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Optimized procedure for the determination of P species in soil by liquid-state ^{31}P-NMR spectroscopy

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Abstract

Background: Liquid-state ^{31}P-NMR spectroscopy becomes progressively an important role for studying phosphorus (P) dynamics in soil. Soils of different origin and organic matter content were used to optimize sample preparation and re-dissolution procedures to improve characterization of P species in soil by ^{31}P-NMR spectroscopy. The efficiency of P extraction from an untreated fresh soil was compared to that from freeze-dried and air-dried soil samples.

Results: A freeze-drying pretreatment not only provided the greatest extraction yields of total and organic P from both farmland and forest soils but also enhanced the intensity of signals for inorganic and organic P species in ^{31}P-NMR spectra, except for polyphosphates. Re-dissolution of freeze-dried soil extracts in relatively dilute alkaline solution and addition of a small aliquot of concentrated HCl to the NMR tube prior to analysis improved the quality of NMR spectra. Finally, the visibility of relatively weak P signals, such as for phosphorus diesters, phosphonates, polyphosphate, phospholipids, and DNA were reproducibly enhanced when ^{31}P-NMR spectra were generated after at least 15 h of acquisition time.

Conclusion: The optimized procedure presented here ensured the greatest detectability of inorganic and organic P species by liquid-state P-NMR spectroscopy in soil extracts.

Keywords: Liquid-state ^{31}P-NMR spectroscopy; Soil pretreatment before extraction; Re-dissolution of P extracts; Inorganic and organic P species

Background

Solution-state ^{31}P-NMR spectroscopy represents a useful tool to follow the cycle of phosphorus (P) in the environment [23]. However, due to the relatively low concentration of P compounds present in environmental compartments (soils and sediments), natural organic matter (NOM), and microbial biomass, the procedures for sample preparation for the detection of P content by NMR require a careful setup of experimental conditions, including the removal of paramagnetic species [25,27,11]. In fact, an optimization of sample pretreatment and NMR experimental parameters become critical to improve the final quality of ^{31}P NMR spectra and minimize variations in the determination of P compounds [7,18,21].

Several works were devoted to improve procedures in order to obtain meaningful and reproducible liquid-state ^{31}P-NMR spectra. In particular, the extracting solution may affect the quantitative solubilization of P species [3,20]. Cade-Menun and Preston [5] found that an aqueous solution containing both NaOH and EDTA extracted the greatest diversity of P forms and the largest percentage of total phosphorus in soil. Recently, it has been shown that a 0.25 M NaOH and 0.05 M EDTA extracting solution was most efficient in solubilizing a maximum range of organic P forms from sediments [26,28], while minimizing the co-extraction of Fe (III) and Mn (II) metals, which may significantly affect the resolution and intensity of ^{31}P NMR spectra [14, 1]. Addition of HCl or Chelex to extracts was also found to be useful in reducing interferences [13,24]. Recently, the capacity of bicarbonate dithionate (BD), EDTA, Chelex-100, and 8-hydroxyquinoline

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(HQ) were compared in order to prevent solubilization of Fe and Mn from environmental samples [9]. It was found that HQ was the most efficient treatment, although it was also observed that excessive HQ in the extracting solution produced an insoluble residue during subsequent freeze-drying of extracts.

Extracted matter is normally concentrated prior to solution-state ^{31}P-NMR analysis by either rotary evaporation or freeze-drying [10]. However, the latter procedure was reported to lead to considerable degradation of polyphosphate and glucose 6-phosphate and significant reduction of NMR signals of phospholipids extracted from a calcareous sediment [4]. The disappearance of phopholipids and pyrophosphate in extracts from a sediment from Taihu Lake in China was also noted after freeze-drying of samples [26]. On the other hand, while rotary evaporation of aqueous extracts is a time-consuming enrichment procedure as compared to freeze-drying, it also fails to sufficiently enrich P content in samples with very low P concentration. This becomes evident for soil-water leachates or dissolved organic matter, for which a 1,000- to 2,000-fold enrichment is usually required [4,19,8]. In these cases, sample freeze-drying followed by re-dissolution in the proper solution for NMR analysis may be the most useful method.

The aim of this work was to set up a reproducible procedure to extract and concentrate organic P species solubilized from soil samples of different organic matter content. Sample extraction and re-dissolution and spectral acquisition time were assayed to optimize detection of P species by ^{31}P-NMR spectroscopy.

Methods

Materials and chemical properties

A surface (0 to 20 cm) layer of an alluvial clayey loam agricultural soil was collected at the Experimental Farm of the University of Naples Federico II at Castel Volturno (CE). This soil was used without (sample A) and with (sample B) addition of 125 q ha^{-1} of farm compost as P source. A volcanic silty loam soil sample (0 to 20 cm) was collected from the forested area around the Royal Palace of Portici (NA) (sample C). The overlaying litter material was collected from the same forest soil (sample D). The compost used here to amend the agricultural soil and extract P (sample E) was derived from a pilot composting plant build at the Experimental Farm of the University of Naples Federico II at Castel Volturno (CE). The pH of the samples was determined by a pH meter (HANNA Instruments, Padova, Italy) in a soil:water suspension of 1:2.5 ratio. The C, H, and N content of samples was determined by an elemental analyzer (Eager 200, Fisons, Ipswich, UK), while the content of the organic matter (OM) was achieved by the Walkley-Black method. Organic P was calculated in soils and compost by difference in P content before and after ignition at 550°C and followed by extraction with 1 M HCl [2]. Total P in both original sample materials and their extracts (TP_E) was obtained by first digesting samples in concentrated H_2SO_4 and $HClO_4$ for 2 h and the measuring total dissolved P in digested solutions by a UV-vis spectrometer (Lambda 25, Perkin Elmer, Waltham, MA, USA) by the molybdate colorimetry method [17].

Sample treatments and preparation for NMR acquisition

Soils, litter, and compost samples used in this study were cleaned from plant remains, passed through a 1-mm sieve, and divided into three aliquots. One aliquot (fresh) was subjected to direct extraction of P species, the second aliquot was first freeze-dried, and a third aliquot was left to air-dry (20°C to 25°C) for 2 weeks before extraction.

Extractions

About 4 g of each aliquot of soil and compost was mixed with 0.25 M NaOH and 0.05 M EDTA solution at a 1:8 soil:solution ratio and shaken at 20°C for 16 h. The mixture was centrifuged at 12,000 *g* (20°C) for 30 min, and the clear supernatant solution was collected into a 50-mL centrifuge tube. An amount (1.1 mL for each 10 mL extract) of a 3% aqueous 8-hydroxyquinoline (HQ) solution was added to the tube to remove Fe and Mn metals [9]. The solution pH was adjusted to 9.0 ± 0.1, and, after at least 30 min, the solution was centrifuged. Thirty millimeters of the supernatant was separated, frozen at −80°C, and freeze-dried. The resulting powder was finely ground before further analysis.

Re-dissolutions

The freeze-dried powder obtained with samples extraction was divided into two aliquots of about 1 g each. One aliquot was re-dissolved in 2 mL of a 1 M NaOH solution, while a second aliquot was re-dissolved in 2 mL of a 10 M NaOH solution. After about 2 h, the samples were centrifuged at 12,000 *g* for 30 min. Then, 930 μL of the supernatant were transferred into a 5-mm NMR tube, together with a deuterated solution of metylendiphosphonic acid-P,P′-disodium salt (MDP) (Epsilon Chimie, Guipavas, France) as internal standard (δ = 16.62 ppm), for a final 2.65 mM concentration. Moreover, in order to flocculate possible suspended particles, which may be present in the redissolved extracts and interfere with NMR analyses, 100 μL of concentrated HCl was further added to the NMR tube prior to acquisition of ^{31}P-NMR spectra. All samples were prepared in triplicates for NMR analyses.

^{31}P-NMR analysis

The P containing solutions were analyzed by a 400-MHz Bruker Avance spectrometer (Bruker AXS, Inc., Madison,

WI, USA), equipped with a 5-mm Bruker broadband inverse (BBI) probe, operating at ^{31}P resonating frequency of 161.81 MHz, applying 6 s initial delay and a 45° pulse length ranging between 8.5 and 9.5 μs (–2 dB power attenuation). The ^{31}P-NMR spectra of sample solutions were acquired from 5, 10, and 15 h of acquisition time. The 5 h of acquisition time comprised 3,000 transients and 5,461 time domain points, while the ^{31}P spectra for the 15-h acquisition time consisted in 9,000 transients, 16,384 time domain points, and a spectral width of 250 ppm (40,650 Hz). Except for the samples used to determine the best acquisition time, the rest of the ^{31}P-NMR spectra of this study were acquired with 15 h of acquisition time. An inverse gated pulse sequence, with 80-μs length Waltz16 decoupling scheme, with around 15.6 dB as power level, was employed to decouple phosphorous from proton nuclei [16]. All spectra were baseline-corrected and processed by MestReC software (v. 4.9.9.9). The free induction decays (FID) for solution-state ^{31}P-NMR spectra were transformed by applying a fourfold zero filling and a line broadening of 6 Hz. Signals were assigned according to literature [6,15,22,12]. The relative proportions of P species were estimated by integration of ^{31}P-NMR spectral peaks and expressed in respect to the concentration of total P in extracts (TP_E).

Results and discussion

Basic properties

The farmland soils and compost had pH between 8.82 and 8.97, while pH values for litter and forest soil were neutral and weakly alkaline, respectively (Table 1). The largest organic matter (OM) content was found in both compost (522 mg g^{-1}) and litter (284 mg g^{-1}) (Table 1). The content of total P (TP) in compost and litter was significantly greater than that in the forest soil, whereas organic P (OP) was largest in litter and farmland soil. The relatively low amount of both TP and OP in forest soil may be due its lighter texture and sloping topography.

Phosphorus in extracts

The ^{31}P-NMR spectra of extracts obtained from fresh, air-dried, and freeze-dried samples are shown in Figure 1. Spectra reveal that P signals were more visible and intense in extracts from freeze-dried samples than from other sample treatments, being the improvement by freeze-drying more extensive for farmland and forest soils than for forest litter. However, signal enhancement in freeze-dried samples was not as large as that reported by Xu et al. [26], who observed a 50% increase for freeze-dried sediment samples.

TP_E and OP in extracts were larger for freeze-dried than those for either air-dried or fresh samples (Table 2). This difference was generally reflected in the amount of P species measured by ^{31}P-NMR spectra. Exceptions were the phosphonates (18 to 24 ppm) and polyphosphates (–19 to 21 ppm) for the forest soil and the orthophosphates (5.6 ppm) for the forest litter, which were larger in the fresh samples than in both freeze-dried and air-dried samples (Table 2). As for the phosphonates, their reduced content in freeze-dried samples may be explained by their easy degradability, possibly accelerated by samples manipulation.

Air-drying decreased considerably the signals for P monoesters, phospholipids (PL), DNA, and polyphosphates in farmland soil, either with or without compost addition, while these species were not as extensively decreased in the fresh soil (Table 2). Advanced microbial degradation of organic P may be the cause of the reduced values in air-dried samples.

Influence of re-dissolution

The powder materials resulting from freeze-drying soil extracts required to be re-dissolved prior to analysis by liquid-state ^{31}P-NMR spectroscopy. We compared re-dissolution of extracts in either a 1 M or a 10 M NaOH solution.

^{31}P-NMR spectra showed that re-dissolution in a 1 M NaOH solution provided larger values for both TP and OP species than for the 10 M NaOH solution (Figure 2). In particular, approximately 17.0 mg kg^{-1} of organic P and 63.6 mg kg^{-1} of inorganic P were lost during re-dissolution of extract from forest soil with 10 M NaOH. While all P signals showed a general relevant reduction when re-dissolved in 10 M NaOH solution, the phospholipid (PL) signal almost disappeared (Figure 2). This effect was particularly evident in spectra of the humus-rich

Table 1 Some properties of the sample materials used in this study

Materials	pH	OM (mg g^{-1})	TN (mg g^{-1})	C (mg g^{-1})	TP (mg g^{-1})	OP (mg g^{-1})
Farmland soil	8.82	56	0.56	14.7	0.57	0.17
Farmland soil added with compost	8.87	64	0.68	16.5	0.86	0.23
Forest soil	7.47	83	0.36	10.2	0.72	0.08
Forest litter	6.97	284	4.1	50.3	1.18	0.29
Compost	8.97	522	21.9	206	2.18	0.35

OM, organic matter; TN, total nitrogen; C, elemental carbon; TP, total phosphorus; OP, organic phosphorus.

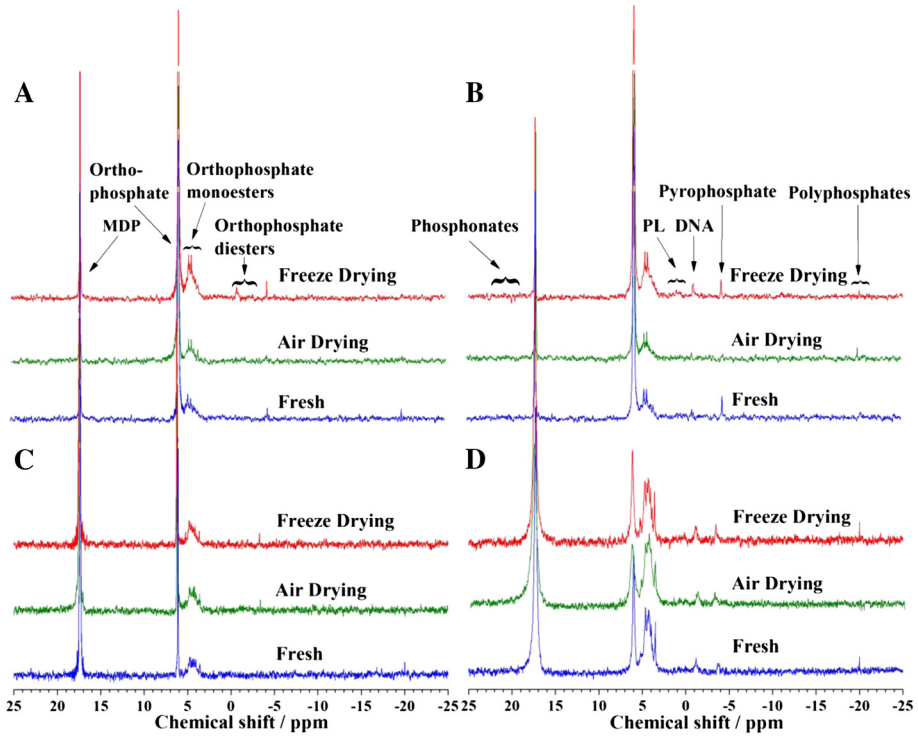

Figure 1 ^{31}**P-NMR spectra of extracts from samples subjected to different treatments and 15 h acquisition time. (A)** Farmland soil. **(B)** Farmland soil added with compost. **(C)** Forest soil. **(D)** Forest litter. MDP, metylendiphosphonic acid-P,P′-disodium salt as internal standard; PL, phospholipids.

Table 2 Total P (TP_E) and organic P (OP_E) in soil extracts (mg kg^{-1}) and relative distribution of P

P species	Phos-P	Orth-P	Monoesters	PL	DNA	Diesters[a]	Pyro-P	Poly-P	OP_E	TP_E
ppm intervals	18 to 24	5.6	5.3 to 3.3	1.6 to −0.3	0.8	−1.1 to −1.6	−4.6	−19 to −21		
Farmland soil										
Freeze-drying	0.5	116.7	14.7	0.6	1.0	1.6	0.2	0.1	16.8	133.8
Air-drying	ND	106.1	7.8	0.5	0.3	0.9	0.2	0.1	8.8	115.1
Fresh	1.4	98.7	8.4	0.9	0.3	1.1	0.2	0.1	11.0	109.9
Farmland soil added with compost										
Freeze-drying	0.5	160.3	16.0	1.7	1.4	3.2	0.4	0.2	19.9	180.7
Air-drying	0.5	153.7	8.6	0.3	0.2	0.4	0.1	0.7	10.3	164.1
Fresh	0.3	153.8	12.8	1.6	0.6	2.2	0.8	0.7	16.0	170.5
Forest soil										
Freeze-drying	4.1	172.0	74.8	3.0	2.5	5.5	0.9	0.4	84.9	257.9
Air-drying	4.5	159.1	70.7	2.0	2.1	4.1	1.0	0.2	79.5	239.7
Fresh	5.2	127.8	52.7	1.2	ND	1.2	ND	2.0	61.1	188.9
Forest litter										
Freeze-drying	8.1	38.6	80.3	2.6	6.7	9.4	2.5	1.4	97.1	140.2
Air-drying	7.8	31.0	83.4	1.9	6.0	7.9	2.5	ND	99.1	132.7
Fresh	3.3	46.2	80.6	2.1	4.9	7.0	1.2	1.0	91.9	139.4

Species in ^{31}P-NMR spectra acquired for 15 h, as referred to TP_E and expressed in milligrams per kilograms. Phos-P, phosphonates; Ortho-P, orthophosphate; Mono-esters, orthophosphate monoesters; PL, phospholipids; Diesters, orthophosphate diesters; Pyro-P, pyrophosphate; Poly-P, polyphosphate; OP_E, organic phosphorus in extracts; TP_E, total phosphorus in extracts; ND, not detected. [a]Extracted orthophosphate diesters were calculated by the sum of phospholipids and DNA.

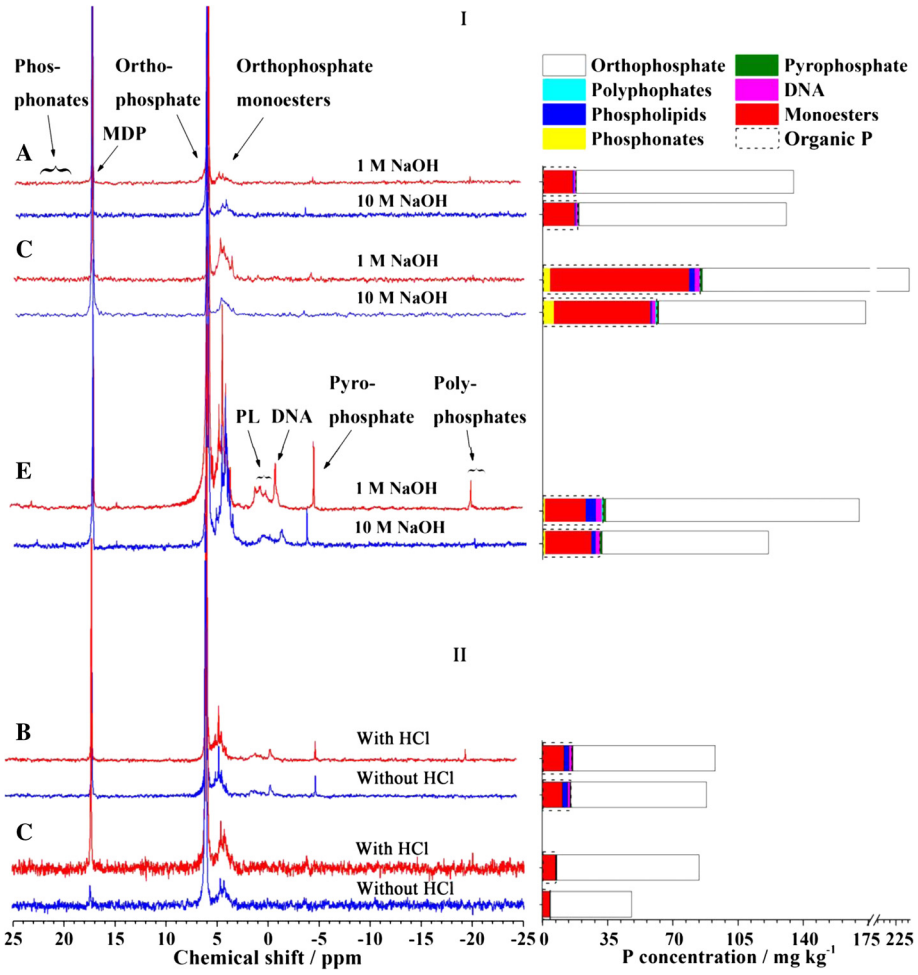

Figure 2 ^{31}P-NMR spectra of extracts from different materials and concentration of various P species (mg kg^{-1}). As re-dissolved in (I) 1 M or 10 M NaOH and (II) 1 M NaOH with and without HCl addition to solution. **(A)** Farmland soil. **(B)** Farmland soil added with compost. **(C)** Forest soil. **(E)** Compost. Spectra were acquired for 15 h. MDP, Metylendiphosphonic acid-P, P′-disodium salt as internal standard; PL, phospholipids.

compost, whereby PL, DNA, and polyphosphate signals were also significantly less intense in the 10 M NaOH solution than those in the dilute alkaline solution. Such lowering of OP signals in the strongly alkaline solution may be due to an enhanced hydrolysis of P from organic compounds.

It was also noted that a displacement of signals chemical shifts in the 10 M NaOH solution (Figure 2). The chemical shift of pyrophosphate was approximately 0.5 ppm downfield as compared to the sample re-dissolved in 1 M NaOH, while those of polyphosphates and DNA were 0.6 ppm downfield. Signals of orthophosphate monoesters were found in the 5.3 to 3.3 ppm range in 1 M NaOH, while they were reduced in a smaller 5.1 to 3.5 ppm interval in 10 M NaOH.

It was also observed that complete sample re-dissolution in alkaline solutions may form suspended particles, which may reduce spectral quality and undermine TP and OP detectability. To alleviate this problem, samples re-dissolved in 1 M NaOH were added with 100 μL HCl before undergoing NMR analyses. This procedure was found to decrease the amount of particles suspended in the alkaline solution and successful in providing more intense signals in ^{31}P-NMR spectra (Figure 2).

NMR acquisition time

As indicated earlier, it is crucial to reach the adequate acquisition time during ^{31}P-NMR experiments in order to generate spectra with sufficient signal intensity to observe differences in sample treatments [6]. Here, we acquired ^{31}P-NMR spectra for 5, 10, and 15 h of acquisition time and compared spectral results (Figure 3). We found that the longest acquisition time (15 h) brought about the greatest response for signals of diesters, phosphonates, and polyphosphate for samples rich in organic matter, such as forest litter and compost (Figure 3). Such long acquisition time is required to increase spectral

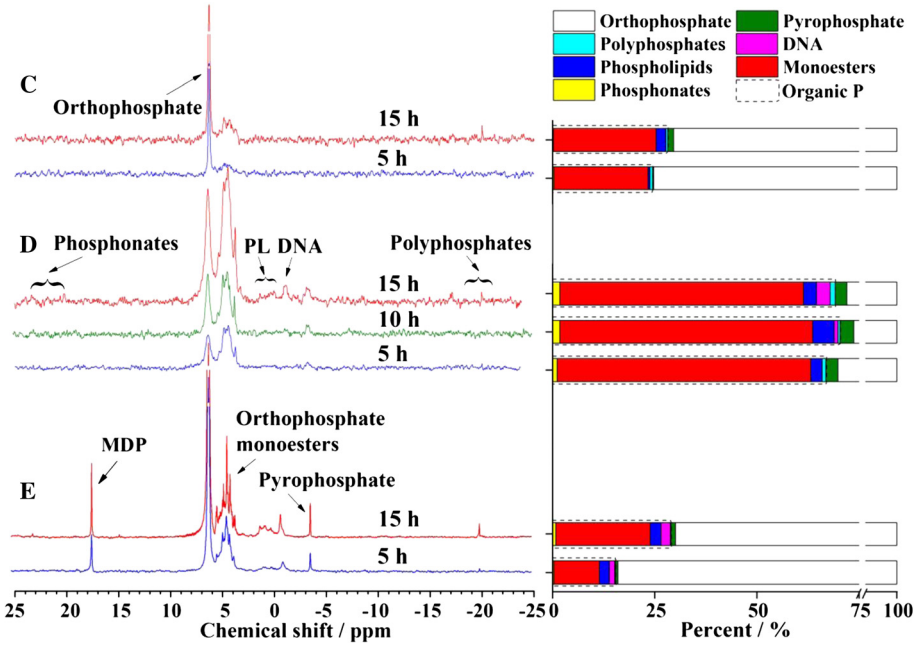

Figure 3 ^{31}**P-NMR spectra of extracts from different materials and relative amount (%) of P species at different NMR acquisition times.** **(C)** Forest soil. **(D)** Forest litter. **(E)** Compost. MDP, metylendiphosphonic acid-P,P'-disodium salt as internal standard; PL, phospholipids.

quality, especially for relatively weak signals. In fact, P signals for DNA and polyphosphate in extracts from forest litter and forest soil, respectively, could not be yet detected after a 5-h long acquisition time, whereas they were visible after 15 h (Figure 3).

Conclusions

Solution-state ^{31}P-NMR spectra obtained in this work revealed that soil and compost samples which were freeze-dried before NMR analysis ensured a larger detectability of total and organic P than samples undergone NMR experiments either after air-drying or directly without pretreatment. Thus, a freeze-drying pretreatment represents the method of choice for natural samples with low P concentration and whose NMR detectability of P signals is poor.

We also showed that re-dissolution of extracts from natural samples into 1 M NaOH solution makes P signals more visible by ^{31}P-NMR spectroscopy than when a stronger alkaline solution is used. Furthermore, when suspended particles may interfere with spectral quality, addition of 100 μL of concentrated HCl to the NMR tube containing the alkaline re-dissolved extracts, the intensity of NMR signals are improved. Finally, we showed that at least 15 h of NMR acquisition time were needed in order to reach the sufficient intensity of P signals and enable distinction among sample treatments. We believe that the procedure optimized here to obtain ^{31}P-NMR spectra for natural samples such as soil and humus-rich materials (litter and compost) may become useful to foster studies on total and organic P dynamics in the environment.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

ML performed soil extraction and sample manipulation before NMR analyses and wrote the first manuscript draft. PM ran the NMR experiments. VC supervised the soil and compost collection and characterization. HM characterized soil and compost properties. ZH contributed to design the experiments. AP supervised the experimental design and finalized the manuscript. All authors read and approved the final manuscript.

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