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A new solution ^{31}P NMR sample preparation scheme for marine sediments

Delphine Defforey,[1]* Barbara J. Cade-Menun,[2] Adina Paytan[1,3]
[1]Department of Earth and Planetary Sciences, University of California Santa Cruz, Santa Cruz, California
[2]Agriculture and Agri-Food Canada, Semiarid Prairie Agricultural Research Centre, Swift Current, Saskatchewan, Canada
[3]Institute of Marine Sciences, University of California Santa Cruz, Santa Cruz, California

Abstract

A new approach for the preparation of marine sediment samples for solution ^{31}P nuclear magnetic resonance spectroscopy (^{31}P NMR) has been developed and tested. This approach addresses important aspects associated with sample pretreatments for marine sediments, including the effects of sample pretreatment on sedimentary P composition. The method increases the signals of low abundance P species in ^{31}P NMR spectra by quantitatively and precisely removing up to 80% of inorganic P (orthophosphate) from sediment samples while causing minimal alteration of the chemical structure of organic P compounds. This method uses a reductive step to solubilize P bound to iron oxyhydroxides, followed by a low pH digestion to extract P from authigenic and biogenic apatite, as well as P bound to calcium carbonate. These P forms combined represent the most abundant inorganic P reservoir in marine sediments. The sample residue is then extracted in an alkaline solvent, 0.25 M NaOH with 0.05 M Na_2EDTA, and processed for ^{31}P NMR spectroscopy. The method was tested on natural marine sediment samples from different localities with high inorganic P content (>85% molybdate reactive P), and allowed for the detection of orthophosphate monoesters and pyrophosphate in samples for which only an orthophosphate signal could be resolved with an NaOH-EDTA extraction alone. This new approach will allow the use of ^{31}P NMR on samples for which low organic P concentrations previously hindered the use of this tool, and will help answer longstanding question regarding the fate of organic P in marine sediments.

Phosphorus (P) is a critical macronutrient for life and thus plays a substantial role in regulating primary productivity in some marine systems and, over geological timescales, in the world's oceans (Krom et al. 1991; Wu et al. 2000; Paytan and McLaughlin 2007). In the open ocean, organic P derived from planktonic and bacterial biomass is the primary form in which P is delivered to marine sediments, and can make up 25–30% of total sedimentary P (Faul et al. 2005; Paytan and McLaughlin 2007; Ruttenberg 2014). Despite its importance to marine P cycling, there is still a limited understanding of the chemical composition of organic P in sediments. This is largely due to low concentrations and analytical difficulties associated with isolating organic matter and determining the chemical speciation of organic P (Ruttenberg 2014). Most studies of marine P cycling in sediments have focused on quantifying total sedimentary organic P concentrations rather than identifying the compounds that make up the sedimentary P pool (Filippelli and Delaney 1996; Anderson and Delaney 2001; Ruttenberg 2014). These studies have shown that the size of the bulk sedimentary organic P pool tends to decrease with depth, consistent with microbial respiration of organic P compounds, until it reaches an asymptotic value that remains low and at constant detectable values. However, mechanisms that would allow for the utilization of sedimentary organic P, the relative reactivity of distinct compounds within this pool and their degree of preservation during diagenesis are still poorly constrained. Numerous studies have proposed possible compounds within the organic P pool (inositol phosphates, phosphonates) that are more refractory and could resist microbial respiration, only to reach the conclusion that these can be utilized for microbial respiration as readily as labile P compounds, such as phosphomonoesters and phosphodiesters (Suzumura and Kamatani 1995; Laarkamp 2000; Benitez-Nelson et al. 2004). This implies that even organic P compounds previously thought to be refractory to microorganisms can be mineralized.

The application of solid-state and solution ^{31}P nuclear magnetic resonance spectroscopy (hereafter referred to as ^{31}P

*Correspondence: ddeffore@ucsc.edu

Additional Supporting Information may be found in the online version of this article.

NMR) to marine sediments and sinking marine particulate matter has substantially improved our understanding of the composition of the bulk organic P sedimentary pool (Carman et al. 2000; Laarkamp 2000; Paytan et al. 2003; Sannigrahi and Ingall 2005). Indeed, ^{31}P NMR studies of marine sediments from different localities indicate that orthophosphate typically constitutes 50–70% of total sedimentary P, pyrophosphate 0–4%, polyphosphate 0–8%, phosphonates 0–15%, P-monoesters ~ 30%, and P-diesters up to 16% (Paytan et al. 2003; Sannigrahi and Ingall 2005). However, successful ^{31}P NMR experiments require high organic to inorganic P ratios and high P concentrations, making ^{31}P NMR difficult to use with widely distributed open ocean low organic matter sediments. A small number of studies have used pretreatments to increase P concentrations and lower paramagnetic ion content in marine sediments. While these studies provided insightful results, they contained some weaknesses in experimental design. Carman et al. (2000) used a two-step pre-extraction (citrate-dithionite-bicarbonate and magnesium chloride) followed by two double-distilled water rinses and a 24 h alkaline extraction (0.5 M NaOH) to prepare samples for solution ^{31}P NMR. The ^{31}P NMR experiments consisted of 30,000 scans (24 h); this, along with the duration of the alkaline extraction, raises the question of possible organic P hydrolysis during sample preparation and analysis. Ingall et al. (1990) and Sannigrahi and Ingall (2005) used an HF/HCl pretreatment to remove mineral phases and concentrate organic matter. However, no information regarding the amount of P removed during pretreatment and the effects of the pretreatment on sample P compounds was provided. Since those studies were published, HF pretreatments have been shown to completely remove inositol phosphates and pyrophosphate from soil samples (Dougherty et al. 2007; Hamdan et al. 2012). This highlights the need for a detailed extraction procedure to prepare marine sediments for solution ^{31}P NMR that includes information about the effects of the sample pretreatment on existing compounds and an evaluation of result reproducibility.

This study focuses on solution ^{31}P NMR because it allows for sample pre-concentration and offers higher spectral resolution than solid-state ^{31}P NMR, and thus has the potential to identify specific P compounds rather than broad compound classes (e.g., aminoethyl phosphonic acid vs. phosphonate; Cade-Menun et al. 2005). Since the output of ^{31}P NMR experiments comes in the form of percentage of total sample P for each P species based on resolvable peak area, samples with high inorganic P content (> 90%) can yield spectra that are dominated by the orthophosphate peak. While other P compounds may be present, their smaller peaks may not be sufficiently resolved from the background noise; as such, the number of collected scans may have to be substantially increased to improve the signal-to-noise ratio enough to see them. The goal of this procedure is to amplify the signal of organic P compounds relative to that of orthophosphate so that low abundance P species may be resolved with ^{31}P NMR without the need for extremely long (> 24 h) ^{31}P NMR experiments. Our sample preparation scheme solubilizes orthophosphate from mineral phases during the first two steps of the procedure before the sediment sample is extracted for solution ^{31}P NMR. First, P bound to iron (Fe) oxyhydroxides is released from sediment samples through a reductive step during which sodium dithionite reduces Fe oxyhydroxides, and citrate complexes with Fe, thus releasing P associated with Fe oxides (Ruttenberg 1992). Following this step, the sediment residue is extracted in a low pH sodium acetate buffer to dissolve authigenic, biogenic apatite, as well as calcium carbonate (Ruttenberg 1992). We chose to use a sodium acetate buffer instead of a stronger acid for this step to minimize the alteration of the chemical structure of organic P compounds during the extraction. This two-step pretreatment quantitatively removes the majority of mineral P from marine sediment samples while also removing polyvalent bridging cations that can interfere with the alkaline extraction and speciation of organic P in marine sediments using ^{31}P NMR (Turner et al. 2005). With this pretreatment, P species with low relative abundance that would not be detected using a single alkaline extraction can be quantified. Our method offers an accessible and reproducible means of preparing samples that would otherwise be unsuitable for ^{31}P NMR analysis.

Materials and procedures

Materials

Laboratory supplies

We used 250 mL high-density polyethylene (HDPE) Nalgene bottles and 2 L HDPE bottles for reagent solutions. All glassware and plasticware were soaked in Decon Neutrad phosphate-free soap, acid-washed in 10% hydrochloric acid and rinsed with 18.2 MΩ·cm deionized water (hereafter referred to as ultrapure water) prior to usage.

Chemical supplies

All chemical supplies used for this study were ACS-grade. These include trisodium citrate (Fisher Chemical), sodium bicarbonate (Fisher Chemical), sodium dithionite (Acros Organics), sodium acetate (Fisher Chemical), sodium hydroxide (Fisher Chemical), disodium ethylenediaminetetraacetic acid (Fisher Chemical), 17.4 M glacial acetic acid (Fisher Chemical), and 18.0 M sulfuric acid (Fisher Chemical). Ancillary information regarding reagent solution preparation is provided in Supporting Information Appendix 1.

Equipment

We used a table-top centrifuge capable of 3000–4000 rpm (Thermo Scientific IEC CL40), a laboratory furnace (Barnstead Thermolyne 62700), an analytical balance (Ohaus Explorer), a shaker table (New Brunswick Scientific C1 Platform Shaker), a freeze-drier (Labconco 75035), an inductively coupled plasma optical emission spectrometer (ICP-OES)

(Perkin-Elmer Optima 4300 DV Inductively Coupled Plasma Optical Emission Spectrometer operated by the University of California, Santa Cruz), a QuikChem 8000 automated ion analyzer (Lachat Instruments), and an NMR spectrometer (600 MHz Varian Unity INOVA spectrometer equipped with a 10 mm broadband probe operated by the Stanford Magnetic Resonance Laboratory at Stanford University).

Extraction procedure

The pretreatment (Fig. 1) uses a reductive step to solubilize reducible P bound to Fe oxyhydroxides (P_{red}), followed by a mild acid digestion to extract P from authigenic and biogenic apatite, as well as P bound to calcium carbonate (P_{CFA}). These P pools combined represent the most abundant inorganic P reservoirs in marine sediments (Ruttenberg 1992). The residue is then extracted in an alkaline solvent, 0.25 M NaOH with 0.05 M Na_2EDTA (hereafter referred to as NaOH-EDTA), which has been shown to be the most effective extractant solution for organic P in soils (Cade-Menun and Preston 1996; Turner et al. 2005).

Initial sample P characterization

We used the ignition method to determine total P and molybdate-reactive P concentrations (MRP, which includes primarily free orthophosphate) for each sediment sample used for this study. Samples for total P analyses were ashed in crucibles at 550°C for 2 h and then extracted in 25 mL of 0.5 M sulfuric acid for 16 h. Samples for MRP analyses were extracted in the same manner, without the ashing step (Olsen and Sommers 1982; Cade-Menun and Lavkulich 1997). We derived molybdate-unreactive P concentrations (MUP, which includes primarily organic P and polyphosphates) in supernatants by subtracting MRP from total P concentrations. For ashed and unashed extracts, MRP was determined as described below in the section titled "Supernatant P analysis."

Sample pretreatment

Prior to the extraction, we freeze-dried, ground and sieved sediment samples to less than 125 μm (Ruttenberg 1992). For a given sample, we weighed four sample replicates (2 g) and placed each in 250 mL HDPE bottles. Sodium dithionite (F.W. 147.12 g mol^{-1}; 7.4 g) was added to each sample split, followed by 200 mL of citrate-bicarbonate solution (pH 7.6). This step produces effervescence, so the solution should be added slowly to the sample. We shook samples for 8 h and then centrifuged them at 3700 rpm for 15 min. We filtered the supernatants with a 0.4 μm polycarbonate filter. We took 20 mL aliquots from the filtrate for each sample split for MRP and total P analyses, and kept them refrigerated until analysis within 24 h. We added 200 mL of ultrapure water to the solid residue for each sample split as a wash step after the above reductive step, shook samples for 2 h, and then centrifuged them at 3700 rpm for 15 min. We filtered the supernatants with 0.4 μm polycarbonate filters and set aside 20 mL of filtrate from each sample split for MRP and total P analyses. We then extracted the solid sample residues in 200 mL of sodium acetate buffer (pH 4.0) for 6 h. At the end of this extraction step, we centrifuged the bottles at 3700 rpm for 15 min, filtered the supernatants with 0.4 μm polycarbonate filters and took a 20 mL aliquot of filtrate from each sample split for MRP and total P analyses. We added 200 mL of ultrapure water to the solid residue for each sample split as a wash step, shook samples for 2 h, and then centrifuged them at 3700 rpm for 15 min. We filtered the supernatants with 0.4 μm polycarbonate filters and set aside 20 mL of filtrate from each sample split for MRP and total P analyses. We repeated the water rinse step, and collected aliquots for MRP and total P analyses as in the previous steps. The concentrations of total P and MRP were determined as described below in the section titled "Supernatant P analysis."

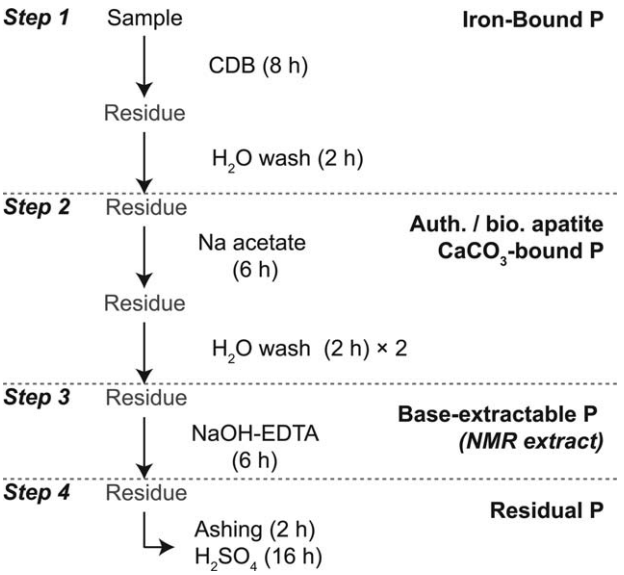

Fig. 1. Stepwise extraction scheme summarizing the preparation of marine sediment samples for solution ^{31}P NMR spectroscopy.

Solution ^{31}P NMR extraction

Solid sediment sample residues following the pretreatment described above were transferred to two 50 mL centrifuge tubes (two sample replicates combined per tube). We added 20 mL of 0.25 M NaOH + 0.05 M Na_2EDTA solution to each tube, vortexed until all sediment was resuspended and then shook samples for 6 h at room temperature (Cade-Menun et al. 2005). We used a solid to solution ratio of 1 : 5 for this step to minimize the amount of freeze-dried material that will need to be dissolved for the ^{31}P NMR experiments. Large amounts of salts from the NaOH-EDTA concentrated in NMR samples lead to higher viscosity and increase line broadening on NMR spectra (Cade-Menun and Liu 2014). We chose an extraction time of 6 h to improve total P recovery while limiting the degradation of natural P compounds in the sample. At the end of the extraction, samples were

centrifuged at 3700 rpm for 15 min and supernatants decanted into 50 mL centrifuge tubes. We collected a 500 μL aliquot from each sample, which we diluted with 4.5 mL of ultrapure water. These were refrigerated until analysis for total P content on the ICP-OES. The sample residues and supernatants were frozen on a slant to maximize the exposed surface area during the lyophilization step; this was done immediately after the removal of the 500 μL aliquot. Once completely frozen, the uncapped tubes containing supernatants and residues were freeze-dried over the course of 48 h. Each tube was covered with parafilm with small holes from a tack to minimize contamination. Freeze-dried supernatants from identical sample splits were combined and dissolved in 500 μL each of ultrapure water, D_2O, NaOH-EDTA, and 10 M NaOH prior to ^{31}P NMR analysis. The D_2O is required as signal lock in the spectrometer (Cade-Menun and Liu 2014). Sample pH was maintained at a pH > 12 to optimize peak separation (Cade-Menun 2005; Cade-Menun and Liu 2014). Sample pH was assessed with a glass electrode, and verified with pH paper to account for the alkaline error caused by the high salt content of our samples (Covington 1985).

Solution ^{31}P NMR analysis

Spectra were acquired immediately following sample preparation on a Varian Unity INOVA 600 MHz spectrometer equipped with a 10-mm broadband probe. We used a 10-mm rather than a 5-mm probe because larger tubes contain a greater concentration of P and thus require fewer scans to achieve similar signal to noise ratios (Cade-Menun and Liu 2014). The analytical parameters used were: 20°C, 90° pulse, 0.48 s acquisition time, 4.52 s delay time, 5600 scans (8 h experiments), no spin, and an external H_3PO_4 standard. We maintained samples at a temperature of 20°C during experiments to achieve optimal spectral resolution (Crouse et al. 2000) and to minimize sample degradation. No proton decoupling was used out of concern for sample degradation (Cade-Menun and Liu 2014). The ratio of P to Fe and manganese (Mn) was used as a proxy for spin-lattice relaxation times (T_1) to ensure adequate delays between pulses and thus quantitative spectra (McDowell et al. 2006). We used 5 s recycle delays, which correspond to three to five times the calculated T_1 values, as recommended by McDowell et al. (2006). Peak identification was based on literature (Turner et al. 2003; Cade-Menun 2015).

Post-experimental processing

^{31}P NMR data were processed using the NMR Utility Transform software (NUTS, Acorn NMR). Peak areas were calculated by integration of spectra processed with a 7 Hz line broadening following baseline correction, peak picking and phasing. We accepted peaks that (1) represented at least 1% of the tallest peak in the total integrated area, (2) were identified by the NUTS software, and (3) were confirmed as signal by visual inspection.

Residual P extraction

Freeze-dried sample residues were ashed in crucibles at 550°C for 2 h and then extracted in 25 mL of 0.5 M sulfuric acid for 16 h (Olsen and Sommers 1982; Cade-Menun and Lavkulich 1997). We centrifuged samples at 3700 rpm for 15 min, filtered supernatants with 0.4 μm polycarbonate filters, and measured P content on an ICP-OES.

Supernatant P analyses

Total P concentrations in sediment extracts were measured using inductively coupled plasma optical emission spectroscopy (ICP-OES). Standards were prepared with the same solutions as those used for the extraction procedure in order to minimize matrix effects on P measurements. Sediment extracts and standards (0 μM, 3.2 μM, 32 μM, and 320 μM) were diluted to lower salt content to prevent salt buildup on the nebulizer (1 : 20 dilution for step 1, 1 : 10 for steps 2–4). Concentration data from both wavelengths (213 nm and 214 nm) were averaged to obtain extract concentrations for each sample. The detection limit for P on this instrument for both wavelengths is 0.4 μM. The MRP concentrations were measured on a QuikChem 8000 automated ion analyzer. Standards were prepared with the same solutions used for the extraction step to minimize matrix effects on P measurements. Sediment extracts and standards (0–30 μM PO_4) were diluted tenfold to prevent matrix interference with color development. The detection limit for P on this instrument is 0.2 μM. We derived MUP concentrations by subtracting MRP from total P concentrations.

Degradation experiments

We verified the effects of our pretreatment on different classes of P compounds by spiking blank CDB and Na-acetate solutions with known standards, and monitoring the degree of hydrolysis over the course of the extraction period. Samples were analyzed by NMR for 13.3 min (16 scans) immediately after preparation of the spiked solutions; this was repeated every after 3 h (sodium acetate experiments) or 4 h (CDB experiments) of storage at room temperature (for 6 h total for the sodium acetate samples and 8 h total for the CDB samples). The standards used to assess hydrolysis due to sample pretreatment were 2-aminoethylphosphonic acid, D-glucose-6-phosphate, DL-α-glycerophosphate, RNA, and ATP. We used methylenedisphosphonic acid (MDP) as an internal standard in these experiments.

Assessment

Evaluation of extraction procedure on natural samples

We evaluated the performance of our method by testing it on natural marine sediment samples and comparing results to those obtained using the commonly used NaOH-EDTA extraction without pretreatment. The sediment samples used were collected during USGS cruises P-1-94-AR (Arctic Ocean) and W-2-98-NC (California Margin) and the JGOFS cruise TT013-06MC (Equatorial Pacific). Samples were

Table 1. Sample location, depth, sampling time, and sedimentary characteristics of the samples used in this study.

Sample ID	Latitude, longitude	Water depth (m)	Sediment depth (cm)	Sampling dates	Sediment composition
Arctic Ocean					
P-1-94-AR P21 0–4 cm	84°5′N, 174°58′W	3180	0–4	07/25/94–08/30/94	<10% $CaCO_3$, high illite, low kaolinite and chlorite*
P-1-94-AR P21 16–20 cm	84°5′N, 174°58′W	3180	16–20	07/25/94–08/30/94	
California margin					
W-2-98-NC TF1 0–4 cm	41°5′N, 125°1′W	3045	0–4	07/17/98–07/24/98	High kaolinite and chlorite, moderate illite†
W-2-98 NC TF1 18–20 cm	41°5′N, 125°1′W	3045	18–20	07/17/98–07/24/98	
Equatorial Pacific					
TT013-06MC 10–12 cm	12°00′S, 134°56′W	4280	10–12	10/30/92–12/13/92	80% $CaCO_3$, 10% opal‡
TT013-06MC 24–26 cm	12°00′S, 134°56′W	4280	24–26	10/30/92–12/13/92	

*Stein et al. (1994).
†Griggs and Hein (1980).
‡Murray and Leinen (1993).

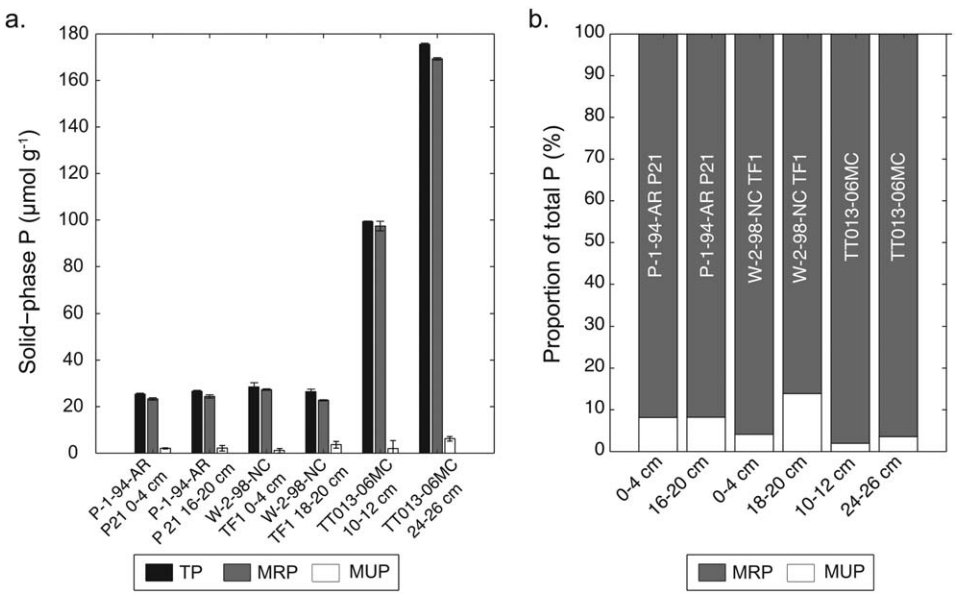

Fig. 2. (**a**) Total P, MRP, and MUP content of the sediment samples used for this study and (**b**) relative proportions of MRP and MUP for each sample. Total P concentrations were determined following the extraction of ignited sediment samples, while MRP concentrations were measured following the extraction of unignited sediment samples. Error bars on the figure show standard deviations. All P concentration measurements were done colorimetrically on a QuikChem 8000 automated ion analyzer. MUP was determined by subtracting MRP from total P concentrations.

selected based on their latitude, differing sediment types, and sedimentary P characteristics (Table 1; Fig. 2). We chose samples with high MRP content (> 85% of total P), and a range of sedimentary mineral composition to assess the performance of our method (Table 1; Fig. 2). For all samples, total orthophosphate content differed between the single NaOH-EDTA extraction and our method due to the fact that orthophosphate is the only compound extracted without the pretreatment. It is worth noting that solution ^{31}P NMR is a poor method to study orthophosphate since extracts remove any associated cations, providing no information about its bonding in the original sample. For the Arctic and Equatorial Pacific samples, our method allowed for the detection of P monoesters and pyrophosphate, which would otherwise not be detectable using a single NaOH-EDTA extraction (Table 2; Fig. 3). For the samples near the California Margin, orthophosphate and P monoesters were detected using both the single NaOH-EDTA extraction and our method (Table 2; Fig. 4). However, our method allowed for the detection of pyrophosphate, which was not measurable with a single alkaline extraction. The total P monoester concentrations were similar when comparing both methods, showing that the

Table 2. Extraction yields for each step. P_{red} refers to P solubilized during the reductive step (step 1), P_{CFA} refers to P released during the mild acid digestion (step 2). NMR extract concentrations are based on total P extracted. Orthophosphate (orthoP), orthophosphate monoester and pyrophosphate (pyroP) content was determined using ^{31}P NMR and the concentrations add up to the NMR extract total P content (step 3). Residual P is the P leftover after the pretreatment and NMR extraction (steps 1–3). Extracted P is the fraction of P that was extracted in steps 1–3. Percentages in parentheses refer to the % P compared to the total sample P content. Dashed lines refer to measurements that were not conducted.

				From ^{31}P NMR spectra				
Sample ID	P_{red} (μmol g^{-1})	P_{CFA} (μmol g^{-1})	NMR extract TP (μmol g^{-1})	OrthoP (μmol g^{-1})	OrthoP monoesters (μmol g^{-1})	PyroP (μmol g^{-1})	Residual P (μmol g^{-1})	Extracted P (%)
Sample pretreatment								
P-1-94-AR P21 0–4 cm	8.10 (40.8%)	8.45 (42.6%)	0.49 (2.5%)	0.40 (2.0%)	0.08 (0.4%)	0.02 (0.1%)	2.81 (14.2%)	85.9
P-1-94-AR P21 16–20 cm	8.86 (42.9%)	8.75 (42.4%)	0.36 (1.8%)	0.32 (1.6%)	0.03 (0.2%)	0.01 (0.1%)	2.68 (13.0%)	86.3
W-2-98-NC TF1 0–4 cm	4.89 (21.8%)	11.9 (53.2%)	1.56 (7.0%)	1.36 (6.1%)	0.16 (0.7%)	0.03 (0.1%)	4.04 (18.0%)	82.0
W-2-98 NC TF1 18–20 cm	3.41 (18.0%)	10.0 (52.4%)	1.54 (8.1%)	1.25 (6.6%)	0.27 (1.4%)	0.02 (0.1%)	4.10 (21.6%)	78.6
TT013-06MC 10–12 cm	14.7 (16.3%)	57.8 (63.8%)	1.61 (1.8%)	1.43 (1.6%)	0.15 (0.2%)	0.03 (0.03%)	16.4 (18.2%)	80.6
TT013-06MC 24–26 cm	31.8 (20.0%)	96.4 (60.7%)	2.79 (1.8%)	2.55 (1.6%)	0.20 (0.1%)	0.04 (0.03%)	27.8 (17.5%)	82.3
No pretreatment								
P-1-94-AR P21 0–4 cm	—	—	2.31 (14.2%)	—	—	—	14.0 (85.8%)	14.2
P-1-94-AR P21 16–20 cm	—	—	3.93 (23.6%)	—	—	—	12.8 (76.4%)	23.6
W-2-98-NC TF1 0–4 cm	—	—	2.87 (14.5%)	2.70 (13.6%)	0.17 (0.9%)	0.00 (0%)	16.9 (85.5%)	14.5
W-2-98 NC TF1 18–20 cm	—	—	1.97 (11.8%)	1.66 (9.9%)	0.31 (1.9%)	0.00 (0%)	14.8 (88.2%)	11.8
TT013-06MC 10–12 cm	—	—	1.73 (2.0%)	—	—	—	83.8 (98.0%)	2.0
TT013-06MC 24–26 cm	—	—	2.53 (1.8%)	—	—	—	141.6 (98.2%)	1.8

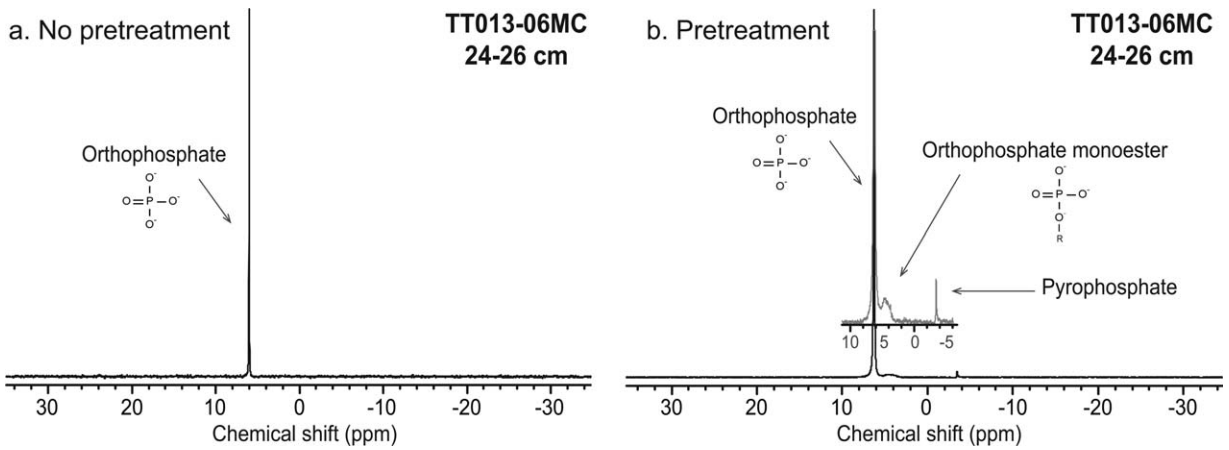

Fig. 3. (**a**) ^{31}P NMR spectrum of sample TT013-06MC (24–26 cm, highest MRP content) following a single NaOH-EDTA with no sample pretreatment and (**b**) spectrum of the same sample after pretreatment to remove orthophosphate. The inset spectrum in (**b**) is enlarged to better show details in the orthophosphate monoester and pyrophosphate regions. Spectra are plotted with 7-Hz line broadening, and are plotted with the orthophosphate peak at the same height.

pretreatment did not remove any measurable P monoesters (Table 2). We attempted to identify specific monoester compounds using spiking, but this was not successful due to low signal to noise ratios and line broadening in the spectra. However, there appear to be some diester degradation peaks (α- and β-glycerophosphate) of similar magnitude in samples treated with the single NaOH-EDTA extraction and in the samples processed with the pretreatment we employed.

Extraction yields for samples with no pretreatment ranged between 1.8–23.6% of total P (Table 2). In comparison, extraction yields for samples with the pretreatment ranged between 78.6–86.3% (Table 2). The P content in the NMR extracts was lower in pretreated samples than in samples with no pretreatment because the pretreatment removed up to 80% of the samples' MRP content (Table 3; Fig. 5). However, adding the pretreatment allowed the quantification of

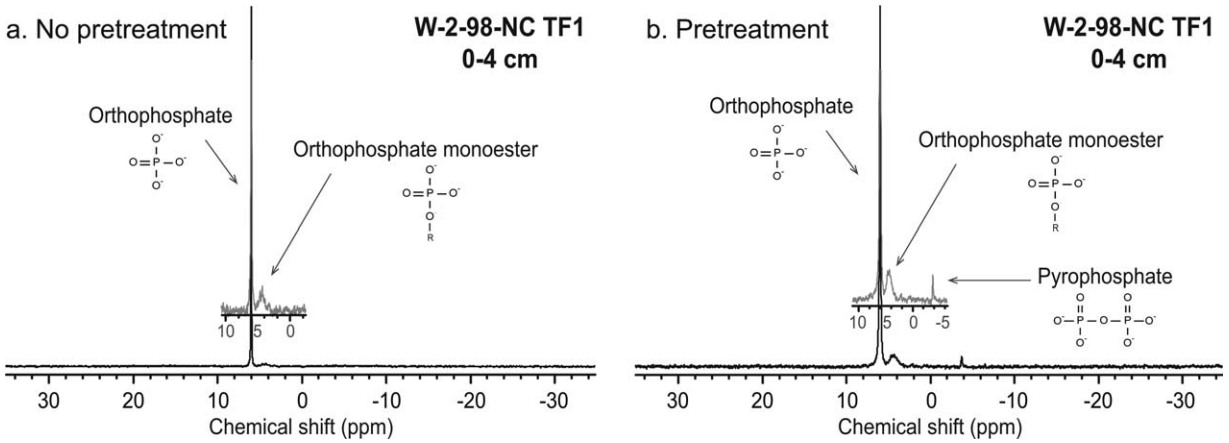

Fig. 4. (**a**) ^{31}P NMR spectrum of sample W-2-98-NC TF1 (0–4 cm, intermediate MRP content) following a single NaOH-EDTA with no sample pretreatment and (**b**) spectrum of the same sample after pretreatment to remove orthophosphate. The inset spectra are enlarged to better show details in the orthophosphate monoester and pyrophosphate regions. Spectra are plotted with 7-Hz line broadening, and are plotted with the orthophosphate peak at the same height.

Table 3. Aluminum (measurement error: 2.1%), calcium (0.9%), iron (2.8%), potassium (7.1%), magnesium (5.1%), manganese (16%), and phosphorus (6.0%) content in ^{31}P NMR extracts. Values in parentheses correspond to measurement errors on the OES determined by replicate analysis of sample TT013-06MC (10–12 cm). Relative error (%) = (SD/mean) × 100. T_1 values are derived from P to iron and manganese ratios (w/v) using the relationship derived by McDowell et al. (2006).

	Al	Ca	Fe	K	Mg	Mn	P		
Sample ID				μmol g^{-1}				P/(Fe + Mn)	Calculated T_1 (s)
Sample pretreatment									
P-1-94-AR P21 0–4 cm	1.31	12.3	0.26	0.28	4.54	0.02	0.49	1.00	0.60
P-1-94-AR P21 16–20 cm	1.70	13.2	0.35	0.25	4.97	0.02	0.36	0.56	0.47
W-2-98-NC TF1 0–4 cm	3.75	28.9	0.61	0.60	3.63	0.09	1.56	1.52	0.75
W-2-98 NC TF1 18–20 cm	2.88	17.3	0.63	0.62	2.68	0.05	1.54	1.28	0.68
TT013-06MC 10–12 cm	0.57	39.8	0.16	0.12	0.32	0.03	1.61	3.95	1.44
TT013-06MC 24–26 cm	0.46	51.4	0.43	0.24	0.36	0.05	2.79	3.23	1.24
No pretreatment									
P-1-94-AR P21 0–4 cm	5.17	58.1	0.09	11.4	35.0	0.00	2.31	16.7	5.09
P-1-94-AR P21 16–20 cm	3.76	120.0	0.06	12.3	43.1	0.01	3.93	38.7	11.4
W-2-98-NC TF1 0–4 cm	4.83	56.0	0.41	14.3	28.0	0.11	2.87	3.86	1.42
W-2-98 NC TF1 18–20 cm	4.84	54.2	0.52	15.0	34.5	0.06	1.97	2.36	0.99
TT013-06MC 10–12 cm	3.06	1244.3	0.00	82.3	0.06	0.02	1.73	105.1	30.4
TT013-06MC 24–26 cm	1.99	1274.1	0.00	103.8	0.08	0.03	2.53	111.7	32.3

extremely low percentages of trace P compounds (as low as 0.1% of total sample P) previously undetectable in the P NMR spectra, and increased overall extraction yields (Table 2).

The pretreatment also resulted in lower concentrations of Al, Ca, K, and Mg in the NaOH-EDTA extracts (Table 3). However, it released additional paramagnetic ions (Fe, Mn) compared to samples treated with a single NaOH-EDTA extraction, causing a decrease in spin-lattice relaxation times (Table 3). Without the pretreatment, the recycle delays in the NMR experiments used were too short to yield quantitative spectra for the Arctic Ocean samples and the Equatorial Pacific samples (Tables 2, 3). The higher paramagnetic ion content of the California Margin samples yielded quantitative spectra even without the pretreatment since the recycle delays used were 3–5 times larger than calculated T_1 times (Tables 2, 3). This supports the argument that the signal in the spectra for the Arctic Ocean samples and the Equatorial Pacific samples was saturated with too short delay times.

It is difficult to conduct replicate analyses of all samples with ^{31}P NMR due to the long experimental times involved and thus the expensive nature of these analyses. For these reasons, we determined the method's reproducibility by

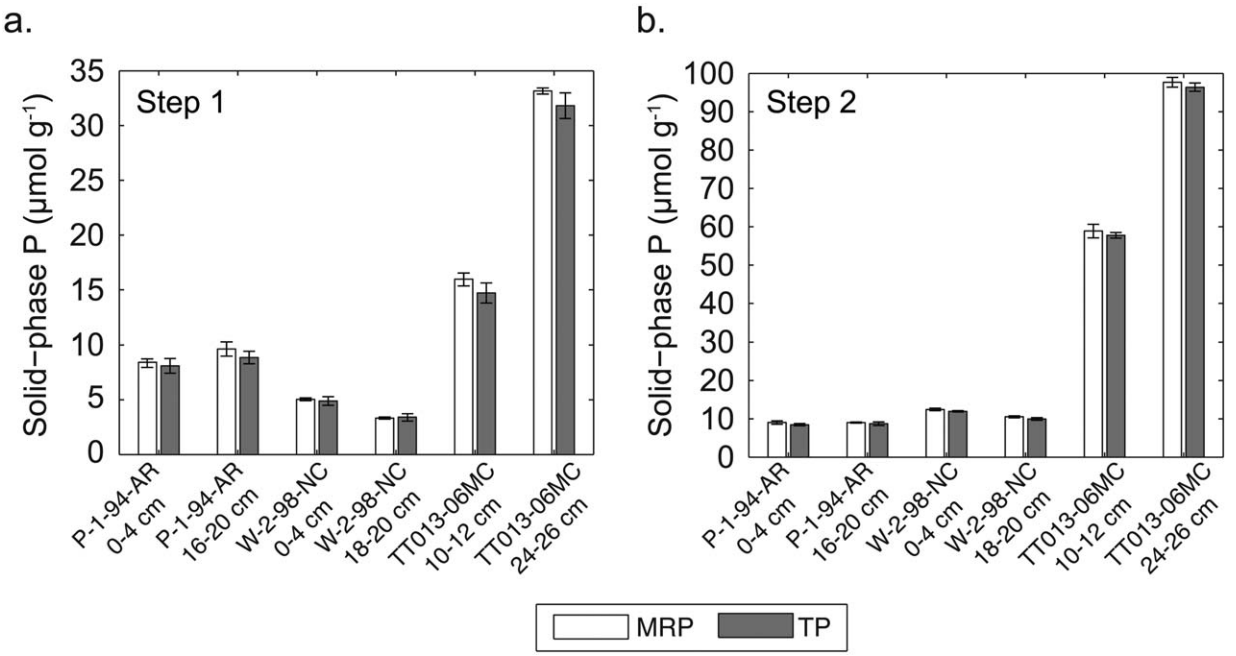

Fig. 5. Mean MRP and total P content in pretreatment extracts from steps 1 (CDB and water extracts, panel **a**) and 2 (sodium acetate and water extracts, panel **b**), along with standard deviations derived from replicate sediment sample splits. Total P concentrations were measured on an ICP-OES, while MRP concentrations were measured colorimetrically on a QuikChem 8000 automated ion analyzer.

extracting multiple replicates of only one of the samples (TT013-06MC 10–12 cm). Measurement uncertainties for MRP for the pretreatment steps ranged from 2.3–2.6% (steps 2 and 1, respectively, results not shown). Total P measurement uncertainties for each extraction step are reported in Table 4. Uncertainties ranged between 1.0–3.8% for the pretreatment steps (highest P concentrations, 14.6 μmol g^{-1} and 52.7 μmol g^{-1}, respectively), and increased to 8–12% for orthophosphate (1.34 μmol g^{-1}) and monoester P (0.16 μmol g^{-1}) determined via P NMR and for the residual P (14.7 μmol g^{-1}) due to the lower P content. The uncertainty associated with measuring pyrophosphate using P NMR is greater (28%) due to the minute amounts of pyrophosphate present in the samples (0.03 μmol g^{-1}, $< 1\%$ of total P), but similar to other published values (Xu et al. 2012). It is worth noting, though, that small changes in pyrophosphate may not be significant due to the high uncertainty.

Table 4. Measurement uncertainties associated with each extraction step using ICP-OES and P NMR spectroscopy determined by replicate analysis of sample TT013-06MC (10–12 cm). Total P relative error is calculated as: relative error (%) = (SD/mean) $\times$ 100.

Extraction step	P fraction	Mean TP (μmol g^{-1})	Standard deviation	TP Rel. error (%)
1	P_{red}	14.6	0.55	3.8
2	P_{CFA}	52.7	0.54	1.0
3	OrthoP	1.34	0.11	7.9
	MonoP	0.16	0.02	11.8
	PyroP	0.03	0.01	27.9
4	Residual P	14.7	1.76	12.0

Pretreatment effects on sample

We monitored the effects of the sample pretreatment on removal of P compounds other than MRP by measuring the MRP and total P content of each extraction step prior to the ^{31}P NMR extraction. We derived MUP content in pretreatment extracts by subtracting MRP from total P concentrations. For all samples, pretreatment extracts contained no detectable levels of MUP (Fig. 5), which indicates that our method does not remove or hydrolyze organic P prior to the ^{31}P NMR extraction step.

We further assessed the effects of the sample pretreatment by monitoring the degree of hydrolysis of eight P compounds representative of naturally occurring organic P. We spiked CDB and sodium acetate blanks with standards, and observed their relative concentrations over the course of the extraction period (8 h for CDB standards, 6 h for sodium acetate standards). We observed no degradation of D-glucose-6-phosphate and DL-α-glycerophosphate during the 8 h CDB extraction (Fig. 6). However, the CDB extraction altered the 2-aminoethylphosphonic acid signal and caused some hydrolysis (change from 2 peaks to 1) over the course of 8 h (Fig. 6). A small decrease (2.2%) in RNA was also observed after 8 h (Fig. 7). We did not observe degradation

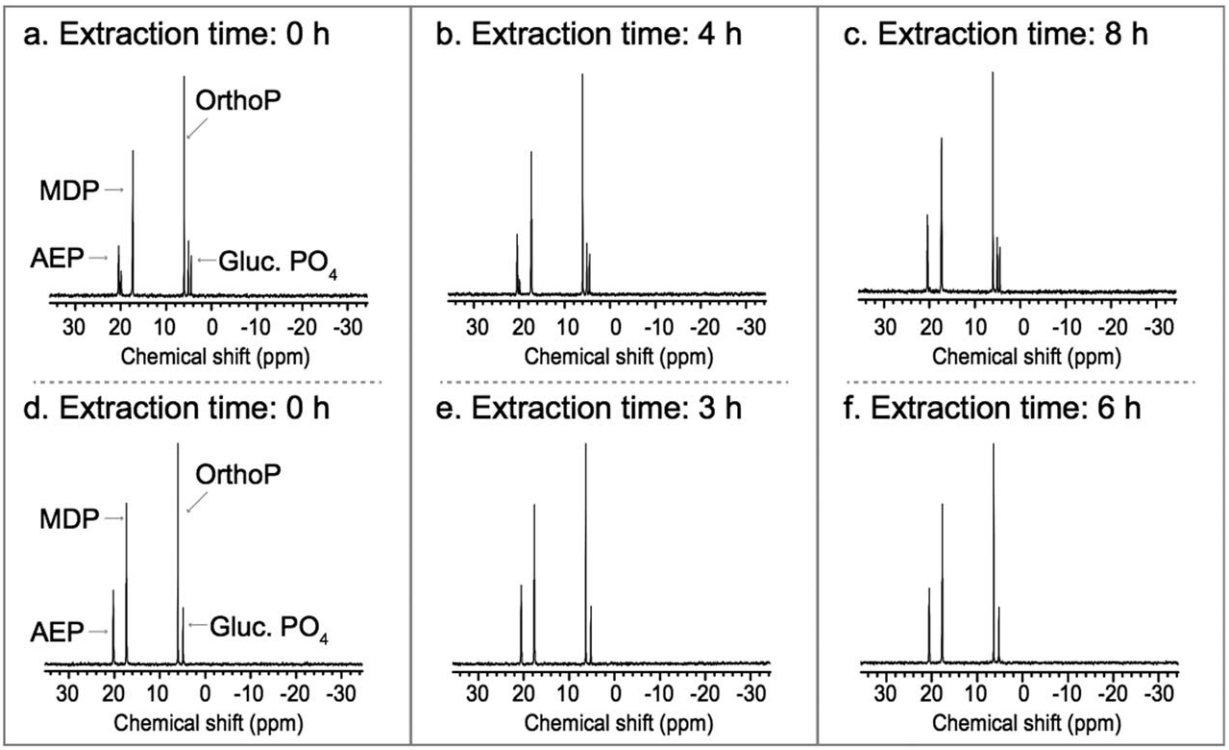

Fig. 6. Spectra from the degradation test for the 8 h CDB extraction (panels a-c) and the 6 h Na-acetate extraction (panels d-f). Spectra in panels a-c are from a blank CDB solution spiked with 2-aminoethylphosphonic acid (AEP), MDP, orthophosphate (orthoP), and D-glucose-6-phosphate (gluc. PO_4). Spectra in panels d-f are from a blank sodium acetate solution spiked with the same compounds. All solutions were pH adjusted to >12. Spectra are plotted with 7-Hz line broadening, and are plotted with the orthophosphate peak at the same height.

in 2-aminoethylphosphonic acid, D-glucose-6-phosphate, ATP, and DL-α-glycerophosphate over the course of the 6 h sodium acetate extraction (Fig. 6). However, there was a slight decrease in the abundance of RNA (2.2%) and ATP (0.8%) (Fig. 7). Overall, the majority of the compounds tested were not rapidly degraded during the pretreatment. It is important to note however, that RNA, and to a lesser extent ATP, hydrolysis ($<5\%$ loss in our hydrolysis experiments) may occur over the course of sample preparation and may cause these compounds to be underestimated. This should be taken into account when interpreting spectra. RNA has been shown to be susceptible to hydrolysis under alkaline conditions during NMR experiments (Smernik et al. 2015), so some degree of degradation with solution ^{31}P NMR using NaOH-EDTA can be expected, with or without any pre-treatment. However, quantification of hydrolysis products can help circumvent the loss of information due to RNA hydrolysis.

Discussion

The new method described here allows for the detection of P forms in marine sediments that were previously below the detection limit by effectively removing up to 80% of mineral P and removing polyvalent bridging cations that can interfere with the alkaline extraction and speciation of organic P in marine sediments, thus increasing the relative signal of low abundance P species (P monoesters, pyrophosphate) in ^{31}P NMR spectra. The reductive step solubilizes orthophosphate bound to Fe oxyhydroxides while removing no measurable MUP, causing no degradation to D-glucose-6-phosphate and DL-α-glycerophosphate, and minimal degradation to 2-aminoethylphosphonic acid, RNA, and ATP. The sodium acetate extraction dissolves authigenic, biogenic apatite, as well as calcium carbonate and extracted no measurable levels of MUP. It is worth noting that Cade-Menun et al. (2015) recently showed that sodium acetate can be used to extract P for NMR analyses in manure samples similarly to NaOH-EDTA. A possible explanation for our different results is that organic P forms are more stabilized and immobile in open ocean sediments than in manure, which affects the degree to which they can be solubilized in the sodium acetate extraction. We recommend future studies monitor the MRP and MUP content of supernatants during extraction to ensure that no significant amount of MUP is solubilized. The sodium acetate extraction did not cause any observable degradation in 2-aminoethylphosphonic acid, D-glucose-6-phosphate, and DL-α-glycerophosphate. However, some degradation of RNA and ATP was observed and could result in underestimations of these compounds.

Without this new pretreatment, spectra from the Equatorial Pacific and Arctic sediment samples were dominated by

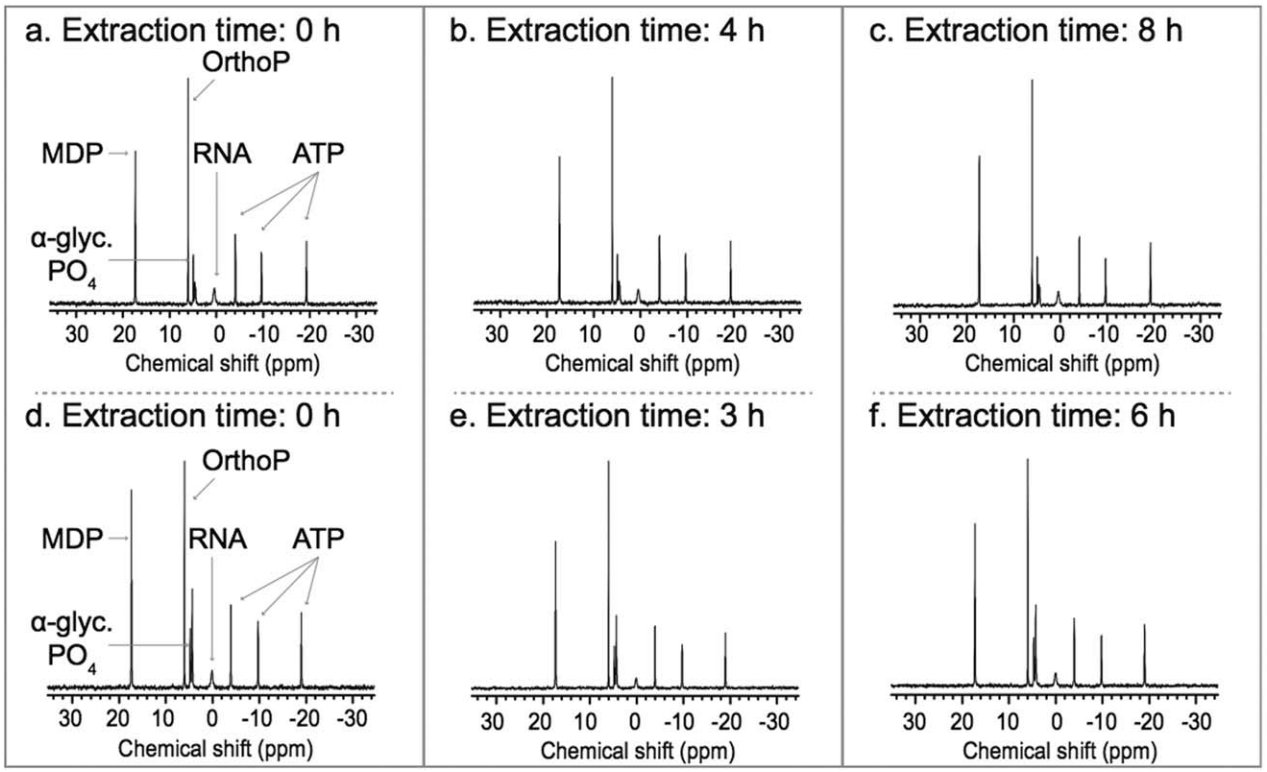

Fig. 7. Spectra from the degradation test for the 8 h CDB extraction (panels a-c) and the 6 h sodium acetate extraction (panels d-f). Spectra in panels a-c are from a blank CDB solution with MDP, orthophosphate, DL-α-glycerophosphate (α –glyc. PO_4), RNA, and ATP. Spectra in panels d-f are from a blank sodium acetate solution spiked with the same compounds. All solutions were pH adjusted to >12. Spectra are plotted with 7-Hz line broadening, and are plotted with the orthophosphate peak at the same height.

orthophosphate, leaving no other peaks visible (Table 2; Fig. 3). The organic P compounds measured in our samples were orthophosphate monoesters (Table 2; Figs. 3, 4). As mentioned above, we were unable to resolve individual peaks in the monoester region due to line broadening and low signal to noise ratios. While identifying peaks in the monoester region was beyond the scope of this methods paper, we recommend future studies correct for degradation peaks to accurately calculate the total amount of orthophosphate monoesters and diesters. Orthophosphate monoesters are generally the dominant group of organic P compounds in both marine and terrestrial environments, and include mononucleotides, sugar phosphates, inositol phosphates, and phosphoproteins (Ingall et al. 1990; Clark et al. 1998, 1999; Carman et al. 2000; Kolowith et al. 2001; Sannigrahi and Ingall 2005; Turner et al. 2005; Sannigrahi et al. 2006). Certain orthophosphate monoesters, particularly the inositol hexakisphosphate stereoisomers, have a high affinity for clays, Fe and aluminum oxide and oxyhydroxide surfaces that leads to the formation of insoluble complexes with polyvalent cations (Celi and Barberis 2005). These complexes may increase their resistance to microbial degradation and lead to their accumulation in sediments (Celi and Barberis 2005). Orthophosphate monoesters are thought to adsorb on the same sites as phosphate anions by exchanging ligands with the mineral surface hydroxyl groups (Baldwin et al. 2002; Celi and Barberis 2005). Orthophosphate diesters, on the other hand, adsorb more weakly on mineral surfaces and are more readily mineralizable, which makes them more vulnerable to microbial degradation (Paytan et al. 2003; Celi and Barberis 2005). Furthermore, organic P remineralization occurs throughout the water column in addition to sediments (Paytan et al. 2003; Benitez-Nelson et al. 2007). These mechanisms combined could account for the absence of orthophosphate diesters in our spectra. Another possibility would be that some orthophosphate diesters were hydrolyzed to orthophosphate monoesters during the extraction process or during NMR analysis (Schneider et al. 2016). Resolving individual monoester peaks in future studies will allow the identification of diester degradation peaks (α- and β-glycerophosphate, mononucleotides) and enable corrections to accurately calculate the total amount of orthophosphate monoesters and diesters separately (Schneider et al. 2016).

We found that all six sediment samples contained small amounts of pyrophosphate, a biogenic form of inorganic P (Table 2). Indeed, pyrophosphate is a product of the biosynthesis of a number of macromolecules, including nucleic acids, amino acids, coenzymes, nucleotides, and activated

precursors to proteins and polysaccharides (Heinonen 2001). Pyrophosphate has been measured in a number of natural samples, including plankton, sediment trap material, and sediments (Sundareshwar et al. 2001; Paytan et al. 2003; Cade-Menun et al. 2005). As in Paytan et al. (2003), sedimentary pyrophosphate concentrations are low and decrease slightly with increasing latitude, supporting the hypothesis of surface water temperature possibly influencing the rate of synthesis or decomposition of this compound.

It is interesting to note that phosphonates, which contain carbon-P bonds, were not detected in any of our sediment samples. Phosphonates have been measured in many marine samples, including sediment trap material, sediments, and dissolved and particulate organic matter (Ingall et al. 1990; Clark et al. 1998, 1999; Carman et al. 2000; Kolowith et al. 2001; Paytan et al. 2003; Benitez-Nelson et al. 2004; Cade-Menun et al. 2005; Sannigrahi et al. 2006). There are two possible hypotheses that could account for the lack of phosphonate detection in our spectra:

1. Phosphonates were remineralized in the water column as particulates sank and in surface sediments, causing sedimentary phosphonates to be either absent or below detection limit despite the pretreatment. Paytan et al. (2003) reported that phosphonates represented 1% of sedimentary P at two sites in the Equatorial Pacific. We did not observe phosphonates in our Equatorial Pacific samples; however, it is important to note that our samples were taken from greater depths (10–12 cm and 24–26 cm below seafloor compared to 1–3 cm below seafloor in Paytan et al. 2003). It is possible that phosphonates were remineralized in the overlying sediments, causing their signal to decrease with depth. Indeed, phosphonate abundance has been shown to decrease significantly between the sediment-water interface and 2 cm below seafloor in anoxic sediments relative to orthophosphate and P esters (Benitez-Nelson et al. 2004). Phosphonate remineralization upon sediment deposition could explain the absence of a phosphonate signal in our spectra.
2. Another possible explanation for the absence of a phosphonate signal is that the alkaline extraction step did not completely solubilize phosphonates. Indeed, while extracting samples with NaOH-EDTA removes the majority of P esters, this alkaline extraction removes a variable portion of phosphonates from marine particulate samples (Cade-Menun et al. 2005). If sedimentary phosphonate levels are low, an incomplete solubilization will result in phosphonate signals below detection limit for ^{31}P NMR spectroscopy. Adding an additional step in the procedure involving solid-state ^{31}P NMR of sediment residues prior to ashing could provide information regarding any organic P compounds that were not extracted.

This new method enhances the use of solution ^{31}P NMR as an effective tool to study organic P in sediments with low organic P content (less than 10% of total P), which represent a large fraction of marine sediments. The use of ^{31}P NMR until now has offered valuable insights into P cycling in sediments with high organic P content, but was not as effective and easily implementable with sediments containing high inorganic to organic P ratios. Using the pre-extraction preparation step, we were able to detect and identify the chemical structure of low abundance organic P species in sediments and demonstrated that orthophosphate monoesters persist in marine sediments despite their potential bioavailability to deep sedimentary subseafloor microorganisms. Furthermore, we observed that pyrophosphate is often present in marine sediment samples and that its abundance varies with latitude. It would be interesting to use this new method to monitor changes in organic P forms and abundance with depth in sediments to investigate the impact of microbial activity and diagenetic processes on organic P compounds as a function of burial depth and age. This will provide valuable insights into the factors controlling the relative reactivity and lability of P compounds and the preservation of organic P in sediments.

Comments and recommendations

Chemical shifts are expressed relative to an external standard and are pH-dependent. For this reason, it is important to consider the standard used and sample pH when comparing results from different studies (Cade-Menun 2015). Maintaining sample pH above 12 and temperature at 20°C during the NMR experiment is optimal for peak separation. We recommend using pH indicator strips in addition to a glass electrode to measure the pH of redissolved NaOH-EDTA extracts to avoid inaccuracies caused by their high salt content, which interferes with standard glass electrode measurements.

Phosphorus NMR requires high sample P content. For this reason, we recommend using at least 2 g of sediment sample in order to resolve the smaller peaks, especially for open ocean sediments where organic P and biogenic P compounds are present in trace amounts.

When possible, spiking with standards should be used to identify specific compounds in P NMR spectra in addition to comparing peaks with compound libraries from published literature. This is the most reliable way to confirm peak identifications. However, given the cost associated with purchasing a large number of pure compounds for spiking, peak libraries have been published and provide a cost-effective means of identifying peaks (Turner et al. 2003; Cade-Menun 2015).

Another important parameter to monitor when preparing marine sediments for ^{31}P NMR analysis is the paramagnetic ion content of the NaOH-EDTA extracts using ICP-OES. Paramagnetic ions affect spectral resolution, as well as recycle delays so their abundance needs to be determined prior to

carrying out an NMR experiment to ensure that spectra are quantitative.

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Conflict of Interest

None declared.

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