Determination of phosphorus in natural waters: A historical review

Paul Worsfold¹, Ian McKelvie¹,², Phil Monbet³

¹ Biogeochemistry Research Centre, Plymouth University, Plymouth, Devon PL48AA, UK.
² School of Chemistry, The University of Melbourne, Victoria 3010, Australia.
³ Pole Mer Bretagne Atlantique, 40 rue Jim Sévellec, 29200 Brest, France

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 $8.4 \times 10^8 - 40 \times 10^8$ 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 $22$ 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
therefore important factors when considering the impact of the element on water quality. In natural waters phosphorus can be found in various “dissolved” forms (operationally defined as the fraction that passes through a 0.2 or 0.45 µm filter [15]), mostly as inorganic orthophosphates and condensed or polyphosphates, but also as organic phosphates (e.g. nucleic acids, proteins, phospholipids, phosphoamides, sugar phosphates, inositol phosphates, aminophosphonates and organic phosphorus pesticides). “Particulate” P (defined as the fraction retained on a 0.2 or 0.45 µm filter [15]) can include clay and silt-associated organic and inorganic P, precipitates of authigenic origin and P-containing biological matter. Colloidal phosphorus is commonly referred to as the P fraction in the 1 nm – 1 µm size range [16-18] and hence both the operationally defined dissolved and particulate fractions can contain colloidal P. This fraction includes both organic and inorganic species of biological and/or mineral origin. The various operationally defined P fractions in natural waters, based on filtration and/or digestion, are shown in Fig. 2, together with examples of the types of phosphorus species found in these fractions.

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

For water quality management purposes it is also informative to determine the loads [21, 22] and fluxes [23] of P species in water bodies in order to investigate, e.g. internal cycling processes [24], the restoration of eutrophic ecosystems [25] and the impacts of P runoff from land on the ecological status of receiving waters [26]. Long-term datasets (≥ 20 years), which
require routine monitoring, are useful for identifying non-hydrological variations in P concentrations and distributions, to contextualise contemporary datasets [27-29] and help to validate catchment models [30].

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

2. Sampling and sample treatment

2.1 Sample collection

Prior to sample collection it is important to ensure that the sampling strategy is fit for purpose [19]. This includes the identification of appropriate sampling locations (with due regard to 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 sub-samples 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 $S_2O_8^{2-}$ is converted to hydrogen sulphate ($HSO_4^{-}$; pKa = 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
Most methods for phosphorus determination are based on the spectrophotometric detection of the intensely coloured phosphomolybdenum blue (PMB). This complex is formed by reaction of phosphate with acidified molybdate producing 12-molybdophosphoric acid (12-MPA), which is subsequently reduced to phosphomolybdenum blue [65], i.e.

\[
\text{PO}_4^{3-} + 12 \text{MoO}_4^{2-} + 27 \text{H}^+ \rightarrow \text{H}_3\text{PO}_4 (\text{MoO}_3)_{12} + 12 \text{H}_2\text{O} \quad (1)
\]

\[
\text{H}_3\text{PMo(VI)}_{12}\text{O}_{40} + \text{Reductant} \rightarrow [\text{H}_4\text{Mo(VI)}_8\text{Mo(V)}_8\text{O}_{40}]^{3-} \quad \text{(Phosphomolybdenum blue)} \quad (2)
\]

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

A variety of reductants (e.g. ascorbic acid, tin(II) chloride, hydrazine sulfate, hydroquinone) and acids have been used in this reaction [50], giving rise to a range of PMB species with different absorbance spectra. Appropriate acid and molybdate concentrations are critical for the formation of PMB; for example, in low acidity conditions, non-linear colour development and self-reduction of the molybdate can occur [68]. The chemistry of phosphomolybdenum blue formation has been comprehensively reviewed by Nagul et al. [50].
The most cited and widely used method is that reported by Murphy and Riley [69] in 1962 which utilised ascorbic acid in the presence of Sb(III) as the reductant. When Sb(III) is present, the rate of formation of molybdenum blue is enhanced compared with ascorbic acid alone (although Sb does not act as a catalyst, as is often claimed), and Sb is incorporated into the complex as \([\text{PSb}_2\text{Mo}_{12}\text{O}_{40}]^-\) which has a \(\lambda_{\text{max}}\) at 880 nm. The optimum conditions for the formation of the molybdenum blue complex was further investigated by Going and Eisenreich [70]. This reaction is less sensitive to salt than others, and colour development is fairly independent of temperature [71], making it the preferred reaction for the determination of phosphorus in natural waters.

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

In addition to the homogeneous reduction methods described above, an on-line UV photo-reduction method has recently been reported [76]. In this system molybdophosphate was reduced to phosphomolybdenum blue in the presence of a hydrogen bond donor (ethanol), using a simple Teflon® tube UV reactor in a FI system. Because the PMB produced by UV 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 µM 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 µg P L$^{-1}$ range, e.g. the APHA standard methods publication quotes a LOD of about 3 µg P L$^{-1}$ with tin(II) chloride reduction (0.1 µM) and 10 µg P L$^{-1}$ with ascorbic acid reduction (0.3 µM) [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 iso-propanol, n/iso-butanol, n/iso-butanol + benzene, n-hexanol and butyl acetate [95]. Detection limits at the sub-µg 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 – cetyl trimethylammonium 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 µg P L\(^{-1}\) at a sampling rate of 5 h\(^{-1}\) was reported [97].
Ion exchange techniques in concert with flow analysis have also been described for preconcentration of phosphate in freshwaters. Freeman et al. [78] used a strong anion exchange resin (AG 1 X-8) in an FI manifold to preconcentrate as much as 3.2 mL of sample. Phosphate and silicate peaks were resolved when the column was eluted with 0.1 M KCl, and an LOD of 0.1 µg P L⁻¹ was achieved with a sample throughput of 6 h⁻¹. Effective pre-concentration was achieved for samples containing less than 200 mg L⁻¹ chloride, making the method suitable for most freshwaters. Udnan et al. reported a similar FI approach for the determination of DRP that used amperometric detection of phosphomolybdate after in-valve anion exchange preconcentration. A LOD of 0.18 µg P L⁻¹ (6 nM) was achieved using a 2 min column loading time, but the method was limited to pristine freshwaters because the preconcentration was adversely affected at chloride concentrations ≥50 mg L⁻¹ [98].

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

Enhancement of detection sensitivity can be achieved by the use of cuvettes with longer optical path lengths. In batch techniques using conventional spectrophotometers, the maximum cuvette length used was conventionally 100 mm. However, Ormaza-González and Statham described the use of a 600 mm long capillary cell with a red LED light source and phototransistor detector for the detection of PMB at low nM concentrations [101]. The development of low refractive 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 µL (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 µg P L\(^{-1}\) (0.006 µM).

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 Moorleghem et al. reported detection limits of 6 µg P L\(^{-1}\) (0.2 µM), at 213.617 nm and 34 µg P L\(^{-1}\) (1.1 µM), 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 digestor 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 ca. 10 h\(^{-1}\) was achieved.

An alternative approach to direct heating was employed by Hinkamp and Schwedt who used a 7.6 m Teflon\(^{\circledR}\) 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\(^{\circledR}\) 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 µg 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
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 Kérouel 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 ± 0.15 µmol 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 µg 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.
Phosphorus speciation provides an additional analytical challenge due to the need for increased selectivity and, often, lower detection limits. Speciation can be defined operationally (e.g. by filtration) or functionally. Functional selectivity can be achieved by the use of selective reagents (e.g. enzymes) or by prior separation (e.g. using ion chromatography). Combining separation strategies with powerful detection techniques such as mass spectrometry is the most promising way forward for a more complete characterisation and quantification of the dissolved phosphorus pool.

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

Abbreviations

- 12-MPA: 12-molybdophosphoric acid
- CRM: certified reference material
- DET: Diffusive Equilibrium in Thin films
- DGT: Diffusive Gradient in Thin films
- DIP: dissolved inorganic P
- DOP: dissolved organic P
- DRP: dissolved reactive phosphorus
- FI: Flow injection
- FRP: filterable reactive phosphorus
- HDPE: high density polyethylene
- IC: ion chromatography
- ICP-AES: inductively coupled plasma-atomic emission spectrometry
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<th>Page</th>
<th>Term</th>
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<tr>
<td>588</td>
<td>ICP-MS</td>
<td>inductively coupled plasma-mass spectrometry</td>
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<td>589</td>
<td>LCW</td>
<td>liquid core waveguide</td>
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<td>590</td>
<td>LED</td>
<td>light emitting diode</td>
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<td>591</td>
<td>LOD</td>
<td>limit of detection</td>
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<td>592</td>
<td>MAGIC</td>
<td>MAGnesium Induced Coprecipitation</td>
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<tr>
<td>593</td>
<td>MRP</td>
<td>molybdate reactive phosphorus</td>
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<td>594</td>
<td>P</td>
<td>phosphorus</td>
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<tr>
<td>595</td>
<td>PMB</td>
<td>phosphomolybdenum blue</td>
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<tr>
<td>596</td>
<td>PTFE</td>
<td>polytetrafluoroethylene</td>
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<tr>
<td>597</td>
<td>SRP</td>
<td>soluble reactive phosphorus</td>
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<tr>
<td>598</td>
<td>TDP</td>
<td>total dissolved phosphorus</td>
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<tr>
<td>599</td>
<td>TP</td>
<td>total phosphorus</td>
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<tr>
<td>600</td>
<td>UV</td>
<td>ultra-violet</td>
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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 FIA 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 PO₄-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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