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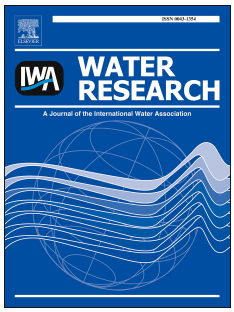

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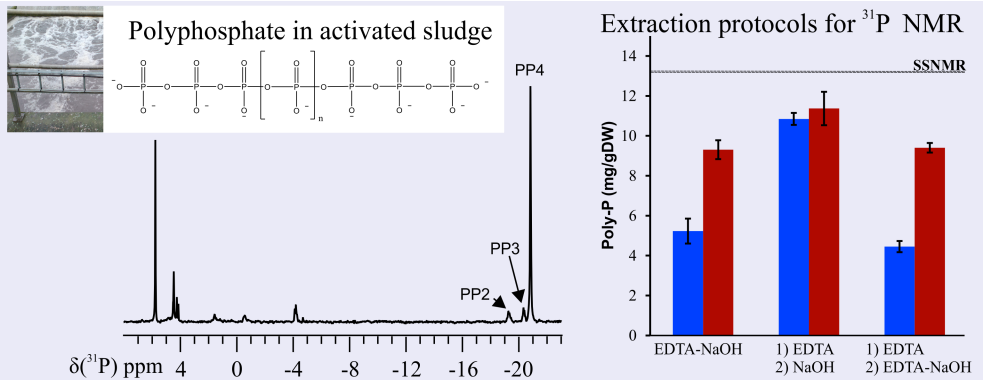

Extraction and quantification of polyphosphates in activated sludge from waste water treatment plants by ^{31}P NMR spectroscopy

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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

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. The aim is 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 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.

Keywords: polyphosphate, identification, quantification, ^{31}P, solid state NMR, EBPR, biological waste water treatment

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). P 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 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 have successfully quantified species-specific poly-P contents by Raman-fluorescence in situ hybridisation (FISH) (Fernando et al. 2018).

^{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

95 and Liu 2013, Cade-Menun 2005a). Lyophilisation of NaOH or EDTA-NaOH extracts of soil
96 followed by dissolution of the lyophilised extract before ^{31}P solution NMR analysis is a very
97 common way to concentrate samples prior to ^{31}P NMR analysis. However, poly-P degradation after
98 lyophilisation of EDTA-NaOH extracts has been observed (Cade-Menun et al. 2006, Reitzel et al.
99 2009), and neutralization of the extract prior to lyophilisation has been suggested as a way to
100 prevent this, as demonstrated for the short-chain poly-P sodium tripolyphosphate (Cade-Menun et
101 al. 2006). Thus far, there is no evidence in the literature for the NMR analysis' ability to accurately
102 quantify the total poly-P content, and the risks of incomplete extraction and/or degradation of poly-
103 P have not been addressed (Hupfer et al. 2008).

104 Solid state ^{31}P magic angle spinning NMR (^{31}P SSNMR) is a non-destructive characterisation
105 technique that only requires minimum pre-analysis treatment of the sample, but is sparingly used
106 for environmental samples as the resolution is lower than for solution NMR (Turner et al. 2005).
107 SSNMR is a useful tool for sludge P characterisation due to relatively high P concentrations in
108 activated sludge from waste water treatment plants compared to, e.g., soil samples (Frossard et al.
109 1994, Hinedi et al. 1989, Huang and Tang 2015). However, analysis by ^{31}P solution NMR is often
110 quicker than by SSNMR and produces spectra with a better resolution that allows identification of
111 specific organic P compounds (Cade-Menun 2005a). The main limitation for quantification of poly-
112 P by ^{31}P solution NMR spectroscopy is the unknown extraction effeciency of the extraction protocol
113 and the possible degradation (hydrolysis) of poly-P by this (Hupfer and Gachter 1995, Hupfer et al.
114 2008). These uncertainties limit the comparability among studies, and to our knowledge, no
115 estimates of the poly-P extraction efficiencies of these protocols have been reported before.

116 In this study, SSNMR was used to quantify the poly-P middle groups in sludge prior to extraction,
117 and this poly-P content was compared to the poly-P extracted by three different extraction protocols
118 and used as a reference for evaluating potential poly-P degradation in the extracts. The advantage of

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

133 **2. Materials and Methods**

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

135 1) A single-step EDTA-NaOH extraction (EN)

136 2) A two-step extraction with EDTA pre-extraction followed by a NaOH extraction (E→N)

137 3) A two-step extraction with EDTA pre-extraction followed by an EDTA-NaOH extraction
138 (E→EN)

139

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 extractionand the E→N extraction protocols were tested, ase both have been developed for extraction of P from sediments, with emphasis on organic P (Ahlgren et al. 2007, Ahlgren et al. 2006) 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 (Figure 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 were 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 hours, respectively. After extraction, the NMR extract was separated from the sludge by centrifugation (3000 rpm, 10 min). The following three protocols were tested (Figure 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 hours.

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 hours.

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 hours.

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 x *g* for 5 min. and diluted with milliQ water before analysis by ICP.

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

186 *2.3 Samples for ^{31}P solid state NMR spectroscopy*

187 ^{31}P SSNMR spectra were recorded on seven sludge samples from Ejby Mølle WWTP (Figure 1).
188 One activated sludge sample was frozen, lyophilised and subsequently analysed by ^{31}P SSNMR
189 spectroscopy ("untreated sludge"). Four samples were extracted by a 0.05 M EDTA solution
190 ("EDTA sludge") or extraction protocol 1 to 3 ("EN_{Res}", "$E \rightarrow N_{Res}$", and "$E \rightarrow EN_{Res}$") to evaluate
191 the effect of EDTA pre-extraction on poly-P recovery and investigate if there was a complete
192 extraction of poly-P by the three extraction protocols. Furthermore, two sludge pellets recovered
193 after a water/hexanol (release of microbial P, called "Hexanol+water") (Cheesman et al. 2010) and
194 a water extraction (a reference to water/hexanol solution, called "water") were analysed
195 (experimental details in supporting information page S5, Figure S2). This was done to establish
196 whether the poly-P resonance in the ^{31}P SSNMR spectra should be ascribed to microbial origin
197 (signal removed after hexanol extraction) or to overlapping Al phosphate resonances (signal present
198 after hexanol extraction).

199 *2.4 Sample concentration for solution NMR spectroscopy*

200 Two different methods used to increase the P concentration in the main extract prior to solution
201 NMR analysis of poly-P containing samples were tested:

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

206 All NMR extracts for rotary evaporation were kept at -20 °C until the day of the NMR analysis,
207 where the samples were thawed at room temperature and concentrated approximately 10-fold by
208 rotary evaporation at 34-38 °C. The concentrated extract was centrifuged at 10,000 x *g* for 5 min. to

209 remove any particles, and 630 µL of the supernatant was mixed with 70 µL deuterium oxide (D_2O)
210 to give a lock signal.

211 The extracts for lyophilisation were neutralized with 1 M HCl to pH of 6.6-7.2 before freezing at -
212 20 °C and lyophilisation at -50 °C. The dried extract was kept at -20 °C until the day of the NMR
213 analysis, where the extract was redissolved by a procedure modified from (He et al. 2009). The
214 dried extract was dissolved in 1 mL of a 0.25M NaOH and 0.05M EDTA solution and 0.2 mL of 10
215 M NaOH and then centrifuged at 10,000 g for 5 min. to remove particles from the extract, and 630
216 µL of the supernatant was mixed with 70 µL D_2O.

217 *2.5 ^{31}P solid state NMR spectroscopy*

218 Quantitative ^{31}P SSNMR spectra were recorded on a 500 MHz Jeol ECZ 500R spectrometer using a
219 3.2 mm triple resonance magic angle spinning (MAS) NMR probe, 15 kHz spinning speed, a 45°
220 pulse, and proton decoupling. Relaxation delays were optimised on each sample, typically 200-300
221 s for sludge-derived samples and 410 s for a synthetic struvite, which served as an external intensity
222 reference for spin counting experiments. The ^{31}P SSNMR spectra were referenced relative to H_3PO_4
223 ($\delta(^{31}P) = 0$ ppm) and were analysed with 100 Hz line broadening in MestReNova (Mestrelab
224 Research) by absolute integration of the spinning side band manifold. The spectra of samples
225 extracted by water/hexanol or water were recorded on a 600 MHz Agilent spectrometer using a 3.2
226 mm triple resonance MAS NMR probe, 15 kHz spinning speed, 22.5° pulse and proton decoupling.

227 ^{31}P spin counting NMR experiments (Dougherty et al. 2005) were acquired to quantify the amount
228 ^{31}P present in paramagnetic species by a modification of the ^{31}P spin counting experiments reported by
229 (Dougherty et al. 2005) . We used a modified version, see supporting information page S7 for
230 further details. P bound in Fe phosphates and other paramagnetic minerals will not be observed in

231 ^{31}P SSNMR under the experimental conditions used, as the chemical shifts are outside the recorded
232 chemical shift range (Kim et al. 2010).

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

236 *2.6 ^{31}P solution NMR spectroscopy*

237 Quantitative ^{31}P solution NMR spectra were recorded on a Jeol ECZ 500R 500 MHz spectrometer at
238 22°C using a 90° pulse (12 μs), 2.16 s acquisition time, a relaxation delay time of 25-30 s
239 (optimised for each extraction protocol) and proton decoupling. Typically, 512 scans were acquired.
240 The carrier frequency was set at -9 ppm to ensure optimal excitation over the chemical shift range 7
241 ppm to -25 ppm.

242 The recycle delay was determined by inversion recovery experiments for representative samples
243 (Figure S4 and Table S2). A recycle delay of minimum five times the longitudinal relaxation time
244 (T_1) was chosen to ensure full relaxation between scans. Spectra were processed with the
245 MestReNova software using a 5 Hz line broadening with an exponential window function and with
246 zero-filling to 64K points (32K points were recorded). The ^{31}P resonances were assigned by
247 comparison with literature (Turner et al. 2003) combined with ^{31}P,^{31}P correlation spectroscopy
248 (COSY) spectra, and a pyro-P spiking experiment to distinguish poly-P terminal groups and pyro-P
249 (Figures S5 and S6, Table S3).

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

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

260 *2.7 Statistical analyses*

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

264 **3. Results**

265 *3.1 Quantification of poly-P middle groups by ^{31}P SSNMR spectroscopy*

266 ^{31}P SSNMR spectroscopy of the lyophilised activated sludge was used to estimate the amount of
267 poly-P middle groups in the sludge prior to any extraction, which is assumed to be the maximum
268 amount of poly-P that can be extracted by the extraction protocols. The ^{31}P SSNMR spectrum of
269 activated sludge from Ejby Mølle contained two broad isotropic resonances along with a series of
270 spinning side bands from each resonance (Figure 2a). The broad resonance at $\delta(^{31}P) \approx 0$ ppm was
271 assigned to a number of overlapping resonances from phosphate containing minerals, e.g., apatite
272 (Aue et al. 1984) and struvite (Bak et al. 2000), as well as biogenic P compounds such as
273 orthophosphate monoesters, orthophosphate diesters, pyrophosphate (pyro-P) and poly-P terminal
274 groups (Frossard et al. 1994, McDowell et al. 2002, Nanzer et al. 2014). The second resonance at

275 $\delta(^{31}P) \approx$ -25 ppm was assigned to poly-P middle groups based on earlier reported ^{31}P solution NMR
276 chemical shifts (Hupfer and Gachter 1995, Turner et al. 2003). Furthermore, extraction of the
277 sludge with hexanol prior to ^{31}P SSNMR removed the resonance at $\delta(^{31}P) \approx$ -25 ppm, which proved
278 the microbial origin of this resonance (Figures 3 and S2).

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

287 *3.2 Identification of poly-P resonances in ^{31}P solution NMR spectra*

288 The resonance in the region $\delta(^{31}P)$ = -4.6 to -4.0 ppm of poly-P terminal P (PP1) was
289 unambiguously assigned to poly-P PP1 from spiking experiments (Figures 4, S5 and S6, Table S3)),
290 and constituted between 0.67 ±0.10 mgP/gDW and 1.2 ±0.4 mgP/gDW (Table 3). The three groups
291 of resonances in the chemical shift range $\delta(^{31}P)$ = -18.4 to -21.2 ppm belonged to PP2, PP3 and
292 PP4 groups (Figure 4) based on earlier studies (Kulaev et al. 2005, Turner et al. 2003, Uhlmann et
293 al. 1990). These three resonances are referred to as "poly-P middle groups", and their relative
294 concentration varied greatly from 4.4 ±0.3 mgP/gDW (E→EN_{Lyo}) to 11.4 ±1.2 mgP/gDW
295 (E→N_{Rot}) (Table 3). The resonances at $\delta(^{31}P)$ = -4.8 to -4.4 ppm was assigned to pyro-P based on
296 spiking experiments, and this resonance often overlap with the end-groups from poly-P, as
297 observed in the NMR spectra of the lyophilised samples (Figure 4). Pyro-P constituted

298 approximately 0.12 ±0.2 mgP/gDW for the rotary evaporated samples (Table 3). The resolution of
299 the ^{31}P solution NMR spectra of the samples concentrated by lyophilisation and dissolution was
300 generally lower than for the samples concentrated by rotary evaporation, resulting in overlap of the
301 poly-P PP1 groups and pyro-P resonances (Figure 4). Furthermore, lyophilisation and dissolution of
302 the main extract resulted in a a higher chemical shift value for the P species, as observed for, e.g.,
303 the orthophosphate resonance, which resonates at $\delta(^{31}P)$ = 5.8 to 5.9 ppm and $\delta(^{31}P)$ 6.1 to 6.4 ppm
304 for the rotary evaporated and lyophilized samples, respectively, c.f., Table S4.

305 *3.3 Effect of the extractant protocol on the quantification of poly-P by ^{31}P solution NMR*

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

313 Even though the EN_{Rot} and E→EN_{Rot} extraction protocols were not statistically different from the
314 E→N_{Lyo} protocol, they extracted less poly-P than the E→N_{Rot} extraction protocol (11.4±1.2
315 mgP/gDW), with 9.3 ±0.3 mgP/gDW extracted by EN_{Rot} and 9.4 ±0.5 mgP/gDW extracted by the
316 E→EN_{Rot} protocol (Table 3). Concentration of the EDTA-NaOH extracts by neutralization and
317 lyophilisation resulted in ^{31}P solution NMR spectra with only 5.2 ±0.4 mgP/gDW (EN_{Lyo}) and 4.4
318 ±0.3 mgP/gDW (E→EN_{Lyo}), which was significantly less than any of the four other protocols
319 (Table 3).

320 *3.4 Efficiency of the extraction protocols*

321 ^{31}P SSNMR analyses were conducted on the sludge pellets remaining after the main extractions to
322 determine whether the lower poly-P recovery in the extracts was due to residual poly-P left in the
323 sludge pellet or hydrolysis of poly-P in the extracts, as none of the extraction protocols extracted
324 100% of the poly-P middle groups based on ^{31}P SSNMR combined with ICP. The resonance at
325 $\delta(^{31}P) \approx 25$ ppm and the associated spinning side bands were completely removed after the E→N
326 extraction (Figure 2d), whereas the 26-31% of the total poly-P remained in the solid phase after
327 extraction (Figure 2c and 2e). Thus, only the E→N extraction protocol extracted all poly-P.

328 EDTA extracts iron-bound P, but did not alter the poly-P and biogenic P, as evident from the ^{31}P
329 SSNMR spectrum and the associated integrals (Figure 2b and Table 1). Thus, EDTA pre-extraction
330 can be safely used for activated sludge without the risk of poly-P removal from the sludge.
331 Extraction with EDTA resulted in an increase in observed intensity in the ^{31}P SSNMR spectrum,
332 and a very distinct decrease in the total Fe and P contents, which dropped from 32.8 ±1.3 mgFe/g
333 DW to 8.5 ±0.2 mgFe/gDW and 32.5 ±0.3 mgP/gDW to 24.3 ±0.3 mgP/gDW, respectively (Table
334 2). Furthermore, the Ca content of the activated sludge was lowered ~10 fold by EDTA extraction
335 of the sludge from 25.3 ±0.5 mgCa/gDW to 2.49 ±0.01 mgCa/gDW, and Zn levels were also
336 slightly decreased from 0.75 ±0.02 mgZn/gDW to 0.33 ±0.02 mgZn/gDW, whereas there was less
337 effect on Al, Mg, and Cu (Table 2). This was also reflected in the concentrations of the metal
338 cations in the main extracts, where the E→N and E→EN extracts contained less Fe, Al, Ca, Mg,
339 Mn, and Zn than the corresponding EN extract, due to the EDTA pre-extraction (Table 3). Despite
340 pre-extraction with EDTA there was still Mg and Mn left in the sludge, which can be chelated by
341 EDTA in the main extract, as evident for the E→EN samples (3.37 ±0.03 mg/g DW and 0.12 ±0.01
342 mg/gDW, respectively) compared with the E→N samples extracts (0.92 ±0.05 mg/gDW and 0.06
343 ±0.01 mg/gDW) (Table 4). Thus, the EDTA pre-extraction of sludge mainly extracts Fe, Ca, Al,

and Zn, which is 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 ofthe 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 closely associated with the paramagnetic species are subject to a decrease in intensity,

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

368 Poly-P middle groups were identified in the ^{31}P SSNMR spectrum by the resonance located at
369 $\delta(^{31}P) \approx -25$ ppm. However, several Al-phosphates have similar $\delta(^{31}P)$ values, e.g., berlinite $AlPO_4$
370 ($\delta(^{31}P) \approx -24.5$ ppm) (Bleam et al. 1989), variscite $AlPO_4 \cdot 2H_2O$ ($\delta(^{31}P) \approx -18.6$ to -19.2 ppm)
371 (Bleam et al. 1989, Hinedi et al. 1989), and augelite $Al_2(OH)_3PO_4$ ($\delta(^{31}P) \approx -29.6$ ppm) (Bleam et
372 al. 1989). If these Al phosphates were present, the poly-P content in the activated sludge would be
373 overestimated. However, the hexanol extraction removed the resonance at ≈ -25 ppm completely,
374 which unambiguously showed that the resonance at ≈ -25 ppm was caused by poly-P rather than Al
375 phosphates.

376 *4.2 Optimal poly-P extraction from activated sludge*

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

382 The E→N extraction protocol resulted in the highest poly-P recovery and performed equally well
383 with both post-extraction concentration methods (E→N_{Rot} and E→N_{Lyo}), although with a tendency
384 for higher recovery when rotary evaporation was used. The efficiency of the two-step E→N
385 extraction protocol was further supported by the complete removal of the poly-P resonance in the
386 ^{31}P SSNMR spectra of the left-over pellet from the extraction, which demonstrates the complete
387 removal of poly-P by this protocol, in contrast to the other protocols. Thus, extraction by the other
388 protocols (i.e. 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 release of metal-bound phosphate (caused by EDTA) and organic P released 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

437 by solution NMR is not significantly different between the E→N_{Lyo} protocol and the E→N_{Rot}
438 protocol, which indicates that poly-P is conserved during the lyophilisation and dissolution of the
439 E→N_{Lyo} samples.

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

454 In sediments and soils, pre-extraction by EDTA or HCl has also been shown to recover more poly-P
455 and pyro-P/poly-P terminal groups than the single step NaOH-EDTA extraction (Ahlgren et al.
456 2007, Ding et al. 2010, Hupfer and Gachter 1995, Turner 2008). Also pre-extraction in a
457 bicarbonate and sodium dithionite solution (BD) may increase the relative recovery of total poly-P
458 and poly-P middle groups (Ahlgren et al. 2007, Cade-Menun et al. 2015, He et al. 2009). However,
459 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

483 speciation and breakdown along the sludge stream at WWTPs, from activated sludge tank to
484 digested sludge.

485 **5. Conclusion**

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

490 • Complete extraction of poly-P from activated sludge was only achieved by a two-step EDTA
491 and NaOH extraction protocol (E→N). A single-step EDTA-NaOH extraction protocol (EN)
492 or a two-step EDTA and EDTA-NaOH (E→EN) extraction protocol both resulted in
493 incomplete extraction of poly-P from activated sludge, as observed by ^{31}P solid state NMR
494 on the residual sludge.

495 • The poly-P quantified by ^{31}P solution NMR constituted up to 86 ±9% of the poly-P middle
496 groups quantified by ^{31}P SSNMR, when a two-step E→N extraction was used followed by
497 concentration by rotary evaporation.

498 • Statistically equal poly-P extraction efficiencies for the two-step E→N protocol result from
499 sample concentration by rotary evaporation or lyophilisation of neutralized extracts prior to
500 ^{31}P solution NMR analysis. However, lyophilisation and dissolution of EN and E→EN
501 extracts resulted in poly-P degradation.

502 • ^{31}P SSNMR is a useful supplement to ^{31}P solution NMR, as it probes the direct speciation of
503 P. However, the better resolution and lower recording time makes ^{31}P solution NMR better
504 suited for quantification and characterisation of poly-P in activated sludge systems.

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Declaration of interests: None

References

Ahlgren, J., De Brabandere, H., Reitzel, K., Rydin, E., Gogoll, A. and Waldeback, M. (2007) Sediment phosphorus extractants for phosphorus-31 nuclear magnetic resonance analysis: A quantitative evaluation. Journal of Environmental Quality 36(3), 892-898.

Ahlgren, J., Reitzel, K., Danielsson, R., Gogoll, A. and Rydin, E. (2006) Biogenic phosphorus in oligotrophic mountain lake sediments: Differences in composition measured with NMR spectroscopy. Water Research 40(20), 3705-3712.

Aue, W.P., Roufosse, A.H., Glimcher, M.J. and Griffin, R.G. (1984) Solid-state phosphorus-31 nuclear magnetic resonance studies of synthetic solid phases of calcium phosphate: Potential models of bone mineral. Biochemistry 23(25), 6110-6114.

Bak, M., Thomsen, J.K., Jakobsen, H.J., Petersen, S.E., Petersen, T.E. and Nielsen, N.C. (2000) Solid-state ^{13}C and ^{31}P NMR analysis of urinary stones. Journal of Urology 164(3), 856-863.

Bleam, W.F., Pfeffer, P.E. and Frye, J.S. (1989) ^{31}P solid-state nuclear magnetic-resonance spectroscopy of aluminum phosphate minerals. Physics and Chemistry of Minerals 16(5), 455-464.

Cade-Menun, B. and Liu, C.W. (2013) Solution phosphorus-31 nuclear magnetic resonance spectroscopy of soils from 2005 to 2013: A review of sample preparation and experimental parameters. Soil Science Society of America journal 78, 19-37.

Cade-Menun, B.J. (2005a) Characterizing phosphorus in environmental and agricultural samples by ^{31}P nuclear magnetic resonance spectroscopy. Talanta 66(2), 359-371.

Cade-Menun, B.J. (2005b) Organic phosphorus in the environment. Turner, B.L., Frossard, E. and Baldwin, D. (eds), pp. 21-44, CABI Publishing, Wallingford, UK.

Cade-Menun, B.J., He, Z.Q. and Dou, Z.X. (2015) Comparison of phosphorus forms in three extracts of dairy feces by solution ^{31}P NMR analysis. Communications in Soil Science and Plant Analysis 46(13), 1698-1712.

Cade-Menun, B.J., Navaratnam, J.A. and Walbridge, M.R. (2006) Characterizing dissolved and particulate phosphorus in water with ^{31}P nuclear magnetic resonance spectroscopy. Environmental Science & Technology 40(24), 7874-7880.

Cade-Menun, B.J. and Preston, C.M. (1996) A comparison of soil extraction procedures for ^{31}P NMR spectroscopy. Soil Science 161(11), 770-785.

Cheesman, A.W., Turner, B.L. and Reddy, K.R. (2010) Interaction of phosphorus compounds with anion-exchange membranes: Implications for soil analysis. Soil Science Society of America journal 74(5), 1607-1612.

Cordell, D., Rosemarin, A., Schroder, J.J. and Smit, A.L. (2011) Towards global phosphorus security: A systems framework for phosphorus recovery and reuse options. Chemosphere 84(6), 747-758.

de-Bashan, L.E. and Bashan, Y. (2004) Recent advances in removing phosphorus from wastewater and its future use as fertilizer (1997-2003). Water Research 38(19), 4222-4246.

Diaz, J.M. and Ingall, E.D. (2010) Fluorometric quantification of natural inorganic polyphosphate. Environmental Science & Technology 44(12), 4665-4671.

Ding, S.M., Bai, X.L., Fan, C.X. and Zhang, L. (2010) Caution needed in pretreatment of sediments for refining phosphorus-31 nuclear magnetic resonance analysis: Results from a comprehensive assessment of pretreatment with ethylenediaminetetraacetic acid. Journal of Environmental Quality 39(5), 1668-1678.

559 Dougherty, W.J., Smernik, R.J. and Chittleborough, D.J. (2005) Application of spin counting to the
560 solid-state ^{31}P NMR analysis of pasture soils with varying phosphorus content. Soil Science
561 Society of America journal 69(6), 2058-2070.
562 Fernando, E.Y., McIlroy, S.J., Nierychlo, M., Herbst, F.-A., Schmid, M.C., Wagner, M., Nielsen,
563 J.L. and Nielsen, P.H. (2018) Resolving the individual contribution of key microbial
564 populations to enhanced biological phosphorus removal with Raman-FISH. bioRxiv.
565 *https://doi.org/10.1101/387795.*
566
567 Florentz, M. and Granger, P. (1983) Phosphorus-31 nuclear magnetic-resonance of activated-sludge
568 - Use for the study of the biological removal of phosphates from wastewater. Environmental
569 Technology Letters 4(1), 9-14.
570 Frossard, E., Tekely, P. and Grimal, J.Y. (1994) Characterization of phosphate species in urban
571 sewage sludges by high-resolution solid-state ^{31}P NMR. European Journal of Soil Science
572 45(4), 403-408.
573 Gomes, F.M., Ramos, I.B., Wendt, C., Girard-Dias, W., De Souza, W., Machado, E.A. and
574 Miranda, K. (2013) New insights into the in situ microscopic visualization and quantification
575 of inorganic polyphosphate stores by 4 ',6-diamidino-2-phenylindole (DAPI)-staining.
576 European Journal of Histochemistry 57(4), 227-235.
577 Harold, F.M. (1966) Inorganic polyphosphates in biology - structure metabolism and function.
578 Bacteriological Reviews 30(4), 772-794.
579 He, Z.Q., Honeycutt, C.W., Griffin, T.S., Cade-Menun, B.J., Pellechia, P.J. and Dou, Z.X. (2009)
580 Phosphorus forms in conventional and organic dairy manure identified by solution and solid
581 state P-31 NMR spectroscopy. Journal of Environmental Quality 38(5), 1909-1918.
582 Hinedi, Z.R., Chang, A.C. and Yesinowski, J.P. (1989) Phosphorus-31 magic-angle spinning
583 nuclear magnetic-resonance of waste-water sludges and sludge-amended soil. Soil Science
584 Society of America journal 53(4), 1053-1056.
585 Huang, R. and Tang, Y. (2015) Speciation dynamics of phosphorus during (hydro)thermal
586 treatments of sewage sludge. Environmental Science & Technology 49(24), 14466-14474.
587 Huang, R.X., Wan, B., Hultz, M., Diaz, J.M. and Tang, Y.Z. (2018) Phosphatase-mediated
588 hydrolysis of linear polyphosphates. Environmental Science & Technology 52(3), 1183-1190.
589 Hupfer, M. and Gachter, R. (1995) Polyphosphates in lake sediments: ^{31}P NMR-Spectroscopy as a
590 tool for its identification. Limnology and Oceanography 40(3), 610-617.
591 Hupfer, M., Gloss, S., Schmieder, P. and Grossart, H.P. (2008) Methods for detection and
592 quantification of polyphosphate and polyphosphate accumulating microorganisms in aquatic
593 sediments. International Review of Hydrobiology 93(1), 1-30.
594 Jing, S.R., Benefield, L.D. and Hill, W.E. (1992) Observations relating to enhanced phosphorus
595 removal in biological-systems. Water Research 26(2), 213-223.
596 Kahiluoto, H., Kuisma, M., Ketoja, E., Salo, T. and Heikkinen, J. (2015) Phosphorus in manure and
597 sewage sludge more recyclable than in soluble inorganic fertilizer. Environmental Science &
598 Technology 49(4), 2115-2122.
599 Kim, J., Middlemiss, D.S., Chernova, N.A., Zhu, B.Y.X., Masquelier, C. and Grey, C.P. (2010)
600 Linking local environments and hyperfine shifts: A combined experimental and theoretical
601 31P and 7Li solid-state NMR study of paramagnetic Fe(III) phosphates. Journal of the
602 American Chemical Society 132(47), 16825-16840.
603 Kulaev, I.S., Vagabov, V. and Kulakovskaya, T. (2005) The Biochemistry of Inorganic
604 Polyphosphates, pp. 26-31, John Wiley & Sons, Incorporated, New York, United Kingdom.
605 Li, W.W., Zhang, H.L., Sheng, G.P. and Yu, H.Q. (2015) Roles of extracellular polymeric
606 substances in enhanced biological phosphorus removal process. Water Research 86, 85-95.

Majed, N., Li, Y. and Gu, A.Z. (2012) Advances in techniques for phosphorus analysis in biological sources. Current Opinion in Biotechnology 23(6), 852-859.

Majed, N., Matthaus, C., Diem, M. and Gu, A.Z. (2009) Evaluation of intracellular polyphosphate dynamics in enhanced biological phosphorus removal process using Raman microscopy. Environmental Science & Technology 43(14), 5436-5442.

Marti, N., Pastor, L., Bouzas, A., Ferrer, J. and Seco, A. (2010) Phosphorus recovery by struvite crystallization in WWTPs: Influence of the sludge treatment line operation. Water Research 44(7), 2371-2379.

McDowell, R.W., Condron, L.M., Mahieu, N., Brookes, P.C., Poulton, P.R. and Sharpley, A.N. (2002) Analysis of potentially mobile phosphorus in arable soils using solid state nuclear magnetic resonance. Journal of Environmental Quality 31(2), 450-456.

Mielczarek, A.T., Nguyen, H.T.T., Nielsen, J.L. and Nielsen, P.H. (2013) Population dynamics of bacteria involved in enhanced biological phosphorus removal in Danish wastewater treatment plants. Water Research 47(4), 1529-1544.

Nanzer, S., Oberson, A., Huthwelker, T., Eggenberger, U. and Frossard, E. (2014) The molecular environment of phosphorus in sewage sludge ash: Implications for bioavailability. Journal of Environmental Quality 43(3), 1050-1060.

O'Connor, G.A., Sarkar, D., Brinton, S.R., Elliott, H.A. and Martin, F.G. (2004) Phytoavailability of biosolids phosphorus. Journal of Environmental Quality 33(2), 703-712.

Reitzel, K., Jensen, H.S., Flindt, M. and Andersen, F.O. (2009) Identification of dissolved nonreactive phosphorus in freshwater by precipitation with aluminum and subsequent ^{31}P NMR analysis. Environmental Science & Technology 43(14), 5391-5397.

Stokholm-Bjerregaard, M., McIlroy, S.J., Nierychlo, M., Karst, S.M., Albertsen, M. and Nielsen, P.H. (2017) A critical assessment of the microorganisms proposed to be important to enhanced biological phosphorus removal in full-scale wastewater treatment systems. Frontiers in Microbiology 8(718).

Turner, B.L. (2008) Soil organic phosphorus in tropical forests: an assessment of the NaOH-EDTA extraction procedure for quantitative analysis by solution ^{31}P NMR spectroscopy. European Journal of Soil Science 59(3), 453-466.

Turner, B.L., Cade-Menun, B.J., Condron, L.M. and Newman, S. (2005) Extraction of soil organic phosphorus. Talanta 66(2), 294-306.

Turner, B.L., Mahieu, N. and Condron, L.M. (2003) Phosphorus-31 nuclear magnetic resonance spectral assignments of phosphorus compounds in soil NaOH-EDTA extracts. Soil Science Society of America journal 67(2), 497-510.

Uhlmann, D., Roske, I., Hupfer, M. and Ohms, G. (1990) A simple method to distinguish between polyphosphate and other phosphate fractions of activated-sludge. Water Research 24(11), 1355-1360.

Yuan, Z.G., Pratt, S. and Batstone, D.J. (2012) Phosphorus recovery from wastewater through microbial processes. Current Opinion in Biotechnology 23(6), 878-883.

Figure 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.

Figure 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.

Figure 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.

Figure 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.

665 **Table 1:** ^{31}P SSNMR results for lyophilised activated sludge and lyophilised activated sludge
666 residues from extraction with 0.05M EDTA and the three different extraction methods tested in this
667 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)

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672 [a]P_{obs} is the percentage of the sample P that is observed in the ^{31}P SSNMR spectrum.

673 [b] $I_{Poly\text{-}P}$ is the integral of the polyphosphate resonance at ca. -25 ppm before correction for $P_{obs.}$

674 [c] Poly-P middle group content of the sludge, not corrected for P_{obs}.

675 [d] Poly-P middle group content of the sludge, corrected for P_{obs}.

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

681

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)

682

683 **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

684 two different concentration methods. Standard deviations (n = 3) given in brackets for P contents. Results of ANOVA analysis (p = 0.05)

685 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)
$E\rightarrow N_{Rot}$	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)
$E\rightarrow N_{Lyo}$	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)
$E\rightarrow EN_{Rot}$	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)
$E\rightarrow EN_{Lyo}$	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)

686 [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.

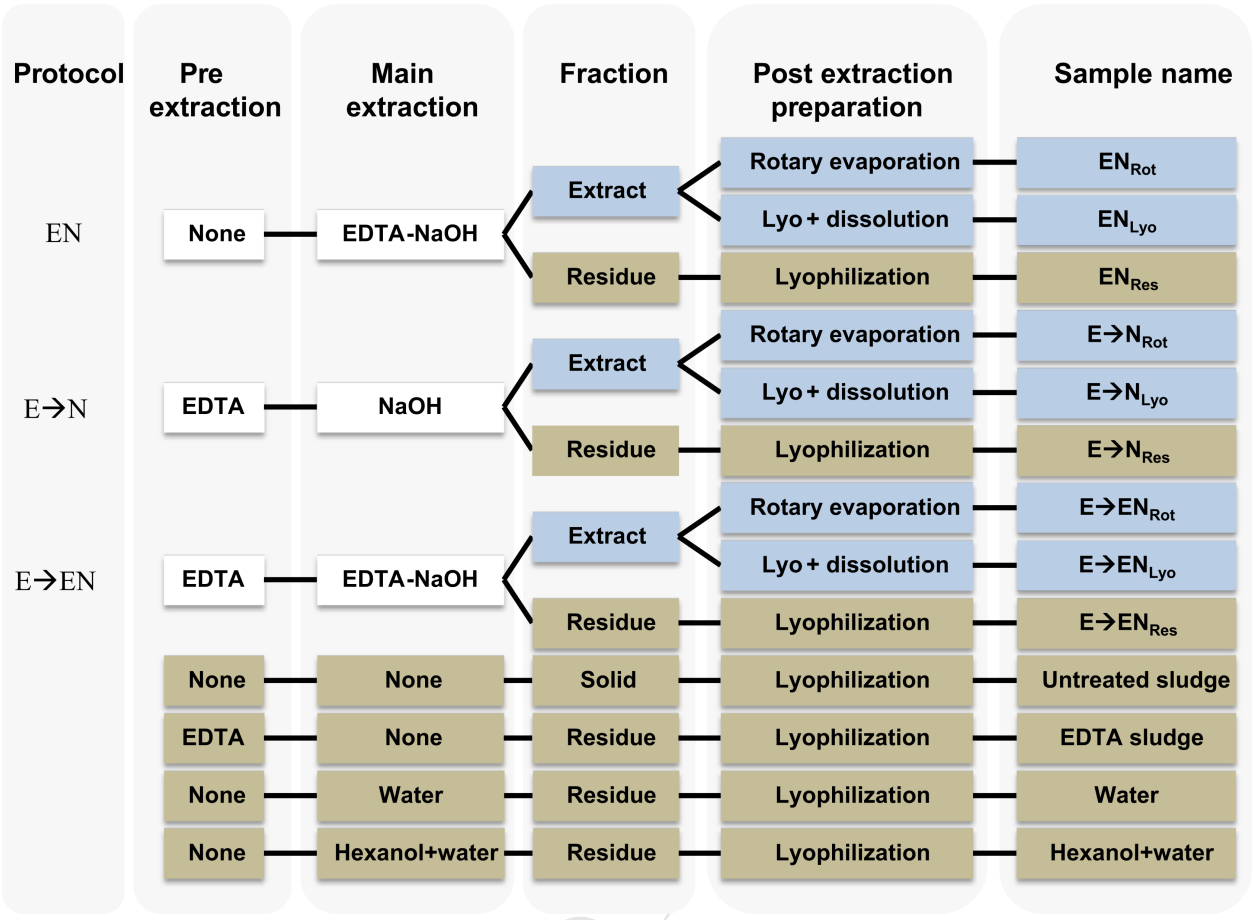
687 [b] Estimated uncertainties are given in brackets.

688 **Table 4:** Metal contents from ICP of the main extracts used for ^{31}P solution NMR (mgP/gDW).

689 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)

690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Fraction
Post extraction preparation
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Extract
Rotary evaporation
ENRot
Lyo + dissolution
ENLyo
Residue
Lyophilization
ENRes
E→N
EDTA
NaOH
Extract
Rotary evaporation
E→NRot
Lyo + dissolution
E→NLyo
Residue
Lyophilization
E→NRes
E→EN
EDTA
EDTA-NaOH
Extract
Rotary evaporation
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Lyo + dissolution
E→ENLyo
Residue
Lyophilization
E→ENRes
None
None
Solid
Lyophilization
Untreated sludge
EDTA
None
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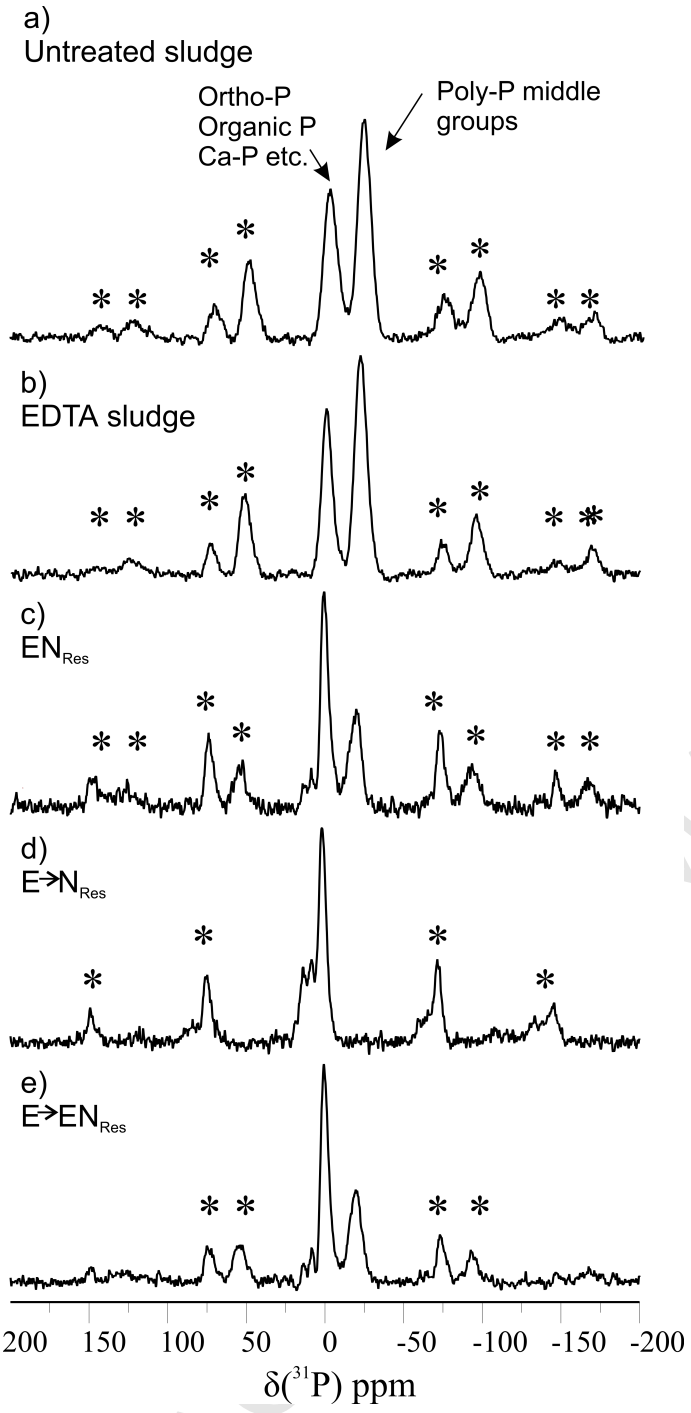
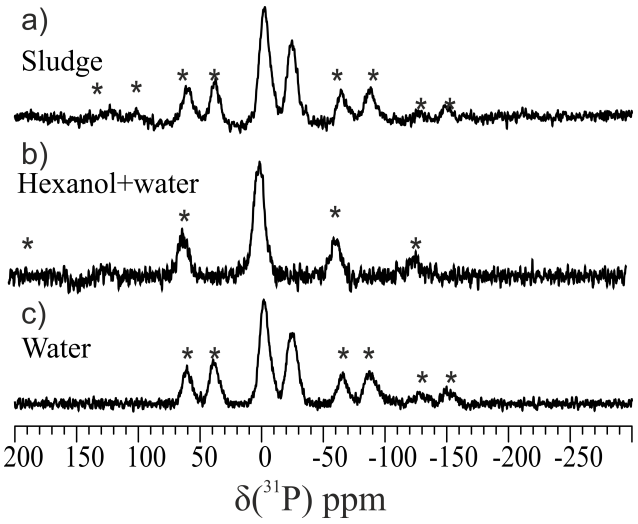
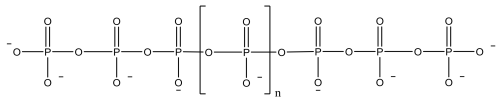
Hexanol+water



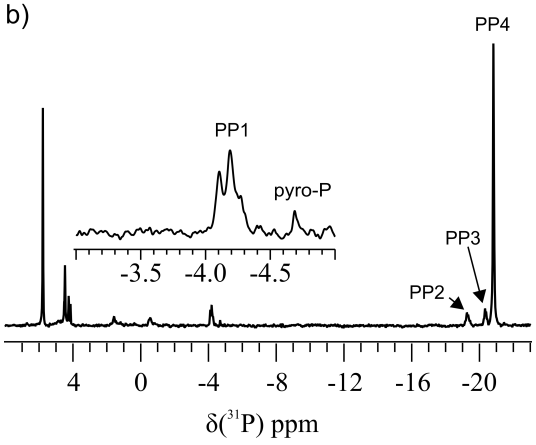


a)

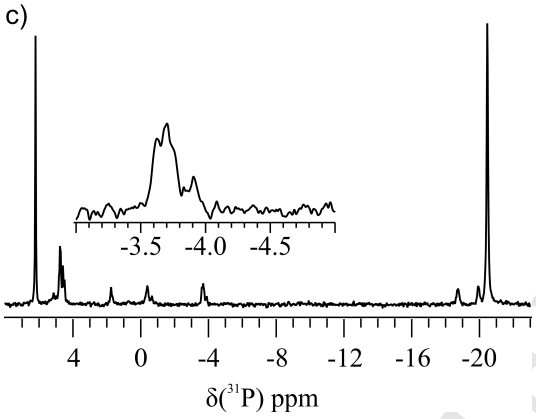


PP1 PP2 PP3 PP4

b)



c)



Highlights:

- ^{31}P solution NMR spectroscopy for quantification of poly-P extracted from activated sludge.
- Three extraction protocols for poly-P from activated sludge were compared.
- Two-step EDTA and NaOH extraction extracts all poly-P from activated sludge.
- Rotary evaporation of extracts gives less poly-P degradation than lyophilisation.
- Poly-P extraction efficiency was evaluated by comparison with solid state NMR results.