

1 Speciation and distribution of P associated with Fe and Al oxides in aggregate-sized fraction
2 of an arable soil

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13 NMR

14

15 **Abstract**

16 To maximize crop productivity fertilizer P is generally applied to arable soils, a significant proportion of
17 which becomes stabilized by mineral components and in part subsequently becomes unavailable to plants.
18 However, little is known about the relative contributions of the different organic and inorganic P bound to
19 Fe/Al oxides in the smaller soil particles. The alkaline (NaOH-Na₂EDTA) extraction with solution ³¹P-
20 nuclear magnetic resonance (³¹P-NMR) spectroscopy is considered as a reliable method for extracting and
21 quantifying organic P and (some) inorganic P. However, any so-called residual P after the alkaline
22 extraction has remained unidentified. Therefore, in the present study, the amorphous (a) and crystalline (c)
23 Fe/Al oxide minerals and related P in soil aggregate-sized fractions (>20, 2-20, 0.45-2 and <0.45 μm) were
24 specifically extracted by oxalate (a-Fe/Al oxides) and dithionite (DCB, both a- and c-Fe/Al oxides). These
25 soil aggregate-sized fractions with and without the oxalate and DCB pre-treatments were then sequentially
26 extracted by alkaline extraction prior to solution ³¹P-NMR spectroscopy. This was done to quantify the P
27 associated with a- and c-Fe/Al oxides both in alkaline extraction and in the residual P of different soil
28 aggregate-sized fractions.

29 The results showed that overall P contents increased with decreasing size of the soil aggregate-sized
30 fractions. However, the relative distribution and speciation of varying P forms were found to be
31 independent of soil aggregate-size. The majority of alkaline extractable P was in the a-Fe/Al oxide fraction
32 (42-47% of total P), most of which was orthophosphate (36-41% of total P). Furthermore, still significant
33 amounts of particularly monoester P were bound to the oxides. Intriguingly, however, Fe/Al oxides were
34 not the main bonding sites for pyrophosphate. Residual P contained similar amounts of total P associated
35 with both a- (11-15% of total P) and c-Fe oxides (7-13% of total P) in various aggregate-sized fractions,
36 suggesting that it was likely occluded within the a- and c-Fe oxides in soil. This implies that with the
37 dissolution of Fe oxides, these P may be released and thus available for plants and microbial communities.

38 **1 Introduction**

39 Phosphorus is after nitrogen the most limiting soil nutrient for agricultural crops and plays an important
40 role in the overall functioning of the agro-ecosystems (Bowman and Moir, 1993; Wei et al., 2014). To
41 increase or maintain crop productivity, P in the form of synthetic and animal based fertilizer is applied to

42 arable soils. However, the long term (over-)application of P in excess of plant crop requirements has
43 increased P in surface and subsurface soils, generally over time transforming it into various unavailable
44 forms (Arai et al., 2005; He, 2009; Sims et al., 2000).

45 Soil organic and inorganic P are in the majority of cases stabilized by association with mineral components.
46 Negatively charged phosphorus compounds attach themselves to mineral components such as
47 aluminosilicates minerals and hydrous Fe or Al oxides (Solomon and Lehmann, 2000; Turner et al., 2005).
48 The P reactivity in Fe/Al oxyhydroxides turns large proportions of soil P into unavailable forms (Arai and
49 Livi, 2013; Solomon and Lehmann, 2000; Solomon et al., 2002). Different Fe/Al oxides however have
50 varying P adsorption capacities and some studies showed amorphous Fe/Al oxides carried the majority of P
51 in soils, despite higher abundance of crystalline Fe oxides (Arai and Livi, 2013; Arai et al., 2005; Rick and
52 Arai, 2011). Few studies have distinguished organic and inorganic P associated with different Fe/Al oxides
53 (i.e. amorphous and crystalline Fe/Al oxides) (Mahieu et al., 2000), despite the fact that P speciation will
54 ultimately determine the different P bioavailability, mobility and mineralization in soil (Dick and Tabatabai,
55 1978; Liu et al., 2014). The study by Mahieu et al. (2000) found that amorphous Fe oxides had strong
56 correlations with some organic P forms in the humic acid and calcium humate extracts of lowland rice soils.
57 Long-terms soil management and development (e.g., cultivation, fertilized management, and forest growth
58 and development) affect soil chemistry, such as the transformation of Fe oxides (Li, et al., 2008). Inevitably,
59 the ecosystem-driven transformation of Fe oxides alters the related P retention and bioavailability in soil.
60 Therefore, the study of various P/Fe-oxides relationship is helpful for understanding soil P dynamics in
61 long time scales. However, little is known about speciation and distribution of P associated with varying
62 Fe/Al oxides. Additionally, finer-sized soil fractions were found to be enriched in highly active and easily
63 mineralized organic P than larger particles (Rubæk et al., 1999). Similarly, organic P forms in general
64 increased with decreasing particle size in forest and cropland soils (Cade-Menun, 2005). It seems that
65 smaller soil particles are more reactive and have higher P retaining capacity due to increased surface area
66 than larger soil particles. In this context, it is important that different soil aggregate-sized fractions are also
67 considered when studying P associated with Fe/Al oxides in soil.

68 Alkaline extraction and solution ^{31}P -NMR spectroscopy are widely thought as reliable methods for
69 quantifying organic phosphorus (Turner, 2008). Inorganic phosphate (Pi) in alkaline soil extracts is
70 assumed to originate from complexes with both Fe and Al (Turner, 2008). However, the distribution of

71 organic P (Po) related to Fe/Al oxides in the alkaline extracts is still unclear. Our study aims to provide a
72 novel method to understand the distribution of Pi and Po on Fe/Al oxides in the alkaline extract of soil.
73 Furthermore, the residual P after alkaline extraction still accounts for significant proportion of P for some
74 soils, most apparent in high-pH soil or for unamended soils with low P concentration (Cade-Menun and Liu,
75 2014; Cheesman et al., 2012; McDowell et al., 2007; Turner et al., 2007; Turner et al., 2003). It is by
76 definition unidentified and is simply presumed to be recalcitrant in the environment (Cheesman et al.,
77 2010). Richter et al. (2006) indicated that recalcitrant P as slowly cycling P did still contribute to P
78 bioavailability on decadal time scales. Therefore, an improved understanding of the residual P
79 distribution, its bonding and “true” recalcitrance in soil is important for developing strategies to exploit this
80 P as an “in part-additional” source for plant uptake or microbial usage. This is probably feasible in line with
81 the observation by He et al. (2008), which suggested that most of this residual P could be orthophosphate.
82 Our current study did aim i) to provide a comprehensive insight on the distribution of P bound to
83 amorphous and crystalline Fe and Al (hydr)oxides in different soil aggregate-sized fractions, ii) to identify
84 the speciation and distribution of Pi and Po associated with amorphous and crystalline Fe/Al (hydr)oxides
85 in NaOH-Na₂EDTA soil extracts and iii) to provide detailed information on residual P distribution after
86 alkaline extraction. It is in order to provide further information on the partition and quantification of these
87 (unavailable) P forms especially for the P/Fe-oxide fraction of the arable soil, which may help to improve
88 current soil P sustainable development management strategies. In this study, we extracted amorphous and
89 crystalline Fe/Al oxides from a bulk soil and different soil aggregate-sized fractions of an arable field.
90 Additionally, the isolated amorphous Fe oxides-free and all Fe oxides-free aggregate-sized fractions were
91 then treated by alkaline extraction. Simultaneously, the original bulk soil and various aggregate-sized
92 fractions were also treated with alkaline extraction and studied by solution ³¹P-NMR spectroscopy.

93 **2 Materials and Methods**

94 **2.1 Site description and soil fractionation**

95 Composite topsoil sample (0-15 cm, pH: 7.64) was collected from a cultivated field in the German Tereno
96 experimental platform site Selhausen (50°52'08"N; 6°26'59"E; arable soil, luvisol) in March 2013. The
97 field was cultivated with winter wheat in October 2011-2012 and winter barley in September 2013. No P
98 fertilization was applied in years 2011-2013. More details about soil physicochemical characteristics and N
99 fertilization of the test field were provided by Supplementary Information in Table S1 and Kupisch et al.

100 (2015). The soil sample was fractionated using the soil particle-size fractionation method (Séguaris and
101 Lewandowski, 2003) which explicitly avoids the destruction of small aggregates in soil (Berns et al., 2008).
102 Concisely, 100 g soil sample was suspended in 200 mL ultrapure water (Milli-Q) and horizontally shaken
103 at 150 rpm for 6 h. After that, 600 mL ultrapure water was added and mixed allowed to settle. Soil phases
104 containing varying soil-aggregated sizes were collected by the pipette method based on Stoke's law.
105 The soil fraction $>20\ \mu\text{m}$ (sand-sized aggregates, with mass ratio of 82.3% of total soil) was obtained
106 by removing supernatant after settling 6 min. The supernatant was sequently settled for 12 h and then
107 remove the new supernatant to obtain soil fraction from 2 to $20\ \mu\text{m}$ (silt-sized aggregates, with mass
108 ratio of 12.9% of total soil). The obtained new supernatant was subsequently separated into particle
109 size of $0.45\text{-}2\ \mu\text{m}$ (with mass ratio of 4.0% of total soil) by spinning the samples at $2525\ g$ for 4 min
110 (Biofuge, Heraeus), according to Stoke's law calculations with an assumed particle density of $2.65\ \text{g}\ \text{cm}^{-3}$.
111 Lastly, $<0.45\ \mu\text{m}$ fraction (with mass ratio of 0.57% of total soil) was obtained by spinning the
112 supernatant at $2525\ g$ for 68 min (Biofuge, Heraeus). The final supernatant only contained the electrolyte
113 phase as well as a small amount of nanoparticles which has been investigated in our other concurrent work
114 (Jiang et al., 2015). The soil aggregate-sized fractions were then freeze-dried for the elements analysis (i.e.
115 Fe, Al, P and Ca) by inductively coupled plasma mass spectrometer (ICP-MS).

116 **2.2 Oxalate and dithionite-citrate-bicarbonate (DCB) treatments**

117 Acid ammonium oxalate was used to dissolve amorphous, poorly crystalline and organo Fe and Al
118 (hydr)oxides (Kleber et al., 2005; Masiello et al., 2004; Neubauer et al., 2013). The bulk soil and different
119 aggregate-sized fractions were treated with acid ammonium oxalate according to the extraction method of
120 Kiem and Kögel-Knabner (2002) and Schwertmann (1964). Briefly, 200 mg soil sample was dispersed in
121 40 mL of 0.2 M acidic oxalate solution (0.113 M ammonium oxalate and 0.087 M oxalic acid) and shaken
122 for 2 hours in the dark, followed by centrifugation. The supernatants were subsequently analyzed for Fe
123 (Fe_{ox}), Al (Al_{ox}), P (P_{ox}) and Ca (Ca_{ox}) by ICP-MS.

124 The DCB mainly extracts organically-bound, amorphous and crystalline Fe oxides and only parts of
125 dissolve oxides for Al and Si. For DCB extraction the protocol of Kiem and Kögel-Knabner (2002) was
126 used. Briefly, 200 mg sample was extracted using 30 mL 0.3 M sodium citrate solution, 10 mL 1 M sodium
127 hydrogen carbonate solution and 1 g sodium dithionite. After shaking for 16 h at room temperature, the soil

128 suspension was centrifuged. The residue was then dispersed in 32 mL magnesium sulfate (0.05 M) to
 129 exchange the adsorbed Fe with magnesium, and centrifuged a second time. The two supernatant phases
 130 were combined and ICP-MS was used to measure Fe (Fe_{DCB}), Al (Al_{DCB}), P (P_{DCB}) and Ca (Ca_{DCB}).

131 2.3 Solution ^{31}P -NMR spectroscopy

132 To highlight the chemical composition of inorganic and organic P species in the bulk soil and different
 133 aggregate-sized fractions (>20 μm , 2-20 μm , 0.45-2 μm and <0.45 μm), these aggregate-sized fractions
 134 without and with oxalate and DCB pre-treatments were shaken 16 h with a mixture of 0.25 M NaOH and
 135 50 mM Na_2EDTA (soil and solution mass ratio is 1:20-25, $pH \geq 13$) (Cade-Menun and Preston, 1996) and
 136 centrifuged at 2525 g for 68 min (Biofuge, Heraeus). The P (P_{NaOH}), Fe (Fe_{NaOH}), Al (Al_{NaOH}) and Ca
 137 (Ca_{NaOH}) were analyzed by ICP-MS for the supernatants. These supernatants were then frozen and
 138 subsequently lyophilized. Na_2S dissolved in D_2O was added to the lyophilized materials to maintain
 139 reducing conditions for the removal of paramagnetic ions (i.e. Fe and Mn) during precipitation (Vestergren
 140 et al., 2012). The solution was centrifuged at 13400 g for 20 min (Centrifuge 5415R, Eppendorf). Solution
 141 ^{31}P -NMR spectras were obtained using a Bruker Avance 600-MHz spectrometer equipped with a prodigy-
 142 probe, operating at 242.95 MHz for ^{31}P (Izarova et al., 2014). Extracts were measured with a D_2O -field
 143 lock at room temperature. Chemical shifts were referenced to 85% orthophosphoric acid (0 ppm). The
 144 NMR parameters generally used were: 32 K data points, 0.5 s repetition delay, 30° pulse width, 24000
 145 scans and 0.7 s acquisition time for the Bruker 600 Avance spectrometers.

146 2.4 Calculations

147 The schematic diagram of whole soil sample preparation is shown in Fig. 1. The P contents in the various
 148 extracted fractions were calculated as follows:

$$149 \quad (P_{a-Fe})_N = P_N - P_{N-I} \quad (1)$$

$$150 \quad (P_{c-Fe})_N = P_{N-I} - P_{N-II} \quad (2)$$

$$151 \quad (P_{other})_N = P_{N-II} \quad (3)$$

152 Where, $(P_{a-Fe})_N$ = NaOH- Na_2EDTA extractable P associated with amorphous Fe/Al oxides;

153 $(P_{c-Fe})_N$ = NaOH- Na_2EDTA extractable P associated with crystalline Fe oxides;

154 $(P_{other})_N$ = NaOH- Na_2EDTA extractable P associated with other matters;

155 P_N = NaOH- Na_2EDTA extractable P in soil samples;

156 P_{N-I} = NaOH- Na_2EDTA extractable P in “amorphous FeO-free” soil samples;

157 P_{N-II} = NaOH-Na₂EDTA extractable P in “FeO-free” soil samples.

158 Relative distribution of Pi and Po in NaOH-Na₂EDTA extracts of soil samples, a-Fe/Al oxides-free and
159 Fe/Al oxides-free soil samples were characterized by solution ³¹P-NMR spectroscopy (Fig. 2 and
160 Supplementary Fig. S1). Here only orthophosphate, phosphate monoesters and pyrophosphate were scaled
161 to 100%. Therefore, the distribution of Pi and Po associated with a- and c-Fe/Al oxides in NaOH-
162 Na₂EDTA extracts (Table 1) was calculated with the combination of NaOH-Na₂EDTA extraction (Table S2)
163 and corresponding solution ³¹P-NMR spectras (Fig. 2 and Fig. S1).

164 The residual P after alkaline extraction was separated into P bound to a-Fe oxides, c-Fe oxides and other
165 matters as follows:

$$166 \quad (P_{a-Fe})_{\text{resi}} = P_{a-Fe} - (P_{a-Fe})_N \quad (4)$$

$$167 \quad (P_{c-Fe})_{\text{resi}} = “P_{c-Fe}” - (P_{c-Fe})_N = (P_{Fe} - P_{a-Fe}) - (P_{c-Fe})_N \quad (5)$$

$$168 \quad (P_{\text{other}})_{\text{resi}} = P_T - P_{Fe} - P_{N-II} \quad (6)$$

169 Where, $(P_{a-Fe})_{\text{resi}}$ = residual P associated with amorphous Fe oxides;

170 $(P_{c-Fe})_{\text{resi}}$ = residual P associated with crystalline Fe oxides;

171 $(P_{\text{other}})_{\text{resi}}$ = residual P associated with other matters;

172 P_{a-Fe} = P associated with amorphous Fe/Al oxides (i.e. oxalate extractable P);

173 P_{Fe} = P associated with amorphous and crystalline Fe/Al oxides (i.e. DCB extractable P);

174 P_{c-Fe} = P associated with crystalline Fe/Al oxides;

175 P_T = total P.

176 3 Results and discussion

177 3.1 Total, oxalate and DCB extractable P, Fe, Al and Ca in soil aggregate-sized fractions

178 Table 2 represents ICP-MS results of elemental contents (i.e. P, Fe, Al and Ca) in the bulk soil and different
179 aggregate-sized fractions, and their oxalate and DCB extracts. Total P was 0.73 g kg⁻¹ in bulk soil with a
180 significant increase from large particles to small particles (range between 0.54 and 2.47 g kg⁻¹). Total Fe
181 and Al in different soil aggregate-sized fractions showed the same gradient with total P. Total P had a
182 strong positive relationship with total Fe and Al ($R^2 > 0.99$; $P < 0.006$) in these aggregate-sized fractions,
183 which indicated that P was, to a large extent, associated with Fe and Al in all aggregate-sized fractions. The
184 high correlation also indicated the cause why smaller particles contained more P. There was more Fe and

185 Al in the smaller size fractions, and the minerals containing these elements (i.e. Fe and Al) acted as
186 important P carriers.

187 Oxalate extractable P, Fe and Al (P_{ox} , Fe_{ox} and Al_{ox}) also had an increasing trend with smaller particle sizes.

188 Oxalate extractable P corresponded to 53-62% of the total P in varying soil aggregate-sized fractions,

189 although the amounts of amorphous Fe and Al oxides fractions were low with respect to total Fe (15-17%)

190 and total Al (2-4%) (Table 2). DCB extractable P (P_{DCB}) was 70-75% of the total P in these aggregate-sized

191 fractions, which implied that Fe/Al oxides were the major carriers of P in the soil (Table 2). The differences

192 between DCB extractable Fe and Al oxides and oxalate extractable Fe and Al oxides represent the amounts

193 of extracted crystalline Fe (c-Fe) and Al (c-Al) oxides. There was a large amount of c-Fe ($6.31-28.4 \text{ g kg}^{-1}$,

194 31-37% of total Fe). In contrast, c-Al content was low (only $0-0.34 \text{ g kg}^{-1}$, 0-0.6% of total Al) in the various

195 aggregate-sized fractions (Table 2). Similarly, high amount of c-Fe and low amount of c-Al were also

196 found in sandy soils (Borggaard et al., 1990). This is probably because organic matter in soil inhibits Al

197 oxides crystallization (Borggaard et al., 1990). Currently there is some ongoing discussion in the literatures

198 (Acebal et al., 2000; Borggaard et al., 1990; Ryan and Gschwend, 1991) with respect to the extent in which

199 c-Al is extracted by DCB and what reasons for such an incomplete extraction of this Al are. Only 12-20%

200 of total P (c-P, the difference between P_{DCB} and P_{ox} , Table 2) was released with crystalline Fe and Al

201 oxides (Table 2). It shows that amorphous Fe/Al oxides are more important for P bonding compared to

202 crystalline Fe/Al oxides. Several researchers did already report that P was dominantly associated with

203 amorphous Fe and Al oxides fractions in various soil types in USA (Arai et al., 2005; Rick and Arai, 2011).

204 It was presumed that specific anion adsorption (e.g. P) suppressed the transformation from amorphous to

205 crystalline Fe oxides (Arai et al., 2005; Biber et al., 1994), which could explain the low amount of P

206 associated with crystalline Fe oxides.

207 Oxalate extracted P also had a strong positive linear correlation with oxalate extracted Fe ($R^2=0.997$) and

208 Al ($R^2=0.976$). The similar high correlation between oxalate extractable P and Fe oxides has already been

209 shown in acidic and neutral soils (Forsmann and Kjaergaard, 2014). Nevertheless, c-Fe and c-Al contents

210 were not strongly correlated with c-P. Although there was a smaller amount of Ca in different aggregate-

211 sized fractions compared to Fe and Al (Table 2), DCB also dissolved between 48 and 100% of total Ca in

212 varying aggregate-sized fractions (Supplementary Table S3). Therefore, it could be assumed that a small

213 amount of the measured c-P (Table 2) was related to Ca minerals (Ca-P precipitates) in the soil.

214 Although there were higher amounts of total Fe, Al and P in smaller particles compared with larger
215 particles, there were no clear discernible proportional differences in oxalate and DCB extractable Fe, Al
216 and P fractions with respect to total Fe, Al and P between various aggregate-sized fractions. Therefore,
217 there was a clear indication that the relative proportional distributions of amorphous, crystalline Fe/Al
218 oxides and related P in this soil were independent on the aggregate-size of soil particles a result not found
219 previously. Kiem and Kögel-Knabner (2002) did suggest that there was a similar Fe_{ox}/Fe_{DCB} ratio across
220 three size separates of arable soil fractions $<6 \mu m$. Fe/Al (hydr)oxides and organic matter bind clay
221 particles together into soil aggregates (Tisdall and Oades, 1982). Therefore, these Fe/Al oxides as inorganic
222 binding agents and associated P were distributed on soil aggregates with all varying sizes proportionally.

223 **3.2 Speciation and distribution of P forms in soil aggregate-sized fractions**

224 There was 58-65% of total P extracted by NaOH- Na_2EDTA in different soil size fractions (Table S2). A
225 similar extraction efficiency (average of 63% of total P) was found in a recent study of dried wetland soil
226 samples (Cheesman et al., 2012). Inorganic and organic P contents in NaOH- Na_2EDTA extracts were
227 characterized by solution ^{31}P -NMR spectroscopy. Inorganic (orthophosphate and pyrophosphate) and
228 organic (phosphate monoesters, phosphate diesters and phosphonates) P forms were present in our soil
229 aggregate-sized fractions.

230 The orthophosphate and phosphate monoesters were major detected inorganic and organic P forms in all
231 soil aggregate-sized fractions (Fig. 3). NaOH- Na_2EDTA extractable orthophosphate (^{31}P -NMR chemical
232 shift of 5-7 ppm) (Cade-Menun, 2005) ranged from 0.25 to 1.27 $g\ kg^{-1}$, accounting for the majority of P
233 (47-51% of total P) in all the aggregate-sized fractions (Fig. 3). Phosphate monoesters (3-6 ppm) (Bol et al.,
234 2006; Cade-Menun, 2005; Fuentes et al., 2012) were always the dominant organic P with the contents of
235 53-332 $mg\ kg^{-1}$ (10-14% of total P) (Fig. 3). Phosphate monoesters are generally the dominant group of
236 organic phosphorus compounds in most soils: they occur mainly as inositol phosphates, a family of
237 phosphoric esters of hexahydroxy cyclohexane (inositol) (Turner et al., 2005). The content of
238 pyrophosphate (-4 to -5 ppm) (Cade-Menun, 2005) was only up to 15 $mg\ kg^{-1}$ (less than 1% of total P).
239 Phosphonates (12 ppm) and phosphate diesters (2.5 to -1 ppm) (Cade-Menun, 2005) did only contribute
240 0.6-1.0% of total P (Fig. 3). We found that there were only little if any P-diesters in fine particles (Fig. 3).
241 Many microbial products such as teichoic acid P or DNA-P would exhibit these structures (Makarov et al.,
242 2002).

243 All the identified P forms were present in higher concentrations in $< 2 \mu\text{m}$ soil fraction when compared to
244 larger-sized particles. Small-sized soil fractions, such as soil colloids isolated in this study, are well-
245 established as being efficient carriers of nutrients (especially phosphorus) (Haygarth et al., 2006).
246 Therefore, soil managements, which control soil colloids loss from productive land (e.g. tillage and
247 wind/water erosion) are crucial to restrain any unwanted strong declines in soil phosphorus levels and thus
248 help to retain the potential bioavailability of phosphorus over longer time periods. However, the
249 distributions of different P forms in our study did seem to be independent of soil aggregate-size (Fig. 3). In
250 contrast, some studies have found that soil size fractions drive the differential P distributions in forest and
251 arable soils (Liu et al., 2014; Solomon et al., 2002). Our novel finding about the similar P distribution in
252 various aggregate-sized fractions of this soil was probably due to the comparable Fe and Al oxides
253 distribution in aggregate-sized fractions when considering that Fe and Al oxides were domain P carriers in
254 the soil.

255 **3.3 NaOH-Na₂EDTA extractable P associated with Fe and Al oxides in soil aggregate-sized fractions**

256 The bulk soil and aggregate-sized fractions after oxalate and DCB pre-treatments were also extracted by
257 NaOH-Na₂EDTA for ³¹P-NMR analysis. We found that the bulk soil (Fig. 2) and other soil aggregate-sized
258 fractions (Fig. S1) after oxalate pre-treatment revealed a higher phosphate monoesters/orthophosphate ratio
259 in NaOH-Na₂EDTA extracts compared to that without oxalate treatment. The bulk soil (Fig. 2) and other
260 soil aggregate-sized fractions (Fig. S1) after DCB pre-treatment had the highest phosphate
261 monoesters/orthophosphate ratio among the three types of samples. This finding implied that relatively
262 high amounts of orthophosphate were removed by oxalate and particularly DCB, i.e., it was mainly
263 orthophosphate that was bound to a- and c-Fe oxides in aggregate-sized fractions rather than organic P.
264 Besides, most pyrophosphate still remained in Fe/Al oxides-free aggregate-sized fractions (Fig. 2 and Fig.
265 S1). Pyrophosphate is perceived to be contained within live fungal tissue (Koukol et al., 2008) or sorbed to
266 the mineral soil matrix (Gunary, 1966). However, the present study suggested that Fe/Al oxides (part of
267 mineral matrix) were not the main sites for pyrophosphate adsorption.

268 Because orthophosphate and phosphate monoesters were the dominant P forms, only they were discussed
269 below (Table 1). The amount of P bound to Fe/Al oxides in NaOH-Na₂EDTA extracts accounted for 49-54%
270 of total P in various aggregate-sized fractions, including orthophosphate (41-47% of total P) and phosphate
271 monoesters (7-9% of total P) (Table 1). The amorphous oxides carried more orthophosphate and phosphate

272 monoesters (36-41% and 6-8% of total P, respectively) than did the crystalline oxides (3-6% and 1-2% of
273 total P, respectively) in the NaOH-Na₂EDTA extracts (Table 1). The P adsorption experiment from
274 Borggaard (1983) showed that a-Fe oxides absorbed more phosphate than c-Fe oxides in soils. Generally,
275 phosphate monoesters are commonly assumed to be stabilized in soils by association with amorphous metal
276 oxides, which have greater sorption affinity compared to crystalline metal oxides (Celi and Barberis, 2007;
277 Turner and Engelbrecht, 2011).

278 Noteworthy, only rare amounts of Fe (0-1% of total Fe) from “untreated” soil aggregate-sized fractions
279 were dissolved by NaOH-Na₂EDTA (Table S3). Although the presence of Na₂EDTA caused some
280 dissolved Fe ions precipitating in humic-iron compounds, the precipitation was negligible at hydroxide ion
281 concentrations ≥ 100 mM (\geq pH 13) (Turner, 2008). The NaOH-Na₂EDTA extracted P was not occluded in
282 the Fe containing minerals (e.g. Fe oxides) but probably associated to the surface of Fe containing minerals
283 and bond to other minerals. Therefore, a- and c-Fe oxides associated P present in NaOH-Na₂EDTA extracts
284 was probably attached to the surface of Fe oxides. Alkaline extraction could remove P held to Fe and Al
285 components of soil surface by chemisorption (Hedley et al., 1982; McLaughlin et al., 1977; Ryden et al.,
286 1977). It is noteworthy that there was unavoidable P released from P-goethite complexes after oxalate
287 treatment by ligand-exchange, the amount of released P was negligible at low initial P level (Johnson and
288 Loeppert, 2006) as such in our soil samples.

289 High contents of dissolved Al were still found in NaOH-Na₂EDTA extracts, and which were similar with
290 those in oxalate and DCB extracts of soil aggregate-sized fractions (Table 2 and Table S3). Similarly, high
291 Al and low Fe contents were found in NaOH-Na₂EDTA extracts from a tropical forest soil (Turner, 2008).
292 Considering that little Si was dissolved in NaOH-Na₂EDTA in all aggregate-sized fractions (data not
293 shown), it indicated that NaOH-Na₂EDTA extracted Al mainly stemmed from amorphous Al oxides (rare
294 c-Al oxides in the soil, Table 2) rather than aluminosilicate minerals in the soil. Therefore, the Al-P in
295 NaOH-Na₂EDTA extracts contained P probably both on surface of Al oxides and occluded in Al oxides.

296 There was still 8-11% of total P (including 4-6% orthophosphate and 4-5% phosphate monoesters) in
297 NaOH-Na₂EDTA extracts which were not associated with Fe/Al oxides of different aggregate-sized
298 fractions (Table 1). The NaOH-Na₂EDTA extraction included P contained within microbial cells (Turner
299 and Blackwell, 2013). Besides, inositol phosphate is stabilized mainly through a strong adsorption on the
300 surface of amorphous metal oxides and clay minerals (Celi and Barberis, 2007), so we assumed that a part

301 of these P compounds were adsorbed on the surfaces of clay minerals. Additionally, it was also believed
302 that there was P associated with Ca minerals in this NaOH-Na₂EDTA extracts considering that 43-98% of
303 total Ca was dissolved in NaOH-Na₂EDTA extracts of different aggregate-sized fractions (Table S3).

304 **3.4 Residual P in soil aggregate-sized fractions**

305 Using our specific separation method, the residual P was separated into P bound to a-Fe oxides, c-Fe oxides
306 and other matters (Equation (4)-(6) and Table 1). The P in residual fractions accounted to 35-42% of total P
307 in aggregate-sized fractions. We found that 11-15% of total P in different aggregate-size fractions was
308 bound to a-Fe oxides and 7-13% of total P was associated with c-Fe oxides (Table 1). The 20-24% of total
309 P associated with Fe oxides (Table 1) was equivalent to 53-66% of the P present in the residual fraction.
310 Therefore, the majority of P in residual fraction was associated with Fe oxides (Table 1). The remaining 12-
311 18% of total P (i.e. 34-47% of the P present in residual fractions) was bound to other minerals or organic
312 matter which was dissolved or released neither in DCB nor in NaOH-Na₂EDTA of different aggregate-
313 sized fractions (Table 1).

314 We believe our novel results suggested that the a- and c-Fe oxides associated P in the residual P fraction
315 were most likely occluded inside of a- and c-Fe oxides. This was in contrast to the a- and c-Fe oxides
316 associated P present in NaOH-Na₂EDTA extracts which were absorbed on the surface of Fe oxides. The P
317 associated with Fe oxides in residual fractions probably was held at the internal surfaces of Fe oxides by
318 chemisorption at protonated surface sites or by the replacement of surface hydroxyls in soil aggregates
319 (Hedley et al., 1982; Ryden et al., 1977), or diffused into the bulk of Fe oxides or into pores and defects
320 (McLaughlin et al., 1977). It means that with the changes in soil conditions (e.g. soil anoxic condition,
321 acidic condition, high concentrations of organic acid or siderophore) (Forsmann and Kjaergaard, 2014;
322 Kraemer, 2004) when Fe oxides become dissolved, then P associated with the Fe oxides may become
323 available to plants or the microbial population. The a-Fe/Al oxides did adsorb more orthophosphate and
324 monoesters than c-Fe/Al oxides (Table 1: NaOH-Na₂EDTA extractable P). However, there were similar
325 amounts of occluded P inside of both a- and c- Fe oxides (Table 1: Residual P).

326 The Ca-P complexes present in residual fractions of soil aggregate-sized fractions should contribute to a
327 proportion of the remaining residual P, ca. 12-18% of total P. Cheesman et.al (2010) suggested that there
328 were inorganic Ca-phosphates in the residual P from detritus and surface soils. In the present study, 12% of
329 total P was not associated with Fe oxides in residual P of <0.45 μ m soil fractions (Table 1), despite that

330 almost all the Ca was already extracted by NaOH-Na₂EDTA and DCB (Table S3). It seemed therefore that
331 the majority of the 12-18% of total P was bound to other materials. In our other concurrent work (Jiang et
332 al., 2015), we found that some P in the soil nanoparticles after DCB treatment was still bound to Fe bearing
333 phyllosilicate minerals (i.e. Fe structurally embedded into the crystalline structure of phyllosilicate minerals
334 via isomorphic substitution) (Regelink et al., 2014). It is possible that these Fe bearing phyllosilicate
335 minerals may also be responsible for the bonding of the remaining P in the NaOH-Na₂EDTA non-extracts.
336 Noteworthy, soil aggregate-sizes do not have a significant effect on the relative distribution of P forms
337 bond to a- and c-Fe/Al oxides for NaOH-Na₂EDTA extracts and residual soil P (Table 1).

338 **4 Conclusions**

339 We concluded for this arable German soil that: 1) With decreasing aggregate-sized fractions soil P content
340 increased, however the distribution and speciation of varying P forms were independent of aggregate-size.
341 2) The majority of NaOH-Na₂EDTA extractable P (Pi and Po) was probably attached to the surface of
342 amorphous Fe oxides and Al oxides, or alternatively inside of the Al oxides. 3) The majority of P in the
343 residual fraction was occluded within Fe oxides, which implies that this P may be released and become
344 available for plants and microbial communities following the dissolution of Fe oxides in soil.

345

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496 Table 1 NaOH-Na₂EDTA extractable P and residual P distribution (% , with respect to total P of each soil
 497 aggregate-sized fraction) associated with amorphous and crystalline oxides, and other matters as shown in
 498 Eqs.1-6.

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	NaOH-Na ₂ EDTA extractable P						Residual P		
	$(P_{a-Fe})_N$		$(P_{c-Fe})_N$		$(P_{other})_N$		$(P_{a-Fe})_{resi}$	$(P_{c-Fe})_{resi}$	$(P_{other})_{resi}$
	Ortho.	Mono.	Ortho.	Mono.	Ortho.	Mono.			
Bulk soil	39	6	5	2	5	4	12	9	18
Sand-sized >20 μm	36	6	6	1	4	4	11	13	18
Silt-sized 2-20 μm	39	7	3	1	6	5	13	7	18
Clay 0.45-2 μm	37	8	4	1	6	5	13	9	16
Clay <0.45 μm	41	6	6	1	6	5	15	8	12

500 Ortho. = orthophosphate; Mono. = P-monoesters.

501 $(P_{a-Fe})_N$ = NaOH-Na₂EDTA extractable P associated with amorphous Fe/Al oxides.

502 $(P_{c-Fe})_N$ = NaOH-Na₂EDTA extractable P associated with crystalline Fe oxides.

503 $(P_{other})_N$ = NaOH-Na₂EDTA extractable P associated with other matters.

504 $(P_{a-Fe})_{resi}$ = Residual P associated with amorphous Fe oxides.

505 $(P_{c-Fe})_{resi}$ = Residual P associated with crystalline Fe oxides.

506 $(P_{other})_{resi}$ = Residual P associated with other matters.

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524 Table 2 The amounts of P, Fe, Al and Ca in bulk soil and soil aggregate-sized fractions, and extracted by oxalate and dithionite (DCB) (g kg^{-1}). Fraction in bracket (%)

525 with respect to total P, Fe, and Al values of each aggregate-sized fraction shown in Table 2 are given in parentheses. Data are the mean values of three replicates with

526 standard deviations.

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Aggregate-sized fractions	P_T^\dagger	Fe_T^\dagger	Al_T^\dagger	Ca_T^\dagger	$\text{P}_{\text{ox}}^\ddagger$	$\text{Fe}_{\text{ox}}^\ddagger$	$\text{Al}_{\text{ox}}^\ddagger$	P_{DCB}^\S	$\text{Fe}_{\text{DCB}}^\S$	$\text{Al}_{\text{DCB}}^\S$	$\text{c-P}^\bar{\bar{}}$	$\text{c-Fe}^\bar{\bar{}}$	$\text{c-Al}^\bar{\bar{}}$	*1 *2
Bulk soil	0.73±0.03	24.0±0.7	44.6±2.1	4.03±0.12	0.41±0.01 (56)	3.75±0.10 (16)	0.88±0.03 (2)	0.53±0.02 (72)	11.6±0.5 (48)	1.03±0.08 (2)	0.12 (16)	7.80 (33)	0.15 (0.3)	↓ ↓
Sand-sized >20 μm	0.54±0.05	19.5±2.9	37.4±2.3	3.63±0.32	0.29±0.01 (53)	2.92±0.24 (15)	0.59±0.04 (2)	0.40±0.04 (73)	9.24±0.53 (47)	0.81±0.07 (2)	0.11 (20)	6.31 (32)	0.22 (0.6)	
Silt-sized 2-20 μm	1.40±1.00	44.7±3.9	73.8±0.9	5.63±0.40	0.82±0.04 (58)	7.00±0.23 (16)	1.83±0.07 (2)	0.98±0.04 (70)	20.7±0.5 (46)	1.93±0.04 (3)	0.17 (12)	13.7 (31)	0.10 (0.1)	
Clay 0.45-2 μm	2.37±0.06	69.3±3.5	108±2	7.10±0.30	1.38±0.01 (58)	11.6±0.1 (17)	3.28±0.05 (3)	1.72±0.06 (73)	36.8±0.7 (53)	3.62±0.31 (3)	0.33 (14)	25.2 (36)	0.34 (0.3)	
Clay <0.45 μm	2.47±0.06	76.4±3.3	114±2	8.23±0.35	1.54±0.07 (62)	12.4±0.5 (16)	4.36±0.28 (4)	1.85±0.09 (75)	40.8±1.1 (53)	4.11±0.35 (4)	0.31 (13)	28.4 (37)	ND	

528 *1 Amounts increase with decreasing particle size.

529 *2 Relative proportions of isolated fractions are independent of decreasing particle size.

530 † Total P, Fe, Al and Ca.

531 ‡ Oxalate extractable P, Fe and Al.

532 § DCB extractable P, Fe and Al.

533 $\bar{\bar{}}$ The c-(P, Fe, Al) means the difference between $(\text{P, Fe, Al})_{\text{DCB}}$ and $(\text{P, Fe, Al})_{\text{ox}}$.

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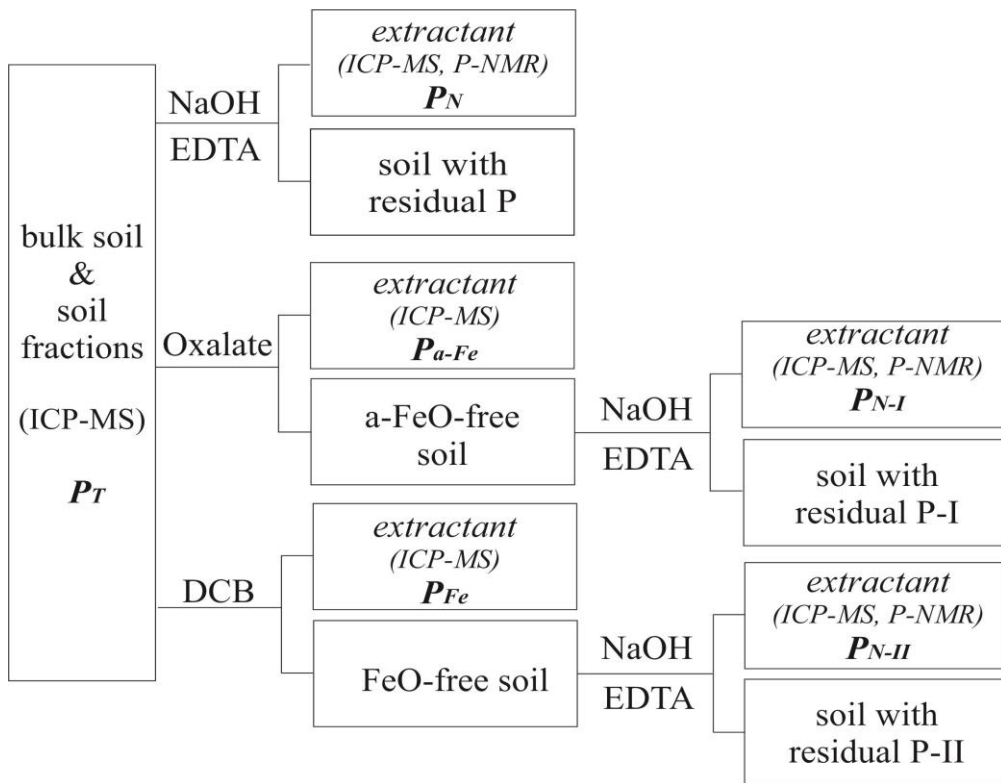
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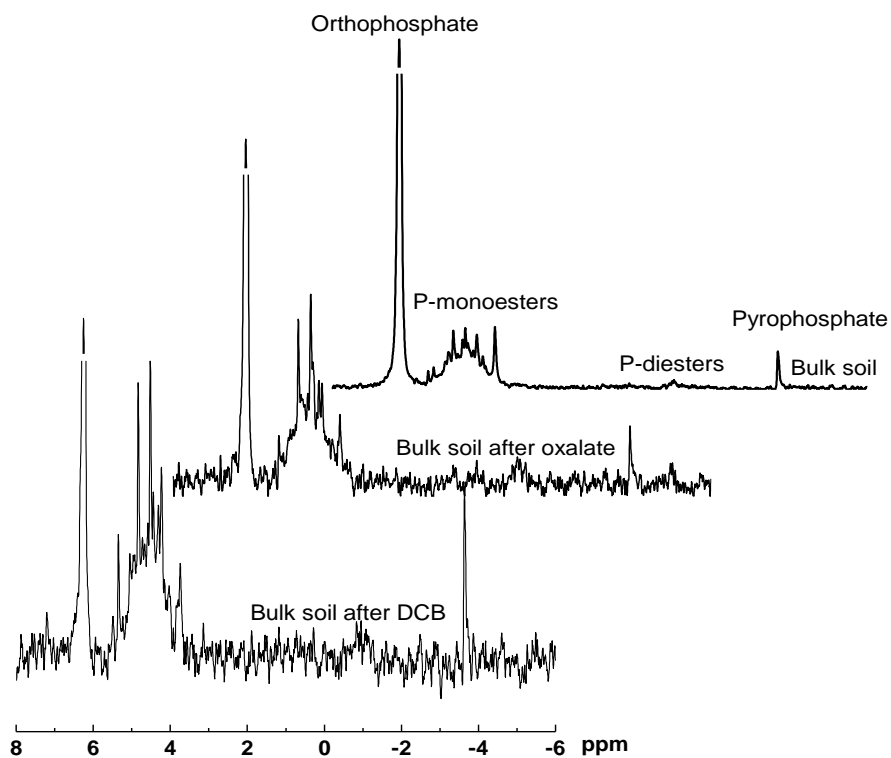


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Fig. 1 A schematic diagram of the soil sample treatments.

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	Orthophosphate (%)	P-monoesters (%)	Pyrophosphate* (%)
Bulk soil	79.4	19.1	1.4
Bulk soil after oxalate	60.8	35.0	4.2
Bulk soil after DCB	51.5	43.0	5.5

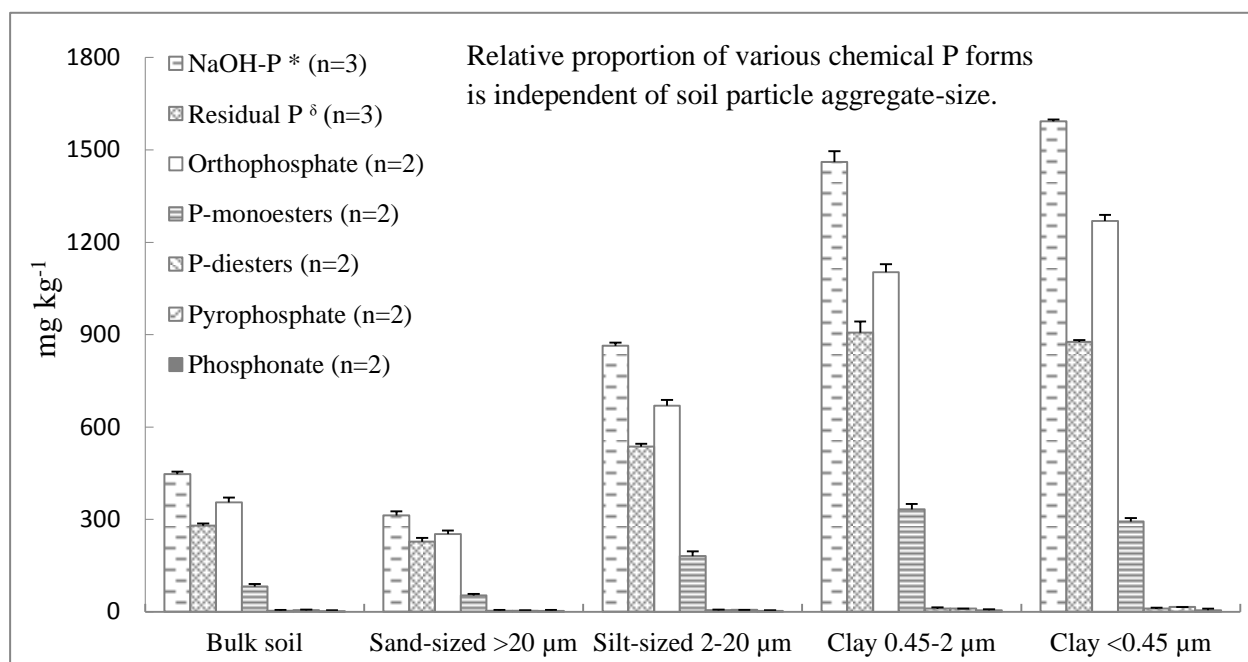
549 *All other P forms are not included.

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551 Fig. 2 Solution phosphorus-31 nuclear magnetic resonance spectra of NaOH-Na₂EDTA extracts of bulk
 552 soil without and with oxalate and dithionite (DCB) pre-treatments (all subfigures have been reduced by the
 553 same factors). The associated table only shows the relative mass distribution (%) of orthophosphate, P-
 554 monoesters and pyrophosphate in NaOH-Na₂EDTA extracts of bulk soil without and with oxalate and DCB
 555 pre-treatments.

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560 * NaOH-Na₂EDTA extractable P. δ NaOH-Na₂EDTA non-extractable P.

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562 Figure 3. NaOH-Na₂EDTA extractable P, residual P content, and phosphorus forms / contents (mg kg⁻¹)
 563 from the NaOH-Na₂EDTA extracts in different aggregate-sized fractions as identified by solution
 564 phosphorus-31 nuclear magnetic resonance spectroscopy. The varying chemical P contents were
 565 determined by multiplying the proportion of spectral area by total concentration of the NaOH-Na₂EDTA
 566 extractable P.

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