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

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1	Sensitive determination of total particulate phosphorus and particulate inorganic phosphorus in
2	seawater using liquid waveguide spectrophotometry
3	
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### 12 Abstract

Determining the total particulate phosphorus (TPP) and particulate inorganic phosphorus 1314(PIP) in oligotrophic oceanic water generally requires the filtration of a large amount of water 15sample. This paper describes methods that require small filtration volumes for determining the 16 TPP and PIP concentrations. The methods were devised by validating or improving 17conventional sample processing and by applying highly sensitive liquid waveguide spectrophotometry to the measurements of oxidized or acid-extracted phosphate from TPP and 1819PIP, respectively. The oxidation of TPP was performed by a chemical wet oxidation method 20using 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 2122for decreasing acidity. While the conventional procedure for acid removal requires a ten-fold 23dilution of the 1 M HCl extract with purified water, the improved procedure proposed in this 24study uses 8 M NaOH solution for neutralizing 1 M HCl extract in order to reduce the dilution 25effect. An experiment for comparing the absorbances of the phosphate standard dissolved in 260.1 M HCl and of that dissolved in a neutralized solution [1 M HCl : 8 M NaOH = 8:1 (v:v)]27exhibited a higher absorbance in the neutralized solution. This indicated that the improved 28procedure completely removed the acid effect, which reduces the sensitivity of the phosphate 29measurement. Application to an ultraoligotrophic water sample showed that the TPP

30	concentration in a 1075 mL-filtered sample was 8.4 nM with a coefficient of variation (CV) of
31	4.3% and the PIP concentration in a 2300 mL-filtered sample was 1.3 nM with a CV of 6.1%.
32	Based on the detection limit (3 nM) of the sensitive phosphate measurement and the ambient
33	TPP and PIP concentrations of the ultraoligotrophic water, the minimum filtration volumes
34	required for the detection of TPP and PIP were estimated to be 15 and 52 mL, respectively.
35	
36	Keywords
37	Total particulate phosphorus; Particulate inorganic phosphorus; Liquid waveguide
38	spectrophotometry

### 40 **1. Introduction**

Phosphorus (P) is an essential element for all life forms. P is a constituent of genetic 4142materials (DNA and RNA) and cellular compounds (phosphoproteins and phospholipids), and it 43is 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 44filtration through 0.2–0.7 µm filters [1, 2]. Total particulate P (TPP) retained on the filter 45consists of particulate inorganic P (PIP) and particulate organic P (POP). PIP exists in mineral 46phases, as P adsorbed onto particles [3] and as intracellular storage products [4] such as 47orthophosphate, pyrophosphate and polyphosphate [5]. In contrast, POP comprises P 48incorporated in organic molecules of biochemical origin, and it is generally defined as the 49difference between the TPP and PIP concentrations [6, 7]. Because inorganic and organic 5051forms of both particulate and dissolved P transform each other through biological activity [2, 8], 52understanding the size and the dynamics of each pool is necessary to characterize their role in the P cycle. 5354Oligotrophic oceans occupy nearly 60% of the global ocean [9]. The oligotrophic regions are characterized by low chlorophyll a (Chl a) concentrations ( $\leq 0.1 \ \mu g \ L^{-1}$ ) [10] as well as low 5556TPP concentrations (<30 nM) [5, 11–13]. Despite these low concentrations of particulate 57matter 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.

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# 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	

111

# 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 $\sim$ 5 mL of 0.17 M Na <sub>2</sub> SO <sub>4</sub> 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 $\mu$ m syringe filter (Millex-HV, Millipore, Massachusetts, USA). Because >2% K <sub>2</sub> S <sub>2</sub> O <sub>8</sub>
138	inhibits color development in the sample after autoclaving [24], the digested solutions were
139	diluted to $1.5\%$ K <sub>2</sub> S <sub>2</sub> O <sub>8</sub> 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 $+ 3\% K_2 S_2 O_8 +$ 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

in order to draw a calibration curve. Each standard that was dissolved in 1.5% K<sub>2</sub>S<sub>2</sub>O<sub>8</sub> was 147prepared by mixing phosphate standards dissolved in purified water (40, 100, 200, 400, 1000 148and 2000 nM) with 3% autoclaved K<sub>2</sub>S<sub>2</sub>O<sub>8</sub> [1:1 (v:v)]. 149150The reproducibility of TPP determination was obtained by analyzing field samples. Sampling was conducted at a station (30°00'S, 120°00'W), which is found within the 151152ultraoligotrophic eastern South Pacific, on January 11 2011 during the KH-11-10 cruise of R/V 153Hakuho-maru. This area has one of the lowest oceanic Chl a concentrations in the world [34]. During the cruise, low surface concentrations of Chl a at the station were confirmed (0.021  $\mu$ g 154155 $L^{-1}$ ). Given the Chl *a* concentrations, extremely low TPP concentrations were expected. Seawater samples for TPP were collected at surface layer using an acid-clean bucket. The 156samples were poured into five polycarbonate bottles (Thermo Scientific Nalgene, Rochester, NY, 157158USA). Each sample with a volume of 1075 mL was filtered. The filters were stored at -20°C 159until ashore analysis. 160

161 2.3. PIP protocol

Particulate P was collected on the GF/F filter through the same sampling procedure as that
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 20°C. The residue that was found in
166	the extract was removed using the Millex-HV 0.45 $\mu m$ 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 $-20^{\circ}$ 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  $\pm$  standard deviation (SD) of the absorbances of the procedural and reagent

200 blanks were  $0.009 \pm 0.001$  and  $0.009 \pm 0.003$ , respectively (n = 3) (Table 1). The mean

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 <sub>2</sub> S <sub>2</sub> O <sub>8</sub> (Fig. 1). The regression equation obtained is $y = 0.0010x - 0.0089$ , with $r^2 =$

- $214 \quad 0.9997 \ (n = 14)$ , 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–

1000 nM), V<sub>r</sub> is the reagent volume (20 mL), DR is the dilution ratio (2) and V<sub>f</sub> is the filtration
volume (100 mL).

- 222
- 223 *3.1.3.* Concentration and reproducibility of the field sample
- TPP concentrations of the field samples were  $8.4 \pm 0.36$  nM (mean  $\pm$  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 (26°05'S, 114°00'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
- volume required is estimated to be 15 mL, according to the following equation:

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

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  $\pm$  SD of the absorbances of procedural and reagent blanks were  $-0.016 \pm 0.002$  and -

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

239	was not significantly different ( <i>t</i> 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 <sub>r</sub> : 20 mL, DR:
254	9/8, and V <sub>f</sub> : 100 mL).
255	

#### 3.2.3. Absorbance comparison with the conventional protocol 256

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.

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

277	PIP concentrations of the field samples were $1.3 \pm 0.08$ nM (mean $\pm$ 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 <sub>r</sub> = 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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309	

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360 Table 1

361 Absorbances of procedural and reagent blanks in the determinations of TPP and PIP (improved362 protocol).

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

368	Table	2

TPP and PIP concentrations in the ultraoligotrophic eastern South Pacific, and the minimum 369 filtration volume calculated from the DL of the LWCC (3 nM), and ambient particulate P 370371concentrations. Mean concentration  $\pm$  SD (nM) CV (%) P pool Minimum filtration volume (mL) TPP  $8.4 \pm 0.36$  (*n* = 5) 4.3 15 PIP  $1.3 \pm 0.08$  (*n* = 4) 6.1 52

## 373 Figure captions

- Figure 1. Calibration curve ranging from 0 to 1000 nM phosphate dissolved in 1.5% K<sub>2</sub>S<sub>2</sub>O<sub>8</sub>.
- 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
- and the original protocols (nM).





Ehama et al. Fig. 2



Ehama et al. Fig. 3