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Determination of phosphorus in natural waters: A historical review

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Abstract

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

Keywords

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

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 8.4×10^8 – 40×10^8 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 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 μm 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 μm 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 μm 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 (≥ 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

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

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

Clean sample containers and sample collection apparatus (including filters) are an essential prerequisite for minimising contamination and most cleaning methods involve acid washing, e.g. soaking items overnight in a nutrient free detergent, rinsing with ultra-pure water, followed by soaking overnight in 10% HCl, and finally rinsing with ultra-pure water [42]. Polytetrafluoroethylene (PTFE) or high density polyethylene (HDPE) are the preferred materials for containers but quartz, borosilicate glass, low density polyethylene and polypropylene have

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

2.2 Sample pretreatment and storage

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

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

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

2.3 Sample digestion

For the determination of total phosphorus (TP; unfiltered samples) and total dissolved phosphorus (TDP; filtered samples) it is necessary to convert all of the phosphorus-containing species into a detectable form. For natural waters the most common method of detection is the “molybdenum blue” chemistry with spectrophotometric detection which determines molybdate-reactive orthophosphate (PO_4^{3-}) using either a batch or flow-based approach (see §3.1) [50]. Inductively coupled plasma based methods (ICP-AES and ICP-MS) can also be used if the concentration of P is sufficiently high. The process of conversion, which involves the breaking of P-O-P, C-O-P and C-P bonds in condensed and organic P compounds is called *digestion* and is typically achieved by thermal oxidation with hydrolysis using either an autoclave, digestion block or microwave [51] or by UV photo-oxidation with or without heating.

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

for the simultaneous determination of TP and total nitrogen (TN) and for the digestion of marine waters due to the oxidation of chloride to free chlorine by peroxydisulfate in acidic media, which reduces its oxidising power [55]. When alkaline peroxydisulfate digestion is used, autoclaving or thermal heating should be continued until $\text{S}_2\text{O}_8^{2-}$ is converted to hydrogen sulphate (HSO_4^- ; $\text{pK}_a = 1.99$) so that a low pH is reached in the latter stages of the digestion and acid hydrolysis of condensed phosphate species is achieved [51]. An acidic peroxydisulfate method was reported by Gales *et al.* [56] and simplified by Eisenreich *et al.* [57]. The method gives good recoveries and is simple and easy to use and is therefore recommended for the determination of TP and TDP in fresh waters [58].

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

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

3. Analytical methods

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 λ_{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 λ_{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[®] 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

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

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

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

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

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

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

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

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

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

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

Flow-based systems readily facilitate the automation of solid phase extraction methods, minimizing the use of solvent. For example, Liang *et al.* have reported an example of a solid phase extraction FI preconcentration technique that is suitable for detection of DRP in marine waters. This involved solid phase extraction of the phosphomolybdenum blue – cetyltrimethylammonium bromide ion pair on a C_{18} column followed by elution with ethanol and sulfuric acid before spectrophotometric detection, giving a detection limit of 1.6 nM but with a sample throughput of only 2 h^{-1} [95]. In a related approach, Nagul *et al.* used polymer inclusion membranes (PIMs) in combination with a FI manifold to perform on-line extraction of phosphate directly from low ionic strength fresh waters before stripping and detection as PMB. A detection limit of 0.04 $\mu\text{g P L}^{-1}$ at a sampling rate of 5 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 h^{-1} . Effective pre-
354 concentration was achieved for samples containing less than 200 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[®] AF2400 has enabled the construction of liquid core waveguide

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

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

3.2 Total and total dissolved phosphorus

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

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

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

An alternative approach to direct heating was employed by Hinkamp and Schwedt who used a 7.6 m Teflon[®] digestion coil in a microwave oven for digestion of organic and condensed phosphates. Amperometric detection was performed in FI mode, with a limit of determination of 0.1 mg P L^{-1} , a precision of 3% RSD at 5 mg P L^{-1} and a sample throughput of 20 h^{-1} [112]. Benson *et al.* described the application of a flow analysis system in which digestion was performed continuously off-line in a 6 m Teflon[®] reactor. Digestate was passed through a microporous debubbler to remove gas bubbles prior to injection into a spectrophotometric FI system for detection of the PMB formed [63]. As an alternative to the use of continuous coil digestors, Almeida *et al.* [113] described the use of a micro-batch reactor for microwave TP digestion which was coupled with a multi-syringe FI analysis system.

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

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

3.3 Phosphorus speciation

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

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

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

3.3.1 Operational speciation

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

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

3.3.2 Functional speciation

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

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

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

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

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

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

4. Quality assurance of phosphorus data

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

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

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

5. Conclusions and future perspectives

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

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

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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 μM $\text{PO}_4\text{-P}$ obtained using a LWCC of 1 m path length. Error bars ± 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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