Colloid-bound and dissolved phosphorus species in topsoil water extracts along a grassland transect from Cambisol to Stagnosol

Xiaoqian Jiang[1], Roland Bol[1], Barbara J. Cade-Menun[2*], Volker Nischwitz[3], Sabine Willbold[3], Sara L. Bauke[4], Harry Vereecken[1], Wulf Amelung[1,4], Erwin Klumpp[1]

[1] Institute of Bio- and Geosciences, Agrosphere Institute (IBG-3), Forschungszentrum Jülich GmbH, Jülich, Germany

[2] Swift Current Research and Development Centre, Agriculture and Agri-Food Canada, Box 1030, 1 Airport Rd. Swift Current, SK, S9H 3X2 Canada

[3] Central Institute for Engineering, Electronics and Analytics, Analytics (ZEA-3), Forschungszentrum Jülich GmbH, Jülich, Germany

[4] Institute of Crop Science and Resource Conservation, Soil Science and Soil Ecology, Nussallee 13, University of Bonn, 53115 Bonn, Germany

*Corresponding author

Barbara J. Cade-Menun, Email: Barbara.Cade-Menun@AGR.GC.CA

Abstract

Phosphorus (P) species in colloidal and "dissolved" soil fractions may have different distributions. To understand which P species are potentially involved, we obtained water extracts from the surface soils of a gradient from Cambisol, Stagnic Cambisol to Stagnosol from temperate grassland, Germany. These were filtered to < 450 nm, and divided into three procedurally-defined fractions: small-sized colloids (20-450 nm), nano-sized colloids (1-20 nm), and "dissolved P" (< 1 nm), using asymmetric flow field flow fractionation (AF4), as well as filtration for solution ^{31}P-NMR spectroscopy. The total P of soil water extracts increased in the order Cambisol< Stagnic Cambisol< Stagnosol due to increasing contributions from the dissolved P fraction. Associations of C-Fe/Al-PO_4^{3-}/pyrophosphate were absent in nano-sized (1-20 nm) colloids from the Cambisol but not in the Stagnosol. The ^{31}P-NMR results indicated that this was accompanied by elevated portions of organic P in the order Cambisol > Stagnic Cambisol > Stagnosol. Across all soil types, elevated proportions of inositol hexakisphosphate species (e.g. *myo*-, *scyllo*-, and D-*chiro*-IHP) were associated with soil mineral particles (i.e. bulk soil and small-sized soil colloids) whereas other orthophosphate monoesters and phosphonates were found in the 'dissolved' P fraction. We conclude that P species composition varies among colloidal and "dissolved" soil fractions after characterization using advanced techniques, i.e. AF4 and NMR. Furthermore, stagnic properties affect P speciation and availability by potentially releasing dissolved inorganic and ester-bound P forms as well as nano-sized organic matter-Fe/Al-P colloids.

Keywords: colloidal phosphorus; dissolved phosphorus; field flow fractionation; ^{31}P-NMR; grassland; Cambisol; Stagnosol.

Abbreviations: AEP, 2-Aminoethyl phosphonic acid; AF4, asymmetric flow field flow fractionation; Al, aluminum; Ca, calcium; DNA, deoxyribonucleic acid; EDTA, Ethylenediaminetetraacetic; Fe, iron; FFF, field flow fractionation; ICP-MS, inductively coupled plasma mass spectrometer; myo-IHP, myo-inositol hexakisphosphate; N, nitrogen; NMR, nuclear magnetic resonance; OC, organic carbon; OCD, organic carbon detector; OM, organic matter; PES, polyethersulfone; Pi, inorganic P species; Po,

organic P species; Si, silicon; UV, ultraviolet; WDCs, water dispersible colloids; WDFCs, water dispersible fine colloids.

1. Introduction

Phosphorus (P) is an essential nutrient for plant growth and limits terrestrial ecosystem productivity in many arable and grassland soils (Vance et al., 2003). The availability and transport of P depend on the speciation and concentration of P in the soil solution, which contains both 'dissolved' and colloidal P forms (Shand et al., 2000; Hens and Merckx, 2002; Toor and Sims, 2015). Dissolved orthophosphate is generally the main P species in solution and can be directly taken up by plant roots (Condron et al., 2005; Pierzynski et al., 2005). However, colloidal P in the size range of 1-1000 nm (Sinaj et al., 1998) may also contribute significantly to total P content in the soil solution (Haygarth et al., 1997; Shand et al., 2000; Hens and Merckx, 2001). Recent studies found that fine colloids (< 450 nm fraction) in soil water extracts consisted of nano-sized (< 20 nm) and small-sized (20 < d < 450 nm) particles with different organic matter and elemental composition (Henderson et al., 2012; Jiang et al., 2015a). Very fine nano-sized P colloids, around 5 nm are even prone to plant uptake (Carpita et al., 1979). In addition, the presence of fine colloids alters the free ionic P content in the soil solution through sorption processes (Montalvo et al. 2015). After diffusion-limited uptake depletes the free ionic P in the soil solution, these fine colloids disperse in the diffusion layer and therewith re-supply free ionic P species for roots (Montalvo et al., 2015). Because water-dispersible colloids (WDCs) can be easily released from soil in contact with water (Jiang et al., 2012; Rieckh et al., 2015), they have also been suggested as model compounds for mobile soil colloids (de Jonge et al., 2004; Sequaris et al., 2013). However, little is known about the chemical composition of P species in different-sized WDCs.

Recent studies have started to characterize natural fine colloidal P in freshwater samples and soil water extracts using asymmetric flow field flow fractionation (AF4) coupled to various detectors (e.g. ultraviolet [UV] and inductively coupled plasma mass spectrometer [ICP-MS]) for improved size fractionation of colloids and online analysis of their elemental composition (Henderson et al., 2012; Regelink et al., 2013; Gottselig et al., 2014; Jiang et al., 2015a). These analyses are increasingly combined with solution ^{31}P-nuclear magnetic resonance (NMR) spectroscopy, which offers low detection limits and can quantify different inorganic and organic P compound groups (Cade-Menun, 2005; Cade-Menun and Liu, 2014) in isolated colloidal materials (e.g. Liu et al., 2014; Jiang et al., 2015a, b; Missong et al., 2016). However, we are not aware of studies that have applied these methods

systematically to WDCs obtained from different major reference soils. Here, we focus on the comparison of Cambisols and Stagnosols. In contrast to Cambisols, Stagnosols are soils with perched water forming redoximorphic features. Due to temporary water saturation and resulting oxygen limitation, the reduction of iron (Fe^{III}) is accompanied by the dissolution of its oxides and hydroxides (Rennert et al. 2014), and the P associated with these Fe-minerals should correspondingly be redistributed in soil solution.

The objective of this study was to elucidate how stagnant water conditions alter the potential release of different P compounds in colloidal and 'dissolved' fractions of soil solution. For this purpose, water-extractable P was obtained from a transect of Cambisols to Stagnosols in a German temperate grassland, and characterized using both solution ^{31}P-NMR and AF4 coupled online with UV and organic carbon detector (OCD) or ICP-MS analyses.

2. Materials and methods

2.1 Site description

The grassland test site in Rollesbroich is located in the northern part of the Eifel in North Rhine-Westphalia, Germany (50 ° 62 ´ N, 06 ° 30 ´ E). The grassland vegetation is dominated by perennial ryegrass (*Lolium perenne* L.) and smooth meadow grass (*Poa pratensis* L.). According to the soil map of the geological service of North Rhine-Westphalia (Fig. 1), the dominant soil types on the test site are Cambisols (extensive meadow with three to four cuts per year, no cattle grazing), Stagnic Cambisols (cattle pasture but with less frequent grazing than the Stagnosols), and Stagnosols [intensively used as pasture with frequent cattle grazing followed by harrowing with a tire-drag harrow and application of organic manure (cattle slurry)]; classification according to IUSS Working Group WRB (2015). The elevation along the transect generally decreases from south to north, with the highest elevation of 512.9 m a.s.l. at plot 1 and the lowest point of 505.1 m a.s.l. at plot 3 (Fig. 1, Table 1). The catchment mean annual precipitation was 103.3 cm for the period from 1981 to 2001, and the highest runoff occurred during winter due to high precipitation and low evapotranspiration rates, as well as overland flow due to saturation excess (Gebler et al., 2015). The topsoil samples (2-15 cm) of plot 1 (S1-1, S1-2, and S1-3, Cambisol), 2 (S2, Stagnic Cambisol), and 3 (S3-1, S3-2, and S3-3,

Stagnosol) were taken as a representative transect across the site in early March, 2015 (Fig. 1). It is worth noting that Stagnic water conditions do not mean that the soils are under reduced conditions for the whole year – only for some significant time of the year. We sampled a Stagnosol, but only the topsoil (2-15 cm) which was not under perching water, i.e., it was aerobic at time of sampling. As such, the Stagnols used for this study were oxic at various times each year, but also experienced periods of reducing conditions that did not occur in the other samples along the transect. Surface turf (0-2 cm) was removed as it contained predominantly grass roots and little mineral soil. Removal of this very surface turf may also help minimizing effects from recent manure input on soil properties. Stones and large pieces of plant material were removed by hand. All samples were sieved immediately to < 5 mm and stored at 5 ℃.

2.2 Water dispersible fine colloids (WDFCs) separations and AF4-UV-ICP-MS / AF4-UV-OCD analyses

The WDCs of Rollesbroich grassland soil samples with three field replicates in S1 and S3 were fractionated using the soil particle-size fractionation method of Séquaris and Lewandowski (2003), but with moist soils. In brief, moist soil samples (100 g of dry soil basis) were suspended in ultrapure water (Mill-Q, pH: 5.5) in a soil: solution mass ratio of 1:2, and shaken for 6 h. Thereafter, 600 mL of ultrapure water were added and mixed. The WDCs suspensions were collected using a pipette after a 12-h sedimentation period. These WDCs suspensions were subsequently centrifuged for 15 min at 10,000 × *g* and filtered through 0.45-μm membranes (cellulose mixing ester) to produce the suspension containing WDFCs sized below 0.45 μm. It is worth noting that Mill-Q water was used here to extract soil colloids instead of rain water or pore water, since total amounts of WDFCs will likely be larger when using Mill-Q water, i.e., we consider these WDFCs as potentially water-dispersible colloids. In addition, the use of Mill-Q water facilitates subsequent sample processing with AF4 and NMR. It is inevitable that Mill-Q water would result in the release of P due to desorption and dissolution of poorly crystalline authigenic mineral phases. Additionally，living cells within the soil would also certainly undergo significant osmotic stress, likely resulting in osmotic rupture and releasing organic and inorganic P found in intracellular components. It is also worth noting that the

131 experimental procedure with Mill-Q water under oxic conditions may have an impact on oxidation of
132 aqueous iron (Fe^{2+}) and colloidal ferrous particles. However, at time of sampling, the very surface
133 soils were not fully water saturated as allowed even for Stagnosols for time of the year. As such, the
134 analyzed species and size fractions are representative of differences in response to the extraction
135 procedure based on different soil redox conditions that reflect a kind of legacy of former redox cycle,
136 but at time of sampling and analyses the soils were aerobic.
137
138 An AF4 system (Postnova, Landsberg, Germany) with a 1 kDa polyethersulfone (PES) membrane and
139 500 µm spacer was used for size-fractionation of the soil sample WDFCs. It is a separation technique
140 that provides a continuous separation of colloids. The retention time of the colloids can be converted
141 to hydrodynamic diameters of the colloids using AF4 theory or calibration with suitable standards
142 (Dubascoux et al., 2010). The AF4 was coupled online to an ICP-MS system (Agilent 7500, Agilent
143 Technologies, Japan) for monitoring of the Fe, aluminum (Al), silicon (Si), and P contents of the size-
144 separated particles (Nischwitz and Goenaga-Infante, 2012) and to OCD and UV detectors for
145 measuring organic carbon (OC). These elements were analyzed as part of the main soil minerals (e.g.
146 clay minerals and Fe oxides) that can be associated with P (Jiang et al., 2015a). The OCD is a
147 promising technique for monitoring OC concentrations for liquid-flow based separation systems with
148 the advantages of high selectivity and low detection limits (Nischwitz et al., 2016). Briefly, the
149 operation principle is that the acidification of the sample flow removes inorganic C and subsequently
150 the OC is oxidized in a thin film reactor to carbon dioxide, which can be quantified by infrared
151 detection (Nischwitz et al., 2016). A 25 µM NaCl solution at pH 5.5, which provided good separation
152 conditions for the WDFCs, served as the carrier. The injected sample volume was 0.5 mL and the
153 focusing time was 15 min with 2.5 mL min^{-1} cross flow for the AF4-UV-OCD system while 2 mL
154 injected volume and 25 min focusing time were used for the AF4-ICP-MS system. Thereafter, the
155 cross flow was maintained at 2.5 mL min^{-1} for the first 8 min of elution time, then set to decrease
156 linearly to 0.1 mL min^{-1} within 30 min, and maintained for 60 min. It then declined within 2 min to 0
157 mL min^{-1}, and remained at this rate for 20 min to elute the residual particles. The detection limit of the

ICP-MS system was 0.1-1 μg L^{-1} for the elements analyzed in this study. The AF4 characteristics of WDFCs did not change significantly in the 6 months period of the investigation.

2.3 Particle separations of WDFCs and solution ^{31}P-NMR spectroscopy

The soil samples were treated as described in section 2.2 to obtain the suspension containing WDFCs < 450 nm. We pooled the WDFCs suspensions of the field replicates in order to receive sufficient samples for solution ^{31}P-NMR. The first peak fraction after AF4 separation has a particle size smaller than ~20 nm (approximately 300 kDa; Jiang et al., 2015a; Fig. 2). Therefore, the suspension containing WDFCs < 450 nm of these three samples were separated into three size fractions: 300 kDa-450 nm, 3-300 kDa, and < 3 kDa (nominally 1 nm; Erickson, 2009). The 300 kDa-450 nm particle fractions were separated by passing ~600 mL of the WDFCs suspension through a 300 kDa filter (Sartorius, Germany) by centrifugation. The 3-300 kDa particle fractions were subsequently isolated by passing the < 300 kDa supernatant through a 3 kDa filter (Millipore Amicon Ultra) by centrifugation. Finally, the final supernatant containing the < 3 kDa particles as well as the electrolyte phase was frozen and subsequently lyophilized.

The bulk soil samples (1 g) and the three fractions of soil water extracts were respectively mixed with 10 mL of a solution containing 0.25 M NaOH and 0.05 M Na_2EDTA (ethylenediaminetetraacetate) for 4 h, as a variation of the method developed to extract P for ^{31}P-NMR (Cade-Menun and Preston, 1996; Cade-Menun and Liu, 2014; Liu et al., 2014). Extracts were centrifuged at 10,000 × *g* for 30 min and the supernatant was frozen and lyophilized. Each NaOH-Na_2EDTA-treated lyophilized extract, and the < 3 kDa fraction without NaOH-Na_2EDTA treatment, was dissolved in 0.05 mL of deuterium oxide (D_2O) and 0.45 mL of a solution containing 1.0 M NaOH and 0.1 M Na_2EDTA (Turner et al. 2007). A 10 μL aliquot of NaOD was added to the < 3 kDa fraction without NaOH-Na_2EDTA treatment to adjust the pH. The prepared samples were centrifuged at 13,200 × *g* for 20 min (Centrifuge 5415R, Eppendorf).

Solution ^{31}P-NMR spectra were obtained using a Bruker Avance 600-MHz spectrometer equipped with a prodigy-probe (a broadband CryoProbe which uses nitrogen [N]-cooled RF coils and preamplifiers to deliver a sensitivity enhancement over room temperature probes of a factor of 2 to 3

for X-nuclei from ^{15}N to ^{31}P), operating at 242.95 MHz for ^{31}P. Extracts were measured with a D_2O-field lock at room temperature. Chemical shifts were referenced to 85% orthophosphoric acid (0 ppm). The NMR parameters generally used were: 32 K data points, 3.6 s repetition delay, 0.7 s acquisition time, 30 ° pulse width and 10,000 scans. Compounds were identified by their chemical shifts after the orthophosphate peak in each spectrum was standardized to 6.0 ppm during processing (Cade-Menun et al., 2010; Young et al., 2013). Peak areas were calculated by integration on spectra processed with 7 and 2 Hz line-broadening, using NUTS software (2000 edition; Acorn NMR, Livermore, CA) and manual calculation. Peaks were identified as reported earlier (Cade-Menun, 2015), and by spiking a select sample with myo-inositol hexakisphosphate (myo-IHP; McDowell et al., 2007).

2.4 Statistical Analyses

Elemental concentrations in bulk soils, soil water extracts, and AF4 fractograms of soil colloidal particles were tested for significant differences (set to $P < 0.05$) using Sigmaplot version 12.5. A t-test was conducted to determine the significance of differences among soil sites, whereas one-way Repeated Measurements (RM) ANOVAs with Fisher LSD were performed with Fisher LSD post-hoc test to test for significant differences among soil fractions and AF4 fractograms for the Cambisol and Stagnosol. Data were assessed with Shapiro-Wilks and Brown-Forsythe-tests to meet the criteria of normal distribution and homogeneity of variances respectively; those which had unequal variance data were $\log_{10}$ transformed before statistical analyses.

3. Results and discussion

3.1 Colloid and colloidal P distribution in different size fractions based on AF4-fractograms

The AF4-UV-OCD and AF4-ICP-MS results of the WDFCs showed different OC, Si, P, Fe, and Al concentrations in different-sized colloid fractions as a function of elution time (Fig. 2). Before the first peak, an initial small void peak occurred at 1 min (Fig. 2 D, E, F). Thereafter, three different colloid-size fractions occurred individually as three peaks in the WDFCs of all samples (Fig. 2). The first peak of the fractograms corresponded to a particle size below 20 nm according to the calibration result using latex standards (Jiang et al., 2015a). The third peak, which was eluted without cross flow,

214 contained only small amounts of residual particles or particles possibly previously attached on the
215 membrane during focus time; it had similar OC and element distributions as the second peak in all
216 samples (Fig. 2). Therefore we considered these two fractions together as a whole. As such, the size
217 ranges from 20 to 450 nm from here onward are described as the "second size fraction".
218 For the first fraction representing nano-sized colloids of the three field sites, the OCD and UV signals
219 indicated increasing OC concentration in the order of S1 (Cambisol; Fig. 2A), S2 (Stagnic Cambisol;
220 Fig. 2B), and S3 (Stagnosol; Fig. 2C). Distinct peaks of Fe, Al, and P in the first size fraction (< 20 nm)
221 were only present in the Stagnosol (S3; Fig. 2 F), suggesting that under stagnant water conditions,
222 Fe/Al may more readily be involved in nano-sized soil particles than under other soil conditions. In
223 contrast, negligible amount of P, Al, and Fe were detected in the first fraction of S1 and S2 (Fig. 2 D
224 and E, Table S1). While it is sometimes difficult to determine whether this peak is real or just the
225 tailing of the void signal (Fig. 2 D and E), solution ^{31}P-NMR results confirmed the presence of P in
226 this size fraction (see next section). The nano-sized colloids from the Cambisol contained OC and
227 negligible P, Fe, and Al; those from the Stagnosol contained significantly higher concentrations of OC,
228 P, Fe, and Al (Table S1). We therefore assumed that the nano-sized colloidal P forms in the Stagnosol
229 mainly consisted of OC-Fe(Al)-P associations. Nanoparticulate humic (organic matter)-Fe (Al) (ions
230 /(hydr)oxide)-phosphate associations have recently been identified both in water and soil samples
231 (Gerke, 2010; Regelink et al., 2013; Jiang et al., 2015a). Our results suggest that the formation of these
232 nano-sized specific P-associations is favoured by the stagnant water conditions with high OC and
233 water contents in Stagnosol but not in the other soil types along the grassland transect.
234 The second size fraction (Fig. 2 A, B, C, i.e. the small-sized colloids) contained significantly more OC
235 than the smaller nano-sized colloids for all studied soils (Table S1). Notably, the OC contents of the
236 second fraction increased in the order Cambisol < Stagnic Cambisol < Stagnosol; the UV signal
237 therein supporting the results obtained with the OC detector. The larger-sized colloids were
238 significantly richer in Al, Fe, Si, and P than the smaller-sized ones (Table S1), though again with
239 differences among subsites: the stagnic Cambisol showed the largest Fe, Al, and Si contents in the
240 second fraction, as if there were a gradual change from low WDFC release in the Cambisol to the
241 formation of larger WDFC in the stagnic Cambisol and finally to the formation of smaller WDFC in

242 the Stagnosol. Though this trend warrants verification by more sites, it appeared at least as if the
243 increasing oxygen limitation from Cambisols via stagnic Cambisols to Stagnosols promoted an
244 increasing formation of small C-rich P-containing nanoparticles with additional contributions from Fe-
245 and Al-containing mineral phases. Stagnosols like S3 are characterized by a dynamic reduction regime
246 with dissolution of reactive Fe oxides (Rennert et al. 2014), which led to a decrease in the content of
247 Fe oxides in the second colloidal fraction (Table S1). Correspondingly, the dissolution of Fe oxides in
248 the second fraction under stagnant water may also liberate OC from the organo-Fe mineral
249 associations, thus releasing some OC to the nano-sized first fraction (Jiang et al., 2015a). This could
250 be an additional reason for the higher concentration of OC in the first peak of S3 (Table S1), apart
251 from a generally slower degradation of organic matter under limited oxygen supply (Rennert et al.
252 2014). Hence, the AF4 results indicated that the composition and distribution of particulate P varied
253 among the different-sized colloidal particles, and that its properties were impacted by the soil type and
254 related properties. However, AF4-ICP-MS results do not provide information about the elemental
255 concentrations of the 'dissolved' P fraction of these grassland soils. We cannot rule out any effects
256 from sample storage or from the use of Mill-Q water, as discussed in the Methods section, However,
257 although all samples were treated the same way, differences among the samples were consistent with
258 soil characteristics at each site. This suggests that the influences of treatment and storage were
259 minimal, but further investigation is warranted in future studies.
260
261 **3.2 Soil total, colloidal and dissolved P contents based on fractionation by filtration**
262 Soil water extracts < 450 nm, < 300 kDa, and < 3 kDa were obtained by filtration to determine total
263 elemental contents by ICP-MS analysis. Data did not have to be pooled for these analyses; as such, we
264 could test statistical differences. We considered the soil water extract < 3 kDa in this paper to be the
265 'dissolved' fraction. Significant differences ($P < 0.05$) were ascertained for elevated concentrations of
266 TOC, total P, as well for lower concentrations of total Al and Fe in the Stagnosol relative to the
267 Cambisol (Table 1). Furthermore, the Stagnosol had significantly higher concentrations of Si and P in
268 the individual size fractions of soil water extracts (except marginally significantly higher P in < 3 kDa,
269 $p = 0.06$), as well as higher Fe and Al concentrations in < 300 kDa and < 3kDa fraction than the

corresponding fractions of the Cambisol (Table 2). The stagnic Cambisol generally resembled the Cambisol rather than the Stagnosol in bulk soil analysis, but this was not the case for the soil water extracts. This implied that the stagnic properties have a greater impact on the colloidal particles and "dissolved" fraction compared to bulk soil.

The oxygen limitation and reduction regime of the Stagnosol probably also favored the accumulation of OC and dissolution of Fe oxides both in bulk soil and colloids (Rennert et al. 2014). Dissolution of Fe oxides in turn results in a disaggregation of colloidal particles (Jiang et al., 2015a). As the released oxides are main carriers for P, these processes may explain why the distribution of colloidal and dissolved P also changed across the different grassland soils. As Table 2 shows, large proportions of P in the < 450 nm fraction of the Stagnosol were dissolved P (i.e. recovered here in the < 3 kDa fraction), whereas colloidal P dominated in the Cambisol and Stagnic Cambisol.

3.3 Inorganic and organic P species in the different-sized soil colloidal and the 'dissolved' fractions

Solution ^{31}P-NMR was used to elucidate the speciation of P in bulk soil and soil water extracts separated by ultrafiltration into the size fractions 300 kDa-450 nm, 3-300 kDa, and < 3 kDa for each of the three soils (Fig. 3 and S1, Table 3). The identified P included inorganic P forms (orthophosphate, pyrophosphate, and polyphosphate), and organic P in phosphonate, orthophosphate monoester and diester compound classes. Phosphonates included 2-aminoethyl phosphonic acid (AEP) and several unidentified peaks (Table S3). Orthophosphate monoesters included four stereoisomers of inositol hexakisphosphate (*myo*-, *scyllo*-, *neo*-, and D-*chiro*-IHP), diester degradation products (α-glycerophosphate, β-glycerophosphate and mononucleotides), choline phosphate, and unidentified peaks at 3.4, 4.2, 4.7, 5.0, 5.3, and 5.9 ppm. Orthophosphate diesters were divided into deoxyribonucleic acid (DNA) and two categories of unknown diesters (OthDi1 and OthDi2, respectively). Orthophosphate, pyrophosphate, orthophosphate monoesters, and diesters have also been detected in other studies of grassland, arable, and forest Cambisols and Stagnosols (e.g., Murphy et al., 2009; Turrion et al., 2010; Jarosch et al., 2015).

297 For the bulk soil samples and colloidal fractions of 300 kDa-450 nm of our soil samples,
298 orthophosphate and orthophosphate monoesters (mainly *myo*-IHP) were the main P compounds in all
299 samples (Fig. 3 and S1, Table 3 and S2). These main P compounds in these two soil fractions showed
300 similar trends among the soil samples: the proportions of organic P (e.g. orthophosphate monoesters
301 and diesters) decreased in the order of Cambisol > Stagnic Cambisol > Stagnosol (Table 3). The
302 similarity in this trend for the different organic P forms can likely be attributed to similarities in the
303 mineral components of bulk soil and colloidal fractions: i.e., similar element concentrations and thus
304 likely also similar clay mineralogy, Fe oxide signature and OC content of bulk soil and respective
305 colloid fraction according to the AF4-OCD and AF4-ICP-MS results (Fig. 2 and Table S1).
306 Orthophosphate, orthophosphate monoesters and diesters are predominantly stabilized by association
307 with these mineral components (Solomon and Lehmann, 2000; Turner et al., 2005; Jiang, et al, 2015a).
308 We assume that most of the relatively higher proportion of orthophosphate and lower percentage of
309 organic P in the Stagnosol may be attributed to the dissolution of Fe oxides, which likely released
310 organic P. Additionally, the higher concentrations of OC in both bulk soil (Table1) and large colloids
311 of the Stagnosol probably favored the formation of OC-Fe/Al-PO_4^{3-} complexes (see above). However,
312 we cannot rule out the effects of differences in grazing and manure application on the P forms in these
313 soils. Cattle grazing and the application of cattle slurry would be expected to add P that is
314 predominantly orthophosphate, with lower concentrations of organic P forms including *myo*-IHP
315 (Cade-Menun 2011 and references therein). As such, this may have contributed to the increased
316 orthophosphate and decreased organic P we observed on these sites.
317 Our study is the first to distinguish the chemical P composition in colloidal fractions of 3-300 kDa and
318 300 kDa-450 nm. We found different P speciation and distribution between these two fractions. This is
319 probably related to differences in their element composition, which are dominated by OC-P/ OC-
320 Fe(Al)-P associations in the 3-300 kDa soil fraction and by clay-Fe oxides-OC-P associations in the
321 300 kDa-450 nm size fraction (Fig. 2). Intriguingly, we did not find any organic P but only inorganic P
322 in the 3-300 kDa of all three soils (orthophosphate in Cambisol and Stagnic Cambisol, orthophosphate
323 and pyrophosphate in the Stagnosol; Table 3). Furthermore, the Stagnosol nanoparticle fraction 3-300
324 kDa had a higher proportion of pyrophosphate than the 300 kDa-450 nm size fraction.

When comparing the solution ^{31}P-NMR results of the < 3 kDa soil fractions with and without NaOH-Na_2EDTA treatments (Fig. 3 and Fig. S1), we observed that most of the phosphonates, orthophosphate monoesters and diesters were lost after NaOH-Na_2EDTA treatment (Fig. 3 and Fig. S1). There were two possible explanations: 1) 'dissolved' organic P in the NaOH-Na_2EDTA solution is sensitive and easily hydrolyzed to orthophosphate (Cade-Menun and Liu, 2014); or 2) in absence of NaOH-Na_2EDTA, most orthophosphate was removed by adsorption on sedimentary material in the re-dissolved solution after centrifugation when preparing the samples for NMR analysis (Cade-Menun and Liu, 2014), resulting in elevated portions of organic P in the NMR sample. The second possibility may also explain the observation that there was no orthophosphate in the 'dissolved' fraction of the Cambisol without NaOH-Na_2EDTA treatment (Fig. S1). Almost all the orthophosphate may have been removed with the sedimentary phase due to the extremely low concentration of dissolved P in this soil. Therefore, we will focus on the discussion of results obtained from the < 3 kDa soil fractions without NaOH-Na_2EDTA treatment, as they provide better information on the origin of Po-species than the other samples that received this treatment.

The composition of P species in the < 3 kDa soil fractions (i.e. "truly" dissolved P) differed among the three soils (Table 3). The majority of observed P in the < 3 kDa soil fraction of the Cambisol was organic P, comprised mainly of phosphonates and orthophosphate monoesters. The < 3 kDa soil fraction of the Stagnic Cambisol contained various P species from all compound classes, including orthophosphate, orthophosphate monoesters, orthophosphate diesters, pyrophosphate, polyphosphates, and phosphonates. The < 3 kDa soil fraction of the Stagnosol contained similar P species as the Stagnic Cambisol, with relatively higher proportions of orthophosphate monoesters and phosphonates, but a lower proportion of orthophosphate diesters (Table 3). It is worth noting that there were more species of phosphonates in the < 3 kDa fraction than other fractions of each soil (Fig. 3 and S1). The larger signal at ~ 21-23.5 ppm was assigned to AEP (Doolette et al., 2009; Cade-Menun, 2015), which occurred in both the soil particles and the < 3 kDa fraction. However, the small signals at ~ 36-39 ppm and 45-46 ppm existed only in the < 3 kDa fraction of soil samples (Fig. 3 and S1). The resonance at 36-39 ppm might be assigned to dimethyl methyl phosphonic acid, based on Cade-Menun

(2015). However, spiking experiments were not conducted to identify peaks in this region, so their specific identity and origins remain unknown.

The solution ^{31}P-NMR results showed that P species composition in the two colloidal fractions and the electrolyte phase differed among all three soil samples, with more phosphonates potentially existing in the electrolyte phase. However, in the study of Missong et al. (2016), more phosphonates and orthophosphate diesters were found in colloidal fractions rather than the electrolyte phase of two forest Cambisols. Missong et al. (2016) used centrifugation while we used filtration to separate these particle sizes and phases. Additionally, Missong et al. (2016) worked with forest soils while we worked with grassland soils. McLaren et al. (2015) recently confirmed that the speciation of organic P is markedly different between high (> 10 kDa) and low (< 10 kDa) molecular weight fractions of soil extracts. In any case, both colloidal aggregation and changes in soil order paralleled P forms. However, also other soil properties but former redox state (like pH), as well as variations in anthropogenic, site-adapted management may be additional covariates affecting P colloids and composition.

3.4 Distribution of orthophosphate monoesters and pyrophosphate

With variations in overall P species composition, the proportions of certain species of orthophosphate monoesters were also distributed differently among the investigated fractions of the three soils. For example, the proportion of various IHP stereoisomers (i.e. *myo*-, *scyllo*-, D-*chiro*-IHP) decreased with decreasing colloid size (Table S2). This suggests that the majority of IHP was associated with soil mineral particles but did not exist in the dissolved form in our soil samples. The *myo*-IHP stereoisomer is the principal input of inositol phosphate to soil in the form of plant material (Turner et al. 2002) and the other stereoisomers may come from plants or may be synthesized by soil organisms (Caldwell and Black, 1958; Giles et al., 2015). Inositol phosphate is stabilized mainly through strong adsorption on the surface of amorphous metal oxides and clay minerals (Celi and Barberis, 2007). Shang et al. (1992) found *myo*-IHP sorbed onto Al and Fe oxides to a greater extent than glucose 6-phosphate. Several orthophosphate monoesters such as unknown peaks at 3.4, 4.7 and 5.9 ppm were only detected in the electrolyte phase of soil samples (Table S2). The differences in orthophosphate monoester species distribution between soil particles and the electrolyte phase show that soil minerals such as clay

minerals and Fe (Al) oxides are only associated with certain species of orthophosphate monoesters such as IHP, while other species of orthophosphate monoesters exist only in the electrolyte phase. Further research is warranted to fully understand the factors controlling Po in these different size fractions.

It is worth noting that although the proportion of pyrophosphate in bulk soil was very low, there was more pyrophosphate in the colloidal and electrolyte phases of the Stagnic Cambisol and the Stagnosol than in the Cambisol, and mostly in the electrolyte and nano-sized colloidal fraction (Table 3). Our former study (Jiang et al., 2015b) indicated that Fe/Al oxides were not the main bonding site for pyrophosphate adsorption in different-sized fractions of an arable soil. Considering that a high proportion of pyrophosphate (38.5%) existed in the 3-300 kDa fraction of the Stagnosol, which contained P mainly in OC-Fe(Al) $^{2/3+}$-P associations (see above), it seems reasonable to assume that pyrophosphate existed as a colloidal OC-Fe(Al)$^{2/3+}$-pyrophosphate complex. In this regard, the accumulation of pyrophosphate may have been favored by the larger OC contents in this soil (Fig. 2 C).

This study shows for the first time that P species composition varies among the electrolyte phase and colloids of different size, with the specific distribution being related to the stagnic water regime of the soil. It could potentially promote P availability by a mechanism that results in a loss of colloids, thus providing less surface area for the immediate bonding of inorganic P to minerals, while at the same time potentially releasing organic P from mineral bonding so that it is more prone to decomposition. Relating the static differences in P species composition among the different soils and fractions to true dynamics of P transformations, e.g., by performing controlled mesocosm experiments, now warrants further attention.

Appendix A. Supplementary data

The elemental concentrations in AF4 fractograms, phosphorus spectra and species determined by solution ^{31}P-NMR as well as solution ^{31}P-NMR chemical shifts of the P compounds were shown in supporting information.

Acknowledgments

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Table 1 General soil characteristics and concentrations (g kg^{-1} soil) of total organic carbon (TOC), total Fe, Al, P, and Si in bulk S1 (Cambisol), S2 (Stagnic Cambisol), and S3 (Stagnosol). The lowercase letters indicate significant differences among soil sites (significant difference of soil site 1 and 3 was tested by t-test, $p < 0.05$).

Soil	pH[IV]	Water content (%)	Elevation (m a.s.l.)	TOC (g kg^{-1})	Fe*(g kg^{-1})	Al (g kg^{-1})	P (g kg^{-1})	Si (g kg^{-1})
S1[I]	4.90 ±0.12a	46.5 ±2.9	512.9	35.6 ±2.3a*	23.0 ±1.1a*	52.6 ±2.9a	1.2 ±0.1a	320 ±7.6
S2[II]	4.90	45.3	507.5	35.8	24.0 ±0.4	54.0 ±2.0	1.3 ±0.1	320 ±7.0
S3[III]	5.36 ±0.20b	59.0 ±7.6	505.1	71.1 ±15.1b*	12.8 ±0.4b*	38.7 ±1.1b	1.8 ±0.4b	312 ±12.1

[I] The mean of sample S1-1, S1-2, and S1-3 ± standard deviation.
[II] The mean of three replicate sample S2 ± standard deviation.
[III] The mean of sample S3-1, S3-2, and S3-3 ± standard deviation.
[IV] The mass ratio of soil : water = 1:2.5.
* Data were log transformed before t-test analyses because of unequal variances.

559 Table 2 Concentrations (mg kg^{-1} soil) of P, Al, Fe, and Si in soil water extracts < 450 nm, < 300 kDa, and < 3 kDa, respectively. Different lowercase and
560 uppercase indicate significant differences among soil sites and soil fractions, respectively (significant difference of soil sites 1 and 3 was tested by t-test, One
561 Way RM ANOVA for soil fractions with Fisher LSD post-hoc test, P <0.05).

Soil	DOC (g kg^{-1})	P (mg kg^{-1})			Al (mg kg^{-1})			Fe (mg kg^{-1})			Si (mg kg^{-1})		
	< 450 nm	<450nm	<300kDa	<3kDa	<450nm	<300kDa	<3kDa	<450nm	<300kDa	<3kDa	<450nm	<300kDa	<3kDa
S1[I]	0.18	0.3 ±0.1a*	0.2 ±0.2a*	0.1 ±0.1	2.0 ±0.4A °	0.6 ±0.0 $^{\alpha}$aB °	0.6 ±0.0$^{\alpha}$aB °	2.1 ±0.5A	0.2 ±0.0$^{\alpha}$aB	0.2 ±0.0$^{\alpha}$a*B	8.1 ±0.6aA	6.8 ±0.3aB	6.6 ±0.4aB
S2[II]	0.17	1.3 ±0.9	0.5 ±0.6	0.4 ±0.3	7.3 ±0.3	1.1 ±0.2	1.1 ±0.2	9.2 ±0.5	0.4 ±0.1	0.4 ±0.1	14.1 ±0.5	7.3 ±0.0$^{\alpha}$	7.8 ±0.8
S3[III]	0.23	4.4 ±2.0b*	3.3 ±2.7b*	4.1 ±2.6	4.1 ±3.1	0.7 ±0.1b	0.7 ±0.0b	4.6 ±3.3	0.4 ±0.1b	0.5 ±0.1b*	14.6 ±1.3b	10.6 ±2.1b	11.4 ±2.5b

562 [I] The mean of sample S1-1, S1-2, and S1-3 (Cambisol) ± standard deviation.
563 [II] The mean of three replicate extracts of sample S2 (Stagnic Cambisol) ± standard deviation.
564 [III] The mean of sample S3-1, S3-2, and S3-3 (Stagnosol) ± standard deviation.
565 [α] Standard deviation of 0.0 means value <0.05.
566 *Data were log transformed before t-test analyses because of unequal variances.
567 °Data were log transformed before One Way RM ANOVA analyses because of unequal variances.

Table 3 the proportion (%) of phosphorus species[a] determined by solution ^{31}P-NMR for the different soil fractions of S1 (Cambisol), S2 (stagnic Cambisol), and S3 (Stagnosol).

Soil fractions	Pi	Po	Ortho-P	Pyro-P	poly	P-mono	P-mono*	P-diest	P-diest*	Phon-P
	----------%----------									
S1 bulk	43.4	56.6	41.2	1.5	0.7	52.9	44.5	2.2	10.6	1.5
S2 bulk	47.8	52.2	46.4	0.9	0.5	48.6	43.7	1.4	6.3	2.2
S3 bulk	63.7	36.3	63.0	0.2	0.5	31.2	27.0	1.5	5.7	3.6
S1 300 kDa-450 nm	22.8	77.2	22.8	-[y]	-	56.7	49.5	11.1	18.3	9.4
S2 300 kDa-450 nm	56.8	43.2	53.1	1.0	2.7	29.9	26.9	5.2	8.2	8.1
S3 300 kDa-450 nm	70.2	29.8	59.7	9.2	1.3	24.2	19.9	2.8	7.1	2.8
S1 3-300 kDa	100	-	100	-	-	-	-	-	-	-
S2 3-300 kDa	100	-	100	-	-	-	-	-	-	-
S3 3-300 kDa	100	-	61.5	38.5	-	-	-	-	-	-
S1 < 3 kDa	13.5	86.5	-	-	13.5	26.9	26.9	1.9	1.9	57.7
S2 < 3 kDa	21.3	78.7	9.5	5.1	6.7	29.3	13.8	24.2	34.6	25.2
S3 < 3 kDa	22.2	77.8	8.8	6.0	7.4	29.4	27.4	8.2	10.2	40.2

[a] inorganic P (P_i), organic P (P_o), orthophosphate (Ortho-P), pyrophosphate (Pyro-P), polyphosphate (poly), orthophosphate monoesters (P-mono), orthophosphate diesters (P-diest), phosphonates (Phon-P). * recalculation by including diester degradation products (α glycerophosphate, β glycerophosphate, and mononucleotides) with P-diest rather than P-mono (Liu et al. 2014; Young et al. 2013). [y] below detection limit, i.e. <0.05%.

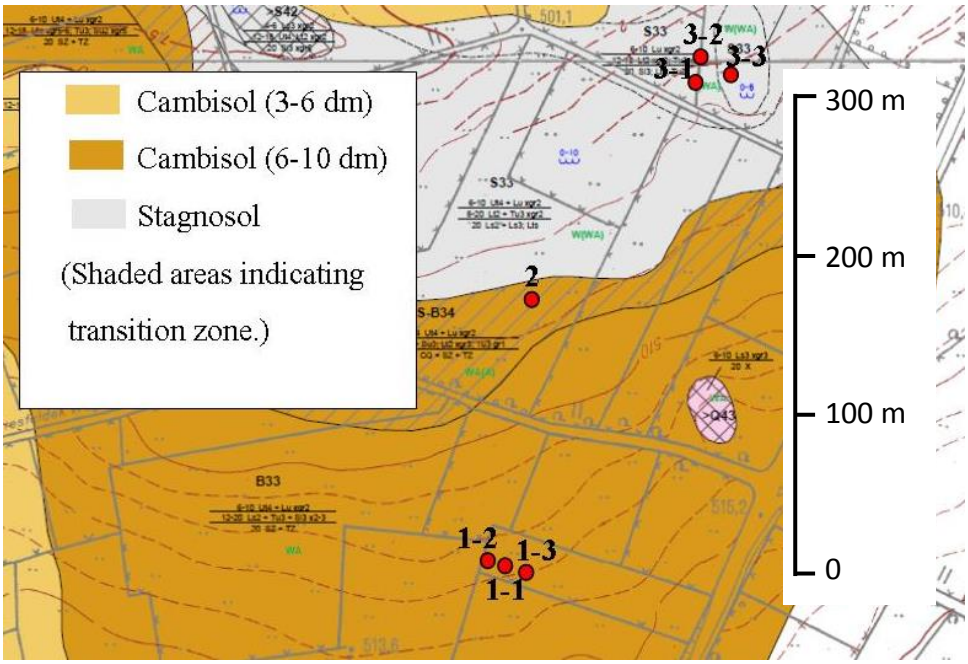

Fig. 1 Excerpt from the soil map of the test site at Rollesbroich (*modified from Geologischer Dienst Nordrhein-Westfalen, 2008*). Numbered red dots indicate location of plots.

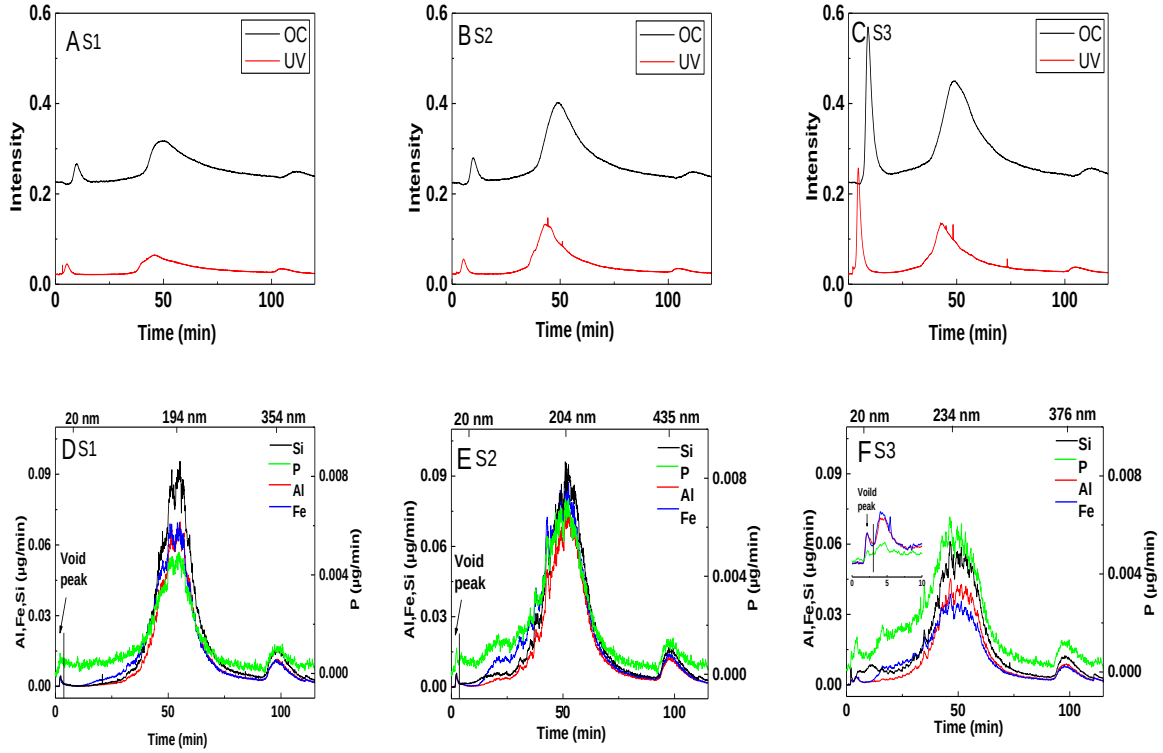

Fig. 2 Asymmetric flow field-flow fractionation (AF4) fractograms of water dispersible fine colloids (WDFCs) of S1, S2, and S3. The fractograms show the organic carbon (OC) and ultraviolet (UV) signal intensities (A, B, and C) and the Fe, Al, P, and Si mass flow (D, E, and F) monitored by inductively coupled plasma mass spectrometer (ICP-MS) of S1 (Cambisol), S2 (Stagnic Cambisol), and S3 (Stagnosol). The sizes of peaks were according to the AF4 result of sulfate latex standard particles and dynamic light scattering results. The OC and UV peaks occurred with elements (ICP-MS) peaks at the same time and the slight delay among these peaks is due to the different length of tubes to different detectors which cause slightly different internal volume and retention time.

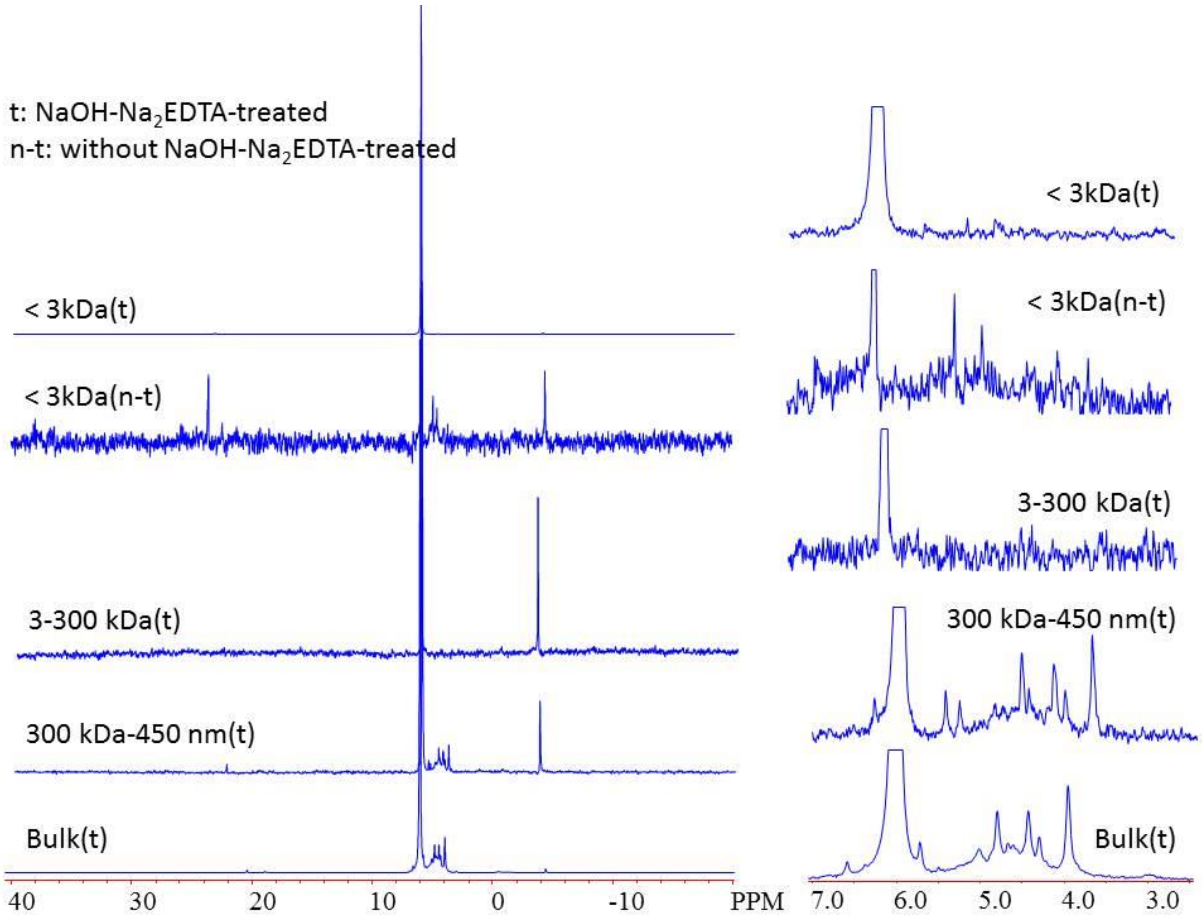

Fig. 3 Solution phosphorus-31 nuclear magnetic resonance spectra of NaOH–Na_2EDTA extracts of bulk soil, 300 kDa-450 nm, 3-300 kDa and < 3 kDa fractions in soil water extracts < 450 nm of S3 (Stagnosol).

Reviewer 1

We thank the referee for the detailed comments which helped a lot to improve the manuscript. In this manuscript, we stated a new hypothesis, revised some explanations and conclusion as follows:

Line 18: We changed the sentence "Stagnant water conditions may release phosphorus (P) in soil solution that was formerly bound to Fe oxides" into "Phosphorus (P) species in colloidal and "dissolved" soil fractions may have different distributions".

Lines 31-34: We changed the last sentence into "We conclude that P species composition varies among colloidal and "dissolved" soil fractions after characterization using advanced techniques, i.e. AF4 and NMR. Furthermore, stagnic properties affect P speciation and availability by potentially releasing dissolved inorganic and ester-bound P forms as well as nano-sized organic matter-Fe/Al-P colloids.".

Q: The extract used (MQ water) is quite harsh compared to natural waters such as rain water or pore water and would result in significantly greater release of P than that possible by contact with water in a natural environment, due to desorption and dissolution of poorly crystalline authigenic mineral phases. Living cells within the soil would also certainly undergo significant osmotic stress likely resulting in osmotic rupture and release organic and inorganic P found in intracellular components. The potential ramifications of these effects on the results should be clearly stated and discussed, as there are clearly implications as to the origin and mobility of identified P species in a natural context. Are the species identified in the size fractions indicated present in the natural soil or a result of alterations during the extraction procedure?

A: We agree that a contact of soil to rain and pore water would provide a more realistic scenario; yet, rain and pore water chemistry is variable and thus hard to standardize. As a result, the release of natural nanoparticles from soil could also be variable. Using MQ water for extraction instead of aqueous solutions with higher ionic strength, however, has also two advantages. On the one hand, as also stated by the reviewer, it increases sample dispersion in that we get access to potentially dispersible colloids. We stated this more clearly now. On the other hand, there are analytical advantages, because we avoid interference of additional ions with the retention of particles on the membrane in the channel of FFF, and because MQ water better allows to freeze-dry large amounts of soil solution. Natural water would increase viscosity for the re-dissolved solution, which could increase line broadening and thus decrease the ability to differentiate peak resonances from one another (Cade-Menun and Liu, 2014). We agree that MQ water has potential ramifications of the effects on the results as the reviewer mentioned; as such we added related discussion in the main manuscript as follows:

Lines 123-130: It is worth noting that Mill-Q water was used here to extract soil colloids instead of rain water or pore water, since total amounts of WDFCs will likely be larger when using Mill-Q water, i.e., we consider these WDFCs as potentially water-dispersible colloids. In addition, the use of Mill-Q water facilitates subsequent sample processing with AF4 and NMR. It is inevitable that Mill-Q water would result in the release of P due to desorption and dissolution of poorly crystalline authigenic mineral phases. Additionally，living cells within the soil would also certainly undergo significant osmotic stress, likely resulting in osmotic rupture and releasing organic and inorganic P found in intracellular components.

Q: I have concerns with the way in which the results are framed within the context of oxygen availability and iron redox cycling. The first sentence of the abstract "Stagnant water conditions may release phosphorus (P) in soil solution that was formerly bound to Fe oxides" implies that the P release investigated is due to reductive dissolution of ferric oxides in the absence of oxygen. Undoubtedly, oxygen availability differences between the soil samples selected resulted in differences to iron speciation, particle size, organic carbon content and P speciation. The handling of the soil samples in the laboratory does not appear to have preserved the field redox conditions and likely resulted in considerable oxidation of reduced iron species during processing, particularly in the sampled Stagnosols. Oxidation of aqueous Fe^{2+} and colloidal ferrous particles can be very fast (seconds to minutes) therefore the extraction in presumably oxic MQ water for 18 hours almost certainly changed the composition and speciation of the colloids, which were later characterized. Although the importance of Fe oxidation and reduction processes on P speciation generally is highlighted in the manuscript, the impact of these processes during sample processing and on the final dataset is not discussed. The differences between the three soil types are convincing but I question whether the analyzed species and size fractions are representative of the soils themselves or of differences in response to the extraction procedure based on different initial soil redox conditions. Extracting soils in MQ water, under oxic conditions, is not representative of P released during reductive dissolution, as implied in the abstract and in fact would result in the opposite process (oxidative precipitation of Fe hydroxides).

A: We agree. However, we also have to annotate hear that stagnant water conditions do not mean that there was stagnant water to the very top of the land surface at time of sampling. By definition, stagnant water dominates for most time of the year and most parts of the soil profile, but it must not (and was) not present in the very surface soil at each time of sampling. When we sampled, the soils were not saturated, i.e., they must have been aerobic already (as common in these surface soils, also in Stagnosols). Hence, the experiment process with Mill-Q water under oxic conditions has potential impact on oxidation of aqueous Fe^{2+} and colloidal ferrous particles, but we do not see this risk as very severe, because we sampled (and stored) the soils in aerobic conditions. We mentioned it in the manuscript as follows:

Line 130-136: It is worth noting that the experimental procedure with Mill-Q water under oxic conditions may have an impact on oxidation of aqueous iron (Fe^{2+}) and colloidal ferrous particles. However, at time of sampling, the very surface soils were not fully water saturated as allowed even for Stagnosols for time of the year. As such, the analyzed species and size fractions are representative of differences in response to the extraction procedure based on different soil

redox conditions that reflect a kind of legacy of former redox cycle, but at time of sampling and analyses the soils were aerobic.

Q: 89. Inclusion of a site map would be useful here in the main manuscript rather than in the supporting information. A scale should also be included to establish the distance between the sampling sites.

A: We added the map with a scale in the main manuscript as follows:

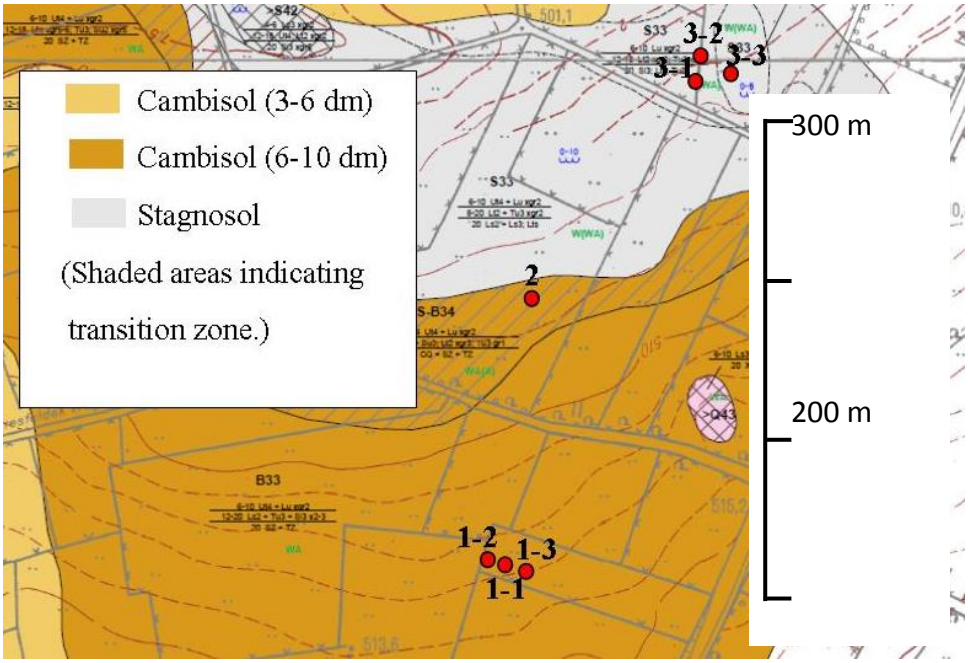

Fig. 1 Excerpt from the soil map of the test site at Rollesbroich (*modified from Geologischer Dienst Nordrhein-Westfalen, 2008*). Numbered red dots indicate location of plots.

Q: 101. How long were the samples stored at 5°C? Long storage times prior to extraction and preservation could result in significant speciation changes. It is impossible to evaluate the importance of these changes if the storage time is not provided.

A: The samples were sieved immediately to < 5 mm and stored at 5°C for less than 6 months before the extraction. All samples were stored in similar manner. The FFF characteristics of WDFCs did not change significantly in the 6 months period of the investigation. We added this information in the manuscript (lines 158-159).

Long storage time under oxic condition have potential impact on the forms of Fe-minerals in soil. However, it is also worth noting that we sampled topsoil (2-15 cm) from Stagnosol which is not the horizon where water is actually stagnating. Additionally, stagnic water conditions do not

mean that the soils are under reduced conditions for the whole year – only for some significant time of the year. Although all samples were treated the same way, differences among the samples were consistent with soil characteristics at each site. This suggests that the influences of treatment and storage were minimal.

We gave related discussion as follows:

Lines 103-106: It is worth noting that Stagnic water conditions do not mean that the soils are under reduced conditions for the whole year – only for some significant time of the year. We sampled a Stagnosol, but only the topsoil (2-15 cm) which was not under perching water, i.e., it was aerobic at time of sampling.

Lines 255-259: We cannot rule out any effects from sample storage or from the use of Mill-Q water, as discussed in the Methods section, However, although all samples were treated the same way, differences among the samples were consistent with soil characteristics at each site. This suggests that the influences of treatment and storage were minimal, but further investigation is warranted in future studies.

Q: 112. Please list the material of the 0.45 micron membranes.

A: The material of membrane was cellulose mixing ester and we added it in the manuscript (line 122).

Q: 119. There is no justification for the choice of analytes - Fe, Al, Si and Ca? The rationale for this may not be clear to some readers.

A: These elements containing minerals (e.g. clay minerals and Fe oxides) were main soil minerals which can be associated with P. We added information about this to the text (lines 145-146).

118. What were the limits of detection and precision for the analytes measured by ICP-MS?

A: The limits of detection (LOD) depend highly on the element, matrix, possible interferences and last but not least the daily performance. The precision, on the other hand, depends mostly on the concentration but also on the element and matrix. Analytes with a concentration close to the LOD have a rather poor precision, whereas higher concentrated analytes achieve a precision of typically 3-10% (relative standard Deviation) depending on the matrix and homogeneity of the samples. The LOD of measured elements in this manuscript is typically around 0.1 to 1 ug/L. We added information about this to the text (lines 157-158).

146. Was neutralization of the NaOH-Na_2EDTA extracts with HCl performed to avoid break down of polyphosphate species? Perhaps the rationale for not doing so could be included here?

A: We did not neutralize the NaOH-Na_2EDTA extracts with HCl prior to lyophilization, although this was recommended by Cade-Menun et al. (2006, EST 40:7874-7880). Neutralization of samples has not been widely adopted, and was not used by Liu et al. 2014 in their study of WDCs. However, it is something to consider for future studies.

169 – "for identify" I believe this should read "to test for significant differences" or "to identify significant differences".

A: Yes, we changed it into "to test for significant differences among soil fractions" in the manuscript (line 201).

170 – Which tests were employed to determine distribution normality?

A: We used a Shapiro-Wilks test for normality. This is now indicated in the text (line 202).

178 – Analysis of Ca is not previously mentioned. Either calcium analysis should be included in the methods section, and the statement clarified i.e. what constitutes a low concentration? Or this statement could be removed here.

A: We removed this statement.

202 – The effect of pH, and differences between the sampled soils should probably be discussed here.

A: Please see our response to Reviewer 2 on this topic.

216 – This discussion needs to take into account the effect of the reactions that likely occurred during the oxic extraction procedure.

A: We added the following comment on lines 255-259: We cannot rule out any effects from sample storage or from the use of Mill-Q water, as discussed in the Methods section, However,

although all samples were treated the same way, differences among the samples were consistent with soil characteristics at each site. This suggests that the influences of treatment and storage were minimal, but further investigation is warranted in future studies.

280 – clay-Fe oxides is an interpretation based on elemental analyses, it is not certain that the colloids identified contain clay minerals from the analyses conducted.

A: We cannot identify clay minerals according to FFF and element analyses. However, clay minerals with Si and Al elements and Fe oxides are common minerals for soils. We did TEM experiments for arable soils in a prior study (Jiang et al., 2015) and found clay minerals and Fe oxides in soil colloids.

Lines 145-146: These elements were analyzed as part of the main soil minerals (e.g. clay minerals and Fe oxides) that can be associated with P (Jiang et al., 2015a).

Table 1 – Dissolved or total organic carbon? If this refers to the bulk soil it is not dissolved organic carbon but total organic carbon? The table caption refers to uppercase letters but the letters indicating significant differences are lower case.

A：We changed the table caption as suggested.

Table 2 – To help distinguish between bulk solid analyses and analysis of water extracts I suggest using mg kg-1 for bulk soil analysis and mg L^{-1} for water analysis. Also –TOC for bulk solid analysis and DOC for aqueous and colloidal analyses.

A: We changed TOC into DOC as suggested. With respect to the unit of water analysis, we still prefer mg kg^{-1} because mg L^{-1} cannot directly tell readers the concentrations of colloidal and dissolved elements compared to those in bulk soil.

Table 3 – The formatting and alignment issues make this quite hard to read. “below detection limit” is noted but the detection limit is not defined?

A: Below detection limit <0.05%. We added it in the Table 3.

Reviewer 2:

We thank the referee for the detailed comments which helped a lot to improve the manuscript. In this manuscript, we stated a new hypothesis, revised some explanations and conclusion as follows:

Q：Comment 1: The paper does not give any information about the agricultural use of the sampled grassland sites. Are these sites used for cattle breeding. Do they receive P-containing manure? Are they, or were they, subject to inorganic P fertilization? Were the land use or P fertilization, if any, similar in the cambisol and stagnosol plots? The authors should provide information on those different points.

A: As we know, the grassland vegetation is dominated by perennial ryegrass (*Lolium perenne L.*) and smooth meadow grass (*Poa pratensis L.*). We do not have any information about the amount of P fertilization but we know that there were different managements among these there soils according to personal observation as follows:

-for the Cambisols: extensive meadow with three to four cuts per year, no cattle grazing.
-for the Stagnic Cambisols: cattle pasture but with less frequent grazing than the Stagnosols
-for the Stagnosols: intensively used as pasture with frequent cattle grazing followed by harrowing with a tire-drag harrow and application of organic manure (cattle slurry).

We added this information in manuscript (lines 90-96).

We also added the following sentence to the discussion of the NMR results (lines 311-316):

However, we cannot rule out the effects of differences in grazing and manure application on the P forms in these soils. Cattle grazing and the application of cattle slurry would be expected to add P that is predominantly orthophosphate, with lower concentrations of organic P forms including *myo*-IHP (Cade-Menun 2011 and references therein). As such, this may have contributed to the increased orthophosphate and decreased organic P we observed on these sites.

Comment 2: Could the authors specify why they use Mill-Q and a pH set at 5.5 in their extraction experiments. Although I can understand that the aim of their study is to compare the behavior of different soils to colloidal extraction and not the impact of the nature of a given extractant and a given pH, I know from my own experience that the nature of the solution and the pH used during colloidal extraction may have great impacts on the composition of the colloids extracted and on the partitioning of P between the colloidal and dissolved phases. Therefore, a justification of the choices made regarding the extracting solution is necessary.

A: Please see our response to Reviewer 1 on this topic.

Comment 3: Too little information is given with respect to the ultrafiltration procedure used for preparing the ^{31}P-NMR spectroscopy samples. First, 600 ml is quite a large volume to ultrafiltrate. Most probably, more than one filter had to be used to ultrafiltrate such big volumes. Could the number of filters used be given? Second, of which

material are the filters made of? Are they made of cellulose acetate and if so what are their organic (and phosphorus) blank(s) and the blanks of the overall ultrafiltration procedure?

A：Around 6 filters were used to ultrafiltrate 600 mL solution. The filter was made of regenerated cellulose membrane. Before the ultrafiltration of samples, we washed the filter by filtering Mill-Q water. The P concentration of < 3 kDa fraction of sample 1 is 0.1 ±0.1 mg/kg, which means there was negligible P concentration in sample 1. It also indicated that the filter material did not affect the P concentration of soil samples. Therefore, although we did not perform the blank experiment, we do not think that there was any P from the filtration. Also according to the NMR results, there was no organic P in 3-300 kDa fractions of soil samples which means there was no potential organic P from the filters into soil samples.

Q: Comment 4 : Could the authors explain why, in parallel of the ultrafiltration procedure they used to prepare ^{31}P-NMR spectroscopy samples, they also mixed soil water extracts with 0.025M NaOH and 0.05 M Na_2EDTA solution. Was their purpose in doing so to compare these two preparation techniques to check if they were going to give similar (or different) results? Was there a risk that the ultrafiltration technique on its own could fail to give reliable results?

A: Comment 4: We did not explain it clearly here. The different-sized soil water extracts were obtained by the ultrafiltration procedure and then each size-range soil water extract was mixed to receive sufficient samples for the ^{31}P-NMR characterization. Each size-range soil water extract was then mixed with 0.025 M NaOH and 0.05 M Na_2EDTA to extract P for ^{31}P-NMR (see section 2.3). This was done so that all P-NMR experiments were conducted in the sample matrix for bulk soil and soil water extracts.

Q: Comment 5: The authors should add a size scale in the top diagrams of Fig. 1, in which the OC and UV peaks are portrayed. Anyhow, the first peak on the left of these diagrams seems to occur for a particle size slightly higher than 20. Why then quote in the text (line 181) that the particles corresponding to this first peak are <20 nm in size.

Why then also consider in line 186 that only two fractions are higher in particle size than 20 nm? If the same size scale as that shown in the bottom diagrams in Fig. 1 is transposed to the top ones, all the three OC and UV peaks occurring in the latter seem to be for particles of > 20 nm size. Therefore, I am not convinced in the current state

of Fig. 1 that the first peak recorded in the analyzed samples correspond to particles below 20 nm.

Comment 6: In all the top diagrams of Fig. 1 there is a shift between OC and UV peaks, the UV peaks occurring systematically at a lower elution time than the OC ones. What is the reason for this shift?

A: Comment 5 and 6: Actually the OC and UV peaks occurred with element (ICP-MS) peaks at the same time. The AF4 is connected with UV, OCD and ICP-MS detector with different-length

tubes. The slight delay among these peaks is due to the different length of tubes to different detectors which cause slightly different internal volume and retention time (see lines 585-587).

Q: Comment 7: I have problems with the idea promoted by the authors that the OC concentration of the first particle fraction would increase from samples 1 to 3. Indeed, the information provided by the top diagrams in Fig. 1 to which they refer lines 188 to 190 are intensities not concentrations. Would it not be equally possible that the OC concentration of the <20 nm particles is in fact constant in the three samples, but that the concentration of this size class of particles in the water extracts increases from sample 1 to 3 ?

A: Comment 7: We did the calibration of different OC concentrations and found the OC concentrations had a linear positive relation with intensities. It cannot be determined if the concentration of OC or the particles increases from samples 1 to 3. Here the OC concentration is the ratio of OC mass to bulk soil mass but not to <20 nm soil particle fraction.

Comment 8: The authors argue that the occurrence of distinct Al and Fe peaks in the first size fraction of the Stagnosol could suggest that oxides are more readily involved in nano-sized soil particles under stagnant soil conditions. I find this interpretation surprising as stagnant conditions are expected to limit the stability of iron oxides. Should it be possible that Fe and Al peaks found in this fraction correspond not to oxides but to Fe and Al ions adsorbed onto, or complexed by organic matter? What proves that the Fe found in this fraction is Fe^{3+} and not Fe^{2+}. The authors should consider alternative hypotheses of that type here as they do not provide any direct (e.g. spectroscopic) evidence of the presence of Al and Fe oxides in their samples?

A: Comment 8: It is correct that we need to consider alternative hypotheses with Fe and Al ions besides the iron oxides (see lines 229-231: Nanoparticulate humic (organic matter)-Fe (Al) (ions /(hydr)oxide)-phosphate associations have recently been identified both in water and soil samples (Gerke, 2010; Regelink et al., 2013; Jiang et al., 2015a)). Some published studies have shown the existence of P-Fe/Al-OC complexes with size of ~5 nm (Regelink et al., 2013). In our previous studies with Luvisols (Jiang et al., 2015), we found amorphous Fe/Al oxides in the smaller-sized fractions. However, we did not undertake such specific experiments in the case of Cambisol and Stagnosol.

Q: Comment 9 : The concentrations quoted in Table S1 are in mg/kg. How these concentrations were calculated? To what refer Â´n kg Â¨z in this table? To the amount of solution in which the particles are eluted ? To something else ? Please, give precision on that.

A: Comment 9: mg/kg soil particles.

Q: Comment 10 : The mechanism promoted by the author of a higher OC concentration in the first peak of the Stagnosol sample due to the release of OC from the larger colloidal fraction because of reductive dissolution of the iron oxides present in this fraction is plausible. However, the AF4 experiments were not performed under reducing

conditions. Although this mechanism could probably occur in the true soil solutions under the water saturated conditions that usually prevail in the field in Stagnosol-type soils, I am sceptical about the fact that it could developed in the present case, as the experiments were apparently performed under aerobic conditions.

A：Comment 10: It is correct that the experiments were performed under aerobic conditions. However, the AF4 experiment showed the current properties of the Stagnosol and Cambisols. The Stagnosol soils had higher OC concentration in the first peak than the Cambisol. We only say the high OC content in the first peak is apart from the reductive dissolution of iron oxides. A generally slower degradation of organic matter under limited oxygen supply in Stagnosol was another factor for the high OC content. Although the experiments were performed under aerobic conditons, we do not think all the iron ions will be oxidized into iron oxides in our experimental conditions. Stagnic water conditions do not mean that the soils are under reduced conditions for the whole year – only for some significant time of the year. We sampled a Stagnosol, but only the topsoil (2-15 cm) which is not the horizon where water is actually stagnant. As such, the Stagnols used for this study were oxic at various times each year, but also experienced periods of reducing conditions that did not occur in the other samples along the transect.

(see lines 103-106).

Q: Comment 11 : The hypothesis brought about by the authors that the oxygen limitation and reduction regime of the Stagnosol would favor the dissolution of Fe oxides in Stagnosol colloids is not entirely convincing to me. Indeed, Table 2 shows higher Fe concentrations in S3 than in S1 sample colloids. Could it be possible that the Fe found in S3 colloids is in part Fe2+ and not Fe oxides?

A: Comment 11: Table 2 shows higher Fe concentrations in S3 but the standard deviation is also extremely high (4.6±3.3), suggesting there were no significant difference between S3 and S1 (2.1±0.5). As shown in the FFF result in Table S1, Fe concentrations in the second peaks in Cambisol and Stagnosol were 7.60±2.11 and 7.34±0.53 mg/kg. It is also possible that the Fe found in S3 colloids were in part Fe^{2+} absorbed onto the surface of particles.

Q: Comment 12: I agree with the authors that the dissolution of Fe oxides in the Stagnosol could release Po in the soil solution, but the fate of this Po puzzle me. It seems implicit for the authors that this Po should be readily mineralized and transformed into Pi. However, Stagnosol being waterlogged soils, we expect a reduction of the microbial activity in these soils and thus of the mineralization rate of Po. I am also not convinced by the hypothesis promoted by the authors on line 276 that the formation of OC-Fe/Al-PO_4^{3-} should be favored in these soils. Why should it be so, particularly if iron

oxides are expected to be dissolved due to the reducing conditions that characterized Stagnosol as suggested by the authors earlier in the paper. I see a lot of contradictions and approximations here.

A: Comment 12: The comment about the Po is reasonable and we have no proof that this Po will be readily mineralized and deleted it in the manuscript. The reason why the formation of OC-Fe/Al-PO_4^{3-} should be favored in Stagnosol soil is that more OC in Stagnosol will bind to more PO_4^{3-} to form the complex of OC-Fe/Al-PO_4^{3-}. Although iron oxides are expected to be dissolved in Stagnosol, some Fe oxides are expected to exist in this soil. Stagnic water conditions do not mean that the soils are under reduced conditions for the whole year – only for some significant time of the year. We sampled a Stagnosol, but only the topsoil (2-15 cm) which is not the horizon where water is actually stagnant. On the other hand, Fe/Al ions could also form the complex of OC-Fe/Al-PO_4^{3-}. (see lines 229-231)

Q: Comment 13: I have difficulties to understand the conclusion of section 3.3 stating that "pedogenesis also affects the redistribution of different P species among different P colloids and the electrolyte phase". I do not see in which respects the results presented in this study allow to put constrains on the pedogenesis of the studied soils and on the impact of this pedogenesis on the present P speciation results. In my opinion, other variables like land use, anthropogenic P inputs or the methodology used to extract colloids are likely to be as important as, and maybe more important than the pedogenetical history of soils in creating difference in P speciation among soils.

A: Comment 13: It is correct and we revised it as suggested:

Lines 361-364: In any case, both colloidal aggregation and changes in soil order paralleled soil P forms. However, also other soil properties but former redox state (like pH), as well as variations in anthropogenic, site-adapted management may be additional covariates affecting P colloids and composition.

Q: Comment 14: Why pyrophosphate of microbial origin should it be more abundant in Stagnosol than Cambisol, considering that the microbial activity should be enhanced in the more oxygenated Cambisol? I do not pick up authors' arguments here.

A: Comment 14: we deleted the sentence: Pyrophosphate may be of microbial origin (Condron et al., 2005).

Q: Line 85 : How an organic carbon detector works. Could you specify or quote a refrence in which the principle of the method is described.

A: The OCD is a promising technique for monitoring organic carbon concentrations for liquid-flow based separation systems with the advantages of high selectivity and low detection limits (Nischwitz et al., 2016). Briefly, the operation principle is that the acidification of the sample flow removes inorganic carbon and subsequently the organic carbon is oxidized in a thin film reactor to carbon dioxide which can be quantified by infrared detection (Nischwitz et al., 2016). We added these sentences in the materials and method section (lines 146-151).

Q: Line 116 : Replace " ; " by " . "

Line 119 : Replace " …for monitoring of Fe, aluminum (Al)…" by Â´n: : : for monitoring

of iron (Fe), aluminum (Al): : : "

A: We changed the first sentence. However, iron was defined as Fe earlier in the manuscript, so we did not change the second sentence.

Line 132-133. What do the authors mean by "the nano-sized colloidal particles after AF4 separation were smaller than < 20 nm" ? According to Fig. 1, the colloidal particles recovered by the AF4 indeed ranged in size from 20 to 435 nm with peaks at 204 and 435 nm ! Do you mean that the AF4 technique separated all colloids with a nominal size > 20 nm

A: We defined the first peak fraction as nano-sized colloidal particles. Definitely it misleads readers. We changed the sentence as follows:

Lines 164-165: The first peak fraction after AF4 separation has the particle size smaller than ~20 nm.

Q: Line 150. I suggest the authors start a new paragraph from "Solution 31P-NMR…:" as they change of topics from that point, shifting from the description of how the samples were prepared to how the NMR spectra were obtained.

Line 169 : Replace "…test to test foridentify…" by " …test to identify… "

A: We changed them as suggested.

Q: Lines 183-184 : What more direct evidence have the authors that the third peaks in the fractograms could correspond to particle previously attached to the mebrane during focus time ?

A: The third peaks occurred after cross flow was zero. There was no force to bring the particles closed to the membrane. Therefore, the former particles attached to the membrane have possibly been eluted from the channel by the carrier flow.

Lines 195-196 : Is the claim made here that the nano-sized colloids from the cambisol contain P, Fe and Al in lower (negligible) concentrations compared to the same fraction in the stagnosol so true ? Indeed, I calculated the OC/Al and OC/Fe ratios of both soil types and they are not so different: 58 and 93 for Al, and 74 and 105 for Fe. Everything looks like if the nano-sized colloid fractions were equivalent in composition in both soil types, the fraction being simply more concentrated in the stagnosol compared to the cambisol.

A: It is correct. We also just mention that there were higher concentrations of OC, P, Fe, and Al in the nano-sized colloids from the Stagnosol compared to the Cambisol. The concentrations here were the ratio of elemental mass to bulk soil mass. We indicated it in the Tables.

Line 202: Replace “Stagnols” by “Stagnosol”

A: We changed it as suggested.

Line 205: I agree that the UV signal is consistent with the OC peak distribution. However, I once again wonder about the reason why the UV peaks are shifted to somewhat lower elution time compared to the OC ones. Could the authors comment on that and provide explanation for this shift?

A: That is because the AF4 is connected with UV detector and the OCD detector were then connected with UV. The slight delay among the two peaks is due to the different length of tubes to UV and OC detectors which cause slightly different internal volume and retention time (see lines 585-587).

Q: Line 208-209: I agree that the second-size fraction of the stagnic Cambisol present the highest Fe, A, Si and P concentrations of the three analyzed second size-fractions. Considering however ratios of OC/Al, OC/Fe, and OC/P

A: The concentrations here mean different elements amount per kg soil not per kg OC. We indicated it in the Tables.

Q: Line 218 : It is not clear to me why OC sorbed on iron oxides materials should be of nanometric size? Could the authors cite papers which prove this to be so.

A: We found more OC in the first peak fraction, and assumed this additional OC was partly derived from the OC formerly sorbed on iron oxides. When iron oxides were dissolved, this sorbed OC was released as nano-size particles and was eluted in the first peak. We do not say all the OC sorbed on iron oxides should be of nanometric size. We think a part of nano-size OC in the Stagnosol was derived from the OC formerly sorbed on iron oxides. As found in our previous study with soil colloids of Luvisols (Jiang et al., 2015), higher OC content were found in the first peak fraction after the dissolve of Fe oxides with DCB treatment. Lines 247-249: Correspondingly, the dissolution of Fe oxides in the second fraction under stagnant water may also liberate OC from the organo-Fe mineral associations, thus releasing some OC to the nano-sized first fraction (Jiang et al., 2015a).

Q: Lines 237-238 : "This implies that the assignment of stagnic properties is related to its behavior in the colloidal particles and dissolved fraction". I find this sentence badly constructed. Do the authors mean that soils are classified according to the composition of the colloids they can release? I cannot believe that.

A: We changed it as follows:

Lines 272-273: This implied that the stagnic properties have a greater impact on the colloidal particles and "dissolved" fraction compared to bulk soil.

Q: Line 243 (as regards Table 2): I wonder why TOC concentrations were not measured in the <300 KDa and < 5KDa fractions. Could the authors give an explanation for that?

A: The OC detector cannot give valid values to distinguish <300 kDa and <3 kDa fractions because of the extremely low concentration of OC in 3-300 kDa fractions.

Q: Lines 280-281. I do not see how Fig. 1 can be used to infer the proportion of clay-Fe oxides-OC-P associations in the 300 KDa-450 nm fractions. Could you explain?

A: The FFF results showed that there were Si, Al, Fe, OC and P in the 300 kDa-450 nm fraction. We measured another arable soil sample with TEM in former work (Jiang et al., 2015) and found clay minerals and Fe oxides in these fractions from soil (see lines 145-146).

Q: Line 300: The statement made here that the majority of P in the <3KDa fraction of the Cambisol was Po is quite "funny" in the light of what is said page 11 about the fact that the absence of NaOH-Na2EDTA most of the Pi is removed from the solution through sorption on the soil mienrals. Quite clearly, the data cannot be used to assess the proportions of Pi and Po as the methodology used biased these proportion. They just can be used to inventory the organic species present, which is already an innovative and very important objective.

A: We agree and changed this sentence to: the majority of observed P in the < 3 kDa fraction of the Cambisol was organic P (lines 340-341).

Q: Line 348-352. What direct poof do the authors have that pyrophosphates is bound to Fe oxides? Could and alternative interpretation be that orthophosphates form ternary complexes with Fe^{3+} or Fe^{2+} ions themselves bound to OC?

A: Here Fe/Al means ions and we will emphasis it in the main text as follows:

Lines 388-391: Considering that a high proportion of pyrophosphate (38.5%) existed in the 3-300 kDa fraction of the Stagnosol, which contained P mainly in OC-$Fe(Al)^{2/3+}$-P associations (see above), it seems reasonable to assume that pyrophosphate existed as a colloidal OC-$Fe(Al)^{2/3+}$-pyrophosphate complex.