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Review

# Characterisation and quantification of organic phosphorus and organic nitrogen components in aquatic systems: A Review

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ABSTRACT

This review provides a critical assessment of knowledge regarding the determination of organic phosphorus (OP) and organic nitrogen (ON) in aquatic systems, with an emphasis on biogeochemical considerations and analytical challenges. A general background on organic phosphorus and organic nitrogen precedes a discussion of sample collection, extraction, treatment/conditioning and preconcentration of organic phosphorus/nitrogen from sediments, including suspended particulate matter, and waters, including sediment porewaters. This is followed by sections on the determination of organic phosphorus/nitrogen components. Key techniques covered for organic phosphorus components are molecular spectrometry, atomic spectrometry and enzymatic methods. For nitrogen the focus is on the measurement of total organic nitrogen concentrations by carbon hydrogen nitrogen analysis and high temperature combustion, and organic nitrogen components by gas chromatography, high-performance liquid chromatography, gel electrophoresis, mass spectrometry, nuclear magnetic resonance spectrometry, X-ray techniques and enzymatic methods. Finally future trends and needs are discussed and recommendations made.



## Contents



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## 1. Introduction

### 1.1. *Analytical context*

As limiting nutrients for algal growth, phosphorus (P) and nitrogen (N) play an essential role in the biological productivity of aquatic ecosystems [1,2]; therefore elevated P and N inputs to the aquatic environment increase the risk of eutrophication and impact on water quality. There are many analytical methods and review articles on the determination of inorganic P and N in aquatic systems, e.g. [3–9], but the emphasis of this review is on the determination of the organic phosphorus (OP) and organic nitrogen (ON) pools and the individual components within these pools. These pools are intrinsically linked within the aquatic environment, as shown in Fig. 1, but because different analytical approaches have been developed for OP and ON they are considered separately in this review. All abbreviations used in this review are listed in Table 1.

Research into the environmental chemistry of OP and ON has lagged far behind that of the larger organic carbon (OC) pool. As for OC, the OP/ON pool comprises a range of compounds of different functionality and molecular mass, from small molecules to proteins, in dissolved, colloidal and particulate phases, as shown in Fig. 2, and this complexity presents a significant analytical challenge. These components represent potentially bioavailable sources of P and N but their characterisation and quantification have been largely ignored in favour of inorganic P and N, principally due to a lack of suitable analytical methods. Hence this review describes approaches for the collection, extraction, treatment/conditioning and preconcentration of OP/ON from sediments and aqueous media, bulk OP/ON determinations, separation techniques for OP/ON and the characterisation and selective measurement of OP/ON components, derived from the growth and decay of living organisms, in aquatic environments. The determination of P and N in micro-organic contaminants (e.g. pharmaceuticals, pesticides, herbicides and personal care products) that are found in aquatic systems is not considered here. Reviews of the determination of these OP and ON compounds can be found elsewhere (e.g. [10–15]).

The fractionation of P and N components within the total P and N pools in aquatic systems is usually determined by applying operationally defined approaches. These are shown in Fig. 3 for P and Fig. 4 for N and are referred to throughout the review. However, the key driver for this review is the need to further characterise the OP/ON components within these operationally defined fractions in order to better understand their role in aquatic biogeochemical cycling and ecosystem function.

### 1.2. *Phosphorus*

The predominant inorganic species (i.e. mono- or di-protonated orthophosphate) is the most bioavailable P form and the emphasis for many decades has been on the study of its abundance and dynamics. However in many waters and sediments, the OP fraction, which includes nucleic acids, phospholipids, inositol phosphates, phosphoamides, phosphoproteins, sugar phosphates, amino phosphoric acids and organic condensed P species, is at least as abundant as inorganic P [16,17]. There is strong evidence that some organisms are adapted to access P directly from organic compounds via enzymatic hydrolysis and/or bacterial decomposition [18–23]. Abiotic hydrolysis and photolysis can also mineralise OP compounds to phosphate [24,25]. Despite the now acknowledged abundance of the organic P fraction in aquatic systems, the importance of this fraction is not widely recognised as a potentially large pool of bioavailable P [26].

Most dissolved organic P (DOP) compounds are non-reactive with molybdate, which is the basis of the classical and widely used phosphomolybdenum blue spectrophotometric method for phosphate determination [27]. Thus, information on DOP often results from indirect measurements, either as

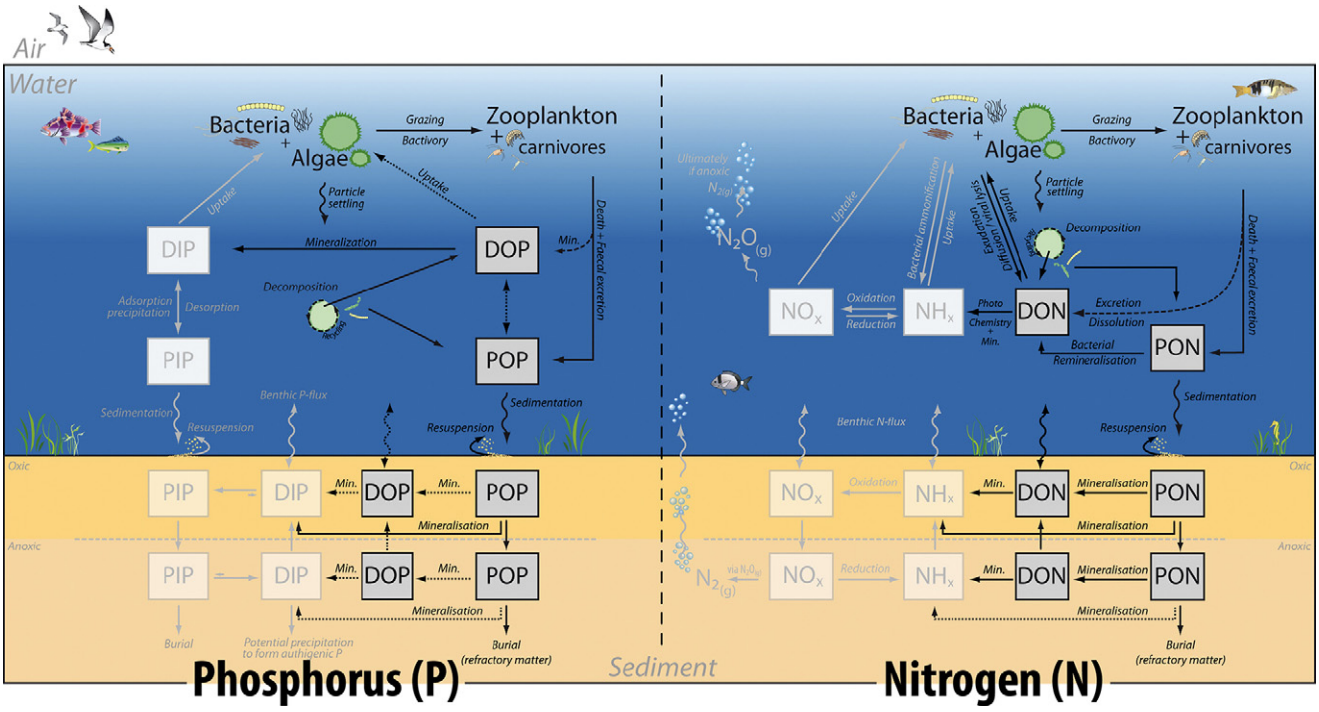


**Fig. 1 – Biogeochemical cycles of P and N highlighting the roles of OP and ON in the sediment and the water column. DOP: dissolved organic phosphorus; POP: particulate organic phosphorus; DIP: dissolved inorganic phosphorus; PIP: particulate inorganic phosphorus; DON: dissolved organic nitrogen; PON: particulate organic nitrogen; DIN: dissolved inorganic nitrogen.**

the difference between the total dissolved P (TDP) and dissolved reactive phosphorus (DRP), or as a measurement of DRP produced by a preliminary extraction or digestion step. Enzymatic hydrolysis procedures and techniques such as $^{31}$P nuclear magnetic resonance (NMR) spectrometry and soft X-ray fluorescence have only become readily available in recent years, thus enabling better determination and characterisation of organic P species [28–30].

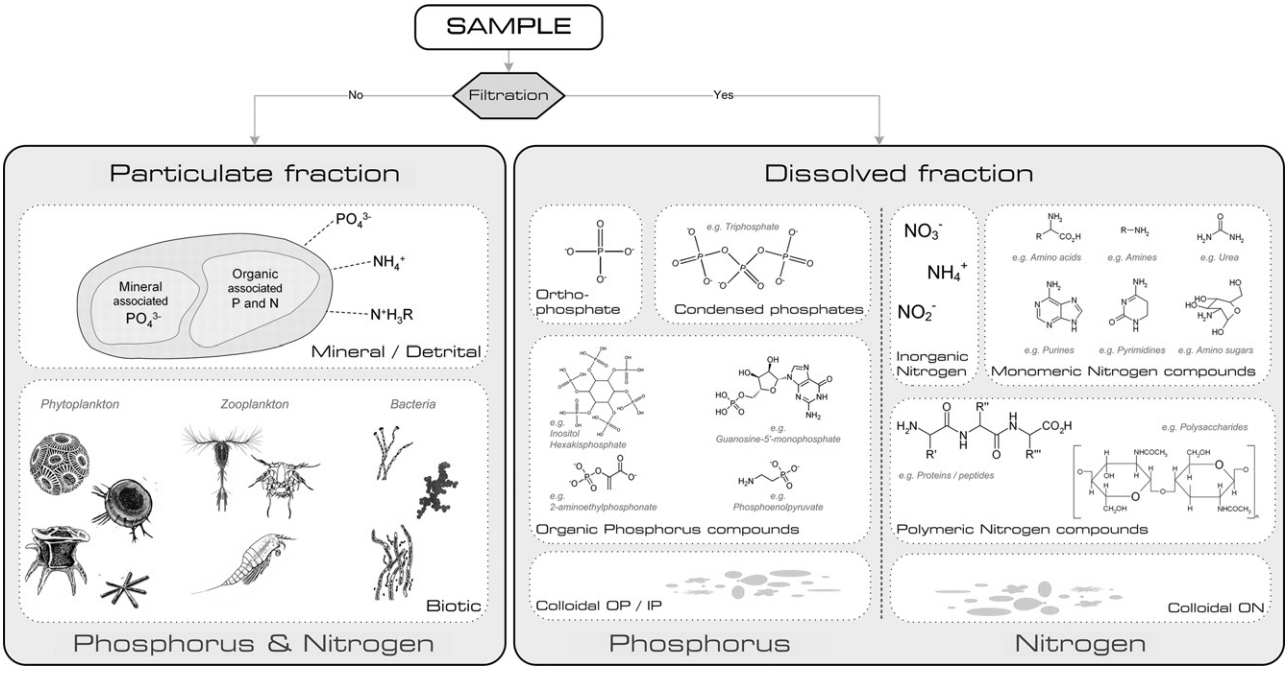


**Fig. 2 – Representation of typical P and N components in the operationally defined dissolved and particulate fractions of a total sample.**

**Table 1 – List of abbreviations used**

| | |
|---|---|
| 2D-PAGE | Two-dimensional polyacrylamide gel electrophoresis |
| AQC | 6-Aminoquinolyl-N-hydroxysuccinimidyl carbamate |
| CBB | Coomassie blue |
| CFF | Cross-flow filtration |
| CHN | Carbon hydrogen nitrogen |
| CP-MAS | Cross polarization magic angle spinning |
| CRM | Certified reference material |
| DCAA | Dissolved combined amino acids |
| DCP-MAS | Double cross polarization magic angle spinning |
| DFAA | Dissolved free amino acids |
| DGT | Diffusive gradients in thin films |
| DIN | Dissolved inorganic nitrogen |
| DIP | Dissolved inorganic phosphorus |
| DNA | Deoxyribose nucleic acid |
| DOC | Dissolved organic carbon |
| DOM | Dissolved organic matter |
| DON | Dissolved organic nitrogen |
| DOP | Dissolved organic phosphorus |
| DRP | Dissolved (molybdate) reactive phosphorus |
| EDTA | Ethylenediaminetetraacetic acid |
| ESI-MS | Electrospray ionisation mass spectrometry |
| FIA | Flow injection analysis |
| FID | Flame ionisation detector |
| FL | Fluorescence |
| FOP | Filterable organic phosphorus |
| FRP | Filterable reactive phosphorus |
| GC | Gas chromatography |
| GF/F | Glass (micro)fibre filter |
| GPC | Gel permeation chromatography |
| HMM | High molecular mass |
| HPLC | High-performance liquid chromatography |
| HPSEC | High-performance size-exclusion chromatography |
| HTCC | High-temperature catalytic combustion |
| ICP-AES | Inductively coupled plasma-atomic emission spectrometry |
| ICP-MS | Inductively coupled plasma-mass spectrometry |
| IEF | Isoelectric focussing |
| IPG | Immobilised pH gradient |
| kDa | Kilodalton |
| LC | Liquid chromatography |
| LC–$MS^n$ | Liquid chromatography–mass spectrometry |
| LMM | Low molecular mass |
| MAGIC | MAGnesium-Induced Coprecipitation |
| MISPE | Molecularly imprinted solid-phase extraction |
| MS | Mass spectrometry |
| NIRS | Near-infrared spectrometry |
| NMR | Nuclear magnetic resonance |
| NOM | Natural organic matter |
| NPD | Nitrogen-phosphorus detector |
| OC | Organic carbon |
| ON | Organic nitrogen |
| OP | Organic phosphorus |
| PHAA | Particulate hydrolysable amino acid |
| PHP | Phytase hydrolysable phosphorus |
| PIP | Particulate inorganic phosphorus |
| PN | Particulate nitrogen |
| PON | Particulate organic nitrogen |
| POP | Particulate organic phosphorus |
| SBSE | Stir-bar sorptive extraction |
| SDS | Sodium dodecyl sulphate |
| SDS-PAGE | Sodium dodecylsulphate-polyacrylamide gel electrophoresis |
| SEDEX | Sedimentary extraction |
| SIA | Sequential injection analysis |
| SPE | Solid phase extraction |
| SPM | Suspended particulate matter |
| SPME | Solid phase microextraction |
| TDN | Total dissolved nitrogen |
| TDP | Total dissolved phosphorus |
| TFF | Tangential flow filtration |
| TFP | Total filterable phosphorus |
| THAA | Total hydrolysable amino acid |
| TOP | Total organic phosphorus |
| TPN | Total particulate nitrogen |
| TPP | Total particulate phosphorus |
| TP | Total phosphorus |
| TRP | Total reactive phosphorus |
| UF | Ultrafiltration |
| UV | Ultraviolet |
| XANES | X-ray absorption near edge structure |
| XPS | X-ray photoelectron spectrometry |

### 1.3. *Nitrogen*

Nitrogen in aquatic environments comprises dissolved inorganic nitrogen (DIN, the sum of nitrate, nitrite and ammonium), dissolved organic nitrogen (DON) and total particulate nitrogen, TPN, which is almost wholly organic (i.e. TPN = PON). Most studies have focused on DIN, although it is now known that DON frequently comprises the largest part (60–69%) of total dissolved N (TDN) in lakes, rivers, estuarine and surface ocean waters. Reviews on DON in aquatic systems can be found in Bronk [31] and Berman and Bronk [32], building on an earlier review by Antia et al. [33]. A large fraction of DON is now known to be bioavailable [34–36] and provides the majority of N requirements in certain oligotrophic systems [34]. The low molecular mass (LMM) (<1 kDa) DON pool is quantitatively important, comprising 70–80% of marine DON [37,38]. In oceanic environments, ≤14% of the DON is identifiable at the molecular level, and this fraction includes monomeric and small polymeric compounds such as urea, amino acids, amines, amino sugars, purines, pyrimidines, nucleosides and nucleotides. Proteins and polypeptides, important components of the high molecular mass (HMM) polymeric ON pool (>1 kDa), have been quantified indirectly via hydrolysis to amino acids, or by non-specific assays [39]. In recent years enzyme-based microsensors have been developed to measure protein in river water and effluent [40]. One consequence of this general approach has been that molecular information has been lost, and it is only recently, through the development and adoption of better separations and instrumentation, that measurement of individual protein moieties has become possible. In the very large molecular mass humic and fulvic acids found in freshwater dominated environments, N is found either as an integral part of the acid molecule, or as part of a relatively low molecular mass molecule sorbed to the acid. The PON fraction comprises mainly proteinaceous compounds [41].

## 2. Sample collection, extraction, treatment and preconcentration

### 2.1. *Sample collection and initial treatment*

Various options are available for sample collection, depending on the location, sample type and fraction to be collected. For

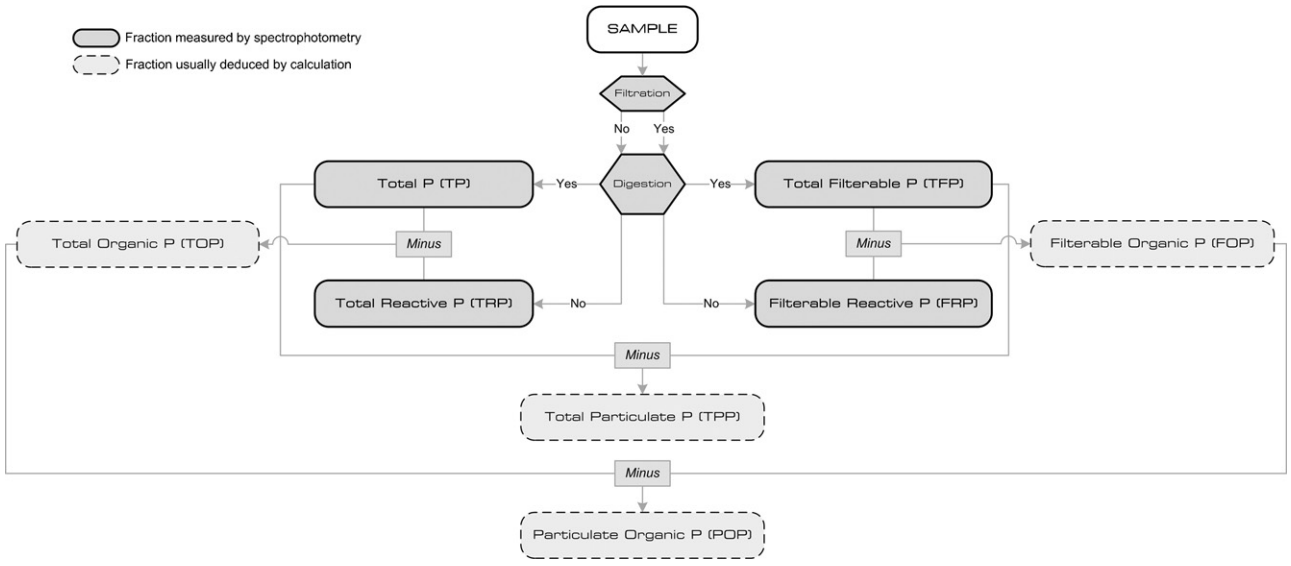


**Fig. 3 – Operationally defined P fractions in aquatic samples. TP: total phosphorus; TFP: total filterable phosphorus; TOP: total organic phosphorus; TRP: total reactive phosphorus; FRP: filterable reactive phosphorus; FOP: filterable organic phosphorus; TPP: total particulate phosphorus; POP: particulate organic phosphorus.**

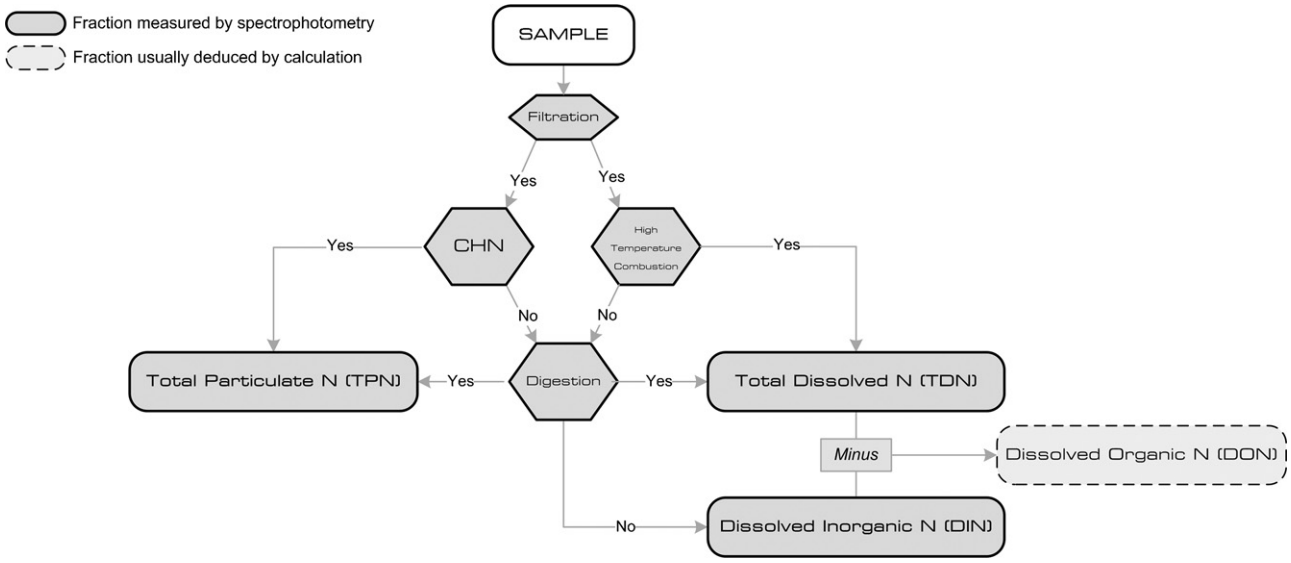


**Fig. 4 – Operationally defined N fractions in aquatic samples. TPN: total particulate nitrogen; TDN: total dissolved nitrogen; DON: dissolved organic nitrogen; DIN: dissolved inorganic nitrogen.**

sediments, surface scrapes using spatula-like devices are the easiest to use but provide limited sample mass or depth resolution. Montluçon and Lee [42] collected sediments from an estuarine saltmarsh using corers to obtain 10 cm deep cores. Within estuaries and shallow bays, grab samplers and box corers can be used, including Soutar corers [43,44]. Box corers and piston corers are suitable for continental shelf and open ocean environments [45–48]. Cores can be sub-sampled using acrylic or other plastic tubing, preferably within an inert atmosphere of $N_2$ or Ar gas, and then sliced [44,47,48]. Oxic, upper core sediment can also be sieved [48].

Suspended particulate matter (SPM) can be collected using a sampling bottle, the volume of which will depend on the turbidity encountered. Medium to high turbidity rivers, lakes and other surface waters can normally be sampled using containers of 10 L volume or less [49], while larger volume samplers (Niskin or Go-Flo type) are more appropriate for deployment in low turbidity marine waters [50]. Separation of SPM from whole water samples can be undertaken by centrifugation or filtration. For filtration, combusted glass fibre filters of nominal pore diameter 0.7 μm (GF/F) are typically used. While these filters do not separate all of the bacteria, viruses and small colloids from the filtrate, advantages include low contamination and relatively high flow rate [51,52]. If a smaller pore size is required (e.g. 0.45 μm or less), then aluminium oxide, cellulose acetate or polycarbonate filter membranes can be used.

Sediment traps or submersible pumps, suspended in the water column, can also be deployed [49,53,54]. Mesh nets of

64 μm and 355 μm size have been used for collecting water column suspended plankton for the determination of their PON content [44]. Another study, specifically for N, compared measured concentrations of PON in seawater samples collected by Niskin bottles and *in situ* pump/filtration systems [55]. The higher values of PON in SPM obtained from bottle-collected samples were thought to be because samples collected by pumping are biased away from larger or motile particulate matter including zooplankton.

Sediments and SPM collected for subsequent determination of the particulate P and N pools, or their individual components, can be stabilised for short periods by refrigeration [42] or longer periods by freezing at −20 °C or −70 °C [38,44] or freeze drying [44,46,56]. However, both freezing and freeze-drying of particulate matter can give rise to changes in operationally defined N pools, relative to determinations undertaken on fresh material [131]. Preserved sediment and SPM samples can be ground if required before further treatment, either lightly [48] or to some pre-determined size (e.g. <35 μm) [44].

For the determination of dissolved components, water from shallow freshwater and estuarine systems can be collected in cleaned glass or plastic bottles as described above for SPM [51,57,58]. Niskin, Go-Flo and stainless steel samplers are commonly used for sampling in deeper estuarine and marine waters [59]. Niskin and Go-Flo bottles are designed to minimise contamination and can pass through the DOM rich air–sea interface closed [60]. When collecting and treating water samples for the determination of DOP/DON it is recommended that the apparatus is cleaned overnight in nutrient-free detergent, leached for at least 24 h in 1.2 M HCl and rinsed using high purity water. Glassware should also be muffled (450 °C, 6 h) to remove surface bound organic matter [5,51]. Where DOP/DON or their individual components are at low or trace concentrations, clean techniques designed for trace metal analysis should be used in order to avoid sample contamination [61,62].

Preliminary sample treatment to collect the dissolved phase (operationally defined as that fraction which passes through a 0.7, 0.45 or 0.2 μm filter) usually involves filtration to remove particulate matter [5]. It is essential that filtration is carried out immediately after the sample is collected to prevent short term changes in P/N speciation. Polycarbonate or cellulose acetate membrane filters are recommended for dissolved constituents in natural waters. Filtration with a 0.2 μm filter is preferred as it removes the majority of bacteria and plankton that could otherwise alter dissolved OP/ON concentrations and speciation during storage [5]. Membranes used for filtration, including ultrafiltration (UF), must also be cleaned prior to use [63].

### 2.2. *Treatment and preconcentration for organic phosphorus determination*

#### 2.2.1. *Sediments and SPM*

The isolation of selected OP components more directly necessitates a pretreatment procedure, especially for sediments, usually by means of extraction. Depending on the study objective, the selected procedure might therefore quantitatively extract the total OP, fractionate the total P into discrete pools (including organic P) based on relative solubility, or extract a single P component. The nature of the extractant solution is then of primary importance as it not only influences the recovery of the organic fraction but also the composition of the extracted compounds. The choice of the extractant solution may also be dictated by the post-extraction analytical technique. For example with $^{31}$P NMR measurements, alkaline solutions are preferred in order to maximise the spectral resolution, although this may also have an effect on the stability of extracted species. When the conventional spectrophotometric DRP detection method is used, the nature of the extractant is less important because hydrolysis/oxidation is employed post-extraction to ensure that the OP is in the molybdate reactive, detectable form.

Sediment extraction procedures for P are numerous and most have been adapted from soil science [64,65]. Typically the OP fraction is determined by the difference between total P and inorganic P, which can be done using a simple extraction procedure or a more complex sequential extraction scheme. A major challenge is to avoid hydrolysis and oxidation of OP during the procedures used to extract inorganic P. In a study of five extraction procedures for the determination of the OP fraction in sediment, Sommers et al. [66] compared methods differing in principle and complexity. One method involving successive extraction with hot HCl, cold NaOH and hot NaOH was shown to be most suitable for the routine determination of OP in lake sediments. Later Aspila et al. [67] developed a much simpler, rapid and semi-automated method for the determination of inorganic, organic and total P in lake and river sediment. Organic P was determined by the difference in P content of a 1 M HCl extract measured before and after ignition (550 °C) of the dry sediment. The main errors arising with this technique are likely to be (1) potential hydrolysis of organic P species in 1 M HCl, leading to an overestimation of inorganic P, and (2) incomplete solubilisation of some inorganic P species in 1 M HCl (e.g. those associated with iron oxides), which would thus be defined as OP. However, the method is widely accepted and used in routine sediment and SPM analysis [68–76].

More complex sequential extraction procedures using a series of extractants, each chosen to selectively dissolve a single phase, have also been widely used for characterisation of the different P fractions, including OP, in sediments. Usually the specificity and efficiency of the different extraction steps, particularly for OP, are tested by extraction experiments on a wide range of inorganic and organic analogs to mimic natural sedimentary constituents. The different fractions that are obtained are operationally defined on the basis of the dissolution of a particular phase in the given extractant. The most commonly extracted P forms are (1) labile, exchangeable or loosely sorbed P, (2) Fe and Al-bound P, (3) Ca-bound P, (4) apatite and non-apatite P, (5) detrital P and (6) organic P. Usually the sequence is designed to remove the most labile phases first, and the chemical severity of the extraction increases with each following step. The OP fraction is usually considered to be the residual or refractory P containing fraction that remains after all other extractions had been performed. Rather than assigning a chemical designation, some fractionation schemes which use reagents that cannot specifically target a single chemical fraction designate the extracted fractions by the nature of the extractant (e.g. NaOH–P, [79]). Moreover,

**Table 2 – Most widely recognised extraction schemes for P speciation in soils and sediments (the targeted fraction for each extractant is shown in italics)**

| Author | Step 1 | Step 2 | Step 3 | Step 4 | Step 5 | Step 6 | Developed for | Reference |
|---|---|---|---|---|---|---|---|---|
| Chang and Jackson (1957) | 1 M $NH_4Cl$ | 0.5 M $NH_4F$ pH 8.2 | 0.1 M NaOH | 0.5 M $H_2SO_4$ | CDB | 0.1 M NaOH | Soil | [64] |
| | *Labile P* | *Al bound P* | *Fe bound P* | *Ca Bound P* | *Reductant soluble P* | *Refractory P* | | |
| Williams et al. (1976) | CDB at 85 °C | 1 M NaOH | 0.5 M HCl | | | | Soil | [65] |
| | *Non-apatite P* | *Fe and Al bound P* | *Apatite P* | | | | | |
| Aspila et al. (1976) | 1 M HCl | Ignition at 550 °C 1 M HCl | | | | | Freshwater sediment | [67] |
| | *Inorganic P* | *Total P* | | | | | | |
| Hieltjes and Lijklema (1980) | 1 M $NH_4Cl$ pH 7.0 | 0.1 M NaOH | 0.5 M HCl | | | | Freshwater sediment | [77] |
| | *Labile P* | *Fe and Al bound P* | *Ca bound P* | | | | | |
| Psenner et al. (1988) | 1 M $NH_4Cl$, pH 7.0 | CDB | 1 M NaOH | 0.5 M HCl | 1 M NaOH at 85 °C | | Freshwater sediment | [78] |
| | *NH4Cl extractable P* | *Buffer dithionite extractable P* | *NaOH extractable P* | *HCl extractable P* | *Refractory P* | | | |
| Ruttenberg (1992) | 1 M $MgCl_2$ pH 8.0 | CDB, pH 7.6 | 1 M Na-acetate buffer in acetic acid pH 4.0 | 1 M HCl | Ignition at 550 °C 1 M HCl | | Marine sediment | [81] |
| | *Exchangeable or loosely sorbed P* | *Easily reducible or reactive Fe bound P* | *CFAP + biogenic apatite + CaCO3 bound P* | *Detrital apatite + other inorganic P* | *Organic P* | | | |
| Jensen and Thamdrup (1993) | 0.46 M NaCl | BD, pH 7.0 | 0.1 M NaOH | 0.5 HCl | Ignition 520 °C 1 M HCl (boiling) | | Marine sediment | [275] |
| | *Loosely sorbed P* | *Fe and Mn bound P* | *Clays and Al bound P* | *Ca bound P* | *Residual/refractory organic P* | | | |
| De Groot and Golterman (1993) | 0.02 M Ca-NTA, 0.045 M dithionite, pH 8 | 0.05 M Na-EDTA pH 8 | 0.25 M $H_2SO_4$ | 2 M NaOH 90 °C | Digestion 1 g $K_2S_2O_8$ −2 mL $H_2SO_4$ | Phytase | Estuarine sediment | [79] |
| | *Fe bound P* | *Ca bound P* | *Acid soluble organic P* | *Fulvic acid P* | *Humic acid-P* | *Phytate P* | | |
| Vink et al. (1997) | 1 M $MgCl_2$ pH 8.0 | 1% SDS $HCO_3$-buffer pH 8.6 at 80 °C | CDB, pH 7.6 | 1 M Na-acetate buffer in acetic acid pH 4.0 | 1 M HCl | Ignition 550 °C 1 M HCl | Estuarine sediment | [83] |
| | *Exchangeable or loosely sorbed P* | *Organic P* | *Easily reducible or reactive Fe bound P* | *CaCO3 bound P + CFAP + biogenic apatite* | *Detrital fluoroapatite* | *Residue* | | |

CDB: citrate dithionite bicarbonate; BD: bicarbonate dithionite; NTA: nitrilotriacetic acid; EDTA: ethylenediaminetetraacetic acid.

some fractionation schemes not only determine DRP in each extract but also TP, thus allowing the determination of OP (or at least molybdate unreactive P) in each extract by difference [275]. Some of the most widely recognised schemes are shown in Table 2. Some extraction methods, e.g. [65,81], rely on an ignition step to oxidise the organic matter at 550 °C in a manner identical to the procedure of [67] who reported no serious loss of P by volatilisation for ignition times ranging from 2 to 16 h. However, De Groot and Golterman [79] raised the possibility that a significant portion of OP could be hydrolyzed by strongly alkaline or acidic solutions. Moreover, Ruttenberg's SEDEX scheme [81] clearly showed that a substantial fraction of P could be extracted from phytoplankton and zooplankton analogs by $MgCl_2$ (1 M), since 80% of the P in phytoplankton is soluble in distilled water. Even if the OP that reaches sediments is more refractory, having lost the more soluble and labile components in transit to the sediments, the selectivity of appropriate extractants with respect to OP is still a matter of debate. In order to better preserve the OP fraction, the extraction schemes of [79,80] use chelating agents such as EDTA (ethylenediaminetetraacetic acid). This enables the selective extraction of inorganic P associated with metal phases with less alteration of the OP fraction than with stronger extractants. Organic P is first extracted with 0.25 M $H_2SO_4$ and the remaining organic P with 2 M NaOH at 90 °C. Subsequent acidification of the alkaline extract enables separation of P associated with humic and fulvic acids by precipitation. Further hydrolysis using the enzyme phytase enables the determination of phytate (*myo*-inositol hexakisphosphate) in the extract containing P associated with the fulvic acids. The organic P fraction has also been extracted with surfactants such as sodium dodecyl sulphate (SDS) by a method originally developed by Robbins et al. [82] for deep sea sediments, and more recently adapted to estuarine materials [83]. In the latter study, SDS extraction was included in the early stages of the SEDEX sequential scheme, and was shown to efficiently extract OP, allowing efficient separation of the organic and inorganic P fractions. However, its utilisation in routine analysis has been limited because of the repeated treatments that are required to fully extract OP.

Organic solvents have also been used to extract some OP compounds and Suzumura [84] recently reviewed extraction procedures for phospholipids in marine samples. For sediment, a solvent mixture of chloroform, methanol and water [85] or dichloromethane and methanol [86,87] has been used. Procedures involving pressurised hot solvent [88] or a surfactant [89] have also been described. Other procedures for extraction of specific OP compounds, such as inositol phosphates, adenosine triphosphate, nucleic acids and nucleotides, are available for soils ([90] and references therein) but they have not been applied to sediments, possibly because of limitations in terms of extraction efficiency.

#### 2.2.2. *Waters (including sediment pore waters)*

As many OP compounds contain P–O–P and C–O–P bonds that need to be broken down in order to release inorganic P (as phosphate) for subsequent detection, the determination of OP in waters often necessitates a digestion step. This procedure is not selective and thus targets the total P fractions, i.e. TP for unfiltered samples and TDP for filtered samples, rather than individual OP components. The organic fraction is therefore usually calculated by subtraction of the DRP fraction from the total P concentration (see Fig. 3). A wide range of digestion methods is available, including acid digestion, fusion, dry ashing and, more recently, autoclaving, UV (ultraviolet) photo-oxidation and microwave heating. In all cases, high temperatures, high acidity and/or an oxidising environment are required to efficiently complete the conversion of OP and the release of inorganic P. Further details on bulk digestion procedures can be found in articles by Worsfold et al. [5] and Maher and Woo [91].

In water, the concentration of organic compounds can be much lower than in sediment. The determination of the OP fraction and its characterisation can therefore be difficult, especially when the characterisation tools are not highly sensitive (e.g. chromatography or $^{31}P$ NMR). To overcome this difficulty, different preconcentration approaches have been developed. As for sediments, there is the potential for alteration and degradation of some of the most labile OP compounds to occur during this pretreatment step.

For the determination of DOP samples are initially filtered as described in Section 2.1 and stored at 4 °C or −20 °C [5]. With an appreciation of the occurrence and importance of smaller sub-micrometre particles within the filtrate following this initial step, various approaches have been used to further separate and/or preconcentrate this material. Ultrafiltration based on tangential-flow or cross-flow filtration (CFF) has been used to fractionate dissolved organic matter on the basis of size. Typical UF membranes have a nominal 1 kDa cut-off (equivalent to $10^3$ nominal molecular mass or ca. 1 nm pore size), although membranes up to 30 kDa have been utilised [92]. The component retained by the chosen membrane (i.e. the fraction that does not pass through the membrane) is often referred to as the colloidal fraction, although how much of the retained organic matter is truly colloidal [93] has not been quantified. Reported advantages of UF include relatively rapid processing of large volumes of water, large concentration factors, minimum alteration of colloidal matter and the availability of a wide range of membrane types, surface areas and molecular mass cut-offs [94,95]. The permeation and retention behaviour of colloids has been extensively studied over the last 10–15 years, and has lead to more consistent performance of UF systems. However, there is conflicting evidence concerning the optimal operating conditions for UF [94,96,97] and validation. Calibration of each UF system must be undertaken before its use with environmental samples, including determination of actual membrane molecular mass cut-off, blank control, colloid retention, recovery and mass balance, and optimal concentration factors [94,95]. The deployment of molecular probes has been a key factor in this respect although most of the focus and effort has been on the examination of colloidal organic carbon [97].

CFF has been widely used for trace metal and radionuclide studies [95,98,99] but application to P has been much less common [100–105]. Samples separated by CFF can be further concentrated by rotary evaporation [102], freeze-drying or reverse osmosis [104,106]. Freeze-drying is not recommended as it can potentially involve some physical and chemical modification of the sample [107]. The main problems associated with the CFF technique for P studies have been described

in detail by Bauer et al. [100] and mainly consist of contamination and scavenging effects, both prior to and during sample processing. Contamination can arise from the different components of the various CFF systems and the presence of contaminants in the CFF cleaning reagents that have not been adequately flushed from the system prior to sample processing. For P, scavenging effects arise mainly from sorption by organic or inorganic (e.g. Fe- or Mn-oxyhydroxides) colloids during sample processing. Another problem associated with the CFF technique is the possible production of colloidal OP artefacts via the association of inorganic P with organic molecules. Recommendations on cleaning and preconditioning procedures have been suggested. Buesseler [108] and Guo et al. [109] recommended that a concentration factor (i.e. the ratio of initial sample volume to final retentate volume), of >40 should be achieved in order to minimise the retention of dissolved P with a molecular mass less than the ultrafiltration membrane cut-off.

Significant preconcentration of dissolved P can be obtained by coprecipitation techniques, including the MAGIC procedure (MAGnesium-Induced Coprecipitation) [110,111] although they are not often used for DOP. Stevens and Stewart [112] used an adsorption–precipitation technique involving lanthanum hydroxide at pH 6.5 to preconcentrate DOP by up to 100-fold. More recently, Rumhayati et al. [113] developed a diffusive gradient in thin film (DGT) binding gel with lanthanum hydroxide to accumulate DIP and DOP from sediment pore and overlying waters. Lee and Lal [114] developed a technique for binding and concentrating DIP and DOP using iron(III) hydroxide-coated acrylic fibres from large volumes of water. However, the efficiency and accuracy of this technique is questionable following reports showing quantitative hydrolysis of DOP compounds at the mineral surface [24,25,115]. Nucleic acids have been determined by precipitation with cetyltrimethylammonium bromide [116] and inositol phosphates can be precipitated with iron and barium salts after selective oxidation of DOP with hypobromite [117].

Preconcentration of OP compounds can be achieved using liquid–liquid extraction methods. Cloud-point extraction with non-ionic surfactants offers simple and fast extraction and preconcentration of OP compounds. A detailed description of this technique is outside the scope of this review and readers are directed to the work of Carabias-Martinez [118]. In order to minimise or eliminate the use of organic solvents, sorption techniques based on solid phase extraction (SPE) of DOM onto macroporous resins, based on the chemical, rather than physical, properties of the organic compounds, can be used. Non-ionic styrene-divinylbenzene (XAD-1, XAD-2, XAD-4) and acrylic-ester (XAD-7, XAD-8) Amberlite resins have generally been used to separate the DOM pool in both fresh and marine waters into broadly hydrophilic and hydrophobic components, which can then be separated into acidic, neutral and basic fractions using sequential elution [119–121]. Related techniques, e.g. solid phase microextraction (SPME), molecularly imprinted solid-phase extraction (MISPE) and stir-bar sorptive extraction (SBSE) have also been developed. These techniques allow the preconcentration of OP compounds from large volumes of aqueous sample, including drinking water [67,122–125]. One example of the use of SPE for the preconcentration of natural OP compounds is the isolation of phospholipids from DOM in coastal waters [126].

### 2.3. *Treatment and preconcentration for organic nitrogen determination*

#### 2.3.1. *Sediments and SPM*

Whilst PON can be determined using an elemental analyser (Section 3.2.1), other methods for the determination of PON incorporate a digestion step to convert N into an inorganic species, which is then quantified. The two most common approaches are the Kjeldahl digestion and the peroxodisulfate (also referred to as persulphate) digestion (also referred to as wet oxidation). In the Kjeldahl method, N in the −3 oxidation state is converted to ammonia, which is subsequently distilled and measured by titration, spectrophotometry or with an ion-selective electrode. The N in azide, azine, azo, hydrazone, nitrate, nitrite, nitrile, nitroso, oxime and semicarbazone components is not determined and particulate ON is determined after the removal of ammonia [9]. This has the advantage of ensuring optimum measurement precision because of the need, otherwise, to subtract the ammonia content. Peroxodisulfate digestion is generally undertaken under alkaline conditions and converts all inorganic and organic N components to nitrate [9]. For a 10 mL sample a typical detection limit is ca.10 $\mu$mol N $L^{-1}$, depending on the method chosen for nitrate detection. Particular challenges with this method include control of the blank, the amount of sample handling and the time-consuming nature of the process. Reported applications include the determination of PON in marine SPM collected on a range of filter types, with the method being adapted for shipboard use [52,127,128].

Major components of PON include amino acids, either free or combined as proteins and peptides. These moieties are generally associated with living and detrital material, although some will be sorbed to mineral phases within the sediment [47]. The standard method for examining the amino acid component of the PON involves extraction with 6 M HCl at 100–110 °C for 20–24 h or at 150–170 °C for 1–4 h [129]. This method hydrolyses peptide bonds [38] and solubilises non-protein amino acids, to yield amino acids which can then be determined by HPLC. Hexosamines can also be extracted using this approach [56]. The ON extracted into the hydrolysate, termed the total or particulate hydrolysable amino acid (THAA or PHAA), is typically <50% of the total PON. Losses of amino acid during hydrolysis and sample handling are estimated by spiking the sample with charge-matched amino acids [44,46,130]. Nunn and Keil [48] examined six methods for extracting PON from coastal marine sediments to identify reagents that could remove proteins without destroying the integrity of the molecules, thereby allowing further structural analysis by advanced macromolecular techniques, such as gel electrophoresis or mass spectrometry (MS). The extraction methods were compared with the standard HCl digest for amino acid recovery. Treatment of the sediment with 0.5 M NaOH at 37 °C for 2 h extracted the largest fraction of amino acids (ca. 60% of the total recovered by acid hydrolysis) while yielding a similar amino acid profile. The other reagents were less efficient in terms of total amino acid recovery (Triton X-100 ≥ hot water > $NH_4HCO_3$ > HF) but the extracts contained

distinctly different amino acid profiles, enriched in acidic amino acids and depleted in basic amino acids compared with the HCl extract. It was suggested that these reagents were preferentially extracting hydrophilic portions of proteins that were probably in solution, while more hydrophobic portions were too strongly attached to the sediment surface to be recovered under these conditions. A combination of reagents may therefore be helpful for identifying the bioavailable and recalcitrant portions of protein molecules in sediments. THAA may not represent the bioavailable PON if not all the amino acid is proteinaceous [47] and digestion methods using enzymes have been developed to provide a better estimate of bioavailable PON [131]. Other methods have been developed to isolate particulate protein directly; particularly in plankton dominated suspended sediments [39].

#### 2.3.2. *Waters (including sediment pore waters)*

Centrifugation is a common method for collecting pore waters from sediments for subsequent bulk DON analysis, although DOM can be released by fauna in heavily bioturbated sediments [132]. Centrifugation is not recommended for pore water extraction if dissolved free amino acids (DFAAs) are to be measured, due to the introduction of artefacts, and squeezing may be a preferred option, followed by filtration of the pore water [43,133,134]. Filtration of samples collected for all types of DON determinations should be undertaken immediately, or as soon as practicable, after collection to minimise changes to the DON pool from biological and other activity [60]. Filtration should be undertaken at low pressure (<20 kPa) to avoid cell lysis at the filter surface and subsequent loss of cell ON to the filtrate. Samples for the determination of DON are typically filtered through ashed glass fibre filters of 0.7 μm nominal pore diameter [135], or other membrane types with pore sizes in the range of 0.02–0.45 μm [51,63], as noted earlier. For dissolved amino acids, Fuhrman and Bell [62] recommended filtration using 0.2 μm pore diameter membranes, although dissolved combined amino acids (DCAAs), including proteins, have been isolated using 0.45 μm and GF/F filters [136,137].

Filtered samples for the determination of TDN are typically acidified and then stored cold or frozen [138] although rapid freezing alone also appears to be acceptable [139,140]. Acidification to pH 2–3 is achieved using 10 μL 50% (v/v) HCl or $H_3PO_4$ per 10 mL sample. Artefacts arising from acidification, including sorption of atmospheric ammonia and loss of volatile organic compounds [60], are likely to be small. Sealed glass vials are preferred to plastic containers, as DOM may leach from the latter over time and contaminate the sample. Acidified and cold-stored (at 4 °C) samples are stable for at least 4 years [59]. $HgCl_2$ should not be used to stabilise the analyte if the TDN is going to be measured by high temperature catalytic combustion [141].

Samples for the determination of dissolved free amino acids and dissolved combined amino acids are generally stored frozen (−20 °C) in the dark [136]. Pore waters can be flushed with $N_2$ prior to freezing [47]. Samples for the determination of individual DON components, including urea, methylamines and protein are filtered (0.2 μm, 0.45 μm, GF/F) and stored frozen (−20 and −30 °C) or freeze-dried prior to further separation steps and/or analysis [134,142,143]. There are few data on the isolation and preservation of other DON components, e.g. purines, pyrimidines [32].

Ultrafiltration, discussed in detail in Section 2.2.2, has also been applied to DON. Systematic studies on the efficacy of UF for the investigation of marine DON were first reported in the last decade [100]. The use of UF to isolate DON and its components has been reported for lake [92,144], river, estuary [145,146] and marine waters [147,148].

Other separation methods based on size include reverse osmosis and gel-permeation chromatography (GPC; also known as high-performance size exclusion chromatography, HPSEC), but these have not been used to any great extent for the isolation of colloidal DON. In GPC, molecules larger than the gel pores move rapidly through the column while the smaller molecules penetrate into the pores of the gel; different effective travel path lengths lead to separation of compounds with different sizes. Molecular mass calibration graphs must be made using appropriate standards. Gjessing et al. [149] described the deployment of a field-based reverse osmosis system on a Norwegian lake and reported recoveries of DOC of 90–93%. DON recovery was not given, although the retained water was subsequently analysed for total and organic N and free and hydrolysable amino acids [149,150]. Additional Norwegian freshwaters were subject to reverse osmosis and determinations made of total N, hydrolysable amino acids, proteinaceous materials and *N*-acetylamino sugars [151,152]. Egeberg et al. [92] and Tuschall and Brezonik [144] used GPC on filtered lake water and subsequently determined peptides and proteins on the separated fractions. Egeberg et al. [92] undertook GPC on filtered and UF fractionated (<0.5 kDa) lake waters for the subsequent determination of DON by peroxodisulfate digestion and spectrophotometry.

Information on the use of SPE techniques for the isolation of DON is sparse. Lara et al. [121] used sorption onto XAD-2 to examine changes in concentration and organic N composition of hydrophobic acid, hydrophobic neutral and hydrophilic DON pools during the growth cycle of a marine alga. Using mass balance, they reported quantitative (90–110%) recovery of sorbed DON from this resin. Silica-based C-18 stationary phases can also be used to separate DOM from waters, but there are few reports of their use to isolate DON. Schwede-Thomas et al. [153] isolated DOM from a range of fresh waters using SPE C-18 cartridges, XAD columns and UF, and found that there were no significant differences in the molecular properties of the DOM separated by each method when the DOM was primarily allochthonous, but that differences did occur when the DOM was from autochthonous sources.

Electrophoresis can also be used to separate individual N containing proteins. Schmitt-Kopplin and Junkers [154] provide an overview of the role of electrophoresis in the study of natural organic material. In freshwater and marine studies, individual proteins are commonly separated and detected using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) [142,155]. This separation technique is discussed in more detail, in conjunction with various detection systems, in Section 3.2.5. Precipitation is also used as a concentration and purification step during the analysis of proteins in seawater. Tanoue et al. [142] used trichloroacetic acid as the co-precipitate, whilst Powell et al. [148] employed methanol/chloroform/water precipitation. Schulze [143] used

ethanol to precipitate proteins in reconstituted freeze-dried stream and lake water samples.

Filtered samples can be digested in order to determine DON or a component thereof. Kjeldahl digestions have been employed for the subsequent determination of DON in lake waters by Tuschall and Brezonik [144], whilst Raimbault et al. [53] adapted the method for seawater on board ship, reporting ca. 5% precision for lagoon and coastal waters. However, the method is not suitable for human-impacted freshwaters containing high concentrations of nitrate ($\geq$700 $\mu$mol $L^{-1}$), because nitrate interferes with the measurement of organic N through the oxidation of ammonia released from organic compounds. Dafner et al. [156] employed microwave-assisted peroxodisulfate digestion in the determination of DON in samples from the North Sea and Scheldt Estuary. Egeberg et al. [92] used in-line peroxodisulfate digestion of UF dissolved organic matter from Norwegian lakes in order to examine the distribution of DON among different size classes. UV photo-oxidation is also used for the determination of TDN in waters, with all N species being converted to nitrate that is detected by the Griess method [157] or UV absorbance [158].

Mace and Duce [159] outlined the advantages and disadvantages of peroxodisulfate digestion and UV oxidation in relation to the determination of DON in rainwater. Results from intercomparison exercises of these methods with high temperature combustion for the determination of DON in aquatic samples are discussed in Section 3.2.2. Other digestion methods for TDN include photo-catalytic combustion with and without a catalyst ($TiO_2$ or Pt/$TiO_2$) [160,161].

DCAA are usually determined following acid hydrolysis in 6 M HCl at 110 °C for ca. 20 h [162]. A more rapid (23 min) method was subsequently developed by Tsugita et al. [163], using acid hydrolysis (HCl plus trifluoroacetic acid) at 156 °C. Keil and Kirchman [136] applied this method, known as vapour phase hydrolysis, to a range of estuarine and marine samples and found that DCAA concentrations were 0.8–3 times higher using vapour phase hydrolysis. They concluded that the latter method recovered amino acids in an unknown chemical form that was recalcitrant under the usual hydrolysis conditions. Recovery of the amino acid tryptophan was shown not to be reproducible or quantitative during the acid hydrolysis of protein samples without the addition of a reducing agent, and an alkaline hydrolysis is used instead [164,165]. In waters with >40 $\mu$M nitrate, ascorbic acid is added prior to hydrolysis to prevent oxidation of amino acids by nitric acid [130,166]. Digestion methods for other components of the DON pool have not been reported.

## 3. Determination of organic phosphorus and organic nitrogen components

### 3.1. *Organic phosphorus*

#### 3.1.1. *Molecular spectrometry*

In the past two decades NMR spectrometry, especially $^{31}$P NMR, has become an increasingly powerful tool for the characterisation of OP compounds in soils [17] and aquatic systems [24,29,73,167–175], as shown in Fig. 5. In sediment for example, characterisation of OP from lakes and estuaries revealed a

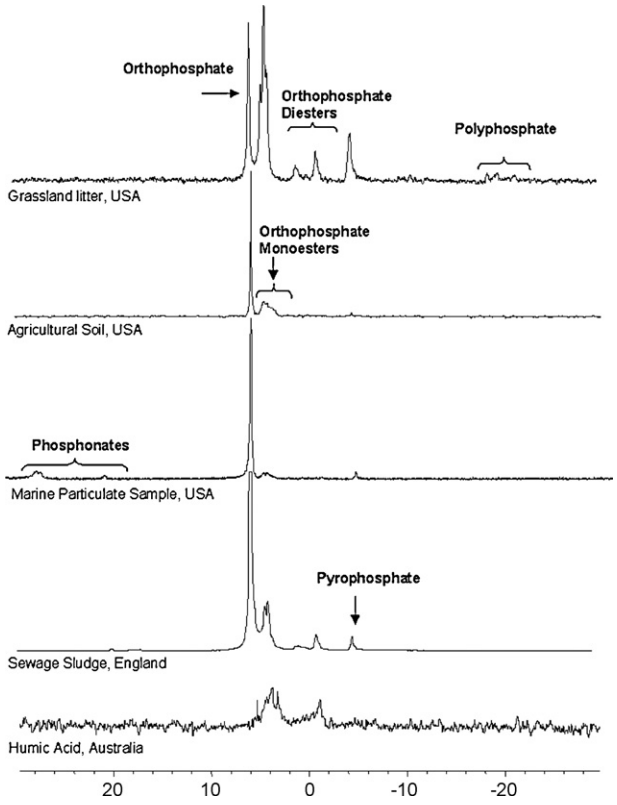


**Fig. 5 – 31P-NMR spectra of different phosphorus species in a range of environmental and agricultural matrices. Reprinted from B.J. Cade-Menun, Talanta 66 (2005) 361, with permission from Elsevier.**

wide range of P compounds (e.g. orthophosphate, polyphosphate, pyrophosphate, phosphate monoesters and diesters) but not phosphonate [174,176]. The presence of pyrophosphate at high concentrations in some estuarine sediments has been attributed to human activities [175]. In water, $^{31}$P NMR spectrometry has identified a range of P compounds, including phosphonates, pyrophosphate, polyphosphate, phosphate monoesters and diesters with spatial and temporal variability [103]. $^{31}$P NMR has also shown that marine DOP is chemically distinct from particulate OP [171].

An extensive description of NMR spectrometry is beyond the scope of this review but is available elsewhere [177,178]. However, the basic principle of NMR lies in exploiting the magnetic properties of the atomic nucleus, providing information about the bonding characteristics of the P atom and therefore the nature of the P-containing species present in the sample. Due to its natural abundance as a single isotope of spin 1/2, $^{31}$P is ideally suited for NMR studies. In principle any P-containing species can be detected, the amount of each being determined from the area of an individual signal peak which is proportional to the number of P nuclei present. However, the natural abundance of $^{31}$P is comparatively low and this, together with chemical shift anisotropic line broadening and the presence of any paramagnetic species such as iron in the sample, reduces resolution dramatically [54]. Thus, sample preparation plays a key role in achieving good resolution NMR spectra. Phosphorus nuclei in both organic and inor-

ganic species generally give signals between 25 and −25 ppm, including phosphonate (20 ppm), orthophosphate (5–7 ppm), phosphate monoesters (3–6 ppm), phosphate diesters (2.5 to −1 ppm), pyrophosphate (−4 to −5 ppm) and polyphosphate (−20 to −22 ppm). A detailed description of the NMR spectrum of each P-containing species has been reported [17,90].

$^{31}$P NMR spectrometry can be performed either in the solid-state or in solution (high resolution $^{31}$P NMR). With the exception of drying, very little sample preparation is usually required with solid-phase $^{31}$P NMR. Chemical changes in the sample are minimised during pretreatment although the drying step has been identified as causing changes in the intrinsic P composition of samples, most likely due to degradation of pyrophosphate [179]. In solid-state $^{31}$P NMR only the phosphonate peak is resolved, with the remainder of the spectrum consisting of a broad signal of overlapping peaks from other P containing species such as orthophosphate, phosphate monoesters and diesters.

With solution $^{31}$P NMR, the extraction step enables OP to be concentrated as well as allowing removal of interfering paramagnetic ions. Aqueous samples usually provide much better resolved spectra than solid samples, and give a better separation of peaks from different P compound classes (e.g. phosphate monoester and diesters) and sometimes specific P species (e.g. deoxyribose nucleic acid (DNA)). Extraction and solubilisation generally use NaOH alone or in combination with either the cation exchange resin Chelex, NaF or EDTA. Chelex and EDTA are both used to release P from the interfering paramagnetic ions. While the Chelex removes any paramagnetic ions from solution before measurement, extraction with EDTA leaves the paramagnetic ions in solution in a complexed form, and thus enhances the potential for line broadening. These extraction procedures are also sometimes supplemented by pre- or post-treatments involving acid or dithionite [169,180] and dialysis, or ion exchange resins [181,182]. Because of the different protocols used as well as the potential risk of species hydrolysis [183], the form of P extracted is likely to be significantly different, both quantitatively and qualitatively, from any P-containing species present in the original sample. This makes comparison between studies limited and sometimes irrelevant. Aware of these issues, Cade-Menun et al. [179] has investigated the effects of sample collection, storage and preparation for marine particulate samples. Significantly, these authors found that NaOH–EDTA extraction removed the majority of organic esters, but only a variable portion of phosphonates (39–67%). A preferential extraction of Ca-associated phosphate-containing species over Mg-, Fe- and Al-associated phosphates was also observed. Despite the fact that solution $^{31}$P NMR gives much better resolved spectra than solid state $^{31}$P NMR, they recommend that both variations of $^{31}$P NMR be undertaken on a given sample whenever possible, but especially when examining inorganic P-containing species and when studying the abundance of phosphonates. While solution and solid-state $^{31}$P NMR are well-proven techniques for P speciation, detection limits are generally about 1000-fold higher than methods based on spectrophotometry.

Near infrared reflectance (NIR) spectrometry has rarely been used to characterise OP compounds although Malley [184] and Malley et al. [185] determined the organic carbon, ON, and OP content of sediments from the Great Lakes. Calibration was acceptable for carbon and N but the determination of OP was more difficult because particle size and mineralogy affected the NIR spectra [186].

Flow injection analysis (FIA) and its variants, e.g. sequential injection analysis (SIA), coupled with spectrophotometric detection, have been widely used to determine DIP in marine, river, and estuarine water, sediments and leachates [187–190] but has been less frequently applied to the determination of specific DOP components [30,191]. FIA has the advantage of allowing determinations to be carried out *in situ* and in real time [188] with detection limits in the $\mu g\,P\,L^{-1}$ (30 nM) range with excellent precision. Two reviews concerning the use of FIA (and other flow techniques) to determine P species in the environment (predominantly inorganic species) have been published recently. That by Estela and Cerda [192] covers OP determinations in the environment, including signal detection processes, while Worsfold et al. [5] discuss sampling and quality assurance issues.

### 3.1.2. *Atomic spectrometry*

Inductively coupled plasma-atomic emission spectrometry (ICP-AES) has become a standard analytical method for determining total and inorganic P concentrations in soils [193]. This study reported experiments on soils from several archaeological digs and compared results obtained using ICP-AES with those obtained using spectrophotometry. They concluded that the concentration of OP (TP–TDP) at each of the three sites depended more on the method used to extract the P from the soil than on the method used for P determination. Inductively coupled plasma-mass spectrometry (ICP-MS), particularly when coupled with HPLC, is a powerful technique for the determination and speciation of P in nucleic acids, proteins and other biologically important molecules containing this element [194] while high resolution MS enhances this capability [195]. Mass spectrometry will continue to develop as a technique for P speciation, as more sophisticated and precise methods of ionisation in the mass spectrometer, e.g. electrospray, permit a more varied suite of P-containing compounds to be determined.

Soft X-ray fluorescence spectrometry in the form of X-ray absorption near edge structure spectrometry (XANES) adds a further dimension to the determination of OP containing species [28,196] because, as well as being able to provide quantitative and qualitative information, it has sub-micrometre spatial resolution and can therefore aid in determining the atomic environment of the P atom. For single moieties such as inorganic phosphates, pyrophosphates and phosphonates, where P is in the +5 valence state, the XANES spectrum is well defined and characterises the type of P-containing compound. However, for samples where there is considerable heterogeneity, such as marine sediments, P-species identification is more difficult and comparison with standard spectra is required [28].

One important advantage of XANES spectrometry over other forms of OP determination is that it can be used *in situ* to study heterogeneous environments, i.e. it is possible to determine P-containing species in mixtures of two substrates at the same time without drying out or cooling the sample prior to analysis. However, although XANES spectrom-

etry is potentially a powerful tool for the speciation of OP compounds it is more commonly used to determine bonding characteristics associated with inorganic phosphates and OP species adsorbed on aluminium hydroxides, phytic acid, and complex iron- and calcium-containing compounds [197] and aluminium and iron oxide minerals [198].

#### 3.1.3. *Enzymatic methods*

Phosphatases catalyse chemical reactions that release inorganic P (as phosphate) from OP molecules containing P–O–P and C–O–P bonds which is subsequently detected. The use of substrate-specific phosphatase enzymes therefore allows the OP fraction to be characterised and sometimes classified into several functional groups. Despite their widespread use for the characterisation of OP in soil and manure [183,199–204], enzymatic hydrolysis techniques have had limited application to natural waters and sediments [30,105,205–211]. This is due to the difficulties involved in accurate detection of orthophosphate produced by enzymatic hydrolysis from the usually low concentrations of DOP in natural waters.

Alkaline phosphomonesterase (also called alkaline phosphatase) is the most commonly used enzyme, but other phosphatases, including phosphodiesterase, phospholipase and phytase, have also been used to characterise the OP pool. Studies have investigated the substrate specificity of commercially available phosphatases with a wide range of OP model compounds [30,204]. They have shown that most of these enzymes are substrate specific with alkaline phosphatases quantitatively hydrolysing condensed P compounds and phosphate monoesters (with the exception of phytate). Alkaline phosphodiesterase was shown to successfully target phosphate diesters such as DNA and bis-(para-nitrophenyl) phosphate, but no activity was detected toward phosphate diesters like phospholipids. Finally phytase was not substrate-specific and hydrolysed all ester–P bonds. Negligible hydrolysis was always observed for all these enzymes towards the phosphonate. Turner et al. [204] showed that combining these phosphatase enzymes allows the identification and classification of functional classes of OP in soil water-extracts. More recently, Monbet et al. [30] developed a similar protocol to assess the enzymatically hydrolysable fraction of DOP in estuarine sediment porewater and sewage effluent. The various DOP components characterised by this approach are shown schematically in Fig. 6. These authors highlighted the potential interferences of multivalent metallic cations on enzymatic methods and recommended the use of tri-sodium citrate for prevention. A surfactant such as sodium dodecycl sulphate was also used to prevent coating on the walls of the tubing and the formation of insoluble, ion association complexes between enzyme proteins and phosphomolybdenum blue during the determination of DRP.

Enzymes can be used either in conventional batch reactions or immobilised in reactors. With the batch method, enzymes are usually prepared in a buffered solution with pH adjusted to give the maximum enzyme activity. The main buffers used are generally Tris–HCl and glycine–HCl. However, Tris buffer is not recommended when using phospholipase C as it may inhibit enzyme activity [212]. All buffer solutions are usually supplemented with a natural activator such as $Mg^{2+}$. Some authors recommend the use of a bacterial inhibitor such as sodium azide ($NaN_3$) to prevent microbial growth in the final assay step. Suzumura et al. [105] and Monbet et al. [30] found no difference in the results of hydrolysis with or without $NaN_3$ for filtered samples (<0.2 μm). Whilst older enzymatic protocols used a high concentration of enzymes, probably to minimise the product inhibition effect, the most recent protocols have markedly reduced the enzyme concentration in the final assay and no product inhibition is observed, even in the presence of high concentrations of fully hydrolysed OP compounds.

The immobilised enzyme reactor approach has been used in a number of studies. For example, Shan et al. [210] used immobilised alkaline phosphatase in a flow injection manifold to perform rapid on-line enzymatic hydrolysis of DOP and detection of the released phosphate as DRP. Amini and McKelvie [213] developed a sensitive and selective flow injection method for the determination of phosphatidylcholine in sediment pore waters and extracts. This involved the use of phospholipase C, alkaline phosphatase and choline oxidase co-immobilised on controlled pore glass in a packed column reactor. McKelvie et al. [3] and more recently Omaka et al. [191] developed flow injection methods for the determination of phytase hydrolysable phosphorus (PHP) using immobilised phytase. The immobilised enzyme approach has several advantages compared with conventional batch or automated flow methods using soluble enzyme reagents, including (1) the possibility to use the reactor for several hundred enzymatic hydrolysis experiments before activity declines, (2) reaction time is extremely rapid (minutes) compared with batch method (sometimes hours) and (3) there is little risk of phosphate inhibition of the enzyme as the reacted sample and products are usually quickly transported away from the active sites. However, when soil extracts are analysed, adsorption of iron hydroxides or natural organic matter to the enzyme reactor can cause rapid loss of enzyme activity.

### 3.2. *Organic nitrogen*

#### 3.2.1. *Carbon hydrogen nitrogen (CHN) analysis*

Particulate N in sediments and SPM can be determined directly by elemental analysis. The N is converted to nitrogen gas at high temperature and quantified using thermal conductivity detection. Analysis is undertaken on SPM retained on low-organic matter filter membranes or on 'loose' sediment. Because of the relatively small sample mass or volume that can be introduced into some elemental analysers, SPM on filter membranes needs to be sub-sampled a number of times (by cutting circles using a cork borer for example) and the sub-samples combined for the analysis. If there is marked heterogeneity in the distribution of PON over the membrane then the resulting concentration measurement can become inaccurate and/or imprecise. A number of certified reference materials (CRMs) are available for quality control. If organic C is to be simultaneously determined then inorganic C must be removed; this is particularly important if the sediments are carbonate or sulphide-rich. Nieuwenhuize et al. [214] and Yamamuro and Kayanne [215] have developed methods for eliminating the inorganic C in a wide range of sediment types and carbonate contents using HCl or HCl vapour.

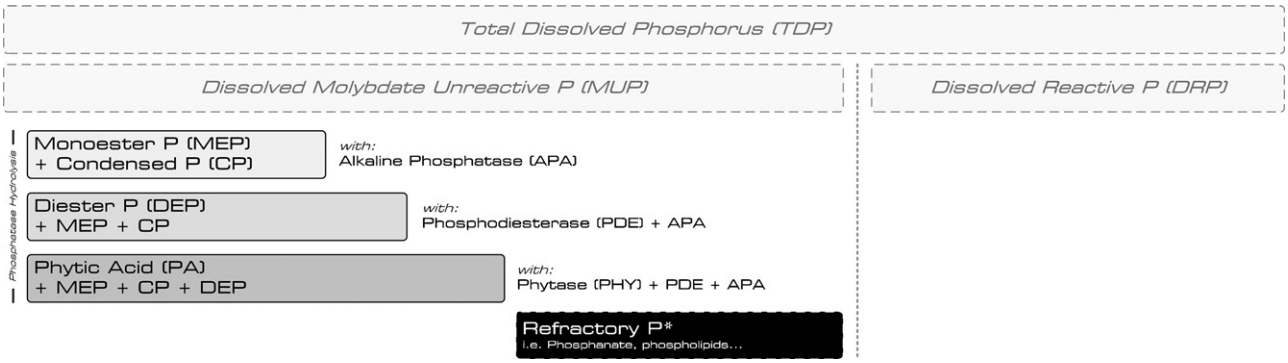


**Fig. 6 – Schematic diagram of the different components of the DOP pool determined using enzyme hydrolysis under environmentally relevant conditions.**

### 3.2.2. *High temperature combustion (HTC)*

The determination of TDN is achieved through combustion of filtered samples at high temperature (680 °C with catalyst, 850 °C with catalyst and 1000 °C without catalyst) to yield gaseous N oxides which are detected by chemiluminescence [135]. Whilst this approach is frequently referred to as high temperature oxidation, particularly when DOC is simultaneously analysed, the combustion of TDN to NO, the measured species, involves both oxidation and reduction. For this reason we refer to the technique in relation to TDN determination as high temperature combustion. High temperature combustion has been applied to freshwater, estuarine and marine samples [57,216–218]. In some cases, measurements have been made in the field [219]. The detection limit can be as low as 1 μM N [51]. The method suffers from the same disadvantage as other approaches (peroxodisulfate digestion, UV oxidation) that are used to determine TDN in that DON is calculated after subtraction of DIN, resulting in reduced precision for the DON concentration in waters with high DIN [135]. To counter this, Lee and Westerhoff [220] employed dialysis pretreatment on surface and drinking waters to decrease the loss of precision in the DON measurements by high temperature catalytic combustion (HTCC) by reducing the DIN concentration; it was particularly effective at DIN:TDN ratios >0.6. Sharp et al. [135] reported an instrument comparison for the determination of TDN in seawater (estuarine, coastal and oceanic). They observed that the measurements undertaken at 680 °C with catalyst were the most precise, perhaps because of reduced salt interference. At the higher temperatures noted above, sublimation of NaCl occurred, resulting in damage to the instrument and reduced analytical precision [221]. Sharp et al. [135] also concluded that instruments based on combustion at 680 °C with catalyst yielded the most accurate data. However, they found that the peroxodisulfate method also included in the intercomparison gave slightly higher TDN concentrations, suggesting incomplete conversion of some N compounds by the HTCC instruments and/or incorrect integration of the analytical peaks. Nevertheless, a number of studies have examined the oxidation efficiencies of HTCC methods [219,221–224] and have shown that the approach quantitatively recovers N from a range of compounds with different degrees of refractivity, including humic materials.

Rogora et al. [217] measured TDN in 800 freshwater samples by peroxodisulfate digestion and HTCC and found no significant differences between the two methods. In a more extensive study, Bronk et al. [223] compared peroxodisulfate digestion, UV oxidation and HTCC for the determination of TDN using representative model DON compounds and natural water types. Model compounds were recovered to a similar extent by each of the methods, 93 ± 13% by peroxodisulfate digestion, 91 ± 10% by the HTCC method and 91 ± 12% by UV oxidation when the sample was augmented with an alkaline peroxodisulfate reagent. The three approaches also gave similar results for the natural water samples. However, due to the poor recovery of some of the model compounds with each of the methods, the authors concluded that concentrations of TDN, and subsequently DON, determined in real samples using these methods may lead to an underestimation of the true concentration in some cases, the extent of the discrepancy depending on the TDN composition of the sample.

### 3.2.3. *Gas chromatography (GC)*

Gas chromatography with appropriate detection is a sensitive analytical technique for the separation and determination of molecules within the ON pool. Suitable analytes can be routinely detected using either a flame ionisation detector (FID) or a thermionic detector (N–P detector, NPD), which is 50 times more sensitive for N-species than a FID [225]. GC can be used for the determination of volatile ON species, or those that can be rendered volatile by derivatisation. To date, it has not been widely applied to studies of the DON pool, which mainly comprises non-volatile species, such as amino acids, peptides and proteins. Aliphatic and aromatic amines ($RNH_2$) can be analysed by GC as both underivatised and derivatised molecules. An example is the methylamines, which are ubiquitous in aquatic systems (up to μM) and can be used as substrates by algae and bacteria [226,227]. Historically these analytes tended to adsorb onto glass columns and solid-phase material, leading to poor reproducibility [228]. However, the column solid phase can be adapted to eliminate adsorption problems and poor peak shape through treatment with KOH or NaOH. Derivatisation may also reduce adsorption problems, though tertiary amines cannot be derivatised without first being converted to secondary amines [229]. Most GC techniques for the determination of amines have focussed on free amines

(i.e. unprotonated or underivatised). Fitzsimons et al. [230] used a combination of static microdiffusion and packed column GC-NPD for analysis of methylamines, based on work by Abdul-Rashid et al. [231]. The analytes and internal standards were resolved within 8 min and detection limits were in the range of 2–12 nM. This preconcentration step was further developed by Yang et al. [232] using a two-step circulation diffusion method for detection of amines at concentrations below 10 nM.

Recent developments in capillary GC have included its adaption to the analysis of volatile, basic compounds. For example, the use of polyethylene glycol columns doped with an alkaline metal hydroxide or thick-film methylpolysiloxane columns now enables operation at higher column temperatures [233]. However, caustic additives, which minimise adsorption of amines onto packed columns, cannot be used to coat polysiloxane stationary phases as this promotes column degradation. To this end, advances in deactivation technologies have included the stabilisation of base-deactivated porous polymers [234]. SPME can be combined with GC-NPD for amine determination [235]. As a solventless, headspace technique, SPME eliminates the need to inject aqueous samples at high pH, which might cause accelerated deterioration of the GC column. A polydimethylsiloxane fibre SPME was used in combination with a PoraPLOT capillary GC column for the detection of amines in wastewater and sewage-polluted water samples [235]. The method is both fast and reproducible but may have limited application, in its current form, to samples with trace amine concentrations.

#### 3.2.4. *High-performance liquid chromatography (HPLC)*

High-performance liquid chromatography can be used for the separation and detection of volatile and non-volatile ON species. Samples are introduced in aqueous solution and analytes resolved on a packed column using either isocratic or gradient elution. A range of HPLC detectors are available but most aquatic methods use fluorescence (FL) detection for maximum sensitivity as FL detectors are at least an order of magnitude more sensitive (fmol) than other detectors, and excellent qualitative and quantitative results have been achieved for a range of compound classes. Samples are usually derivatised prior to FL detection to ensure a uniform response. Dissolved free amino acids can be detected by HPLC after derivatisation with a fluorescing reagent, such as ortho-phthaldialdehyde (OPA) [236], dimethylaminoazobenzene sulfonyl chloride (DABS-Cl) [237] or 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate (AQC) [238]. The selection of the reagent usually depends on its stability and the presence of interfering substances that can react with the reagent. AQC detects both primary and secondary amines but is an insensitive method, with a limit of detection of 200 nM [238]. Amino acids and other primary amines form highly fluorescent isoindoyl derivatives after reaction with OPA and a thiol compound. Although OPA is the FL reagent of choice for aquatic studies of amino acids, it does not react with the secondary amino acids proline and hydroxyproline, leading to criticism that it underestimates concentrations of both DFAA and DCAA. Both DFAA and DCAA (peptides and proteins) can be quantitatively determined by HPLC, but the latter cannot be measured at the molecular level as samples must be hydrolysed prior to analysis to cleave peptide bonds [136,238]. Nonetheless it is a highly sensitive method which can detect amino acids at the fmol level.

Chiral analysis of amino acids can increase the information provided by acid hydrolysis. It has been used in attempts to age oceanic dissolved organic carbon [239] and assess bacterial contributions to the DON pool [240]. The technique is based on the fact that all amino acids except glycine are chiral, with proteins and peptides in living organisms consisting almost exclusively of L-isomer amino acid residues. An exception to this is peptidoglycan, a component of bacterial cell walls, which has been identified in a range of aquatic environments [241,242]. Analytes are derivatised using the OPA method but the thiol compound (e.g. mercaptoethanol) is replaced by a chiral thiol compound to ensure chromatographic separation of the D- and L-enantiomers. A number of compounds have been tested and *N*-iso-L-butyryl-L-cysteine appears to provide best resolution. It has been used in recent studies [240,242,243] and detection limits are reported to be in the nM range [243].

A HPLC method was developed for the determination of underivatised, dissolved amines [226]. Water samples were collected in gas-tight bottles and aliquots transferred into a flow injection-gas diffusion system coupled to an ion chromatograph. The analytes were preconcentrated by diffusion across a gas-permeable membrane before separation and analysis on an ion chromatograph. Limits of detection for methylamines were 3–5 nM. Ammonia and trimethylamine oxide (a quaternary amine) can also be detected in this way, the latter after enzymatic reduction to trimethylamine [244]. This method has been applied to water column, atmospheric and pore-water measurements of ammonia and amines.

#### 3.2.5. *Gel electrophoresis*

Successful characterisation of HMM DON, such as peptides and proteins, has lagged behind improvements in amino acid analysis. However, although amino acids comprise the largest identifiable pool of DON in the oceans [245], their distributions may reflect sources other than peptides or proteins (e.g. peptidoglycan) and hydrolysis provides little information on the biogeochemistry of the parent molecules. Analytical advances in the field of proteomics, such as gel electrophoresis, have been adopted to identify and characterise intact proteins in aquatic systems [155,246,247] providing insight into the cycling of the HMM DON fraction.

Sodium dodecyl-polyacrylamide gel electrophoresis and two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) have been used to detect and identify proteins in oceanic and coastal waters [148,155,246,248] as shown in Fig. 7. The method requires a large water sample (20–60 L), which is collected and ultrafiltered, usually after pre-filtration (GF/F or 0.2 μm filters). The nominal molecular mass cut-off for ultrafiltration ranges from 10 to 14 kDa and samples are reduced in volume to below 1 L, then chemically treated to remove proteins from solution in pellet form. This pellet is dissolved in a buffered solution. The limit of detection for both SDS-PAGE and 2D-PAGE is 10 pg [248]. Total protein concentrations are determined prior to gel electrophoresis to ensure standard loading across the gel, using the Lowry protein assay [155] or densitographically [248]. Samples are diluted to an appropri-

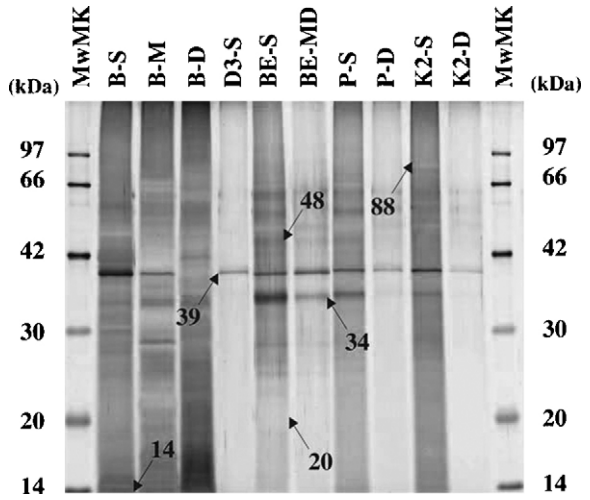


**Fig. 7 – Profiles of dissolved proteins in oceanic waters using SDS-PAGE. Reprinted from Yamada and Tanoue, Progress in Oceanography 247 (2006) 6, with permission from Elsevier.**

ate protein concentration (~0.25–1 μg) and heated to achieve complete denaturation and reduction of disulfide bonds.

For sample preparation biocide is added to prevent biodegradation during sample transport, e.g. 0.01% $NaN_3$ and 0.004% protease inhibitor solution [155] and SDS (0.01%) added to retain proteins in solution during preconcentration [148,248,249]. This is followed by pre-filtration [148,249] and concentration of proteins by a TFF system with a nominal molecular mass cut-off at 10 kDa. The next steps are desalting with a rinsing solution (35 mM $NH_4HCO_3$ + 0.01% SDS) or by dialysis [155], precipitation of a proteinaceous pellet through addition of trichloroacetic acid (TCA) followed by standing for 12 h at 4 °C [249] or −20 °C [155], centrifugation to remove TCA-soluble material and, finally, dissolution of the proteinaceous pellet in SDS-PAGE buffer for analysis. It is recommended that all post-sampling steps are performed at 4 °C and samples frozen (−20 °C) if sample preparation is interrupted at any stage [155]. For SDS-PAGE, samples (5–20 μL) are dissolved in gel loading buffer [155] and electrophoresis performed at 200 V for 45 min [155] to 6 h [148]. For 2D-PAGE, samples are dissolved in a buffer solution then loaded onto pre-prepared immobilised pH gradient (IPG) strips. The first dimension involves isoelectric focussing (IEF) in three steps at 20 °C [155] rehydration (250 V) for 15 min, linear gradient voltage ramping (250–4000 V) over 2 h and final focussing (4000 V) for 5 h. Once IEF has been completed, the IPG strip is soaked in equilibration buffer. The second dimension involves placing the IPG strip across the gel used for SDS-PAGE, and SDS-PAGE is then performed as described.

Proteins are visualised for both gel electrophoresis methods by staining using Coomassie blue (CBB), fluorescence or silver ions. Silver staining, the most sensitive of the techniques (0.1–1.0 ng), has been preferred in recent studies [148,155,247]. CBB is at least an order of magnitude less sensitive (10–100 ng), while fluorescence staining has a detection limit of 1–10 ng [155]. After staining the gels are scanned using a densitometer [155]. SDS-PAGE and 2D-PAGE have been used to successfully identify proteins on the basis of molecular mass (SDS-PAGE), and molecular mass and isoelectric points (2D-PAGE). Proteins isolated on the gels can be subjected to further treatments and analyses to aid characterisation [148,155,248]. However, samples typically show strong background staining [155,247] and the sensitivity of silver staining can cause contamination. For example, despite using clean techniques at every stage, Jones et al. [155] detected ubiquitous protein bands between 50 and 68 kDa, which were interpreted as skin keratin [249]. In addition, silver staining has been criticised as identifying polysaccharides and DNA as well as proteins, although Tanoue [249] did not detect non-proteinaceous molecules by SDS-PAGE.

A rapidly expanding proteomics library means that proteins isolated using gel electrophoresis can be further characterised through combination with liquid chromatography–tandem mass spectrometry (LC–$MS^n$), after enzymatic treatment [148] or N-terminal amino acid sequencing using Edman's method [247]. As the typical molecular mass cut-off for gel electrophoresis is 10 kDa, molecules with molecular masses between those of amino acids and proteins (e.g. peptides) cannot be identified or characterised using this technique.

### 3.2.6. *Mass spectrometry (MS)*

Mass spectrometry is routinely used as a detection technique for organic molecules in combination with GC. However, the low volatility of most ON molecules has limited the applicability of GC–MS to ON studies. HPLC mobile phase solvents routinely contain non-volatile, ionic species, such as acetate or phosphate buffers, which are incompatible with detection by MS [250,251] where the analyte ions must in the gaseous form. This has precluded structural determination of dissolved, non-volatile analytes, especially if the solvent is also non-volatile [252,253]. Electrospray ionisation (ESI) was developed for the determination of macromolecules [254]. It allows intact molecules to be transferred to the gas phase and for structural information to be obtained from molecular or pseudomolecular ions by MS. It is applicable to polar, non-volatile compounds and, as such, is ideal for molecular characterisation of DON [255,256]. HPLC–ESI-MS has been combined with gel electrophoresis for the characterisation of HMM DON molecules after isolation [148]. As protein molecules (>10 kDa) are too large to be analysed directly, and hydrolysis would destroy the molecular structure, enzymes are used to cleave the protein into smaller fragments that can be separated and detected by HPLC–ESI-MS. *De novo* sequencing of the peptide tandem mass spectra ($MS^n$) generates short amino acid sequences (peptide tags), which can be used to search databases for protein class and source information [148,257].

LC–ESI-MS has also been successfully applied to the determination of amino acids and small (two residue) peptides [251,258]. The 20 protein amino acids were detected, underivatised, at low levels (fmol to pmol) using this method [251]. Application of the method to saline samples would require a desalination step, however, which has not yet been reported.

#### 3.2.7. *Nuclear magnetic resonance spectrometry (NMR)*

$^{13}C$ NMR spectrometry has been used to partially characterise complex organic structures (e.g. humic acids) in natural systems [181,259,260] though the majority of these studies have been performed in the solid-state using cross polarization and magic angle spinning (CP-MAS) techniques [261,262]. Solid-state $^{15}N$ NMR has been used to detect a range of N bonding arrangements such as: amide-N, including peptides, indoles, lactames and carbazoles (220–285 ppm); amino-N of terminal amino acids or aliphatic amino groups of peptides, amino sugars and amino acids (348 ppm); primary and secondary amino-N of guanidines, aromatic amines or aniline (285–325 ppm); heterocyclic-N in purines, indoles, imidazoles, pyrole-like compounds or proline-N in peptides (145–220 ppm); pyride-N and imine structures (25–90 ppm). As ON is a subset of natural organic matter (NOM) and $^{15}N$ has a low natural abundance relative to $^{14}N$ (0.36% compared with 1% for $^{13}C$ relative to $^{12}C$), large dry-masses of NOM are required. Knicker and Hatcher [263] analysed humic isolates from different layers of an organic-rich algal sapropel from Mangrove Lake, Bermuda, using solid-state $^{15}N$ NMR spectrometry in order to examine delineation of diagenetic pathways. Amide-N, most likely from peptide-like material, was found to represent the main ON in all humic fractions at all depths of the sapropel. Amides could also be identified in the residues from 6 M HCl hydrolysis of the alkaline insoluble extract (formerly called humin).

The sensitivity of $^{15}N$ investigations using CP-MAS techniques decreases as the distance to $^{1}H$ nuclei increases within a structure [264]. In addition, the overlap in chemical shift regions of amide-N and pyrrole- and indole-N can make unambiguous assignments difficult. This problem can be overcome through the application of solid-state double cross polarization (DCP) MAS, which generate spectra that show only signals relating to intra- and inter-molecular interaction of $^{13}C$ and $^{15}N$. It has been used to directly analyse dried bulk solids, revealing the chemical environment of N within organic structures. The DCP-MAS technique was used in conjunction with correlation spectrometry (COSY) by Knicker [264] to investigate the chemical nature of N in degraded algae. COSY interrogates NMR spectra acquired for different nuclei in two dimensions ($^{15}N$ and $^{13}C$ in this case). Cross peaks indicating coupling between $^{13}C$ and $^{15}N$ were detected for carboxyl/amide-C and N-substituted-alkyl-C; heteroaromatic-N was not detected. A concurrently acquired $^{15}N$ spectrum using the single pulse excitation (Bloch decay) technique (direct excitation of the $^{15}N$ spin system) confirmed the lack of aromatic or imine N in the sample.

In the solution phase, NMR spectrometry has been used to characterise environmentally relevant organic chemicals [265,266], though typical environmental concentrations and the prevalence of complex mixtures have limited this type of investigation. McCarthy et al. [38] used $^{15}N$ NMR spectrometry to characterise oceanic HMM DON and found it to be largely amide in form. Isolated HMM DOM was analysed in water samples from rivers and estuaries within the Everglades coastal ecosystem [146]. In this study $^{15}N$ CP-MAS NMR spectrometry was used in combination with X-ray photoelectron spectrometry (XPS); data indicated that most N was in the amide form but that aromatic forms were also significant.

#### 3.2.8. *X-ray spectrometry*

X-ray photoelectron spectrometry is a surface chemical analysis technique that can be used to analyse the surface chemistry of a material. It has not been widely applied to organic matter analysis, but can provide information on pyrolic, pyridinic, quaternary and aromatic amine N. A study by Patience et al. [267] combined XPS with elemental analysis, biochemical analysis, and pyrolysis-gas chromatography with atomic emission detection to investigate ON speciation in surface sediments from the Peru upwelling region. These samples were shown to contain at least four different ON-containing functional groups: amino, pyrrole, pyridine and (tentatively) quaternary N. In another study Vairavamurthy and Wang [268] used K-edge XANES as a selective, sensitive and non-destructive method to investigate N-speciation in humic substances and sediments. They found amide-N to be the dominant form, although pyridinic N was also significant (20–30% of total N), with a sub-fraction consisting of its oxidised derivatives. An unidentified form of highly oxidised N was also identified, mainly associated with the sediments studied.

#### 3.2.9. *Enzymatic methods*

The detection of ON using enzymatic methods has mainly focussed on urea ($(NH_2)_2CO$), which can be a significant source of N for marine bacteria and freshwater and marine phytoplankton [32,33]. Urea has been implicated in coastal eutrophication, linked to an increase in the usage of urea-based fertilisers over the last four decades [269]. Urea is typically detected in aquatic systems using a urease assay, which converts the molecule into $NH_4^+$, with subsequent spectrophotometric detection; thus samples must be corrected for $NH_4^+$. The urease method was compared with a diacetyl monoxime method for measurement of dissolved urea in seawater by Price and Harrison [270]. The study was conducted using an artificial seawater matrix and natural and urea-spiked field samples from coastal and oceanic environments. Seawater-type and time of sample collection were important variables affecting urea measurement by the urease method, and recovery of internal standards ranged from 40 to 100%. Increasing the heating time of the urease assay, or the concentration of urease added to the seawater samples, increased the amount of urea determined. However, measured values were still less than the concentration of the urea internal standards.

## 4. Future trends and recommendations

One of the key requirements for the introduction and acceptance of any new method is a robust demonstration of its accuracy, e.g. by the analysis of CRMs (if available) and intercomparison exercises. The area of OP/ON component determinations is no exception. Therefore the development and wide availability of a range of CRMs that are certified for TDP/TDN and DOP/DON at environmentally relevant concentrations in natural waters and sediments would be

advantageous. Methods are also required to completely (ion exchange, volatilisation) or partially (dialysis) remove DIP/DIN prior to DOP/DON determination in order to improve accuracy [271]. Furthermore, there is no direct analytical method for DOP/DON quantification, and research efforts are needed to address the inadequacies of existing methods.

Given the emerging ecological importance of organic nutrients, there is a real need for improved measurement techniques for determining both the total organic N and P concentrations and the concentrations of individual organic moieties. Much of our current understanding of the aquatic N and P cycles is built on an over reliance on techniques for the inorganic and total forms, with little regard for the important organic fraction. The balance of material in this review suggests that techniques for organic N are currently more developed than those for organic P but there are still many analytical challenges for both elements.

Another area requiring attention is the difficult issue of sampling. Alteration of the sample is implicit in the process of sampling, and it is not a matter of whether or not sample alteration occurs, but to what extent. Changes in temperature, moisture content, light and redox conditions can all affect the speciation of OP and ON. Thus, the application of *in situ* techniques that minimise perturbation of the sample is highly desirable. Use of passive, diffusive sampling devices, such as peepers or diffusive gradients in thin films (DGT) [272] is one possible approach that can be applied to sediments, soils and waters. To date application of these devices has focussed on sampling and measurement of the most bioavailable ionic forms of metals or nutrients (e.g. DRP), but there is certainly a role for their use in the study of organic nutrients. However, this will require the development of DGT systems with binding phases suitable for organic nutrients.

Wastewaters are a challenging matrix for the determination of the DOP and DON pools and LC–MS, coupled with more selective sample pretreatment, will be an important tool for their characterisation. Wastewater derived DON accounts for up to 80% of dissolved N in nitrified–denitrified effluent [273] but the sum of all identified N-containing compounds is only 10% of the DON with the remainder probably consisting of polymerised biological compounds. Similarly, recent studies showed that a high percentage (>50%) of the residual TDP in the final effluent from a wastewater treatment plant was comprised of DOP that survived anaerobic digestion, some of which was potentially bioavailable [30].

The routine determination of proteins from DOM has the potential to develop into a novel field of environmental research [143]. With respect to enzymes, a combination of mass spectrometry with immunoprecipitation or affinity purification is a potential tool for studying specific enzymes of interest. Proteomics technology has been successfully transferred to environmental research, with gel electrophoresis used to isolate intact proteins from seawater. This approach preserves essential information on the nature of the original molecules, which can be used for source fingerprinting. However, the isolation and preconcentration procedure has a molecular mass cut-off of 1–10 kDa [155,248]. With regard to N, HMM DON is thought to comprise only 20–30% of total DON [37,38], which means that the quantitatively dominant LMM DON fraction remains largely uncharacterised [31]. A recently developed analytical procedure to detect and characterise dissolved LMW peptides in saline water [274] comprises SPE of filtered water samples, and separation and detection of eluted analytes by LC–ESI-MS. Tandem MS can then be performed on molecular ions of interest. The method has been tested using peptides up to 2 kDa and offers the potential to characterise unknown analytes through *de novo* sequencing. The application of such techniques to the DOP pool is less well developed [195] but represents an important and emerging field.

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