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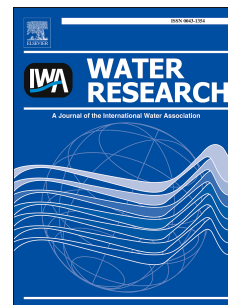
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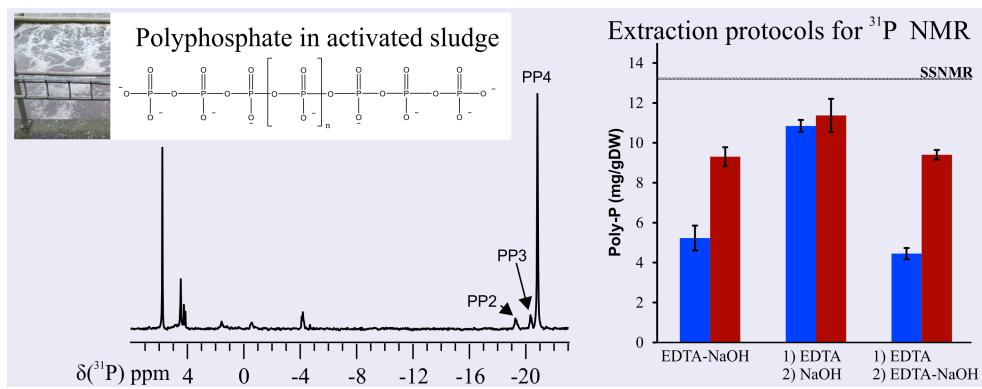
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2 waste water treatment plants by  $^{31}\text{P}$  NMR spectroscopy

3

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14 **Abbreviations:**

15	NMR	nuclear magnetic resonance
16	EDTA	ethylenediaminetetraacetic acid
17	EBPR	enhanced biological phosphorus removal
18	EPS	extracellular polymeric substances
19	ICP	inductively coupled plasma
20	poly-P	polyphosphate
21	ppm	parts per million
22	$\delta(^{31}\text{P})$	$^{31}\text{P}$ chemical shift
23	PAO	polyphosphate accumulating organism
24	SSNMR	solid state nuclear magnetic resonance
25	TP	total phosphorus

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

45 **Keywords:** polyphosphate, identification, quantification,  $^{31}\text{P}$ , solid state NMR, EBPR, biological  
46 waste water treatment

## 47 **1. Introduction**

48 Phosphorus (P) recovery from waste water is an alternative P resource that becomes increasingly  
49 important as global P reserves are limited (Cordell et al. 2011). P recovery from domestic waste  
50 water can cover up to 20% of the global phosphorus consumption (Yuan et al. 2012). Phosphorus  
51 and nitrogen are removed during the treatment of waste water in order to protect the recipient from  
52 excess nutrients. Today, the most common methods of P removal from municipal waste water  
53 include enhanced biological P removal (EBPR) (Jing et al. 1992) and precipitation by  
54 aluminum(III) ( $\text{Al}^{3+}$ ) or iron(III) ( $\text{Fe}^{3+}$ ) compounds. Enhanced biological P removal relies on  
55 aerobic uptake of phosphate and conversion to internal inorganic polyphosphate (poly-P) by poly-P  
56 accumulating organisms (PAOs) (Yuan et al. 2012). The use of EBPR is cost-effective, as it saves  
57 chemicals and enhances the value of the sludge as a fertilizer (Kahiluoto et al. 2015, O'Connor et al.  
58 2004). Furthermore, poly-P might also be used to recover P, e.g., as struvite if the degradation of  
59 poly-P and the subsequent release of orthophosphate from PAOs can be controlled (Yuan et al.  
60 2012). Optimisation of the P uptake in PAOs by EBPR systems and control of the subsequent  
61 phosphate release requires correct identification and quantification of the total amount of poly-P in  
62 the sludge. In order to better understand and optimise the EBPR process, and retain more P, one  
63 should be able to precisely quantify and identify the poly-P formed by the PAOs to, e.g., monitor  
64 changes in the poly-P accumulation under different conditions. However, reliable methods for the  
65 quantification of the poly-P species are needed as current methods have several shortcomings such  
66 as inefficient extraction and poly-P degradation (Hupfer et al. 2008).

67 Although several methods exist for poly-P identification and quantification, none of these methods  
68 have been proven to reliably quantify the total poly-P content of bulk activated sludge. One of the  
69 most common methods for quantification of poly-P in environmental samples is staining followed  
70 by fluorometry (Hupfer et al. 2008, Majed et al. 2012), which often includes an alkaline extraction

71 with NaOH (Diaz and Ingall 2010, Majed et al. 2012) or a permeabilisation step which allows the  
72 dye to cross cell membranes (Gomes et al. 2013). Thus, absolute quantification of poly-P by  
73 staining techniques may be hindered due to, e.g., insufficient extraction/permeabilisation and the  
74 risk of degradation of poly-P in the extract (Majed et al. 2012). Furthermore, many dyes only bind  
75 to longer poly-P chains ( $>10 P_i$ ) (Diaz and Ingall 2010, Hupfer et al. 2008), which excludes short-  
76 chain poly-P from the quantification. Raman micro-spectroscopy allows for identification and  
77 quantification of poly-P on a cellular level in activated sludge, but this has so far not been  
78 transferred into absolute, bulk quantities (Majed et al. 2009), even though a recent study have  
79 successfully quantified species-specific poly-P contents by Raman-fluorescence in situ  
80 hybridisation (FISH) (Fernando et al. 2018).

81  $^{31}\text{P}$  NMR analyses have been used for investigations of poly-P in sludge since 1983 (Cade-Menun  
82 2005b, Florentz and Granger 1983), the  $^{31}\text{P}$  chemical shift reflects the position of the phosphate  
83 group in the poly-P chain: Terminal phosphate at the end of the chain (PP1 group) can be  
84 distinguished from penultimate phosphate groups near the end of the chain (PP2 and PP3) and  
85 phosphate groups inside the poly-P chain (PP4). These groups can be directly quantified by  $^{31}\text{P}$   
86 solution NMR spectroscopy (Hupfer et al. 2008). However, comparisons among studies are  
87 hampered by the large differences in sludge preparation, extraction procedures, and preparation of  
88 the extracts for the  $^{31}\text{P}$  NMR analysis. Hence, previous  $^{31}\text{P}$  solution NMR studies of organic P and  
89 poly-P from different environmental samples including sludge used a wide range of combinations  
90 of pre-treatment (air-drying, freezing/lyophilisation etc.), pre-extractant (ethylenediaminetetraacetic  
91 acid (EDTA), trichloroacetic acid, etc.), main extractant (EDTA-NaOH, NaOH, etc.) and post-  
92 treatments of the extracts (e.g., lyophilisation or rotary evaporation) (Cade-Menun and Liu 2013). A  
93 list with examples of extraction protocols including references is given in supporting information  
94 (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  $^{31}\text{P}$  solution NMR analysis is a very  
97 common way to concentrate samples prior to  $^{31}\text{P}$  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 triphosphate (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  $^{31}\text{P}$  magic angle spinning NMR ( $^{31}\text{P}$  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  $^{31}\text{P}$  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  $^{31}\text{P}$  solution NMR spectroscopy is the unknown extraction efficiency 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  $^{31}\text{P}$   
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  $^{31}\text{P}$  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  $^{31}\text{P}$  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,  $^{31}\text{P}$  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

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

147 <sup>31</sup>P solution NMR was used to identify and quantify poly-P in the extracts of the activated sludge,  
148 and <sup>31</sup>P SSNMR was used to estimate the total poly-P content of the sludge prior to extraction and  
149 to examine the sludge residues after extraction to establish whether all the poly-P was extracted.  
150 Finally, the poly-P middle group content determined from <sup>31</sup>P solution NMR and <sup>31</sup>P SSNMR were  
151 compared to calculate the poly-P extraction efficiencies of the different extraction protocols.

### 152 *2.1 Activated sludge sample from Ejby Mølle waste water treatment plant*

153 Activated sludge was sampled from Ejby Mølle waste water treatment plant (WWTP) in Odense,  
154 Denmark. The plant (corresponding to ca. 210 000 person equivalents) receives a mixture of  
155 domestic and industrial waste water, and P is removed by a combination of precipitation with  
156 iron(III) chloride (FeCl<sub>3</sub>) and biological P removal (Stokholm-Bjerregaard et al. 2017). The  
157 activated sludge sample was taken from the aerated activated sludge tank and was kept refrigerated  
158 in a 10 L plastic bottle until analysis (maximum four hours after sampling). All sludge samples used  
159 for NMR extractions and SSNMR were centrifuged and decanted.

### 160 *2.2 Protocols for extraction of poly-P from activated sludge*

161 30 mL of activated sludge (5.7 g DW/L) was centrifuged 10 min. at 2000 rpm and decanted prior to  
162 extraction. The resulting sludge pellet (approx. 0.17 g DW) was used for the NMR extractions. The

163 pellet was resuspended in 40 mL solution (details below) at a shaking table (speed 54-60 rpm). The  
164 duration of the pre-extraction step and main extraction was one hour and 16 hours, respectively.  
165 After extraction, the NMR extract was separated from the sludge by centrifugation (3000 rpm, 10  
166 min). The following three protocols were tested (Figure 1):

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

169 Protocol E→N. The activated sludge pellet was extracted using a two-step extraction, with a pre-  
170 extraction by 40 mL by a 0.05 M EDTA solution for one hour followed by centrifugation at 3000  
171 rpm for 10 min, followed by decanting of the EDTA extract. The resulting pellet was extracted with  
172 40 mL of 0.25 M NaOH for 16 hours.

173 Protocol E→EN. The activated sludge pellet was extracted using a two-step extraction, with a pre-  
174 extraction by 40 mL of a 0.05 M EDTA solution for one hour followed by centrifugation at 3000  
175 rpm for 10 min followed by decanting of the EDTA extract. The resulting pellet was extracted with  
176 40 mL of an EDTA-NaOH solution (0.25 M NaOH and 0.05 M EDTA) for 16 hours.

177 Subsamples (5 mL) of the resulting main extracts were used for analysis of total P by inductively  
178 coupled plasma optical emission spectroscopy (ICP-OES). The subsample was centrifuged at  
179 10,000 x g for 5 min. and diluted with milliQ water before analysis by ICP.

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

### 186 2.3 Samples for $^{31}\text{P}$ solid state NMR spectroscopy

187  $^{31}\text{P}$  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  $^{31}\text{P}$  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<sub>Res</sub>”, “E→N<sub>Res</sub>”, and “E→EN<sub>Res</sub>”) 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  $^{31}\text{P}$  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  $\mu\text{L}$  of the supernatant was mixed with 70  $\mu\text{L}$  deuterium oxide ( $\text{D}_2\text{O}$ )  
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  $^\circ\text{C}$  and lyophilisation at -50  $^\circ\text{C}$ . The dried extract was kept at -20  $^\circ\text{C}$  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  $\mu\text{L}$  of the supernatant was mixed with 70  $\mu\text{L}$   $\text{D}_2\text{O}$ .

#### 217 2.5 $^{31}\text{P}$ solid state NMR spectroscopy

218 Quantitative  $^{31}\text{P}$  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 $^\circ$   
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  $^{31}\text{P}$  SSNMR spectra were referenced relative to  $\text{H}_3\text{PO}_4$   
223 ( $\delta(^{31}\text{P}) = 0$  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 $^\circ$  pulse and proton decoupling.

227  $^{31}\text{P}$  spin counting NMR experiments (Dougherty et al. 2005) were acquired to quantify the amount  
228  $^{31}\text{P}$  present in paramagnetic species by a modification of the  $^{31}\text{P}$  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  $^{31}\text{P}$  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 $^{31}\text{P}$ solution NMR spectroscopy*

237 Quantitative  $^{31}\text{P}$  solution NMR spectra were recorded on a Jeol ECZ 500R 500 MHz spectrometer at  
238 22°C using a 90° pulse (12  $\mu\text{s}$ ), 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  $^{31}\text{P}$  resonances were assigned by  
247 comparison with literature (Turner et al. 2003) combined with  $^{31}\text{P}$ ,  $^{31}\text{P}$  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  $^{31}\text{P}$  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  $^{31}\text{P}$  SSNMR leaving  
255 out the contribution from the poly-P terminal groups. In contrast, both groups were visible in  $^{31}\text{P}$   
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  $^{31}\text{P}$  solution NMR spectroscopy if a similar content of poly-P middle  
259 groups can be obtained through  $^{31}\text{P}$  solution and  $^{31}\text{P}$  SSNMR.

## 260 *2.7 Statistical analyses*

261 For the poly-P middle group content determined from  $^{31}\text{P}$  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 $^{31}\text{P}$ SSNMR spectroscopy*

266  $^{31}\text{P}$  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  $^{31}\text{P}$  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  $\delta(^{31}\text{P}) \approx 0$  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  $\delta(^{31}\text{P}) \approx -25$  ppm was assigned to poly-P middle groups based on earlier reported  $^{31}\text{P}$  solution NMR  
276 chemical shifts (Hupfer and Gachter 1995, Turner et al. 2003). Furthermore, extraction of the  
277 sludge with hexanol prior to  $^{31}\text{P}$  SSNMR removed the resonance at  $\delta(^{31}\text{P}) \approx -25$  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  $66 \pm 2\%$   
281 P was visible in the  $^{31}\text{P}$  SSNMR due to the high Fe content ( $32.8 \pm 1.3$  mgFe/gDW, Tables 1 and 2).  
282 Thus, the measured concentration of poly-P middle groups was adjusted with a factor of  $P_{\text{obs}}$ , which  
283 gives a total poly-P concentration of  $13.2 \pm 0.3$  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  $^{31}\text{P}$  solution NMR spectroscopy. The total P in  
286 the sludge was  $32.5 \pm 0.3$  mgP/gDW, so the poly-P made up 41% of all P in the sample.

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

288 The resonance in the region  $\delta(^{31}\text{P}) = -4.6$  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  $0.67 \pm 0.10$  mgP/gDW and  $1.2 \pm 0.4$  mgP/gDW (Table 3). The three groups  
291 of resonances in the chemical shift range  $\delta(^{31}\text{P}) = -18.4$  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  $4.4 \pm 0.3$  mgP/gDW ( $\text{E} \rightarrow \text{EN}_{\text{LyO}}$ ) to  $11.4 \pm 1.2$  mgP/gDW  
295 ( $\text{E} \rightarrow \text{N}_{\text{Rot}}$ ) (Table 3). The resonances at  $\delta(^{31}\text{P}) = -4.8$  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  $0.12 \pm 0.2$  mgP/gDW for the rotary evaporated samples (Table 3). The resolution of  
299 the  $^{31}\text{P}$  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 higher chemical shift value for the P species, as observed for, e.g.,  
303 the orthophosphate resonance, which resonates at  $\delta(^{31}\text{P}) = 5.8$  to  $5.9$  ppm and  $\delta(^{31}\text{P}) 6.1$  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 $^{31}\text{P}$ solution NMR*

306 The three different extraction protocols showed significantly different poly-P middle group  
307 concentrations in the  $^{31}\text{P}$  solution NMR analysis of the extracts with the E $\rightarrow$ N extraction being the  
308 most efficient protocol for poly-P. Up to  $86 \pm 9\%$  of the poly-P observed by SSNMR (Table 3 and  
309 Figure 4) was extracted,  $10.8 \pm 0.4$  mgP/gDW (E $\rightarrow$ N<sub>Ly</sub>) and  $11.4 \pm 1.2$  mgP/gDW (E $\rightarrow$ N<sub>Rot</sub>), (Table  
310 3). For the E $\rightarrow$ N extraction protocol, there was no statistical difference in poly-P middle group  
311 content in  $^{31}\text{P}$  solution NMR for the two concentration protocols (E $\rightarrow$ N<sub>Rot</sub> and E $\rightarrow$ N<sub>Ly</sub>), 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<sub>Rot</sub> and E $\rightarrow$ EN<sub>Rot</sub> extraction protocols were not statistically different from the  
314 E $\rightarrow$ N<sub>Ly</sub> protocol, they extracted less poly-P than the E $\rightarrow$ N<sub>Rot</sub> extraction protocol ( $11.4 \pm 1.2$   
315 mgP/gDW), with  $9.3 \pm 0.3$  mgP/gDW extracted by EN<sub>Rot</sub> and  $9.4 \pm 0.5$  mgP/gDW extracted by the  
316 E $\rightarrow$ EN<sub>Rot</sub> protocol (Table 3). Concentration of the EDTA-NaOH extracts by neutralization and  
317 lyophilisation resulted in  $^{31}\text{P}$  solution NMR spectra with only  $5.2 \pm 0.4$  mgP/gDW (EN<sub>Ly</sub>) and  $4.4$   
318  $\pm 0.3$  mgP/gDW (E $\rightarrow$ EN<sub>Ly</sub>), which was significantly less than any of the four other protocols  
319 (Table 3).

### 320 *3.4 Efficiency of the extraction protocols*

321  $^{31}\text{P}$  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  $^{31}\text{P}$  SSNMR combined with ICP. The resonance at  
325  $\delta(^{31}\text{P}) \approx 25$  ppm and the associated spinning side bands were completely removed after the E $\rightarrow$ 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 $\rightarrow$ 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  $^{31}\text{P}$   
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  $^{31}\text{P}$  SSNMR spectrum,  
332 and a very distinct decrease in the total Fe and P contents, which dropped from  $32.8 \pm 1.3$  mgFe/g  
333 DW to  $8.5 \pm 0.2$  mgFe/gDW and  $32.5 \pm 0.3$  mgP/gDW to  $24.3 \pm 0.3$  mgP/gDW, respectively (Table  
334 2). Furthermore, the Ca content of the activated sludge was lowered  $\sim 10$  fold by EDTA extraction  
335 of the sludge from  $25.3 \pm 0.5$  mgCa/gDW to  $2.49 \pm 0.01$  mgCa/gDW, and Zn levels were also  
336 slightly decreased from  $0.75 \pm 0.02$  mgZn/gDW to  $0.33 \pm 0.02$  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 $\rightarrow$ N and E $\rightarrow$ 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 $\rightarrow$ EN samples ( $3.37 \pm 0.03$  mg/g DW and  $0.12 \pm 0.01$   
342 mg/gDW, respectively) compared with the E $\rightarrow$ N samples extracts ( $0.92 \pm 0.05$  mg/gDW and  $0.06$   
343  $\pm 0.01$  mg/gDW) (Table 4). Thus, the EDTA pre-extraction of sludge mainly extracts Fe, Ca, Al,

344 and Zn, which is also reflected in lower concentrations of these metals in the main NMR extracts,  
345 and EDTA in the main extract enhances Mg and Mn extraction from the activated sludge.

#### 346 **4. Discussion**

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

##### 355 *4.1 Quantification of poly-P middle groups by $^{31}\text{P}$ SSNMR*

356  $^{31}\text{P}$  SSNMR spectroscopy allowed for quantification of the total poly-P middle group content in the  
357 activated sludge, and thereby served as a reference for calculating the extraction efficiency based on  
358  $^{31}\text{P}$  solution NMR. Quantitative analysis of the  $^{31}\text{P}$  SSNMR spectra is complicated by the presence  
359 of paramagnetic ions such as  $\text{Fe}^{3+}$  applied for precipitation of P from waste water (Hinedi et al.  
360 1989, Huang and Tang 2015), but was corrected by spin counting. These paramagnetic ions induce  
361 faster relaxation of the NMR nuclei, as well as a large change in chemical shift for P directly  
362 associated with the paramagnetic centre. For soil studies, it has been shown that the effect of  
363 paramagnetic ions on the NMR signal intensity is primarily due to close association of the  
364 paramagnetic ions and the P, and not a bulk effect (Dougherty et al. 2005). We therefore assume  
365 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  $\delta(^{31}\text{P}) \approx 0$  ppm is  
367 not affected by the presence of paramagnetic species in the sludge.

368 Poly-P middle groups were identified in the  $^{31}\text{P}$  SSNMR spectrum by the resonance located at  
369  $\delta(^{31}\text{P}) \approx -25$  ppm. However, several Al-phosphates have similar  $\delta(^{31}\text{P})$  values, e.g., berlinite  $\text{AlPO}_4$   
370 ( $\delta(^{31}\text{P}) \approx -24.5$  ppm) (Bleam et al. 1989), variscite  $\text{AlPO}_4 \cdot 2\text{H}_2\text{O}$  ( $\delta(^{31}\text{P}) \approx -18.6$  to  $-19.2$  ppm)  
371 (Bleam et al. 1989, Hinedi et al. 1989), and augelite  $\text{Al}_2(\text{OH})_3\text{PO}_4$  ( $\delta(^{31}\text{P}) \approx -29.6$  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  $\approx -25$  ppm completely,  
374 which unambiguously showed that the resonance at  $\approx -25$  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  $^{31}\text{P}$  SSNMR shows that poly-P middle  
381 groups remain in the solid phase after extraction.

382 The E $\rightarrow$ N extraction protocol resulted in the highest poly-P recovery and performed equally well  
383 with both post-extraction concentration methods (E $\rightarrow$ N<sub>Rot</sub> and E $\rightarrow$ N<sub>Ly</sub>), although with a tendency  
384 for higher recovery when rotary evaporation was used. The efficiency of the two-step E $\rightarrow$ N  
385 extraction protocol was further supported by the complete removal of the poly-P resonance in the  
386  $^{31}\text{P}$  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 $\rightarrow$ EN) is not recommended for quantification of poly-P in activated sludge.

389 The reason for incomplete extraction of poly-P by EN and E→EN cannot be conclusively  
390 established from our experimental setup. However, the inefficiency of the EN protocol indicates  
391 that some other mechanism of poly-P extraction is in play here as opposed to extraction protocols  
392 used in soil research, where the EN protocol is commonly used for soil samples due to the high  
393 extraction efficiency (Cade-Menun and Preston 1996). The high extraction efficiency of the EN  
394 protocols for soil P is ascribed to a combination of release of metal-bound phosphate (caused by  
395 EDTA) and organic P released from the surface of minerals and organic matter, when NaOH creates  
396 electrostatic repulsion between the organic P compound and mineral or organic matter surface  
397 (Turner et al. 2005). Furthermore, organic P associated with minerals or organic matter through  
398 bridging ions as  $\text{Ca}^{2+}$  or  $\text{Fe}^{3+}$  can be released by replacement of the bridging ions with  $\text{Na}^+$  (Turner  
399 et al. 2005). However, poly-P is present inside bacterial cells in activated sludge, and perhaps also  
400 in the extracellular polymeric substance (EPS) surrounding the cells (Li et al. 2015). Since the  
401 binding of poly-P in activated sludge is very different from P binding found in soils this could  
402 explain why the EN extraction protocol optimised for soil samples is not efficient for poly-P in  
403 activated sludge. Even though extraction of poly-P from activated sludge by NaOH has been  
404 reported in many studies, e.g., (Huang and Tang 2015, Uhlmann et al. 1990), the efficiency of poly-  
405 P extraction has not been addressed in previous studies, and it remains unknown whether all poly-P  
406 was extracted during these procedures. From our results, it appears that the combination of EDTA  
407 and NaOH in the main extract retards poly-P extraction from sludge, rather than promoting poly-P  
408 hydrolysis. However, our experimental setup does not allow a conclusive explanation of these  
409 findings.

#### 410 *4.3 The effect of pre-extraction of activated sludge*

411 Pre-extraction with EDTA has been suggested to increase the amount of poly-P detected in NMR  
412 extracts by removal of divalent cations from the sludge or sediment (Hupfer and Gachter 1995).

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

#### 426 *4.4 Degradation of poly-P during post-extraction sample concentration*

427 Poly-P middle group contents were significantly lower when lyophilisation was used for  
428 concentration of the NMR extract in the EN and E→EN protocols, which implies that rotary  
429 evaporation is preferable for these protocols. Whereas the low poly-P content in the EN<sub>Rot</sub> and  
430 E→EN<sub>Rot</sub> extracts can be attributed to insufficient poly-P extraction from the activated sludge, the  
431 very low poly-P extraction efficiencies of EN<sub>Ly</sub> and E→EN<sub>Ly</sub> cannot be explained by insufficient  
432 poly-P extraction alone. Hence, degradation of the poly-P to orthophosphate during the  
433 lyophilisation or dissolution steps seems very likely for these two protocols, as indicated by an  
434 increase in the relative orthophosphate content in the NMR extracts during the lyophilisation  
435 procedure. However, poly-P does not always degrade during lyophilisation/dissolution, as seen by  
436 the high poly-P recovery of 82(3)% of the E→N<sub>Ly</sub> protocol, where the poly-P content determined

437 by solution NMR is not significantly different between the  $E \rightarrow N_{Ly0}$  protocol and the  $E \rightarrow N_{Rot}$   
438 protocol, which indicates that poly-P is conserved during the lyophilisation and dissolution of the  
439  $E \rightarrow N_{Ly0}$  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  $E \rightarrow N_{Ly0}$  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  $EN_{Ly0}$  and  $E \rightarrow EN_{Ly0}$  samples. The  $E \rightarrow N$  extract  
447 contained four times less Mg, and only half as much Mn as the EN and  $E \rightarrow EN$  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  $EN_{Ly0}$  and  $E \rightarrow EN_{Ly0}$  and not for  
451  $EN_{Rot}$  and  $E \rightarrow EN_{Rot}$ , 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

460 seems to result in degradation of poly-P, as seen from a higher concentration of PP1 compared to  
461 PP2-PP4 in the study by (Ahlgren et al. 2007).

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

#### 466 4.5 Perspectives

467 The recommended extraction protocol for  $^{31}P$  NMR analyses of activated sludge allowed direct  
468 identification and absolute quantification of poly-P in the activated sludge. In contrast to lab-scale  
469 phosphate release/uptake studies, this bulk quantification of poly-P can be used as a direct measure  
470 of the amounts of poly-P associated with the bacteria in the activated sludge under *in situ*  
471 conditions. Our quantification method can thereby serve as a direct indicator of the phosphate  
472 removal efficiency of the PAO community present in the activated sludge. Improved efficiency of  
473 the EBPR treatment of the waste water can potentially reduce the application of Al and Fe in the  
474 WWTP needed to reduce the effluent P concentration below the limits set by the authorities, and  
475 may also increase P recovery in P synthesizing units as struvite recovery units (de-Bashan and  
476 Bashan 2004, Marti et al. 2010). In this study, the poly-P in activated sludge constituted ca. 13  
477 mgP/gDW (1.3 wt% of dry sludge), with a TP of the sludge of 32.5 mgP/gDW. Our poly-P  
478 measurements are in the same range as the  $8.8 \pm 1.4$  to  $14.0 \pm 0.6$  mgP/gDW found in phosphate  
479 release studies on EBPR sludge from a range of Danish WWTPs (Mielczarek et al. 2013). It is  
480 possible that the poly-P content can become even higher as EBPR sludge may contain up to 50-70  
481 mgP/gDW while non-EBPR sludge only contains 10-20 mgP/gDW (Yuan et al. 2012). In addition,  
482 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  $^{31}\text{P}$  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  $^{31}\text{P}$  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 $\rightarrow$ N). A single-step EDTA-NaOH extraction protocol (EN)  
492 or a two-step EDTA and EDTA-NaOH (E $\rightarrow$ EN) extraction protocol both resulted in  
493 incomplete extraction of poly-P from activated sludge, as observed by  $^{31}\text{P}$  solid state NMR  
494 on the residual sludge.
- 495 • The poly-P quantified by  $^{31}\text{P}$  solution NMR constituted up to  $86 \pm 9\%$  of the poly-P middle  
496 groups quantified by  $^{31}\text{P}$  SSNMR, when a two-step E $\rightarrow$ N extraction was used followed by  
497 concentration by rotary evaporation.
- 498 • Statistically equal poly-P extraction efficiencies for the two-step E $\rightarrow$ N protocol result from  
499 sample concentration by rotary evaporation or lyophilisation of neutralized extracts prior to  
500  $^{31}\text{P}$  solution NMR analysis. However, lyophilisation and dissolution of EN and E $\rightarrow$ EN  
501 extracts resulted in poly-P degradation.
- 502 •  $^{31}\text{P}$  SSNMR is a useful supplement to  $^{31}\text{P}$  solution NMR, as it probes the direct speciation of  
503 P. However, the better resolution and lower recording time makes  $^{31}\text{P}$  solution NMR better  
504 suited for quantification and characterisation of poly-P in activated sludge systems.

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647

648 **Figure 1:** An overview of the samples. There are six different combinations of extraction protocols  
649 and post-extraction sample concentration (blue) and seven samples for SSNMR analysis (brown).  
650 Samples marked with light blue or dark brown were studied by  $^{31}\text{P}$  solution NMR and  $^{31}\text{P}$  SSNMR,  
651 respectively. Lyo = lyophilisation.

652 **Figure 2:**  $^{31}\text{P}$  MAS SSNMR spectra of sludge and sludge residues after extraction. a) Lyophilised  
653 activated sludge. Residues of activated sludge extracted with b) 0.05 M EDTA, c) first 0.05 M  
654 EDTA followed by 0.25 M NaOH, d) EDTA-NaOH, and e) first 0.05 M EDTA followed by  
655 extraction with a mixed solution with 0.05 M EDTA and 0.25 M NaOH. Spectra were recorded at  
656 11.5 T with spinning speed 15 kHz. Asterisks denote spinning side bands.

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

661 **Figure 4:**  $^{31}\text{P}$  solution NMR spectra. a) Structure of poly-P with indication of poly-P groups that  
662 can be distinguished by  $^{31}\text{P}$  solution NMR, and  $^{31}\text{P}$  solution NMR spectra of b)  $\text{E} \rightarrow \text{N}_{\text{Rot}}$  and c)  
663  $\text{E} \rightarrow \text{N}_{\text{Lyo}}$ . Insets show an expansion of the chemical shift region for PP1 and pyro-P.

664

665 **Table 1:**  $^{31}\text{P}$  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	$P_{\text{obs}}$ <sup>a</sup> (%)	$I_{\text{poly-P}}$ <sup>b</sup> (%)	Poly-P middle groups, not corrected <sup>c</sup> (mgP/gDW)	Poly-P middle groups, corrected <sup>d</sup> (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 <sup>a</sup> $P_{\text{obs}}$  is the percentage of the sample P that is observed in the  $^{31}\text{P}$  SSNMR spectrum.

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

674 <sup>c</sup> Poly-P middle group content of the sludge, not corrected for  $P_{\text{obs}}$ .

675 <sup>d</sup> Poly-P middle group content of the sludge, corrected for  $P_{\text{obs}}$ .

676

677

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

<b>Treatment</b>	<b>TP</b>	<b>Fe</b>	<b>Al</b>	<b>Mg</b>	<b>Ca</b>	<b>Cu</b>	<b>Zn</b>
<b>None</b>	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)
<b>EDTA</b>	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)
<b>EN</b>	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)
<b>E→N</b>	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)
<b>E→EN</b>	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.

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

686 <sup>a</sup>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.

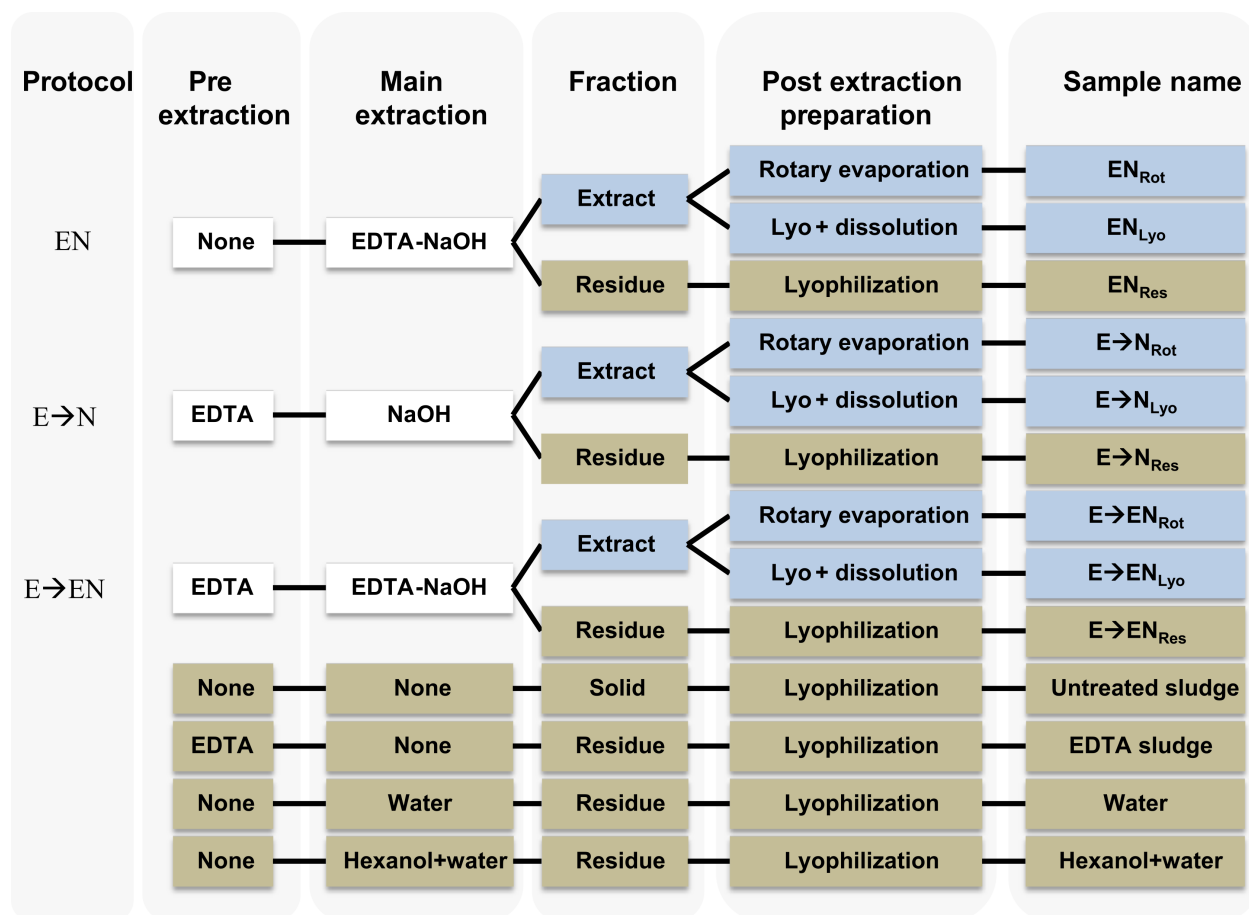
687 <sup>b</sup> Estimated uncertainties are given in brackets.

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

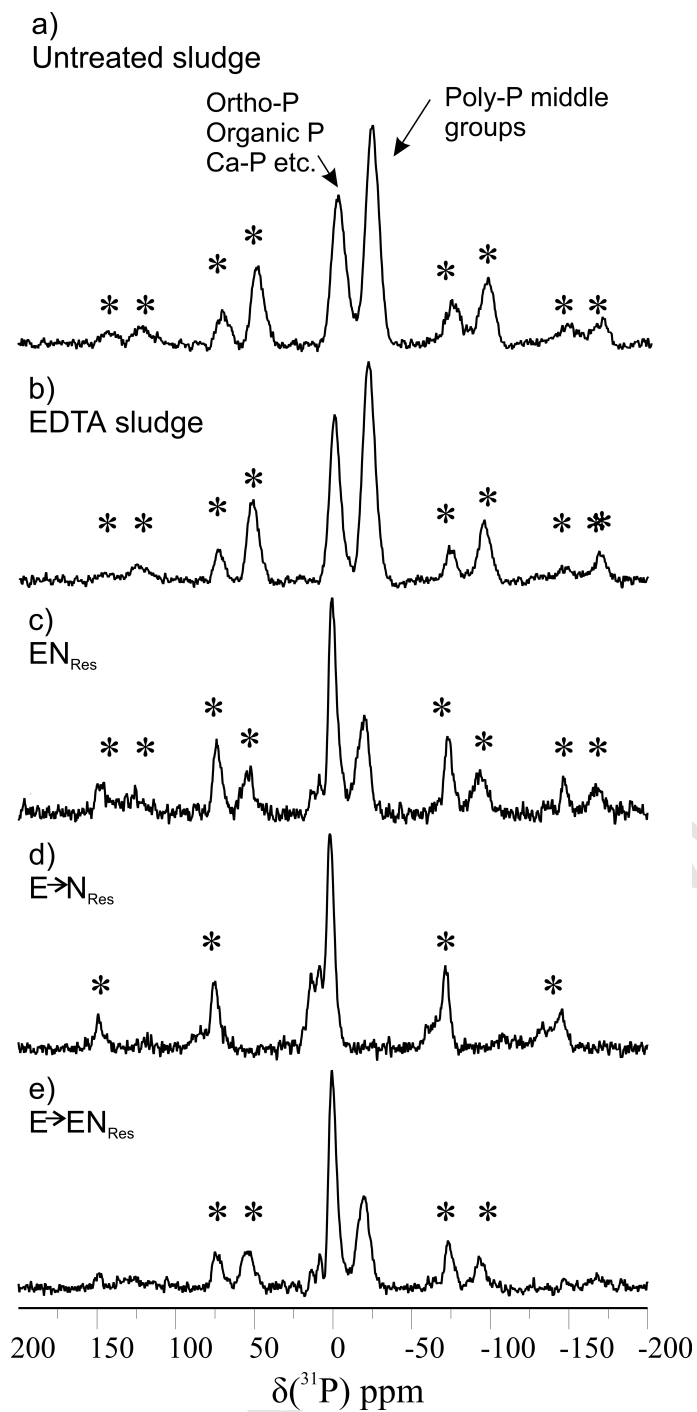
689 Standard deviations ( $n = 3$ ) given in brackets.

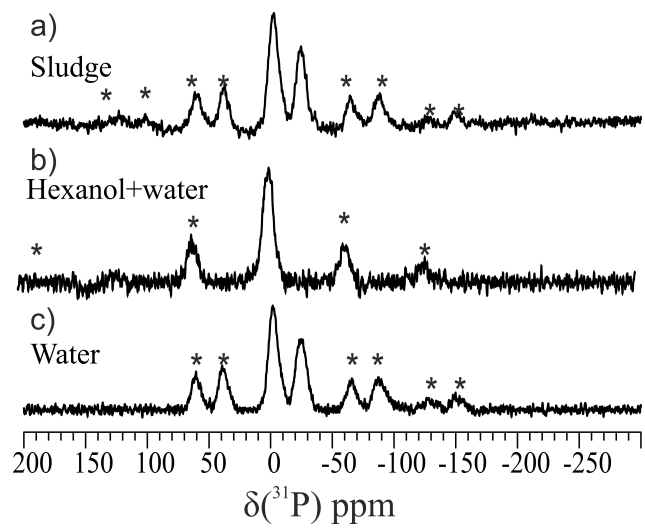
	<b>Fe</b>	<b>Al</b>	<b>Ca</b>	<b>Mg</b>	<b>Mn</b>	<b>Cu</b>	<b>Zn</b>
EN	1.18(0.08)	1.04(0.02)	23.2(0.04)	4.09(0.06)	0.18(0.01)	0.17(0.01)	0.52(0.01)
E→N	0.78(0.07)	0.56(0.02)	2.8(0.8)	0.92(0.05)	0.06(0.01)	0.17(0.01)	0.22(0.02)
E→EN	0.69(0.03)	0.57(0.01)	1.85(0.02)	3.37(0.03)	0.12(0.01)	0.16(0.01)	0.20(0.02)

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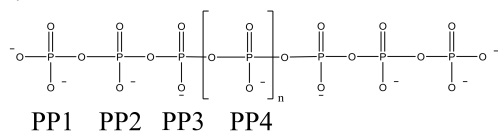


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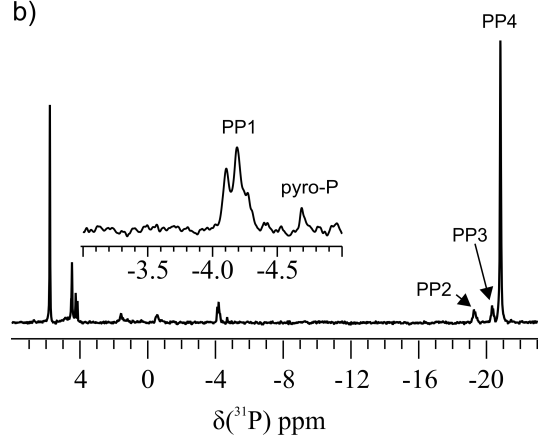




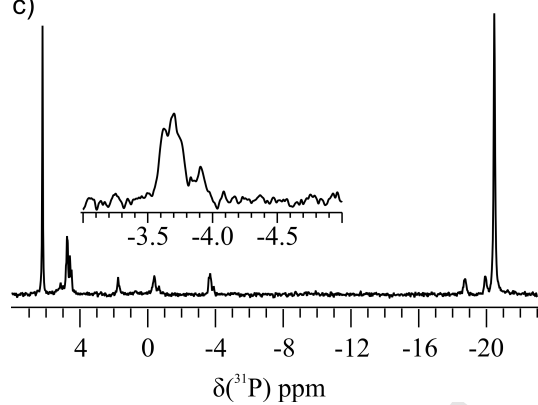
a)



b)



c)



**Highlights:**

- $^{31}\text{P}$  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.