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# Refining $^{31}$P nuclear magnetic resonance spectroscopy for marine particulate samples: Storage conditions and extraction recovery

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**Abstract**

Solution $^{31}$P nuclear magnetic resonance (NMR) spectroscopy has recently been used to characterize phosphorus species within marine particles. However, the effects of sample collection, storage and preparation have not been thoroughly examined. In this study, samples of settling particulates collected from a 1200-m sediment trap located in Monterey Bay, California, were subjected to various storage options (i.e., no storage, refrigeration, freezing, and oven-drying and grinding) prior to extraction for solution $^{31}$P-NMR spectroscopy. Freezing, refrigerating and drying samples for periods of up to 6 months prior to extraction with 0.25 M NaOH+0.05 M $Na_2EDTA$ increased the concentration of extracted P by an average of 16% relative to samples extracted without storage. Pre-extraction storage also introduced some minor changes in P speciation, by increasing the percentage of orthophosphate by up to 15% and decreasing the percentage of pyrophosphate by up to 5%, relative to the abundances of these P species in samples extracted without storage. Drying caused the biggest changes in speciation, specifically decreasing more extensively the relative percentage of pyrophosphate compared to other treatments. Nevertheless, observed changes in speciation due to sample storage within a specific sample were small relative to differences observed among samples collected sequentially in the same area, or reported differences among samples collected at different locations. Samples were also analyzed by solid-state $^{31}$P-NMR spectroscopy before and after extraction, to examine extraction-related changes in P speciation. Comparison of solution with solid-state $^{31}$P NMR indicates that extraction with NaOH–EDTA removes the majority of organic esters, but only a variable portion of phosphonates (39–67%). In addition, there was preferential extraction of Ca-associated phosphate over Mg-, Fe- and Al-associated phosphate. Solution $^{31}$P NMR enables much higher resolution of P species within samples, particularly when it is important to speciate orthophosphate monoesters and diesters, or if polyphosphates are present. However, combining solid-state $^{31}$P NMR with

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solution $^{31}$P NMR spectroscopy for marine particles should be conducted when examining inorganic P speciation and the abundance of phosphonates.


*Keywords:* P speciation; NaOH–EDTA; Nuclear magnetic resonance spectroscopy

## 1. Introduction

Phosphorus (P) is an essential element used by all organisms and may be a limiting nutrient for growth in many regions (Karl et al., 1995; Cotner et al., 1997). Phosphorus is present in the water column in dissolved and particulate phases, and as organic and inorganic forms (Benitez-Nelson, 2000). Although organisms may use both inorganic and organic P forms, orthophosphate is preferred (e.g., Cotner and Wetzel, 1991; Lobban and Harrison, 1994; Karl and Björkman, 2002). Thus, the conversion of particulate P to dissolved forms and organic P to inorganic orthophosphate are crucial for regulating P availability in aquatic systems (Delaney, 1998). However, relatively little is known about the concentrations, turnover rates, transport and fate of particulate P in oceans (Benitez-Nelson, 2000; Paytan et al., 2003). This is, in part, due to current limitations in P analytical techniques. Organic P concentrations in particulate and dissolved samples are determined indirectly by the difference between total P and soluble reactive P (SRP), which is the fraction that reacts to form a blue-colored phosphomolybdate complex under slightly acidic conditions (Koroleff, 1983). However, this procedure may be inaccurate due to hydrolysis of labile organic P compounds with the addition of acid or in samples with high concentrations of polyphosphates, because these complex inorganic P forms are not molybdate reactive (McKelvie et al., 1995; Thomson-Bulldis and Karl, 1998). Thus, organic compounds may be considered inorganic, or vice versa. In addition, these indirect methods provide no information about P speciation. Because P forms vary in their chemical and biological reactivity, it is difficult to understand oceanic P cycling without identifying the P species present and their relative abundance. One tool that has been helpful in recent years for the identification and quantification of P species in environmental samples is $^{31}$P-NMR spectroscopy.

Phosphorus NMR is a non-destructive, non-invasive technique that uses the magnetic resonance of a nucleus to identify the chemical forms of that nucleus within a sample. It has the advantage that all P species can be characterized simultaneously, without the need for complex purification and chromatographic separation procedures (Cade-Menun, 2004). Although widely used for studies of terrestrial ecosystems, studies of aquatic P using $^{31}$P-NMR spectroscopy are few. Solid-state and solution $^{31}$P-NMR spectroscopy have been used to characterize P forms in sediments from oceans (Ingall et al., 1990; Carman et al., 2000), estuaries (Halls, 2002; Sundareshwar et al., 2001) and freshwater systems (e.g., Hupfer et al., 1995, 2004; Khoshmanesh et al., 2002; Selig et al., 2002; Watts et al., 2002).

In the ocean water column, dissolved, high-molecular-weight (HMW) P in seawater has been characterized with solid-state $^{31}$P-NMR spectroscopy after tangential flow ultrafiltration (Clark et al., 1998, 1999; Kolowith et al., 2001). A prominent peak that includes both orthophosphate and orthophosphate esters was observed (75% of P), as well as a smaller peak for phosphonates (25%), with the relative proportion of these two compound classes invariant with depth and sampling location. In a study of settling particulate matter collected by sediment traps, solution $^{31}$P-NMR spectroscopy revealed a range of P compounds, including phosphonates, orthophosphate, pyrophosphate, polyphosphate, orthophosphate monoesters and orthophosphate diesters (Paytan et al., 2003). This research also showed regional, temporal and depth-dependent variations in P composition and demonstrated that the relative distribution of P forms in particulate organic P differs from that in plankton material and in most sediments. Unlike HMW dissolved organic P, however, phosphonates in the particulate P samples examined by Paytan et al. (2003) were never greater than 6% of the extracted P. In contrast, phosphonates

comprised 3–23% of the particulate P pool in sinking particles collected from the Cariaco Basin that were analyzed with solid-state $^{31}$P-NMR spectroscopy (Benitez-Nelson et al., 2004). These dissimilarities may be due to regional differences in P cycling in the different ocean basins, as Cariaco samples were collected at shallower depths and many of the samples were from anoxic waters. However, this dissimilarity may also demonstrate a greater detection of phosphonates with solid-state $^{31}$P-NMR, or incomplete or preferential extraction of specific P species by the NaOH–EDTA extractant used for solution $^{31}$P-NMR spectroscopy.

Solid-state and solution $^{31}$P-NMR each have inherent strengths and weaknesses. Solid-state $^{31}$P-NMR allows samples to be examined directly, with minimal preparation (with the exception of drying). However, the presence of paramagnetic ions such as Fe and Mn and chemical shift anisotropy reduce spectral resolution, producing broad peaks (Cade-Menun, 2004; Benitez-Nelson et al., 2004). Although the phosphonate peak is separate, peaks for orthophosphate and orthophosphate monoesters and diesters overlap, hampering the characterization of many of the P forms. In contrast, spectral resolution is much better with solution $^{31}$P-NMR spectroscopy. Compound classes such as orthophosphate monoesters and diesters are clearly separated, and specific P species such as deoxyribose nucleic acid (DNA) can often be identified (Cade-Menun, 2004). However, as with any extraction procedure for P analysis, there is always the risk of hydrolysis (Turner et al., 2003b), or that certain P forms are preferentially extracted over others. There is also the concern that differences in sample preparation or storage can affect P speciation and/or P extraction, limiting comparisons among studies. For example, freezing and drying, which are the most common protocols used for sediment trap sample storage, are known to lyse cells in soil samples (Turner and Haygarth, 2001; Turner et al., 2003d). Addressing these concerns is essential in order to ensure that the results obtained by $^{31}$P-NMR analysis of marine particulate samples are reliable and reproducible.

The objective of this study is to address some of these questions with respect to solution $^{31}$P NMR spectroscopy of marine particulate samples. Specifically, this study investigates (1) the effects of storage treatments such as drying and freezing on P speciation in sample extracts; and (2) preferential extraction of P species, by comparing solid-state and solution $^{31}$P NMR spectroscopy.

## 2. Materials and methods

### 2.1. Samples and treatments

The marine particulate samples used in this study were collected in a 13-cup Honjo-type sediment trap (Pilskaln et al., 1996) deployed in Monterey Bay, California (36.66°N longitude, 122.37°W latitude, 1200 m depth) between March 12, 2003 and September 10, 2003. After the trap was retrieved, the material in each sampling cup, representing a 2-week period, was split into 10 sub-samples of identical weight. The first sub-sample split from each cup was extracted within 48 h of retrieval for solution $^{31}$P-NMR spectroscopy (method described below). The second sub-sample was stored in the refrigerator (4 °C) in cup solution for 6 months prior to extraction. A third sub-sample was centrifuged at approximately 1500×$g$ for 20 min, weighed, oven-dried at 40 °C, and reweighed to determine dry weight and moisture content prior to extraction. One sub-sample was also taken from the first cup collected in the 6-month deployment and from the last sediment trap cup (last 14 days before recovery) (S201 and S213, respectively), frozen immediately, and stored frozen for 1 month before extraction. The remaining sample splits from each cup were oven-dried at 40 °C and then stored at room temperature prior to further analysis.

Five of the cup samples were chosen for our study based on their mass collected in the trap cups and their carbon (C), nitrogen (N) and P composition as seen in oven-dried samples (Table 1). Total mass flux in these samples ranged from 0.47 to 1.4 g m$^{-2}$ day$^{-1}$. Total C ranged from 3.5 to 4.4 mmol g$^{-1}$, and N varied from 0.41 to 0.51 mmol g$^{-1}$. Sample S204 had the highest total P concentration (53.7 μmol g$^{-1}$); S211 the lowest (34.1 μmol g$^{-1}$). The concentration of organic P was similar among the samples (21–25 μmol g$^{-1}$), representing 41.5–67.1% of total P. There was considerable variation among the five samples with respect to the distribution of P forms as determined after a sequential leaching extraction

Table 1
Characteristics of the sediment trap samples used in this study

| | Method | Units | S201 | S204 | S206 | S211 | S213 |
|---|---|---|---|---|---|---|---|
| Initial date | | | 3/12/03 | 4/23/03 | 5/21/03 | 7/30/03 | 8/27/03 |
| Final date | | | 3/26/03 | 5/7/03 | 6/4/03 | 8/13/03 | 9/10/03 |
| Flux | | g $m^{-2}$ $day^{-1}$ | 0.468 | 1.36 | 0.708 | 1.40 | 0.547 |
| Total mass for 2-week collection | | g | 3.28 | 9.50 | 4.96 | 9.93 | 3.83 |
| Total C | | mmol $g^{-1}$ | 4.12 | 4.37 | 3.54 | 3.68 | 4.31 |
| Total N | | mmol $g^{-1}$ | 0.493 | 0.505 | 0.409 | 0.452 | 0.512 |
| *Phosphorus* | | | | | | | |
| Total | Digest[a] | μmol $g^{-1}$ | 35.7 | 53.7 | 42.0 | 34.1 | 45.8 |
| Organic | Ignition[b] | μmol $g^{-1}$ | 24.0 | 22.3 | 23.1 | 21.0 | 22.8 |
| | | % total P | 67.1 | 41.5 | 55.0 | 61.5 | 49.8 |
| Loosely bound ($MgCl_2$) | SEDEX[c] | % total P | 10.3 | 10.6 | 10.8 | 9.5 | 15.5 |
| Oxide (dithionite, citrate, bicarbonate) | SEDEX | % total P | 15.6 | 14.8 | 15.6 | 14.9 | 15.1 |
| Authigenic (Na-acetate) | SEDEX | % total P | 22.6 | 31.8 | 30.0 | 35.0 | 8.92 |
| Detrital (HCl) | SEDEX | % total P | 7.70 | 12.8 | 8.43 | 6.72 | 8.92 |
| Organic (ashing and HCl) | SEDEX | % total P | 43.9 | 30.4 | 35.2 | 33.9 | 24.7 |

Mass and nutrient concentrations are per gram dry weight basis.
[a] $H_2SO_4$–$H_2O_2$ digestion (Parkinson and Allen, 1975).
[b] Ignition and extraction (Saunders and Williams, 1955).
[c] Sequential extraction (Ruttenberg, 1992).

(SEDEX), which separates P into five operationally defined pools (Ruttenberg, 1992): loosely sorbed or exchangeable P (extracted with $MgCl_2$, pH 8); Fe-oxide-bound P (dithionite in citrate–bicarbonate, pH 7.6); authigenic carbonate fluorapatite, biogenic apatite and $CaCO_3$-associated P (Na-acetate, pH 4); detrital P, including apatite (HCl); and organic P (ashing and HCl extraction). However, the authigenic and organic fractions dominated the P composition in all samples (Table 1).

### 2.2. Solution $^{31}P$ nuclear magnetic resonance spectroscopy

Samples S201, S204 and S213 were used to study the effects of storage on P speciation with solution $^{31}$P-NMR spectroscopy. Fresh, refrigerated for 6 months and frozen for 1 month (thawed overnight in the refrigerator) sample splits were centrifuged at approximately 1500×*g* for 20 min, the supernatant was removed, and the remaining solids (0.33, 0.95 and 0.38 g, respectively, for samples S210, S204 and S213) and the oven-dried and ground sample split for these cups (total of 12 splits, four treatments for each of the three cups) were extracted with 20 mL of 0.25 M NaOH+0.05 M $Na_2EDTA$ at room temperature for 6 h on an end-over-end shaker (Cade-Menun and Preston, 1996). After extraction, the samples were centrifuged at approximately 1500×*g* for 20 min. A 1-mL aliquot of supernatant was removed for ICP analysis of P, Al, Ca, Fe, Mg and Mn concentrations after dilution to 10 mL, and the remainder of the supernatant was frozen overnight and then freeze-dried for 24–48 h. Lyophilized extracts were dissolved in 0.4 mL 10 M NaOH and 2.1 mL $D_2O$ and allowed to stand for 30 min with occasional vortexing. Samples were then centrifuged for 20 min at approximately 1500×*g*. The supernatant was transferred to NMR tubes and stored at 4 °C prior to analysis within 24 h. Solution $^{31}$P NMR spectra were acquired at 202.45 MHz on a Varian Unity Inova 500-MHz spectrometer equipped with a 10-mm broadband probe, using a 90° pulse, 0.68-s acquisition, 4.32-s pulse delay and 15-Hz spinning. Temperature was regulated at 20 °C (Cade-Menun et al., 2002; Turner et al., 2003b). Total acquisition time per sample was 7–17 h (4700–12000 scans), depending on the sample P concentration. Compounds were identified by their chemical shifts (ppm) relative to an external orthophosphoric acid standard. After standardizing the orthophosphate

peak in all samples to 6 ppm, peak assignments were based on Tebby and Glonek (1991), Cade-Menun and Preston (1996) and Turner et al. (2003b,c). Line broadening of 1 and 5 Hz was used to separate overlapping peaks. The spectra were processed with NUTS software (Acorn NMR, Livermore, CA), using automated peak analysis tools for peak-picking and spectral integration, and the percentages were calculated based on total peak area.

The limited mass of material collected in sample cups prevented duplicate analysis for this study, thus preventing direct determination of analytical error. However, based on the solution $^{31}$P NMR literature (Turner, 2004, and references therein), error for larger peaks such as orthophosphate is estimated at 5% (e.g., $41.2 \pm 2.1\%$ for orthophosphate in S201 fresh) and 10% for smaller peaks (e.g., $2.5 \pm 0.25\%$ ATP in S201 fresh).

### 2.3. Solid-state $^{31}$P NMR

Samples S204, S206 and S211 were used to determine possible changes in P speciation due to extraction. Oven-dried unextracted sub-samples and the dried residue remaining after fresh sub-sample extraction for solution $^{31}$P-NMR spectroscopy were analyzed by solid-state $^{31}$P-NMR spectroscopy. Spectra were recorded on a Varian Inova 500 spectrometer operating at 202.489 MHz using a Doty Scientific 4 mm/XC magic angle spinning (MAS) probe. Bloch decays of 50 ms were collected with a 200-ppm window after 30° excitation pulses. Continuous wave proton dipolar decoupling with a field strength of 45 kHz was applied during acquisition. A MAS speed of 15 kHz was used, and 8000 to 81,000 scans were collected for each run.

The spectra were processed with NUTS software, using 60 Hz line broadening. Automated analysis tools were used to select peaks, and peak areas were calculated with a line-fitting subroutine. The best line fit was obtained by allowing the line fitting subroutine to determine the ratio of Lorentzian or Gaussian lineshape, and each result was visually inspected to determine that the line fitting result was valid. Results were determined as relative peak areas (%). Prominent spinning side bands were observed only for peaks at ~7 and 2.5 ppm. Peak areas of spinning side bands were added to the areas of the peaks from which they originated (Kinchesh et al., 1995). Error was estimated at 5% for peak areas determined by line-fitting, based on Hunger et al. (2004).

## 3. Results

### 3.1. NaOH–EDTA extraction

Extraction of samples with NaOH–EDTA recovered 34–57% of total P (Table 2). These recoveries are similar to those reported for dried sediment trap material (Paytan et al., 2003) and for calcareous soils (Turner et al., 2003a; Hansen et al., 2004). Phosphorus recovery was lowest in the samples extracted fresh and was more than 10% higher after storage by refrigeration, freezing or drying.

### 3.2. Designation of chemical shifts in solution $^{31}$P-NMR spectroscopy

Designation of chemical shifts is based on Cade-Menun (2004) and references therein. Orthophosphate was present in all samples (Fig. 1). Other detected inorganic P forms include pyrophosphate (−4.2 ppm), long-chain polyphosphates (−4.0, −17.5, −25.3 ppm) and the inorganic anhydrous orthophosphate chain of ADP and ATP (−4.5, −7.8, −10.9, and −19.8 ppm).

Extracted organic P forms included phosphonates and orthophosphate monoesters and diesters. In the phosphonate region (28–14 ppm), peaks for aromatic phosphonates were detected at 14.1 ppm, for phosphonolipids at 18.5 ppm, for aminoethyl phosphonates at 20.3 and 23.3 ppm, and other unidentified phosphonates at 25.9, 26.4, 26.9 and 27.9 ppm. Or-

Table 2
Sample recovery of total P after extraction with NaOH–EDTA for each storage treatment

| Sample | % Total P | | | |
|---|---|---|---|---|
| | Fresh | Frozen | Dried | Refrigerated |
| S201 | 34.4 | 49.1 | 45.9 | 54.6 |
| S204 | 35.5 | nd[a] | 46.1 | 50.9 |
| S213 | 36.5 | 50.2 | 57.1 | 54.8 |

[a] Not determined.

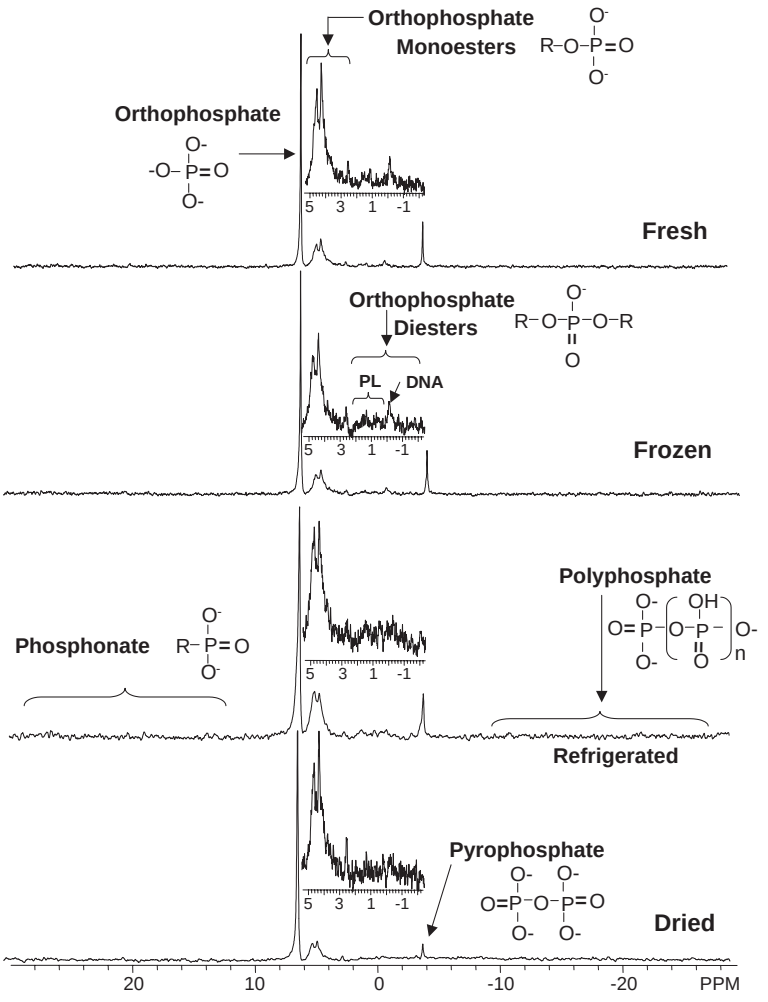


Fig. 1. $^{31}$P-NMR spectra for sediment trap sample S201, showing the effect of either no storage (fresh) or storage (frozen, refrigerated, or dried) prior to extraction with 0.25 M NaOH+0.05 M $Na_2EDTA$. All spectra are plotted with the orthophosphate peak at the same height. The full spectra were processed with 5 Hz line broadening, while the insets were processed with 1 Hz line broadening.

thophosphate monoesters were observed from 5.5 to 2.9 ppm. These were grouped into inositol phosphates (5.9, 5.5, 5.3, 5.1, 4.7, 4.6, 4.4 and 4.0 ppm), sugar phosphates such as glucose-6-phosphate (2.9, 3.1, 3.3, 3.5 and 3.7 ppm) and other orthophosphate monoesters (4.2 and 4.8 ppm). The inositol phosphates were so designated because they were still present after alkaline bromination, a hydrolysis technique that destroys other orthophosphate monoesters but not inositol phosphates (Turner and Richardson, 2004). No attempt was made to distinguish between the different orthophosphate monoester P forms such as inositol mono-, di-, tri-, tetra-, penta-, and hexakisphosphate (Turner and Richardson, 2004). The other orthophosphate monoesters include products from orthophosphate diester degradation, such as from RNA at 4.2 ppm and phospholipids at 4.8 ppm. Within the orthophosphate diesters, peaks were designated as phospholipids (0.9 and 1.8 ppm), RNA (0.5 ppm) and DNA (−0.9 ppm). Other orthophosphate diesters include teichoic acid (2.3 ppm), aromatic orthophosphate diesters (6.1, 6.6 and 8.9 ppm), and undetermined P forms (−0.1, −0.5, −1.3 and −2.2 ppm).

Table 3
Distribution of inorganic P forms (% of extracted P) in $^{31}$P-NMR spectra after storage treatment (fresh, frozen, refrigerated, dried)

| Sample | Treatment | Orthophosphate | Pyrophosphate | Polyphosphates | | Total inorganic P |
|---|---|---|---|---|---|---|
| | | | | Poly[a] | ATP | |
| S201 | Fresh | 41.2 | 6.2 | 1.4 | 2.5 | 51.3 |
| | Frozen | 37.5 | 5.0 | 4.8 | 2.2 | 49.5 |
| | Dried | 47.7 | 2.0 | 2.1 | 3.6 | 55.4 |
| | Refrigerated | 35.8 | 4.6 | 2.8 | 3.9 | 47.1 |
| S204 | Fresh | 58.4 | 2.9 | 0.7 | 2.0 | 64.0 |
| | Dried | 65.5 | 1.8 | 0.7 | 2.0 | 70.0 |
| | Refrigerated | 56.5 | 2.3 | 0.7 | 2.0 | 61.5 |
| S213 | Fresh | 35.3 | 7.7 | 2.0 | 2.0 | 47.0 |
| | Frozen | 49.4 | 6.0 | 3.4 | 2.8 | 61.6 |
| | Dried | 50.8 | 2.7 | 0.0 | 1.5 | 55.0 |
| | Refrigerated | 49.1 | 3.4 | 2.9 | 1.5 | 56.9 |
| S206 | Fresh | 41.2 | 8.5 | 1.4 | 2.1 | 53.2 |
| S211 | Fresh | 48.4 | 6.7 | 1.4 | 3.5 | 60.0 |

[a] Abbreviations: Poly, polyphosphate; ATP, adenosine triphosphate.

### 3.3. Distribution of P forms in solution $^{31}$P-NMR spectroscopy

In all samples, 35–65% of extracted P was orthophosphate (Table 3), with the lowest recovery of orthophosphate in the fresh extract of the most recently collected sample (S213). Total inorganic P was highest for the dried samples of S201 and S204, and for the frozen sample of S213. Inositol phosphates (Table 4) comprised the next highest percentage of extracted P (8.9–24.8%). The ratio of orthophosphate monoesters to orthophosphate diesters was highest for the dried sample of S201 and S204, but not S213, where the frozen sample had the highest ratio. For all three samples used in the storage test, orthophosphate was highest after drying, and DNA was lowest for that treatment. Pyrophosphate was lowest in dried samples and highest in fresh samples.

When individual P compounds were grouped into compound classes, total polyphosphates were lowest, and orthophosphate was highest in the dried subsample splits in all tested samples (Fig. 2).

Table 4
Distribution of organic P forms (% of extracted P) in $^{31}$P-NMR spectra after storage treatment (fresh, frozen, refrigerated, dried)

| Sample | Treatment | Monoesters | | | Diesters | | | | Phosphonates | | | Total Organic P | Monesters: Diesters |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | IP | Sgr | Oth | Lpd | RNA | DNA | Oth | PnL | AEP | Oth | | |
| S201 | Fresh | 23.4 | 5.4 | 7.4 | 1.8 | 1.2 | 1.8 | 4.9 | 0.7 | 0.7 | 1.4 | 48.7 | 3.73 |
| | Frozen | 24.8 | 4.2 | 7.1 | 2.1 | 1.4 | 2.8 | 5.4 | 0.7 | 1.3 | 0.7 | 50.5 | 3.09 |
| | Dried | 13.7 | 6.2 | 13.5 | 1.4 | 0.7 | 1.4 | 4.9 | 0.7 | 0.7 | 1.4 | 44.6 | 3.98 |
| | Refrigerated | 19.4 | 6.2 | 9.4 | 3.4 | 1.4 | 2.0 | 7.1 | 0.8 | 0.8 | 2.4 | 52.9 | 2.52 |
| S204 | Fresh | 10.5 | 2.9 | 1.2 | 0.6 | 0.0 | 1.2 | 3.1 | 0.0 | 3.4 | 13.1 | 36.0 | 2.98 |
| | Dried | 8.9 | 2.4 | 5.3 | 0.6 | 0.6 | 0.6 | 2.5 | 0.0 | 2.1 | 7.0 | 30.0 | 3.86 |
| | Refrigerated | 10.5 | 4.1 | 2.3 | 0.6 | 0.6 | 1.2 | 1.7 | 0.0 | 2.8 | 12.7 | 38.5 | 2.77 |
| S213 | Fresh | 23.8 | 3.1 | 6.4 | 0.6 | 0.6 | 2.6 | 10.9 | 0.0 | 0.7 | 4.2 | 53.0 | 2.27 |
| | Frozen | 16.8 | 3.4 | 5.3 | 1.3 | 0.7 | 1.3 | 6.8 | 0.0 | 0.7 | 2.1 | 38.4 | 2.52 |
| | Dried | 21.4 | 2.7 | 4.0 | 1.3 | 0.0 | 0.7 | 11.0 | 0.0 | 0.8 | 3.1 | 45.0 | 2.16 |
| | Refrigerated | 16.8 | 2.8 | 6.1 | 1.3 | 1.3 | 1.3 | 9.5 | 0.0 | 0.8 | 3.2 | 43.1 | 1.92 |
| S206 | Fresh | 18.5 | 4.9 | 7.1 | 2.1 | 0.7 | 1.4 | 10.0 | 0.7 | 0.7 | 0.7 | 46.8 | 2.15 |
| S211 | Fresh | 12.8 | 4.1 | 6.0 | 2.0 | 1.3 | 2.7 | 8.1 | 0.0 | 0.7 | 2.3 | 40.0 | 1.62 |

Abbreviations: IP, inositol phosphates; Sgr, sugar phosphates; Oth, other; Lpd, Phospholipid; RNA, ribonucleic acid; DNA, deoxyribonucleic acid; PnL, phosphonolipids; AEP, aminoethyl phosphonic acid.

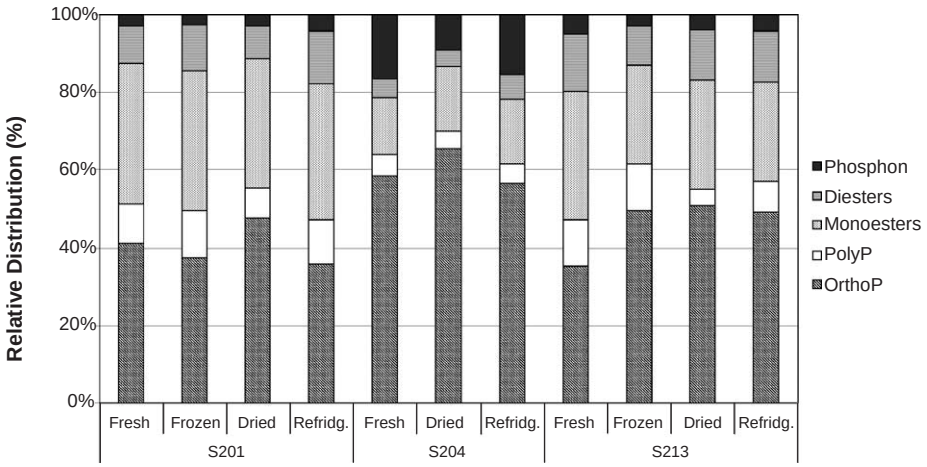


Fig. 2. The relative distribution of P into the five major P form classes determined by $^{31}$P-NMR spectroscopy: phosphonates (phosphon), orthophosphate diesters (diesters), orthophosphate monoesters (monoesters), orthophosphate (orthoP) and polyphosphates, including pyrophosphate (polyp). The samples used were the sediment trap samples S201, S204 and S213, extracted with 0.25 M NaOH+0.05 M $Na_2EDTA$ without storage (fresh) or after storage (dried, frozen, or refrigerated).

In order to further separate trends related to storage procedures, principal components analysis, without rotation, of the solution $^{31}$P-NMR data was conducted (Fig. 3). For sample S201, the $^{31}$P NMR spectra for the fresh and frozen treatments are most similar and are separated from the refrigerated sample along PC1 and from the dried sample along PC2. For sample S204, the fresh sample is separated along PC1 and PC2 from the dried and refrigerated samples. The greatest spread among treatments is seen for sample S213, the sample with the shortest residence in the trap cup. The frozen and refrigerated samples are separated from the fresh sample along PC1. The dried treatment is separated from the other treatments along both PC1 and PC2.

For the samples used in the test of solid-state and solution $^{31}$P-NMR spectroscopy (S204, S206 and S211), extraction with NaOH–EDTA removed about

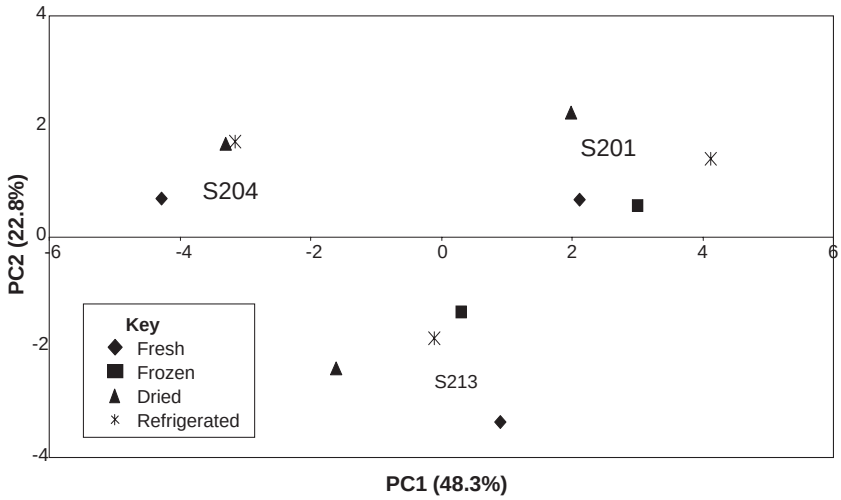


Fig. 3. Principal components analysis of $^{31}$P-NMR spectra for the pre-extraction treatments (fresh, frozen, dried and refrigerated) of the sediment trap samples (S201, S204 and S213).

Table 5
Concentrations of P, Ca, Fe, Al, Mg, and Mn in the initial samples (per g dry wt.) analyzed by solid-state $^{31}$P-NMR spectroscopy prior to extraction, the concentrations of each element (and percentage recovery) extracted with NaOH–EDTA, and the concentrations of each element (per g dry wt.) in the residues after extraction

| | | | Initial solid | Extract | Final residue |
|---|---|---|---|---|---|
| P | S204 | μmol g$^{-1}$ | 53.7 | 19.1 (35.6)[a] | 34.7 |
| | S206 | | 42.0 | 13.3 (31.7) | 28.6 |
| | S211 | | 34.1 | 11.0 (32.3) | 23.1 |
| Ca | S204 | mmol g$^{-1}$ | 4.31 | 0.38 (35.3) | 0.69 |
| | S206 | | 6.24 | 0.72 (46.5) | 0.83 |
| | S211 | | 4.64 | 0.51 (44.3) | 0.64 |
| Al | S204 | mmol g$^{-1}$ | 2.07 | 0.001 (0.01) | 2.07 |
| | S206 | | 1.73 | 0.000 (0.00) | 1.73 |
| | S211 | | 1.38 | 0.000 (0.00) | 1.38 |
| Fe | S204 | μmol g | 643.2 | 1.35 (0.21) | 641.9 |
| | S206 | | 526.0 | 0.79 (0.15) | 525.2 |
| | S211 | | 398.7 | 1.41 (0.35) | 397.2 |
| Mg | S204 | μmol g$^{-1}$ | 883.7 | 1.50 (0.17) | 882.2 |
| | S206 | | 733.4 | 0.68 (0.09) | 732.7 |
| | S211 | | 722.6 | 84.8 (11.7) | 637.8 |
| Mn | S204 | μmol g$^{-1}$ | 5.40 | 0.12 (2.19) | 5.28 |
| | S206 | | 4.42 | 0.08 (1.80) | 4.34 |
| | S211 | | 3.29 | 0.13 (3.87) | 3.17 |

These residues were dried and analyzed by solid-state $^{31}$P-NMR spectroscopy.
[a] Percentage of total removed by extraction with NaOH–EDTA.

30% of the total P, 40% of the total Ca, 2% of Mn and only traces of Fe and Al, with little variation among the samples (Table 5). Extraction removed less than 1% of Mg from S204 and S206, but more than 11% of total Mg was extracted from S211.

The distribution of P species in extracted samples, determined by solution $^{31}$P NMR spectroscopy, is shown in Tables 3 and 4. Samples S206 and S211 differ from S204 in the relative percentage of phosphonates, which is higher in S204, and in the relative percentages of polyphosphates, orthophosphate monoesters and orthophosphate diesters, which are lower in S204.

### 3.4. Solid-state $^{31}$P-NMR spectroscopy

Phosphorus speciation determined using solid-state NMR for samples S204, S206 and S211 before extraction and on the residues after extraction is given in Tables 6 and 7, and solid and solution spectra for sample S204 are shown in Fig. 4, with spinning side-bands noted by an asterisk (*). The solid line in each solid-state spectrum shows the original spectrum, and the dotted lines show the peaks determined by the line-fitting software during processing. For all three

Table 6
Solid-state NMR results, showing chemical shifts determined by line-fitting (ppm), the concentration of total P (μmol g$^{-1}$ dry wt), and the proportion of total P (%)

| | S204 unextracted | S204 extracted | S206 unextracted | S206 extracted | S211 unextracted | S211 extracted | Designation |
|---|---|---|---|---|---|---|---|
| ppm | 27.1 | | | 23.0 | | | Phosphonates |
| μmol g$^{-1}$ (%) | 0.77 (1.4) | | | 0.87 (3.0) | | | |
| ppm | 21.3 | 20.4 | 20.4 | | 20.5 | 20.4 | Phosphonates |
| μmol g$^{-1}$ (%) | 2.6 (4.7) | 0.75 (2.0) | 0.64 (2.0) | | 0.19 (0.6) | 0.10 (0.4) | |
| ppm | | 15.5 | 12.1 | | 12.0 | | Phosphonates |
| μmol g$^{-1}$ (%) | | 0.75 (2.1) | 0.62 (1.5) | | 0.12 (0.4) | | |
| ppm | 7.4 | | 6.9 | 5.9 | 6.9 | 7.0 | Ca-phosphates |
| μmol g$^{-1}$ (%) | 15.7 (29.2) | | 12.3 (29.2) | 0.20 (0.7) | 7.89 (23.1) | 4.71 (20.4) | |
| ppm | 2.5 | 2.3 | 2.6 | 2.6 | 2.5 | 2.5 | Ca-phosphates |
| μmol g$^{-1}$ (%) | 28.5 (53.1) | 29.4 (80.2) | 20.4 (48.6) | 21.0 (73.4) | 21.2 (62.2) | 13.0 (56.3) | |
| ppm | −0.6 | −0.5 | −0.2 | −0.7 | −0.5 | −0.5 | Organic esters[a] |
| μmol g$^{-1}$ (%) | 5.38 (10.0) | 3.00 (8.2) | 5.21 (12.4) | 1.33 (4.7) | 3.66 (10.7) | 0.44 (1.9) | |
| ppm | | −3.1 | −4.0 | −3.7 | −4.4 | −1.4 | Ca, Al or Mg phosphates |
| μmol g$^{-1}$ (%) | | 2.70 (7.3) | 0.94 (2.2) | 1.29 (4.5) | 0.56 (1.6) | 3.36 (14.6) | |
| ppm | −6.5 | | −7.8 | −6.3 | −8.8 | −8.5 | Mg phosphates |
| μmol g$^{-1}$ (%) | 0.80 (1.5) | | 0.55 (1.3) | 3.94 (13.8) | 0.48 (1.4) | 1.47 (6.4) | |
| ppm | | −10.5 | −10.5 | | | | Al phosphates |
| μmol g$^{-1}$ (%) | | 0.08 (0.2) | 1.18 (2.8) | | | | |

[a] Organic esters include orthophosphate monoesters and orthophosphate diesters.

Table 7
Concentration of P (μmol P $g^{-1}$ dry wt) of specific compounds before and after extraction, the difference between extracted and unextracted, and the concentration of P (μmol P $g^{-1}$ dry wt.) in P forms in extracts

| | S204 | | | | S206 | | | | S211 | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Before | After | Difference | Extract | Before | After | Difference | Extract | Before | After | Difference | Extract |
| Orthophosphate and Polyphosphate[a] | 45.0 | 32.2 | 12.8 ± 0.61 | 12.2 | 35.4 | 26.5 | 8.92 ± 0.45 | 7.10 | 30.1 | 22.6 | 7.55 ± 0.38 | 6.61 |
| Phosphonates | 3.40 | 1.51 | 1.89 ± 0.09 | 2.20 | 1.43 | 0.87 | 0.56 ± 0.01 | 0.28 | 0.31 | 0.10 | 0.21 ± 0.01 | 0.33 |
| Organic Esters[b] | 5.30 | 3.00 | 2.30 ± 0.12 | 2.60 | 5.21 | 1.33 | 3.88 ± 0.19 | 5.97 | 3.66 | 0.44 | 3.22 ± 0.16 | 4.07 |
| Total Pi | 45.0 | 32.2 | 12.8 ± 0.61 | 12.2 | 35.4 | 26.5 | 8.92 ± 0.45 | 7.10 | 30.1 | 22.6 | 7.55 ± 0.38 | 6.61 |
| Total Po | 8.70 | 4.51 | 4.19 ± 0.21 | 4.81 | 6.64 | 2.20 | 4.44 ± 0.22 | 6.25 | 3.97 | 0.54 | 3.43 ± 0.17 | 4.40 |
| Ca phosphates | 44.2 | 29.4 | 14.8 | | 32.7 | 21.2 | 11.5 | | 29.1 | 17.7 | 11.4 | |
| Mg phosphates | 0.80 | 0.00 | 0.80 | | 0.55 | 3.94 | −3.39 | | 0.48 | 1.47 | −0.99 | |
| Al phosphates[c] | 0.00 | 2.78 | −2.78 | | 2.12 | 1.29 | 0.83 | | 0.56 | 3.36 | −2.80 | |

[a] Orthophosphate and polyphosphate were grouped together here due to overlapping peak regions.
[b] Organic esters include orthophosphate monoesters and orthophosphate diesters.
[c] The concentrations calculated from peaks with chemical shifts in a region corresponding to Ca, Al or Mg phosphates in Table 6 are included with the Al phosphates.

samples, more peaks were detected in the unextracted samples than the extracted residue (Table 6). Peaks from 28 to 12 ppm were designated as phosphonates (Benitez-Nelson et al., 2004). Peaks between 12 and 2 ppm were classed as inorganic Ca-associated phosphates. Compounds known to have chemical shifts in this region include dicalcium phosphate dihydride (9 ppm) and hydroxyapatite and octacalcium phosphate at 3 ppm (Frossard et al., 1994; McDowell et al., 2002). The peaks for organic esters ranged from 0.5 to −0.7 ppm. The peaks from −1.4 to −4.6 ppm were designated as Ca-, Al- or Mg-associated phosphates, as compounds that have peaks in this region include monetite (2 ppm), crandallite (−5 ppm) and amorphous magnesium phosphates (−2.4 ppm) (Bleam et al., 1989; Frossard et al., 1994; Hunger et al., 2004). The peaks at −7.1 to −8.0 ppm correspond to the region in which Mg-associated phosphates show peaks (e.g., newberryite at −7.2 ppm; Hunger et al., 2004), and so are designated as Mg-associated phosphates. Compounds that show peaks in the range of −10.1 to −19 include Al phosphates such as brazilianite (−10.2 ppm), wavelite (−11 ppm), metavariscite (−13.2 ppm), senegalite (−16 ppm) and variscite (−19 ppm) (Bleam et al., 1989; Duffy and vanLoon, 1995). Phosphorus associated with paramagnetic ions such as Fe or Mn will not be visible in $^{31}$P-NMR spectra. It should also be noted that the chemical shifts for pyrophosphate and long-chain polyphosphates would also be in the −4 to −25 ppm region. Thus, all of these peak designations should only be considered as our best estimates. We do not wish to imply that any of these specific P compounds such as wavelite are present in our samples, but rather to demonstrate the criteria on which our peak designations were based.

For sample S204, peaks were detected in the phosphonate region at 27.1 and 21.3 ppm in the unextracted sample and at 20.4 and 15.5 ppm in the extracted sample (Table 6). Peaks at 7.4 and −6.5 ppm are present in unextracted but not extracted samples, while peaks at −3.1 and −10.5 ppm are present after extraction but not before. Peaks at ~2.4 and −0.6 ppm are present both before and after extraction, but the P concentration changes. For sample S206, peaks are present at 20.4, 12.1 and 10.5 ppm before, but not after extraction, and a peak at 23.0 is visible after extraction. For sample S211, only the peak at 12.0 ppm is no longer present after extraction.

To allow a better comparison of compound classes detected by solution $^{31}$P-NMR spectroscopy to those detected in extracted and unextracted samples after solid-state $^{31}$P-NMR spectroscopy, the results for samples S204, S206 and S211 from Tables 3 and 6 are grouped in Table 7. Please note that orthophosphates and polyphosphates are grouped together, because they cannot be separated in the solid-state spectra. The difference between P concentrations in extracted and unextracted solid samples is similar to that found

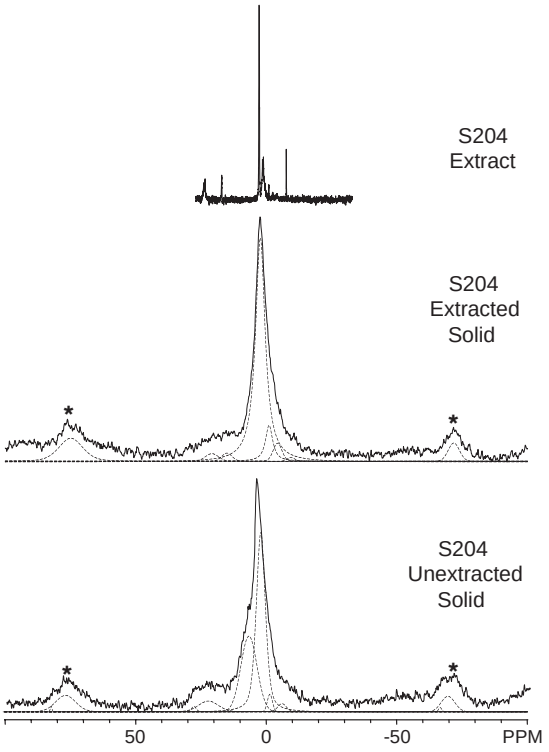


Fig. 4. $^{31}$P-NMR spectra for sediment trap sample S204. Bottom: solid-state spectrum of the unextracted sample. Middle: solid-state spectrum of the extracted residue. Top: solution spectrum of the NaOH–EDTA extract. Solid lines show the acquired spectrum; dashed lines show peaks determined with line-fitting. Spinning side-bands indicated with *.

in the "extract" using solution $^{31}$P-NMR spectroscopy in all three samples, despite large differences in the magnitude of P concentrations in various compounds. For S204, the P concentrations of orthophosphate and organic esters are slightly lower in the extracts, and the phosphonates are slightly higher. For S206 and S211, the orthophosphate and phosphonate concentrations are lower in the extracts, and higher for the organic esters, especially for S211.

When the inorganic P compounds are grouped together, with the peaks that could be Ca-, Al- or Mg-associated phosphates classed as Al-associated phosphates, the greatest decrease after extraction for all three samples was in the Ca phosphates. There was a net decrease in Mg phosphates for S204 and in Al phosphates in S206 after extraction. There appears to be a net gain of Al phosphates in S204 and S211 residues and Mg phosphates in S206 and S212.

## 4. Discussion

### 4.1. NaOH–EDTA extraction

There were some increases in extracted P concentrations of refrigerated, frozen and dried samples relative to those extracted immediately after collection. This increased P recovery is consistent with results from soil analysis, where increases in extractable P occurred after freezing and thawing of soils, which changes osmotic potential and lyses microbial cells (Turner and Haygarth, 2001; Turner et al., 2003d). It also indicates that extraction of fresh samples with NaOH–EDTA may not quantitatively lyse all cells. Hydrolysis of organic compounds also appears to occur with increased storage, especially with drying, because orthophosphate was always highest, and pyrophosphate and DNA lowest in dried samples. Other orthophosphate monoesters also increased after drying for samples S201 and S204, but not for sample S213, the most recently collected sample. This compound class includes products from the degradation of orthophosphate diesters to monoesters, after the removal of C moieties. This suggests that degradation of P forms may occur naturally as samples sit for months in trap cups. Trap poisons such as $HgCl_2$ have been shown to kill organisms within the trap, but do not prevent exogenous enzyme activity (Lee et al., 1992; Lee and Fisher, 1992).

Immediately after trap recovery, the P concentration of the cup solution in samples S201, S204 and S204 was 41, 413 and 161 μM and increased to 55, 426 and 248 μM after cold but not frozen storage. There were no differences in P concentrations when measured colorimetrically or by ICP, indicating that all P in solution was orthophosphate. This increase in solution P corresponds to the increased P recovery in extracted solid samples after refrigeration and is likely due to the slow, natural degradation of labile organic P forms such as simple sugar phosphates and RNA to more easily leachable forms.

Most of the differences among storage treatments were small and were within the estimated error of ±5–10% reported for the detection of the different compounds (Turner and Richardson, 2004). This is supported by the principal components analysis, which resulted in groupings by sample rather than by treatment, suggesting that the treatment-induced

differences were smaller than natural differences among samples. Thus, in studies using samples from different sources such as that of Paytan et al. (2003), the comparisons among regions or even within regions at different times should be valid despite treatment and storage differences. It is not always practical to extract samples immediately after traps are recovered, and in many cases previously collected and archived samples are used. Nonetheless, our results suggest that storing samples in the freezer or refrigerator is preferential to drying for $^{31}$P-NMR analysis, because the spectra of the frozen and refrigerated samples were more similar to those of the samples extracted immediately after trap recovery. On the other hand, the P extraction yield is higher for dried samples; thus a larger fraction of the total P is evaluated.

### 4.2. Solid-state $^{31}$P-NMR spectroscopy

We used line-fitting software to identify a number of peaks in our solid-state spectra. This has not been done previously for spectra of marine samples (e.g., Kolowith et al., 2001; Benitez-Nelson et al., 2004), but is a common practice for solid-state $^{31}$P-NMR spectra of other environmental samples (e.g., McDowell et al., 2002; Hunger et al., 2004). This allows us to separate specific organic and inorganic P compounds. Our results show that a large percentage of the P present before and particularly after extraction is inorganic P. This was confirmed by the good agreement between inorganic P concentrations in the extracts and the calculated difference between unextracted and extracted samples from the solid-state spectra.

The extraction procedure removed 48%, 67% and 86% of the organic P from samples S204, S206 and S211, respectively. Recovery of organic esters was 74–88% from samples S206 and S211, but was only 43% for S204. Recovery of phosphonates was generally poorer than that of organic esters, ranging from 39% to 67%. There was no consistent trend among the samples with respect to recovery of specific phosphonate compounds. However, it is interesting to note that phosphonates within the extracts comprised about the same relative percentage of total P as in the unextracted sample, indicating that despite incomplete recovery the relative distribution of species using solution NMR may be preserved.

Most of the extracted inorganic P was from Ca-associated phosphates, which was confirmed by the high concentration of Ca in the extracts relative to other cations. We cannot say anything about the extraction of Fe phosphates, because they are not visible with $^{31}$P-NMR spectroscopy. The low Fe concentration in the extracts, however, suggests that they are either not extracted or that their abundance is below our detection limits; sequential extraction showed that Fe-oxide-associated P was only a small proportion of the total P (~15%). Only small concentrations of Al-phosphates appear to have been extracted, and in fact, there appears to be a net increase in Al-phosphates in samples S204 and S211. Increases in Al-phosphates after extraction were also reported by Frossard et al. (1994) and Hunger et al. (2005). This is most likely not a net increase but rather due to a greater visibility of Al-phosphates in the extracted residues after the removal of broader, overlapping peaks. It may also be due to slight changes in chemical shifts resulting from the change in pH after extraction, a precipitation of Al-phosphates during extraction (Hunger et al., 2005), or inaccuracies in our assignment of peaks in this region.

One point to note is that dried, ground samples are used for solid-state $^{31}$P-NMR spectroscopy. Given the observed differences in solution $^{31}$P-NMR spectra after drying, relative to samples extracted immediately after trap recovery, it is possible that some of the observed inorganic P forms are artifacts of the drying process. The inability to separate polyphosphates from orthophosphate in $^{31}$P-NMR solid-state spectroscopy may also be a drying artifact, as pyrophosphate concentrations were reduced in solution $^{31}$P-NMR spectra during extractions of dried samples.

## 5. Conclusions

Our results suggest that freezing, drying or storing samples in the refrigerator for long periods prior to extraction for solution $^{31}$P-NMR spectroscopy will increase the concentration of extracted P, but will introduce only small changes to the relative abundance of compounds in spectra, relative to samples extracted immediately after trap recovery. Drying appeared to cause the biggest changes, most likely due to increased degradation of P species such as

pyrophosphate. However, as mentioned above, these changes are small when compared to the differences among samples, even for samples collected at the same location over a 6-month period.

Extraction with NaOH–EDTA removes the majority of organic esters, but does not remove quantitatively all of the phosphonates from some samples. There also appeared to be preferential extraction of Ca-associated phosphates over those associated with Fe and Al. Pyrophosphates, which are clearly distinguished in solution $^{31}$P NMR spectra, could not be separated from orthophosphate compounds in solid-state spectra. This may an artifact of sample drying, which is required to analyze samples with solid-state $^{31}$P NMR spectroscopy.

Our results suggest that solution $^{31}$P NMR spectroscopy enables much higher resolution of P-species within samples, particularly when examining differences between orthophosphate monoesters and diesters, or if polyphosphates are present. However, combining solid-state $^{31}$P NMR with solution 31P NMR spectroscopy for marine particles can provide additional information about inorganic P speciation and abundance of phosphonates.

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