**Title:**

# $^{18}O$ isotope labeling combined with $^{31}P$ nuclear magnetic resonance spectroscopy for accurate quantification of hydrolysable phosphorus species in environmental samples

**Liming Wang [1, 2*], Wulf Amelung [2, 3], and Sabine Willbold [1*]**

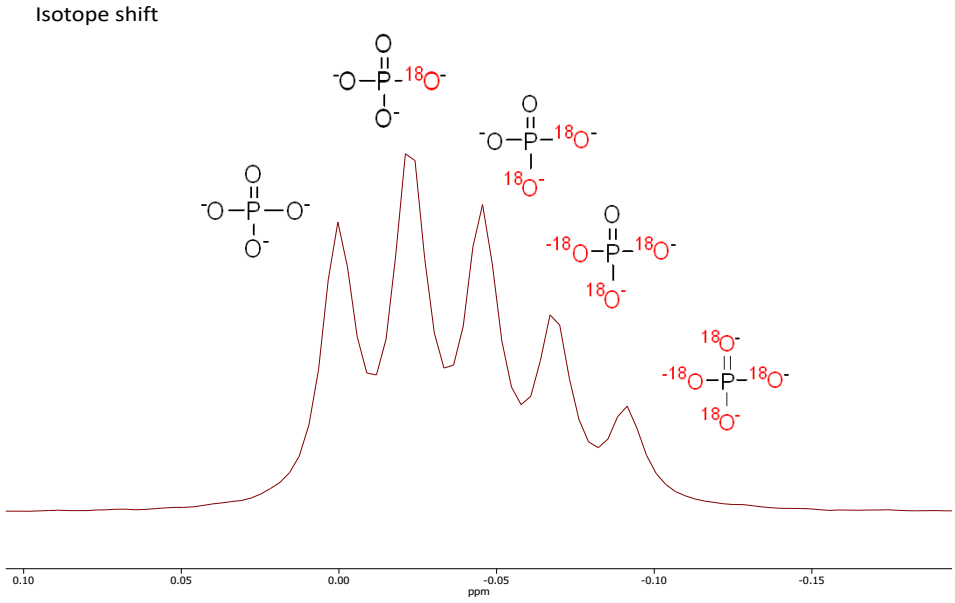
1 Central Institute for Engineering, Electronics and Analytics, Analytics (ZEA-3), Forschungszentrum Jülich GmbH, 52425 Jülich, Germany

2 Institute of Bio- and Geosciences, Agrosphere (IBG-3), Forschungszentrum Jülich GmbH, 52425 Jülich, Germany

3 Institute of Crop Science and Resource Conservation, Soil Science and Soil Ecology, University of Bonn, Nußallee 13, D-53115 Bonn, Germany

* Correspondence to: Liming Wang, lim.wang@fz-juelich.de, tel: +49 2461618964, fax: +49 2461611970. Sabine Willbold, s.willbold@fz-juelich.de, tel: 49 2461616063.

13 **Table of contents**




14

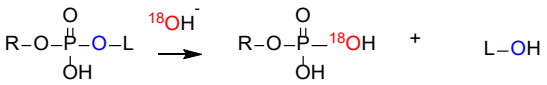
**Abstract**

$^{31}$P nuclear magnetic resonance (NMR) spectra can be biased due to the hydrolysis of labile P species during sample treatment and NMR analysis. This paper offers an approach to circumvent this problem by performing sample preparation and analysis in $^{18}$O-enriched medium. Heavy $^{18}$O isotope atoms were introduced into the resulting artificial hydrolysis products. The NMR signal of $^{18}$O-labeled P was shifted upfield relative to the unlabeled P nuclei in natural metabolites. This isotope shift enabled an immediate differentiation of artificial hydrolysis products from natural metabolites. Moreover, the hydrolysis products could be accurately quantified. Our data suggest that the extent to which artificial hydrolysis alters NMR spectra varies among different types of environmental samples. For instance, 72–84 percent of the detected monoesters in the organic soils of this study were actually artificially hydrolyzed diesters. By contrast, artificial hydrolysis products in the mineral soils used for this study accounted for less than 6 percent of the total monoesters. Polyphosphate was also hydrolyzed to yield $^{18}$O-labeled products in algal biomass.

Phosphorus (P) is a key element for the functioning of ecosystems. Understanding P cycling is essential for maintaining adequate crop yields while minimizing environmental risks.[1,2] $^{31}$P-NMR has been employed for nearly four decades to investigate organic P in soil samples.[3] Improved NMR spectral quality due to refinement of acquisition parameters[4,5] and evolution of NMR instrumentation[6] further promoted universal application of the procedure for P studies. The combination of NaOH-EDTA extraction with $^{31}$P NMR spectroscopy was reported for the first time in 1996 [7] and subsequently refined with regard to sample preparation and experimental parameters.[4,8,9] Then, NaOH-EDTA extraction followed by NMR analysis has been the standard procedure for P studies.[5,10,11] However, the alkaline condition was also known to hydrolyze labile P species, such as polyphosphate,[12] phospholipids, and RNA.[13-18]

Organic phosphorus compounds occur in a variety of forms, mainly including monoesters, diesters, phosphonates, and polyphosphates.[19] A critical feature of phosphate is that it has three ionizable hydroxyl groups. Organic P compounds, which have one or two alkylated hydroxyls, can still have negatively charged oxygen (O) atom(s) repelling nucleophilic attack. Thus, organic P compounds should, in principle, be highly resistant to spontaneous hydrolysis.[20] Nevertheless, certain P compounds, such as phospholipids, RNA, and polyphosphate are unstable. The hydrolysis pathways of these labile P compounds have been identified,[20-22] as illustrated in Figure S1. The breakdown of phosphodiester linkage in phospholipids and RNA is initiated by the formation of a cyclic intermediate, which is then opened by a nucleophilic hydroxyl group. Eventually, two isomers are produced. Alkaline hydrolysis of polyphosphate involves nucleophilic attack and cleavage of terminal P groups to produce orthophosphates with concomitant formation of a ring intermediate, trimetaphosphate, which can be rapidly broken down to tripolyphosphates.[23] Below 30 $^{o}$C, alkaline hydrolysis of tripolyphosphate is very slow and yields orthophosphate and hydrolytically more

stable pyrophosphate.[24] In particular, two aspects should be noted. First, ring formation by intramolecular hydroxyl group attack is an essential step for the cleavage of P-O bond. This fundamentally explains the high hydrolytic reactivity of these labile P compounds. For example, RNA hydrolysis is approximately two orders of magnitude faster than that of DNA,[20] because the absence of the 2'-hydroxyl group in DNA makes formation of a ring intermediate impossible. This fact forms the basis for DNA as a storage material for genetic information in higher organisms.[20] Second, all hydrolysis pathways involve O atom exchange, as illustrated in a simplified manner in Figure 1. The leaving group takes away one O from the phosphate group, and a new O from the solution matrix is attached to the hydrolysis products ($S_N2$ (P) mechanism).[25,29]




R: alkyl group

L: Leaving group

**Figure 1. Simplified scheme for alkaline hydrolysis of P ester linkage involving oxygen exchange by a nucleophilic attack.**

The hydrolysis of labile organic P compounds alters structural information. As a result, some of the obtained spectra may lead to misinterpretation in terms of P fate in the environment. For instance, previous soil P studies reported that the content of diesters and polyphosphates were usually underestimated due to the artificial hydrolysis.[4,14,30] A possible way to correct the quantification error is to suppress artificial hydrolysis by reducing the extraction time and/or NMR analysis time, since the extent of P hydrolysis is proportional to the exposure time of P compounds to nucleophilic agent (e.g., $OH^-$). However, both P substrates and $OH^-$ are greatly concentrated after reconstituting the freeze-dried extracts

in alkaline solution, which means the P hydrolysis rate will be substantially accelerated.[21] Moreover, in order to acquire quantitative $^{31}$P spectra, NMR analysis time cannot be reduced indefinitely and usually takes hours, which allows considerable P hydrolysis such as RNA (Figure S2). Consequently, it is hardly possible to alleviate P hydrolysis to an ignorable extent even using minimum extraction time and NMR analysis time. Recent studies have re-quantified diesters by including all mononucleotides and α-, β-glycerophosphate,[17,31-34] but this methodology may risk inclusion of α- and β-glycerophosphate and mononucleotides that are present in the monomer form (for example, following *in situ* natural degradation of diesters) as diesters. Therefore, for a correct understanding of P transformation in the environment, we urgently need to differentiate true natural degradation from artificial hydrolysis.

The aim of the present study is to treat samples in $^{18}$O-enriched medium, in order to label the hydrolysis products with $^{18}$O if hydrolytic cleavages occurs. Heavy isotope substitution shortens the P-O bond and increases the shielding effect on the P nucleus. The corresponding P signal will shift about 0.02 ppm upfield in $^{31}$P NMR spectra,[35,36] which is known as isotope shift.[37] Then the artifacts ($^{18}$O labeled) can be principally differentiated from natural metabolites (unlabeled) via the effect of isotope shift. Adding up the content of $^{18}$O labeled artifacts will allow an accurate quantification of their precursors. The ultimate purpose is to correct the P quantification error in environmental samples.

## MATERIALS AND METHODS

**Reference standards and chemicals.** Reference standards of RNA, phosphatidylcholine, *myo*-inositol hexakisphosphates (IHP), α-, and β-glycerophosphate were purchased from Sigma-Aldrich.

Methylenediphosphonic acid (MDPA) was supplied by Alfa Aesar. $^{18}O$-enriched water (98 percent) was obtained from Campro Scientific GmbH.

**Sample information.** A variety of samples were selected for testing. The whole O layer, including the L, Of, Oh1, and Oh2 soil horizons of a Tangelhumus profile (Histosol) located at Wettersteinwald in the Bavarian limestone Alps (denoted as **WETT**), was sampled. More information on WETT sites have been reported elsewhere.[28] Two mineral soils were used: one is from the surface A horizon (Cambisol, **ROLL**) of the Rollesbroich grassland belonging to the TERENO long-term field observatory network in Germany; and the other is an A horizon (Leptosol, **TUTT**) from Tuttlingen forest in southern Germany. Detailed information on ROLL and TUTT can be found in previous studies.[38,39] Two other environmental samples were also included: a desert vegetation sample (*Cristaria*, **VEGE**) from the Paposo transect of the Atacama Desert in Chile and a microalgae sample (*Chlorella vulgaris*, **ALGA**) cultivated in the lab, as reported earlier.[40] Sample information and elemental content are given in Table S1. The samples were dried in oven at 40 °C. Hydrolysis of P compounds during sample drying cannot be totally excluded and may affect the 18O labelling; however, we assume that this effect is unlikely significant because P compounds were not exposed to $OH^-$.

**Experimental procedure.** NaOH and $Na_2EDTA$ were dissolved to a concentration of 0.25 M NaOH and 50 mM $Na_2EDTA$ in 98 % $H_2{}^{18}O$ ($^{18}O$-matrix) and normal water ($^{16}O$-matrix), respectively. In the $^{18}O$-matrix, $H_2{}^{18}O$ is under constant autodissociation[41] and exchanges O with $^{16}OH^-$ to produce $^{18}OH^-$. The final equilibrium involves dominant amount of $^{18}OH^-$ along with a slight amount of $^{16}OH^-$, as with a similar case involving hydrogen-deuterium exchange in liquid water.[42]

107 The hydrolysis of labile organic P compounds can occur at all stages of experiment, including NaOH-
108 EDTA extraction,[14-16] lyophilization,[43] redissolution of freeze-dried solids,[21] and NMR analysis. Each
109 sample was prepared and analyzed in the $^{18}O$- and $^{16}O$-matrix, separately. The $^{18}O$ labeled signals can be
110 differentiated through direct comparison between $^{18}O$ and $^{16}O$ spectra of the same sample. For sample
111 extraction, 100 mg of dried samples was dispersed in 2 mL of $^{18}O$- and $^{16}O$-matrix, respectively, and
112 shaken for 16 h. The suspension was then centrifuged at 14000 g for 30 min and the resultant
113 supernatant was then frozen and subsequently lyophilized at -80 °C. The freeze-dried solids were re-
114 dissolved in the $^{18}O$- and $^{16}O$-matrix, respectively, and then centrifuged again at 14000 g for 30 min. The
115 supernatant was decanted into the NMR tube and spiked with 10 µL MDPA solution (7 $mg \cdot mL^{-1}$ in $^{18}O$-
116 and $^{16}O$-matrix, separately) for quantification purpose. $D_2O$ in a capillary tube was placed into the NMR
117 tube for field lock.

118 **NMR parameters.** NMR data were acquired at a $^{31}P$ frequency of 242.95 MHz on a Bruker 600 MHz
119 spectrometer, equipped with 5mm broadband CryoProbe Prodigy$^{TM}$. The 1D spectra were acquired with
120 the following parameters: 90° pulse calibrated at 12 µs, 0.99 s acquisition time, no spinning, 298 K, proton
121 inverse-gated decoupling, 3088 scans. The isotope shift after $^{18}O$ labeling is merely 0.02 ppm, and the $^{18}O$
122 labeling essentially introduced additional lines to the crowded monoester region. Therefore, special care
123 was taken to boost spectral resolution in order to differentiate labeled and unlabeled signals. For instance,
124 the samples are conductive and can be heated by the decoupler coil during acquisition.[47] Thus, the spectral
125 resolution suffers from disturbance of the shimming of the static magnetic field by volume change.
126 Therefore, an excessively long pulse delay of up to 18 s was used to cool down the samples and restore

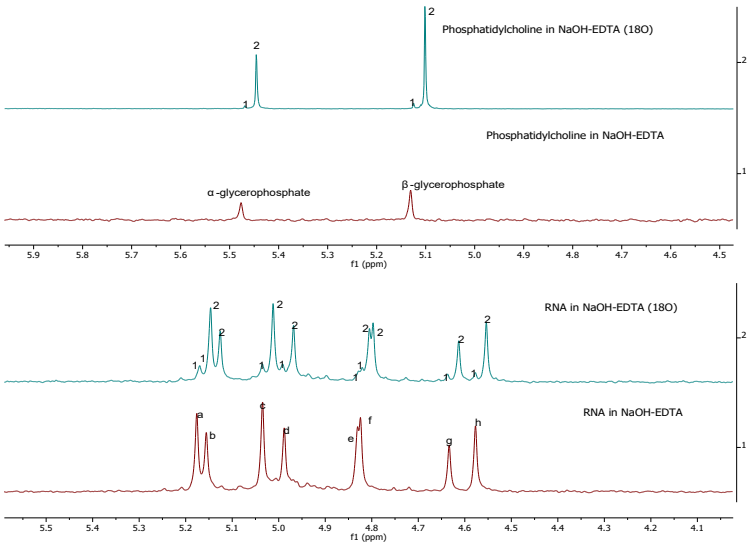
the shimming before each scan. Moreover, no line broadening was applied for data processing (MestReNova software, version 8.1.2-11880). Line-fitting was also performed with MestReNova. Due to the expense and time constraint, NMR measurements of replicate extracts have not been performed regularly.[48] The error for NMR analysis of soil and similar samples reportedly ranged from 5–10 percent.[9,49] Precision was also evaluated here using a mixture of RNA and phosphatidylcholine. We acquired NMR spectra in triplicate and accumulated a similar signal-to-noise ratio to real samples. The quantification errors were 2.4 percent and 1.8 percent for phosphatidylcholine and RNA, respectively.

## RESULTS AND DISCUSSION

**Model compounds.** The NMR spectra of phosphatidylcholine and RNA reference standards in two matrices are shown in Figure 2. The two reference standards in the $^{18}O$ matrix give more signals than those in the $^{16}O$ matrix. Phosphatidylcholine in the normal $^{16}O$ matrix, for example, yields two monoester signals, α-, β-glycerophosphate with the α-form downfield [21] (Figure 2, second spectrum from the top). When measured in the $^{18}O$ matrix, the spectrum clearly shows two more signals that are $^{18}O$ isotope labeled, upfield by approximately 0.02 ppm. The ratio of α- to β-form is 0.6, identical to the data in previous work.[21] The appearance of two unlabeled signals is due to $^{16}O$ residues in $^{18}O$-enriched water plus the $^{16}O$ introduced by NaOH, causing a systematic error in quantifying the artificially hydrolyzed phosphorus compounds. It is referred to here as $^{16}O$ error and estimated as 4.5 percent based on the integration ratio, $^{16}O$ / ($^{16}O$+$^{18}O$).

RNA hydrolysis is complete in 37 hours (Figure S2) and its degradation products contribute to four resonances of 2'- nucleotides and the other four from 3' forms (Figure 2, bottom spectrum). More

147 specifically, the eight signals can be assigned to guanosine 3'-monophosphate (**a**, 3'-GMP), adenosine 3'-
148 monophosphate (**b**, 3'-AMP), uridine 3'-monophosphate (**c**, 3'-UMP), cytidine 3'-monophosphate (**d**, 3'-
149 CMP), cytidine 2'-monophosphate (**e**, 2'-CMP), uridine 2'-monophosphate (**f**, 2'-UMP), adenosine 3'-
150 monophosphate (**g**, 2'-AMP), and guanosine 2'-monophosphate (**h**, 2'-GMP).[18] By comparison, hydrolysis
151 in the $^{18}$O-matrix produces two groups of signals. Unlabeled signals downfield are denoted as Group 1,
152 showing an identical chemical shift to the signals in the $^{16}$O-matrix. Group 2 is comprised of eight signals
153 from $^{18}$O-labeled isotopologues, and featured by 0.02 ppm isotope shift upfield. The $^{16}$O error of RNA was
154 8.4 percent, greater than phosphatidylcholine because the RNA was more unstable,[15] and hydrolysis
155 products were visible from the start of the experiment in the reference standard used (see Figure S2).



156

157 **Figure 2. NMR spectra of RNA and phosphatidylcholine in $^{18}$O- (in green) and $^{16}$O- (in brown) matrix.**
158 **Resonances in Groups 1 and 2 were unlabeled and $^{18}$O-labeled, respectively. a: 3'-GMP; b: 3'-AMP; c:**
159 **3'-UMP; d: 3'-CMP; e: 2'-CMP; f: 2'-UMP; g: 2'-AMP; h: 2'-GMP.**

**Organic soil samples.** The thick Tangelhumus profile is formed as a result of the long-term accumulation of organic materials. A large P reservoir is also built up simultaneously. Our earlier study found that all predominant monoesters originated from degradation of RNA and phospholipids.[28] However, whether these degradation products are natural metabolites or artifacts remained unknown. Figure 3 shows the monoester region of the NMR spectrum of the WETT Of layer in the $^{18}O$ and $^{16}O$-matrices (L, Oh1, and Oh2 given in Figure S3-5). The MDPA signal is set as the reference signal at 17.39 ppm. Signals **c** and **e** are from α- and β-glycerophosphate (see spiking spectra in Figures S6–7), respectively. Full spectra of all samples are also given in the Supporting Information (Figures S8–S15). The other eight resonances were identified as four each of 2' and 3' nucleotides from RNA, according to signal assignment results by 2D heteronuclear single quantum coherence (HSQC) $^{31}P$ NMR spectroscopy.[28] In the $^{18}O$ spectrum, the signals can be divided into two groups. Signals in Group 1 resonated at exactly the same chemical shift as signals in the $^{16}O$ spectrum, while signals in Group 2 were observed 0.02 ppm upfield relative to those of Group 1, indicating that the former were $^{18}O$-labeled isotopologues and thus artifacts. A corrected monoester region of the spectrum (in blue) can be constructed by deleting all $^{18}O$ labeled signals, but keeping the unlabeled Group 1 signals. In the corrected spectrum, the signal intensity of each monoester corresponds to the unlabeled portion of the respective compound. For instance, the signal of α-glycerophosphate is 76 percent of its original intensity in the corrected spectrum, while the intensity of all other monoester signals decreased to about 10 percent.

After line-fitting and quantitative calculation (in Table 1, quantification results of hydrolytically stable P compounds in Table S2), the proportion of inherent α-glycerophosphate (52–76 percent) is larger than its $^{18}O$-labeled isotopologue (24–48 percent), suggesting that it is mostly inherent in soil. By contrast, the vast

majority of β-glycerophosphate (90–95 percent) and mononucleotides (88–98 percent) detected by NMR spectroscopy are actually artifacts. Therefore, ignoring the artifacts from sample preparation and NMR analysis will lead to severe misinterpretation of the NMR spectra.

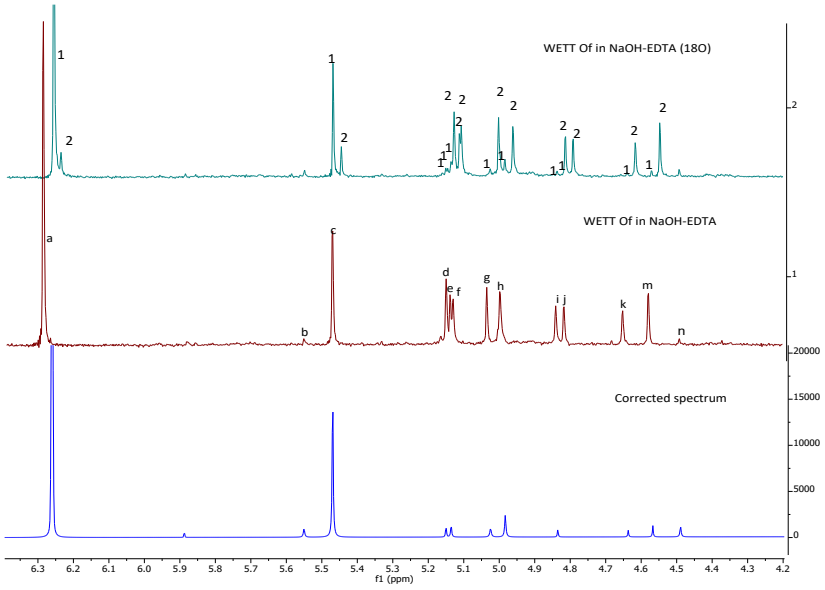



**Figure 3. Monoester region of WETT Of spectra in $^{18}O$ (in green) and $^{16}O$ (in brown) matrix, and corrected spectrum of monoester region (in blue). Resonances in Groups 1 and 2 were unlabeled and $^{18}O$-labeled, respectively. Signal a: orthophosphate; c, e: α- and β-glycerophosphate; d, and signal f to m: 2' and 3' mononucleotides; b, n: unknown.**

**Table 1. Measured and corrected concentration of P compounds. Values in parentheses are the proportions (%) of unlabeled content relative to total measured content of degradation products.**

| Sample | Orthophosphate | α-glycerophosphate | β-glycerophosphate | Total nucleotides | Total monoesters | Phospholipids | RNA | Polyphosphate |
|---|---|---|---|---|---|---|---|---|
| | | | | mg P $kg^{-1}$ | | | | |
| ***WETT L*** | | | | | | | | |
| Measured | 41.1 | 18.7 | 17.5 | 55.0 | 92.5 | 0.0 | 0.0 | 0.0 |
| Corrected | 41.1 | 9.8 (52) | 0.8 (5) | 3.3 (6) | 15.2 (16) | 25.6 | 51.7 | 0.0 |
| ***WETT Of*** | | | | | | | | |
| Measured | 35.6 | 14.7 | 5.3 | 59.2 | 82.0 | 0.0 | 0.0 | 29.5 |
| Corrected | 32.3 | 11.3 (76) | 0.5 (10) | 6.2 (10) | 20.7 (25) | 8.3 | 53.1 | 32.8 |
| ***WETT Oh1*** | | | | | | | | |
| Measured | 32.6 | 2.5 | 3.3 | 15.9 | 23.6 | 0.0 | 0.0 | 33.0 |
| Corrected | 32.6 | 1.3 (53) | 0.3 (9) | 1.9 (12) | 5.6 (24) | 4.1 | 13.3 | 33.0 |
| ***WETT Oh2*** | | | | | | | | |
| Measured | 19.6 | 0.6 | 1.2 | 6.0 | 10.2 | 0.0 | 0.0 | 8.0 |
| Corrected | 19.6 | 0.4 (55) | 0.1 (8) | 0.1 (2) | 2.9 (28) | 1.4 | 5.8 | 8.0 |
| ***ROLL*** | | | | | | | | |
| Measured | 272.8 | 1.5 | 2.6 | 15.7 | 174.6 | 0.0 | 0.0 | 0.0 |
| Corrected | 272.8 | 1.5 (100) | 0 (0) | 13.9 (88) | 170.2 (97) | 2.6 | 1.8 | 0.0 |
| ***TUTT*** | | | | | | | | |
| Measured | 81.9 | 3.1 | 5.8 | 38.5 | 109.5 | 0.0 | 0.0 | 0.0 |
| Corrected | 81.9 | 3.1 (100) | 0 (0) | 37.4 (97) | 102.7 (94) | 5.8 | 1.0 | 0.0 |
| ***VEGE*** | | | | | | | | |
| Measured | 873.3 | 59.2 | 13.8 | 126.8 | 366.1 | 0.0 | 0.0 | 0.0 |
| Corrected | 873.3 | 48.7 (82) | 2.2 (16) | 20.2 (16) | 237.4 (65) | 22.1 | 106.6 | 0.0 |
| ***ALGA*** | | | | | | | | |
| Measured | 1309.5 | 360.5 | 442.5 | 1509.4 | 2833.7 | 0.0 | 0.0 | 732.9 |
| Corrected | 1216.6 | 223.9 (62) | 238.2 (54) | 251.6 (17) | 1235 (44) | 340.9 | 1257.8 | 832.6 |

**Mineral soil samples.** The two studied mineral soils show distinct spectral profile and $^{18}O$ labeling behavior compared with organic soils above. In the NMR spectra of TUTT and ROLL (Figure 4), *myo*-IHP (signal **m1-4**), α-, and β-glycerophosphate (signal **b**, **d**) were confirmed by spiking experiment (Figures S16–S19). Mononucleotides (4.3–4.9 ppm)[18] and other monoester signals such as *scyllo*- (**s**) and isomerized IHP (**i1-2**) were also identified.[39,51] Only two signals are $^{18}O$ labeled: one at 4.87 ppm from β-glycerophosphate, and the other at 4.41 ppm, likely from mononucleotide.

Mineral soils consist mainly of weathering-derived minerals and organic matter at advanced stages of decomposition. During such decomposition processes, hydrolysable RNA and phospholipids are exposed to the extracellular environment and degraded to hydrolytically more stable mononucleotides and glycerophosphates. These degradation products can be further stabilized through association with soil minerals.[52] Therefore, they are intrinsic components of the soil P pool and will not be labeled during sample preparation and NMR analysis in the $^{18}O$ matrix. Consequently, $^{18}O$-labeled artifacts contribute just 3 percent of the total mononucleotides, as shown in Table 1. It should be noted that the proportion of $^{18}O$-labeled mononucleotides can be underestimated here because other tiny $^{18}O$- labeled signals may be concealed by signal overlapping.

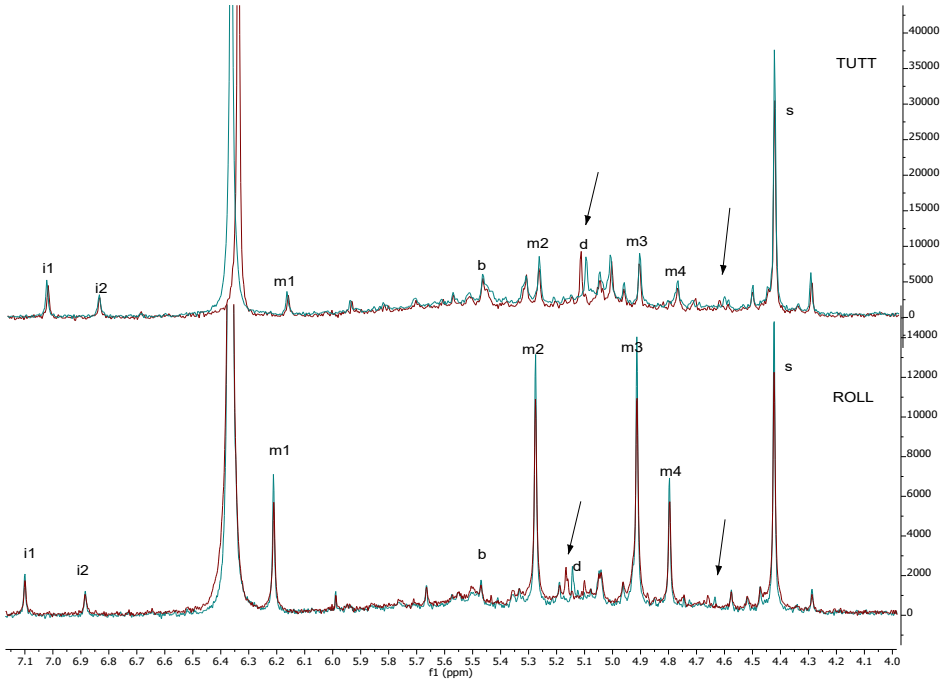



**Figure 4. Monoester region of TUTT and ROLL spectra in $^{18}O$ (in green) and $^{16}O$ (in brown) matrix. Signal b: α-glycerophosphate; d: β-glycerophosphate; m1-4: *myo*-IHP; s: *scyllo*-IHP; i1-2: isomerized IHP; signals between 4.5–5.2 ppm except m3: mononucleotides.**

**Vegetation sample.** At specific sites along the Paposo transect of the Atacama Desert, some rare rainfall and fog from the South Pacific Ocean support growth of certain vegetation species. The studied vegetation (*Cristaria*) accumulates up to 1895 mg P per kilogram biomass, substantially higher than in the underlying soil (3.5 mg P. $Kg^{-1}$ soil). More intriguingly, a very diverse P pool with 21 monoester signals is detected (Figure 5). When comparing the two spectra in the $^{18}O$ and $^{16}O$ matrices, all monoester signals can be again divided into two groups: 11 unlabeled signals, and 10 signals labeled with $^{18}O$. These 10 signals are

**Kommentiert [ED1]:** Okay? More than one matrix?

218 also two glycerophosphates (**e, j**) and eight mononucleotides (**i**, **k**, **l**, **m**, **p**, **q**, **s**, and **t**) from phospholipids
219 and RNA, respectively.

220 The signal assignment of $^{31}$P-NMR spectra is usually a laborious task because degradation products of
221 phospholipids and RNA complicate the crowded monoester region and lead to signal misidentification.[53] For
222 example, the signals of α- and β-glycerophosphate are deceptively similar in chemical shift and intensity to
223 the two strongest *myo*-IHP signals.[21] Therefore, spiking experiments were inevitable for confident signal
224 assignment.[54] Here, after excluding the interference of the 10 resonances from the $^{18}$O-labeled artifacts,
225 signal **c**, **g**, **n**, **r** clearly reflect the 1:2:2:1 pattern of the four *myo*-IHP signals.[10] Finally, only seven signals
226 remain unidentified after a simple 1D NMR measurement; thus, the monoester region is substantially
227 simplified. The quantification results in Table 1 reveal that $^{18}$O-labeled mononucleotides from artificial
228 hydrolysis of RNA account for 84 percent of the total mononucleotides. Up to 84 percent of β-
229 glycerophosphate is present as $^{18}$O isotopologue, which is much higher than that of α form, of which only 18
230 percent is $^{18}$O-labeled.

**Kommentiert [WS2]:** 10 resonances minus 4: 6 are left not 7

**Kommentiert [LW3R2]:** 21 monoester signals in total, 10 resonances are 18O labeled, 4 are myo-IHP. So 21-10-4=7 remain unidentified.

**Kommentiert [WS4]:** c, g, or, please check in fig. 5 you mention c, g, n, r

**Kommentiert [LW5R4]:** revised to c, g, n ,r
thanks

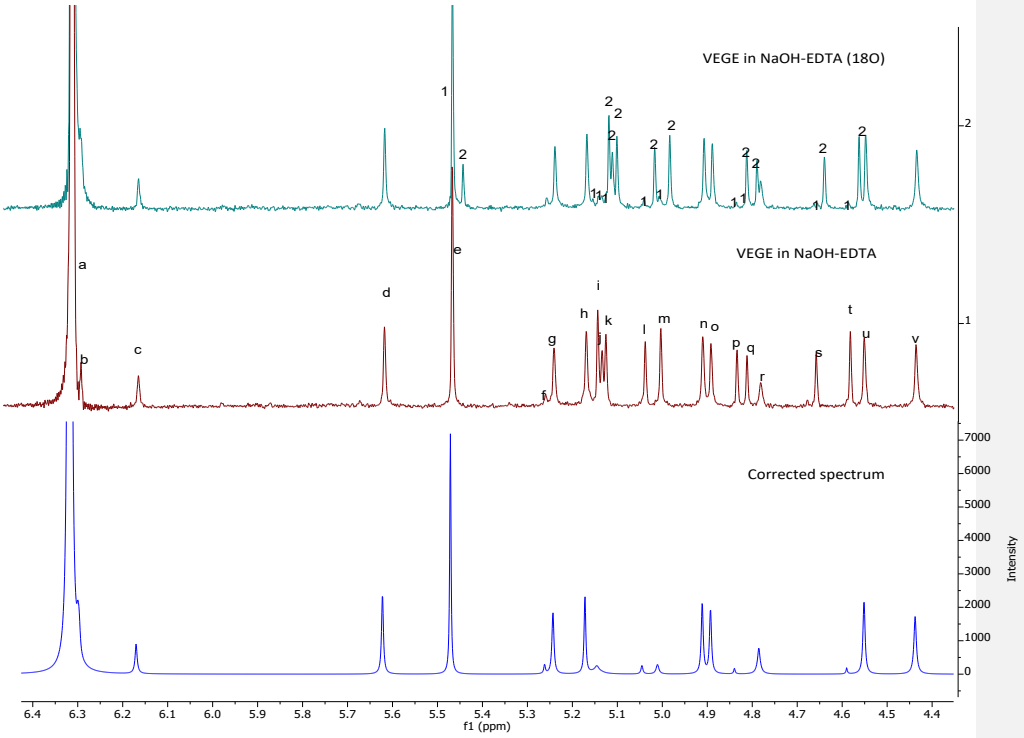



**Figure 5. Monoester region of VEGG spectra in $^{18}O$ (in green) and $^{16}O$ (in brown) matrix. Resonances in Groups 1 and 2 were unlabeled and $^{18}O$-labeled, respectively. Signal a: orthophosphate; e, j: α- and β-glycerophosphate; i, k, l, m, p, q, s, and t: 2' and 3' mononucleotides; signal c, g, n, r: myo-IHP, other signals: unknown.**

**Algae sample.** A self-cultivated alga (*Chlorella vulgaris*) was selected to test the method for sample from aqueous ecosystem. Algae are known to rapidly take up P from wastewater and accumulate large amounts of P, especially in the form of polyphosphates in cells.[55,56] The P-rich algal biomass can be potentially converted to biofertilizers,[57] which represents a promising opportunity to recover P from wastewater for crops. The characterization and quantification of P compounds in algae, particularly

241 polyphosphates, is of central interest, but also problematic due to the unknown degree of hydrolysis. After
242 isotopic extraction, 10 resonances of isotopologues (Group 2) are observed again in the monoester region
243 in Figure 6 ($^{18}$O spectrum). In the $^{16}$O spectrum, signals **e** and **l** are assigned as two glycerophosphates, and
244 signals **h**–**p** are from 2' and 3' nucleotides. Quantification results after correction show that artifacts
245 contribute to 56 percent of the total detected monoesters. Moreover, the $^{18}$O-labeled pyrophosphates and
246 the terminal P of tripolyphosphates[10] are also detected (Figure 6). After line-fitting and quantitative
247 calculation, it is found that 12 percent of polyphosphates were hydrolyzed in the course of sample
248 preparation and analysis. Interestingly, a considerable proportion of α-glycerophosphate remained
249 unlabeled by $^{18}$O in algae and all other studied samples. A possible origin of inherent α-glycerophosphate is
250 the enzymatic degradation of phospholipids to phosphatidic acid mediated by phospholipase D[58] and
251 subsequent cleavage of the fatty acids of phosphatidic acid (Figure S20).

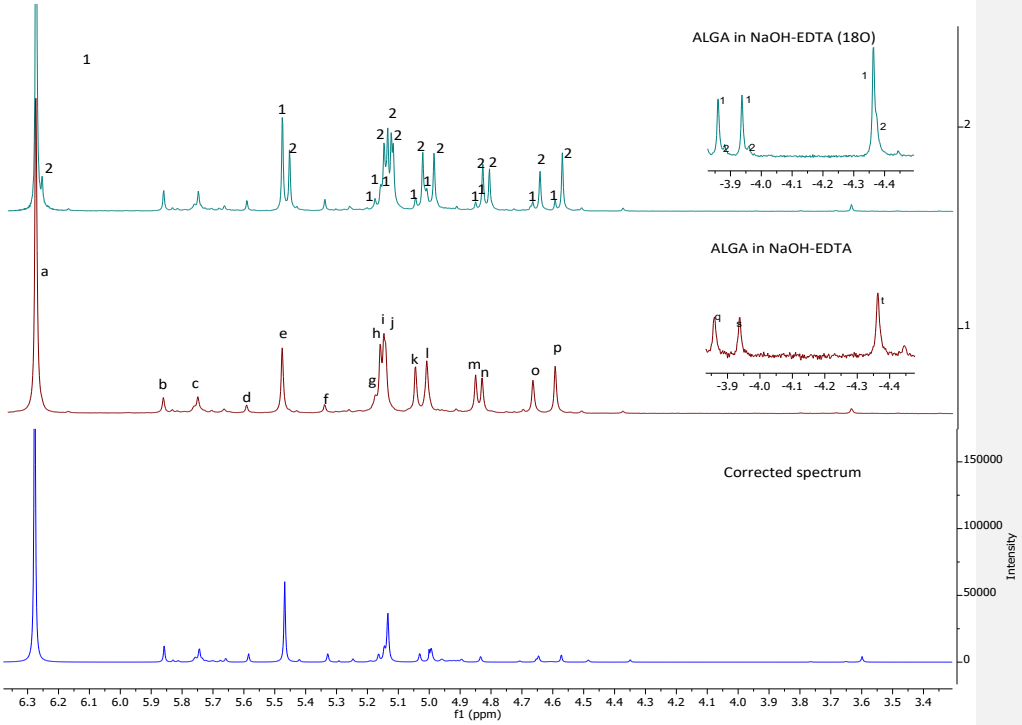



**Figure 6. Regional presentation of ALGA spectra in $^{18}O$ (in green) and $^{16}O$ (in brown) matrix. The zoomed insets show signals of pyrophosphate and tripolyphosphate between δ = -3.9 and -4.4 ppm. Resonances in Groups 1 and 2 were unlabeled and $^{18}O$-labeled, respectively. Signal a: orthophosphate; e, i: α- and β-glycerophosphate; h, and j to p: 2' and 3' mononucleotides; q and r: terminal P of tripolyphosphate; signal s: pyrophosphate other signals: unknown.**

Kommentiert [ED6]: matrices?

## CONCLUSION

The spontaneous hydrolysis of the labile P species can be attributed either to natural degradation *in situ* or to artificial hydrolysis during sample treatment and sample analysis. Differentiation of these two processes

was difficult because the resulting degradation products were identical. For example, hydrolysis of phospholipids such as phosphatidylcholine yields α- and β-glycerophosphates, while 2' and 3' mononucleotides are formed from RNA. Hydrolysis of polyphosphate produces ortho-, pyro-, and tripolyphosphate. Here, we proposed this $^{18}O$ labelling procedure for the quantification of hydrolysable P compounds, which is more accurate than either presenting NMR data[59] as obtained or than simply attributing all degradation products to the corresponding assumed precursors.[28,31] Previous studies have employed the ratio of monoesters to diesters as an indicator of P cycling in soilsm,[59-61] or when correlating the activity of various enzymes in soils to the content of monoesters and diesters.[62] All of these interpretations would have been more meaningful if they were based on accurate NMR data. Our method now opens up novel opportunities to reevaluate P turnover in the environment.

## ACKNOWLEDGMENTS

The authors are grateful to the China Scholarship Council (CSC), Deutsche Forschungsgemeinschaft (DFG) SPP1685 and CRC 1211 for financial support of personal and lab costs, respectively. We thank Prof. Jörg Prietzel, Prof. Erwin Klumpp, Mrs. Ramona Mörchen, and Dr. Christina Kuchendorf for providing the samples. We acknowledge Dr. Volker Nischwitz for ICP-OES measurement and Ms. Claudia Walraf for lyophilizing the samples.

**Author contributions:** LW conceived the experimental design, carried out all measurements. SW and WA completed the design and coordinated the study. The manuscript was written by LW with input from SW and WA.

**Competing interests:** The authors declare that they have no conflict of interest.

**Supporting Information**. Brief descriptions in non-sentence format listing the contents of the files supplied

as Supporting Information.

## REFERENCE


(1) Stutter, M. I.; Shand, C. A.; George, T. S.; Blackwell, M. S.; Bol, R.; MacKay, R. L.; Richardson, A. E.; Condron, L. M.; Turner, B. L.; Haygarth, P. M. *Environmental Science & Technology* **2012**, *46*, 1977-1978.

(2) Ticconi, C. A.; Abel, S. *Trends in Plant Science* **2004**, *9*, 548-555.

(3) Newman, R.; Tate, K. *Communications in Soil Science & Plant Analysis* **1980**, *11*, 835-842.

(4) Cade-Menun, B.; Liu, C. W. *Soil Science Society of America Journal* **2014**, *78*, 19.

(5) Cade-Menun, B. J. In *Organic phosphorus in the environment*, Turner, B. L.; Frossard, E.; Baldwin, D. S., Eds.; CABI Publishing: Wallingford, UK, 2005, pp 21-44.

(6) Kovacs, H.; Moskau, D.; Spraul, M. *Progress in Nuclear Magnetic Resonance Spectroscopy* **2005**, *46*, 131-155.

(7) Bowman, R.; Moir, J. *Soil Science Society of America Journal* **1993**, *57*, 1516-1518.

(8) Cade-Menun, B.; Liu, C.; Nunlist, R.; McColl, J. *Journal of Environmental Quality* **2002**, *31*, 457-465.

(9) Cade-Menun, B. J.; Benitez-Nelson, C. R.; Pellechia, P.; Paytan, A. *Marine Chemistry* **2005**, *97*, 293-306.

(10) Cade-Menun, B. J. *Geoderma* **2015**, *257–258*, 102-114.

(11) Cade-Menun, B. J. *Talanta* **2005**, *66*, 359-371.


**Kommentiert [WA7]:** Please, check againmanually whether numbners correspond to the correct positioning in the text! Cannot do so since I do not know all papers.

**Formatiert:** Deutsch (Deutschland)

(12) Cade-Menun, B. J.; Navaratnam, J. A.; Walbridge, M. R. *Environmental Science & Technology* **2006**, *40*, 7874-7880.

(13) Cade-Menun, B. J.; Preston, C. M. *Soil Science* **1996**, *166*.

(14) Turner, B. L.; Mahieu, N.; Condron, L. M. *Soil Science Society of America Journal* **2003**, *67*, 497-510.

(15) Makarov, M.; Haumaier, L.; Zech, W. *Soil Biology and Biochemistry* **2002**, *34*, 1467-1477.

(16) McDowell, R.; Stewart, I. *Chemistry and Ecology* **2005**, *21*, 211-226.

(17) Turner, B. L.; Newman, S. *Journal of Environmental Quality* **2005**, *34*, 1921-1929.

(18) Smernik, R. J.; Doolette, A. L.; Noack, S. R. *Communications in Soil Science and Plant Analysis* **2015**, *46*, 2746-2756.

(19) Condron, L. M.; Turner, B. L.; Cade-Menun, B. J. In *Phosphorus: Agriculture and the environment*, Sims, J. T., and Sharpley, A.N., Ed.; Agron. Monogr. 46. ASA, CSSA, and SSSA, 2005, pp 87-121.

(20) Westheimer, F. H. *Science* **1987**, *235*, 1173-1178.

(21) Doolette, A.; Smernik, R.; Dougherty, W. *Soil Science Society of America Journal* **2009**, *73*, 919-927.

(22) Brockerhoff, H. *Journal of Lipid Research* **1963**, *4*, 96-99.

(23) McCullough, J.; Van Wazer, J.; Griffith, E. *Journal of the American Chemical Society* **1956**, *78*, 4528-4533.

(24) Crowther, J. P.; Westman, A. *Canadian Journal of Chemistry* **1954**, *32*, 42-48.

(25) Kirby, A. J.; Nome, F. *Accounts of chemical research* **2015**, *48*, 1806-1814.

(26) Turner, B. L.; Cade-Menun, B. J.; Condron, L. M.; Newman, S. *Talanta* **2005**, *66*, 294-306.

(27) Vestergren, J.; Vincent, A. G.; Jansson, M.; Persson, P.; Ilstedt, U.; Gröbner, G.; Giesler, R.; Schleucher, J. *Environmental Science & Technology* **2012**, *46*, 3950-3956.

(28) Wang, L.; Amelung, W.; Prietzel, J.; Willbold, S. *Geoderma* **2019**, *340*, 192-205.

(29) Kirby, A. J.; Manfredi, A. M.; Souza, B. S.; Medeiros, M.; Priebe, J. P.; Brandão, T. A.; Nome, F. *ARKIVOC: Online Journal of Organic Chemistry* **2009**, 28-38.

(30) McDowell, R.; Cade-Menun, B.; Stewart, I. *European Journal of Soil Science* **2007**, *58*, 1348-1357.

(31) Jiang, X.; Amelung, W.; Cade-Menun, B. J.; Bol, R.; Willbold, S.; Cao, Z.; Klumpp, E. *Scientific Reports* **2017**, *7*, 10818.

(32) Young, E. O.; Ross, D. S.; Cade-Menun, B. J.; Liu, C. W. *Soil Science Society of America Journal* **2013**, *77*, 1636-1647.

(33) Cade-Menun, B. J. *Chemical and Biological Technologies in Agriculture* **2017**, *4*, 19.

(34) Liu, J.; Hu, Y.; Yang, J.; Abdi, D.; Cade-Menun, B. J. *Environmental Science & Technology* **2014**, *49*, 168-176.

(35) Webb, M. R.; McDonald, G. G.; Trentham, D. R. *Journal of Biological Chemistry* **1978**, *253*, 2908-2911.

**Formatiert:** Deutsch (Deutschland)

(36) Cohn, M.; Hu, A. *Proceedings of the National Academy of Sciences* **1978**, *75*, 200-203.

(37) Batiz-Hernandez, H.; Bernheim, R. A. *Progress in Nuclear Magnetic Resonance Spectroscopy* **1967**, *3*, 63-85.

(38) Jiang, X.; Bol, R.; Cade-Menun, B. J.; Nischwitz, V.; Willbold, S.; Bauke, S. L.; Vereecken, H.; Amelung, W.; Klumpp, E. *Biogeosciences* **2017**, *14*, 1153-1164.

(39) Wang, L.; Missong, A.; Amelung, W.; Willbold, S.; Prietzel, J.; Klumpp, E. *Geoderma* **2020**, *375*, 114507.

(40) Schreiber, C.; Behrendt, D.; Huber, G.; Pfaff, C.; Widzgowski, J.; Ackermann, B.; Müller, A.; Zachleder, V.; Moudříková, Š.; Mojzeš, P.; Schurr, U.; Grobbelaar, J.; Nedbal, L. *Bioresource Technology* **2017**, *234*, 140-149.

(41) Geissler, P. L.; Dellago, C.; Chandler, D.; Hutter, J.; Parrinello, M. *Science* **2001**, *291*, 2121-2124.

(42) Salomon, M. *National Aeronautics and Space Administration, NASA TN D-5223, Washington, DC* **1969**.

(43) Staal, L. B.; Petersen, A. B.; Jørgensen, C. A.; Nielsen, U. G.; Nielsen, P. H.; Reitzel, K. *Water Research* **2019**, *157*, 346-355.

(44) Vincent, A. G.; Vestergren, J.; Gröbner, G.; Persson, P.; Schleucher, J.; Giesler, R. *Plant and Soil* **2013**, *367*, 149-162.

(45) Vincent, A.; Schleucher, J.; Gröbner, G.; Vestergren, J.; Persson, P.; Jansson, M.; Giesler, R. *Biogeochemistry* **2012**, *108*, 485-499.

Formatiert: Deutsch (Deutschland)

Formatiert: Deutsch (Deutschland)

(46) Doolette, A.; Smernik, R.; Dougherty, W. *Soil Research* **2011**, *49*, 152-165.

(47) Alderman, D. W.; Grant, D. M. *Journal of Magnetic Resonance (1969)* **1979**, *36*, 447-451.

(48) Turner, B. L.; Condron, L. M.; Richardson, S. J.; Peltzer, D. A.; Allison, V. J. *Ecosystems* **2007**, *10*, 1166-1181.

(49) Turner, B. L.; Newman, S.; Cheesman, A. W.; Reddy, K. R. *Soil Science Society of America Journal* **2007**, *71*, 1538-1546.

(50) Prietzel, J.; Christophel, D. *Geoderma* **2014**, *221*, 28-39.

(51) Wang, L.; Amelung, W.; Willbold, S. *Environmental science & technology* **2017**, *51*, 13256-13264.

(52) Lehmann, J.; Kleber, M. *Nature* **2015**, *528*, 60-68.

(53) Bünemann, E. K.; Smernik, R. J.; Doolette, A. L.; Marschner, P.; Stonor, R.; Wakelin, S. A.; McNeill, A. M. *Soil Biology and Biochemistry* **2008**, *40*, 1908-1915.

(54) Smernik, R. J.; Dougherty, W. J. *Soil Science Society of America Journal* **2007**, *71*, 1045-1050.

(55) Shilton, A. N.; Powell, N.; Guieysse, B. *Current Opinion in Biotechnology* **2012**, *23*, 884-889.

(56) Powell, N.; Shilton, A.; Chisti, Y.; Pratt, S. *Water Research* **2009**, *43*, 4207-4213.

(57) Mulbry, W.; Westhead, E. K.; Pizarro, C.; Sikora, L. *Bioresource Technology* **2005**, *96*, 451-458.

Formatiert: Deutsch (Deutschland)

(58) Jenkins, G. M.; Frohman, M. A. *Cellular and Molecular Life Sciences CMLS* **2005**, *62*, 2305-2316.

(59) Lang, F.; Kruger, J.; Amelung, W.; Willbold, S.; Frossard, E.; Bunemann, E. K.; Bauhus, J.; Nitschke, R.; Kandeler, E.; Marhan, S.; Schulz, S.; Bergkemper, F.; Schloter, M.; Luster, J.; Guggisberg, F.; Kaiser, K.; Mikutta, R.; Guggenberger, G.; Polle, A.; Pena, R., et al. *Biogeochemistry* **2017**, *136*, 5-29.

(60) Condron, L. M.; Turner, B. L.; Cade-Menun, B. J. *Phosphorus: Agriculture and the environment* **2005**, *46*, 87-121.

(61) Condron, L.; Frossard, E.; Tiessen, H.; Newmans, R.; Stewart, J. *Journal of Soil Science* **1990**, *41*, 41-50.

(62) Wei, K.; Chen, Z.; Zhu, A.; Zhang, J.; Chen, L. *Soil and Tillage Research* **2014**, *138*, 35-43.

**Formatiert:** Deutsch (Deutschland)