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Sensitive determination of total particulate phosphorus and particulate inorganic phosphorus in seawater using liquid waveguide spectrophotometry

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Abstract

Determining the total particulate phosphorus (TPP) and particulate inorganic phosphorus (PIP) in oligotrophic oceanic water generally requires the filtration of a large amount of water sample. This paper describes methods that require small filtration volumes for determining the TPP and PIP concentrations. The methods were devised by validating or improving conventional sample processing and by applying highly sensitive liquid waveguide spectrophotometry to the measurements of oxidized or acid-extracted phosphate from TPP and PIP, respectively. The oxidation of TPP was performed by a chemical wet oxidation method using 3% potassium persulfate. The acid extraction of PIP was initially carried out based on the conventional extraction methodology, which requires 1 M HCl, followed by the procedure for decreasing acidity. While the conventional procedure for acid removal requires a ten-fold dilution of the 1 M HCl extract with purified water, the improved procedure proposed in this study uses 8 M NaOH solution for neutralizing 1 M HCl extract in order to reduce the dilution effect. An experiment for comparing the absorbances of the phosphate standard dissolved in 0.1 M HCl and of that dissolved in a neutralized solution [1 M HCl : 8 M NaOH = 8:1 (v:v)] exhibited a higher absorbance in the neutralized solution. This indicated that the improved procedure completely removed the acid effect, which reduces the sensitivity of the phosphate measurement. Application to an ultraoligotrophic water sample showed that the TPP

concentration in a 1075 mL-filtered sample was 8.4 nM with a coefficient of variation (CV) of 4.3% and the PIP concentration in a 2300 mL-filtered sample was 1.3 nM with a CV of 6.1%. Based on the detection limit (3 nM) of the sensitive phosphate measurement and the ambient TPP and PIP concentrations of the ultraoligotrophic water, the minimum filtration volumes required for the detection of TPP and PIP were estimated to be 15 and 52 mL, respectively.

Keywords

Total particulate phosphorus; Particulate inorganic phosphorus; Liquid waveguide spectrophotometry

1. Introduction

Phosphorus (P) is an essential element for all life forms. P is a constituent of genetic materials (DNA and RNA) and cellular compounds (phosphoproteins and phospholipids), and it is essential for energy transmission in living cells (in the form of ATP). P in natural water exists in both particulate and dissolved forms. These fractions can be defined operationally by filtration through 0.2–0.7 μm filters [1, 2]. Total particulate P (TPP) retained on the filter consists of particulate inorganic P (PIP) and particulate organic P (POP). PIP exists in mineral phases, as P adsorbed onto particles [3] and as intracellular storage products [4] such as orthophosphate, pyrophosphate and polyphosphate [5]. In contrast, POP comprises P incorporated in organic molecules of biochemical origin, and it is generally defined as the difference between the TPP and PIP concentrations [6, 7]. Because inorganic and organic forms of both particulate and dissolved P transform each other through biological activity [2, 8], understanding the size and the dynamics of each pool is necessary to characterize their role in the P cycle.

Oligotrophic oceans occupy nearly 60% of the global ocean [9]. The oligotrophic regions are characterized by low chlorophyll *a* (Chl *a*) concentrations ($\leqq$0.1 μg L^{-1}) [10] as well as low TPP concentrations (<30 nM) [5, 11–13]. Despite these low concentrations of particulate matter prevail, the integrated dynamics of particulate P over oligotrophic regions are likely to

58 have a significant impact on global oceanic P cycling because of the vastness of the oligotrophic
59 habitats. However, few studies exist on particulate P dynamics in oligotrophic regions (e.g. [5,
60 11–15]), as opposed to the large number of recent studies on dissolved P dynamics (e.g. [16–
61 19]). Furthermore, information on the POP and PIP fractions is particularly limited among the
62 particulate P studies [5, 14]. This is mainly due to the large amount of water sample required
63 for filtration (1–12 L) [5, 11–15], which hampers the accumulation of data on particulate P
64 pools.
65 The chemical methods for TPP measurements are based on the oxidative and acid
66 hydrolytic liberation of organically bound inorganic P and the subsequent determination of
67 phosphate with the phosphomolybdenum blue method [20, 21]. TPP digestion has been
68 carried out by various methods, including chemical wet oxidation (CWO) [22] and
69 high-temperature dry combustion (HTDC) [7]. Although the CWO method is simpler and less
70 time consuming than the HTDC method, it was reported that P recovery was generally lower in
71 the CWO method than in the HTDC method [23, 24]. Suzumura [24] improved the CWO
72 method by using 3% potassium persulfate ($K_2S_2O_8$). The P recovery in this method is the same
73 as that in the HTDC method when measuring the samples from oceanic and riverine suspended
74 particulate matters, plankton, and marine sediments with exception of clay minerals. Although
75 high contents of clay minerals in samples potentially decrease the P recovery in the improved

76 CWO method, mineral supplies from landmass to oceanic water are generally very small and
77 the decrease in the P recovery due to minerals is likely unobservable in oceanic water [24].
78 The analytical protocol of Aspila et al. [6] has been used for the determination of PIP in
79 seawater [5, 14, 25]. In this protocol, phosphate is extracted from particulate P by acid
80 treatment with 1 M HCl, and its concentration is determined by the phosphomolybdenum blue
81 method. While the acid treatment successfully extracts most of the PIP compounds in seawater,
82 it is not so effective with the decomposition of many POP compounds [25]. In the original
83 protocol of Aspila et al. [6], the 1 M HCl extract is diluted ten-fold with purified water before
84 phosphate determination, because the development of color through the phosphomolybdenum
85 blue reaction is inhibited in the highly acidic conditions [6, 26, 27]. However, in the
86 oligotrophic regions where PIP concentrations are frequently below 5 nM [5, 14], considerable
87 amounts of seawater are needed for filtration, in order to compensate for the dilution.
88 A liquid waveguide capillary cell (LWCC) has been recently used for the automated
89 analysis of phosphate in natural water [19, 28–31]. With the use of a long-pathlength flow cell,
90 ranging from 1–2.5 m, the LWCC system performed the measurement of nanomolar
91 concentration of phosphate with a low detection limit (DL) ranging from 0.5–3 nM. The
92 application of the LWCC system to the determination of trace particulate P could decrease the

93 filtration volume. However, to the best of our knowledge, the LWCC system has never been
94 utilized for the determination of particulate P.
95 In this study, the LWCC system was applied in order to measure the concentration of TPP
96 and PIP. Sample processing for TPP was based on the method of Suzumura [24]. For the PIP
97 procedure, the sample processing method of Aspila et al. [6] was modified by using 8 M NaOH
98 instead of purified water for decreasing acidity, in order to minimize the dilution effect.
99 Contamination of trace P in the filter and the reagents of sample processing was carefully
100 monitored, because the highly sensitive LWCC system can potentially detect such a
101 contamination. The established methods were applied to TPP and PIP determination in
102 ultraoligotrophic seawater.
103
104 **2. Experimental**
105 All reagents used in this study were of analytical reagent grade obtained from Wako Pure
106 Chemical Industries (Osaka, Japan) and Sigma Aldrich (St Louis, MO, USA). The purified
107 water for preparing the reagents and diluting the samples was obtained with the use of a reverse
108 osmosis and deionization system (Millipore Auto Pure WEX3 and WR600A, Yamato, Tokyo,
109 Japan). All instruments were washed using Merck Extran MA03 detergent (Merck Ltd, Tokyo,
110 Japan) and then rinsed with 0.3 M HCl and purified water prior to use.

111

112 *2.1. Spectrophotometric measurement of nanomolar phosphate*

113 The analysis for phosphate concentration was based on a LWCC method devised by
114 Hashihama et al. [19, 32]. A gas-segmented continuous flow analytical system (AutoAnalyzer
115 II, Technicon, now Seal Analytical, Hampshire, UK) was used for an automated analysis of
116 phosphate. A schematic diagram of this system was previously shown in Fig. 1 of Hashihama
117 et al. [32]. Spectrophotometric analysis was performed by using a tungsten fiber optic light
118 source (L7893, Hamamatsu Photonics, Shizuoka, Japan), a 1 m long path LWCC (LWCC-2100;
119 World Precision Instruments, Sarasota, FL, USA), and a miniature fiber optic spectrometer
120 (USB4000, Ocean Optics, Dunedin, FL, USA). The spectrometer was connected to a
121 computer, and an absorbance at 708 was operated using Spectra Suite software (Ocean Optics,
122 Dunedin, FL, USA). The analytical reagents (molybdate and ascorbic acid solutions) were
123 prepared by using the methodology of Hansen and Koroleff [21], with the exception of the
124 ascorbic acid solution [32]. Acetone and 15% sodium dodecyl sulfate solution were added to
125 the ascorbic acid solution to eliminate baseline drift [32, 33]. Potassium dihydrogen phosphate
126 was used to prepare standard solutions. The DL of this method was 3 nM [32].

127

128 *2.2. TPP protocol*

129 A pre-combusted, acid-washed glass fiber filter (Whatman GF/F, 2.5 cm in diameter, Kent,
130 UK) was used to collect particulate P. Filtration was carried out with the use of an aspirator
131 (A-3S, TOKYO RIKAKIKAI, Tokyo, Japan) under vacuum at <0.02 MPa. Just after filtration,
132 the filter was rinsed with ~5 mL of 0.17 M Na_2SO_4 to remove any dissolved P that was absorbed
133 onto it. Then, the filter was dried and placed into a digestion glass bottle (GL32, Duran,
134 Wertheim/Main, Germany). The TPP on the filter was digested with 20 mL of 3% $K_2S_2O_8$ at
135 120°C for 30 minutes using an autoclave (KTS-2322, ALP, Tokyo, Japan) [24]. The bottle was
136 shaken before and after autoclaving. The residue in the digested solution was removed using a
137 0.45 μm syringe filter (Millex-HV, Millipore, Massachusetts, USA). Because >2% $K_2S_2O_8$
138 inhibits color development in the sample after autoclaving [24], the digested solutions were
139 diluted to 1.5% $K_2S_2O_8$ with purified water. Phosphate concentration in the diluted solution
140 was determined by the LWCC method.
141 The absorbances of procedural blank (GF/F filter + 3% $K_2S_2O_8$ + purified water) and
142 reagent blank (3% $K_2S_2O_8$ + purified water) were compared to check P contamination of GF/F
143 filter. In this case, the absorbance of purified water (+colorimetric reagent) was set to zero.
144 The procedural blank was prepared by filtering 1L of purified water and it was processed
145 following the outlined digestion procedure.
146 The absorbance of standard solutions (20, 50, 100, 200, 500 and 1000 nM) was measured

147 in order to draw a calibration curve. Each standard that was dissolved in 1.5% $K_2S_2O_8$ was
148 prepared by mixing phosphate standards dissolved in purified water (40, 100, 200, 400, 1000
149 and 2000 nM) with 3% autoclaved $K_2S_2O_8$ [1:1 (v:v)].
150 The reproducibility of TPP determination was obtained by analyzing field samples.
151 Sampling was conducted at a station (30º00'S, 120º00'W), which is found within the
152 ultraoligotrophic eastern South Pacific, on January 11 2011 during the KH-11-10 cruise of R/V
153 *Hakuho-maru*. This area has one of the lowest oceanic Chl *a* concentrations in the world [34].
154 During the cruise, low surface concentrations of Chl *a* at the station were confirmed (0.021 μg
155 L^{-1}). Given the Chl *a* concentrations, extremely low TPP concentrations were expected.
156 Seawater samples for TPP were collected at surface layer using an acid-clean bucket. The
157 samples were poured into five polycarbonate bottles (Thermo Scientific Nalgene, Rochester, NY,
158 USA). Each sample with a volume of 1075 mL was filtered. The filters were stored at –20ºC
159 until ashore analysis.
160
161 *2.3. PIP protocol*
162 Particulate P was collected on the GF/F filter through the same sampling procedure as that
163 carried out for the obtainment of TPP samples. The filter was placed in a 30 mL
164 polypropylene tube and 20 mL of 1 M HCl was added. The tube was placed in the dark on a

165 shaker bath (EP-1; TAITEC, Saitama, Japan) for 24 h at 20ºC. The residue that was found in
166 the extract was removed using the Millex-HV 0.45 μm syringe filter. To neutralize the extract,
167 2.5 mL of 8 M NaOH were added [1 M HCl : 8 M NaOH = 8:1 (v:v)]. Phosphate
168 concentration of the neutralized solution was measured by the LWCC method.
169 The absorbances of the procedural blank (GF/F filter + 1 M HCL + 8 M NaOH) and the
170 reagent blank (1 M HCL + 8 M NaOH) were compared to check P contamination on the filter.
171 In this case, the absorbance of purified water (+colorimetric reagent) was set to zero. The
172 procedural blank was prepared by filtering 1L of purified water and it was processed through the
173 outlined extraction procedure.
174 The absorbances of standard solutions (20, 50, 100, 200, 500 and 1000 nM) were measured
175 to draw a calibration curve. Each standard was prepared by dissolving phosphate standards in
176 a mixed solution of 1 M HCl and 8 M NaOH [8:1 (v:v)]. To confirm the difference between
177 absorbances of phosphate in the conventional and improved protocols, the absorbances of the
178 phosphate standards (20, 50, 100, 200, 500 and 1000 nM), which were dissolved in 0.1 M HCl
179 (prepared by diluting 1 M HCl by 10% with purified water, i.e. the conventional protocol of
180 Aspila et al. [6]), were also measured.
181 In order to compare the ambient PIP concentrations as determined through the conventional
182 and improved protocols, the two protocols were applied to the water samples collected around a

183 station (34°36'N, 139°06'E) from the Sagami Bay on May 30, 2013 during the SE-13-05 cruise
184 of RT/V *Seiyo-maru*. Five samples were collected at the surface at different times using an
185 acid-clean bucket, and then filtered. The filtration volume of each sample was 1230 mL. The
186 filter was extracted with 1 M HCl and the extract was dispensed into duplicate tubes, one for the
187 conventional protocol (ten-fold dilution with purified water) and another for the improved
188 protocol (neutralization with 8 M NaOH). After the ten-fold dilution and neutralization, the
189 two types of solutions were analyzed by the LWCC method.
190 The reproducibility of PIP determination through the improved protocol was obtained by
191 analyzing field samples, which were collected at the same station as the TPP samples. Sample
192 collection and filtration were done in the same way as for the TPP samples, apart from the
193 filtration volume, which was 2300 mL (n = 4). The filters were stored at –20°C until ashore
194 analysis.
195
196 **3. Results and discussion**
197 *3.1. TPP determination*
198 *3.1.1. Filter blank*
199 The mean ± standard deviation (SD) of the absorbances of the procedural and reagent
200 blanks were 0.009 ± 0.001 and 0.009 ± 0.003, respectively (n = 3) (Table 1). The mean
201 absorbances between two blanks were not significantly different (t test, $p > 0.05$), indicating that

202 P contamination in the GF/F filter was negligible. This result was consistent with the results of
203 Suzumura [24], Labry et al. [25], and Raimbault et al. [35], who reported that P contamination
204 in the GF/F filter was substantially low. Furthermore, this study confirmed that there was no
205 significant contamination even for nanomolar phosphate determination. The absorbance of
206 reagent blank was higher than that of purified water. Labry et al. [25] reported significant P
207 contamination of $K_2S_2O_8$ in their CWO method. P contamination of $K_2S_2O_8$ used in the present
208 study was probably responsible for the higher absorbance. As a result, it was necessary to
209 include the absorbance derived from the $K_2S_2O_8$ in the analytical blank.
210
211 *3.1.2. Calibration curve*
212 A calibration curve was obtained from the absorbance of each duplicate standard dissolved
213 in 1.5% $K_2S_2O_8$ (Fig. 1). The regression equation obtained is y = 0.0010x – 0.0089, with r^2 =
214 0.9997 (n = 14), where y is the absorbance and x is the concentration of phosphate. The wide
215 linear dynamic range could be applicable to various oceanic samples. For example, if a 100
216 mL filtration volume is used, then 3–1000 nM phosphate corresponds to 1.2–400 nM of ambient
217 TPP, according to the following equation:
218 $C_a = C_p \times V_r \times DR / V_f$ (1)
219 where C_a is the ambient TPP concentration (1.2–400 nM), C_p is the phosphate concentration (3–

220 1000 nM), V_r is the reagent volume (20 mL), DR is the dilution ratio (2) and V_f is the filtration

221 volume (100 mL).

222

223 *3.1.3. Concentration and reproducibility of the field sample*

224 TPP concentrations of the field samples were 8.4 ± 0.36 nM (mean ± SD, n = 5) (Table 2).

225 Because of the low coefficient of variation (CV) (4.3%), this method provides high-precision

226 measurements even for ultraoligotrophic water. Moutin et al. [12] investigated surface TPP

227 concentrations in the eastern South Pacific (26º05'S, 114º00'W) and reported concentrations of

228 5–10 nM, which is consistent with the results of this study. Given the DL of the LWCC

229 method (3 nM) and the low concentrations of ambient TPP (8.4 nM), the minimum filtration

230 volume required is estimated to be 15 mL, according to the following equation:

231 $V_f = \mathrm{DL} \times V_r \times \mathrm{DR} / C_a$ (2)

232 The filtration volume estimated was 67–800 times lower than that of previous studies (1–12 L)

233 [5, 11–13, 15].

234

235 *3.2. PIP determination*

236 *3.2.1. Filter blank*

237 Mean ± SD of the absorbances of procedural and reagent blanks were –0.016 ± 0.002 and –

238 0.018 ± 0.002, respectively (n = 3) (Table 1). The mean absorbance between the two blanks

239 was not significantly different (*t* test, $p > 0.05$), as was the case for the filter blank test for TPP.
240 This indicates that P contamination of the GF/F filter was also negligible in the case of PIP
241 determination. The absorbances of both procedural and reagent blanks were lower than that of
242 purified water. This is probably due to the difference in refractive index between ionic
243 solutions (1 M HCl + 8 M NaOH) and purified water [28]. Therefore, it is necessary to use the
244 neutralized solution as an analytical blank.
245
246 *3.2.2. Calibration curve*
247 A calibration curve was obtained from the absorbances of each duplicate standard dissolved
248 in the neutralized solution (Fig. 2). The regression equation obtained is y = 0.0011x – 0.0034,
249 with $r^2 = 1.0000$ ($n = 7$), where y is the absorbance and x is the concentration of phosphate. The
250 strong correlation of the linear regression line indicates a wide linear dynamic range of up to
251 1000 nM phosphate, which is able to measure the PIP concentrations in various oceanic waters.
252 For example, if 100 mL of the filtration volume is assumed, 3–1000 nM phosphate corresponds
253 to 0.68–225 nM PIP according to equation 1 (C_a: 0.68–225 nM, C_p: 3–1000 nM, V_r: 20 mL, DR:
254 9/8, and V_f: 100 mL).
255
256 *3.2.3. Absorbance comparison with the conventional protocol*

257 A calibration curve for the conventional protocol was also obtained from the absorbances
258 of each pair of phosphate standards that were dissolved in 0.1 M HCl (Fig. 2). The curve
259 showed a strong linear correlation up to 1000 nM (r^2 = 0.9998), which was the same as that by
260 the improved protocol. However, the absorbances of the standards in the conventional
261 protocol were significantly lower than those of the improved protocol (paired *t* test, $p < 0.05$, n
262 = 7). Aspila et al. [6] used the ten-fold dilution of 1M HCl with purified water to remove the
263 effect of acidity on phosphate analysis. However, the incomplete removal of acid could be the
264 reason behind the lower absorbances in the conventional protocol [26, 27]. In addition to 8.9
265 times higher sensitivity in the improved protocol than the conventional protocol by decreasing
266 dilution ratio from 10 to 9/8, sensitivity of the improved protocol further increased by 2.3%
267 compared to that of the conventional protocol when taking into account a slope ratio of two
268 regression lines (0.001069/0.001045).
269
270 *3.2.4. Comparison with the conventional protocol using natural samples*
271 The PIP concentrations of the natural samples derived from the conventional and improved
272 protocols are shown in Fig.3. These concentrations were not significantly different from each
273 other (paired *t* test, $p > 0.05$, $n = 5$). The result confirmed that the use of NaOH had no
274 influence on PIP determination for the natural samples.

275

276 *3.2.5. Concentration and reproducibility of the field sample*

277 PIP concentrations of the field samples were 1.3 ± 0.08 nM (mean ± SD, n = 4) (Table 2).
278 Because of the low CV (6.1%), this method provides high-precision measurements even for
279 ultraoligotrophic water. Yoshimura et al. [5] reported that typical proportions of PIP to TPP in
280 subtropical and subarctic regions range between 10 and 20%. In this study, the proportion of
281 PIP to TPP was 15%, which is within the typical range, and the concentration of POP (which is
282 obtained by subtracting PIP from TPP) was estimated to be 7.1 nM. Taking into account the
283 DL of the LWCC method (3 nM), the low PIP concentration (C_a = 1.3 nM), the reagent volume
284 (V_r = 20 mL), and the dilution ratio (DR = 9/8), the minimum filtration volume required (V_f) is
285 estimated to be 52 mL according to equation 2. The estimated filtration volume is 38 times
286 lower than that used in the previous PIP studies in the oligotrophic ocean (2 L) [5].

287

288 **4. Conclusions**

289 The present study established sensitive methods for the determination of TPP and PIP in
290 the oligotrophic oceans. The proposed methods possess two distinct advantages over the
291 conventional methods. Firstly, significant decreases in filtration volumes for TPP and PIP
292 were performed through the application of the LWCC method. Secondly, the improved PIP

293 protocol was more sensitive than the conventional protocol in terms of the decrease in the
294 dilution ratio of 1 M HCl extract and the increase in the absorbance of the colorimetric
295 determination of phosphate. This also contributes to the decrease in the filtration volume.
296 The small filtration volumes enable rapid sample accumulation in the field. Field observations
297 revealed that the methods could detect very low concentrations of TPP and PIP with high
298 precisions even in ultraoligotrophic water. The methods are considered to be valuable in
299 understanding the role of particulate P in the oceanic P cycle.
300

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360 Table 1

361 Absorbances of procedural and reagent blanks in the determinations of TPP and PIP (improved

362 protocol).

363

Type of blank	Absorbance ± SD (n = 3)
TPP procedural blank (GF/F filter + $K_2S_2O_8$ + pure water)	0.009 ±0.001
TPP reagent blank ($K_2S_2O_8$ + pure water)	0.009 ±0.003
PIP procedural blank (GF/F filter + HCl + NaOH)	–0.016 ±0.002
PIP reagent blank (HCl + NaOH)	–0.018 ±0.002

364

365

366

367

Table 2

TPP and PIP concentrations in the ultraoligotrophic eastern South Pacific, and the minimum filtration volume calculated from the DL of the LWCC (3 nM), and ambient particulate P concentrations.

P pool	Mean concentration ± SD (nM)	CV (%)	Minimum filtration volume (mL)
TPP	8.4 ± 0.36 (n = 5)	4.3	15
PIP	1.3 ± 0.08 (n = 4)	6.1	52

373 Figure captions

374 Figure 1. Calibration curve ranging from 0 to 1000 nM phosphate dissolved in 1.5% $K_2S_2O_8$.

375 Concentrations of the assumed TPP indicate the estimated values if filtration volume was 100

376 mL.

377 Figure 2. Calibration curve ranging from 0 to 1000 nM phosphate dissolved in the neutralized

378 solution (open circle) and 0.1 M HCl (closed circle). The assumed concentrations of PIP

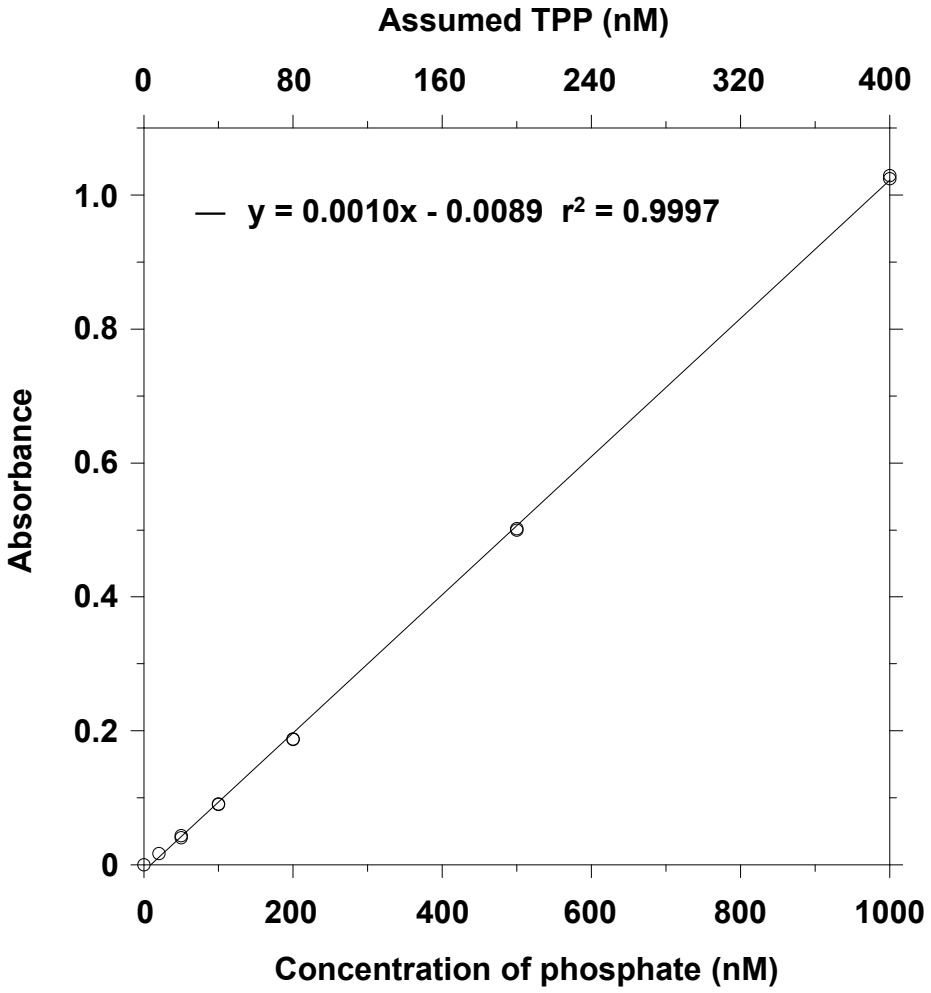
379 indicate the ambient PIP concentrations if the filtration volume was 100 mL in the improved

380 protocol.

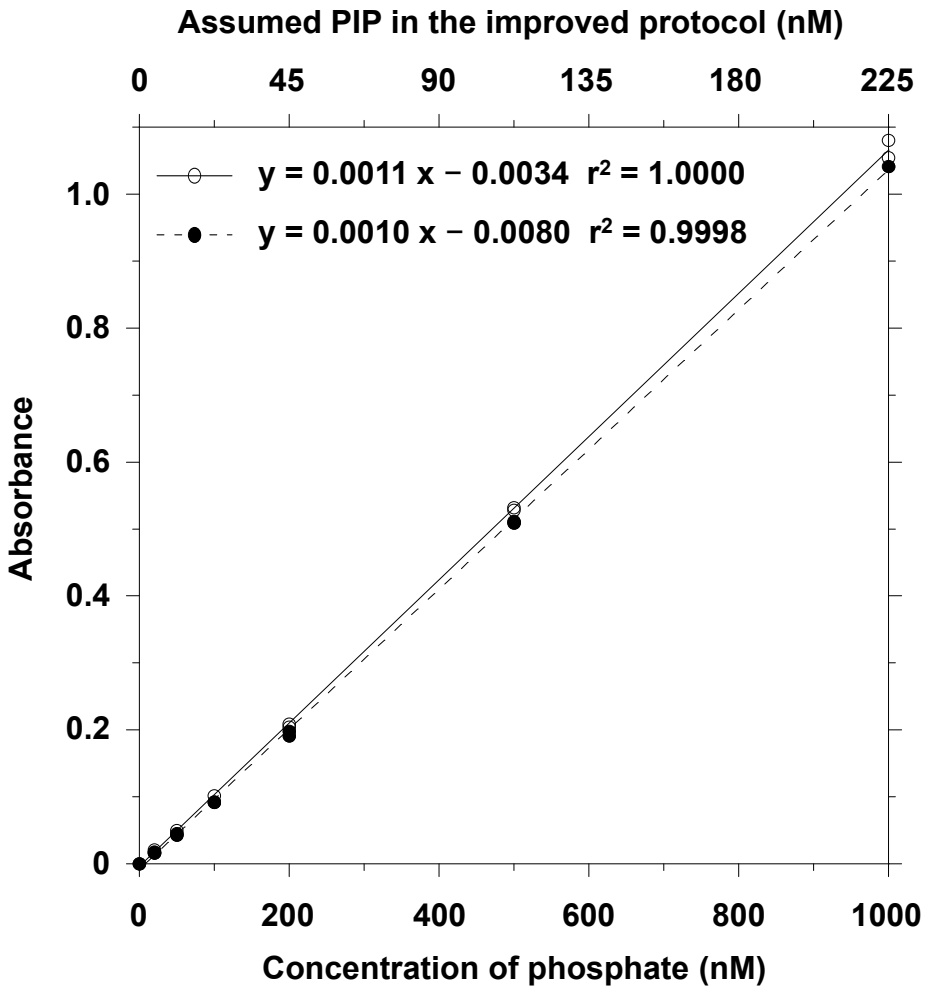
381 Figure 3. PIP concentrations of the natural samples (Sagami Bay) derived from the improved

382 and the original protocols (nM).

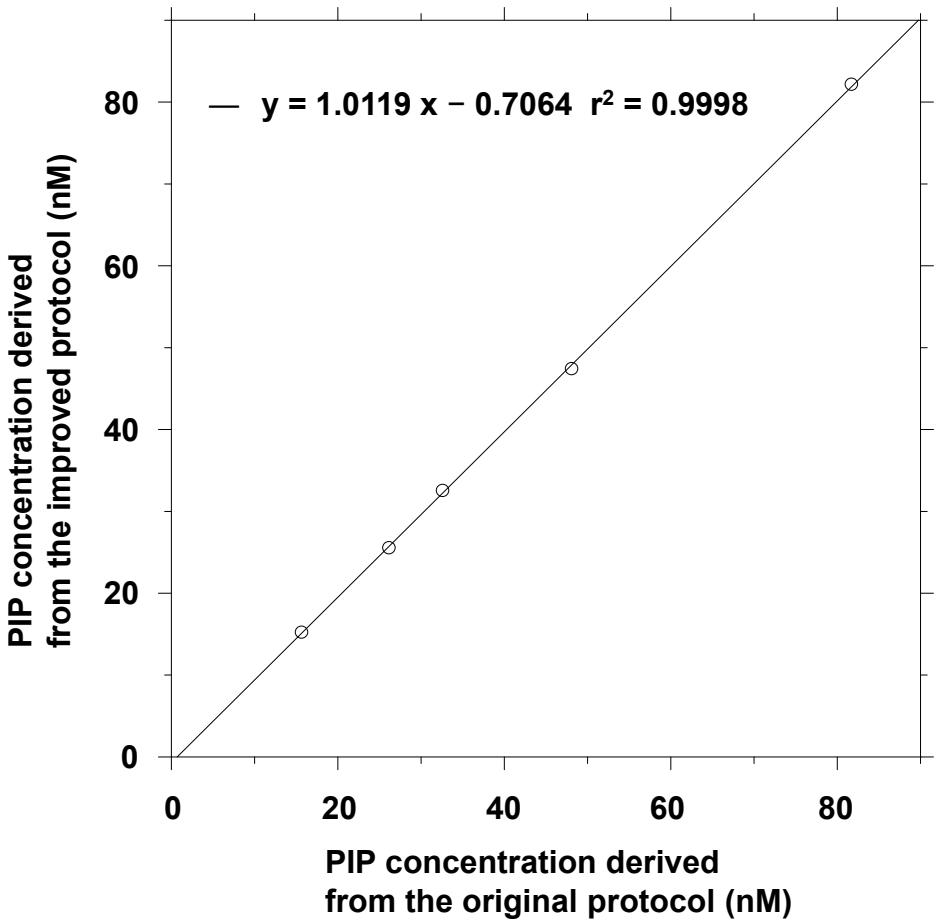
383



Ehama et al. Fig. 1



Ehama et al. Fig. 2



Ehama et al. Fig. 3