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A simple and cost-effective manual solid phase extraction method for the determination of nanomolar dissolved reactive phosphorus in aqueous samples

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Abstract

Phosphate is a typical limiting nutrient in oligotrophic oceans and its concentration can be as low as the nanomolar level, especially in surface seawater. Different techniques for nanomolar level phosphate determination have been studied, most of which are automatic and high throughput methods based on flow analysis. However, these techniques need expensive commercial or laboratory-made instruments, and experienced operators. This study reports an operationally convenient, sensitive and practical method for the determination of nanomolar dissolved reactive phosphorus in aqueous samples, based on classic phosphomolybdenum blue chemistry and solid phase extraction methodology. Under acidic conditions, the formed phosphomolybdenum blue can be preconcentrated on a hydrophilic-lipophilic balance solid phase cartridge, eluted with 0.2 M NaOH, and detected at 700 nm after color recovery with the addition of more reagents. After optimization of the recovery reagents volumes, the effectiveness of the method was evaluated. This simple manual method is sensitive (detection limit of 3.0 nM using 50 mL sample, which can be lowered to $\sim$ 1 nM by preconcentrating more sample), reproducible (relative standard deviation of 2.6%, $n = 81$, with different operators), free of interferences from salinity (0–35), silicate (up to 200 μM) and arsenate (up to 100 nM) and suitable for different aqueous matrixes. The effects of a series of phosphorus-containing compounds ($n = 13$) were evaluated, with more than half showing hydrolysis during the experiment. This method was applied to determine the phosphate concentration in samples collected from the Western Pacific.

Phosphorus is an essential nutrient required to maintain the normal metabolism of organisms and could be a limiting nutrient in marine and freshwaters (Benitez-Nelson 2000; Paytan and McLaughlin 2007; Karl 2014). In some oligotrophic areas, the concentration of phosphate exists at nanomolar levels due to the low solubility of phosphorus minerals and the uptake of phosphorus through phytoplankton photosynthesis (Zhang and Chi 2002). Due to its importance in marine systems, measurements of nanomolar phosphate (and other nutrient) concentrations is one of the most commonly performed analyses in oceanographic research (Patey et al. 2008; Ma et al. 2014a). Various terms regarding phosphate measurements are used in scientific literature. Phosphate measurements based on the classic phosphomolybdenum blue (PMB) method (Murphy and Riley 1962) are referred to as dissolved reactive phosphorus (DRP) or soluble reactive phosphorus (SRP), which contains the orthophosphate and easily hydrolyzed dissolved organic phosphorus (DOP) or condensed phosphorus compound (part of the dissolved inorganic phosphorus) (DIP) (Karl and Tien 1992). These fractions are operationally defined by filtration through a 0.2 μm or 0.45 μm membrane (Jarvie et al. 2002; Worsfold et al. 2008). To clarify the nomenclature, it is suggested to use the term filterable reactive phosphorus (FRP, membrane pore-size in μm), for example, FRP (0.2 μm) (Nollet and Gelder 2013).

Reviews specific to phosphate analysis include the works of Estela and Cerdà (2005), Motomizu and Li (2005), Villalba et al. (2009), and Berchmans et al. (2012). However, for trace phosphate measurements in seawater matrices, only a few methods have been widely used: MAGnesium Induced Coprecipitation (MAGIC), solid-phase extraction (SPE), and liquid waveguide capillary cell (LWCC) (Patey et al. 2008; Ma et al. 2014a). The MAGIC method is based on the coprecipitation of phosphate with $Mg(OH)_2$, which is induced by the addition of NaOH into the seawater sample. The precipitate can be concentrated by centrifugation, dissolved in

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

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diluted acid and measured with the PMB method (Karl and Tien 1992). Following its wide application by marine scientists (e.g. Thomson-Bulldis and Karl 1998; Wu et al. 2000), the MAGIC protocol has been improved (Rimmelin and Moutin 2005) and applied to freshwater analysis (Anagnostou and Sherrell 2008). The LWCC method is based on sensitivity amplification using a long-path (up to 5 m) optical cell, normally made of Teflon AF I/II (Gimbert and Worsfold 2007; Páscoa et al. 2012). Since its first application by Zhang and Chi (2002), the LWCC method has been widely utilized in DRP analysis in combination with different flow analysis techniques, as summarized by Zimmer and Cutter (2012). Normally, for LWCC-based flow analysis, phosphate-free seawater is needed for compensating the refractive index between the carrier/wash solution and seawater sample, which is time-consuming and requires extremely meticulous operation (Ma et al. 2014a). The SPE-based methods use either two sorption materials: C18 for the concentration of the PMB-CTAB (cetyltrimethylammonium bromide) ion-pair complex (Liang et al. 2007; Ma et al. 2008b), or HLB (hydrophilic-lipophilic balance, Waters Oasis) which concentrates PMB directly without the use of CTAB (Ma et al. 2008a). The successful replacement of C18 with HLB reduces the reaction time and organic solvent consumption. More importantly, no salinity effect is observed with the HLB method, therefore, deionized water can be used as the matrix, eliminated the need to prepare "phosphate-free" seawater. This method has been applied by other research group (e.g. Han et al. 2012, 2013; Du et al. 2013).

Except for the MAGIC method, most of the published papers for nanomolar phosphate analysis are based on expensive commercial or laboratory-made automatic analyzers, which is important for laboratory or field work dealing with a large number of samples. Conversely, for laboratories which don't have so many samples and adequately experienced operators, manual operation is more straightforward and can reduce costs. In this study, a method for manual determination of nanomolar DRP based on solid phase concentration of PMB on HLB sorbent was established and comprehensively evaluated to prove its sensitivity, accuracy, stability, and practicability.

Materials and procedures

Reagents and standards

Most of the chemicals, purchased from Sinopharm Chemical Reagent, China, unless stated elsewhere, were reagent grade or better and used without further purification. A phosphate stock solution of 8.0 mM was prepared by dissolving 54.40 g predried KH_2PO_4 in 500 mL ultra-pure water, collected from a Millipore water purification system (Millipore, MA, U.S.A., 18.2 ΩM). A silicate stock solution of 10 mM was prepared from predried Na_2SiF_6. An arsenate stock solution of 13.33 mM was purchased from ChemService, U.S.A. Working standards were prepared as required using suitable stepwise dilutions. A stock mixed reagent (MR) solution for color development was prepared by mixing 100 mL of 130 g L^{-1} $(NH_4)_6Mo_7O_{24}{\cdot}4H_2O$ solution, 100 mL of 3.5 g L^{-1} potassium antimony tartrate and 300 mL of 1 + 1 diluted H_2SO_4 (Ma et al. 2008b). The solution was stored at 4°C in a refrigerator while not in use. An ascorbic acid (AA) solution of 100 g L^{-1} was prepared daily. The eluent, 0.2 M NaOH solution, was prepared by dissolving appropriate amount of solid NaOH in water and sealed carefully to avoid the dissolution of atmospheric CO_2. Five national phosphate standard seawater samples (GBW08623) were purchased from the Second Institute of Oceanography, State Oceanic Administration, China. One environmental phosphate reference material (GSBZ500028-94, Batch No. 203417) was purchased from the Institute for Environmental Reference Materials, the Ministry of Environmental Protection (http://www.ierm.com.cn), China. Two international nutrient reference materials, Lot.BV and Lot.CA, were purchased from the General Environmental Technos. (KANSO Technos), Japan.

All the glassware used in this study was precleaned by soaking in 2 M HCl solution overnight, and thoroughly rinsing with pure water. The glassware containing PMB solution should be cleaned immediately after the experiment to reduce the possibility of the adsorption of PMB on the inner wall.

Procedures for sample analysis

Standard solutions and samples were analyzed according to the following procedures. For 50 mL samples, 1 mL of AA and MR were added sequentially and mixed thoroughly. The combined solution was left for color formation at room temperature for 5 min. Afterward, the solution was loaded onto the front part of a preconditioned Oasis HLB cartridge (3 cc/60 mg, P/N: WAT094226, www.waters.com) via a peristaltic pump (BT100-2J, www.lgpump.com.cn) at a flow rate of 40.0 mL min^{-1}. After loading, the PMB on the cartridge was back-flushed into a 1 cm cuvette with 1.5 mL eluent solution (0.2 M NaOH) via a syringe, from the rear of the cartridge, to reduce the diffusion of PMB in the sorbent. Then, 0.06 mL of MR solution and 0.03 mL of AA solution were added into the cuvette and thoroughly mixed with the eluent to recover the PMB (*vide infra*). Finally, the absorbance of the complex was measured at 700 nm using a UV–visible spectrophotometer (723-PC, www.spectrum-cn.com).

The pump tubing was connected to the front of the cartridge via a Luer connector. The rear part of the cartridge was cut and connected to a push-fit type PTFE adapter furnished with a 3-cm long 1.0-mm-diameter PTFE tubing at one end, which can be easily connected to a syringe filled with NaOH solution. The simple schematic diagram of the manual protocol and a photo of the cartridge and fittings were shown in Supporting Information (Figs. S1, S2). A

similar connection was also reported by other researchers for on-line monitoring (Anthemidis et al. 2011).

Sampling

Bottled water was purchased from the local market and analyzed within 2 h at the laboratory. Seawater samples were collected using Niskin bottles attached to a Rosette sampler during a cruise in April 2015 in the Western Pacific. The samples were frozen at −20°C immediately after collection and were analyzed within one month. Before analysis, the samples were completely thawed and mixed. The temperature and salinity were recorded using an SBE 911 plus CTD recorder (Sea-Bird, U.S.A).

Assessment and discussion

In the previous study, several parameters related to this research were studied: the effects of MR and AA concentrations and the reaction times on the PMB formation (Ma et al. 2014b), the effect of flow rate on sample loading on the HLB cartridge, the choice and concentration of eluent on the elution of PMB from HLB cartridge (Ma et al. 2008a). The optimized parameters were verified and used in the same manner as the published values. Only parameters related to this manual SPE procedure were evaluated and optimized in the following study.

Recovery of PMB from NaOH solution

The PMB formation is effected by several parameters, including pH, $[H^+]$: [Mo] ratio, reagent concentrations, etc. (Ma et al. 2014b). In this study, the PMB concentrated on HLB was eluted with NaOH solution, and the absorbance of the eluted compound under the alkaline condition decreased with time. It was found that readjusting the eluent with MR solution could re-establish a normal PMB level (Ma et al. 2008a). However, the stability of the reformed PMB, which was crucial for a manual operation method, was not further studied. Therefore, the effects of the addition of more MR and AA solutions to the eluted PMB, in NaOH solutions, were studied. It was found for 1.5 mL NaOH eluent, adding 0.06 mL MR and 0.03 mL AA could return the PMB to the theoretical calculated value in less than 1 min. The absorbance of this recovered PMB solution was stable for at least 30 min, which was long enough for the manual operation. The detailed data of net absorbances of 300 nM samples measured at different times by adding different volumes of reagent can be found in Supporting Information (Fig. S3). The spectrums of PMB (Fig. 1) in different matrixes illustrate the high efficiency of this recovery procedure, and 700 nm was therefore selected as the detection wavelength. It should be noted that the absorbances were calibrated by volume changes following reagent additions.

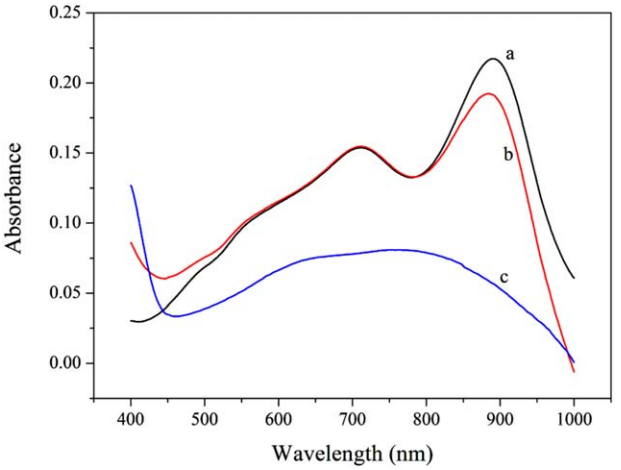


Fig. 1. Spectrum of 10 μM PMB (a) in pure water and in 0.15 M NaOH solution with (b) and without (c) the addition of reagents.

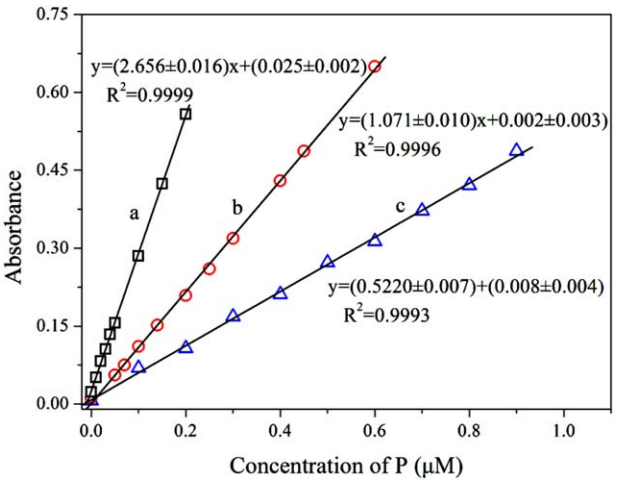


Fig. 2. Calibration curves obtained using different sample volume (a. 250 mL, b. 100 mL, c. 50 mL)

Method performance: linearity, detection limit, and reproducibility

Under the procedure described above with different sample volumes, three calibration curves ranging from 10 nM to 900 nM were obtained and shown in Fig. 2. These curves were obtained at different days with good linearity ($R^2 > 0.9993$). The determination range can be easily broadened by decreasing the sample loading volumes, depending on the phosphate concentrations of the seawater samples. A photo of five HLB cartridges with varying phosphate concentrations is provided in Supporting Information (Fig. S4).

The detection limit for 50-mL sample was 3.0 nM, which was estimated as three times the standard deviation of the measured blanks ($n = 12$). The detection limit could be further lowered with more loading sample, but the analysis time will be longer.

The average absorbance and standard deviation for repetitive determinations of 50 mL of 300 nM phosphate solutions was 0.159 ± 0.004 (RSD = 2.6%, $n = 81$). This experiment was conducted by three individuals over several days. It should be noted that two of them had no experience on trace nutrient analysis and only a 30-min training session was conducted before obtaining the data shown in Fig. 3. The highly repetitive results illustrate the potentially wide applicability of this method and the stability of the SPE cartridge, as the entire experiment was conducted using the same cartridge. Results obtained using different cartridges from the same manufacturer's batch also showed good reproducibility, as indicated by the slopes of interday calibration curves (Fig. 3).

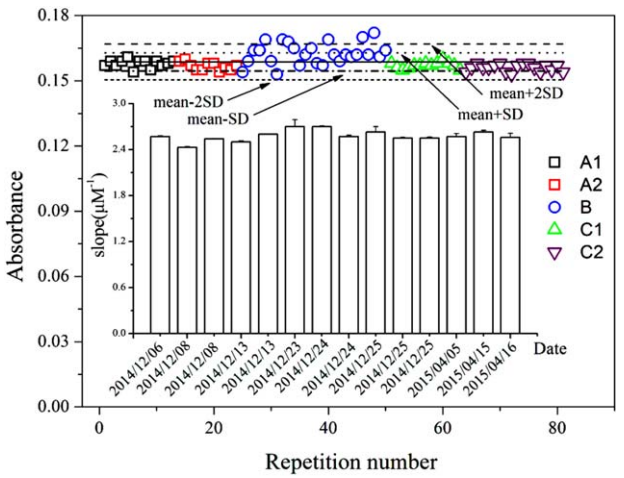


Fig. 3. The reproducibility of determination and the slopes of calibration curves in different days. The letters (A, B, C) present different individuals, and the corner marks (1, 2) mean different days.

Salinity effect

To study the effects of salinity, samples (200 nM) and blanks were measured in different salinity matrices (0, 7, 14, 21, 28, and 35). The samples of different salinity were prepared by diluting artificial seawater with Milli-Q water. No significant differences were observed in the samples at different salinities (data shown in Supporting Information Fig. S5). Therefore, this method can be applied to seawater samples of different salinities without any further calibration. More importantly, pure water can be used for preparing calibration curves and quality control samples without preparing phosphate-free seawater as reported by other by other authors (e.g., Li et al. 2008).

The interference of silicate and arsenate

Since silicate and arsenate are two of the main species interfering with the determination of DRP using the PMB method (Patey et al. 2010), the effects of silicate and arsenate on DRP determination using this method were studied, with samples of 0 nM, 50 nM, and 100 nM phosphate spiked with different concentration of silicate and arsenate. As shown in Fig. 4, the absorbances using this method showed no significant differences with silicate concentrations from 0 μM to 200 μM. There was no obvious variation with arsenate concentrations from 0 nM to 100 nM, but the arsenate inference did become serious at concentrations higher than 100 nM. As arsenate concentrations in the open ocean are very low, mostly between 10 nM and 50 nM (Johnson 1971; Karl and Tien 1997), for most applications, the influence from arsenate could be ignored. In areas with arsenate pollution, the addition of reducing reagents to reduce arsenate to arsenite was recommended (Johnson 1971).

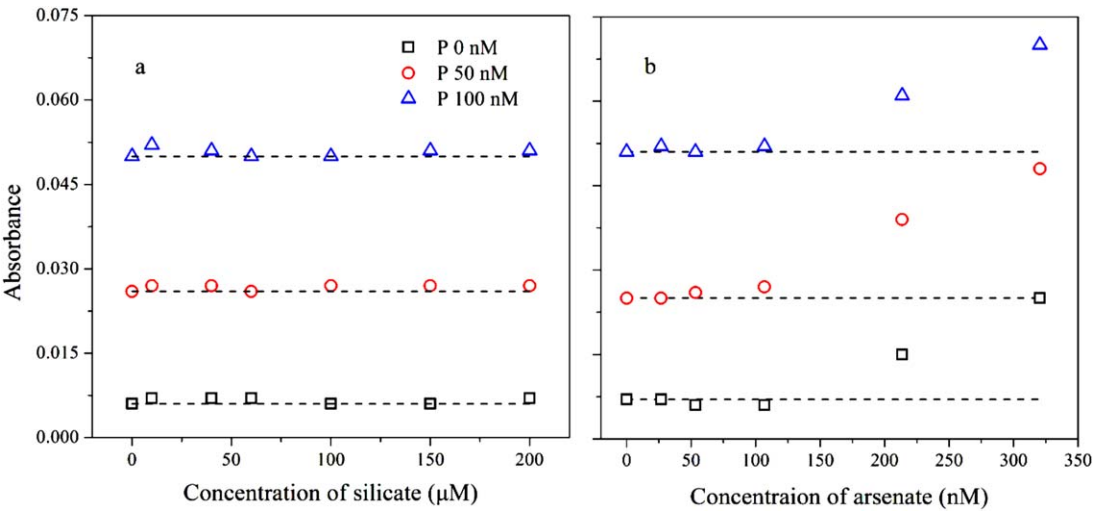
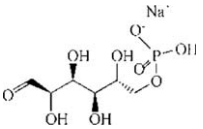
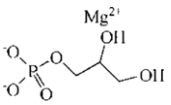
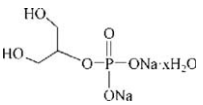
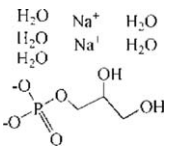
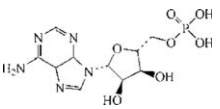
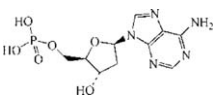
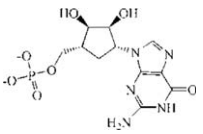
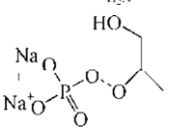
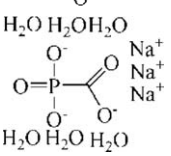
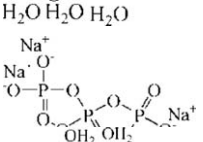
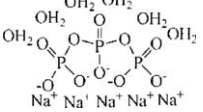
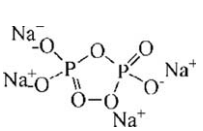


Fig. 4. The influence of silicate (left) and arsenate (right) on 0 nM, 50 nM and 100 nM phosphate solution.

Table 1. Information and hydrolysis rates for a series of phosphorus compounds.

#	CAS	Name	Manufactures	Purity (%)	Molecular formula	Molecular structural formula	Percentage degradation (%)
1	66778-08-3	Phenyl phosphate, disodium salt dihydrate	Acros	98	$C_6H_5Na_2O_4P \cdot 2H_2O$		16.4 ± 0.3
2	54010-71-8	D(+)-Glucose 6-phosphate sodium salt,	Acros	98	$C_6H_{12}NaO_9P$		18.3 ± 0.3
3	927-20-8	DL-α-Glycerol phosphate magnesium salt hydrate	Sigma	85	$C_3H_7MgO_6P \cdot xH_2O$		64.6 ± 1.3
4	55073-41-1	Glycerol phosphate disodium salt hydrate	Sigma	99	$C_3H_7Na_2O_6P \cdot xH_2O$		57.9 ± 0.8
5	154804-51-0	β-Glycerophosphate disodium salt hydrate	Sigma	99	$(HOCH_2)_2CHOP(O)(ONa)_2 \cdot xH_2O$		ND*
6	61-19-8	Adenosine-5′-monophosphoric acid	Alfa	99	$C_{10}H_{14}N_5O_7P$		ND*
7	653-63-4	2′-Deoxyadenosine-5′-monophosphate	Alfa	98	$C_{10}H_{14}N_5O_6P$		41.0 ± 1.0
8	5550-12-9	Guanosine 5′-monophosphate disodium salt hydrate	Acros	97	$C_{10}H_{12}N_5Na_2O_8P \cdot xH_2O$		70.2 ± 2.2
9	13408-09-8	β-glycerol- phosphate pentahydrate	Acros	98	$C_3H_7Na_2O_6P \cdot 5H_2O$		ND*
10	34156-56-4	Sodium Phosphonoformate hexahydrate	Adamas	98	$Na_2O_3PCO_2Na \cdot 6H2O$		ND*
11	7758-29-4	Sodium tripolyphosphate	Adamas	95	$Na_5P_3O_{10}$		ND*
12	231-838-7	Tripolyphosphate pentasodium salt hexahydrate	Aldrich	98	$Na_5P_3O_{10} \cdot 6H_2O$		ND*
13	7722-88-5	Sodium pyrophosphate	Alfa	98	$Na_4O_7P_2$		34.5 ± 1.3

*ND, not detected

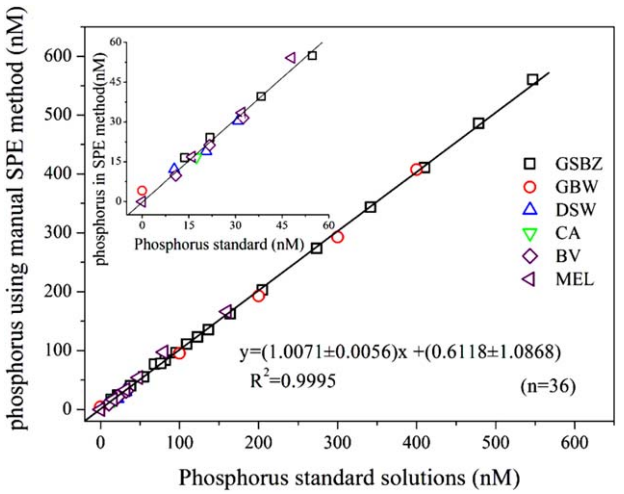


Fig. 5. Determination of different kinds of phosphate standard solutions. GSBZ: phosphate standards from Institute for Environmental Reference Materials; GBW: phosphate standards solution from Second Institute of Oceanography; DSW: reference in seawater collected in the North Pacific Ocean at 1400 m; CA and BV: phosphate standards in seawater Lot. CA and BV from the General Environmental Technos; MEL: phosphate standards prepared from the State Key Laboratory of Marine Environmental Science.

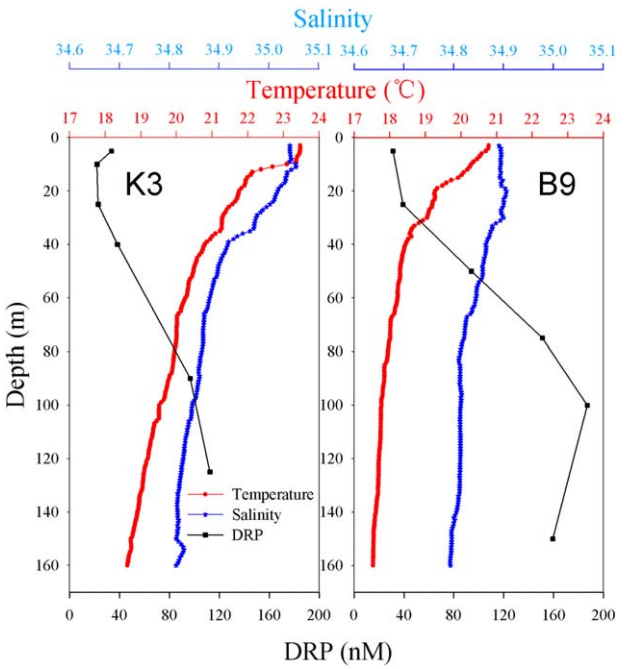


Fig. 6. Vertical profiles of temperature (red circle), salinity (blue star) and DRP concentration (black square) in two stations (K3 136° 21′ E, 26° 7′ N, B9 146° 33′ E, 29° 32′ N) in the Western Pacific.

Interferences from P-containing compounds

There are various forms of phosphorus in the open oceans, and the determination of DRP would be affected by the hydrolysis of these compounds. Therefore, the interference experiment was conducted with similar idea of Li and Hansell (2008). A set of P-containing compounds (0–1000 nM) were prepared and determined under the same protocol for DRP measurement. Increase in measured DRP could be assumed due to the hydrolysis of these P-containing compounds. The percentage degradation was calculated as the ratios of calibration curves slopes of the P-containing compound and phosphate using this method. As shown in Table 1, different P-containing compounds exhibited varying hydrolysis percentage, such as β-glycerophosphate disodium salt hydrate, Adenosine-5′-monophosphoric acid, β-glycerolphosphate pentahydrate, sodium phosphonoformate hexahydrate, sodium tripolyphosphate, and tripolyphosphate pentasodium salt hexahydrate having nearly undetectable degradation, while others exhibited varying detectable percentage degradation, ranging from $16.4\% \pm 0.3\%$ to $70.2\% \pm 2.2\%$. The percentage degradation should be related to the physicochemical property and structure of these compounds, but more model compounds are needed for further study.

Validation of the method

The use of certified materials is the best way to evaluate the accuracy of an established method, but due to limitations related to sample storage and preparation, to the best of the authors' knowledge, no certified nanomolar phosphate solution is currently available (Ma et al. 2014a). Therefore, different types of phosphate standard/certified solutions at the μM level (GSBZ500028-94 Batch No.203417, GBW08623, deep seawater, Lot. CA and Lot. BV) were diluted and measured using this method. This evaluation work was valid based on two assumptions: (1) the method is not affected by matrix differences, as the dilution will change the sample matrix and (2) the dilution procedure introduces insignificant error. As shown before (salinity effect section) and below (recovery section), this method can be used for samples of varying salinities and matrices, which meets the first assumption. For the second assumption, an experienced senior technician from a certified nutrient analysis laboratory (State Key Laboratory of Marine Environmental Science, Xiamen University) prepared a series of diluted "blind" phosphate standard solutions for further analysis using this method. As shown in Fig. 5, the detected values and the certified values were very close for these different standard solutions, illustrating the high accuracy of this method.

Recovery

Bottled water with various matrices and seawater samples collected from the Western Pacific were analyzed before and after the spike with standard solutions. The recoveries for seawater ($n = 3$) were between 96.6% and 99.9%, the recoveries for bottled water ($n = 4$) were between 94.5% and 102.6%. Excellent recoveries were observed for the various

sample types, indicating that this method has little matrix effect (more data can be found in Supporting Information Table S1).

Application

This manual operational method was applied using 42 seawater samples collected from nine stations in the Western Pacific in April, 2015. The vertical profiles of DRP, temperature and salinity for two stations are shown in Fig. 6. The DRP concentrations ranged from 21.9 nM to 348.0 nM in the study region. The DRP concentrations in the upper layer were very low due to active biological uptake.

Comments and recommendations

As the purpose of this study is to establish a simple, practical and universal nanomolar phosphate analysis procedure for all laboratories, only the very basic equipment (e.g., spectrophotometer) was utilized. Therefore, any analytical laboratories can utilize this method within a limited budget. The only consumable in this experiment is the HLB cartridge, which costs only several dollars and can be used more than 100 times, based on our experience, although the label calls for "single use." The necessity to recondition the cartridges was also evaluated and it was found that a single reconditioning with an organic solvent (e.g., ethanol) in the beginning of the experiment was sufficient to maintain the performance of the method, which greatly simplified the analytical procedures, compared with previous research (Liang et al. 2007, Ma et al. 2008a,b).

Although this method is sensitive, accurate, easy to operate and budget friendly, the flow-based method is still the mainstay for nanomolar nutrient analysis, especially for standard nutrient analysis laboratories dealing with a large number of samples (Ma et al. 2014a). This method can be further improved with minor revisions, if necessary. The sensitivity of the method can be easily increased using micro-volume cuvettes with longer path lengths, but a better and more expensive spectrophotometer with a suitable beam to focus on the special cuvette may be needed. The cost of this procedure can be further reduced by using syringes instead of pumps for loading samples on cartridges and/or using a DIY photometer instead of a commercial model (Ma et al. 2012; Yang et al. 2014). As well, more DOP compounds should be studied to evaluate the relationship between the hydrolysis percentage and compound structure.

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