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Marine sediment pore-water profiles of phosphate $\delta^{18}O$ using a refined micro-extraction

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Abstract

Phosphorus cycling in the ocean is influenced by biological and geochemical processes that are reflected in the oxygen isotope signature of dissolved inorganic phosphate (P_i). Extending the P_i oxygen isotope record from the water column into the seabed is difficult due to low P_i concentrations and small amounts of marine porewaters available for analysis. We obtained porewater profiles of P_i oxygen isotopes using a refined protocol based on the original micro-extraction designed by Colman (2002). This refined and customized method allows the conversion of ultra-low quantities (0.5 – 1 µmol) of porewater P_i to silver phosphate (Ag_3PO_4) for routine analysis by mass spectrometry. A combination of magnesium hydroxide co-precipitation with ion exchange resin treatment steps is used to remove dissolved organic matter, anions, and cations from the sample before precipitating Ag_3PO_4. Samples as low as 200 µg were analyzed in a continuous flow isotope ratio mass spectrometer setup. Tests with external and laboratory internal standards validated the preservation of the original phosphate oxygen isotope signature ($\delta^{18}O_p$) during micro extraction. Porewater data on $\delta^{18}O_p$ has been obtained from two sediment cores of the Moroccan margin. The $\delta^{18}O_p$ values are in a range of +19.49 to +27.30‰. We apply a simple isotope mass balance model to disentangle processes contributing to benthic P cycling and find evidence for P_i regeneration outbalancing microbial demand in the upper sediment layers. This highlights the great potential of using $\delta^{18}O_p$ to study microbial processes in the subseafloor and at the sediment water interface.

Phosphorus (P) is one of the essential nutrients for life on Earth. The oceanic cycle of phosphate (P_i) controls marine primary productivity on both geologic and recent timescales (Benitez-Nelson 2000; Froelich et al. 1982; Paytan and McLaughlin 2007), and is linked to global carbon biogeochemistry and atmospheric oxygen levels (Colman et al. 2000). In those regions of the oceans where input of inorganic P_i is limited, it must be biologically recovered from organic matter (OM) to maintain the supply to the pelagic and benthic communities. Pathways and rates of biological P_i recycling thus directly control the P supply state of the marine ecosystem. So far, research has focused on budgets and standing stocks of P_i in the water column and marine sediments and provided insight into P_i partitioning between different sedimentary fractions. However, transformations and fluxes in and between those compartments are still poorly understood (Benitez-Nelson 2000). Despite the recognition of the seabed's decisive role for OM remineralization and P_i recycling, as well as for effective burial of P_i in the marine sediments, it remains difficult to characterize the biological contribution to P_i turnover in this main reservoir of the marine P cycle. Phosphorus is resistant to redox transformations (Schink and Friedrich 2000) and P_i is its main molecular form, either inorganic or incorporated as esters in organic molecules. Phosphonates may be important in particular environments (Kolowith et al. 2001). Because most assessments of marine P pools include the quantification of P that was released as P_i in the process, it has been almost impossible to reconstruct transitions from P_i concentration data only.

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Only recently, the oxygen isotopic signature of dissolved inorganic phosphate ($\delta^{18}O_p$) has been proposed to yield information on biological P cycling (Blake et al. 2001). The P-O bond is stable under ambient conditions of the Earth's surface and ocean, and only the activity of enzymes of living organisms can alter the phosphate oxygen isotope signature by exchanging oxygen atoms with surrounding media (Blake et al. 1997; Tudge 1960). Hydrolysis of phosphoesters is the most crucial process (Blake et al. 2005; Liang and Blake 2009). Experimental studies elucidated enzymatic mechanisms involved in this hydrolysis and determined the respective isotope effects for different cultures (Blake et al. 2005; Liang and Blake 2006), reconstructed signatures of different P_i substrates (Liang and Blake 2009), and discriminated biological processes from geochemical reactions (Blake et al. 1998; Liang and Blake 2007). Field investigations of marine, estuary, and riverine water $\delta^{18}O_p$ have disentangled the balance of P_i release and uptake (Colman et al. 2005; McLaughlin et al. 2006b), and identified external P_i sources (McLaughlin et al. 2006a). This new "inorganic biomarker" (Blake et al. 2001) should thus prove useful for characterizing P_i dynamics in marine sediments, where the porewater P_i pool connects diagenetic mineralization of organic matter, microbial uptake and release, adsorption/desorption and precipitation/dissolution reactions with solid phase minerals.

For analysis of $\delta^{18}O_p$ by isotope ratio mass spectrometry (IRMS), it is necessary to convert P_i into a pure phase without isotopic alteration. Ideally, that compound excludes external oxygen sources, and is non-hygroscopic and stable under laboratory conditions. Silver phosphate (Ag_3PO_4) has been proven a convenient P_i phase for this purpose (Firsching 1961; O'Neil et al. 1994) and has since then gradually substituted the earlier fluorination technique (Kolodny et al. 1983; Tudge 1960). Commonly, Ag_3PO_4 is carbothermically reduced with carbon at high temperature (>1200°C) in a thermal combustion elemental analyzer (TCEA) to yield carbon monoxide (CO) for analysis by IRMS (Colman 2002; McLaughlin et al. 2004; O'Neil et al. 1994). TCEA and mass spectrometer are linked via a continuous flow interface, and the CO gas is measured instantaneously after formation (Kornexl et al. 1999).

Two detailed protocols for isolating, purifying, and precipitating small quantities of P_i from complex matrix solutions such as fresh and ocean waters have been published lately (Colman 2002; McLaughlin et al. 2004). Unlike the open ocean water column, where sample size is only restricted by pump performance and bottle capacity, porewater of marine sediments is difficult to obtain and sample volumes are very limited. Retrieving undisturbed sediment involves drilling a core with typical diameter of a few centimeters, yielding porewater samples around a few tens of milliliters. For common sedimentary P_i concentrations (10^0– 10^2 µM), this results in not more than 1 µmol P_i in the initial sample, which is at the low end of what aforementioned protocols may handle. Based upon similar principles, the procedures outlined by Colman (2002) and McLaughlin et al. (2004) involve a series of precipitations, resin treatments, and concentration steps to remove dissolved organic phosphorus (DOP) and interfering ions from the sample. With respect to obtaining P_i from marine porewaters, neither of the two methods shows a considerable advantage over the other, and we assume that both may be equally appropriate. Here we present a refined version of the protocol by Colman (2002), tailored for the conversion of ultra-low quantities (<1 µmol) of porewater P_i to Ag_3PO_4 for routine IRMS analysis. We tested the method with external reference materials and internal laboratory standards, both pure and in matrices simulating natural samples, and successfully obtained data on porewater $\delta^{18}O_p$ from two sediment cores of the Northwest African continental shelf.

Materials and equipment

Labware

Here, we list the laboratory equipment that is needed for processing a single sample at a time, unless mentioned in the text. For sample collection and storage, we use one or two Rhizon suction samplers with Luer connector (Rhizosphere Research, Wageningen) and three-way Luer valves, two to four 20 mL syringes, and a 60-mL HDPE sample bottle (Nalgene). Processing in the laboratory requires one or two 30 mL PPCO Oak ridge centrifuge flasks (Nalgene), two 50-mL PP Falcon centrifuge tubes (Sarstedt), seven 26-mL PS test tubes with stoppers (Sarstedt), and one 2.0-mL Eppendorf reaction cup. All centrifuge tubes are triple-washed with nitric acid (HNO_3, 1 mol L^{-1}) and rinsed with deionized water (H_2O, Milli-Q) prior to use. The anion separation line (Fig. 1) comprises one 60 mL syringe barrel, a 6 mL reservoir cartridge (Varian Bond Elut) with frits and Luer connector, Luer stopcock and tube adaptors, Tygon tubing (ID 1.6 mm, OD 3.2 mm), and two-stop pump tubes (Ismatec, color code orange-orange, ID 0.89 mm). Further consumables include a filter cartridge (0.22 µm, Millipore Steriflip Express Plus Membrane), a filter membrane (0.2 µm, Ø20 mm, cellulose acetate, Sartorius), nine disposable PS cuvettes (2.5 mL, Brand), a disposable Petri dish (Greiner). IRMS sample preparation requires a silver cartridge (3.5 mm × 5 mm, Hekatech) annealed at 600°C prior to use. Powder-free latex gloves should be worn to avoid sample contamination.

Chemicals and reagents

For preparation of reagents, we use potassium phosphate (KH_2PO_4, ACS grade, Merck), sodium bicarbonate ($NaHCO_3$, ACS grade, Merck), silver nitrate ($AgNO_3$, puriss. p.a., Riedel-de Haën), ammonium nitrate (NH_4NO_3, Fluka Ultra BioChemika), sodium hydroxide solution (NaOH, 1 mol L^{-1}, Merck), phosphate standard solution (1000 mg KH_2PO_4 L^{-1}, Merck CertiPur), and ultrapure HNO_3 (67 %) freshly prepared by sub-boiling distillation. The required working solutions are cleaning of equipment: 1 mol L^{-1} HNO_3; Step 1: 1

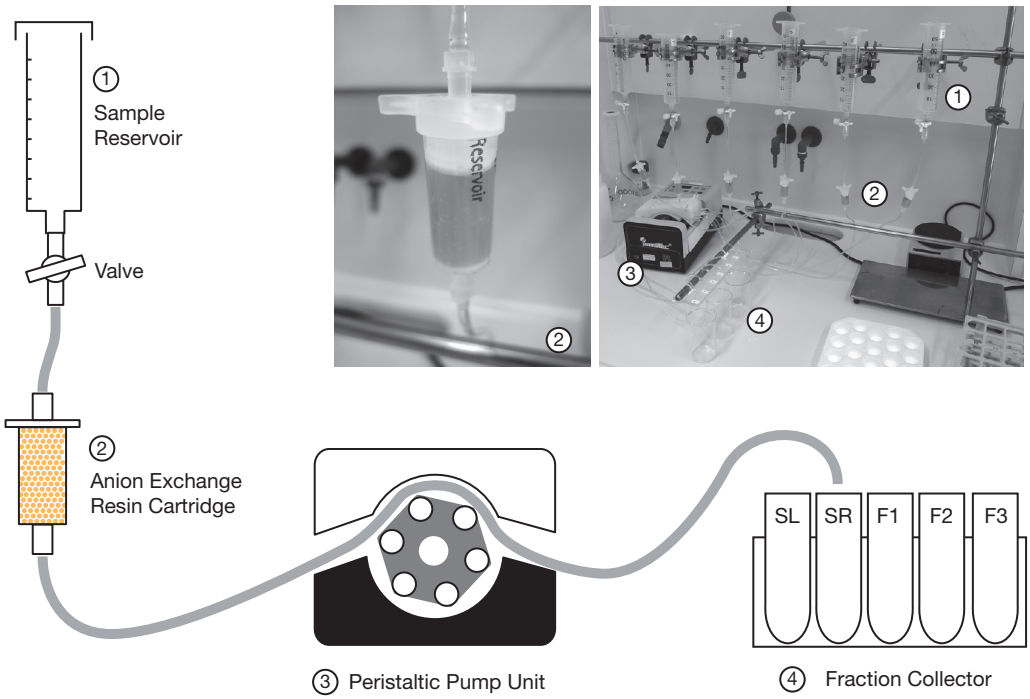

Fig. 1. Preparative anion chromatography setup. The sample is transferred into the 60 mL syringe barrel reservoir (1), loaded onto the anion exchange resin column (2), mounted before the peristaltic pump (3), and sample fractions are collected with a tube rack fraction collector (4). The preparation line comprises six parallel units in simultaneous operation (photograph on top right) and could be extended according to the maximum capacity of the peristaltic pump.

mol L^{-1} NaOH and 0.1 mol L^{-1} HNO_3; Step 2: freshly prepared 1 and 0.15 mol L^{-1} $NaHCO_3$; Step 3: 1 mol L^{-1} HNO_3; Step 5: a silver ammine solution containing 0.2 mol L^{-1} $AgNO_3$ buffered in 0.35 mol L^{-1} NH_4NO_3 and 0.74 mol L^{-1} NH_4OH (O'Neil et al. 1994).

We further use a batch of artificial seawater (ASW, Kester et al. 1967) for method testing on ASW samples containing amendments of KH_2PO_4. Sample processing also requires pH indicator strips (Merck), anion exchange resin AG1-X8 (hydroxide form, 100-200 mesh, biotechnology grade, Biorad), and cation exchange resin AG50W-X8 (hydrogen form, 100-200 mesh, biotechnology grade, Biorad).

Equipment

Equipment used in this study included a tabletop centrifuge accommodating 30 mL centrifuge tubes and capable of spinning up to 15,000 rpm (Sigma 2-16), a test tube vortexer (IKA Basic), a peristaltic pump (Ismatec IPC 12), a horizontal shaker plate (Heraeus), a drying cabinet (Memmert), a thermostatic heating block with 25 mL PTFE beakers and argon blow-down line, a thermostatic heating plate (diameter 14 cm) with a quartz bath, a spectral photometer (Merck SQ 118), a micro balance (Sartorius), and a temperature conversion elemental analyzer (Thermo Finnigan TC/EA) linked via a continuous flow interface (Thermo Finnigan ConFlo II) to an isotope ratio mass spectrometer (Thermo Finnigan Delta plus).

Refined separation protocol for porewater phosphate after Colman (2002)

Sample handling and aqueous phase analyses

It is first essential to obtain filtered, uncontaminated porewater samples for analysis of the oxygen isotope composition of P_i and concentrations of P_i and other ions. Porewater samples from sediment cores are taken with Rhizon suction samplers (Rhizosphere Research, Wageningen) immediately after core retrieval. These micro porous polymer tubes (5 cm length, 3.5 mm diameter) are inserted into the sediment core, and porewater is extracted by attaching an evacuated syringe to the Luer coupling. The polymer has a filter size of 0.1 µm. In contrast to conventional squeeze sampling of the porewater, the Rhizon technique yields a practically sterile sample, leaves intact cells behind, and avoids the pressure-related release of adsorbed P_i (Dickens et al. 2007; Seeberg-Elverfeldt et al. 2005). The samples are stored at 4°C until further processing.

Dissolved P_i is routinely quantified by spectral photometry (Merck SQ 118, 820 nm), using the phosphomolybdenum blue method modified after Hansen and Koroleff (1999) and Murphy and Riley (1962). Anions were determined by ion chromatography (Metrohm 861 Advanced Compact IC, column A Supp 5, conductivity detection after chemical suppression). Cations were measured by inductively coupled plasma optical emission spectrometry (ICP-OES, Perkin Elmer Optima 3300R). The oxygen isotope composition of water ($\delta^{18}O_w$) was

determined on selected samples after equilibration with CO_2 using mass spectrometry.

Step 1: Multiple P_i co-precipitation with magnesium hydroxide

The first step of the micro extraction procedure isolates P_i from other ions and dissolved organic matter (DOM) in solution and concentrates P_i in a small volume. Strict separation of porewater P_i from other P pools is a prerequisite for the proper interpretation of the isotopic signatures. In this protocol, we strip P_i from the solution by co-precipitation with magnesium hydroxide ("MagIC," Colman 2002; Karl and Tien 1992). This step is repeated at least three times, each time reducing the volume and further cleaning the sample. The details of this step are summarized in cookbook style below.

- Transfer the porewater sample to a 30-mL centrifuge tube. When the sample volume exceeds 30 mL, split the sample between two tubes and recombine before the second repetition.
- Add 1 mL of a 1 mol L^{-1} NaOH solution to each tube. This raises the pH to around 10 (check with pH test strip) and induces precipitation of magnesium hydroxide [$Mg(OH)_2$]. Further NaOH solution may be added in case the initial addition did not suffice.
- Shake the sample for 30 s using a vortex. The $Mg(OH)_2$ floc adsorbs dissolved P_i quantitatively.
- To separate floc from solution, centrifuge the sample at 10,000 rpm for 15 min.
- Re-dissolve the pellet with 10 mL of 0.1 M HNO_3.
- Repeat the above procedure including co-precipitation of P_i with $Mg(OH)_2$, vortexing, centrifugation, and re-dissolution three times. The resulting pellet should be of white color. In presence of high DOM, which is indicated by coloration of the porewater sample or a stained pellet, the procedure has to be repeated until the discoloration disappears. This may take up to five repetitions.
- After final re-dissolution of the pellet, carefully adjust sample pH to 6 with NaOH. At this pH, the main P_i species is $H_2PO_4^-$.
- Determine the P_i concentration of the processed sample spectrophotometrically.
- In the centrifugation steps, the high rotation speed ensures complete settling of the fine crystalline $Mg(OH)_2$. This is not achieved at lower speeds (e.g., 3000 rpm, Colman 2002).
- The supernatant solution of each centrifugation step is discarded after checking for absence of P_i. The resulting sample has an approximate volume of 10 mL.

Step 2: Preparative anion chromatography

The second step of the micro extraction procedure leads to a further purification of the phosphate samples by removing residual DOM and anions. We here use a simple, pump-based anion-exchange chromatography setup for easy control of flow rates and volumes of collected fractions (Fig. 1). Sample P_i is recovered with a $NaHCO_3$ eluent, and solution exchanged with Na^+ (Colman 2002). The detailed instructions for this step are as follows:

- Wash 3.0 g anion exchange resin AG1 × 8 into a cut-to-fit SPE cartridge and mount it into the pump system (Fig. 1). Gas bubble-free packing of the columns ensures consistent flow rates.
- Before sample processing, condition each column with 60 mL of 1 M $NaHCO_3$ solution, and flush with 40 mL H_2O at a flow rate of 1.5 mL min^{-1}. The standard flow rate in all following steps is 1.5 mL min^{-1}, and the pump timer is used to control fraction volumes.
- Transfer the sample into the 60 mL syringe barrel reservoir and load it onto the column at a flow rate of 1.5 mL min^{-1} and collect the sample load fraction (SL, Fig. 1) in a 26-mL tube. This step is successful when the spectral photometric check for P_i in SL is negative.
- Flush the sample reservoir with 10 mL of H_2O, collect sample rinse fraction SR and again check for P_i.
- Fill approximately 45 mL of a freshly prepared 0.15 M $NaHCO_3$ solution into the 60 mL syringe barrel reservoir (Fig. 1). Elute P_i from the column in three fractions, each time collecting the eluate: F1, 8 mL pre-run, in 26 mL tube; F2, 20 mL sample run, in 50 mL Falcon tube; F3, 12 mL post-run, in 26 mL tube. Check the P_i concentration in the fractions. The proper performance of Step 2 is confirmed by absence of P_i in fractions F1 and F3 and quantitative recovery of P_i from F2. Only F2 is kept for the next step.

Depending on sample matrix and P_i concentration, and especially for very high P_i loads, it may be necessary to adjust the amount of resin used. We have determined calibration curves for typical P_i amounts in porewater samples prepared in artificial seawater (Fig. 2), and found that our setup is well suited for the range of P_i encountered in the investigated natural samples (10-200 µmol L^{-1}). It may be possible to further simplify the setup by using gravity-driven elution, but the pump system facilitates control of elution volume and time and enhances sample throughput when processing six or more samples in parallel.

Step 3: Cation exchange in batch mode

The third step of the micro extraction procedure removes HCO_3^- and Na^+ from the samples and converts the sample P_i from $H_2PO_4^-$ to H_3PO_4. This is achieved by the exchange of Na^+ for H^+, which subsequently reacts with HCO_3^- to form CO_2 that bubbles off spontaneously (Colman 2002). The procedure is as follows:

- Convert the cation exchange resin AG50WX8 to H^+ form by 30 min of batch reaction with 1 mol L^{-1} HNO_3 on a horizontal shaker. Rinse the resin three times with H_2O.
- Add approximately 3.0 g of the converted resin to the sample. This induces a rapid release of CO_2. After the initial CO_2 pulse, close the sample tube and agitate it for 2 h on the horizontal shaker. Every 20-30 min, it will be necessary to open the tube to release excess CO_2.
- Use a Steriflip® membrane filter cartridge and a second

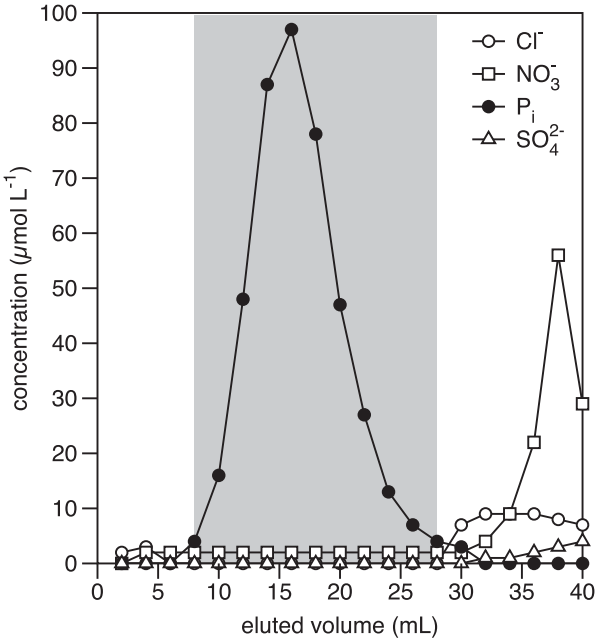

Fig. 2. Breakthrough curves for chloride (Cl^-), nitrate (NO_3^-), phosphate (P_i), and sulfate (SO_4^{2-}) in the preparative anion chromatography setup. Shown is data from a test sample of 1 µmol P_i in 10 mL artificial seawater. The gray background indicates the part of the eluate that is collected within fraction F2 and recovers the sample P_i quantitatively.

tube to separate the resin from the dissolved sample. Rinse the filter with 2 mL H_2O that is collected with the sample.

Two hours of reaction guarantee complete removal of $NaHCO_3$ from the sample. This is a crucial prerequisite for the subsequent clean precipitation of Ag_3PO_4 (Step 5). At the end of Step 3, the sample of around 22 mL has a mildly acidic pH of 5–6. Proper handling, fresh conditioning, and rinsing of the resin before every application turned out to be crucial to the successful precipitation of Ag_3PO_4. Using resin prepared the previous day resulted in a reddish discoloration of the sample and made proper precipitation of Ag_3PO_4 impossible. We have not yet resolved the cause of this complication.

Step 4: Sample concentration

The fourth step of the micro extraction procedure serves to concentrate P_i to a level where it can be precipitated as silver phosphate (Ag_3PO_4). This step reduces the sample volume from approximately 25 mL to 0.5-1 mL. The details for this step are as follows:

- Carefully transfer the 20 mL sample into the PTFE beaker of the heating block.
- Evaporate sample at 60°C, under a gentle argon stream. The volume reduction to 0.5-1 mL is completed after 8–10 h.
- Pipette the sample into a 2.0 mL micro reaction cup followed by a 200 µL H_2O rinse of the PTFE beaker. The sample is now ready for precipitation of Ag_3PO_4 (Step 5).

The sample concentration step may be streamlined considerably. The original protocol by Colman (2002) uses an automatic evaporator that concentrates the sample much faster. We recommend using a similar device when available.

Step 5: Silver phosphate precipitation

The fifth step of the micro extraction procedure is the precipitation of Ag_3PO_4. Here, Ag_3PO_4 crystals are produced that can easily be handled for oxygen isotope analysis by IRMS. Excess Ag^+ is added to the samples in the form of silver ammine solution (O'Neil et al. 1994). The details of the precipitation step are as follows:

- Add silver ammine solution to the sample, in a Ag:P_i ratio of approximately 100:1. For example, this corresponds to 0.5 mL silver ammine solution for a 1 mL sample containing 1 µmol P_i.
- Incubate the precipitation cups in a 50°C sand bath under a dust-protection cabinet. Under these conditions, NH_3 slowly evaporates and liberates Ag^+ that reacts with P_i in solution. After a few hours, crystals of Ag_3PO_4 start to form on the liquid surface and the walls of the reaction cup. Complete precipitation of Ag_3PO_4 takes up to 60 h.
- Carefully detach the crystals from the walls of the reaction cup using a 2 mL Eppendorf pipette and transfer them onto a 0.2 µm membrane filter on a vacuum filtration unit.
- Wash the crystals thoroughly with H_2O to remove any nitrate that may have persisted in the sample.
- Place the crystals on the filters into a small Petri dish and dry them at 60°C for at least 12 h. The dry crystals can be kept in envelopes of annealed aluminum foil in a dessicator for subsequent IRMS analysis.

For successful precipitation of Ag_3PO_4, it is crucial to maintain a high Ag:P_i ratio. The 10:1 ratio used in Colman (2002) did not prove successful for small sample P_i quantities. However, we suggest further testing here if the results are not satisfactory at first instance. In general, stronger buffering with NH_4NO_3 retards crystallization. The slow precipitation technique (Colman 2002) yields bigger and easier-to-handle crystals than the comparatively more rapid precipitation described by Dettmann et al. (2001). Thorough removal of remaining nitrate from the crystals in the washing step is essential because nitrate, as an oxygen-bearing moiety, compromises oxygen isotope analysis by IRMS.

Isotope ratio mass spectrometry

We use a standard continuous flow TC/EA IRMS setup that follows the scheme used in Colman (2002), Laporte et al. (2009), and McLaughlin et al. (2004). Silver phosphate samples of a minimum weight of 200 µg are weighed into silver capsules and tightly crimped to minimize air inclusion (Vennemann et al. 2002). The samples are introduced to the TC/EA via the autosampler, where the Ag_3PO_4 is carbothermically reduced in a glassy carbon reactor at 1450°C for complete conversion of sample oxygen to CO. We achieve better results by operating the reactor without an additional graphite crucible in the reaction zone (Colman et al. 2000), but with an amendment of approximately 7 g nickelized carbon to the glassy carbon granules (Kornexl et al. 1999). With

this setup, very small amounts of Ag_3PO_4 (around 200 µg) can be analyzed. The capacity of the reactor is up to 150 consecutive samples, after which silver residues have to be removed from the glassy carbon granules. The sample gas is carried by a continuous Helium (He) stream at 80 mL min^{-1}, passes a H_2O trap filled with hygroscopic magnesium perchlorate [$Mg(ClO_4)_2$], and a gas chromatography (GC) column held at 90°C. The GC column separates gases that potentially interfere with the measurement (i.e., N_2 from CO). An open split (ConFlo) serves as interface between the high pressure system (TC/EA) and vacuum system (IRMS). The sample CO is measured after two injections of reference CO gas from a tank. The peak integration of mass 28, 29, and 30 bases on a time-based background average. Raw $\delta^{18}O_p$ values are calculated by the Isodat software (Isodat NT version 2.0) and normalized to VSMOW by a linear three-point calibration with external standards TU1, TU2, and USGS35 (Table 1). Daily routine includes running the three standards in duplicate for calibration of sample $\delta^{18}O_p$ to VSMOW, then a batch of 15 to 20 samples, and finally again the three standards for instrument drift control. Usually drift can be neglected during a day's run.

Assessment

Mass spectrometer performance for low sample weights

We evaluated the performance of our IRMS setup with external oxygen isotope standards TU1, TU2, and USGS35 (Table 1) to test the reproducibility and accuracy of the measurements at low sample weights. We found no remarkable difference in variability of uncalibrated $\delta^{18}O_p$ values of the standard TU1 for weight classes 100-200 µg, 200-300 µg, and 300-400 µg (Fig. 3). Samples with lower weights showed higher deviations and were not considered for oxygen isotope measurements.

Preservation of isotopic signature during P_i micro extraction

We tested our method to ensure that the presented purification procedure does not entail any alteration of the oxygen isotopic signature, neither by contamination nor by isotopic exchange. The micro extraction procedure was applied to materials of known (TU1, TU2) and unknown (BR2) isotopic composition that were dissolved in water and artificial sea water (ASW) to assess accuracy and reproducibility of the method (Table 2). Additional tests were performed with artificial samples (standard BR1, dissolved potassium phosphate from Merck Certipur® solution) varying in P_i concentration (0.5 µmol L^{-1} to 100 µmol L^{-1}), pH (4.5 and 7.5), and matrix composition (water and North Atlantic Sea Water; Table 2).

The test series comparing low pH (4.5) to high pH (7.5) was carried out to evaluate potential acid hydrolysis of DOP, which would result in incorporation of oxygen isotopes from water (Blake et al. 1997). We prepared samples of P_i standard BR1 in sterile filtered stock North Sea water (NSW) with a P_i blank below detection limit of the phosphomolybdenum blue method (< -1 µmol L^{-1}) in two concentrations (50 and 100 µmol L^{-1}) and adjusted pH to 4.5 and 7.5, respectively, and subjected them to the micro extraction protocol. The $\delta^{18}O_p$ values of resulting Ag_3PO_4 were compared with those of directly precipitated Ag_3PO_4 from BR1 (Table 2). Samples with pH 4.5 deviate from the direct reference, while the mean values of replicates from pH 7.5 were statistically identical to the

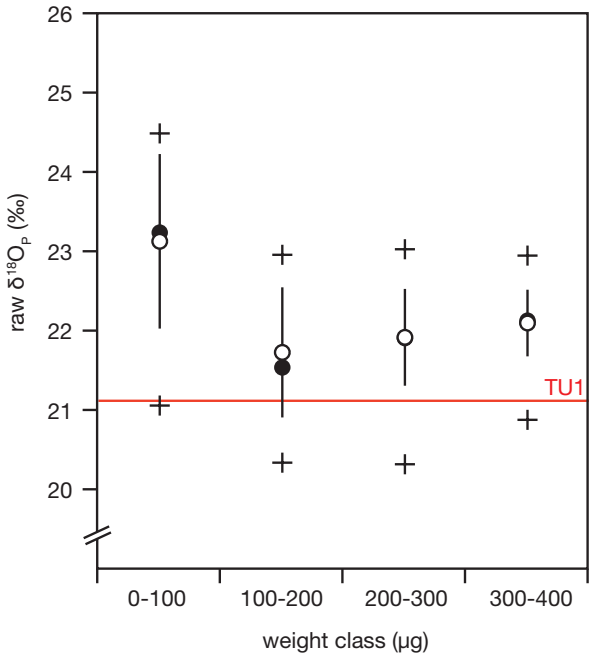

Fig. 3. Low Ag_3PO_4 weights do not compromise IRMS analyses. Shown are arithmetic mean (circle) with 1σ standard deviation (lines), median (dots), and minimum/maximum (crosses) of uncalibrated $\delta^{18}O_p$ values of repeated measurements of standard TU1 (referenced value: red line) in different weight classes. Weight classes of 100-200 (n = 12), 200-300 (n = 35), and 300-400 µg (n = 30) appear similar, whereas 0-100 µg (n = 14) show considerably higher variability and were thus considered too small for sample analysis.

Table 1. External standard materials for calibration of measurements to VSMOW

Standard	Compound	$\delta^{18}O$ (‰VSMOW)	Method	Reference
TU1	Ag_3PO_4	+21.11 ± 0.57	high-temperature reduction IRMS	Vennemann et al. (2002)
TU2	Ag_3PO_4	+5.35 ± 0.62	high-temperature reduction IRMS	Vennemann et al. (2002)
USGS35	$NaNO_3$	+57.5 ± 0.4	high-temperature reduction IRMS	National Institute of Standards and Technology (2008)

Table 2. Test series for evaluation of the microextraction protocol. Oxygen isotope values ($\delta^{18}O_P$) and recoveries are given as arithmetic means μ with standard deviation σ, each for *n* replicates.

Material	Source PO_4	Matrix and concentration	Preparation	*n*	$\delta^{18}O_P$ μ ± σ ‰	recovery μ ± σ %
BR1	Merck Certipur PO_4^{3-} solution	in H_2O 0.50 μmol L^{-1}	direct precipitation reference	7	8.29 ± 0.74	
		in NSW 50 μmol L^{-1} pH 4.5	micro extraction	9	6.97 ± 0.09	92 ± 2
		in NSW 100 μmol L^{-1} pH 4.5	micro extraction	6	7.41 ± 0.20	91 ± 3
		in NSW 50 μmol L^{-1} pH 7.5	micro extraction	6	7.95 ± 0.19	94 ± 5
		in NSW 100 μmol L^{-1} pH 7.5	micro extraction	6	8.83 ± 0.21	96 ± 4
BR2	Merck KH_2PO_4 ACS reagent	in H_2O 100 μmol L^{-1}	direct precipitation reference	12	12.19 ± 0.29	
		in ASW 100 μmol L^{-1}	micro extraction	4	12.08 ± 0.20	93 ± 3
TU1	Ag_3PO_4	in ASW 100 μmol L^{-1}	micro extraction	4	20.88 ± 0.04	93 ± 3
TU2	Ag_3PO_4	in ASW 100 μmol L^{-1}	micro extraction	4	5.50 ± 0.25	93 ± 2

direct reference. This discrepancy may reflect acid hydrolysis of DOP from NSW. We infer that the initial sample pH is crucial for the conservation of the original sample $\delta^{18}O_P$. Therefore, samples must be kept at pH higher than 4.5 until complete DOM removal after steps 1 and 2. When dissolving sample pellets with HNO_3 in step 1, it needs to be considered that excessive use of this acid, which is also an oxidizing agent, could have negative effects on the reliability of the presented method.

We also tested the full experimental protocol with a set of phosphate that covers a range of oxygen isotope compositions, BR2 (KH_2PO_4, Merck ACS grade reagent, $\delta^{18}O_P$ = 12.2 ‰), TU1 (Ag_3PO_4 standard, Vennemann et al. [2002], $\delta^{18}O_P$ = 21.1 ‰), and TU2 (Ag_3PO_4 standard, Vennemann et al. [2002], $\delta^{18}O_P$ = 5.4 ‰). Solution of BR2 was prepared as a 100 μmol L^{-1} solution in sterile filtered artificial seawater (ASW, Kester et al. 1967) that did not contain DOP. The standards TU1 and TU2 were dissolved in a solution of NH_4NO_3 and NH_4OH. From those solutions, we prepared 100 μmol L^{-1} samples in ASW analogous to BR2. The isotope composition of BR2 from micro extraction was isotopically indistinguishable from directly precipitated reference Ag_3PO_4 (Table 2), and both dissolved TU1 and TU2 matched the referenced $\delta^{18}O_P$ values (Table 2, referenced values in Table 1).

Mass recovery of P_i after extraction

Mean sample P_i recoveries were higher than 90% for artificial test samples (93 ± 4%, *n* = 39), and in a similar range for natural samples (91 ± 19 %, *n* = 47). In light of the results of the test series, we conclude that the preparative loss of sample P_i is isotopically nonselective and does not compromise data quality.

Natural samples

The investigation area offshore Cape Ghir is located in a trade-wind driven upwelling system, where nutrient-rich deep waters sustain high productivity in the coastal zone, and nutrients are transported several hundred kilometers to the open ocean in filaments in the surface waters (Freudenthal et al. 2002). During the R/V *M.S. Merian* cruise 04/4a in March 2007, two sites on the Moroccan continental shelf were drilled on two locations just offshore Cape Ghir (GeoB11804-4, 30°50.73′N, 10°5.90′W and GeoB11807-2, 30°51.02′N, 10°16.10′W) using a remotely operated drill rig (MARUM MeBo). Drilling reached depths of 39 mbsf (GeoB11804-4) and 17 mbsf (GeoB11807-2). Immediately after retrieving the cores from the rig, we took porewater samples that were stored at 4°C and shipped back to Bremen for further processing. We successfully extracted P_i with the micro extraction procedure and precipitated Ag_3PO_4 even from samples containing only 0.4 μmol P_i, which corresponds to final Ag_3PO_4 weights of

around 150 µg. These sample amounts are a factor of 0.2 to 0.5 lower than previously used for IRMS analyses (Colman 2002; McLaughlin et al. 2004), but yield similar variability as higher weights in a comparison of standard measurements (Fig. 3). It is thus feasible to use these low amounts of Ag_3PO_4 for determination of $\delta^{18}O_P$.

Discussion

Downcore profiles

At both investigated sites, porewater phosphate concentrations reach a maximum where sulfate profiles show a distinct change in their slope and sulfate values become low (Fig. 4). These concentration profiles indicate that P_i is released from the sediments to the overlying water column whereas sulfate is consumed in the upper sediment by sulfate reduction during degradation of organic matter, and at 7 m below surface (mbsf) by sulfate reduction related to the anaerobic oxidation of methane. There are two major sources for P_i in the porewater, release of P_i during remineralization of organic matter (OM) and release of P_i adsorbed to mineral phases during early diagenesis. During microbial respiration of OM, P is preferentially regenerated and the C:P ratio of OM increases with depth (Ingall and Van Capellen 1990). Sulfide produced during microbial sulfate reduction induces transformation of mineral phases, i.e., the reduction of iron and manganese. Phosphate incorporated or adsorbed to such phases is released to the porewater. The accumulation of dissolved phosphate in porewater, and the reflux to overlying bottom waters suggests that in the topmost sediments, P_i release exceeds microbiological demand and geochemical sequestration.

Examination of the disequilibrium in the phosphate oxygen isotope composition provides more insight into the balance between biological demand, sequestration, and flux of P in the sediments. We can calculate the theoretical oxygen isotope composition of P_i at equilibrium with water according to the equation of Longinelli and Nuti (1973) by using a gradient of 0.01°C m^{-1}, and the measured $\delta^{18}O_W$ that had an average of 0.26 ‰ (Eq. 1).

$$\delta^{18}O_P = [(111.4 - T)\ 4.3] + \delta^{18}O_W \quad (1)$$

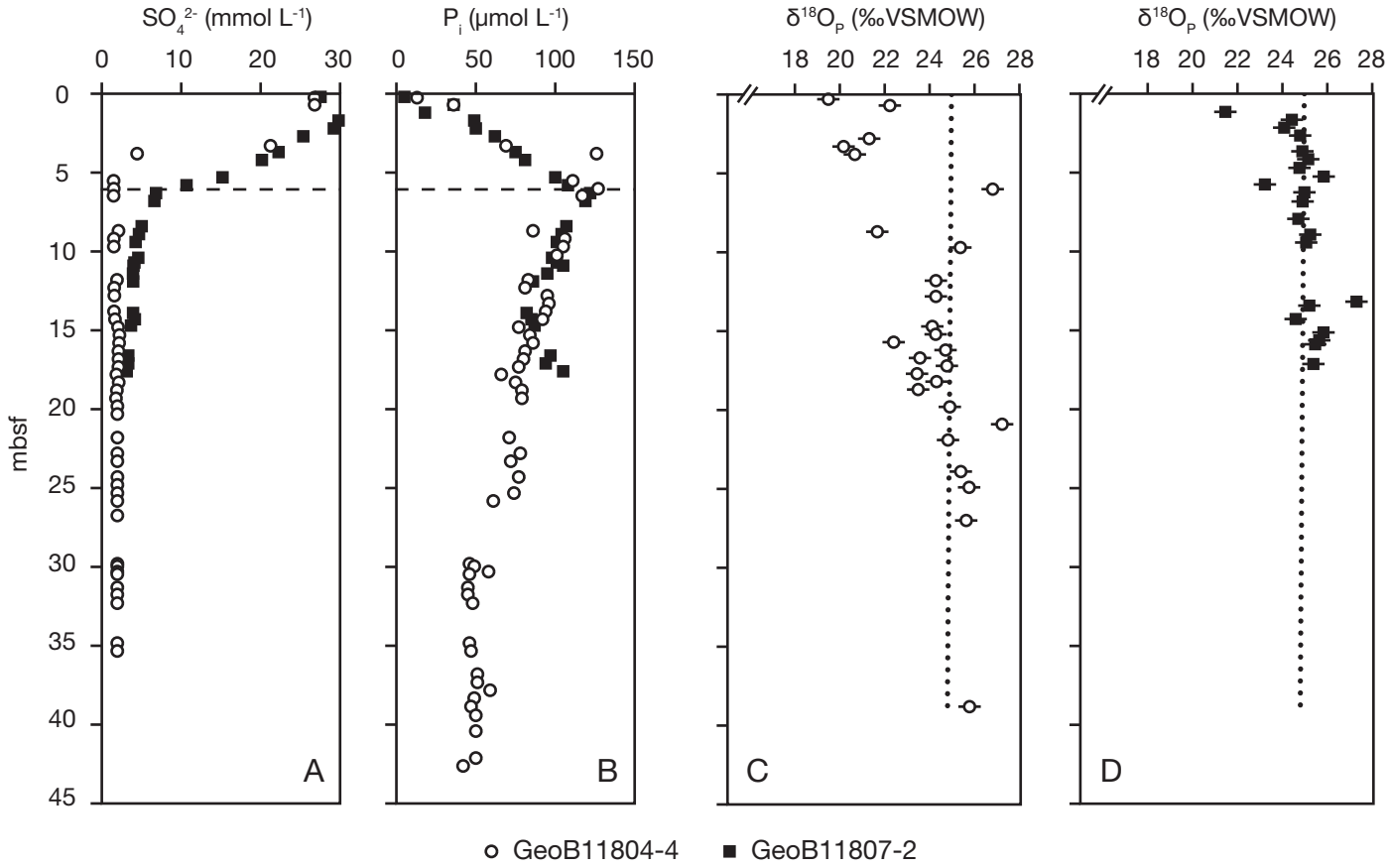

Fig. 4. Porewater profiles for sediment cores GeoB11804-4 and GeoB11807-2. Left panels: dissolved sulfate (A) and phosphate concentrations (B) for both cores. The dashed line at 6 mbsf indicates the sulfate penetration depth (SPD) at both sites. Right panels: porewater phosphate $\delta^{18}O_P$ referenced to VMSOW for GeoB11804-4 (C) and GeoB11807-2 (D). For reason of clarity, the $\delta^{18}O_P$ have been corrected for water $\delta^{18}O$, and the temperature-dependent isotopic equilibria for a geothermal gradient of 0.05°C m^{-1} have been calculated from the empirical relationship of Longinelli and Nuti (1973) and a water $\delta^{18}O$ of 0‰ (Eq. 2). Error bars are 1σ standard deviation from concomitant external standard analyses.

The comparison of the actual measurements to the calculated equilibrium isotope composition shows a remarkable offset at the core tops, whereas measured values overlap with the theoretical values at greater sediment depths (Fig. 4). The offset of pore $\delta^{18}O_p$ from the equilibrium isotope composition indicates that this disequilibrium is either caused within the sediment, or by exchange of P_i with the overlying water column. In the latter case, P_i from the overlying water column is in disequilibrium with the theoretical equilibrium values. Offsets in the oxygen isotope compositions of P_i to values lower than the theoretical equilibrium have been shown to be caused by the activity of extracellular enzymes such as alkaline phosphatase (APase) that liberate P_i from OM by phosphoester hydrolysis (Blake et al. 2005). These processes are in competition with the activity of intracellular enzymes such as pyrophosphatase (PPase), that equilibrate P_i oxygen with ambient water, and activities of phosphoenzymes involved in the synthesis and degradation of P biomolecules (e.g., ADP, ATP, polyphosphates, phospholipids; cf. Blake et al. 2005).

In sediments, bioavailable P_i is depleted whereas C:P ratios of OM increase with depth. This imposes a need for efficient P_i recycling within the microbial community. Such circumstances are expected to favor rapid microbial turnover of P_i and to drive $\delta^{18}O_p$ toward oxygen isotope equilibrium with water. This expectation is supported by the observed isotope trends in the investigated cores (Fig. 4). However, it is intriguing that this rapid equilibration does not lead to equilibrium oxygen isotope values in the top of the sediments. This implies that the influx of P_i depleted in ^{18}O relative to the equilibrium value is larger than the microbial turnover causing isotope equilibration, a hypothesis supported by the fact that P_i concentrations are elevated in the upper part of the sediment column.

Simple isotope mixing model

We can use a simple two-endmember isotope mixing model to obtain a rough estimate of the relative importance of production of P_i by OM by phosphoester hydrolysis (APase pathway; offset from the equilibrium value) compared with the production of P_i that is equilibrated with porewater. We assume that inorganic processes, such as adsorption and desorption of P_i with iron and manganese oxyhydroxides (Blake et al. 2001) or precipitation and dissolution of apatite (Blake et al. 1998; Liang and Blake 2007) can be neglected because of minor long-term isotope effects (Jaisi et al. 2010). We further assume that all P_i fed to the system by sinking OM from the photic zone has an oxygen isotope composition of 21‰, which is inferred from Eq. 1, using a temperature of 10°C. Considering the uncertainties for those assumptions, together with additional complications such as different forms of organic bound P_i (mono- and diesters), enzyme systems, or P_i residence times, it is evident that such a simple model cannot yield quantitative information. However, it can be used to illustrate general patterns, i.e., allows assessing semi-quantitatively the relative importance of equilibration versus disequilibrium processes.

Now, we can determine one end-member of the isotope-mixing model. The oxygen isotope effect of APase regeneration pathway can be calculated after Liang and Blake (2006) as follows:

$$\delta^{18}O_{P,APase} = 0.25(\delta^{18}O_W - 30‰) + 0.75(\delta^{18}O_{Porg}) \quad (2)$$

The other end-member of the mixing model (PPase activity) corresponds to the temperature-dependent isotope equilibrium between P_i and water (Eq. 1).

The two end-members of the isotope-mixing model can now be combined in an isotope mass balance, which results in the measured isotope composition of P_i at a particular sediment depth:

$$\delta^{18}O_P = x(\delta^{18}O_{P,APase}) + (1 - x)(\delta^{18}O_{P,PPase}) \quad (3)$$

The parameter x corresponds to the relative contribution of the two end-members in the isotope mixing model. Thus, by calculating the value of x, we can estimate the relative importance of the respective pathways (Fig. 5). As expected, the supply of P_i from APase activity is outcompeted by PPase toward core bottom, whereas up to 32% of P_i in the upper sediment column exhibits the imprint of APase activity. This value may even be underestimated because of unconstrained isotopic rebound of released P_i toward equilibrium. However, consider-

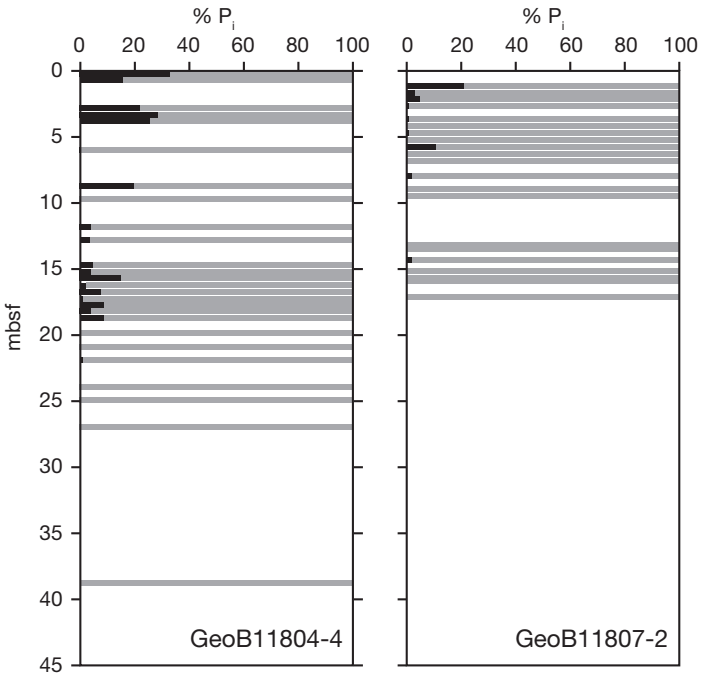

Fig. 5. Fraction of porewater P_i in isotopic disequilibrium with ambient H_2O (black) and in isotopic equilibrium with ambient H_2O (gray), derived from a two end-member mixing model. The disequilibrium fraction represents regenerated P_i that is not been equilibrated with porewater by microorganisms.

ing that P_i concentrations decrease strongly toward the interface to the water column, where microbial activity is highest, it is striking that the oxygen isotope composition of P_i is not fully overprinted by equilibrium isotope exchange. Upward diffusion of P_i with distinct isotope composition from sources below, OM mineralization or redissolution of solid-bound P_i, would potentially contribute to this effect. At the investigated sites, however, the P_i concentration profiles suggest a diffusive source from a depth where P_i is more close to the isotopic equilibrium (Fig. 4). This indicates that the oxygen isotope composition of P_i in the porewater close to the sediment water interface is not just the result of mixing of P_i from the water column with P_i from deeper sediments, but that the microbial benthic P turnover is an important contributor to marine P cycling.

Using oxygen isotope tools, we now stand on the cusp of new insights into the modern marine P cycle. At the moment though, we lack data on porewater $\delta^{18}O_P$ from other marine sites. Consequently, our novel data may represent a general or a site-specific pattern. Unlike the presented simple end-member mixing approach, more elaborate reactive transport models will provide quantitative information about sedimentary P cycling. To gain a more thorough understanding of the P_i oxygen isotope system in marine sediments, we need better knowledge about sources and sinks of inorganic P_i, and about sedimentary processes that potentially affect the oxygen isotope composition of P_i pools. The role of transformations between dissolved and solid-bound P_i in various phases, such as iron minerals and phosphorites, needs further investigation in natural systems, and high turnover rates and fluxes in the benthic boundary layer, where water column and sediments are coupled, may also contribute to yet unforeseen isotopic effects.

Comments and recommendations

In line with earlier studies, we found sample pH being the important control on P_i recovery and isotope integrity (McLaughlin et al. 2004). Thus, monitoring pH during sample handling is prerequisite for successful extraction of P_i for isotope analysis. Considering the extremely low P_i quantities in porewater samples, we suggest employing a pH microelectrode with short response time instead of pH indicator strips that may induce sample loss and can be a source of sample contamination. Though the micro extraction method is manually elaborate, it was possible to achieve throughput rates of approximately 6 porewater samples per day. The volume reduction of the sample (Step 4) is very time-consuming and the bottleneck in achieving higher sample throughputs. Therefore, we recommend the use an automated evaporator to speed up this step as suggested in the original method by Colman (2002).

We have not evaluated whether the micro extraction protocol of McLaughlin et al. (2004) can be modified for the application on porewater samples. However, we believe that this method could be equally refined as the protocol from Colman (2002).

We expect that innovations in mass spectrometry will further decrease the sample amounts needed for isotope analysis, allowing the use of oxygen isotope composition of P_i as a tracer for P cycling in yet unexplored environments. Our refined micro extraction protocol is a first step in the direction to make very small amounts of P_i accessible for isotope analysis.

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