

2016-04-28

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<http://hdl.handle.net/10026.1/4476>

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10.1016/j.aca.2016.02.047

Anal Chim Acta

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2 published version for accuracy and citation. Published in **Analytica Chimica Acta**, 918, 8-20  
3 (2016) doi: 10.1016/j.aca.2016.02.047.  
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5 Determination of phosphorus in natural waters: A historical review

6

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14

15 Abstract

16 The aim of this paper is to introduce a virtual special issue that reviews the development of  
17 analytical approaches to the determination of phosphorus species in natural waters. The focus  
18 is on sampling and sample treatment, analytical methods and quality assurance of the data. The  
19 export of phosphorus from anthropogenic activities (from diffuse and point sources) can result in  
20 increased primary production and eutrophication, and potentially the seasonal development of  
21 toxic algal blooms, which can significantly impact on water quality. Therefore the quantification  
22 of phosphorus species in natural waters provides important baseline data for studying aquatic  
23 phosphorus biogeochemistry, assessing ecosystem health and monitoring compliance with  
24 legislation.

25

26 Keywords

27 Phosphorus, natural waters, water quality, sampling, sample treatment, analytical methods

28

## 1. Phosphorus biogeochemistry

Phosphorus (P) is an essential nutrient element that is used by all living organisms for growth and energy transport [1] and is often the limiting nutrient for primary production in terrestrial and aquatic ecosystems [2-4]. The terrestrial environment is a major P reservoir, with  $8.4 \times 10^8$  –  $40 \times 10^8$  Tg in sediments, 96,000 – 200,000 Tg in soils (<60 cm deep) and 2,600 – 3,000 Tg in terrestrial biota [5]. In the aquatic environment the major reservoirs are the surface (0 – 300 m) ocean (2,700 Tg), the deep (300 – 3,000 m) ocean (87,000 Tg) and oceanic biota (50 – 140 Tg). The atmospheric environment is a relatively small reservoir (0.03 Tg) [5] but can be an important source for oligotrophic ecosystems [6]. The major P fluxes are between marine biota and ocean water, between soil biota and soil, from soil to the surface ocean and erosion / weathering of rocks. A schematic diagram of the aquatic phosphorus cycle, showing the major reservoirs and fluxes, is shown in Fig. 1.

The intensification of agriculture has resulted in a global demand for P of about  $22 \text{ Tg y}^{-1}$  from mined fossil phosphate resources [7], with minable reserves estimated at 10,000 – 20,000 Tg [5]. Current agricultural practices give rise to significant impacts on water quality due to P losses to water bodies, e.g. from agricultural run-off [8], e.g. elevated levels of P can lead to eutrophication [3, 9], harmful algal blooms, oxygen depletion and mortality of biota. Population growth and increasing industrialisation are also drivers of elevated P inputs to natural waters [10] from both diffuse and point (e.g. sewage treatment works) sources [11]. This has led to “cultural eutrophication”, which is the accelerated anthropogenic enrichment of the environment with nutrients and the concomitant production of undesirable effects [12].

Dissolved inorganic P (DIP), in the form of orthophosphate, is easily utilized by primary producers and is therefore the major bioavailable form of P, but some dissolved organic P (DOP) species can also be utilized [13, 14]. The fractionation and speciation of phosphorus are

56 therefore important factors when considering the impact of the element on water quality. In  
57 natural waters phosphorus can be found in various “dissolved” forms (operationally defined as  
58 the fraction that passes through a 0.2 or 0.45  $\mu\text{m}$  filter [15]), mostly as inorganic  
59 orthophosphates and condensed or polyphosphates, but also as organic phosphates (e.g.  
60 nucleic acids, proteins, phospholipids, phosphoamides, sugar phosphates, inositol phosphates,  
61 aminophosphonates and organic phosphorus pesticides). “Particulate” P (defined as the fraction  
62 retained on a 0.2 or 0.45  $\mu\text{m}$  filter [15]) can include clay and silt-associated organic and  
63 inorganic P, precipitates of authigenic origin and P-containing biological matter. Colloidal  
64 phosphorus is commonly referred to as the P fraction in the 1 nm – 1  $\mu\text{m}$  size range [16-18] and  
65 hence both the operationally defined dissolved and particulate fractions can contain colloidal P.  
66 This fraction includes both organic and inorganic species of biological and/or mineral origin. The  
67 various operationally defined P fractions in natural waters, based on filtration and/or digestion,  
68 are shown in Fig. 2, together with examples of the types of phosphorus species found in these  
69 fractions.

70

71 The ultimate analytical challenge is therefore to develop reliable analytical methods that are  
72 sufficiently sensitive and accurate to determine the concentrations of individual phosphorus  
73 species in each of these fractions. Given the spatially and temporally dynamic nature of P  
74 transport, both frequent and widespread measurements are also desirable. For reliable  
75 measurements robust sampling strategies are also required [19]. In the longer term remote  
76 monitoring networks may overcome the need for sampling [20] but *in situ* sample treatment  
77 remains a challenge.

78

79 For water quality management purposes it is also informative to determine the loads [21, 22]  
80 and fluxes [23] of P species in water bodies in order to investigate, e.g. internal cycling  
81 processes [24], the restoration of eutrophic ecosystems [25] and the impacts of P runoff from  
82 land on the ecological status of receiving waters [26]. Long-term datasets ( $\geq 20$  years), which

83 require routine monitoring, are useful for identifying non-hydrological variations in P  
84 concentrations and distributions, to contextualise contemporary datasets [27-29] and help to  
85 validate catchment models [30].

86

87 The potentially adverse impact of elevated phosphorus concentrations in natural waters has led  
88 to the inclusion of phosphorus standards in various national and international legislative  
89 frameworks and guidelines. In Europe the Water Framework Directive (WFD - 2000/60/EC) [31]  
90 covers river basins, estuaries and coastal margins and the Marine Strategy Framework  
91 Directive (MSFD, 2008/56/EC) covers marine waters [32]. In the UK the specific legislation  
92 relating to phosphorus is discussed in reports by the UK Technical Advisory Group on the Water  
93 Framework Directive (UKTAG) [33, 34]. Revised standards proposed in the 2013 report [34]  
94 build on *“improvements in understanding of the relationship between phosphorus  
95 concentrations and the response of river plant communities”* and were derived from *“a new  
96 approach to setting phosphorus standards that produces site-specific estimates of natural  
97 phosphorus concentrations, taking account of a site’s alkalinity and altitude”*. The key driver for  
98 emerging legislative P concentrations for defining river water quality (high, good, moderate,  
99 poor or bad) is therefore a closer linkage with local biological responses. Reliable  
100 measurements of phosphorus species in natural waters, as discussed above, will be a  
101 prerequisite for further refinement of legislative guidelines for P.

102

## 103 2. Sampling and sample treatment

104

### 105 2.1 Sample collection

106

107 Prior to sample collection it is important to ensure that the sampling strategy is fit for purpose  
108 [19]. This includes the identification of appropriate sampling locations (with due regard to  
109 access and safety issues), the frequency of sample collection and the P species to be

110 determined. This requires a clear statement of the objectives of the sampling programme and  
111 an understanding of P biogeochemistry and the stability of the P determinands [35]. The figures  
112 of merit of the intended analytical methods should also be specified, e.g. detection limit, linear  
113 range, selectivity, accuracy and precision, together with any constraints on time and cost, in  
114 order to select the most appropriate sample collection and detection strategies [36]. Samples  
115 can be collected manually (discrete grab samples or integrated cross-sectional samples) or by  
116 deploying an automatic sampler for time series acquisition [37]. In the latter case, appropriate  
117 quality assurance is necessary to allow for the fact that different samples will be stored  
118 unfiltered for different lengths of time [38]. Replicate sampling at each location/time is strongly  
119 recommended and in situations where only one sample can be collected, at least three sub-  
120 samples should be analysed for robust quantification [39].

121  
122 The sampling strategy should take account of temporal and spatial variability in P  
123 concentrations and ensure that collected samples are representative of the water body being  
124 sampled. Spatial variability is influenced by point and diffuse inputs, in-water processes (e.g.  
125 plant, algal and bacterial turnover), mixing zones, thermal stratification with depth in lakes or  
126 salinity stratification in estuaries. Temporal variability is influenced by seasonal (e.g. summer  
127 base flow compared with higher autumn and winter flows) and short-term (e.g. rain events)  
128 changes in river flow [40] and physico-chemical gradients (e.g. temperature and salinity) [41].

129  
130 Clean sample containers and sample collection apparatus (including filters) are an essential  
131 prerequisite for minimising contamination and most cleaning methods involve acid washing, e.g.  
132 soaking items overnight in a nutrient free detergent, rinsing with ultra-pure water, followed by  
133 soaking overnight in 10% HCl, and finally rinsing with ultra-pure water [42].

134 Polytetrafluoroethylene (PTFE) or high density polyethylene (HDPE) are the preferred materials  
135 for containers but quartz, borosilicate glass, low density polyethylene and polypropylene have

136 also been used. The analytical requirements of the detection method will dictate the minimum  
137 sample volume. Use of containers with a larger size [43] and lower surface area to volume ratio  
138 [44] should minimise adsorptive losses. Cleaned sample containers should be rinsed three  
139 times with the sample prior to filling. When collecting samples it is good practice to utilise  
140 sample blanks to monitor the sampling process.

## 142 2.2 Sample pretreatment and storage

143  
144 After the collection of representative water samples it is essential that they are effectively  
145 treated and stored in order to maintain sample integrity. Filtration is the most common form of  
146 sample pretreatment to separate the dissolved and particulate phases, as defined above. This  
147 step should ideally be carried out at the time of sample collection to prevent changes in P  
148 fractionation and speciation. Polycarbonate (e.g. Nuclepore<sup>®</sup>), cellulose acetate or cellulose  
149 acetate/nitrate 47 mm diameter membrane filters are commonly used but larger diameter and  
150 capsule filters have also been reported [45]. A 0.45  $\mu\text{m}$  filter provides faster filtration than a 0.2  
151  $\mu\text{m}$  filter but the latter has the advantage of removing the majority of bacteria, picoplankton and  
152 colloidal species that could impact on dissolved P concentrations during storage [35, 46, 47].  
153 Filtration can be carried out under vacuum or positive pressure but an excessive pressure  
154 gradient across the membrane can rupture algal cells, releasing intracellular contents into the  
155 sample, and/or disaggregate colloidal material. Highly turbid samples can lead to rapid clogging  
156 of the filter, particularly with smaller nominal pore size filters.

157  
158 The characteristics of selected storage/preservation methods for the determination of  
159 phosphorus are summarised elsewhere [42, 44]. A variety of physical (e.g. refrigeration,  
160 freezing (-4°C), deep-freezing (-20°C)) and chemical (e.g. addition of chloroform, mercuric  
161 chloride) preservation techniques have been used to maintain the original phosphorus

162 concentration and speciation during storage but there is not a generic treatment protocol that is  
163 ideal for all environmental situations. Factors such as phosphorus concentration, water  
164 hardness, salinity, dissolved organic matter, particulate matter and biological conditions need to  
165 be considered. For example, in lowland chalk catchments freezing samples can lead to  
166 phosphate being co-precipitated with calcite when samples are thawed [42, 48, 49] and for such  
167 samples storage at 4 °C, with chloroform addition to prevent biological growth, was  
168 recommended [42].

### 170 2.3 Sample digestion

171  
172 For the determination of total phosphorus (TP; unfiltered samples) and total dissolved  
173 phosphorus (TDP; filtered samples) it is necessary to convert all of the phosphorus-containing  
174 species into a detectable form. For natural waters the most common method of detection is the  
175 “molybdenum blue” chemistry with spectrophotometric detection which determines molybdate-  
176 reactive orthophosphate ( $\text{PO}_4^{3-}$ ) using either a batch or flow-based approach (see §3.1) [50].  
177 Inductively coupled plasma based methods (ICP-AES and ICP-MS) can also be used if the  
178 concentration of P is sufficiently high. The process of conversion, which involves the breaking of  
179 P-O-P, C-O-P and C-P bonds in condensed and organic P compounds is called *digestion* and is  
180 typically achieved by thermal oxidation with hydrolysis using either an autoclave, digestion block  
181 or microwave [51] or by UV photo-oxidation with or without heating.

182  
183 Autoclaving is generally simple and quick, gives reproducible results and is carried out in sealed  
184 vessels to minimise contamination [44, 47]. Peroxydisulfate is the preferred oxidant and was  
185 first used for the determination of TP in seawater in the 1960s [52]. Since then a variety of  
186 peroxydisulfate methods, in either acidic [53] or basic [54] media, have been reported [47].  
187 Autoclaving with alkaline peroxydisulfate, rather than acidic peroxydisulfate, is recommended

188 for the simultaneous determination of TP and total nitrogen (TN) and for the digestion of marine  
189 waters due to the oxidation of chloride to free chlorine by peroxydisulfate in acidic media, which  
190 reduces its oxidising power [55]. When alkaline peroxydisulfate digestion is used, autoclaving or  
191 thermal heating should be continued until  $S_2O_8^{2-}$  is converted to hydrogen sulphate ( $HSO_4^-$ ;  $pK_a$   
192 = 1.99) so that a low pH is reached in the latter stages of the digestion and acid hydrolysis of  
193 condensed phosphate species is achieved [51]. An acidic peroxydisulfate method was reported  
194 by Gales *et al.* [56] and simplified by Eisenreich *et al.* [57]. The method gives good recoveries  
195 and is simple and easy to use and is therefore recommended for the determination of TP and  
196 TDP in fresh waters [58].

197  
198 UV photo-oxidation can be used for the digestion of marine and freshwaters [41, 59] but if the  
199 sample contains condensed polyphosphates, heating with HCl or peroxydisulfate after UV  
200 irradiation is recommended [60]. UV photo-oxidation also gives good recoveries when  
201 incorporated into a flow injection (FI) manifold [61, 62]. Microwave digestion has also been used  
202 in flow systems in conjunction with spectrophotometric detection [51, 63] and ICP-MS detection  
203 [64].

204  
205 The above methods provide a quantitative measurement of TP or TDP but if information on  
206 specific classes of P compounds is required, a more selective method of sample treatment is  
207 necessary, e.g. the use of phosphate cleaving enzymes such as acid and alkaline  
208 phosphatases, and this is discussed in § 3.3.

### 210 3. Analytical methods

#### 212 3.1 Dissolved reactive phosphorus

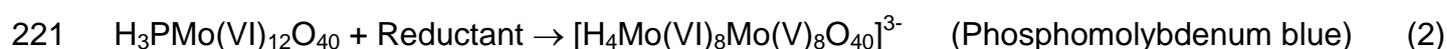
213

214 Most methods for phosphorus determination are based on the spectrophotometric detection of  
215 the intensely coloured phosphomolybdenum blue (PMB). This complex is formed by reaction of  
216 phosphate with acidified molybdate producing 12-molybdophosphoric acid (12-MPA),  
217 which is subsequently reduced to phosphomolybdenum blue [65], i.e.

218



220



222

223 “Molybdate reactive” phosphorus (MRP) in the dissolved fraction is variously described as  
224 *dissolved reactive phosphorus* (DRP), *soluble reactive phosphorus* (SRP) and *filterable reactive*  
225 *phosphorus* (FRP) in recognition that this fraction may also include some acid labile, molybdate-  
226 reactive organic and condensed and colloid-associated phosphorus species. This can lead to  
227 overestimation of free phosphate [65]. While some have suggested that ion chromatography  
228 gives a better estimate of free orthophosphate [66] than DRP, Hens and Merx used gel filtration  
229 to demonstrate that in 0.45 µm filtered soil solution, both MRP and ion chromatographic P  
230 measurements overestimated the free orthophosphate concentration by up to 2.3- and 1.4-fold,  
231 respectively [67]. Despite this, DRP is still the most widely used surrogate measure of readily  
232 bioavailable P because it is practically convenient to measure.

233

234 A variety of reductants (e.g. ascorbic acid, tin(II) chloride, hydrazine sulfate, hydroquinone) and  
235 acids have been used in this reaction [50], giving rise to a range of PMB species with different  
236 absorbance spectra. Appropriate acid and molybdate concentrations are critical for the  
237 formation of PMB; for example, in low acidity conditions, non-linear colour development and  
238 self-reduction of the molybdate can occur [68]. The chemistry of phosphomolybdenum blue  
239 formation has been comprehensively reviewed by Nagul *et al.* [50].

240

241 The most cited and widely used method is that reported by Murphy and Riley [69] in 1962 which  
242 utilised ascorbic acid in the presence of Sb(III) as the reductant. When Sb(III) is present, the  
243 rate of formation of molybdenum blue is enhanced compared with ascorbic acid alone (although  
244 Sb does not act as a catalyst, as is often claimed), and Sb is incorporated into the complex as  
245  $[\text{PSb}_2\text{Mo}_{12}\text{O}_{40}]^-$  which has a  $\lambda_{\text{max}}$  at 880 nm. The optimum conditions for the formation of the  
246 molybdenum blue complex was further investigated by Going and Eisenreich [70]. This reaction  
247 is less sensitive to salt than others, and colour development is fairly independent of temperature  
248 [71], making it the preferred reaction for the determination of phosphorus in natural waters.

249

250 Tin(II) chloride has also been used as a reductant, especially for the determination of phosphate  
251 in freshwaters, because the reaction is rapid and the absorptivity is greater than that for  
252 ascorbic acid/Sb(III). In the reduction process, Sn(IV) is substituted for Mo(VI) in the PMB  
253 complex, to form  $\alpha\text{-}[\text{Pmo}_{10}\text{Sn}_2\text{O}_{37}]^{5-}$ , shifting  $\lambda_{\text{max}}$  to 700 nm and enhancing the absorptivity. At  
254 this wavelength, absorbance can be measured using simple solid state detectors that utilise red  
255 light emitting diodes as the light source [72, 73], and this approach has been widely adopted for  
256 flow analysis systems intended for field application [74]. However, chloride inhibits the reduction  
257 process by complexing with Sn(IV), and a decrease in sensitivity of ca. 15% occurs in a  
258 seawater matrix compared with that of deionized water [75]. Despite this limitation, reduction  
259 with tin(II) chloride has been favoured for automatic flow analysis of non-saline waters because  
260 of the faster reaction kinetics and higher sensitivity.

261

262 In addition to the homogeneous reduction methods described above, an on-line UV photo-  
263 reduction method has recently been reported [76]. In this system molybdophosphate was  
264 reduced to phosphomolybdenum blue in the presence of a hydrogen bond donor (ethanol),  
265 using a simple Teflon<sup>®</sup> tube UV reactor in a FI system. Because the PMB produced by UV  
266 photo-reduction is transient, the reaction is best performed under the highly controllable and

267 reproducible conditions achieved using a flow system. Unstable chemical reducing reagents  
268 such as ascorbic acid or tin(II) chloride are not required, making this approach more suitable for  
269 longer term field based measurements.

270

271 Species that can interfere with the formation of the PMB complex include silicate, arsenate,  
272 nitrite, nitrate, sulphide, chromium and copper [50, 77]. Silicate interference can be minimised  
273 by careful adjustment of the acid and molybdate concentrations [71], and by the inclusion of  
274 tartrate in the reagent mixture [78]. Interferences from arsenate can be eliminated by reduction  
275 of As(V) to As(III) by the addition of sodium thiosulfate [71, 79].

276

277 Flow analysis techniques can be advantageous in treating or avoiding interferences in the  
278 determination of DRP. For example, Grace *et al.* described a FI method for DRP measurement  
279 in anoxic estuarine sediment pore waters, using on-line pre-oxidation of sulphide with  
280 permanganate prior to spectrophotometric detection [80], in which the selectivity was not  
281 compromised by the oxidation of organic P species commonly found in this matrix. Additionally,  
282 the use of FI enabled the analysis to be performed without exposure of samples to the  
283 atmosphere, thus avoiding changes in the redox condition.

284

285 An early (1982) application of FI to DRP measurement in marine systems involved the use of  
286 reagent injection or “reverse” FI analysis [81]. In this approach, a defined volume of a combined  
287 colorimetric reagent, containing ascorbic acid and antimonyl tartrate as the reductant, was  
288 injected into a flowing stream of sample with heating to 50 °C. This approach resulted in  
289 arguably higher sensitivity, more economical reagent use and a better suitability for on-line or  
290 under way monitoring applications than conventional FI. A similar multiple reagent injection  
291 approach (multi-commutation) was described by Lyddy-Meaney *et al.* [74] for DRP  
292 determination in a portable FI system, using tin(II) chloride reduction at ambient temperature.  
293 This system included on-line 0.2 µm tangential-flow filtration and a specially designed multi-

294 reflection optical flow cell that minimised Schlieren effects caused by variations in the refractive  
295 index of estuarine and marine waters [82]. The system was used for chemical mapping and  
296 spatial resolution of one measurement per 250 m was achieved at an injection rate  $225 \text{ h}^{-1}$  and  
297 a boat speed of 30 knots (Fig. 3).

298

299 Non-spectrophotometric detection methods involving the formation of molybdophosphoric acid  
300 have also been investigated for the determination of phosphate, e.g. amperometric detection of  
301 phosphate as molybdophosphate [83-86]. Determination of phosphate based on the  
302 fluorescence quenching of the molybdate ion association complex with fluorophores such as  
303 rhodamine B, rhodamine 6G or thiamine by phosphate is reportedly more sensitive than  
304 spectrophotometric methods based on phosphomolybdenum blue [87, 88]. For example, Frank  
305 *et al.* [89] demonstrated a sequential injection analysis system for DRP determination using the  
306 fluorescence quenching of rhodamine 6G-molybdate to achieve a LOD of  $0.05 \mu\text{M}$  at a high  
307 sample throughput of  $270 \text{ h}^{-1}$ . This system was used effectively for surface water transect  
308 measurements in the North Sea, Wadden Sea and Elbe estuary. More recently, Kröckel *et al.*  
309 reported a reverse FI method using the same chemistry that achieved a LOD of  $7 \text{ nM}$  for  
310 phosphate measurements in seawater [90]. A FI method with chemiluminescence detection  
311 based on luminol oxidation by 12-MPA has also been used to determine phosphate in  
312 freshwaters [91].

313

314 The LOD of batch spectrophotometric phosphomolybdenum blue methods is typically in the low  
315  $\mu\text{g P L}^{-1}$  range, e.g. the APHA standard methods publication quotes a LOD of about  $3 \mu\text{g P L}^{-1}$   
316 with tin(II) chloride reduction ( $0.1 \mu\text{M}$ ) and  $10 \mu\text{g P L}^{-1}$  with ascorbic acid reduction ( $0.3 \mu\text{M}$ ) [92].  
317 These LODs are inadequate for the determination of DRP in low nutrient (oligotrophic) waters. A  
318 number of approaches have therefore been described to enhance the sensitivity of  
319 spectrophotometric PMB methods, *viz*, by modification of the chromophore detected, e.g. by ion  
320 pairing of molybdophosphate with a dye, preconcentration by extraction (both liquid and solid

321 phase), ion exchange or coprecipitation, or the use of spectrophotometric cells with an extended  
322 optical path length.

323

324 As an alternative to the direct measurement of PMB, some authors have used the formation of  
325 ion association complexes between basic dyes and molybdophosphoric or vanadate-  
326 molybdophosphoric acid as the basis for DRP determination, with sensitivity arguably better  
327 than the PMB method [93]. However, a surfactant must be used to avoid precipitation of the ion-  
328 association complex, and this can be a major source of blank contamination [79].

329

330 Solvent extraction can be performed to enhance sensitivity, either by extracting  
331 molybdophosphate followed by reduction, e.g. with tin(II) chloride [92], or by extracting after the  
332 formation of PMB as described by Strickland and Parsons [94]. Solvents used include iso-  
333 propanol, n/iso-butanol, n/iso-butanol + benzene, n-hexanol and butyl acetate [95]. Detection  
334 limits at the sub- $\mu\text{g L}^{-1}$  level were achieved for river water measurements by Motomizu and  
335 Oshima, who performed solvent extraction on the ion-association pair formed between  
336 molybdophosphoric acid and Malachite Green, without reduction [96].

337

338 Flow-based systems readily facilitate the automation of solid phase extraction methods,  
339 minimizing the use of solvent. For example, Liang *et al.* have reported an example of a solid  
340 phase extraction FI preconcentration technique that is suitable for detection of DRP in marine  
341 waters. This involved solid phase extraction of the phosphomolybdenum blue – cetyl  
342 trimethylammonium bromide ion pair on a  $\text{C}_{18}$  column followed by elution with ethanol and  
343 sulfuric acid before spectrophotometric detection, giving a detection limit of 1.6 nM but with a  
344 sample throughput of only  $2 \text{ h}^{-1}$  [95]. In a related approach, Nagul *et al.* used polymer inclusion  
345 membranes (PIMs) in combination with a FI manifold to perform on-line extraction of phosphate  
346 directly from low ionic strength fresh waters before stripping and detection as PMB. A detection  
347 limit of  $0.04 \mu\text{g P L}^{-1}$  at a sampling rate of  $5 \text{ h}^{-1}$  was reported [97].

348

349 Ion exchange techniques in concert with flow analysis have also been described for  
350 preconcentration of phosphate in freshwaters. Freeman *et al.* [78] used a strong anion  
351 exchange resin (AG 1 X-8) in an FI manifold to preconcentrate as much as 3.2 mL of sample.  
352 Phosphate and silicate peaks were resolved when the column was eluted with 0.1 M KCl, and  
353 an LOD of 0.1  $\mu\text{g P L}^{-1}$  was achieved with a sample throughput of 6  $\text{h}^{-1}$ . Effective pre-  
354 concentration was achieved for samples containing less than 200  $\text{mg L}^{-1}$  chloride, making the  
355 method suitable for most freshwaters. Udnan *et al.* reported a similar FI approach for the  
356 determination of DRP that used amperometric detection of phosphomolybdate after in-valve  
357 anion exchange preconcentration. A LOD of 0.18  $\mu\text{g P L}^{-1}$  (6 nM) was achieved using a 2 min  
358 column loading time, but the method was limited to pristine freshwaters because the  
359 preconcentration was adversely affected at chloride concentrations  $\geq 50 \text{ mg L}^{-1}$  [98].

360

361 The MAGIC (MAGnesium Induced Coprecipitation) method proposed by Karl and Tien [99] has  
362 been utilized by chemical oceanographers for the determination of DRP in low-nutrient ocean  
363 waters. It involves the coprecipitation of phosphate when magnesium hydroxide (brucite) is  
364 precipitated by the addition of NaOH. Then, after separation by centrifugation, the precipitate is  
365 dissolved in 0.1 M HCl and the phosphate concentration determined as PMB. A modified  
366 method reported by Rimmelin and Moutin [100] gave a typical preconcentration factor of 25 and  
367 resulted in a detection limit of a 0.8 nM.

368

369 Enhancement of detection sensitivity can be achieved by the use of cuvettes with longer optical  
370 path lengths. In batch techniques using conventional spectrophotometers, the maximum cuvette  
371 length used was conventionally 100 mm. However, Ormaza-González and Statham described  
372 the use of a 600 mm long capillary cell with a red LED light source and phototransistor detector  
373 for the detection of PMB at low nM concentrations [101]. The development of low refractive  
374 index polymers such as Teflon<sup>®</sup> AF2400 has enabled the construction of liquid core waveguide

375 (LCW) cells that offer the possibility of even longer optical path lengths, and these can be  
376 readily configured as flow cells for use in flow analysis. For example, Gimbert *et al.* [102]  
377 evaluated the suitability of a 1000 mm LCW cell for detection of phosphate as tin(II)-reduced  
378 PMB at 710 nm using a FI manifold, and achieved a LOD of 10 nM (Fig. 4). Optimal results  
379 were obtained using an injection volume of 500  $\mu\text{L}$  (twice the internal volume of the LCW cell)  
380 and background correction at 470 nm. Similarly Zhang *et al.* used a 2000 mm LCW cell in a  
381 segmented continuous flow system, with an LOD of 1.5 nM [103], for underway analysis of more  
382 than 1000 samples from the west Florida continental shelf and the oligotrophic Sargasso Sea  
383 [104]. As a generality, background correction or other compensation strategies should be used  
384 to avoid Schlieren effects in these LCW cells [73].

385

386 Passive sampling techniques such as Diffusive Gradient in Thin films (DGT) can be used for the  
387 *in situ* preconcentration of DRP [105-108]. Mohr *et al.* determined both DRP and low molecular  
388 weight organic P species such as adenosine monophosphate (AMP) and myo-inositol  
389 hexakisphosphate (IP6) using DGT with an iron oxide based binding gel [107]. Monbet *et al.*  
390 deployed both DGT and Diffusive Equilibrium in Thin films (DET) *in situ* to obtain high spatial  
391 resolution (mm scale) DRP sediment porewater profiles in two lagoons of the Gippsland Lakes  
392 (SE Australia). DRP concentrations were determined using the PMB method with tin(II) chloride  
393 in an automatic FI manifold [82] and the detection limit was  $0.2 \mu\text{g P L}^{-1}$  ( $0.006 \mu\text{M}$ ).

394

### 395 3.2 Total and total dissolved phosphorus

396

397 The determination of TP or TDP requires that the sample must first be digested to convert all P  
398 forms to detectable orthophosphate, as described in § 2.3, before detection, usually as PMB.  
399 Proposed methods should be thoroughly validated using model phosphorus compounds that  
400 range in stability from labile to refractory [109], certified reference materials and comparison  
401 with a reference method. Inductively coupled plasma – optical emission spectroscopy (ICP-

402 OES) can also be used for detection if concentrations are sufficiently high. For example, Van  
403 Moorleghem *et al.* reported detection limits of  $6 \mu\text{g P L}^{-1}$  ( $0.2 \mu\text{M}$ ), at  $213.617 \text{ nm}$  and  $34 \mu\text{g P L}^{-1}$   
404 ( $1.1 \mu\text{M}$ ), at  $178.221 \text{ nm}$  [110].

405

406 A number of automatic methods, based mainly on flow analysis, have been described for the  
407 determination of TP and/or TDP. For example, Ayoagi *et al.* [111] used a  $10 \text{ m}$  long capillary  
408 digester containing a Pt wire as catalyst in an FI system to perform thermal digestion with  
409 peroxydisulfate at  $160 \text{ }^\circ\text{C}$ . Orthophosphate produced by digestion was detected using the  
410 Malachite green-molybdophosphate chemistry and a sample throughput of  $\text{ca. } 10 \text{ h}^{-1}$  was  
411 achieved.

412

413 An alternative approach to direct heating was employed by Hinkamp and Schwedt who used a  
414  $7.6 \text{ m}$  Teflon<sup>®</sup> digestion coil in a microwave oven for digestion of organic and condensed  
415 phosphates. Amperometric detection was performed in FI mode, with a limit of determination of  
416  $0.1 \text{ mg P L}^{-1}$ , a precision of 3% RSD at  $5 \text{ mg P L}^{-1}$  and a sample throughput of  $20 \text{ h}^{-1}$  [112].

417 Benson *et al.* described the application of a flow analysis system in which digestion was  
418 performed continuously off-line in a  $6 \text{ m}$  Teflon<sup>®</sup> reactor. Digestate was passed through a  
419 microporous debubbler to remove gas bubbles prior to injection into a spectrophotometric FI  
420 system for detection of the PMB formed [63]. As an alternative to the use of continuous coil  
421 digestors, Almeida *et al.* [113] described the use of a micro-batch reactor for microwave TP  
422 digestion which was coupled with a multi-syringe FI analysis system.

423

424 Other automatic flow systems have used UV photo-oxidation either alone [114-116] or in  
425 combination with thermal digestion [115] for the determination of TP and TDP. However, while  
426 these methods were suitable for waste waters and freshwaters, Peat *et al.* found that for  
427 samples such as soil waters, acidic photo-oxidation was required to avoid interference from the  
428 higher concentrations of Fe and Al found in the matrix that complexed with phosphate [114].

429 Aminot and Kerouel [59] reported similar matrix problems in natural seawater using a  
430 segmented continuous flow system, presumably due to complexation with Ca and Mg ions, and  
431 suggested that sample dilution by a factor of 5 - 6 was required to obtain complete digestion.  
432 Gentle *et al.* found that the problem of incomplete digestion in seawater could be overcome  
433 across the full salinity range (0 – 35) in a FI system if photo-oxidation combined with thermal  
434 digestion when acidic peroxydisulfate was adopted. This FI system was capable of 115  
435 measurements per hour, with a LOD of  $1 \mu\text{g P L}^{-1}$  (0.03  $\mu\text{M}$ ), and used to perform 2499  
436 underway TP measurements during a cruise in the coastal waters of Victoria, SE Australia  
437 [117].

438

### 439 3.3 Phosphorus speciation

440

441 The chemical species or forms of aquatic phosphorus can be determined using either  
442 *operational* or *functional* approaches. In the former, species are defined by the chemical  
443 operation involved, e.g. by the formation of PMB to give “molybdate reactive P” (*cf.* § 3.1),  
444 whereas in the latter, highly specific assays may be applied to quantify species with particular  
445 functionality, e.g. by specific enzymatic assays or chromatographic separation. The distinction  
446 between operational and functional measurement is illustrated by Baldwin [118] who compared  
447 spectrophotometric PMB (*operationally* defined) and ion chromatographic (*functionally* defined)  
448 methods to show that only a small fraction of the filterable MRP present in eutrophic waters was  
449 comprised of dissolved orthophosphate. The difference was ascribed to the hydrolysis of labile  
450 organic P or desorption from colloidal material [118] which occurred as an artefact of the PMB  
451 method.

452

453 The organic P fraction, which comprises nucleic acids, phosphoproteins and amino phosphoric  
454 acids, phospholipids, inositol phosphates, phosphonates and organic condensed P compounds,  
455 such as adenosine triphosphate, can be at least as abundant as inorganic P in some natural

456 waters [119, 120]. There is compelling evidence that in the absence of orthophosphate, some  
457 algae and cyanobacteria can utilize phosphorus from organic P compounds *via* enzymatic  
458 hydrolysis [5, 70, 121-127]. Consequently there is growing interest in methods for the  
459 determination of organic P [128], which hitherto was considered unavailable, and hence was  
460 ignored as a source of bioavailable P.

### 462 3.3.1 Operational speciation

463  
464 The most common operational delineation of phosphorus species is that based on filtration to  
465 discriminate between the so-called dissolved or filterable fraction and particulate forms (*cf.* § 2.2  
466 and Fig 2). Thereafter, digestion (with strong acids/bases and oxidants (*cf.* § 3.2), or dilute acid  
467 hydrolysis can be performed to obtain estimates of the amount of *total* or *condensed*  
468 phosphorus forms present within either the filterable or particulate size fractions. The *organic*  
469 fraction has conventionally been determined as the unreactive residual fraction after the  
470 reactive and condensed fractions have been subtracted from the total P concentration [92, 129].

471  
472 A convenient, operational, non-specific measure of organic P can be achieved using alkaline  
473 UV photo-oxidation in a flow system with a low wattage lamp [59, 62, 116]. Under alkaline, rapid  
474 photo-oxidation conditions, minimal hydrolysis of condensed P occurs, and what is detected is  
475 the sum of (DOP + DRP). DOP is therefore determined by subtracting the DRP, with the caveat  
476 that DRP may already include some labile DOP.

### 478 3.3.2 Functional speciation

479  
480 Strickland and Solorzano [130] and Herbes *et al.* [131] were among the first to determine  
481 alkaline phosphatase-hydrolysable phosphate in sea and lake waters as a means of measuring  
482 the phosphomonoester fraction. A similar approach has been applied using a suite of different

483 phospho-enzymes to characterise organic P in natural waters. Turner *et al.* [132] performed a  
484 series of enzymatic reactions utilising alkaline phosphomonoesterase (labile monoester P),  
485 phosphodiesterase + alkaline phosphomonoesterase (diester-P compounds) and phytase  
486 (inositol hexaphosphate) to characterise organic P in soil solution, and a similar approach was  
487 adopted by Monbet *et al.* in a study of coastal lake [133] and estuarine [13] waters using the FI  
488 manifold shown in Fig. 5.

489  
490 The use of FI techniques with immobilised enzyme reactors enables convenient and rapid  
491 measurement of these enzymatically available organic P fractions. Shan *et al.* determined  
492 alkaline phosphatase-hydrolysable phosphorus using immobilized alkaline phosphatase from *E.*  
493 *coli* [134, 135], while similar approaches have been applied for phytase-hydrolysable  
494 phosphorus with 3-phytase [136] in natural waters using FI systems. However, since the  
495 detection step for all of these reactions involves the measurement of PMB, the enzymatic  
496 selectivity may be compromised because some labile P species may also be molybdate  
497 reactive. Ideally, a more selective technique such as ion chromatography could be used to  
498 overcome this deficiency.

499  
500 Arguably chromatographic separations can be included in the category of “functional”  
501 techniques because the detected species are separated on the basis of their hydrophobicity or  
502 charge prior to detection using a variety of techniques. Ion chromatography (IC) with  
503 suppressed electrical conductivity detection is widely and routinely used for the quantification of  
504 orthophosphate in fresh waters. IC has also been applied to speciation of both inorganic and  
505 organic phosphorus moieties using a variety of post separation detection methods [137]. For  
506 example, separation of ortho-, di- and tri-phosphate in wastewaters was described by Jolley *et al.*  
507 *et al.* using ion exchange chromatography with post column detection of PMB following autoclave  
508 digestion [138]. Interestingly, Jolley *et al.* did not detect any condensed phosphate in  
509 wastewaters. However, Halliwell *et al.* [139] using IC coupled to on-line post column digestion

510 using a FI system, demonstrated that the half-life of triphosphate was less than 10 h, thus  
511 accounting for its apparent absence [139]. Espinosa *et al.* [125] used a similar ion  
512 chromatographic approach, but with off-line TP digestion and detection, to study P speciation in  
513 soil leachate waters.

514

515 Ion chromatography has also been coupled with ICP-AES for the on-line determination of  
516 orthophosphate and glyphosate [140] and phosphite, hypophosphite, pyrophosphate and  
517 tripolyphosphate [141]. Similarly, mass spectrometry has been coupled with IC for the  
518 determination of hypophosphite, phosphite, and phosphate [142] and dialkyl phosphinate acids  
519 [143] in water. These hyphenated separation-detection techniques show the greatest potential  
520 for selective speciation of the plethora of organic and inorganic P species that may occur in  
521 natural waters (and wastewaters).

522

#### 523 4. Quality assurance of phosphorus data

524

525 Phosphorus, particularly in the form of DRP, is a key determinand in many environmental  
526 monitoring programmes and it follows that accurate data are required to implement water quality  
527 management strategies and monitor compliance with environmental standards [47]. Total  
528 phosphorus (TP) is also determined, although less frequently than DRP, and is important for  
529 monitoring discharges from, e.g. wastewater treatment plants and determining P loads [29].  
530 General guidelines on data quality can be found in ISO/IEC 17025 [144] and specific discussion  
531 of nutrient data quality (including P) in marine waters, together with a practical illustration of how  
532 to determine an uncertainty budget, is presented by Worsfold *et al.* [145].

533

534 Certified reference materials (CRMs) are an important component of any quality assurance  
535 programme for the determination of dissolved phosphorus in natural waters and this was

536 discussed by Aminot and K  rouel in 1995 [146]. A key challenge is to provide a stable natural  
537 water CRM and this has been achieved for seawater by the National Research Council Canada  
538 using gamma irradiation [147]. Their current seawater CRM for nutrients (MOOS-3) has a  
539 certified quantity value for phosphate of  $1.60 \pm 0.15 \mu\text{mol L}^{-1}$  based on analysis by  
540 spectrophotometry and ion exchange ICP-MS. The stability of a seawater reference material  
541 has also been investigated by the Meteorological Research Institute of Japan [148] and it has  
542 been used in intercomparison exercises to improve the comparability of oceanic orthophosphate  
543 determinations [149].

544

## 545 5. Conclusions and future perspectives

546

547 The role of phosphorus as an essential nutrient, coupled with the potential of excess  
548 phosphorus to have a negative impact on water quality, makes it crucial to have reliable  
549 analytical methods for the determination of phosphorus species in natural (and waste) waters.  
550 This requires appropriate methods for sample collection (minimising contamination and sample  
551 degradation) and sample treatment (e.g. filtration and digestion). Analytical methods for the  
552 determination of phosphorus are dominated by spectrophotometric methods based on direct  
553 detection of the intensely coloured phosphomolybdenum blue species, which determines the  
554 “molybdate reactive phosphorus” fraction and has typical detection limits in the low  $\mu\text{g P L}^{-1}$   
555 range. Sensitivity can be enhanced by a variety of chemical (e.g. ion pair formation) and  
556 instrumental (e.g. use of long path length liquid core waveguides) means. Instrumental  
557 developments such as waveguides are a relatively low cost way to achieve detection limits in  
558 the nM range and, in conjunction with flow analysis methods, can be deployed at sea and in  
559 remote environments.

560

561 Phosphorus speciation provides an additional analytical challenge due to the need for increased  
562 selectivity and, often, lower detection limits. Speciation can be defined operationally (e.g. by  
563 filtration) or functionally. Functional selectivity can be achieved by the use of selective reagents  
564 (e.g. enzymes) or by prior separation (e.g. using ion chromatography). Combining separation  
565 strategies with powerful detection techniques such as mass spectrometry is the most promising  
566 way forward for a more complete characterisation and quantification of the dissolved  
567 phosphorus pool.

568

569 Rigorous quality assurance procedures are required in order to ensure compliance with the  
570 legislative requirements for phosphorus in natural waters as well as supporting water quality  
571 management strategies and biogeochemical studies. This in turn generates a need for stable,  
572 matrix specific reference materials that are certified for phosphorus species at environmentally  
573 relevant concentrations.

574

#### 575 Abbreviations

576	12-MPA	12-molybdophosphoric acid
577	CRM	certified reference material
578	DET	Diffusive Equilibrium in Thin films
579	DGT	Diffusive Gradient in Thin films
580	DIP	dissolved inorganic P
581	DOP	dissolved organic P
582	DRP	dissolved reactive phosphorus
583	FI	Flow injection
584	FRP	filterable reactive phosphorus
585	HDPE	high density polyethylene
586	IC	ion chromatography
587	ICP-AES	inductively coupled plasma-atomic emission spectrometry

588	ICP-MS	inductively coupled plasma-mass spectrometry
589	LCW	liquid core waveguide
590	LED	light emitting diode
591	LOD	limit of detection
592	MAGIC	MAGnesium Induced Coprecipitation
593	MRP	molybdate reactive phosphorus
594	P	phosphorus
595	PMB	phosphomolybdenum blue
596	PTFE	polytetrafluoroethylene
597	SRP	soluble reactive phosphorus
598	TDP	total dissolved phosphorus
599	TP	total phosphorus
600	UV	ultra-violet
601		
602		

Fig. 1. A schematic diagram of the aquatic phosphorus cycle. Flux and reservoir data obtained from [5, 121, 150-158].

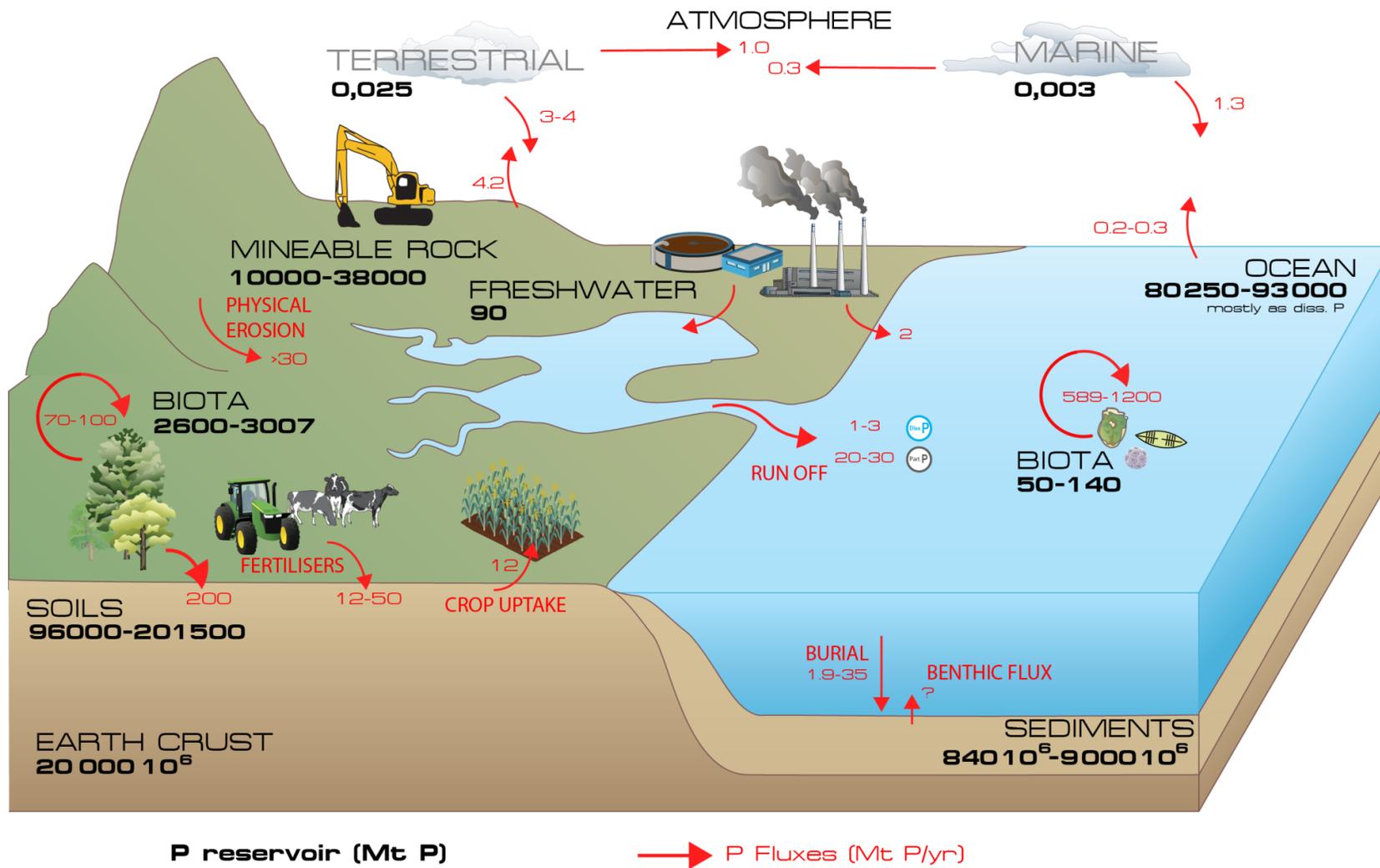


Fig. 2. The various operationally defined P fractions in natural waters, based on filtration and/or digestion.

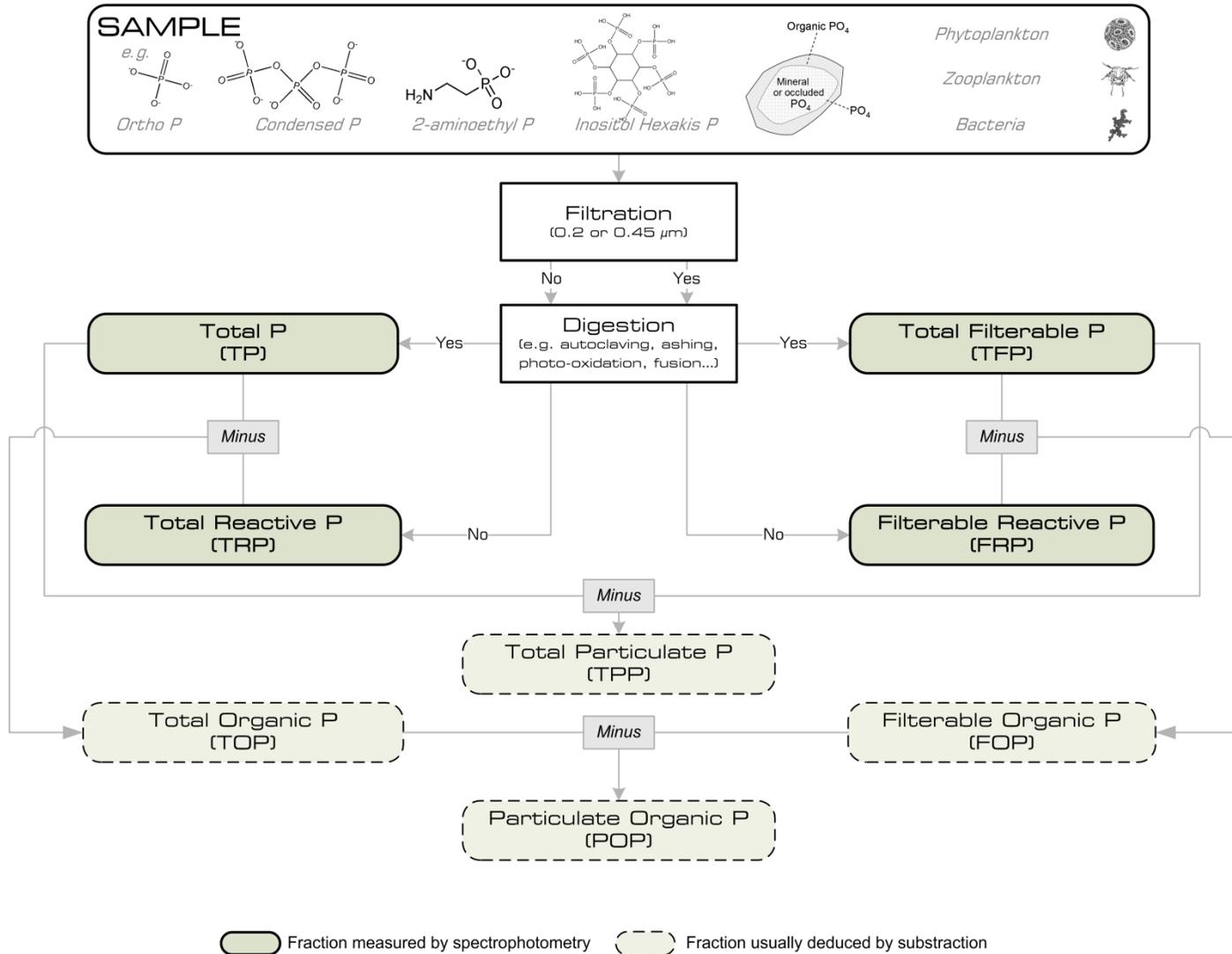


Fig. 3 (a) Schematic diagram of a compact, portable FI analyser for the determination of phosphate. PP, peristaltic pump; TFF, 0.2 mm tangential flow filter; FS, differential flow splitter; PG, propellant gas and regulator; MC, mixing coil; FC, flow cell; V0, 2-way valve; V1, V2 and V3, miniature solenoid valves; R1, ammonium molybdate reagent; R2, tin(II) chloride reagent; Std, standard. (b) Phosphate concentrations obtained underway in Port Phillip Bay using the portable FIA system compared with those obtained for samples collected by hand and analysed in the laboratory. Adapted, with permission, from A.J. Lyddy-Meaney, P.S. Ellis, P.J. Worsfold, E.C.V. Butler and I. D. McKelvie, A compact flow injection analysis system for surface mapping of phosphate in marine waters, *Talanta*, 58 (2002) 1043-1053.

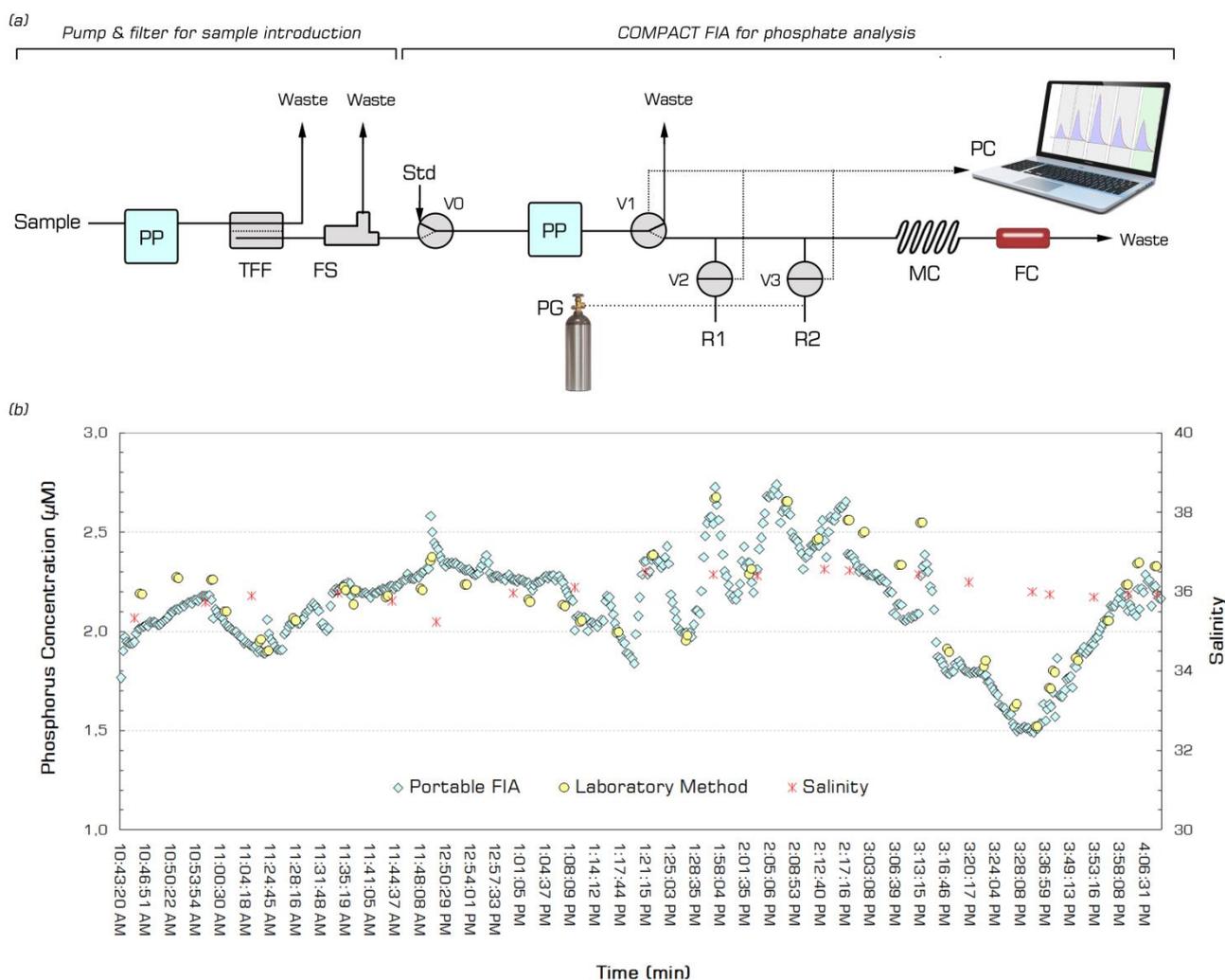


Fig. 4. FI manifold incorporating a liquid core waveguide (LWCC) for the determination of molybdate reactive phosphorus (top). Typical detector trace and calibration using standards in the range 0.01 to 1  $\mu\text{M}$   $\text{PO}_4\text{-P}$  obtained using a LWCC of 1 m path length. Error bars  $\pm 3$  standard deviations,  $n = 3$  (bottom). Reproduced, with permission, from L.J. Gimbert, P.M. Haygarth, P.J. Worsfold, Determination of nanomolar concentrations of phosphate in natural waters using flow injection with a long path length liquid waveguide capillary cell and solid-state spectrophotometric detection, *Talanta*, 71 (2007) 1624-1628.

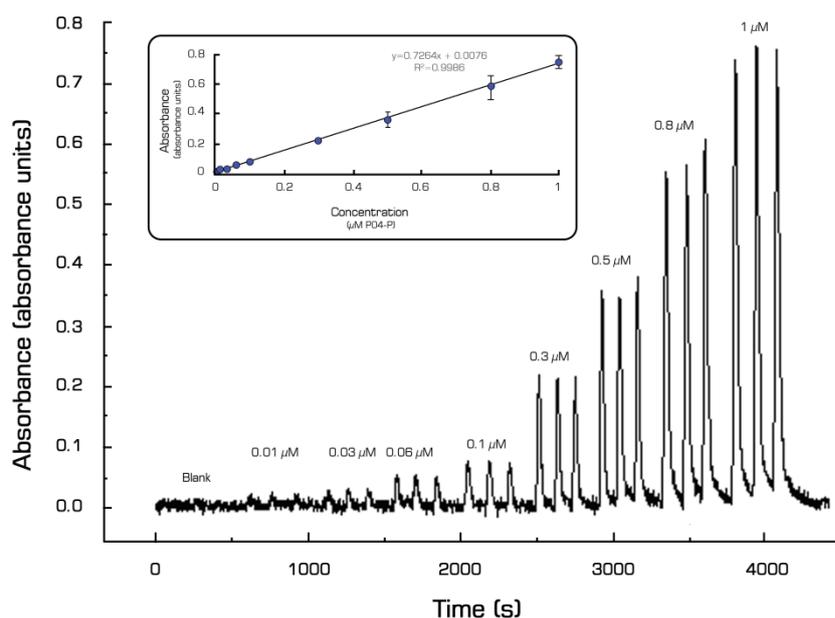
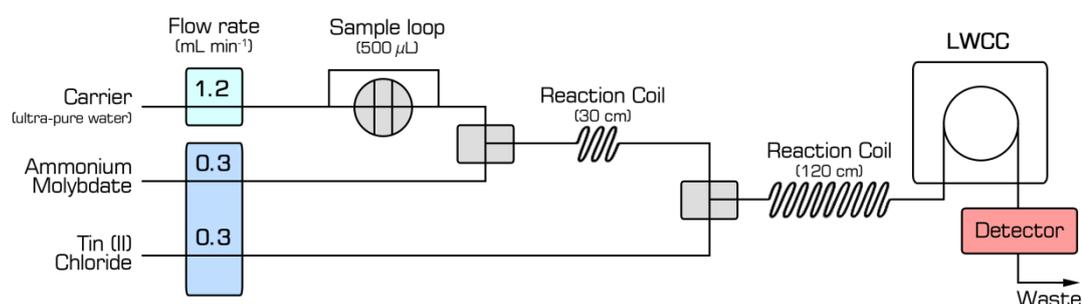
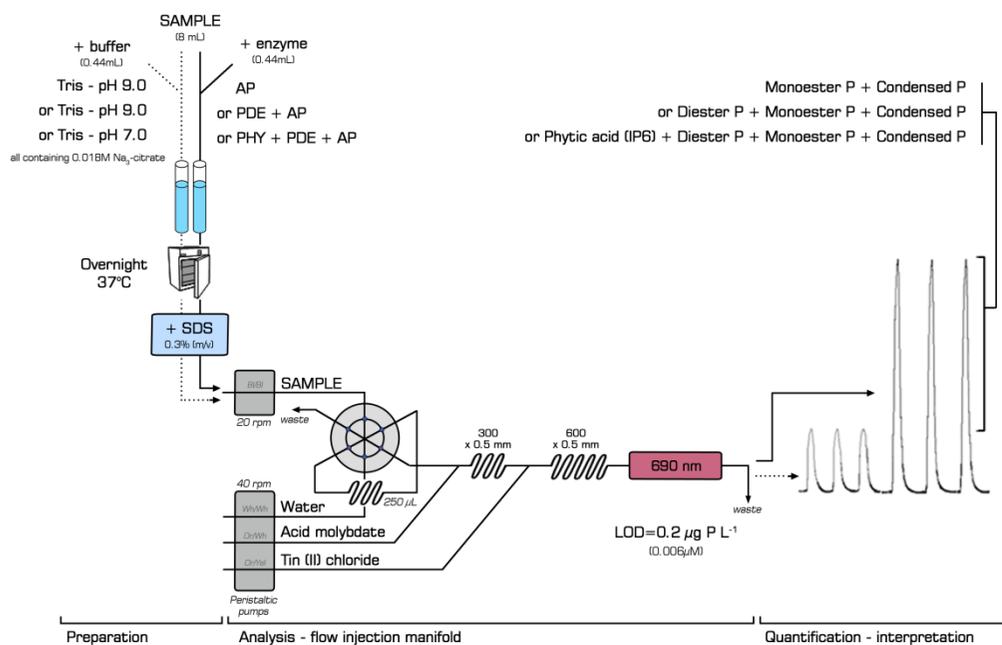


Fig. 5. Schematic of the experimental design for the speciation of dissolved organic phosphorus in natural waters. The left-hand side shows the assay preparation (with/without enzyme), incubation and surfactant addition. The centre describes the flow injection manifold used for DRP measurement. The right-hand side shows an example of triplicate peaks obtained with and without enzyme added. The difference represents the fraction of enzymatically hydrolysable P (EHP). Adapted, with permission, from P. Monbet , I.D. McKelvie , A. Saefumillah and P.J. Worsfold, A protocol to assess the enzymatic release of dissolved organic phosphorus species in waters under environmentally relevant conditions, *Environmental Science and Technology*, 41 (2007) 7479-7485. Copyright 2007, American Chemical Society.



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