rotary evaporation at 34–38 °C. The concentrated extract was centrifuged at 10,000×*g* for 5 min to remove any particles, and 630 μL of the supernatant was mixed with 70 μL deuterium oxide (D_2O) to give a lock signal.

The extracts for lyophilisation were neutralized with 1 M HCl to pH of 6.6–7.2 before freezing at −20 °C and lyophilisation at −50 °C. The dried extract was kept at −20 °C until the day of the NMR analysis, where the extract was redissolved by a procedure modified from (He et al., 2009). The dried extract was dissolved in 1 mL of a 0.25 M NaOH and 0.05 M EDTA solution and 0.2 mL of 10 M NaOH and then centrifuged at 10,000 g for 5 min to remove particles from the extract, and 630 μL of the supernatant was mixed with 70 μL D_2O.

2.5. ^{31}P solid state NMR spectroscopy

Quantitative ^{31}P SSNMR spectra were recorded on a 500 MHz JEOL ECZ 500R spectrometer using a 3.2 mm triple resonance magic angle spinning (MAS) NMR probe, 15 kHz spinning speed, a 45° pulse, and proton decoupling. Relaxation delays were optimised on each sample, typically 200–300 s for sludge-derived samples and 410 s for a synthetic struvite, which served as an external intensity reference for spin counting experiments. The ^{31}P SSNMR spectra were referenced relative to H_3PO_4 ($\delta(^{31}P) = 0$ ppm) and analysed with 100 Hz line broadening using MestReNova (Mestrelab Research) by absolute integration of the spinning side band manifold. The ^{31}P SSNMR spectra of samples extracted by water/hexanol or water were recorded on a 600 MHz Agilent spectrometer using a 3.2 mm triple resonance MAS NMR probe, 15 kHz spinning speed, 22.5° pulse and proton decoupling.

^{31}P spin counting NMR experiments (Dougherty et al., 2005) were acquired to quantify the amount ^{31}P present in paramagnetic species by a modification of the ^{31}P spin counting experiments reported by (Dougherty et al., 2005). We used a modified version, see supporting information page S7 for further details. P bound in Fe phosphates and other paramagnetic minerals will not be observed in ^{31}P SSNMR under the experimental conditions used, as the chemical shifts are outside the recorded chemical shift range (Kim et al., 2010).

The uncertainties associated with data-analysis were estimated by processing (phase and baseline correction, and integration) each spectrum thrice and the uncertainties are given as an estimated standard deviation.

2.6. ^{31}P solution NMR spectroscopy

Quantitative ^{31}P solution NMR spectra were recorded on a JEOL ECZ 500R 500 MHz spectrometer at 22 °C using a 90° pulse (12 μs), 2.16 s acquisition time, a relaxation delay time of 25–30 s (optimised for each extraction protocol) and proton decoupling. Typically, 512 scans were acquired. The carrier frequency was set at −9 ppm to ensure optimal excitation over the chemical shift range 7 ppm to −25 ppm.

The recycle delay was determined by inversion recovery experiments for representative samples (Fig. S4 and Table S2). A recycle delay of minimum five times the longitudinal relaxation time (T_1) was chosen to ensure full relaxation between scans. 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). The ^{31}P resonances were assigned by comparison with literature (Turner et al., 2003) combined with ^{31}P,^{31}P correlation spectroscopy (COSY) spectra, and a pyro-P spiking experiment to distinguish poly-P terminal groups and pyro-P (Figs. S5 and S6, Table S3).

The relative concentrations of the soluble P species extracted from the sludge found by ^{31}P solution NMR spectroscopy were converted into mgP/gDW based on the TP found from the ICP-OES measurement of the extracts.

The total amount of poly-P present in the sludge could not be directly quantified by SSNMR, as only the poly-P middle group resonances can be unambiguously quantified by ^{31}P SSNMR leaving out the contribution from the poly-P terminal groups. In contrast, both groups were visible in ^{31}P solution NMR spectra. However, due to the non-invasive nature of the SSNMR technique the chain length of poly-P is unaffected by this technique. Consequently, it is assumed that the total poly-P content can be quantified by ^{31}P solution NMR spectroscopy if a similar content of poly-P middle groups can be obtained through ^{31}P solution and ^{31}P SSNMR.

2.7. Statistical analyses

For the poly-P middle group content determined from ^{31}P solution NMR, a one-factor ANOVA (significance level $p = 0.05$) was performed followed by Tukey's test in Sigmaplot v. 14.0. Normality of the data was checked by a Kolmogorov-Smirnoff test.

3. Results

3.1. Quantification of poly-P middle groups by ^{31}P SSNMR spectroscopy

^{31}P SSNMR spectroscopy of the lyophilised activated sludge was used to estimate the amount of poly-P middle groups in the sludge prior to any extraction, which is assumed to be the maximum amount of poly-P that can be extracted by the extraction protocols. The ^{31}P SSNMR spectrum of activated sludge from Ejby Mølle contained two broad isotropic resonances along with a series of spinning side bands from each resonance (Fig. 2a). The broad resonance at $\delta(^{31}P) \approx 0$ ppm was assigned to a number of overlapping resonances from phosphate containing minerals, e.g., apatite (Aue et al., 1984) and struvite (Bak et al., 2000), as well as biogenic P compounds such as orthophosphate monoesters, orthophosphate diesters, pyrophosphate (pyro-P) and poly-P terminal groups (Frossard et al., 1994; McDowell et al., 2002; Nanzer et al., 2014). The second resonance at $\delta(^{31}P) \approx -25$ ppm was assigned to poly-P middle groups based on earlier reported ^{31}P solution NMR chemical shifts (Hupfer and Gachter, 1995; Turner et al., 2003). Furthermore, extraction of the sludge with hexanol prior to ^{31}P SSNMR removed the resonance at $\delta(^{31}P) \approx -25$ ppm, which proved the microbial origin of this resonance (Figs. 3 and S2) (see Fig. 3).

Spin counting experiments were performed on the SSNMR samples in order to correct for missing intensity due to iron in the samples. For the activated sludge sample from Ejby Mølle, only $66 \pm 2\%$ P was visible in the ^{31}P SSNMR due to the high Fe content (32.8 ± 1.3 mgFe/gDW, Tables 1 and 2). Thus, the measured concentration of poly-P middle groups was adjusted with a factor of P_{obs}, which gives a total poly-P concentration of 13.2 ± 0.3 mgP/gDW (Table 1). This value served as a reference for calculation of extraction efficiencies for the three extraction protocols, by comparison with the sum of the poly-P middle groups found by ^{31}P solution NMR spectroscopy. The total P in the sludge was 32.5 ± 0.3 mgP/gDW, so the poly-P made up 41% of all P in the sample.

3.2. Identification of poly-P resonances in ^{31}P solution NMR spectra

The resonance in the region from $\delta(^{31}P) = -4.6$ to -4.0 ppm of poly-P terminal P (PP1) was unambiguously assigned to poly-P PP1 from spiking experiments (Figs. 4, S5, S6, Table S3), and constituted between 0.67 ± 0.10 mgP/gDW and 1.2 ± 0.4 mgP/gDW (Table 3). The three groups of resonances in the chemical shift range $\delta(^{31}P) = -18.4$ to -21.2 ppm belonged to PP2, PP3 and PP4 groups (Fig. 4) based on earlier studies (Kulaev et al., 2005; Turner et al., 2003; Uhlmann et al., 1990). These three resonances are referred to as "poly-P middle groups", and their relative concentration varied greatly from 4.4 ± 0.3 mgP/gDW ($E \rightarrow EN_{Lyo}$) to 11.4 ± 1.2 mgP/gDW ($E \rightarrow N_{Rot}$) (Table 3). The resonances at $\delta(^{31}P) = -4.8$ to -4.4 ppm was assigned to pyro-P based on spiking experiments, and this resonance often overlap with the end-groups from poly-P, as observed in the NMR spectra of the lyophilised samples (Fig. 4). Pyro-P constituted approximately 0.12 ± 0.2 mgP/gDW for the rotary evaporated samples (Table 3). The resolution of the ^{31}P solution NMR spectra of the samples concentrated by lyophilisation and dissolution was generally lower than for the samples concentrated by rotary evaporation, resulting in overlap of the poly-P PP1 groups and pyro-P resonances (Fig. 4). Furthermore, lyophilisation and dissolution of the main extract resulted in a higher chemical shift value for the P species, as observed for, e.g., the orthophosphate resonance, which resonates at $\delta(^{31}P) = 5.8–5.9$ ppm and $\delta(^{31}P)$ 6.1–6.4 ppm for the rotary evaporated and lyophilised samples, respectively, c.f., Table S4.

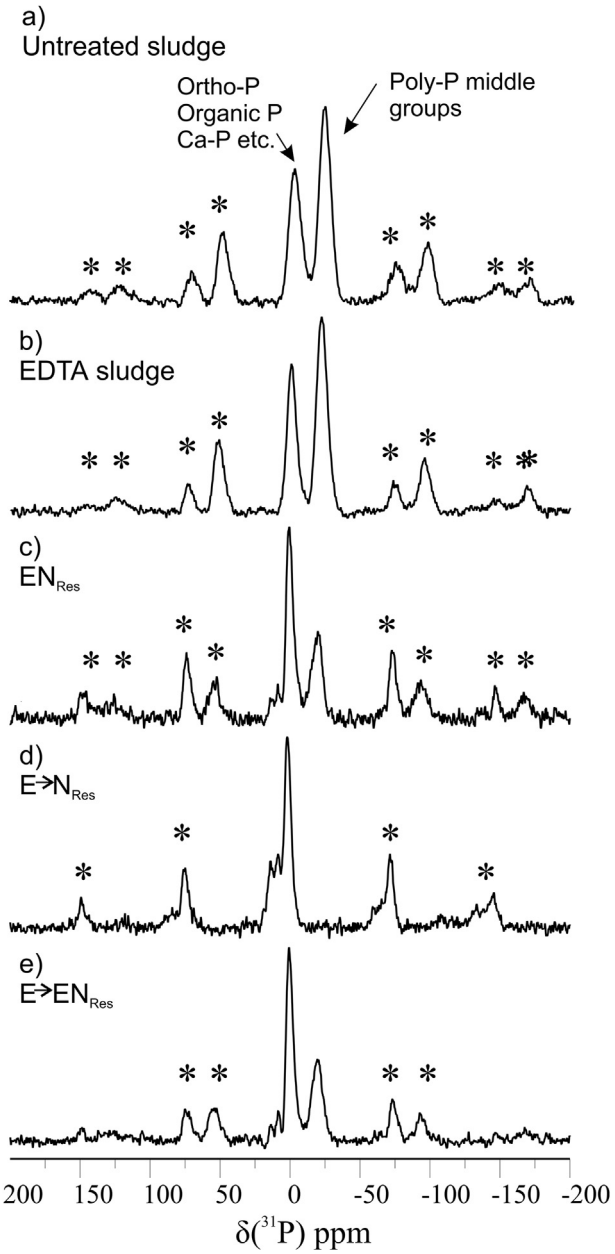

Fig. 2. ^{31}P MAS SSNMR spectra of sludge and sludge residues after extraction. a) Lyophilised activated sludge. Residues of activated sludge extracted with b) 0.05 M EDTA, c) first 0.05 M EDTA followed by 0.25 M NaOH, d) EDTA-NaOH, and e) first 0.05 M EDTA followed by extraction with a mixed solution with 0.05 M EDTA and 0.25 M NaOH. Spectra were recorded at 11.5 T with spinning speed 15 kHz. Asterisks denote spinning side bands.

3.3. Effect of the extractant protocol on the quantification of poly-P by ^{31}P solution NMR

The three different extraction protocols showed significantly

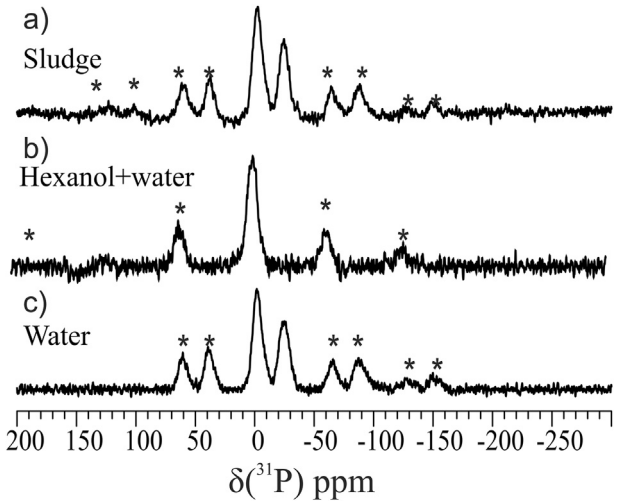

Fig. 3. ^{31}P MAS SSNMR spectra of sludge samples. a) Lyophilised activated sludge, b) Activated sludge pre-treated by an extraction in water and hexanol or c) pre-treated by a single extraction in water. The spectra were recorded at 14.1 T with spinning speed 15 kHz. Asterisks denote spinning side bands.

different poly-P middle group concentrations in the ^{31}P solution NMR analysis of the extracts with the E→N extraction being the most efficient protocol for poly-P. Up to 86 ± 9% of the poly-P observed by SSNMR (Table 3 and Fig. 4) was extracted, 10.8 ± 0.4 mgP/gDW (E→N_{Lyo}) and 11.4 ± 1.2 mgP/gDW (E→N_{Rot}), (Table 3). For the E→N extraction protocol, there was no statistical difference in poly-P middle group content in ^{31}P solution NMR for the two concentration protocols (E→N_{Rot} and E→N_{Lyo}), when analysed by an ANOVA analysis (p = 0.05) followed by Tukey's test (Fig. 4 and Table 3).

The EN_{Rot} and E→EN_{Rot} extraction protocols extracted less poly-P than the E→N_{Rot} extraction protocol (11.4 ± 1.2 mgP/gDW), with 9.3 ± 0.3 mgP/gDW extracted by EN_{Rot} and 9.4 ± 0.5 mgP/gDW extracted by the E→EN_{Rot} protocol, though not statistically different (Table 3). Concentration of the EDTA-NaOH extracts by neutralisation and lyophilisation resulted in ^{31}P solution NMR spectra with only 5.2 ± 0.4 mgP/gDW (EN_{Lyo}) and 4.4 ± 0.3 mgP/gDW (E→EN_{Lyo}), which was significantly less than any of the four other protocols (Table 3).

3.4. Efficiency of the extraction protocols

^{31}P SSNMR analyses were conducted on the sludge pellets remaining after the main extractions to determine whether the lower poly-P recovery in the extracts was due to residual poly-P left in the sludge pellet or hydrolysis of poly-P in the extracts, as none of the extraction protocols extracted 100% of the poly-P middle groups based on ^{31}P SSNMR combined with ICP. The resonance at δ(^{31}P) ≈ 25 ppm and the associated spinning side bands were

Table 1
^{31}P SSNMR results for lyophilised activated sludge and lyophilised activated sludge residues from extraction with 0.05M EDTA and the three different extraction methods tested in this study. Estimated deviations of the data analysis are given in brackets.

Treatment	P_{obs} [a] (%)	$I_{poly\text{-}P}$ [b] (%)	Poly-P middle groups, not corrected [c] (mgP/gDW)	Poly-P middle groups, corrected [d] (mgP/gDW)
None	66(2)	62(2)	19.9(0.3)	13.2(0.3)
EDTA	91(2)	64(1)	15.8(0.3)	14.1(0.3)
EN	73(2)	39(2)	4.8(0.1)	3.4(0.1)
E→N	73(3)	0	0	0
E→EN	84(2)	39(3)	5.2(0.1)	4.1(0.1)

[a] P_{obs} is the percentage of the sample P that is observed in the ^{31}P SSNMR spectrum.
[b] $I_{Poly\text{-}P}$ is the integral of the polyphosphate resonance at ca. −25 ppm before correction for P_{obs}.
[c] Poly-P middle group content of the sludge, not corrected for P_{obs}.
[d] Poly-P middle group content of the sludge, corrected for P_{obs}.

Table 2
ICP-OES (Total P, Fe, Al, Mg, Ca, Cu and Zn) results for lyophilised activated sludge and lyophilised activated sludge residues from extraction with 0.05 M EDTA and the three different extraction methods tested in this study. Standard deviation (n = 2) given in brackets. Unit: mg/gDW.

Treatment	TP	Fe	Al	Mg	Ca	Cu	Zn
None	32.5(0.3)	32.8(1.3)	2.48(0.04)	5.49(0.007)	25.2(0.5)	0.16(0.004)	0.75(0.002)
EDTA	24.3(0.3)	8.5(0.2)	2.08(0.003)	4.60(0.02)	2.49(0.01)	0.17(0.01)	0.33(0.02)
EN	11.8(0.2)	49.0(1.3)	2.38(0.1)	1.41(0.03)	1.58(0.03)	0.15(0.02)	0.23(0.01)
E→N	10.5(0.003)	24.7(0.4)	3.56(0.01)	8.65(0.03)	1.47(0.03)	0.18(0.01)	0.26(0.004)
E→EN	12.4(0.3)	12.6(0.2)	2.63(0.07)	1.39(0.04)	0.71(0.002)	0.18(0.01)	0.15(0.001)

Table 3
Contents (mgP/gDW) of poly-P end group and poly-P middle group in main extracts of the three tested extraction methods and two different concentration methods. Standard deviations (n = 3) given in brackets for P contents. Results of ANOVA analysis (p = 0.05) followed by Tukey's test for the poly-P middle groups are indicated by superscript capital letters.

	TP extracted (mg/gDW)	TP extraction efficiency (%)	PP1	Pyro-P [a]	PP2	PP3	PP4	PP2-PP4	PP2-PP4 extraction efficiency (%) [b]
EN_{Rot}	28.2	86.9	0.86(0.08)	0.11(0.02)	0.68(0.07)	0.61(0.1)	8.0(0.3)	**9.3(0.3)^{A}**	71(3)
EN_{Lyo}	29.7	91.3	0.67(0.1)	–	0.29(0.1)	0.27(0.2)	4.7(0.4)	**5.2(0.4)^{B}**	40(3)
E→N_{Rot}	23.0	70.9	1.2(0.4)	0.12(0.2)	1.0(0.2)	0.91(0.2)	9.4(1.2)	**11.4(1.2)^{C}**	86(9)
E→N_{Lyo}	21.5	66.2	1.1(0.2)	–	0.95(0.2)	1.1(0.3)	8.8(0.1)	**10.8(0.4)^{AC}**	82(3)
E→EN_{Rot}	18.4	56.7	0.87(0.2)	0.12(0.04)	0.71(0.1)	0.80(0.1)	7.9(0.5)	**9.4(0.5)^{A}**	71(4)
E→EN_{Lyo}	18.2	56.1	0.40(0.2)	–	0.17(0.07)	0.27(0.2)	4.0(0.2)	**4.4(0.3)^{B}**	34(2)

[a] Pyro-P could not be separated from poly-P PP1 groups in all spectra, and is therefore included in the integral of PP1 for the Lyo spectra.
[b] Estimated uncertainties are given in brackets.

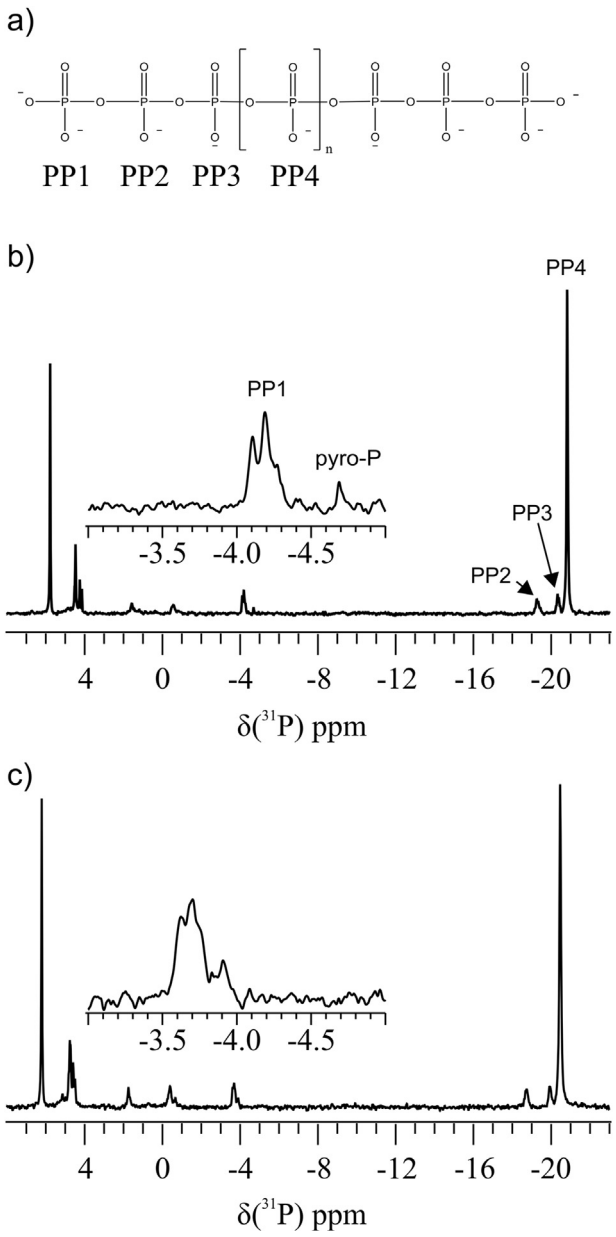

Fig. 4. ^{31}P solution NMR spectra. a) Structure of poly-P with indication of poly-P groups that can be distinguished by ^{31}P solution NMR, and ^{31}P solution NMR spectra of b) E→N_{Rot} and c) E→N_{Lyo} Insets show an expansion of the chemical shift region for PP1 and pyro-P.

completely removed after the E→N extraction (Fig. 2d), whereas the 26–31% of the total poly-P remained in the solid phase after extraction (Fig. 2c and e). Thus, only the E→N extraction protocol extracted all poly-P.

EDTA extracts iron-bound P, but did not alter the poly-P and biogenic P, as evident from the ^{31}P SSNMR spectrum and the associated integrals (Fig. 2b and Table 1). Thus, EDTA pre-extraction can be safely used for activated sludge without the risk of poly-P removal from the sludge. Extraction with EDTA resulted in an increase in observed intensity in the ^{31}P SSNMR spectrum, and a very distinct decrease in the total Fe and P contents, which dropped from 32.8 ± 1.3 mgFe/g DW to 8.5 ± 0.2 mgFe/gDW and 32.5 ± 0.3 mgP/gDW to 24.3 ± 0.3 mgP/gDW, respectively (Table 2). Furthermore, the Ca content of the activated sludge was lowered ~10 fold by EDTA extraction of the sludge from 25.3 ± 0.5 mgCa/gDW to 2.49 ± 0.01 mgCa/gDW, and Zn levels were also slightly decreased from 0.75 ± 0.02 mgZn/gDW to 0.33 ± 0.02 mgZn/gDW, whereas there was less effect on Al, Mg, and Cu (Table 2). This was also reflected in the concentrations of the metal cations in the main extracts, where the E→N and E→EN extracts contained less Fe, Al, Ca, Mg, Mn, and Zn than the corresponding EN extract, due to the EDTA pre-extraction (Table 3). Despite pre-extraction with EDTA there was still Mg and Mn left in the sludge, which can be chelated by EDTA in the main extract, as evident for the E→EN samples (3.37 ± 0.03 mg/g DW and 0.12 ± 0.01 mg/gDW, respectively) compared with the E→N samples extracts (0.92 ± 0.05 mg/gDW and 0.06 ± 0.01 mg/gDW) (Table 4). Thus, the EDTA pre-extraction of sludge mainly extracted Fe, Ca, Al, and Zn, which was also reflected in lower concentrations of these metals in the main NMR extracts, and EDTA in the main extract enhances Mg and Mn extraction from the activated sludge.

4. Discussion

The combination of ^{31}P SSNMR and solution NMR, successfully allowed for identification of the optimum extraction protocol for identification and quantification of poly-P in activated sludge. Thus, the two-step E→N extraction showed an almost complete recovery of poly-P from the sludge with no signs of post-extraction hydrolysis of poly-P. Rotary evaporation and lyophilisation of the neutralized extracts resulted in comparable poly-P content for the E→N extraction protocol, but rotary evaporation gave a better separation of the poly-P terminal groups and pyro-P in the ^{31}P solution NMR spectra. Thus, the best protocol for extraction of poly-P from activated sludge is the two step E→N extraction protocol based on our ^{31}P NMR results.

4.1. Quantification of poly-P middle groups by ^{31}P SSNMR

^{31}P SSNMR spectroscopy allowed for quantification of the total poly-P middle group content in the activated sludge, and thereby served as a reference for calculating the extraction efficiency based on ^{31}P solution NMR. Quantitative analysis of the ^{31}P SSNMR spectra is complicated by the presence of paramagnetic ions such as Fe^{3+} applied for precipitation of P from waste water (Hinedi et al., 1989; Huang and Tang, 2015), but was corrected by spin counting. These paramagnetic ions induce faster relaxation of the NMR nuclei, as well as a large change in chemical shift for P directly associated with the paramagnetic centre. For soil studies, it has been shown that the effect of paramagnetic ions on the NMR signal intensity is primarily due to close association of the paramagnetic ions and the P, and not a bulk effect (Dougherty et al., 2005). We therefore assume that only P in close proximity to the paramagnetic species are subject to a decrease in intensity, i.e. the relative

Table 4
Metal contents from ICP of the main extracts used for ^{31}P solution NMR (mgP/gDW). Standard deviations (n = 3) given in brackets.

	Fe	Al	Ca	Mg	Mn	Cu	Zn
EN	1.18(0.08)	1.04(0.02)	23.2(0.04)	4.09(0.06)	0.18(0.01)	0.17(0.01)	0.52(0.01)
E→N	0.78(0.07)	0.56(0.02)	2.8(0.8)	0.92(0.05)	0.06(0.01)	0.17(0.01)	0.22(0.02)
E→EN	0.69(0.03)	0.57(0.01)	1.85(0.02)	3.37(0.03)	0.12(0.01)	0.16(0.01)	0.20(0.02)

intensities of the poly-P resonances and the group of resonances at $\delta(^{31}P) \approx 0$ ppm is not affected by the presence of paramagnetic species in the sludge.

Poly-P middle groups were identified in the ^{31}P SSNMR spectrum by the resonance located at $\delta(^{31}P) \approx -25$ ppm. However, several Al phosphates have similar $\delta(^{31}P)$ values, e.g., berlinite $AlPO_4$ ($\delta(^{31}P) \approx -24.5$ ppm) (Bleam et al., 1989), variscite $AlPO_4 \cdot 2H_2O$ ($\delta(^{31}P) \approx -18.6$ to -19.2 ppm) (Bleam et al., 1989; Hinedi et al., 1989), and augelite $Al_2(OH)_3PO_4$ ($\delta(^{31}P) \approx -29.6$ ppm) (Bleam et al., 1989). If these Al phosphates were present, the poly-P content in the activated sludge would be overestimated. However, the hexanol extraction removed the resonance at $\delta \approx -25$ ppm completely, which unambiguously showed that the resonance at $\delta \approx -25$ ppm is due to poly-P rather than Al phosphates.

4.2. Optimal poly-P extraction from activated sludge

The variation in poly-P content from different extraction protocol has previously been ascribed to hydrolysis of poly-P during sample preparation (Ahlgren et al., 2007; Hupfer and Gachter, 1995). However, our results unambiguously show that incomplete extraction of poly-P is the main reason for the poor performance of some extraction protocols, as ^{31}P SSNMR shows that poly-P middle groups remain in the solid phase after extraction.

The E→N extraction protocol resulted in the highest poly-P recovery and performed equally well with both post-extraction concentration methods (E→N_{Rot} and E→N_{Lyo}), although with a tendency for higher recovery when rotary evaporation was used. The efficiency of the two-step E→N extraction protocol was further supported by the complete removal of the poly-P resonance in the ^{31}P SSNMR spectra of the left-over pellet from the extraction, which demonstrates the complete removal of poly-P by this protocol, in contrast to the other protocols. Thus, extraction by the other protocols (EN and E→EN) is not recommended for quantification of poly-P in activated sludge. The reason for incomplete extraction of poly-P by EN and E→EN cannot be conclusively established from our experimental setup. However, the inefficiency of the EN protocol indicates that some other mechanism of poly-P extraction is in play here as opposed to extraction protocols used in soil research, where the EN protocol is commonly used for soil samples due to the high extraction efficiency (Cade-Menun and Preston, 1996). The high extraction efficiency of the EN protocols for soil P is ascribed to a combination of the release of metal-bound phosphate (caused by EDTA) and organic P liberated from the surface of minerals and organic matter, when NaOH creates electrostatic repulsion between the organic P compound and mineral or organic matter surface (Turner et al., 2005). Furthermore, organic P associated with minerals or organic matter through bridging ions as Ca^{2+} or Fe^{3+} can be released by replacement of the bridging ions with Na^+ (Turner et al., 2005). However, poly-P is present inside bacterial cells in activated sludge, and perhaps also in the extracellular polymeric substance (EPS) surrounding the cells (Li et al., 2015). Since the binding of poly-P in activated sludge is very different from P binding found in soils this could explain why the EN extraction protocol optimised for soil samples is not efficient for poly-P in activated sludge. Even though extraction of poly-P from activated sludge by NaOH has been reported in many studies, e.g., (Huang and Tang, 2015; Uhlmann et al., 1990), the efficiency of poly-P extraction has not been addressed in previous studies, and it remains unknown whether all poly-P was extracted during these procedures. From our results, it appears that the combination of EDTA and NaOH in the main extract retards poly-P extraction from sludge, rather than promoting poly-P hydrolysis. However, our experimental setup does not allow a conclusive explanation of these findings.

4.3. The effect of pre-extraction of activated sludge

Pre-extraction with EDTA has been suggested to increase the amount of poly-P detected in NMR extracts by removal of divalent cations from the sludge or sediment (Hupfer and Gachter, 1995). Poly-P has been reported to be stable in alkaline solutions (Hupfer and Gachter, 1995), but the presence of divalent metal cations may catalyse the degradation of poly-P (Harold, 1966). (Hupfer and Gachter, 1995) showed that sediment addition to an alkaline solution of a synthetic poly-P induced a degradation of the poly-P, which was attributed to cations which catalysed poly-P degradation. The catalysing effect was also observed for extracts of sediments where sediment particles were removed by centrifugation, which indicated that the catalysing agent responsible for poly-P degradation is soluble (Hupfer and Gachter, 1995). As mentioned above, our results demonstrate that it is not poly-P degradation that causes a lower content of poly-P in the EN and E→EN extracts, but rather incomplete poly-P extraction from the sludge. However, these metal cations may promote poly-P degradation in the extracts after extraction, as observed for the lyophilised extracts in this study. Recently, Ca^{2+} has been reported to decrease the rate of poly-P degradation by phosphatase enzymes (Huang et al., 2018), which together with our results indicates that metal cations other than Ca^{2+} are involved in catalysis of poly-P breakdown.

4.4. Degradation of poly-P during post-extraction sample concentration

Poly-P middle group contents were significantly lower when lyophilisation was used for concentration of the NMR extract in the EN and E→EN protocols, which implies that rotary evaporation is preferable for these protocols. Whereas the low poly-P content in the EN_{Rot} and E→EN_{Rot} extracts can be attributed to insufficient poly-P extraction from the activated sludge, the very low poly-P extraction efficiencies of EN_{Lyo} and E→EN_{Lyo} cannot be explained by insufficient poly-P extraction alone. Hence, degradation of the poly-P to orthophosphate during the lyophilisation or dissolution steps seems very likely for these two protocols, as indicated by an increase in the relative orthophosphate content in the NMR extracts during the lyophilisation procedure. However, poly-P does not always degrade during lyophilisation/dissolution, as seen by the high poly-P recovery of 82(3)% of the E→N_{Lyo} protocol, where the poly-P content determined by solution NMR is not significantly different between the E→N_{Lyo} protocol and the E→N_{Rot} protocol, which indicates that poly-P is conserved during the lyophilisation and dissolution of the E→N_{Lyo} samples.

Both synthetic and naturally occurring poly-P have been reported to degrade during lyophilisation of the NMR extract (Cade-Menun et al., 2006; Reitzel et al., 2009). Neutralisation prior to lyophilisation has been reported to reduce poly-P breakdown during lyophilisation of tripolyphosphate extracts (Cade-Menun et al., 2006). Our E→N_{Lyo} samples confirm this where the poly-P middle group recovery by ^{31}P solution NMR spectroscopy was similar to the poly-P middle group content determined from ^{31}P SSNMR. Neutralisation of the NMR extracts did, however, not completely prevent breakdown of poly-P in the EN_{Lyo} and E→EN_{Lyo} samples. The E→N extract contained four times less Mg, and only half as much Mn as the EN and E→EN extracts, and the presence of these two divalent cations in high concentrations could play a role in catalysing the degradation of poly-P during lyophilisation of these extracts. However, this possible effect of Mg and Mn catalysis of poly-P fragmentation was only observed for EN_{Lyo} and E→EN_{Lyo} and not for EN_{Rot} and E→EN_{Rot}, indicating that it is the combination of cations and lyophilisation that catalyses degradation of poly-P. As a consequence, we do not recommend the use of lyophilisation for

concentration of NMR extracts which contain EDTA.

In sediments and soils, pre-extraction by EDTA or HCl has also been shown to recover more poly-P and pyro-P/poly-P terminal groups than the single step NaOH-EDTA extraction (Ahlgren et al., 2007; Ding et al., 2010; Hupfer and Gachter, 1995; Turner, 2008). Also pre-extraction in a bicarbonate and sodium dithionite solution (BD) may increase the relative recovery of total poly-P and poly-P middle groups (Ahlgren et al., 2007; Cade-Menun et al., 2015; He et al., 2009). However, the reported spectra resulting from extractions with BD pre-extraction and a NaOH main extraction seems to result in degradation of poly-P, as seen from a higher concentration of PP1 compared to PP2-PP4 in the study by (Ahlgren et al., 2007).

Hence, we recommend using E→N_{Rot} for extraction of poly-P from fresh sludge since it leads to an almost complete recovery of the total amount of poly-P in the sludge, limited fragmentation/degradation of poly-P and a good separation of poly-P PP1 resonances and pyro-P in the NMR spectrum.

4.5. Perspectives

The recommended extraction protocol for ^{31}P NMR analyses of activated sludge allowed direct identification and absolute quantification of poly-P in the activated sludge. In contrast to lab-scale phosphate release/uptake studies, this bulk quantification of poly-P can be used as a direct measure of the amounts of poly-P associated with the bacteria in the activated sludge under *in situ* conditions. Our quantification method can thereby serve as a direct indicator of the phosphate removal efficiency of the PAO community present in the activated sludge. Improved efficiency of the EBPR treatment of the waste water can potentially reduce the application of Al and Fe in the WWTP needed to reduce the effluent P concentration below the limits set by the authorities, and may also increase P recovery in P synthesizing units as struvite recovery units (de-Bashan and Bashan, 2004; Marti et al., 2010). In this study, the poly-P in activated sludge constituted ca. 13 mgP/gDW (1.3 wt% of dry sludge), with a TP of the sludge of 32.5 mgP/gDW. Our poly-P measurements are in the same range as the 8.8 ± 1.4 to 14.0 ± 0.6 mgP/gDW found in phosphate release studies on EBPR sludge from a range of Danish WWTPs (Mielczarek et al., 2013). It is possible that the poly-P content can become even higher as EBPR sludge may contain up to 50–70 mgP/gDW while non-EBPR sludge only contains 10–20 mgP/gDW (Yuan et al., 2012). In addition, quantification of poly-P by ^{31}P NMR spectroscopy could also be useful in studies of the poly-P speciation and breakdown along the sludge stream at WWTPs, from activated sludge tank to digested sludge.

5. Conclusion

An efficient protocol to quantitatively extract poly-P from activated sludge was identified. Two large limitations of the application of ^{31}P solution NMR spectroscopy for reliable quantification of poly-P (unknown extraction efficiencies and risk of poly-P hydrolysis) are addressed in this study by a combination of ^{31}P solution and solid state NMR spectroscopy. The main findings are:

- Complete extraction of poly-P from activated sludge was only achieved by a two-step EDTA and NaOH extraction protocol (E→N). A single-step EDTA-NaOH extraction protocol (EN) or a two-step EDTA and EDTA-NaOH (E→EN) extraction protocol both resulted in incomplete extraction of poly-P from activated sludge, as observed by ^{31}P solid state NMR on the residual sludge.
- The poly-P quantified by ^{31}P solution NMR constituted up to 86 ± 9% of the poly-P middle groups quantified by ^{31}P SSNMR, when a two-step E→N extraction was used followed by concentration by rotary evaporation.
- Statistically equal poly-P extraction efficiencies for the two-step E→N protocol result from sample concentration by rotary evaporation or lyophilisation of neutralized extracts prior to ^{31}P solution NMR analysis. However, lyophilisation and dissolution of EN and E→EN extracts resulted in poly-P degradation.
- ^{31}P SSNMR is a useful supplement to ^{31}P solution NMR, as it probes the direct speciation of P. However, the better resolution and lower recording time makes ^{31}P solution NMR better suited for quantification and characterisation of poly-P in activated sludge systems.

Declaration of interests

None.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.watres.2019.03.065.

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