FLOW INJECTION TECHNIQUES FOR INVESTIGATING THE BIOGEOCHEMISTRY OF NUTRIENTS IN NATURAL WATERS

By

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ABSTRACT

Flow injection techniques for investigating the biogeochemistry of nutrients in natural waters

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This thesis describes the development of robust flow injection (FI) techniques for the determination of nutrients, with a focus on phosphorus species, in natural waters. Chapter one presents a general overview of nutrients (P, N and Si) and their aquatic biogeochemistry and analytical methods for nutrient determinations in natural waters.

Chapter Two reports a four channel FI manifold incorporating a thiosulphate stream for on-line masking of arsenate, which is potentially a major interferent in the determination of low concentrations of phosphate in natural waters. The method is suitable for the determination of filterable reactive phosphorus (FRP) in anthropogenically impacted waters such as the Tamar Estuary. The effects of $[H^+]:[molybdate]$ ratio, temperature and matrix interferences were systematically investigated. Typical figures of merit were: typical RSDs of 1.5%, a practical limit of detection of 2 μg P L$^{-1}$ and a linear range of 2 - 100 μg P L$^{-1}$ at 30 °C. This was the optimum temperature to minimise interference from silicate. The method was applied to the determination of FRP in the Tamar Estuary and the results were in good agreement (paired t-test; P = 0.05) with those obtained using a segmented flow analyser reference method.

In Chapter Three, a four channel FI manifold, incorporating a thiosulphate stream and two micro-columns (an iminodiacetate resin to mask trace metals and a strong anion exchange resin to mask phosphate) is described. The figures of merit at 60 °C were a practical limit of detection of 10 μg Si L$^{-1}$, a linear range of 10 - 1000 μg Si L$^{-1}$ and typical RSD of 1.5%. The method showed good agreement (P = 0.05) with a segmented flow analyser reference for Tamar Estuary samples.

Chapter Four reports the effect of pH on the recovery of dissolved organic phosphorus (DOP) from fresh waters after batch autoclave digestion using acidic peroxydisulphate. A final pH of 1.5 - 2.0 after digestion and an acid to molybdate mole ratio of 62 - 65 in the FI manifold gave the best recoveries, especially at low DOP concentrations (10 - 50 μg P L$^{-1}$). Peroxydisulphate in the absence of acid gave the best results for the determination of DOP in high salinity waters.

Chapter Five extends the applicability of FI to investigate phosphorus biogeochemistry by incorporating a micro-column containing immobilized phytase in the FI manifold to determine a single species (phytic acid) within the DOP pool. The optimised method was applied to the determination of phytase hydrolysable phosphorus in the Tamar Estuary and results showed that this fraction constituted 20 - 25% of the DOP pool.

The results from six surveys (five transects and one tidal cycle) of the Tamar Estuary are reported in chapter six and highlight the temporal and spatial variability of SRP, silicate and nitrate. Other chemical (As, Cu, Ni, Co, Al, Fe, Mn, Cr) and physical (pH, dissolved oxygen, salinity, conductivity and suspended particulate matter) parameters are also presented. Results were generally in agreement with historical data for nutrient concentrations in the Tamar Estuary.
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Relevant scientific seminars and conferences were regularly attended at which work was often presented and a paper was prepared for publication.

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PRESENTATIONS AND CONFERENCES ATTENDED

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Table 6.9 Effect of diverse ions on the determination of 20 μg P L⁻¹ using the Skalar instrumentation. Results are mean of three replicates for each ion.
LIST OF ABBREVIATIONS

\([H^+]\) - hydrogen ion concentration
3s – (3 times standard deviation of the blank)
4-MUP – 4-methylumbelliferyl phosphate
AAS – atomic absorption spectrometry
ADP – adenosine-5-diphosphate
AMP – aminoethy phosphoric acid, AMP
ATP – adenosine-5-triphosphate di-sodium salt
BAP – bioavailable phosphorus
CFA – continuous flow analysis
Chl a – chlorophyll a
CL – chemiluminescence
CNBr – cyanogen bromide
COC – thiamine pyrophosphate chloride or cocarboxylase
CPG – controlled porosity glass
CRM – certified reference material
D-G-6-P – D-glucose-6-phosphate
DHP – dissolved hydrolysable phosphorus
DIP – dissolved inorganic phosphorus
DO – dissolved oxygen
DOP - dissolved organic phosphorus
DRP - dissolved reactive phosphorus
DSW – depleted seawater (nutrients P, N and Si)
DUP – dissolved unreactive phosphorus
EA – environmental agency
EC – enzyme commission
EDTA – ethylene diamine tetra acetic acid
EEC – European Economic Community
EHP – enzyme hydrolysable phosphorus
FI – flow injection
FIA – flow injection analysis
FRP - filterable reactive phosphorus
\(g\) – gram
GAP – green analytical procedure
GC – gas chromatography
GCE – glassy carbon electrode
GC-MS – gas chromatography-mass spectrometry
GL – guide level
h – hour
Ha – hectares
HDPE – high density polyethene
HGAAS – hydride generation atomic absorption spectrometry
IC – ion chromatography
ICP-MS – inductively coupled plasma-mass spectrometry
K – degree Kelvin
LC – liquid chromatography
LED – light emitting diode
LNS – low nutrient seawater
LOD – limit of detection
MAC – maximum admissible concentration
MCFIA – multicommutation flow injection analysis
MPA – molybdophosphoric acid
MQ – milli-Q water (deionised water)
MRP – molybdate reactive phosphorus
MTP – methyl triphenyl phosphobromide
MΩ – milli-ohms
N – nitrogen
Ne – neon
°C – degree Celsius
P – phosphorus
p – probability
PIP – particulate inorganic phosphorus
p-NPP – p-nitrophenyl phosphate magnesium salt
POP – particulate organic phosphorus
PP – particulate phosphorus
PPD – phenyl phosphate di-sodium
PTA-Ca – phytic acid calcium salt
PTA-MgK – phytic acid magnesium-potassium salt
PTA – phytic acid
PTFE – polytetra-fluoroethylene (tubing material)
rFIA – reverse flow injection analysis
RFP – flavin mononucleotide (Riboflavin-5’-phosphate di-sodium salt)
RSD – relative standard deviation
s – second
SD – Secchi disk transparency
SE – Schleiren effect
SFA – segmented flow analysis
Si – silicon
SIA – sequential flow analysis
Sn – tin
SRP – soluble reactive phosphorus
STP – pentasodium triphosphate
TDP – total dissolved phosphorus
TFP – total filterable phosphorus
TN – total nitrogen
TON – total oxidized nitrogen
TPP – trisodium trimeta-phosphate
TP – total phosphorus
TRP – total reactive phosphorus
UP – unreactive phosphorus
UPW – ultra pure water
US – United States
UV – ultra-violet
VIS – visible
WHO – World Health Organisation
yr⁻¹ – per year
µg – micro-grams
µM – micro-molar
µS – micro-Siemens
CHAPTER ONE

INTRODUCTION

Main topics in this chapter

Nutrients
Phosphorus
Nitrogen
Silicon
Legislation
Flow injection analysis
Research aims and objectives
1.1. NUTRIENTS
Nutrients are elements required by plants and animals for growth. Deficiency could retard growth and development with adverse effect on plants and animals, and is therefore a necessary index for metabolism [1, 2]. Some of these elements are required in relatively high concentrations (macro-nutrients e.g. phosphorus, nitrogen, potassium) while others are needed only in trace quantities (micro-nutrients e.g. iron, silicon, selenium) [3 - 6]. Their deficiency or excess concentrations can have adverse problems, some very serious to plants and animals alike, hence it is important to monitor their concentrations in the environment [7].

Table 1.1. Effects of eutrophication on estuarine and marine ecosystems. Adapted from reference [11].

<table>
<thead>
<tr>
<th>Effect</th>
</tr>
</thead>
<tbody>
<tr>
<td>Increased biomass of marine phytoplankton and epiphytic algae</td>
</tr>
<tr>
<td>Shifts in phytoplankton species composition to taxa that may be toxic or inedible (e.g. bloom-forming dinoflagellates)</td>
</tr>
<tr>
<td>Increases in nuisance blooms of gelatinous zooplankton</td>
</tr>
<tr>
<td>Changes in microalgal production, biomass and species composition</td>
</tr>
<tr>
<td>Changes in vascular plant production, biomass and species composition</td>
</tr>
<tr>
<td>Reduced water clarity</td>
</tr>
<tr>
<td>Death and losses of coral reef communities</td>
</tr>
<tr>
<td>Decreases in aesthetic value of the water body</td>
</tr>
<tr>
<td>Elevated pH and dissolved oxygen depletion in the water column</td>
</tr>
<tr>
<td>Shifts in composition towards less desirable animal species</td>
</tr>
<tr>
<td>Increased probability of kills of recreationally and commercially important animal species</td>
</tr>
</tbody>
</table>

There are several sources of nutrients to natural waters e.g. erosion, weathering of rocks [8], leaching, agricultural practices and industrial effluents [9]. Plants usually take up nutrients in the form of aqueous ions. Phosphorus (P) and nitrogen (N) are macro-nutrients that have been linked to the eutrophication of natural waters. They are also important as fertilizers for nutrient supplementation to plants in areas of low nutrients. Nutrient enrichment and eutrophication has caused significant effects in affected water bodies. Oxygen is depleted, marine life is lost, the aesthetic and natural value of lakes and rivers is affected, and water quality is
degraded. Fouling of natural waters as a result of algal blooms can produce toxic cyanobacteria [10 - 14]. This raises the cost of water treatment for both domestic and industrial applications. All of these factors exert significant pressures on the economy.

Table 1.2. Characteristics of lakes, streams and marine waters of different trophic states. Adapted from reference [11].

<table>
<thead>
<tr>
<th>Trophic state</th>
<th>TN (mg m⁻³)</th>
<th>TP (mg m⁻³)</th>
<th>chl a (mg m⁻³)</th>
<th>SD (m)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lakes</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Oligotrophic</td>
<td>&lt; 350</td>
<td>&lt; 10</td>
<td>&lt; 3.5</td>
<td>&gt; 4</td>
</tr>
<tr>
<td>Mesotrophic</td>
<td>350 - 650</td>
<td>10 - 30</td>
<td>3.5 - 9</td>
<td>2 - 4</td>
</tr>
<tr>
<td>Eutrophic</td>
<td>650 - 1200</td>
<td>30 - 100</td>
<td>9 - 25</td>
<td>1 - 2</td>
</tr>
<tr>
<td>Hypertrophic</td>
<td>&gt; 1200</td>
<td>&gt; 100</td>
<td>&gt; 25</td>
<td>&lt; 1</td>
</tr>
<tr>
<td>Suspended</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>chl a</td>
<td>&lt; 10</td>
<td>&lt; 10</td>
<td>&lt; 10</td>
<td>&lt; 1</td>
</tr>
<tr>
<td>Benthic</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>chl a</td>
<td>&lt; 10</td>
<td>&lt; 10</td>
<td>&lt; 10</td>
<td>&lt; 1</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Streams</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Oligotrophic</td>
<td>&lt; 700</td>
<td>&lt; 25</td>
<td>&lt; 10</td>
<td>&lt; 20</td>
</tr>
<tr>
<td>Mesotrophic</td>
<td>700 - 1500</td>
<td>25 - 75</td>
<td>10 - 30</td>
<td>20 - 70</td>
</tr>
<tr>
<td>Eutrophic</td>
<td>&gt; 1500</td>
<td>&gt; 75</td>
<td>&gt; 30</td>
<td>&gt; 70</td>
</tr>
<tr>
<td>Marine Waters</td>
<td></td>
<td></td>
<td>chl a</td>
<td>SD (m)</td>
</tr>
<tr>
<td>Oligotrophic</td>
<td>&lt; 260</td>
<td>&lt; 10</td>
<td>&lt; 1</td>
<td>&gt; 6</td>
</tr>
<tr>
<td>Mesotrophic</td>
<td>260 - 350</td>
<td>10 - 30</td>
<td>1 - 3</td>
<td>3 - 6</td>
</tr>
<tr>
<td>Eutrophic</td>
<td>350 - 400</td>
<td>30 - 40</td>
<td>3 - 5</td>
<td>1.5 - 3</td>
</tr>
<tr>
<td>Hypertrophic</td>
<td>&gt; 400</td>
<td>&gt; 40</td>
<td>&gt; 5</td>
<td>&lt; 1.5</td>
</tr>
</tbody>
</table>

The terms oligotrophic, mesotrophic and eutrophic correspond to receiving low, intermediate and high inputs of nutrients. Hypertrophic is the term used for systems receiving greatly excessive nutrient inputs. TN; total nitrogen, TP; total phosphorus, chl a; chlorophyll a and SD; Secchi disk transparency [12,17].

It is therefore necessary to monitor nutrient species in natural waters for legislation, management and planning purposes. Monitoring provides an understanding of biogeochemical processes prevalent in an area. The data generated also help in planning and management policies for recreation [15]. Accurate data collection requires robust and easy to use instrumentation.
Thus development of reliable analytical techniques and understanding of the chemistry of the processes of detection is a *sine qua non* for environmental studies and management of aquatic resources. Eutrophication is a serious problem and poses a threat to global water quality and the management of natural waters (Table 1.1) due to the depletion of floral and faunal communities and this has become one of the challenges for environmental scientists [13,14]. Various parameters are used to compare the trophic status of natural waters, e.g. carbon, nitrogen, phosphorus and chlorophyll [9, 16 - 20]. Different trophic states are shown in Table 1.2.

### 1.2. PHOSPHORUS

Phosphorus (P), atomic number 15, is a highly reactive element and forms compounds with various elements by direct bonding or through oxygen [3, 21- 25]. Phosphorus exhibits nine oxidation states from +5 to −3. Fig. 1.1 shows the structures of various P species, such as orthophosphate, P(+5), hypophosphate (or diphosphate), P(+4) [II], phosphite, P(+3) [III], and hypophosphite, P(+1) [IV]. Sodium diphosphate [V] is a phosphorylation agent for biological substances [1, 2, 25]. Among phosphorus oxo-acids, phosphate and its polymers are important in nature and industry. Orthophosphoric acid is a raw material in fertilizers, detergents, surfactants and flame-retardants. Although cyclopolymermers such as trimetaphosphate [IV] exist in nature, linear polymers of phosphate such as di-(pyro) [VII], tri-poly [VIII] and polyphosphate are the most abundant. However, determination of phosphorus generally refers to the measurement of P(+5).

<table>
<thead>
<tr>
<th>Atomic number</th>
<th>15</th>
</tr>
</thead>
<tbody>
<tr>
<td>Relative atomic mass</td>
<td>30.9738</td>
</tr>
<tr>
<td>Electronic configuration</td>
<td>[Ne] 3s²3p³</td>
</tr>
<tr>
<td>Melting point</td>
<td>White: 44.2°C, Red: 59.0°C</td>
</tr>
<tr>
<td>Boiling point</td>
<td>280 °C (white)</td>
</tr>
<tr>
<td>Density (20 °C)</td>
<td>White: 1.82 g cm⁻³, Red: 2.34 g cm⁻³</td>
</tr>
</tbody>
</table>
Fig. 1.1. Oxo-acids of phosphorus species.

1.2.1. Occurrence in the environment
Phosphorus is an abundant crustal element and is found in natural waters, wastewaters, soils and rocks as minerals in the form of orthophosphate. Many classes of phosphorus compound e.g. orthophosphates, condensed phosphates (pyro-, meta- and polyphosphates) and organic phosphates have been identified [27 - 29]. Phosphorus is also a bio-element and an important constituent of all living cells [30] and consists of 10 % elemental composition of human body (dry weight). The nucleotides in nucleic acids are linked by phosphate bonds through phosphorylation [22, 26] and energy is transferred from one molecule to another in the form of a high-energy phosphate bond, very often in ATP. Many coenzymes also contain phosphate [31 - 33]. Phosphorus is essential to the growth of organisms and in the form of orthophosphate can limit primary production in a body of water [27, 34 - 36]. Excess P concentration arises where discharge of treated wastewater, industrial discharges and agricultural drainage occurs. This will stimulate the growth of photosynthetic aquatic micro- and macro-organisms, which can adversely affect water quality. It is therefore a key parameter for determining the ecological status of any stretch of water for both economic and social reasons [7, 12, 37].

Phosphorus can enter natural water systems from several point and diffuse sources including weathering of the Earth's crust (see Table 1.4), sediment release, animal and plant wastes, agricultural runoff, effluents from industries and sewage treatment works. Storm water runoff is the primary component of diffuse-
source pollution, with the water quality of the discharge being determined by the dominant land use of the catchment. Nutrient contamination can originate from runoff of fertilizers from agricultural lands and from livestock and human waste. Point sources are distinct sources of contamination, i.e., those coming from a concentrated point and flowing directly into water bodies at a discrete point e.g., industrial discharges, municipal sewage treatment facilities and agricultural animal production facilities [11, 12, 37]. Links between nutrient changes in rivers and phytoplankton dynamics due to biogeochemical transformations of nutrients has been established (see Fig. 1.2). Decay of dead organic matter (biological processes) also provides a veritable source of phosphorus to both aquatic and terrestrial ecosystems [27, 29].

Table 1.4. Sources of point and non-point chemical inputs as recognized by US statutes. Adapted from reference [11].

<table>
<thead>
<tr>
<th>Point Sources</th>
<th>Non-point (diffuse) sources</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wastewater effluent (municipal and industrial)</td>
<td>Runoff from agriculture (including return flows from irrigated agriculture)</td>
</tr>
<tr>
<td>Runoff and leachate from waste disposal sites</td>
<td>Runoff from pastures and rangeland</td>
</tr>
<tr>
<td>Runoff and infiltration from animal feedlots</td>
<td>Urban runoff from unsewered areas and sewered areas with population &lt; 100,000</td>
</tr>
<tr>
<td>Runoff from mines, oil fields, and unsewered industrial sites</td>
<td>Septic tank leakage and runoff from failed septic systems</td>
</tr>
<tr>
<td>Storm sewer outfalls from cities with population &gt; 100,000</td>
<td>Runoff from construction sites with an area &lt; 2 ha</td>
</tr>
<tr>
<td>Overflows of combined storm and sanitary sewers</td>
<td>Runoff from construction sites with an area &lt; 2 ha</td>
</tr>
<tr>
<td>Runoff from construction sites with an area &gt; 2 ha</td>
<td>Runoff from abandoned mines</td>
</tr>
<tr>
<td>Overflows of combined storm and sanitary sewers</td>
<td>Atmospheric deposition over a water surface</td>
</tr>
<tr>
<td>Runoff from construction sites with an area &gt; 2 ha</td>
<td>Activities on land that generate contaminants, such as logging, wetland conversion, construction and development of land or waterways</td>
</tr>
</tbody>
</table>
1.2.2. The phosphorus cycle
As can be seen in Table 1.5, much of the world’s phosphorus is stored in the earth’s crust in igneous rocks containing apatite-complexes of phosphate with calcium and is released from the geological pool by weathering, a naturally-occurring process, which also is fundamental to the formation of soil itself. When phosphorus moves from apatite into clay minerals, it is both tightly bound to the clay lattice in place of hydroxyl ions and more reversibly bound by electrostatic attraction to aluminium or iron [38, 39]. Phosphorus in soil occurs in both inorganic and organic forms, and the total concentration of phosphorus held in soil minerals is high, typically 200 mg kg⁻¹. Soil phosphates are characterized by low solubility of phosphate minerals and strong binding to particle surfaces, giving relatively small soil solution concentrations [40].

Fig. 1.2. The aquatic phosphorus cycle. Adapted from ref. [41] with modifications.

Biogeochemical processes lead to degradation of P compounds, releasing phosphate, which is utilized by living organisms. Some bacteria and algae have been found to use P for synthesis in their cell walls and P has been detected in their waste products [42 - 44]. Organic-P compounds participate in rapid terrestrial and aquatic biological P cycles and are widely distributed in nature (see Fig. 1.2
and Table 1.5). The development of robust methods for the determination of various species of phosphorus in natural waters is necessary to ensure effective regulation and control (see Table 1.6 and section 1.2.4). Most of the methods are spectrophotometric and are based on the reduction of orthophosphate in acid media. The choice of a method depends on the sample matrix and sensitivity required.

### Table 1.5. The major global reservoirs and fluxes of phosphorus. Adapted from reference [19].

<table>
<thead>
<tr>
<th>Reservoir</th>
<th>Tg P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Marine sediments</td>
<td>840,000,000</td>
</tr>
<tr>
<td>Soil</td>
<td>96,000 – 160,000</td>
</tr>
<tr>
<td>Crustal rock</td>
<td>19,000</td>
</tr>
<tr>
<td>Biota</td>
<td>2,600</td>
</tr>
<tr>
<td><strong>Flux</strong></td>
<td><strong>Tg P yr(^{-1})</strong></td>
</tr>
<tr>
<td>Marine dissolved → marine biota</td>
<td>600 – 1,000</td>
</tr>
<tr>
<td>Terrestrial biota → soils</td>
<td>200</td>
</tr>
<tr>
<td>Crustal rock → soils (weathering)</td>
<td>14</td>
</tr>
<tr>
<td>Marine detritus → marine sediment</td>
<td>2 – 13</td>
</tr>
</tbody>
</table>

#### 1.2.3. Determination of orthophosphate

The majority of both manual and automated determinations are based on the formation of phosphomolybdenum blue [45], i.e. a heteropoly compound in which antimony is incorporated (e.g. 45 - 52; see Table 1.6). The reaction sequence is;

**First step:**

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

*Molybdate*  
12-*phosphomolybdic acid*

**Second step:**

\[\text{Reducing agent}\]

\[
\text{H}_3\text{PO}_4(\text{MoO}_3)_{12} \rightarrow \text{Phosphomolybdenum Blue Mo(V)} \quad 2
\]

The Murphy and Riley method [45] utilises ascorbic acid as the reductant while potassium antimonyl tartrate is added as a catalyst. This method suffers interference from e.g. silicate, which is however, a common problem in many other phosphomolybdenum blue based procedures.
Table 1.6. Analytical techniques for the determination of phosphorus in different matrices

<table>
<thead>
<tr>
<th>Matrix</th>
<th>Technique</th>
<th>Reagents</th>
<th>Detection method</th>
<th>LOD (µg L$^{-1}$)</th>
<th>Sample throughput (h$^{-1}$)</th>
<th>Detection temp. (°C)</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sea-water</td>
<td>rFIA</td>
<td>Asc-molyb</td>
<td>Spec</td>
<td>0.033</td>
<td>30</td>
<td>40</td>
<td>[46]</td>
</tr>
<tr>
<td>Sea-water</td>
<td>rFIA</td>
<td>Asc-molyb</td>
<td>Spec</td>
<td>0.016</td>
<td>90</td>
<td>50</td>
<td>[47]</td>
</tr>
<tr>
<td>Freshwater</td>
<td>rFIA</td>
<td>Sn-molyb</td>
<td>Led phot</td>
<td>0.024</td>
<td>225</td>
<td>Not stated</td>
<td>[48]</td>
</tr>
<tr>
<td>Estuarine</td>
<td>rFIA</td>
<td>Asc-molyb</td>
<td>Spec</td>
<td>2</td>
<td>Not stated</td>
<td>60</td>
<td>[49]</td>
</tr>
<tr>
<td>Natural water</td>
<td>rFIA</td>
<td>As-molyb</td>
<td>Led phot</td>
<td>12</td>
<td>Not stated</td>
<td></td>
<td>[7]</td>
</tr>
<tr>
<td>River and waste water</td>
<td>FIA</td>
<td>As-molyb</td>
<td>Spec</td>
<td>0.32</td>
<td>20</td>
<td>70</td>
<td>[50]</td>
</tr>
<tr>
<td>Soil leachates</td>
<td>FIA</td>
<td>Sn-molyb</td>
<td>Spec</td>
<td>7</td>
<td>40</td>
<td></td>
<td>[51]</td>
</tr>
<tr>
<td>Natural waters</td>
<td>FIA</td>
<td>Sn-molyb</td>
<td>Spec</td>
<td>3.3</td>
<td>50</td>
<td></td>
<td>[52]</td>
</tr>
<tr>
<td>Tap water</td>
<td>FIA</td>
<td>Sn-molyb</td>
<td>Photo</td>
<td>0.2</td>
<td>6</td>
<td></td>
<td>[53]</td>
</tr>
<tr>
<td>Freshwater</td>
<td>FIA</td>
<td>Am-lum</td>
<td>CL</td>
<td>0.03</td>
<td>180</td>
<td></td>
<td>[59]</td>
</tr>
<tr>
<td>Bottled water</td>
<td>FIA</td>
<td>Am-thiam</td>
<td>Fluor.</td>
<td>0.03</td>
<td>60</td>
<td></td>
<td>[60]</td>
</tr>
<tr>
<td>River water</td>
<td>FIA</td>
<td>Pyroxg-lum</td>
<td>CL</td>
<td>0.3</td>
<td></td>
<td></td>
<td>[61]</td>
</tr>
<tr>
<td>Natural water</td>
<td>FIA</td>
<td>Sn-molyb/</td>
<td>Spec</td>
<td>0.024</td>
<td>40</td>
<td></td>
<td>[62]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>phytase</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Waste water</td>
<td>FIA</td>
<td>Am-GCE</td>
<td>Ampero</td>
<td>32.6</td>
<td>20</td>
<td></td>
<td>[63]</td>
</tr>
<tr>
<td>CRM, water samples</td>
<td>FIA</td>
<td>Am-GCE</td>
<td>Ampero</td>
<td>3.4</td>
<td>70</td>
<td></td>
<td>[64]</td>
</tr>
<tr>
<td>Plant analysis</td>
<td>MCFIA</td>
<td>As-molyb</td>
<td>Spec</td>
<td>80</td>
<td>37</td>
<td></td>
<td>[65]</td>
</tr>
<tr>
<td>Waste water</td>
<td>MCFIA</td>
<td>As-molyb</td>
<td>Spec</td>
<td>180</td>
<td>70</td>
<td></td>
<td>[66]</td>
</tr>
<tr>
<td>Sea-water</td>
<td>CFA</td>
<td>As-molyb</td>
<td>Spec</td>
<td></td>
<td></td>
<td></td>
<td>[67]</td>
</tr>
<tr>
<td>Waste water</td>
<td>SIA</td>
<td>Van-molyb</td>
<td>Spec</td>
<td>65.2</td>
<td>23</td>
<td></td>
<td>[68]</td>
</tr>
<tr>
<td>Waste water</td>
<td>SIA</td>
<td>Mal-green,</td>
<td>Spec</td>
<td>3.26</td>
<td>30</td>
<td></td>
<td>[69]</td>
</tr>
<tr>
<td>Drinking water</td>
<td>SIA</td>
<td>Sn-molyb</td>
<td>Spec</td>
<td>32.6</td>
<td>75</td>
<td></td>
<td>[70]</td>
</tr>
<tr>
<td>Sediments</td>
<td>SIA</td>
<td>As-molyb</td>
<td>Spec</td>
<td>0.016</td>
<td>25</td>
<td></td>
<td>[71]</td>
</tr>
<tr>
<td>Natural water</td>
<td>SFA</td>
<td>As-molyb</td>
<td>Spec</td>
<td>0.33</td>
<td>30</td>
<td>37</td>
<td>[72]</td>
</tr>
<tr>
<td>Freshwater</td>
<td>SFA</td>
<td>As-molyb</td>
<td>Spec</td>
<td>0.032</td>
<td>20</td>
<td></td>
<td>[73]</td>
</tr>
<tr>
<td>Sea-water/ freshwater</td>
<td>SFA</td>
<td>As-molyb</td>
<td>Spec</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Natural water</td>
<td>ICP-MS</td>
<td></td>
<td></td>
<td>6.5</td>
<td></td>
<td></td>
<td>[74]</td>
</tr>
<tr>
<td>Natural water</td>
<td>ICP-MS</td>
<td></td>
<td></td>
<td>0.26</td>
<td></td>
<td></td>
<td>[75]</td>
</tr>
<tr>
<td>UPW</td>
<td>FIA</td>
<td>Solid-Enr</td>
<td>Spec</td>
<td>0.008</td>
<td></td>
<td></td>
<td>[76]</td>
</tr>
</tbody>
</table>

CRM; certified reference material, rFIA; reverse flow injection analysis, FIA; flow injection analysis, MCFIA; multicommutation flow injection analysis, CFA; continuous flow analysis, SIA; sequential injection analysis, SFA; segmented flow analysis, As; ascorbic acid, Sn tin(II) chloride, Molyb; ammonium molybdate reagent, Pyroxg; pyroxdase, Lum; luminol, Spec; spectrophotometry, Led; light emitting diode, phytase; immobilized phytase enzyme, mal-green; malachite green, ICP-MS; inductively coupled plasma-mass spectrometry, Enr; enrichment, UPW; ultra pure water, GCE; glassy carbon working electrode, phot; photometric, CL; chemiluminescence, Fluo; fluorimetry, Ampero; amperometry and LOD; limit of detection.
Many modifications of this method have been reported, usually involving the use of reductants such as tin(II) chloride, 1-amino-2-naphthol-4-sulphonic acid, sodium sulphite, hydrazine sulphate or combinations thereof [53] or acid strengths in order to improve the selectivity and stability of the chromophore produced [54]. The use of ascorbic acid was first reported by Greenfield and Kalber [55] and tin(II) chloride was suggested by Atkins [56]. The main disadvantage of using ascorbic acid alone is the slow colour development. This shortfall is however overcome by the addition of antimony as a catalyst as modified by Murphy and Riley [45]. This single solution reagent reacts rapidly with phosphate ion to give a very stable blue complex, which contains antimony and phosphorus in a 1:1 atomic ratio. Crouch and Malmstadt showed that phosphomolybdic acid acts stoichiometrically as a 2-electron reductant [57]. Ascorbic acid is preferred to tin(II) chloride as the reducing agent in batch analysis because the reaction is less salt and temperature sensitive and a more stable chromophore is produced [18]. The majority of methods are now based on flow injection [e.g. 52 - 76] (Table 1.6).

1.2.4. Phosphorus speciation and fractionation

There are many P species in both organic and inorganic forms [32, 77]. Dissolved P species include orthophosphate e.g., inorganic condensed P (pyro-, meta- and polyphosphates) and organic condensed phosphates (e.g. ATP). Organic P (e.g. sugar P, inositol P, phospholipids, phosphoproteins, phosphoamides) may be found in both dissolved and particulate forms, associated with plant, animal and bacterial cellular material. Particulate P may be sorbed to mixed phases (e.g. clays, clay-organic complexes and metal oxides and hydroxides) [77 - 79].

Analytical determination of P in natural waters is based on the phosphomolybdic acid methodology, as modified by Murphy and Riley [45]. The following determinations are made:

(a) Soluble reactive phosphorus (SRP), a measure of monomeric inorganic phosphorus (orthophosphate) in solution. This is also referred to as dissolved reactive phosphorus (DRP), dissolved inorganic phosphorus (DIP), filterable reactive phosphorus (FRP) and reactive phosphorus for a filtered sample size (e.g. RP (< 0.45 μM)).

(b) Total dissolved phosphorus (TDP). This is also termed ‘total filterable phosphorus’ (TFP) and is a combination of dissolved monomeric inorganic (SRP) and dissolved hydrolysable (polymeric organic) phosphorus (DHP).
(c) Total phosphorus (TP), is the total dissolved phosphorus (TDP) plus particulate phosphorus (PP) in a water sample.

(d) Enzyme hydrolysable phosphorus (EHP), the organic P fractions that are hydrolysed by alkaline or acid phosphatases and phytases which are naturally occurring [80].

Separation of 'dissolved' and 'particulate' P phases is based mainly on filtration using 0.45 μm (mainly) or 0.2 μm membrane filters (see Fig. 1.3).

Fig. 1.3. Operational steps for the determination of various forms of phosphorus in natural waters. C – G = total suspended phosphorus or total particulate phosphorus; A – E = suspended reactive phosphorus or particulate phosphorus; B – F = suspended acid-hydrolyzable P or particulate acid-hydrolysable P; D – H = suspended organic phosphorus or particulate organic phosphorus. Adapted from reference [27].
Dissolved hydrolysable phosphorus (DHP) is the difference between TDP and SRP, and particulate phosphorus (PP) is the difference between TP and TDP. DHP is also referred to in the literature as 'dissolved unreactive phosphorus' (DUP), soluble unreactive phosphorus (SUP), and unreactive phosphorus (UP) for a filtered sample using a defined filter size (e.g. UP (<0.45 μm)). Differentiation between inorganic and organic forms of PP (particulate inorganic phosphorus (PIP)) and particulate organic phosphorus (POP) has also been made [81]. Molybdate reactive phosphorus (MRP) is used in two distinct ways: (a) for filtered samples, MRP is equivalent to SRP measurements, (b) for unfiltered samples, MRP is equivalent to SRP plus a fraction of particulate P which is reactive to the phosphomolybdenum blue method reagents. In the laboratory, orthophosphate PO₄³⁻ as P was determined while field investigations (analysis) were as FRP.

1.2.5. Kinetic studies of determination of FRP

The rate of formation of the phosphomolybdenum blue complex is dependent on reagent and reactant concentrations and the temperature of the solution. At lower P concentrations and low temperatures, it takes longer to reach full colour development. It is therefore essential in batch procedures to take absorbance readings after full colour development has been accomplished under the prevailing conditions [82, 83]. When a solution containing phosphate and molybdate ions is acidified with H₂SO₄, 12-molybdophosphoric acid (MPA) is formed. In the presence of a reducing agent, 12-molybdophosphoric acid is reduced to the phosphomolybdenum blue complex. The intensity of the blue colour is proportional to the amount of orthophosphate ions incorporated in the complex and hence, the reacted orthophosphate can be determined spectrophotometrically [27, 84].

The use of different concentrations of acids (e.g. H₂SO₄, HClO₄ and HNO₃) by different researchers [e.g. 57, 85 - 88] has important consequences on the kinetics of formation of the phosphomolybdenum blue complex. The formation of this complex involves the formation of a mixture of two isomeric heteropoly acids (the α- and β-forms) in proportions that depends on the acidity of the final solution. Consequently, the effects on the reaction rate of changes in the hydrogen ion and molybdate concentrations have been investigated by several authors [e.g. 74, 88]. The β-form is produced when the deca- or dodeca-molybdate ion is the principal molybdate species in the solution. This isomer undergoes spontaneous transformation to the α-form, but can be stabilized by the addition of polar organic
solvents. The α-form is also produced directly from phosphate when the reacting species is the octa-molybdate ion.

It is essential that the acidity and the molybdate concentration are carefully controlled since these, together with the $[\text{H}^+]/[\text{molybdate}]$ ratio, control the species present. Pai et al. [88] found that the ratio was the crucial parameter, influencing the form of the final reduced complex and the reaction kinetics. Jones et al. [89] have shown that if the acid concentration is less than 0.2 M, the molybdate ion suffers some auto-reduction to molybdenum blue in the subsequent reduction stage. Other workers have also reported that the self-reduction of molybdate ion occurs at $[\text{H}^+] < 0.7$ M [27, 71, 87, 90]. At higher acidity, the β-form of the 12-phosphomolybdic acid is the main product. Although they form at acidities up to at least 1.0 M, this is not desirable in practice because the increasing acidity decreases the rate of reduction to molybdenum blue and increases the risk of hydrolyzing labile organic phosphorus compounds and also increases interference. Cotton [90], using ammonium molybdate and 1,2,4-aminonaphthosulfonic acid, reported a range of 0.4-0.8 M H$_2$SO$_4$ throughout which the absorbance of phosphomolybdenum blue complex were constant. Going and Eisenreich [91] reported that normal colour formation occurred for $[\text{H}^+]:[\text{MoO}_4^{2-}]$ molar ratios of between 60 and 80. Below a molar ratio of 60, interference effects may occur due to self-reduction of MoO$_4^{2-}$ ions to form a molybdenum blue colour independent of P concentration. Above a molar ratio of 80, the reaction becomes slow and incomplete, due to the formation of molybdenyl cationic species, which are unreactive with phosphate. This yields a plateau described by Jackson [92] as the acid stability plateau. Rodriguez et al. [93] examined the acid stability plateau for the ascorbic acid molybdenum blue method, while Zhang et al., Pai et al. and Drummond et al. [71, 89, 94] re-examined the optimum $[\text{H}^+]/[\text{MoO}_4^{2-}]$ ratio under which phosphoantimonyl molybdenum blue is formed.

For each pH, there was a $[\text{H}^+]/[\text{MoO}_4^{2-}]$ range which gave a constant analytical response. This range varied with pH but the range (44 - 78) gave a constant analytical response at all the pH values tested (0.36 - 1.06). In this range, colour formation was complete but self-reduction of the molybdate ions did not occur. Further work on the effect of the $[\text{H}^+]/[\text{MoO}_4^{2-}]$ ratio on the reaction rate showed that ratios between 50 and 70 gave fastest reaction rates at all pH values. For pH 0.36 - 0.88, the full colour development occurred within 0.8 - 1.0 minutes. Pai et al.
[88] reported a ratio of 74 at 20 °C to give optimal colour development. It is important to note that the effective \([H^+]\) reagent concentration may be affected by factors such as the acid-neutralization capacity of the sample matrix [89, 90].

From the foregoing, it is clear that spectrophotometric methods may overestimate orthophosphate, because of molybdate and/or acid-induced hydrolysis of labile organic or condensed phosphorus species [95]. Therefore, dissolved reactive phosphate (DRP) as a predictor of readily bioavailable P may be questionable. Other workers have suggested that total reactive phosphorus (TRP), the molybdate reactive phosphorus (MRP) fraction in unfiltered sample, is as good a measure of bioavailable phosphorus (BAP) as DRP [39, 94 - 98], but this may also lead to over estimation because of hydrolysis of labile P-compounds.

Some authors have examined the "DRP overestimation" while others have proposed techniques to minimize the hydrolysis of labile organic and condensed P compounds. Chamberlin et al. [96] reported a "6-second" technique in which the phosphomolybdate-forming reaction was stopped by adjustment of acid strength prior to formation of phosphomolybdenum blue. Tarapchak et al. [99] confirmed that hydrolysis of DOP occurred very rapidly, in both acidic and acidic-free molybdate and Dick et al. [100] reported overcoming this problem by the addition of a citrate-arsenate reagent to complex the excess molybdate.

1.2.6. Interferences
The separation of phosphate from arsenate and silicate ions is an important challenge in analytical chemistry [101]. The separation can be difficult, especially when large amount of silicates or arsenates are involved. Other ions that can interfere in the analysis of orthophosphates are Fe(III), Ca(II), Al(III), Fe(II), Mg(II) and Cu(II) which occur commonly in natural waters and combine with orthophosphate and organic species to form complexes and precipitates [102]. Fe, Cu and As could pose a serious analytical problem where they occur from an anthropogenic source e.g. an area with a past history of mining and mineral processing like the Tamar Valley [103, 104]. However, in most fresh waters, these ions occur in sufficiently low quantities to not affect the determination of phosphorus [105, 106]. Also species like fluoride, chromium [107], oxidizing agents, nitrite, nitrate and sulphide can interfere in the analysis of phosphate [108].
Silica and arsenate form the blue heteropoly acid like phosphate and also absorbs at similar wavelength [109]. The equation below illustrates the formation of heteropoly molybdic acids;

\[ X^{n-}O_a^{(12a-n)-} + 12MoO_4^{2-} + (24+2a-n)H^+ \rightarrow H_{(12-n)}X(Mo_2O_7)_6 + (6+a)H_2O \]

where \( X \) is P(V), As(V), Si(IV). This equation (i) shows that a large excess of molybdate ions or hydrogen ions will force the reaction to the right [101]. This is very important in the formation of the phosphomolybdenum complex, which is the complex that is measured spectrophotometrically.

1.3. NITROGEN

1.3.1. Nitrogen chemistry

Nitrogen is one of the most electronegative elements, forming bonds with many elements by direct bonding or sharing electron pairs. Various oxidation states exist, +1 to +5 and -3 and it is because of its high electronegativity that it forms \( \text{NH}_3 \). The principal oxidation states are +5 and +3 [24, 25]. It has five stable oxides; \( \text{N}_2\text{O}, \text{NO}, \text{N}_2\text{O}_3, \text{N}_2\text{O}_4 \) (\( \text{NO}_2 \)) and \( \text{N}_2\text{O}_5 \) with oxidation states respectively, +1, +2, +3, +4 and +5. The first two are neutral while the others are acidic. Some basic properties of nitrogen are shown in table 1.7.

<table>
<thead>
<tr>
<th>Atomic number</th>
<th>7</th>
</tr>
</thead>
<tbody>
<tr>
<td>Relative atomic mass</td>
<td>14.007</td>
</tr>
<tr>
<td>Electronic configuration</td>
<td>( 1s^22s^22p^3 )</td>
</tr>
<tr>
<td>Melting point</td>
<td>(-196 , ^\circ\text{C} )</td>
</tr>
<tr>
<td>Boiling Point</td>
<td>(-210 , ^\circ\text{C} )</td>
</tr>
</tbody>
</table>

The nitrogen atom of nitrate anion is sp\(^2\) hybridized. This gives the anion a symmetrical planar arrangement of atoms [25] as shown in Fig. 1.4. Naturally occurring isotopes consists of \( ^{14}\text{N} \) and \( ^{15}\text{N} \). \( ^{15}\text{N} \) is used as a tracer and is important in environmental studies.

![Symmetrical planar molecular shapes of nitrate.](image)

Fig.1.4. Symmetrical planar molecular shapes of nitrate.
1.3.2. Occurrence in the environment
Nitrogen as N\textsubscript{2} (g) dinitrogen is an inert gas and comprises 78% of the total air by volume [109]. In terrestrial and aquatic environment it exists as nitrate (NO\textsubscript{3}\textsuperscript{-}), nitrite (NO\textsubscript{2}\textsuperscript{-}), ammonium (NH\textsubscript{4}\textsuperscript{+}) and organic nitrogen with nitrate being the most abundant [111, 112] in many environments. N\textsubscript{2} can be converted to nitrate by bacteria and algae through biogeochemical processes. A biogeochemical cycle results from the interaction of several biological, chemical and physical processes involving fixation, ammonification and nitrification by micro-organisms present in the air, soil or water.

1.3.3 The nitrogen cycle
Nitrogen is a major component of all living cells, controlling the sites and biochemical reactions in which carbon is oxidized or reduced [1, 23, 26, 113 - 116]. Changes in the available concentration of N over a long period of time may affect the activity of micro-organisms in the rapid cycling of nitrogen in the biosphere as can be seen in Fig.1.5. Ammonium ions are important in the nitrogen cycle because they act as a source and pool of organic-N constituents (e.g. amino acids, peptides, nucleic acids).

*Fig. 1.5. The nitrogen cycle and imports and exports. Adapted from [113].*
Biochemical transformations involving N are possible because of the variable oxidation states ranging from −3 (in NH₃) to +5 in (NO₃⁻). Some micro-organisms e.g. nitrogen fixing bacteria such as Rhizobium in root nodules, utilize the ease with which the various species of N transform from one oxidation state to the other and the energy released in this redox process is used to maintain life [115].

1.3.4. Nitrogen fixation
Bacteria and blue-green algae in soil and aquatic environments carry out nitrogen fixation. A typical nitrogen-fixing cyanobacteria is Anabaena. The other types are symbiotic in nature and are found in association with plants like legumes e.g. Rhizobia and Azotobacter. The general reaction is:

4

\[ \text{N}_2 + 6e^- + 6H^+ \rightarrow 2\text{NH}_3 \]

Nitrosomonas bacteria convert NH₄⁺ to NO₂⁻ while Nitrobacter oxidises NO₂⁻ to NO₃⁻. Denitrification occurs when an anaerobic bacterium reduces NO₃⁻ to N₂ [117, 118].

5

\[ 2\text{NH}_3 + 3\text{O}_2 \xrightarrow{\text{Nitrosomonas}} 2\text{NO}_2^- + 2\text{H}^+ + 2\text{H}_2\text{O} \]

6

\[ 2\text{NO}_2^- + \text{O}_2 \xrightarrow{\text{Nitrobacter}} 2\text{NO}_3^- \]

7

\[ \text{NO}_2^- + \text{NO}_3^- \xrightarrow{\text{Denitrifier}} \text{N}_2 + \text{N}_2\text{O} \]

The depletion of oxygen during this process accounts for the harmful effect of nutrient enrichment and has implication during periods of primary production. In man and animals, nitrite can react with amides or amines and produce nitrosamines, which are poisonous and carcinogenic [119]. Nitrogen is a macronutrient and is important for the growth of plants and animals. Plants uptake of nitrogen is in the form of nitrate (NO₃⁻) and ammonium (NH₄⁺) while intake of foods by animals is the major source. Through enzymatic action, the N is converted to amino acids and peptides, which are the building block of proteins [1, 26, 112, 116].

Enzymes also play a major role in the biochemical processes of N because they act as sites and precursors of the reactions [118]. Death and decay of plants and animals leads to nutrient enrichment of aquatic ecosystems because, through
biogeochemical processes, the N component of DNA, ATP or amino acids and peptides can be converted to nitrate and ammonium. Other sources of nitrate include atmospheric deposition (rainfall and dust) and fertilizers.

Also automobile engines produce NO (with short residence time) which is converted to NO₂. NO₂ and can be dry or wet deposited. Manure applications, waste and poultry litter, leaching, sewage discharges, and anthropogenic sources are examples of point and diffuse sources [11, 1120 - 122]. There is a significant contribution from human factors on the global nitrogen cycle [123] and microorganisms which play a significant role in fluxes between compartments (see Table 1.8).

Table 1.8. The major global reservoirs and fluxes of nitrogen. Adapted from reference [19].

<table>
<thead>
<tr>
<th>Reservoir</th>
<th>$10^{15}$ g N</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lithosphere</td>
<td>190,000,000</td>
</tr>
<tr>
<td>Atmosphere</td>
<td>3,900,000</td>
</tr>
<tr>
<td>Oceanic</td>
<td>23,000</td>
</tr>
<tr>
<td>Terrestrial</td>
<td>500</td>
</tr>
<tr>
<td>Flux</td>
<td></td>
</tr>
<tr>
<td>Dry and wet deposition (terrestrial and ocean)</td>
<td>0.160 – 0.450</td>
</tr>
<tr>
<td>Denitrification (terrestrial)</td>
<td>0.043 – 0.390</td>
</tr>
<tr>
<td>Denitrification (ocean)</td>
<td>0.10 – 0.330</td>
</tr>
<tr>
<td>Biologic nitrogen fixation (terrestrial)</td>
<td>0.044 – 0.200</td>
</tr>
<tr>
<td>Biologic nitrogen fixation (ocean)</td>
<td>0.001 – 0.130</td>
</tr>
<tr>
<td>River runoff (terrestrial and oceanic)</td>
<td>0.013 – 0.040</td>
</tr>
</tbody>
</table>

Nitrogen, like P has also been found to be a limiting nutrient for terrestrial plants [12, 17, 111, 115, 124]. In fresh and marine waters, excessive input of nitrate has been linked to algal blooms with adverse consequences on marine life and the quality of receiving waters has been reported [121]. Redfield et al. [125] showed that the major plant nutrients exist in a fixed stoichiometric ratio in seawater (N:P 16:1) [125, 126] by atoms.
1.3.5. Nitrogen speciation and fractionation

The total N concentration in most natural waters varies from 0.1 to 10.0 mg L\(^{-1}\) ammonia-N and nitrite-N levels do not exceed 0.5 mg L\(^{-1}\) in most rivers except in areas of industrial pollution where the concentrations can rise above 2.0 mg L\(^{-1}\).

In Fig.1.6, concentrations are expressed in mg N L\(^{-1}\). Values are quoted as the ranges typically encountered in a variety of waters. The values in brackets represent indicative values at or above which problems have been reported [18]. TON refers to total oxidized nitrogen i.e. \((\text{NO}_3^- + \text{NO}_2^-)\). Sometimes this abbreviation is also used to denote total organic nitrogen.

![Diagram of nitrogen speciation]

**Fig.1.6. Nitrogen speciation in natural waters. Adapted from reference [18].**

1.3.6. Determination of nitrate

The majority of the methods for the determination of nitrate and nitrite are spectrophotometric and are based on the reduction of nitrate to nitrite in a cadmium column and a diazo-coupling reaction [119, 122, 127 - 145]. The main disadvantages of these methods are toxicity of the reagents (cadmium can cause cancer) and low sensitivity. However, many modifications of the basic method have been reported e.g. a solid phase extraction technique followed by a spectrophotometric detection [141] (see Table 1.9).
<table>
<thead>
<tr>
<th>Matrix</th>
<th>Technique</th>
<th>Reagents/ chemistry</th>
<th>Detect. method</th>
<th>LOD (µg N L⁻¹)</th>
<th>Range (µg N L⁻¹)</th>
<th>Nitrate-N</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sea-water, estuarine and coastal waters</td>
<td>FIA</td>
<td>Cadmium reduction/diazotisation</td>
<td>spec</td>
<td>2.8</td>
<td>2.8-100</td>
<td>N-NO₃⁺</td>
<td>[127]</td>
</tr>
<tr>
<td>Natural waters</td>
<td>FIA</td>
<td>Copperized cadmium reduction/diazotisation</td>
<td>spec</td>
<td>50 and 10⁺</td>
<td></td>
<td>&quot;</td>
<td>[129]</td>
</tr>
<tr>
<td>Natural waters</td>
<td>FIA</td>
<td>Catalytic/redox napth., Green B and KBr H₃PO₄</td>
<td>spec</td>
<td>0.5⁺</td>
<td></td>
<td>N-NO₂⁻</td>
<td>[130]</td>
</tr>
<tr>
<td>Water, soil biological samples</td>
<td>FIA</td>
<td>4,4'-methylene bis-m-nitroaniline in HCl</td>
<td>spec</td>
<td>3.96 and 1.48⁺</td>
<td>1.46-36⁺</td>
<td>N-NO₃⁺</td>
<td>[131]</td>
</tr>
<tr>
<td>Natural waters</td>
<td>FIA</td>
<td>Catalytic/redox spec</td>
<td>0.1 and 3⁺</td>
<td>3-150</td>
<td></td>
<td>&quot;</td>
<td>[119]</td>
</tr>
<tr>
<td>Natural waters</td>
<td>FIA</td>
<td>Cadmium reduction</td>
<td>spec</td>
<td>88.6</td>
<td>10-1000</td>
<td>N-NO₃⁻</td>
<td>[132]</td>
</tr>
<tr>
<td>Water samples</td>
<td>FIA</td>
<td>Copperized cadmium reduction/ phloroglucinol</td>
<td>spec</td>
<td>2.3 and 2.9⁺</td>
<td>10-100 and 3-30⁺</td>
<td>N-NO₃⁻</td>
<td>[133]</td>
</tr>
<tr>
<td>Water samples</td>
<td>FIA</td>
<td>GAP (only HClO₄)</td>
<td>spec</td>
<td>50</td>
<td>500-15000</td>
<td>N-NO₃⁻</td>
<td>[134]</td>
</tr>
<tr>
<td>Drinking and surface waters,</td>
<td>SIA</td>
<td>LAV-Ag/AgCl Pot</td>
<td>5-60</td>
<td></td>
<td></td>
<td>&quot;</td>
<td>[135]</td>
</tr>
<tr>
<td>Waste water, aerosol</td>
<td>SIA</td>
<td>Hydrazine reduction in alkaline medium</td>
<td>spec</td>
<td>70 and 200⁺</td>
<td>1200-24800</td>
<td>N-NO₃⁺</td>
<td>[136]</td>
</tr>
<tr>
<td>Natural waters</td>
<td>FIA</td>
<td>Luminol CL</td>
<td>0.7</td>
<td>22-4400</td>
<td></td>
<td>N-NO₃⁻</td>
<td>[137]</td>
</tr>
<tr>
<td>Water samples</td>
<td>FIA</td>
<td>Luminol CL/phot activation</td>
<td>0.3</td>
<td>0.3-443</td>
<td></td>
<td>&quot;</td>
<td>[138]</td>
</tr>
<tr>
<td>Water samples</td>
<td>FIA</td>
<td>Luminol GP/Cl</td>
<td>0.3</td>
<td></td>
<td></td>
<td>N-NO₃⁺</td>
<td>[139]</td>
</tr>
<tr>
<td>Water samples</td>
<td>FIA</td>
<td>Na₂CO₃ and IC</td>
<td>0.3</td>
<td></td>
<td></td>
<td>N-NO₂⁻</td>
<td>[140]</td>
</tr>
<tr>
<td>Sea-water/estuarine</td>
<td>FIA</td>
<td>Sensor/NINED sulphaniamide</td>
<td>spec</td>
<td>1.4</td>
<td>1.4-770</td>
<td>TON</td>
<td>[141]</td>
</tr>
</tbody>
</table>
Table 1.9 continued. Analytical techniques for the determination of nitrate in different environmental matrices

| Natural waters | SPE (Lichrolut EN) | Spec, LC, 10, 6 and 3 | 20-10,000 | N-NO₃⁺ | [124] |
| Natural waters | SPE (PUF) spec | 40 and 5* 0-560 and 0-140* | N-NO₃⁺ N-NO₂[142] |
| Water | Sodium salicylate spec | 10 100-1000 | N-NO₂[143] |
| Natural waters | Quenching of fluo nitrite 5 and 0.1* | 5-1000 N-NO₃⁺ N-NO₂[144] |

FIA; flow injection analysis, SIA; sequential injection analysis, GAP; green analytical procedure, CL; chemiluminescence, Spec; spectrophotometric, Pot; potentiometric, NINED; N-1-naphthyl ethylenediamine dihydrochloride, SPE; solid phase extraction, PUF; polyurethane foam, IC; ion chromatography, GC; gas chromatography, GP; gas phase, Phot; photochemical, TON; total oxidized nitrogen, Phloroglucinol; (1,3,5-trihydroxybenzene) and (*) nitrite concentration.

1.4. SILICON

1.4.1. Silicon chemistry
Silicon is a group (IV) element and possesses +2 and +4 oxidation states. The +3 oxidation state is possible, but occurs within very unstable non-polar compounds [24]. It exhibits covalent character in bonding. The most stable oxidation state is +4 and this can be reduced to the oxidation state of +2 at high temperature [25].

\[
\text{SiO}_2(\text{s}) + \text{Si(\text{s})} \rightarrow 2\text{SiO}_2(\text{s})
\]

Silica (SiO₂), is the major oxide existing in the crystalline forms quartz, tridymite and cristobalite and these can be interconverted at different temperatures. The simplest silicate ion, SiO₄⁴⁻, exists in tetrahedral form. Other compounds of silicate existing in tetrahedral shapes are orthosilicate, SiO₄⁴⁻, e.g. fosterite, Mg₂SiO₄, pyrosilicates, Si₂O₇⁶⁻ e.g. hemmorhite, Zn₄(OH)₂Si₂O₇⁶⁻ and cyclic silicate, Si₃O₉⁶⁻ e.g. wollastonite, Ca₃(Si₃O₉) [24, 146].
1.4.2. Occurrence

Silica (SiO$_2$) accounts for 95% of mineral species in the earth's crust. Typical examples of silicate minerals are augite Ca(MgFeAl)(AlSi)$_2$O$_6$, pyroxene (CaMgFe)Si$_4$O$_{12}$, amphibole (CaMg)Si$_4$O$_{11}$, biotite mica (KFeMg)Si$_4$O$_{10}$, muscovite (KAl)Si$_4$O$_{10}$ and quartz SiO$_2$ [115, 123, 146 - 148]. The biogeochemistry of silicate is centred on (a) dissolution and decomposition of crustal silicate (b) uptake and utilisation of SiO$_2$ and (c) release and redeposition of silica in sedimentary environments. Synthesis and breakdown of organosilicon compounds are fundamental to the uptake and use of siliceous material by micro-organisms. Some basic properties of silicon are shown in table 1.10.

Table 1.10. Some basic physical properties of silicon. Adapted from reference [25].

<table>
<thead>
<tr>
<th>Property</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Atomic number</td>
<td>14</td>
</tr>
<tr>
<td>Relative atomic mass</td>
<td>28.086</td>
</tr>
<tr>
<td>Electronic configuration</td>
<td>[Ne]3s$^2$3p$^2$</td>
</tr>
<tr>
<td>Melting point</td>
<td>1410 °C</td>
</tr>
<tr>
<td>Boiling point</td>
<td>2355 °C</td>
</tr>
</tbody>
</table>

Equations (9) and (10) describe the solubilization of polymeric silica

\[
\text{SiO}_2 + \text{H}_2\text{O} \rightarrow \text{Si} = \text{O} - \text{OH} \quad \text{(9)}
\]

\[
\text{Si} = \text{O} - \text{OH} + \text{H}_2\text{O} \rightarrow \text{HO-Si-OH} \quad \text{(10)}
\]

During chemical weathering, silicate minerals are degraded, releasing SiO$_2$, e.g.

\[
2\text{KAlSi}_3\text{O}_8 + 3\text{H}_2\text{O} \rightarrow \text{Al}_2\text{Si}_2\text{O}_5(\text{OH})_4 + 4\text{SiO}_2 + 2\text{KOH} \quad \text{(11)}
\]

Products of chemical weathering of silicate minerals are hydroxides of Na, K and Ca and this influences the pH of the environment.
1.4.3. The silicon cycle

The biogeochemistry of silicon is influenced by the activity of micro-organisms (see Fig. 1.7). Biological activity is predominant in the marine environment and diatoms, e.g. Bacillariophyceae, control the cycling of silica. Their influence is more predominant in surface waters [148] and could limit the growth of phytoplankton [149]. A low pH increases the rate at which silicate minerals [150-153] dissolve and living organisms can lower pH by producing organic and inorganic acids. CO₂ dissolved in water can also lower pH by forming carbonic acid. Strong acids e.g. H₂SO₄ play a vital role in silicate mineral degradation in soils and rocks. In hot springs, the activity of sulfur-oxidizing bacteria (Thiobacillus thio-oxidans) produces H₂SO₄ from H₂S found in deep thermal water and silicon concentrations as high as 20 mg L⁻¹ have been determined in hot springs [18].

Typical silicate concentrations in natural waters are shown in Table 1.11.

![Fig. 1.7. The silica cycle. Adapted from reference [152].](image-url)
Table 1.11. Silicate concentration in waters. Adapted from reference [18].

<table>
<thead>
<tr>
<th>Water type</th>
<th>Concentration (mg L⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stream</td>
<td>5</td>
</tr>
<tr>
<td>River</td>
<td>1 – 15</td>
</tr>
<tr>
<td>Lake</td>
<td>&lt; 0.5 – 60</td>
</tr>
<tr>
<td>Sea, surface</td>
<td>Trace</td>
</tr>
<tr>
<td>Sea, deep ocean</td>
<td>4</td>
</tr>
<tr>
<td>Brine</td>
<td>Up to 1000</td>
</tr>
</tbody>
</table>

1.4.4. Determination of silicon

Silicon is spectrophotometrically determined as silicate and is based on the formation of a yellow molybdosillic acid having two isomers (α- and β-isomers) depending on the pH [154]. The α-isomer is formed at pH 3.5 - 4.5 and is more stable than the β-isomer, which is formed more quickly at pH 0.8 - 2.5. However determination is favourable at lower hydrogen ion concentration than phosphate which requires a higher hydrogen ion concentration in order to suppress silicate absorption. The yellow molybdosillic acid is converted to a blue heteropoly compound with a reducing agent e.g. tin(II) chloride, ascorbic acid, ascorbic acid-antimonyl (antimonyl facilitates the formation of the blue complex) [155] or iron(II) fluoride [156]. Phosphorus is the major interferent and can be masked with oxalic acid during spectrophotometric determination [145]. Analytical sensitivity can be enhanced by eliminating other interfering species like Fe, Ca, Al and Mg which occur in association with silicate minerals [18]. Many FI techniques for the determination of silicate have been reported [e.g. 110, 157 - 165], based on chemiluminescence [e.g. 157, 158] and spectrophotometric detection [159 - 164]. These techniques have improved both the analysis time and sensitivity (see Table 1.12).

1.5. LEGISLATION RELATING TO NUTRIENTS IN THE AQUATIC ENVIRONMENT

Concerns regarding the concentrations of N and P in the aquatic environment has received serious attention by Governments at all levels, e.g. the Environment Agency (EA) [165], Community of European States (EU) and World Health Organisation (WHO) [166] have all set guide levels (GL) and maximum admissible concentrations (MAC) for phosphorus and nitrogen species. The EU [167 - 170] has strict regulations regarding the discharge of wastewater and use of various
water bodies for domestic and recreational purposes and member states are expected to comply and implement the appropriate legislative directives [171]. Maximum admissible concentrations and guide levels are shown in Table 1.13.

Table 1.12. Analytical techniques for the determination of silicate

<table>
<thead>
<tr>
<th>Matrix</th>
<th>Technique</th>
<th>Reagents/chemistry</th>
<th>Detection method</th>
<th>Temp °C</th>
<th>LOD (μg Si L⁻¹)</th>
<th>Range</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>freshwaters</td>
<td>FIA</td>
<td>Luminol</td>
<td>CL</td>
<td>25</td>
<td>0.35</td>
<td>0.35-140</td>
<td>[157]</td>
</tr>
<tr>
<td>Natural waters</td>
<td>FIA</td>
<td>Luminol</td>
<td>CL</td>
<td></td>
<td>0.1</td>
<td>1-10</td>
<td>[159]</td>
</tr>
<tr>
<td>Sea-water</td>
<td>rFIA</td>
<td>Asc-molyb</td>
<td>Spec</td>
<td>40</td>
<td>1.5</td>
<td>1.5-74</td>
<td>[46]</td>
</tr>
<tr>
<td>Boiler water</td>
<td>FIA</td>
<td>Asc-molyb</td>
<td>Spec</td>
<td>50</td>
<td>19</td>
<td>39-9333</td>
<td>[159]</td>
</tr>
<tr>
<td>Natural waters</td>
<td>FIA</td>
<td>Asc-molyb</td>
<td>LED</td>
<td></td>
<td>15</td>
<td></td>
<td>[160]</td>
</tr>
<tr>
<td>Natural waters</td>
<td>FIA</td>
<td>Asc-molyb</td>
<td>Spec</td>
<td>95</td>
<td>2.8</td>
<td></td>
<td>[120]</td>
</tr>
<tr>
<td>Sea water</td>
<td>FIA</td>
<td>tin(II)-molyb</td>
<td>Spec</td>
<td>&gt;20</td>
<td>0.12</td>
<td></td>
<td>[161]</td>
</tr>
<tr>
<td>Deep ocean</td>
<td>FIA</td>
<td></td>
<td>Scanner</td>
<td></td>
<td>0.04</td>
<td></td>
<td>[162]</td>
</tr>
<tr>
<td>UPW</td>
<td>FIA</td>
<td>rhodamine</td>
<td>Fluores</td>
<td>100</td>
<td>0.06</td>
<td></td>
<td>[163]</td>
</tr>
<tr>
<td>Natural waters,</td>
<td>SIA</td>
<td>Asc-molyb</td>
<td>Spec</td>
<td></td>
<td>73.68</td>
<td>1842-18420</td>
<td>[53]</td>
</tr>
<tr>
<td>sediments</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>wastewater</td>
<td>SIA</td>
<td>Vanado-complex</td>
<td>Spec</td>
<td>900</td>
<td>900-30000</td>
<td></td>
<td>[54]</td>
</tr>
<tr>
<td>water</td>
<td>SFA</td>
<td>Asc-molyb</td>
<td>Spec</td>
<td></td>
<td>0-1000</td>
<td></td>
<td>[164]</td>
</tr>
</tbody>
</table>

UPW; ultra pure water, LED; light emitting diode, rFIA; reverse flow injection, Asc; ascorbic acid, Molyb; ammonium molybdate, SIA; sequential flow analyser, SFA; segmented flow analyser, tin(II); tin(II) chloride, Spec; spectrophotometric, LOD; limit of detection, Temp; temperature, CL; chemiluminescence, Phot; photometric.

Directive 75/440: Abstraction of drinking water in member states.
Surface waters abstracted for drinking water purposes are grouped into three classes; A, B and C, based on the type and level of treatment. A3 is the most advanced. The MAC for nitrate is 11.3 mg N L⁻¹ for all the classes and for phosphate compliance values are 0.17 mg L⁻¹ PO₄-P for A1 and 0.31 mg L⁻¹ for A2 respectively.
This covers all waters intended for drinking, treated or untreated, regardless of origin. GL and MAC values are shown in Table 1.13.

Table 1.13. Maximum admissible concentrations and guide levels
(expressed as elemental concentrations) for various species in water to be used for human consumption. Adapted from reference [18]

<table>
<thead>
<tr>
<th>Species</th>
<th>MAC (mg L(^{-1}))</th>
<th>GL (mg L(^{-1}))</th>
<th>GL (mg L(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nitrate</td>
<td>11.3</td>
<td>5.65</td>
<td>10</td>
</tr>
<tr>
<td>Total ammonia</td>
<td>0.38</td>
<td>0.038</td>
<td>0.1</td>
</tr>
<tr>
<td>Nitrite</td>
<td>0.03</td>
<td>-</td>
<td>1.0</td>
</tr>
<tr>
<td>Khjeldal nitrogen</td>
<td>1</td>
<td>1</td>
<td>-</td>
</tr>
<tr>
<td>Phosphate</td>
<td>1.091</td>
<td>0.087</td>
<td>1</td>
</tr>
</tbody>
</table>

Directive 91/676: Protection of waters from nitrates from agricultural sources.
This prescribes surveys for the identification of sensitive surface waters with respect to nitrogen inputs, especially of agricultural origin. The aim is to promote sound agricultural practices in order to reduce pollution caused by nitrogen inputs from fertilizers.

This relates to the requirements of treatment of urban wastewater before discharge into a given body of water. They are based on the characteristics of the receiving water and sensitivity to eutrophication. For sensitive areas, the target is to reduce N to 10 mg N L\(^{-1}\) and total P to <2 mg P L\(^{-1}\) from sewage works with 10,000 - 100,000 population equivalents (P.E.) and <1 mg P L\(^{-1}\) for those having more than 100,000 P. E. Formal compliance with this Directive was set for 30 June 1993 with the final deadline for technical compliance being 2005.

1.6. FLOW INJECTION ANALYSIS
The previous sections demonstrate the significance of nutrients in the aquatic environment and legislative drivers for reliable analytical methods. Spectrophotometric methods are most commonly used but in order to achieve high sample throughput and field deployment, continuous flow techniques, such as flow injection analysis (FIA), are preferred. FIA is based on the injection of a liquid sample into a moving, non-segmented, continuous carrier stream of a suitable
The injected sample forms a zone that is transported towards a detector that continuously records the absorbance, electrode potential or other physical parameter as it continuously changes due to passage of the sample material through the flow through cell [172 - 175].

Flow injection techniques evolved from air-segmented continuous flow analysers, which came to prominence in the early 1960s with the introduction of commercially available systems [175]. Ever improving automation and data handling capability provides the capability for more rapid analysis with greater numbers of samples being handled by single systems and fewer analysts. Both this and some practical problems experienced with air segmented continuous flow analysis (CFA) encouraged a number of researchers to continue to seek alternative ways of providing continuous flow analysis. What is now known as ‘Flow Injection Analysis’ (FIA) was promoted by Ruzicka and Hansen in the 1970s [173, 174]. FIA differs from earlier air segmented systems in that the sample is injected directly into a moving stream without the addition of air. The sample reagent zone mixes and reacts as it moves downstream towards the detector; the degree of dispersion is controlled by a variety of factors, their impact being specific to the analytical system in use. It is the control of this dispersion which is at the heart of the technique and coupled with short, highly reproducible retention times separates FIA from other CFA techniques and provides the potential for sampling rates of up to 200 per hour [48].

FIA has been applied to spectrophotometric methods using well-established chemistries that are also used in air segmented and discrete analysis systems [e.g. 46 - 49, 119, 128 - 131, 157 - 160]. Such analysis can be simple, involving no more than the addition of the sample to the moving reagent/carer stream and techniques such as dialysis, solvent extraction and multiple reagent addition as intermediate steps. Improved sensitivity can be achieved using a stopped-flow technique or on-line pre-treatment, but at the expense of sampling rate. FIA can also be applied to other detection systems, e.g. ion selective electrodes or as a means of sample introduction to atomic absorption spectrometry (AAS). It is simple and easy to use, requiring minimal operator training, and little maintenance. FIA allows fast changeover from one chemistry to another and easy method development or modification when required [e.g. 62, 65, 76, 134, 135, 165].
1.6.1. Flow injection instrumentation

A flow injection analyser consists of four main components (Fig. 1.8): a propelling system to transport the carrier stream to the detector, an injection system (Fig. 1.9) to introduce the liquid sample into the carrier stream, a reaction zone to introduce reactants and achieve the appropriate mixing in the moving stream, and finally a detector to continuously monitor the flowing stream.

![Fig. 1.8. A simple flow injection system.](image)

![Fig. 1.9. Injection valve for a flow injection system.](image)

1.6.2 Flow of fluids

In Fig. 1.10, the height, H and area, A are related to the concentration of the species being determined. The time between the injection of sample (S) and the height of peak, H is known as the residence time, T and it is the time during which the actual chemical reaction occurs. A well-designed and properly calibrated FIA
manifold can yield readout typically 15 - 60 seconds from the time of sample injection to detection [119, 174].

![Diagram of FIA manifold](image)

Fig. 1.10. Typical FIA detection response output: S is sample injection, W is peak width, A is peak area, H is peak height and T is residence time. Adapted from reference [172].

This property stands FIA apart from other flow techniques as high sample throughput of more than 200 samples per hour with as little as 50 μL of sample and less than 500 μL of reagents per analysis can be achieved [48].

![Diagram of dispersion in FIA](image)

Fig. 1.11. Dispersion of injected solution in FIA.

1.6.3. Dispersion
The dispersion coefficient (D) as proposed by Ruzicka et al. [174] is a ratio,

\[ D = \frac{C_0}{C} \]

where \( C_0 \) is the initial concentration and \( C \) the concentration at the detector after dispersion in the FIA manifold. In Fig. 1.11, as the injected zone advances, it broadens and forms a dispersed profile downstream. The symmetry of the dispersed fluid changes to an asymmetric shape and a continuum of concentrations results. Dispersion affects sample dilution, thus controlled
dispersion will lead to well-defined dilution. The dispersion in FIA systems increases with reaction tube length, diameter, and flow rate and is inversely proportional to the sample volume injected [47, 121, 174].

Many modifications of the flow injection system have been reported, often aimed at improving the rate of sampling and analysis time. Other objectives are the pre-concentration of samples online, analysis of more than one species and the use of very low sample volumes [60, 76].

1.6.4. Detection
Light is an electromagnetic radiation consisting of two sinusoidal waveforms; electric and magnetic vectors. The energy of a photon is related to wavelength by the expression,

\[ E = \frac{hc}{\lambda} = hv = h\nu \]  

\[ E \text{ = discrete quantum energy, } h = \text{Planck constant (6.63 x } 10^{-34} \text{ Js), } \lambda = \text{wavelength of electromagnetic radiation, } c = \text{velocity of light in a vacuum (2.9979 x } 10^8 \text{ ms}^{-1}), \nu = \text{frequency of radiation (hertz) and } \nu, \text{ wavenumber (cm}^{-1}). \]

The energy of electromagnetic radiation (equation 13) is related to the characteristic frequency of absorption of molecular species. According to Beer-Lambert's law, absorbance is directly proportional to the concentration and path length of the absorbing medium. When light passes through a medium containing an absorbing species, a decrease in intensity (attenuation) upon excitation of the analyte occurs. The longer the path length, the more absorption takes place and the larger the decrease in transmitted intensity. In Fig. 1.12, because of the interactions between the photons and absorbing particles, when incident light passes through the sample cell, the radiant power of the beam decreases from \( P_o \) to \( P \). Transmittance (expressed as a percentage) is the fraction of incident radiation transmitted by the solution (see equation 13)

\[ T = \frac{P}{P_o} \]

\[ \%T = \frac{P}{P_o} \times 100\% \]

\( T \) = transmittance, \( P_o \) incident radiant power and \( P \) emitted radiant power.
Absorbance, $A = abc$, where $b$ is the path length, $c$ is the concentration of the absorbing species and $a$ is the molar absorptivity. Absorbance ($A$) is related to transmittance logarithmically by the equation,

$$A = -\log T = \log \frac{P_o}{P}$$

Table 1.14 shows the range of absorption of light of different colours in the visible spectrum. The eye interprets light of different wavelength as different colours, e.g. if a sample emits a blue light (435 - 480), the eye will see it as yellow light. However, a solution containing metal ion in its chromophore complex will absorb yellow light in the same range of wavelength and the eye will see it as yellow. Blue light is therefore complementary to yellow [121, 176].

<table>
<thead>
<tr>
<th>Approximate wavelength range (nm)</th>
<th>Colour</th>
<th>Complementary colour</th>
</tr>
</thead>
<tbody>
<tr>
<td>400 - 435</td>
<td>Violet</td>
<td>Yellow-green</td>
</tr>
<tr>
<td>435 - 480</td>
<td>Blue</td>
<td>Yellow</td>
</tr>
<tr>
<td>480 - 490</td>
<td>Green-blue</td>
<td>Orange</td>
</tr>
<tr>
<td>490 - 500</td>
<td>Blue-green</td>
<td>Red</td>
</tr>
<tr>
<td>500 - 560</td>
<td>Green</td>
<td>Purple</td>
</tr>
<tr>
<td>560 - 580</td>
<td>Yellow-green</td>
<td>Violet</td>
</tr>
<tr>
<td>580 - 595</td>
<td>Yellow</td>
<td>Blue</td>
</tr>
<tr>
<td>595 - 650</td>
<td>Orange</td>
<td>Green-green</td>
</tr>
<tr>
<td>650 - 750</td>
<td>Red</td>
<td>Blue-green</td>
</tr>
</tbody>
</table>

Fig. 1.12. UV/VIS spectrophotometer. Adapted from reference [146].
1.7. AIMS AND OBJECTIVES

The overall aim of this research is to develop simple but robust flow injection analysis (FIA) techniques that can be adapted to the determination (monitoring in situ) of nutrients, especially phosphorus species, in natural waters. These techniques will be used to study the concentrations and aquatic cycling of nutrient species in impacted estuarine environments.

The specific objectives of the research are to:

1. Design and optimise an automated flow injection method for the determination of inorganic-P in rivers, lakes and estuaries with a target detection limit of 1 μg P L⁻¹ in the presence of interfering species such as arsenate and silicate.

2. Design and optimise a flow injection spectrophotometric method for the determination of silicon in anthropogenically impacted natural waters such as the Tamar Estuary with a target detection limit of 20 μg Si L⁻¹.

3 Adapt the inorganic-P flow injection manifold for the determination of organic-P in rivers, lakes and estuaries by off-line or batch autoclaving with particular emphasis on conditions that can significantly affect recovery of dissolved organic-P in unpolluted waters.

4 Design a flow injection manifold for the selective determination of organic-P compounds, e.g. phytic acid, by incorporating immobilized enzymes e.g. phytases. This will assist in understanding of the cycling of phytic acid in an anthropogenically impacted area like the Tamar.

5 Use continuous flow spectrophotometric methods (and related chemical/biological techniques) to determine the inorganic and organic phosphorus species in the Tamar Estuary over different seasons. Other determinants that will aid interpretation include nitrogen species, trace metals (e.g. Fe, Cu), suspended solids, pH, DO, conductivity and salinity.
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CHAPTER TWO

FLOW INJECTION (FI) SPECTROPHOTOMETRIC METHOD FOR THE DETERMINATION OF FILTERABLE REACTIVE PHOSPHORUS (FRP) IN NATURAL WATERS IN THE PRESENCE OF ARSENATE (>300 µg As L⁻¹) AND SILICATE (1.9 mg Si L⁻¹)

Main topics in the chapter

Introduction
Experimental
Optimisation of FI manifold
Application to Tamar Estuary
Conclusions
2. INTRODUCTION

Phosphorus is an essential and often limiting nutrient for phytoplankton growth. Excessive loading of phosphorus in its various physico-chemical forms contributes to the eutrophication of natural waters [1 - 5]. The determination of P species in natural waters is therefore important for monitoring water quality and controlling P in the aquatic environment [6, 7].

Most analytical methods for the determination of P use spectrophotometric detection and are based on the formation of 12-molybdophosphoric acid from the reaction of phosphate with molybdate in acid solution and subsequent reduction with either tin(II) chloride or ascorbic acid [8,9]. Antimonyl tartrate is often used as a catalyst to produce the blue heteropoly phosphomolybdenum compound as first reported by Murphy and Riley in 1962 [9]. This method is also sensitive to silicate and arsenate, which form similar blue heteropoly molybdenum compounds.

First step

\[
PO_4^{3-} + 12MoO_4^{2-} + 27H^+ \rightarrow H_3PO_4(MoO_3)_{12} + 12H_2O
\]

Molybdate  12-phosphomolybdic acid

Second step

Reducing agent

\[
H_3PO_4(MoO_3)_{12} \rightarrow \text{phosphomolybdenum blue Mo(V)}
\]

In addition to the several spectrophotometric methods available for phosphate determination [e.g. 10 - 18], a chemiluminescence method based on the reaction of luminol with phosphomolybdate has been reported [19, 20]. Silicates, phosphates and arsenates have also been determined by on-line column separation [21] and differential kinetics [22, 23] based on the different rates of formation of the silicomolybdenum and phosphomolybdenum complexes, and a solid phase enrichment of phosphomolybdenum without organic solvents [24] has also been reported.

Flow injection (FI) techniques have gained universal acceptance for routine laboratory analysis because of their simplicity, reliability and economy. They are based on the injection of a liquid sample into a flowing, unsegmented carrier stream of a suitable liquid and transported to a detector, which records the transient response or absorbance [25, 26]. One important observation from most of the approaches for P determination is the possibility of over estimation or under
estimation of P as a result of the conditions of determination. Many anions and cations are known to cause interferences in the determination of phosphate [27] and most of the interferences are more pronounced at higher reaction temperatures. The possibility of conversion of organic-P or particulate P species to reactive P in acid media is high when higher temperatures are used [28]. High acid concentrations can induce hydrolysis of organic P species [11] and could increase the effect of interferences by cations e.g. Fe(III), As(III) [10]. Low acid concentrations favour interferences from, for example, Cu [29]. All of these could adversely affect the determination of P, especially in areas that have a history of mineral processing and mining where these mineral elements could be present as an anthropogenic source [30, 31].

There are many reports on the interferences caused by silicate and arsenate in the determination of soluble reactive phosphate (FRP) [e.g. 10 - 12, 28, 29, 32]. High levels of arsenate complicate the analysis, particularly at elevated temperatures, because increased temperature favours the formation of the blue As-complex [14]. The sample matrix is also important as it directly affects the type and magnitude of the interference [13]. Freshwater concentrations of arsenate and silicate may be higher than phosphate depending on the geography/geology of the area. Human activities (sewage and industrial discharges, agricultural run-off and indirectly from mining operations) can exert great influence on water courses such as the Tamar Estuary. Andreae et al. reported typical arsenic concentrations of 54 - 92 mg As L\(^{-1}\) in suspended particulates in the Tejo Estuary, Portugal [33], Seyler et al. reported concentrations of 45 \(\mu\)g As L\(^{-1}\) in some polluted rivers of Europe [34] and Minganti et al. reported arsenic concentrations of 2-310 \(\mu\)g As L\(^{-1}\) in ferns (a type of sea plant having its roots in the water surface) along the Ligurian coast, Italy [35].

The molar ratio of acid to molybdate ([H\(^+\)]:[MoO\(_4\)\(^{2-}\)]) is very important in the spectrophotometric determination of phosphate. Different molar ratios are used [36-39], depending on the nature of the matrix and concentration of phosphate in the sample. A ([H\(^+\)]:[MoO\(_4\)\(^{2-}\)]) ratio of 70, as suggested by Zhang et al. [17] and Pai et al. [39], has a positive affect on the spectrophotometric determination of phosphate, especially when a manual method is used. For Fl, a ratio from 50-80 will have a positive influence on the sensitivity of P determination. Most automated methods however do not follow this trend, as a higher acid concentration compared with molybdate concentration is used [40]. Many investigators have
suggested using a high [H+] with pH < 1.0 in order to avoid interference by silicate [8, 23, 41]. However results presented in this chapter show that high [H+] could be problematic in FI. Colour builds up on the walls of the tubes and the possibility of enhanced interference, especially at elevated temperature, is significant [17]. This will reduce sampling time and increase analysis time.

The objective of this work was to re-optimise a previously reported manifold [42] for the determination of phosphate in the 20 - 100 µg P L⁻¹ concentration range in the presence of silicate averaging 1.9 mg Si L⁻¹ and arsenate at >300 µg As L⁻¹. These concentrations represent typical silicate [43] and arsenate [44] concentrations in the Tamar Estuary. The optimised manifold was then compared with a standard laboratory method for P determination using samples from a Tamar transect.

2.1. EXPERIMENTAL
2.1.1. Reagents
All reagents were prepared using ultrapure-deionised water (resistance = 18.2 MΩ cm⁻¹, UPW, Elga). In order to avoid contamination, all glassware, high-density polyethylene (HDPE) storage bottles for reagent solutions and plastic items were acid cleaned following a standard procedure [45 - 47]. All items for samples and reagents were first washed with distilled water, rinsed three times with ultrapure-deionised water, dried and then soaked in a 1% Nutracon (nutrient-P free reagent) solution for at least 24 hours, rinsed with UPW three times and soaked again in a 10% v/v HCl solution for at least 24 hours and later rinsed three times with UPW. The washed apparatus was dried and stored in zip locked polyethylene bags.

1. Phosphate (K₂HPO₄). A 10 mg P L⁻¹ stock solution was prepared freshly from 1000 mg P L⁻¹ stock solution. P calibration standards in the range of 10 - 100 µg P L⁻¹ were prepared by serial dilution of the stock solution with UPW unless otherwise stated.

2. Ammonium molybdate ((NH₄)₆Mo₇O₂₄·4H₂O). A 0.10 M stock solution was prepared by dissolving 12.358 g of ammonium heptamolybdate in a 100 mL container with UPW. A 0.0129 M working standard was prepared freshly from this stock for use. Both solutions were stable for several weeks. The
0.10 M stock was stored at room temperature in the dark in an HDPE container.

3. Sulphuric acid (H₂SO₄). The AnalaR stock had a specific gravity of 18.4 g dm⁻³. A 2 M stock was prepared by dissolving 27.17 mL from this stock in a flask containing 200 mL UPW. This was made up to the 250 mL mark and stored at room temperature. A 0.90 M solution was made from this stock by dilution unless otherwise stated.

4. L-ascorbic acid (C₆H₇O₆). A 4.6 g of ascorbic acid was dissolved in a 100 mL volumetric flask with UPW and made up to the 100 mL mark. The solution was prepared daily.

5. Antimonyl potassium tartarate (KSbO₄C₄H₄O₆). A 0.10 M stock was prepared by dissolving 3.249 g of antimony potassium tartrate in 100 mL of UPW. A 0.0015 M solution was prepared from this stock and used as required. The stock solution was stable for several weeks.

6. Sodium metasilicate (Na₂O₃Si.9H₂O). A 1000 mg Si L⁻¹ stock was prepared by dissolving 10.14 g in 1 L of UPW. A 10 mg Si L⁻¹ stock was prepared from this and Si calibration standards in the range of 100-1000 µg Si L⁻¹ were prepared by serial dilution of the 10 mg Si L⁻¹ stock unless otherwise stated. This standard was stable for several weeks at room temperature.

7. Sodium thiosulphate (Na₂S₂O₃.5H₂O). A 0.002 M solution was prepared freshly and used immediately by dissolving 0.12 g of sodium thiosulphate in 250 mL of UPW. The thiosulphate standard is very unstable and must be protected from direct sunlight. A black HDPE bottle was always used for storage to minimise decomposition by light.

8. Sodium arsenate heptahydrate (Na₂HAsO₄.7H₂O). An arsenate standard was prepared from the 1000 mg As(V) L⁻¹ stock in 5% (v/v) HNO₃ AnalaR solution. Concentrations in the range 27 - 540 µg As L⁻¹ were prepared freshly from this stock daily with UPW and used immediately. Arsenate is not stable in UPW but is stable in acid solution.

9. Low Nutrient Seawater (LNS) salinity 35. This was used as supplied or as stated. Serial dilutions in the range 10 - 30 salinity were prepared by dilution of the stock in UPW.

2.1.2. Instrumentation and Procedures
The Fl manifold with spectrophotometric detection incorporated 0.75 mm i.d. Teflon tubing, a peristaltic pump (Gilson Minipuls 2, Villiers le Bel, France) and a
Rheodyne six-port switching valve. Data was collected automatically using LabVIEW™ and samples were injected manually. The proposed four-channel flow injection FI system incorporating a thiosulphate stream for the determination of < 100 μg P L⁻¹ is shown in Fig. 2.1. The peristaltic pump was used to propel the carrier/thiosulphate and reagent streams at flow rates of 0.42 and 0.17 mL min⁻¹ respectively. The rotary injection valve was used to inject 440 μL of sample into the carrier stream. Sample and carrier were mixed at a T-piece with the thiosulphate stream. Here As(V) was reduced to As(III). This then mixed with the molybdate reagent in another T-piece and finally with the ascorbic acid reductant in a water bath at 30 °C and was passed into a uv/visible/nir spectrophotometer [48] (Philips PU 8620, Cambridge England) set at 790 nm equipped with a 30 μL flow cell (178.011 - OS 10 mm light path, Optical Glass Windows 320-2500 nm, Hellma, UK). The detector output was automatically recorded using an in-house LabVIEW™ software programme. A uv-visible spectrophotometer [49] (Hewlett Packard HP84553) was used to obtain an absorbance spectrum of the molybdenum blue compound produced using the manifold described in Fig. 2.1. This spectrum was obtained in order to determine the wavelength of maximum absorbance for the FI manifold [24].

Fig. 2.1. Four channel ascorbic acid manifold incorporating a thiosulphate stream for the determination of 20 - 100 μg P L⁻¹ in natural waters in the presence of high arsenate concentrations.

Water samples were filtered in situ using a 0.45 μm filter (cellulose acetate) and stored in pre-cleaned HDPE containers. Water samples were analysed within 24 hours of collection and, when possible, stored at 4 °C prior to analysis. If the samples were stored for longer periods prior to analysis, then they were frozen at
-20°C. Arsenic was analysed using hydride generation atomic absorption spectrometry for saline samples [50, 51] and ICP-MS for freshwater samples.

2.2. RESULTS AND DISCUSSION

2.2.1. Optimisation of various FI parameters

One of the main requirements of continuous-flow techniques is that colour development should be fast to avoid prolonging analysis time, which can lead to increased contamination between reagents and samples and reduced sensitivity. In this work reagent concentrations were optimised to achieve the fastest possible reaction time.

2.2.2. Sample Volume

The effect of sample volume on the determination of phosphate in terms of sensitivity was studied by injecting different volumes from 220 - 500 μL. Fig. 2.2. illustrates the effect of sample volume on the peak height. The response (peak height) of the system increased as the sample volume increased [52, 53], but this decreased sampling rate. A sample volume of 440 μL yielded satisfactory sensitivity and a good sample throughput and was used for all further experiments. At this volume the peak shapes were good and there was no observable refractive index (Schleiren) effect [54].

![Fig. 2.2. Effect of sample volume on response of 33 μg P L⁻¹. Error bars, σ ± 3s (n = 3).](image-url)
2.2.3. Ascorbic Acid Concentration

The ascorbic acid concentration was optimised to achieve a fast reaction time and to utilise the reagent in the most economical manner [8]. The concentration was varied from 30 to 60 g L\(^{-1}\). It was observed that as the concentration increased, there was an increase in response up to 40 g L\(^{-1}\) above which the response reached a plateau (see Fig. 2.3). Thus 46 g L\(^{-1}\) was used throughout the remainder of the work. At this concentration, the time to reach maximum colour development was 40 s at 60 °C and 55 s at 30 °C. This observation is in agreement with the reports by Neal et al. [28] and shows the danger of using an elevated temperature to maximise sensitivity which could lead to interference and overestimation of results. This concentration is higher than concentrations used in slower manual methods [9] where, e.g., 17.6 g L\(^{-1}\) of ascorbic acid was used and time for maximum colour development was 10 min.

Fig. 2.3. Dependence of FI response on ascorbic acid concentration for the determination of 33 μg P L\(^{-1}\). Error bars σ ± 3s (n = 3).

2.3.4. Antimonyl Tartarate Concentration

Antimony (Sb\(^{3+}\)) was introduced into the molybdate reagent stream as antimonyl potassium tartarate to increase the rate of colour development and improve sensitivity [37, 55]. The concentration of antimony necessary to effect this varies from one method to another and from one technique to another. For optimisation, 0.0005 M to 0.002 M was used as shown below.
In Fig. 2.4, the lower concentration resulted in poor colour development and low response while 0.002 M and above led to precipitation. Noisy and toothed peaks were also observed at this higher concentration. The 0.0015 M gave the optimum response and was therefore chosen for this study.

![Graph: Absorbance vs Antimony tartarate concentration (M)]

Fig. 2.4. Effect of antimony tartarate concentration on molybdate on the determination of 33 μg P L⁻¹. Error bars σ ± 3s (n=3). Other conditions as shown in Fig. 2.1.

2.2.5. Effect of [H⁺]:[MoO₄²⁻] mole ratio

The effect of [H⁺] on the phosphomolybdenum blue reaction has been reported previously by many workers [e.g. 56, 57]. Pai et al. reported that 0.9 - 1.3 M H₂SO₄ gave the optimum response in a batch system depending on the concentration of phosphate used in the study. Below 0.8 M [H⁺], self reduction of the molybdate occurs [58]. The [H⁺]:[MoO₄²⁻] ratio is important in the determination of phosphate. The effect of changing [H⁺]:[molybdate] ratio was studied by keeping the concentration of molybdate constant at 0.0089 M, antimony at 0.0015 M and ascorbic acid at 45 g L⁻¹, and varying the [H⁺]. A low phosphate concentration of 16.3 μg P L⁻¹ and 1.40 mg Si L⁻¹ were used and the results obtained are shown in Table 2.1. The low phosphate and high silicate concentrations were used to represent typical conditions found in the Tamar Estuary [59-61] and unpolluted natural waters.

From these investigations, the phosphoantimonymolybdenum reaction is particularly [H⁺] dependent and the progress of the reaction is determined by the ratio of [H⁺]:[molybdate] in the system. In this investigation the high absorbance
as seen in Table 2.1, when using >0.8 M [H⁺] was primarily because the concentration of H⁺ was not sufficiently high to prevent self-reduction. The 0.8 M H⁺ concentration was not chosen despite the high absorbance because it will lead to interference by silicate which absorbs strongly at low acidity as can be seen in Table 2.1.

Table 2.1. Effect of H⁺ concentration on the separate determination of phosphate (16.3 μg P L⁻¹) and silicate (1.9 mg Si L⁻¹ at different P and Si concentrations when the concentration of molybdate was kept constant at 8.9 mM.

<table>
<thead>
<tr>
<th>pH</th>
<th>[H⁺] (M)</th>
<th>[H⁺]:[MoO₄²⁻]</th>
<th>16.3 μg L⁻¹ P</th>
<th>1.9 mg L⁻¹ Si</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.3</td>
<td>0.5</td>
<td>56</td>
<td>0.286 ± 0.002</td>
<td>1.075 ± 0.025</td>
</tr>
<tr>
<td>1.3</td>
<td>0.6</td>
<td>67</td>
<td>0.179 ± 0.005</td>
<td>0.625 ± 0.012</td>
</tr>
<tr>
<td>1.2</td>
<td>0.7</td>
<td>78</td>
<td>0.093 ± 0.001</td>
<td>0.257 ± 0.006</td>
</tr>
<tr>
<td>1.2</td>
<td>0.8</td>
<td>90</td>
<td>0.066 ± 0.002</td>
<td>0.133 ± 0.003</td>
</tr>
<tr>
<td>1.1</td>
<td>0.9</td>
<td>101</td>
<td>0.018 ± 0.001</td>
<td>0.005 ± 0.001</td>
</tr>
<tr>
<td>1.1</td>
<td>1.0</td>
<td>112</td>
<td>0.011 ± 0.000</td>
<td>0.004 ± 0.000</td>
</tr>
<tr>
<td>1.1</td>
<td>1.1</td>
<td>135</td>
<td>0.002 ± 0.000</td>
<td>0.002 ± 0.000</td>
</tr>
</tbody>
</table>

2.2.6. Molybdate concentration.

The [H⁺] was then kept constant at 0.9 M and molybdate concentration varied from 0.008 - 0.015 M. A linear relationship was observed as can be seen in Fig. 2.5. The higher molybdate concentrations were not chosen for future experiments, even though they gave high response, to avoid self-reduction and high blank signals which can lead to overestimation of P and increased interferences. The 0.9 M [H⁺] was better (see Table 2.1), because of minimal interference from silicate in natural waters where the phosphate concentration is <16 μg P L⁻¹ and silicate <1.9 mg Si L⁻¹. The suppressing effect observed at higher [H⁺] is in agreement with reports of other workers in the field [39, 56 - 58, 62]. A higher [H⁺] also resulted in occlusion of the molybdate blue complex on the walls of the tubing and the flow cell, resulting in increased and drifting absorbance.
Fig. 2.5. Effect of increasing molybdate concentration and hydrogen ion kept constant at 0.9 M. Error bars ± 3σ (n=3). Other conditions during the investigation are as shown in Fig. 2.1.

2.2.7. Reaction Temperature
The phosphomolybdenum blue reaction is temperature dependent and so the temperature of the water bath housing the reaction coil was investigated and, as can be seen in Fig. 2.6, there was a gradual increase in response with increasing temperature. A temperature of 60 °C was optimum for the determination of P in this method due to increased noise at higher temperature.

Fig. 2.6. Optimisation of temperature of the reaction coil for the determination of P (33 μg P L⁻¹) at λ = 790 nm.
However, it has been reported that at higher temperatures there is increased interference from silicate and arsenate [63, 64]. Although most reported methods have used 60 °C or above [11, 28] to maximise sensitivity, this temperature is not suitable for the determination of P in samples from areas such as the Tamar Estuary [64].

2.2.8. Major interferences (As and Si) in the determination of low concentrations of phosphorus < 20 μg P L⁻¹.

As shown in Fig. 2.7, an increase in temperature from 30-50 °C leads to a significant increase in sensitivity (response) for phosphate. However the nature of the matrix in the sampling area is important in the choice of temperature required for the determination of low phosphate concentrations. Increasing temperature enhances the relative sensitivity of silicate compared with phosphate (see Figs. 2.7 and 2.8).

![Graph showing effect of temperature on P response.](image)

Fig. 2.7. Effect of temperature on P response.

Fig. 2.8 shows the effect of Si on the determination of phosphate at different temperatures. The lowest sensitivity is at 30 °C and silicate concentrations <1.6 mg Si L⁻¹ will not interfere significantly in the determination of P at low concentrations in natural waters at this temperature.
Fig. 2.8. Effect of temperature on the response for Si on the determination of phosphate as P.

In Fig. 2.9, it can be seen that higher temperatures also promote the formation of the arsenomolybdenum complex. Therefore, the determination of phosphorus at low concentrations e.g. <20 μg P L\(^{-1}\) using 50 °C is not advisable because of increased sensitivity to arsenate [11].

Fig. 2.9. Effect of temperature on the response for As (as arsenate) in the determination of phosphate as P.

2.2.9. Masking Arsenate

Arsenic is a metalloid and its average concentration in the earth's crust is 1.8 mg kg\(^{-1}\). Human activity can generate anthropogenic arsenic, which is a major
environmental pollutant. Arsenic is used in the manufacture of pesticides and herbicides, paints and semi-conductors [65-68]. It is chemically very labile, readily changing its oxidation state. This enhances biological and chemical reactions, which are further influenced by Eh/pH, biological activity, and adsorption or desorption processes. The various oxidation states are; (0, +5, +3 and -3). Arsenate (AsO$_4^{3-}$) and arsenite (AsO$_3^{3-}$) are the most studied species [69].

Most of these oxidation states are found in sediments, ground water, natural waters and mine tailings i.e. anthropogenic sources. Marijin et al. reported four different types of arsenosugars with one of the sugars having a phosphate group [70]. Various reagents have been used to minimise arsenate interference during the determination of phosphate. Ferric(III) chloride and sodium sulphite [10] were investigated in this research but they were not effective in the FI manifold used (see Fig. 2.1). Sodium thiosulphate [11, 71] was also investigated and found suitable for the FI manifold used. Thiosulphate converts arsenate (As(V), AsO$_4^{3-}$) to arsenite (As(III), AsO$_3^{3-}$), thus preventing the formation of the absorbing species i.e. the, blue arsenomolybdate complex which would otherwise interfere with the spectrophotometric determination of phosphate.

$$2\text{AsO}_4^{3-} + 2\text{S}_2\text{O}_3^- \rightarrow 2\text{AsO}_3^{3-} + 2\text{SO}_4^{3-} + 2\text{S}$$

arsenic(V) thiosulphate arsenic(III)

Various concentrations of thiosulphate were used in the thiosulphate stream (see Fig. 2.1) to monitor the effect on the system baseline, and the results are shown in Table 2.2. Low concentrations of thiosulphate did not have an effect on the baseline, but as the concentration of the thiosulphate was increased above 0.006 M, a Schleiren effect was observed. This could be as the result of a breakdown of thiosulphate to sulphide, which forms colloidal particles [11] that could affect detector response. Sulphides also interfere directly in the spectrophotometric determination of P [12]. A Schleiren effect [54], i.e. negative peaks, was observed when 0.006-0.01 M concentrations of thiosulphate were used. Therefore the optimum thiosulphate concentration to give a stable baseline for the FI manifold shown in Fig. 2.1 was 0.002 M. The effectiveness of 0.002 M thiosulphate was then investigated for its ability to mask arsenate over the range 220-540 µg As L$^{-1}$. 

54
Table 2.2. Effect of thiosulphate on the baseline signal of the FI manifold at a temperature of 40 °C.

<table>
<thead>
<tr>
<th>Thiosulphate Conc. (M)</th>
<th>Mean Absorbance (n = 3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.000</td>
<td>0.000 ± 0.000</td>
</tr>
<tr>
<td>0.002</td>
<td>0.000 ± 0.000</td>
</tr>
<tr>
<td>0.004</td>
<td>0.000 ± 0.000</td>
</tr>
<tr>
<td>0.006</td>
<td>-0.002 ± 0.000</td>
</tr>
<tr>
<td>0.008</td>
<td>-0.005 ± 0.000</td>
</tr>
<tr>
<td>0.010</td>
<td>-0.008 ± 0.001</td>
</tr>
</tbody>
</table>

Fig. 2.11 shows that this concentration effectively masked arsenate as compared with the response in the absence of thiosulphate.

![Graph showing effect of thiosulphate on arsenate response](image)

**Fig. 2.10. Effect of thiosulphate concentration on arsenate response at 40 °C.**

It should be noted that arsenate reacts faster than Si but slower than P [11] and from this work, colour development in the FI manifold occurred after 110 s at 40 °C and 138 s at 30 °C. When using thiosulphate it is also important to prepare the reagent fresh each day and store in the dark.

The effects of other potential interferences in the system were also investigated. Some of the species chosen e.g. Fe(III), Al(III), Cr(VI), Mg(II) and Ca(II) have been reported as interferents in the determination of P [72, 73]. These ions are present in soils and natural waters in association with phosphate minerals [73 - 77]. During weathering of rocks or via biogeochemical reactions, these cations are released together in various proportions. Typical phosphate minerals are vivianite, Fe$_3$(PO$_4$)$_2$·8H$_2$O, ludlamite, (Fe,Mn,Mg)$_3$(PO$_4$)$_2$·4H$_2$O, graftonite, (Fe,Mn,Ca)$_3$(PO$_4$)$_2$
and variscite, AlPO₄·2H₂O [73-75]. Al³⁺, Fe³⁺ and Ca²⁺ can remove dissolved phosphate through chemical precipitation [73]. Also Al, Ca, Fe and Mn have been reported to form a sparingly soluble secondary orthophosphate mineral which has reduced mobility due to sorption onto positively charged mineral surfaces. It is therefore necessary to investigate their effects, especially in an anthropogenically impacted area like the Tamar Estuary where several years of mining and mineral processes have impacted significantly on the environment [31]. Concentrations of cations were Fe(II), Fe(III), NH₄⁺ and Cu(II) (1 mg L⁻¹ each); Pb(II), Mg(II), Al(III), Ca(II), Mn(II), Zn(II) and K⁺ (10 mg L⁻¹ each) and anions, NO₂⁻ (1 mg L⁻¹); NO₃⁻ (30 mg L⁻¹), SO₄²⁻ (250 mg L⁻¹), AsO₄³⁻ (0.1 mg) and the results are shown in Figs. 2.11-2.12. Copper interferes to the greatest extent at low acid concentration (<0.8 M) [29]. Iron also interferes but at a higher acid concentration (>0.9 M). EDTA (0.0001 M) can be used to mask the cations and tartaric acid (0.004 M) used to mask silicate in FI when determination is carried out at 60 °C but at low temperatures interferences by trace metals are negligible.

![Graph showing effect of cations on spectrophotometric determination of phosphate](image)

**Fig. 2.11.** Effect of cations on the spectrophotometric determination of phosphate (100 ug = 100 μg P L⁻¹). Error bars σ ± 3s (n=3).

The concentrations of the cations and anions in the Tamar Estuary are lower than those used those used in these investigations [43, 50 - 52, 78] and hence should not pose any problem at optimal conditions of determination e.g. low temperature.
2.2.10. Analytical figures of merit
A calibration was performed with the optimised manifold (see Fig. 2.1) at 30 °C and a practical limit of detection of 2 μg P L⁻¹. Increasing the temperature to 40 °C gave a limit of detection of < 1 μg P L⁻¹. Calibration data are shown in Table 2.3. The limit of detection (LOD) is defined as the concentration, which gives an instrument signal (Y) significantly different from the 'blank' or 'background' signal [79]. LOD = Yb + 3sb; where Yb = blank signal and Sb = standard deviation.

Table 2.3. Analytical figures of merit for the four channel Fl ascorbic acid manifold for the determination of P 2-100 μg P L⁻¹ in the presence of As <300 μg As L⁻¹ in natural waters.

<table>
<thead>
<tr>
<th>Parameter</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Linear range (μg P L⁻¹)</td>
<td>2-100</td>
</tr>
<tr>
<td>Calibration graph</td>
<td></td>
</tr>
<tr>
<td>Slope</td>
<td>0.0003</td>
</tr>
<tr>
<td>Intercept</td>
<td>0.0002</td>
</tr>
<tr>
<td>Correlation coefficient</td>
<td>0.9991</td>
</tr>
<tr>
<td>Limit of detection measured (μg P L⁻¹)</td>
<td>2.0</td>
</tr>
<tr>
<td>R.S.D. % (n = 4)</td>
<td>&lt; 1.5</td>
</tr>
<tr>
<td>Sampling Frequency (h⁻¹)</td>
<td>40</td>
</tr>
<tr>
<td>Detection temperature</td>
<td>30 °C</td>
</tr>
</tbody>
</table>
2.2.11. Application of the FI method to the determination of FRP in natural waters.

The Tamar estuary lies on latitude 50°C 26’14 N and longitude 04°C 11’ 42 W. The Estuary flows into the English Channel and is fed by the rivers Tamar, Lynher and Tavy. The Tamar River provides the main input of fresh water. Several years of settlement and industrial and agricultural activities have exerted great impact on water and sediment quality [80 - 82].

Natural water samples from various locations in the Tamar were collected as grab samples and analysed with the proposed method for P and ICP-MS or HGAAS was used for total dissolved arsenic determination. A segmented flow analyser with spectrophotometric detection was also used as a reference method to compare the results by analysing the same samples at the same time for P. The FI results were compared with the reference method using a paired t-test (p = 0.05) and the results were not significantly different (see Table 2.4). Arsenic concentrations varied considerably compared to those reported by Howard et al. [52] in 1988 (1.8 - 8.8 µg As L⁻¹). This could be as a result of silting which has greatly reduced the depths of the riverbeds leading to greater interaction between the sediments [44,83] and water surface. Concentrations greater than 75 µg As L⁻¹ were recorded in some locations in the fresh water end of the estuary. Tidal influence, wind, rain and pH all affect the distribution of dissolved arsenic in this area.

The results from regular events (sampling campaigns) of the Tamar Estuary using the optimised FI manifold are reported in chapter 6. One of the results (Table 2.4) was compared with a segmented flow analyser reference method with spectrophotometric detection used for the analysis of orthophosphates and this was in good agreement (t-test, P = 0.05), t_{cal} = 1.24; t_{tab} = 2.12.
Table 2.4. Comparison of the Fl method with a reference air-segmented continuous flow analyser (Skalar) method.

<table>
<thead>
<tr>
<th>Location</th>
<th>Grid Reference</th>
<th>Skalar (µg P L⁻¹)</th>
<th>±% (RSD)</th>
<th>Fl (µg P L⁻¹)</th>
<th>±% (RSD)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>North</td>
<td>West</td>
<td>±%</td>
<td>North</td>
<td>West</td>
</tr>
<tr>
<td>Weir Quay</td>
<td>50° 27'.35 N</td>
<td>04° 12'.39 W</td>
<td>94 ± 3.0</td>
<td>94 ± 2.1</td>
<td></td>
</tr>
<tr>
<td>Tamar Bridge</td>
<td>50° 24'.42 N</td>
<td>04° 12'.21 W</td>
<td>91 ± 9.5</td>
<td>90 ± 3.0</td>
<td></td>
</tr>
<tr>
<td>Neal Point</td>
<td>50° 27'.35 N</td>
<td>04° 12'.39 W</td>
<td>70 ± 8.1</td>
<td>73 ± 4.1</td>
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<tr>
<td>Weir Quay</td>
<td>50° 27'.35 N</td>
<td>04° 12'.39 W</td>
<td>103 ± 4.7</td>
<td>94 ± 1.3</td>
<td></td>
</tr>
<tr>
<td>Halton Quay</td>
<td>50° 21'.13 N</td>
<td>04° 13'.87 W</td>
<td>101 ± 3.4</td>
<td>103 ± 2.1</td>
<td></td>
</tr>
<tr>
<td>Southward Farm</td>
<td>50° 21'.13 N</td>
<td>04° 13'.87 W</td>
<td>105 ± 2.8</td>
<td>101 ± 0.0</td>
<td></td>
</tr>
<tr>
<td>Calstock</td>
<td>50° 29'.59 N</td>
<td>04° 12'.39 W</td>
<td>108 ± 1.1</td>
<td>120 ± 8.0</td>
<td></td>
</tr>
<tr>
<td>Rumleigh Farm</td>
<td>50° 27'.35 N</td>
<td>04° 12'.39 W</td>
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<td>148 ± 1.6</td>
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<tr>
<td>Morwellham</td>
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<td>04° 11'.47 W</td>
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<td>81 ± 0.0</td>
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<tr>
<td>Calstock</td>
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<tr>
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<td>107 ± 1.7</td>
<td>96 ± 2.2</td>
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<tr>
<td>Halton Quay</td>
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<td>04° 13'.87 W</td>
<td>107 ± 6.7</td>
<td>103 ± 2.5</td>
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<tr>
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<td>04° 12'.39 W</td>
<td>85 ± 5.8</td>
<td>85 ± 1.1</td>
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</tbody>
</table>

2.4. CONCLUSIONS

The following conclusions can be drawn from the work presented in this chapter:

1. A Fl manifold incorporating a thiosulphate stream can be used for the determination of ≤100 µg P L⁻¹ in samples with high silicate and arsenate concentrations. Freshly prepared thiosulphate should be used in the determination.

2. The mole ratio of [H⁺] to [MoO₄²⁻] is important in the determination of phosphate. A mole ratio of 70 and thiosulphate incorporated on-line is recommended in the presence of high arsenate (>300 µg As L⁻¹).

3. A low temperature of 30 °C is favourable for the determination of phosphate in the presence of high Si and As concentrations in the sample matrix.

4. The results from the optimised Fl manifold were in good agreement with an air-segmented continuous flow analyser reference method.

5. Various cations and anions potentially found in natural waters had no significant interfering effect at the optimised conditions of determination.

6. A calibration was performed with the optimised manifold and the limit of detection was 2 µg P L⁻¹.
2.5. REFERENCES


[77] D. S. Baldwin, A. M. Mitchel and J. M. Olley, Pollutant-Sediment


CHAPTER THREE

FLOW INJECTION SPECTROPHOTOMETRIC METHOD FOR THE DETERMINATION OF SILICATE IN NATURAL WATERS IN THE PRESENCE OF ARSENATE (>300 μg As L⁻¹)

Main topics in this chapter

Introduction
Experimental
Optimisation of FI manifold
Application to Tamar Estuary
Conclusions
3. INTRODUCTION

The determination of silicate and phosphate has gained universal attention because of the role these species play in biogeochemical transformations in natural waters [1, 2]. The concentration of Si in natural waters varies depending on biogeochemical processes. Diatoms require silicon for their cell walls and, in natural waters where such algae are predominant, it can be the limiting nutrient for phytoplankton growth [3 - 5]. The flow rate of a river or an estuary is a factor in the distribution of silicate in that river. The middle section of the river sometimes has higher concentrations of silicate which arise from leaching, erosion and rock weathering. Weathering of silicate minerals and rocks is the main source of silicate to both the marine and freshwater environment [6]. Concentrations of 10 mg Si L\(^{-1}\) have been reported in the surface water of Lake Taupo, New Zealand [7], Galhardo et al. (1997) reported concentrations of 4.8 and 4.9 mg Si L\(^{-1}\), respectively, from the Pirapora and Barra Bonita reservoirs, Spain [8] and >20 mg Si L\(^{-1}\) to trace amounts have been reported in surface waters of hot springs [9]. Casey et al. (1981) reported on the effect of biological production on silicate concentrations in the River Frome and Bere stream, England, with estimates of 1-2 mg Si m\(^{-2}\) day\(^{-1}\) uptake by diatoms during the spring [10]. Areas with previous mining and mineral processing will particularly enhance the concentration of nutrients and trace metals e.g. the Tamar catchment [11, 12].

Many methods for the determination of silicate in natural waters have been reported [e.g. 4, 5, 13 - 21]. Most use spectrophotometry [13 - 19] and are based on the formation of the silicomolybdenum complex and its subsequent reduction with either tin(II) chloride or ascorbic acid in the presence of catalytic amounts of antimony. Ascorbic acid is the most commonly used reductant because it can be used for samples of high salinity e.g. estuarine and coastal waters.

This method is also used for orthophosphate determination, and so some methods rely on the difference in the kinetics of the rate of formation of the silicomolybdenum complex and the phosphomolybdenum complex in an acidic medium [17, 21], with the latter being faster than the former. Other reported methods include voltammetry [22] and ion-exclusion liquid chromatography with chemiluminescence detection based on reduction of the silicomolybdenum complex by luminol [20]. Flow injection (FI) with an anion-exchange resin (TSK-gel SAX) column has also been reported for the determination of silicate [15].
However, for concentrations of P > 1 mg L\(^{-1}\) and for low concentrations of Si, overestimation of Si can arise as complexes formed by both species absorb at the same wavelength and P absorbs more strongly at low acid concentrations [8, 23]. Another interference is from the presence of As, which can be severe if arsenate is present in concentrations exceeding 200 \(\mu g\) As L\(^{-1}\), e.g. from regions with historical mining activity such as the Tamar Estuary [11, 12, 24].

In the spectrophotometric determination of silicate, oxalic acid is used to mask phosphate, and tartaric acid is used to mask silicate when phosphate is being determined [8, 9, 25]. When the concentration of As is >200 \(\mu g\) L\(^{-1}\), many investigators have used sulphite [26] or thiosulphate [7] to reduce As(V) to As(III). Both sulphite and thiosulphate work best in an acidic medium. The effectiveness of these reagents also depends upon suitable ratios of thiosulphate or sulphite to arsenate [7, 26]. Solvent extraction has also been reported for the determination of silicate [27]. This method minimises interference effects but has many drawbacks. It is laborious, prolongs analysis time and, depending on the skill of the analyst, can lead to increased contamination and hence interference.

The determination of Si as silicate is based on the formation of molybdosilic acid and this is the basis of most spectrophotometric determinations of silicate [13-19, 28]. Strickland was the first to recognise this and to report the existence of two isomers, the \(\alpha\)- and \(\beta\)-isomers. 1.5 moles hydrochloric acid per mole of molybdate (\(\text{MoO}_4^{2-}\)) produces the \(\alpha\)-acid while ratios above 2.0 moles of acid per mole of molybdate yield the \(\beta\)-acid [29]. The conditions leading to the formation of both the \(\alpha\)- and \(\beta\)-acids and their relative stabilities has been extensively studied by Truesdale and Smith [18, 19]. They found that the \(\beta\)-form is more stable at pH 1.0-1.8 although the \(\alpha\)-form is produced first in less acidic conditions of pH 2 - 4. For the determination of both \(\alpha\)- and \(\beta\)-isomers as silicate, it was suggested by Miro et al. [3] that a pH of 1 - 2 is optimum. There have been many reports on the effect of the [\(\text{H}^+\)] to [molybdate] ratio that should be used in both the phosphomolybdenum and silicomolybdenum reactions. This is most significant when viewed on the basis that they form similar complexes and hence absorb at similar wavelengths [8].

This chapter reports a simple but robust FI method for the spectrophotometric determination of silicate in natural waters in the presence of concentrations of
arsenate in the range of 300-700 µg As L\(^{-1}\). The proposed method incorporates a thiosulphate stream to reduce arsenate (As(V), AsO\(_4^{3-}\)) to arsenite (As(III), AsO\(_3^{3-}\)), and two micro-columns; an iminodiacetate resin to remove trace metals and a styrene divinylbenzene resin to remove phosphate. The ability of the divinylbenzene resin to remove phosphate ions was demonstrated over the range 1 - 1.6 mg P L\(^{-1}\). The proposed method is simple, highly reproducible (typical RSDs of >1.5, n = 3) and very economical as both columns can be regenerated and used again.

3.1. EXPERIMENTAL

3.1.1. Reagents

All reagents were prepared using ultrapure-deionised water (UPW; Elga, resistance = 18.2 M\(\Omega\) cm\(^{-1}\)). All reagents were of analytical grade and in order to avoid contamination, all glassware and high-density polyethylene (HDPE) storage bottles for reagent solutions were acid cleaned following a standard procedure [30 - 32].

1. Phosphate (K\(_2\)HPO\(_4\)). A 10 mg P L\(^{-1}\) stock solution was prepared freshly from 1000 mg L\(^{-1}\) stock solution. PO\(_4^{-}\)-P calibration standards in the range of 0.1- 0.5 mg P L\(^{-1}\) were prepared by serial dilution of the stock solution with UPW unless otherwise stated.

2. Ammonium molybdate ((NH\(_4\))\(_6\)Mo\(_{12}\)O\(_{44}\).4H\(_2\)O). A 0.100 M stock solution was prepared by dissolving 12.358 g of ammonium heptamolybdate in a 100 mL volumetric flask with UPW. A 8.4 mM working standard was prepared freshly from this stock for use. Both solutions were stable for several weeks. The 0.100 M stock was stored at room temperature in the cupboard in a HDPE container.

3. Sulphuric acid (H\(_2\)SO\(_4\)). The AnalAR stock had a specific gravity of 18.4 g dm\(^{-3}\). 2.00 M stock was prepared by dissolving 27.20 of the AnalAR stock in a flask containing 200 mL UPW. This was made up to the 250 mL mark with UPW and stored at room temperature. A 0.62 M solution was made from this stock by dilution unless otherwise stated.

4. L-ascorbic acid (C\(_6\)H\(_{8}\)O\(_6\)). 4.6 g of ascorbic acid was dissolved in UPW and made up to 100 mL mark. The solution was prepared daily.

5. Antimonyl potassium (+)-tartrate (K\(_2\)SbO.C\(_4\)H\(_4\)O\(_6\)). A 0.100 M stock was prepared by dissolving 3.249 g of antimonyl potassium tartrate in 100 mL of
6. Sodium metasilicate (Na$_2$O$_3$Si.9H$_2$O). A 1000 mg Si L$^{-1}$ stock was prepared by dissolving 1.0136 g stock in 100 mL of (UPW). A 10 mg Si L$^{-1}$ stock was prepared from this and Si calibration standards in the range of 0.4 - 2.0 mg Si L$^{-1}$ were prepared by serial dilution of the 10 mg Si L$^{-1}$ stock unless otherwise stated. This standard was stable for several weeks at room temperature.

7. Sodium thiosulphate (Na$_2$S$_2$O$_3$.5H$_2$O). A 0.002 M solution was prepared freshly and used immediately by dissolving 0.049 g of sodium thiosulphate in 100 mL UPW. The thiosulphate standard is very unstable and must be protected from direct sunlight. Black HDPE containers were always used in the preparation to minimise decomposition by light.

8. Sodium arsenate heptahydrate (Na$_2$HAsO$_4$.7H$_2$O). A 50 mg L$^{-1}$ stock was prepared from the 1000 mg As L$^{-1}$ stock in 5% (v/v) HNO$_3$ AnalAr solution. Concentrations in the range 0.05 - 1.0 mg L$^{-1}$ arsenate were prepared freshly from this stock daily by dilution with 2% HNO$_3$ and used immediately. It should be noted that sodium arsenate is unstable in water.

9. Low Nutrient Seawater (LNS) salinity 35. This was used as supplied or as stated. Serial dilutions in the range 10 - 30 salinity was prepared by dilution of the stock in UPW.

10. A styrene divinyl benzene strong anion exchanger (Dowex 1 x 8 200 - 400 mesh, Cl$^-$ form) and an iminodiacetate chelating resin (Chelex insolubilized on 1 % cross-linked polystyrene (50 - 100) dry mesh sodium form) from Sigma were used in the micro-columns to remove phosphate and metal ions respectively.

3.1.2. Instrumentation

The FI manifold with spectrophotometric detection incorporated 0.75 mm i.d. PTFE tubing, a peristaltic pump (Gilson Minipuls 2, Villiers le Bel, France) and a Rheodyne six-port switching valve. Data collection was done automatically using LabVIEW™ and samples were injected manually. The proposed four-channel flow injection FI system incorporating a thiosulphate stream for the determination of Si is shown in Fig. 3.1.
Fig. 3.1. Four channel flow injection ascorbic acid manifold incorporating a thiosulphate stream for the determination of Si in the presence of <300 µg As L⁻¹ in natural waters. Detector wavelength was 800 nm.

3.1.3. Procedure

The peristaltic pump was used to propel the carrier and thiosulphate at 0.80 mL min⁻¹ and the two reagent streams at 0.16 mL min⁻¹. The rotary injection valve was used to inject 440 µL of sample into the carrier stream. Sample and carrier mixed at a T-piece with the thiosulphate stream. Here arsenate (As(V), AsO₄³⁻) was reduced to arsenite (As(III), AsO₃³⁻). This then mixed with the molybdate at another T-piece and finally with the ascorbic acid reductant in a water bath at 60 °C and then into a uv/visible/nir spectrophotometer (Philips PU 8620 Cambridge, England) set at 800 nm and equipped with a 30 µL flow cell (178.011 - OS 10 mm lightpath, Optical Glass Windows 320 - 2500 nm; Hellma, UK). Detector output was automatically recorded using an in-house LabVIEW™ software programme.

A uv-visible spectrophotometer (Hewlett Packard HP84553) was used to obtain an absorbance spectrum of the molybdenum blue compound produced using the manifold described in Fig. 3.1. This spectrum was obtained in order to determine the wavelength of maximum absorbance for the FI manifold [33]. Water samples were filtered in situ using a 0.45 µm filter (cellulose acetate), 47 mm diameter (Whatman) and stored in acid washed HDPE containers. Water samples were analysed within 24 h and, when possible, stored at 4 °C prior to analysis. If samples were stored for longer periods before analysis they were usually frozen at
-20 °C. Freshly packed columns for both ions were always used. The cotton wool used for the column ends was defatted by soaking in 5 M HNO$_3$ overnight. Total dissolved arsenic was analysed using hydride generation atomic absorption spectrophotometry (HGAAS) for saline samples and ICP-MS for freshwater samples [34, 35].

3.2. RESULTS AND DISCUSSION

3.2.1. Optimisation of Fl variables

One of the major requirements of continuous flow [36, 37] systems is that time of analysis should be short (e.g. >30 s) and that reagent consumption should be low. The objective of the optimisation experiments was to achieve the above requirements. All discussion below refers to the manifold shown in Fig. 3.1.

3.2.2. Wavelength

Wavelength of absorption is important in any spectrophotometric measurement. From these investigations the silicomolybdenum complex absorbs most strongly between 790 and 814 nm. The 800 nm was chosen after the waste from the reacted sample was collected (see Fig. 3.1) and analysed with a uv-visible spectrophotometer [33] and the maximum absorbance was observed at 800 nm.

3.2.3. Length of reaction coils

R1 is the coil where the reduced arsenate (As(V), AsO$_4^{3-}$) mixed with the molybdate reagent. It was observed that as the length of R1 increased from 5 - 20 cm, peak shape and height improved in agreement with Fl theory [38]. However at longer lengths (>20 cm) a downward trend was observed. A 15 cm coil length, which gave the best peak height and resolution, was chosen for all subsequent experiments.

R2 is the reaction coil in which reduction of silicomolybdenum complex occurred, but at a slower rate than that of the phosphomolybdenum complex [39, 40]. As the length of coil increased sensitivity improved (Fig. 3.2) [41, 42] but with longer lengths more time and reagents were required and the peaks broadened. A 1.5 m length was selected for all future work because it gave the best peak shape.
3.2.4. Temperature

The formation of the silicomolybdenum complex is very slow at room temperature [39]. Temperature is therefore important for increasing the energy of the system to make it proceed at a faster rate. The dependence of this reaction on temperature was investigated over the range 25 - 80 °C as shown in Fig. 3.3. As the temperature was increased response and sensitivity also increased [43]. Many researchers have determined silicate at different temperatures. Pai et al. [44] suggested the determination of silicate at 70 °C, Narusawa [15] determined silicate at 95 °C and a segmented flow method (Technicon) [45] uses room temperature. In Fig. 3.3, the response due to temperature increase was gradual and optimum at 70 °C. However, at 70 °C and above, the impact of the As interference increased (from this work) and dual peaks were also observed, especially at 80 °C. Minimal interference effects from arsenate (As(V), AsO₄³⁻) were observed at 60 °C therefore this temperature was chosen for all subsequent experiments despite the lower sensitivity.

3.2.5. Sample volume

The sample volume was optimised to ensure reagent economy and shorter reaction time [46]. In Fig. 3.4., as the sample volume was increased, response and sensitivity also increased [41, 42]. Above 200 µL, the effect of increased volume on response was less significant. Higher volumes (500 - 600 µL) were avoided because they yielded broad peaks, increased reaction times and hence led to an increase in the amount of reagent used. The 440 µL sample loop, which gave the
optimum response in terms of peak resolution and height, was chosen and used in subsequent work.

Fig. 3.3. Effect of temperature (°C) on the determination of silicate using 47 μg Si L⁻¹. Error bars, σ ± 3s (n = 3).

Fig. 3.4. Effect of sample volume on the determination of silicate using 47 μg Si L⁻¹. Error bars, σ ± 3s (n = 3).

3.2.6. H⁺ ion concentration
The concentration of hydrogen ion required to form the silicomolybdenum complex is critical as shown in Figs. 3.5 and 3.6. Very high [H⁺] > 0.7 M is not suitable for the determination of silicate because it suppresses the response in agreement with previous investigations [44]. Also [H⁺] < 0.2 M should be avoided because of very high blank values. Further investigation over two concentration ranges via narrower range (0.6 - 0.7 M) showed (see Fig. 3.6) that the best signal-to-blank
ratio for silicate response was at 0.62 M acid concentration. Therefore 0.62 M acid was used for all subsequent studies.

![Graph showing absorbance vs hydrogen ion concentration](image)

**Fig. 3.5.** Effect of hydrogen ion concentration [H⁺] (broad range) on the determination of silicate using 47 µg Si L⁻¹, Error bars, σ ± 3s (n = 3).

![Graph showing absorbance vs hydrogen ion concentration](image)

**Fig. 3.6.** Effect of hydrogen ion concentration [H⁺] (narrow range) on the determination of silicate using 47 µg Si L⁻¹, Error bars, σ ± 3s (n = 3).

### 3.2.7. Molybdate concentration

The molybdate concentration was then optimised for the formation of the silicomolybdenum complex whilst minimising blank response and a linear relationship was observed (see Fig. 3.7). The [H⁺] concentration used was 0.62 M, i.e. the optimum from section 3.2.6. From Fig. 3.7 the optimum molybdate concentration was 0.0084 M which gave an [H⁺]:[molybdate] ratio of 70,
agreement with literature values [44, 48]. The ratio of \([H^+]\) to molybdate is important in the kinetics of formation of phosphomolybdate and silicomolybdate [18-19, 39, 44, 47]. It has been observed that very low concentrations (<0.005 M molybdate) have little effect on the reaction while high concentrations (>0.01 M) lead to self-reduction and hence an increase in blank response [44].

\[
y = 88.667x - 0.6169 \\
R^2 = 0.9961
\]

Fig. 3.7. Effect of molybdate concentration on the determination of silicate using 47 \(\mu g\) Si L\(^{-1}\). Error Bars, \(\sigma \pm 3s\) (\(n = 3\)).

### 3.2.8. Ascorbic acid concentration

Many reducing agents have been reported for the determination of silicate. Tin(II) chloride is used mostly in fresh waters because the chloride ion concentration is very low in fresh waters and hence there is no common ion effect [3, 18, 19]. 1-amino-2-napthol-sulphonic acid has also been used by some investigators [49]. Ascorbic acid is, however, the most widely used reducing agent. This is because it can be used as a reductant in the determination of silicate in seawater without any matrix interference [50] although the reaction is slower than with tin(II) chloride.

The choice of the reductant can have an impact in the overall sensitivity of the method, hence the ascorbic acid concentration was optimised to obtain the maximum signal-to-blank ratio. The use of ascorbic acid is enhanced by the addition of catalytic amounts of antimony, which helps in the faster formation of the intense blue colour especially as the temperature increases [50-53]. As can be seen in Fig. 3.8, as the concentration increased, response also increased slightly, up to a maximum at 45 g L\(^{-1}\).
Fig. 3.8. Effect of ascorbic acid concentration on the determination of silicate, using 187 μg Si L⁻¹. Error bars, σ ± 3s (n = 3).

3.2.9. Antimonyl tartarate concentration
A pioneering report by Theodore [53] revolutionised the use of ascorbic acid as a reducing agent in the determination of P and Si. It was found that the addition of small amounts of antimonyl tartarate i.e. catalytic amounts facilitated the development of the chromophore. Antimonyl tartrate is therefore important for the fast colour development of the silicomolybdenum complex. This complex forms slowly but the addition of antimonyl tartrate, with increased temperature, facilitates the transformation of the α-form to the β-form. Various concentrations of antimonyl tartrate were investigated and, as shown in Fig. 3.9, with increasing concentration of antimonyl tartrate up to 0.0015 M, the response also increased, above which a plateau was observed. 0.0015 M was chosen for this work as concentrations higher than this resulted in noisy signals and toothed peaks.
Fig. 3.9. Effect of antimonyl tartarate concentration on the determination of silicate using 47 μg Si L⁻¹, error bars, σ ± 3s (n = 3).

3.2.10. Analytical performance
A calibration was performed with the optimised manifold and the limit of detection and other parameters determined are as shown in Table 3.1. The limit of detection (LOD) is defined as the concentration which gives an instrument signal (Y) significantly different from the ‘blank’ or ‘background’ signal [54]. LOD = Yb + 3sb; where Yb = blank signal and Sb = standard deviation of the blank. The detection limit of 3 μg Si L⁻¹ is sufficient for most freshwater environments. The reproducibility and sampling frequency enable reliable results from high throughput analysis of freshwaters. Hence the method can be used for biogeochemical studies.

Table 3.1. Analytical performance of the Fl method at 60 °C.

<table>
<thead>
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<th>Parameter</th>
<th>Value</th>
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<td>Linear range (μg Si L⁻¹)</td>
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<td>0.0005</td>
</tr>
<tr>
<td>Intercept</td>
<td>0.0246</td>
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<tr>
<td>Correlation coefficient</td>
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<td>Limit of detection, (μg Si L⁻¹)</td>
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<tr>
<td>R.S.D. % (n = 4)</td>
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</tr>
<tr>
<td>Sampling frequency (h⁻¹)</td>
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</tr>
<tr>
<td>Detection temperature</td>
<td>60 °C</td>
</tr>
</tbody>
</table>
3.2.11. Cation interferences
Many cations are known to interfere in the spectrophotometric determination of silicate because of their ability to form complexes with molybdate e.g. iron and aluminium derived from leaching [56] and weathering of rocks [57]. Cations of various concentrations were used in the study. The concentrations chosen were above those likely to be encountered in normal conditions in the aquatic environment. Those used were Ca(II) 150 mg L\textsuperscript{-1}, Mg(II) 100 mg L\textsuperscript{-1}, Mn(II) 10 mg L\textsuperscript{-1}, Cu(II) 0.1 mg L\textsuperscript{-1}, Zn(II) 10 mg L\textsuperscript{-1}, Pb(II) 10 mg L\textsuperscript{-1}, Al(III) 10 mg L\textsuperscript{-1}, Ni(II) 1 mg L\textsuperscript{-1}, Co(II) 1 mg L\textsuperscript{-1}, NH\textsubscript{4}\textsuperscript{+} 1 mg L\textsuperscript{-1}, Fe(II) 1 mg L\textsuperscript{-1} and Fe(III) 1 mg L\textsuperscript{-1}. The cations with concentrations as stated above were injected first without Si and then with 47 \(\mu\)g Si L\textsuperscript{-1} and their responses compared and these are shown in Fig. 3.10. Cu(II) is the main potential interferent in agreement with the literature [23, 58]. Iron did not cause interference at 1 mg L\textsuperscript{-1}, which is higher than the concentration of iron in most natural waters [59]. Zn(II), Mn(II) and Pb(II) can be potential negative interferents at 10 mg L\textsuperscript{-1} concentrations because of the suppressing effect observed on the formation of the silicomolybdate ion. However, the concentrations of these ions in most unpolluted natural waters are below 10 mg L\textsuperscript{-1} and as a result pose no problem.

![Fig. 3.10. Effect of cations on the determination of silicate. Error bars, \(\sigma \pm 3s\) (n = 3).](#)

3.2.12. Masking cations with an iminodiacetate resin micro-column
Fig. 3.11 shows the effect of iminodiacetate chelating resin in masking cationic interferences and the concentrations of cations chosen were as follows, Cu(II) 0.1 mg L\textsuperscript{-1}, Fe(II) 0.10 mg L\textsuperscript{-1}, Fe(III) 0.1 mg L\textsuperscript{-1}, Mg(II) 100 mg L\textsuperscript{-1}, Mn(II) 1 mg L\textsuperscript{-1}, Al(III) 10 mg L\textsuperscript{-1}, Co(II) 1 mg L\textsuperscript{-1} and Ni(II) 1 mg L\textsuperscript{-1}. The chelating resin effectively
masked the cations but was not effective for high concentrations of Cu(II) [23]. The chelating resin was effective for Cu(II) concentrations not exceeding 0.05 mg L\(^{-1}\). Copper shows high affinity to the silicomolybdenum complex at low [H\(^+\)] (<0.8 M) and hence could interfere in systems using low hydrogen ion concentrations. This is probably due to the formation of a tetra-amine complex between copper and the ammonium salt and also other Cu(II) salts which are blue in colour.

![Image](image.png)

**Fig. 3.11.** Effect of cation exchange column on cationic species on the determination of silicate. Error bars, \(\sigma \pm 3s\) (n = 3).

### 3.2.13. Effect of anions

Several anions are found in fresh waters and many could interfere in the formation of the silicomolybdenum complex. Phosphate is the major interferent (see Fig. 3.12) because it forms a similar heteropoly acid to silicate. Also arsenate (As(V), AsO\(_4^{3-}\)) is another potential interferent, especially in areas of mineral processing [11, 55] and fluoride (F\(^-\)) has also been reported to interfere in the silicoantimonylmolybdenum blue method [58]. Investigations of the potential effect of these and other anions on the system were carried out.

The concentrations used were based on their likely concentrations in natural waters. These were chloride 2000 mg L\(^{-1}\), fluoride 1 mg L\(^{-1}\), sulphate 500 mg L\(^{-1}\), hydrogen carbonate 100 mg L\(^{-1}\), nitrite 1 mg L\(^{-1}\), nitrate 30 mg L\(^{-1}\), phosphate 0.1 mg L\(^{-1}\), arsenate (As(V), AsO\(_4^{3-}\)) 0.050 mg L\(^{-1}\), 0.05 mg L\(^{-1}\) and urea 1 mg L\(^{-1}\). Fig. 3.12 shows that phosphate and arsenate are the major interferents.
Fig. 3.12. Effect of anions on the determination of silicate. Error bars, $\sigma \pm 3s$ ($n = 3$).

In most natural waters, the concentrations of most of the other anions investigated are low [55, 59 - 63] and hence would not interfere in the determination of low concentrations of Si. In seawater the problem of chloride interference can be overcome by using equivalent low nutrient seawater strength water as a matrix matched carrier. The main conclusion is that phosphate must be completely removed or suppressed during the determination of silicate.

3.2.14. Masking phosphate interference

In Fig. 3.12, phosphate is the major interferent and must be removed in the determination of silicate because the phosphomolybdate complex absorbs more efficiently at low $[H^+]$ which is favourable for the determination of Si (see Fig. 3.5 and 3.6). Oxalic acid is the most commonly used reagent and this removes the interfering effect of phosphate through a ligand exchange reaction. Molybdosilicate is relatively inert and does not take part in this ligand exchange reaction.

This inert species is reduced to the blue heteropoly species by ascorbic acid or the ascorbic acid-antimonyl complex [45]. However, in these studies, the effects of oxalic acid concentrations (0.0001 - 0.001 M) were investigated. As can be seen in Fig. 3.13, it was not suitable for low silicate to high phosphate ratios because it suppressed both phosphate and silicate in the proposed FI method. This implies that the conventional approach of using oxalic acid to mask phosphate does not work effectively at low silicate to phosphate ratios because oxalic acid has a suppressing on the response of both Si and P.
Fig. 3.13. Masking phosphate with oxalic acid in the presence of 47 µg Si L⁻¹
Error bars, σ ± (n = 3). (P = 163 µg P L⁻¹) as used in this figure.

A styrene divinylbenzene anion exchange resin incorporated in a micro-column (0.05 g) was however efficient for the removal of phosphate ions with no suppressing effect on the response of Si at both low and high concentrations, after optimisation of the FI manifold (see Fig. 3.14). Concentrations of P ranging from 1.0 - 1.6 mg P L⁻¹ were injected over 30 times and found to have no effect on the column response as can be seen in Fig. 3.14.

Fig. 3.14 Effect of anion column in masking phosphate. Error bars, σ ± 3s (n = 3).

3.2.15. Masking arsenate interference
Arsenate (As(V), AsO₄³⁻) is a major interferent in the spectrophotometric determination of silicate. There are many reports on its effect in
spectrophotometric methods [e.g. 7, 15, 58]. As can be seen in Fig. 3.12, the iminodiacetate resin micro-column was not able to mask As(V). Its effect must be removed in the determination of Si in order to obtain reliable results. Amongst the most commonly used masking agents for arsenate are sulphite [27] and thiosulphate which reduce arsenate (As(V), AsO$_4^{3-}$) to arsenite (As(III), AsO$_3^{3-}$) [6]. Iodate and iron(II) chloride have also been used because of their reductive properties. Sulphite, iodate and iron(II) chloride were not effective in the flow injection method, but thiosulphate was, probably due to a faster reaction rate. A thiosulphate stream was therefore incorporated online which reduced arsenate to arsenite. In acid media and at temperatures >60 °C, the thiosulphate can be broken down and the released sulphur could cause a Schleiren effect [63].

\[ 2\text{AsO}_4^{3-} + 2\text{S}_2\text{O}_3^{2-} \rightarrow 2\text{AsO}_3^{3-} + 2\text{SO}_4^{3-} + 2\text{S} \]

The concentration of the thiosulphate used was optimised and 0.002 M thiosulphate gave the best results (see Table 3.2).

### Table 3.2. Effect of thiosulphate concentration on the blank signal

<table>
<thead>
<tr>
<th>Thiosulphate Concentration (M)</th>
<th>Absorbance (n=3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.000</td>
<td>0.000 ± 0.000</td>
</tr>
<tr>
<td>0.002</td>
<td>0.000 ± 0.000</td>
</tr>
<tr>
<td>0.004</td>
<td>0.000 ± 0.000</td>
</tr>
<tr>
<td>0.006</td>
<td>-0.002 ± 0.000</td>
</tr>
<tr>
<td>0.008</td>
<td>-0.005 ± 0.000</td>
</tr>
<tr>
<td>0.01</td>
<td>-0.008 ± 0.001</td>
</tr>
</tbody>
</table>

Table 3.3 shows the results obtained when two different thiosulphate concentrations were used to mask various concentrations of arsenate (As(V), AsO$_4^{3-}$) at 60 °C. These data show that thiosulphate can be used to mask high concentrations of arsenate. At this temperature no Schlieren effect was observed.
Table 3.3. Effect of thiosulphate concentration on the response of arsenate at temperature of determination of Silicon (60 °C).

<table>
<thead>
<tr>
<th>µg As L⁻¹</th>
<th>Thiosulphate free (n = 3)</th>
<th>0.003 M thiosulphate Absorbance (n=3)</th>
<th>0.005 M thiosulphate Absorbance (n=3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.000 ± 0.000</td>
<td>0.000 ± 0.000</td>
<td>0.000 ± 0.000</td>
</tr>
<tr>
<td>216</td>
<td>0.013 ± 0.001</td>
<td>0.002 ± 0.000</td>
<td>0.005 ± 0.000</td>
</tr>
<tr>
<td>328</td>
<td>0.031 ± 0.003</td>
<td>0.004 ± 0.000</td>
<td>0.010 ± 0.000</td>
</tr>
<tr>
<td>432</td>
<td>0.071 ± 0.011</td>
<td>0.006 ± 0.000</td>
<td>0.015 ± 0.001</td>
</tr>
<tr>
<td>540</td>
<td>0.168 ± 0.020</td>
<td>0.009 ± 0.001</td>
<td>0.021 ± 0.002</td>
</tr>
<tr>
<td>656</td>
<td>0.227 ± 0.110</td>
<td>0.013 ± 0.001</td>
<td>0.032 ± 0.003</td>
</tr>
</tbody>
</table>

3.2.16. Application of the Fl method to the determination of silicate in natural waters

The Tamar Estuary lies on latitude 50°C 26 14 N and longitude 04°C 11 42 W. The Estuary flows into the English Channel and is fed by the rivers Tamar, Lynher and Tavy. The Tamar River provides the main input of fresh water to the estuary [11, 12]. Several years of settlement and industrial and agricultural activities have exerted great impact on water and sediment quality [64-66]. Natural water samples from various locations in the Tamar were collected as grab samples and analysed with the proposed method for Si and ICP-MS or HGAAS was used for total arsenic determination. A segmented flow analyser with spectrophotometric detection was also used as a reference [45] method to compare the results by analysing the same samples at the same time for Si. The Fl results were compared with the reference method using a paired t-test at 5% probability, \( t_{\text{cal}} = 1.44 \) and \( t_{\text{tab}} = 2.14 \) the results were not significantly different (see Table 3.4).

Arsenic concentrations varied considerably compared to those reported by Howard et. al. [67] in 1988 (1.8 - 8.8 µg As L⁻¹). This could be as a result of silting which has greatly reduced the depths of the riverbeds leading to greater interaction between the sediments [68, 69] and water surface. Also seasonal influences could have a strong affect on the arsenic concentration. Concentrations higher than 75 µg As L⁻¹ were recorded in some locations in the fresh water end of the estuary (see Chapter 6). The method successively removed arsenate and
phosphate interferences and is therefore a robust method for measuring Si, even in waters containing high concentrations of arsenate, 500 µg As L$^{-1}$ and phosphate, 1.6 mg P L$^{-1}$ which can be found in anthropogenically impacted catchments.

Table 3.4. Comparison of the FI method with a reference method (Skalar Flow analyser) for the determination of silicate in the Tamar Estuary. Samples were collected on 29/04/04.

<table>
<thead>
<tr>
<th>Location</th>
<th>Grid Reference</th>
<th>Skalar (mg Si L$^{-1}$)</th>
<th>FI (mg Si L$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>N \ W</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Weir Quay</td>
<td>50°27.35  04°12.39</td>
<td>0.5 ± 1.0</td>
<td>0.4 ± 3.1</td>
</tr>
<tr>
<td>Tamar Bridge</td>
<td>50°24.42  04°12.21</td>
<td>0.4 ± 2.2</td>
<td>0.4 ± 2.7</td>
</tr>
<tr>
<td>Neal Point</td>
<td>50°27.35  04°12.39</td>
<td>0.4 ± 0.7</td>
<td>0.4 ± 2.2</td>
</tr>
<tr>
<td>Weir Quay</td>
<td>50°27.35  04°12.39</td>
<td>0.6 ± 1.3</td>
<td>0.5 ± 3.5</td>
</tr>
<tr>
<td>Halton Quay</td>
<td>50°21.13  04°13.87</td>
<td>1.1 ± 0.9</td>
<td>1.2 ± 2.2</td>
</tr>
<tr>
<td>Southward Farm</td>
<td>50°21.13  04°13.87</td>
<td>1.2 ± 0.2</td>
<td>1.2 ± 2.3</td>
</tr>
<tr>
<td>Calstock</td>
<td>50°29.59  04°12.39</td>
<td>1.3 ± 0.8</td>
<td>1.2 ± 1.4</td>
</tr>
<tr>
<td>Rumleigh Farm</td>
<td>50°27.35  04°12.39</td>
<td>1.2 ± 0.8</td>
<td>1.2 ± 2.5</td>
</tr>
<tr>
<td>Morwellham</td>
<td>50°30.31  04°11.47</td>
<td>2.0 ± 0.5</td>
<td>2.0 ± 2.1</td>
</tr>
<tr>
<td>Weir Head</td>
<td>50°27.35  04°12.39</td>
<td>1.2 ± 0.0</td>
<td>1.3 ± 3.1</td>
</tr>
<tr>
<td>Morwellham</td>
<td>50°30.31  04°11.47</td>
<td>1.2 ± 0.0</td>
<td>1.2 ± 2.1</td>
</tr>
<tr>
<td>Rumleigh Farm</td>
<td>50°27.35  04°12.39</td>
<td>1.3 ± 0.7</td>
<td>1.2 ± 1.3</td>
</tr>
<tr>
<td>Calstock</td>
<td>50°29.59  04°12.39</td>
<td>1.3 ± 0.0</td>
<td>1.3 ± 3.5</td>
</tr>
<tr>
<td>Southward Farm</td>
<td>50°21.13  04°13.87</td>
<td>1.2 ± 0.8</td>
<td>1.0 ± 1.0</td>
</tr>
<tr>
<td>Halton Quay</td>
<td>50°21.13  04°13.87</td>
<td>1.1 ± 0.0</td>
<td>1.1 ± 2.8</td>
</tr>
<tr>
<td>Weir Quay</td>
<td>50°27.35  04°12.39</td>
<td>0.5 ± 0.1</td>
<td>0.4 ± 2.2</td>
</tr>
</tbody>
</table>

Paired t-test results; $t_{cal} = 1.44$ and $t_{tab} = 2.14$ at $p = 0.05$.

3.3. CONCLUSIONS
The following conclusions can be drawn from the work presented in this chapter.
1. A FI manifold with spectrophotometric detection incorporating a thiosulphate stream can be used for the determination of 3-1000 µg Si L$^{-1}$
in samples of high arsenate concentrations up to 500 µg As L\(^{-1}\). Freshly prepared thiosulphate should be used in the determination.

2. The mole ratio of \([H^+]\) to \([\text{MoO}_4^{2-}]\) is important in the determination of silicate. A mole ratio of 70 and thiosulphate incorporated online is recommended in the presence of arsenate concentrations >300 µg As L\(^{-1}\).

3. A styrene divinylbenzene strong anion exchange resin removed phosphate concentrations of 1-1.6 mg P L\(^{-1}\) after optimisation and cationic interferences were eliminated with an iminodiacetate chelating resin. These resins were contained in two different micro-columns on-line.

4. A temperature of 60 °C or less is preferred for the determination of silicate in the presence of arsenate in the range 300-500 µg As L\(^{-1}\) in the sample matrix. At 70-80 °C sensitivity is higher, but there is increased interference from arsenate and noisy toothed peaks are observed. However, at 60 °C, minimal interference effect was observed and therefore was chosen for this work.

5. The results from the optimised FI manifold were in good agreement with an air-segmented continuous flow analyser reference method for a range of estuarine samples (no significant difference at \(p = 0.05\)).

6. For the optimised manifold the limit of detection was 3 µg Si L\(^{-1}\).

3.4. REFERENCES


[33] Hewlett Packard HP 84553, UV-Visible Spectrophotometer.


CHAPTER FOUR

AUTOCLAVE DIGESTION TECHNIQUES FOR THE DETERMINATION OF DISOLVED ORGANIC PHOSPHORUS IN NATURAL WATERS

Main topics in this chapter

Introduction
Experimental
Optimisation of FI manifold
Application to the Tamar Estuary
Conclusions
4. INTRODUCTION
Dissolved organic phosphorus (DOP) constitutes a significant portion of the bioavailable phosphorus (BAP) pool. It is the difference between total dissolved phosphorus (TDP) and filterable reactive phosphorus (FRP), i.e. DOP = TDP-FRP. BAP can be defined as the sum of immediately available P and P that can be transformed into an available form by naturally occurring processes, e.g. physical (desorption), chemical (dissolution) and biological (enzymatic degradation). This fraction of the total phosphorus pool is important for understanding the biogeochemical processes prevalent in any aquatic ecosystem. Several digestion techniques have been reported for the digestion of environmental samples for the determination of DOP (see sections 4.3.1 - 4.3.3).

The aims of this chapter were to investigate:
1. The factors that can lead to low recovery of DOP after batch autoclave digestion.
2. The effect of pH on the recovery of digested samples. This involved the measurement of pH before and after autoclave digestion because of the dependence of phosphomolybdenum complex formation on pH.
3. The effect of matrices on the recovery of DOP after digestion. This also involved dilution of samples before and after digestion.
4. Different digestion reagents to assess their suitability for use in batch autoclave digestion of samples from anthropogenically impacted areas such as the Tamar Estuary.

4.1. EXPERIMENTAL
4.1.1. Reagents
All reagents were of analytical grade and were prepared using ultra-pure water, 'UPW' supplied by Elga, (resistance = 18.2 MΩ cm\(^{-1}\)). All glassware and HDPE (high density polyethylene) bottles used were first washed with a nutrient P-free detergent (Neutracon), rinsed at least three times with UPW and soaked in 10 % v/v HCl overnight and finally rinsed three times with UPW according to standard procedures [1 - 3]. All stock solutions were stored at 4 °C and brought to room temperature before use unless otherwise stated.

1. Trisodium trimeta-phosphate, TPP, (Na\(_3\)P\(_3\)O\(_9\)), FW 305.9 g (Sigma, 95-97%). 100 mg P L\(^{-1}\) stock solution was prepared by dissolving 0.017 g in 50 mL volumetric flask with UPW. Working standards in the range 50 - 100 μg P L\(^{-1}\) were prepared by serial dilution of the stock solution.
2. D-Glucose-6-phosphate barium salt, D-G-6-P, (C$_6$H$_{11}$O$_5$P$_8$Ba), FW 395.5 g (Sigma, 98%). 100 mg P L$^{-1}$ stock solution was prepared by dissolving 0.065 g in 50 mL of UPW. Working standards in the range 50 - 100 µg P L$^{-1}$ were prepared by serial dilution of the stock solution.

3. Phytic acid magnesium-potassium salt, PTA-MgK, (C$_6$H$_{15}$O$_{24}$P$_5$MgK), FW 720.4 g (Sigma 95%). 100 mg P L$^{-1}$ stock solution was prepared by dissolving 0.0204 g in 50 mL UPW. Working standards in the range 50 - 100 µg P L$^{-1}$ were prepared by serial dilution of the stock solution.

4. Phenyl phosphate di-sodium salt, PPD, (C$_6$H$_5$PO$_4$Na$_2$), FW 218.1 g (Sigma 95%). 100 mg P L$^{-1}$ stock solution was prepared by dissolving 0.037 g in 50 mL of UPW. Working standards in the range 50 - 100 µg P L$^{-1}$ were prepared by serial dilution of the stock solution.

5. Thiamine pyrophosphate chloride or cocarboxylase, COC, (C$_{12}$H$_{19}$ClN$_4$O$_7$P$_2$S), FW 460.8 g (Sigma 98%). 100 mg P L$^{-1}$ stock solution was prepared by dissolving 0.037 g in 50 mL of UPW. Working standards in the range 50 - 100 µg P L$^{-1}$ were prepared from the main stock by serial dilution.

6. Adenosine-5-triphosphate di-sodium salt, ATP, (C$_{10}$H$_{14}$N$_5$O$_{13}$P$_3$Na$_2$), FW 551.1 g (Sigma 99%). 100 mg P L$^{-1}$ stock was prepared by dissolving 0.030 g in 50 mL of UPW. Working standards in the range 50 - 100 µg P L$^{-1}$ were prepared by serial dilution of the stock.

7. 4-Methylumbelliferyl phosphate, 4-MUP, (C$_{10}$H$_5$O$_6$P), FW 256.2 g (Sigma). 100 mg P L$^{-1}$ stock was prepared by dissolving 0.041 g in 50 mL of UPW. Working standards in the range 50 - 100 µg P L$^{-1}$ were prepared by serial dilution of the main stock.

8. P-Nitrophenyl phosphate magnesium salt, p-NPP, (C$_6$H$_4$NO$_6$PMg), FW 241.9 g (Sigma). 100 mg P L$^{-1}$ stock was prepared by dissolving 0.078 g in 100 mL of UPW. Working standards in the range 50 - 100 µg P L$^{-1}$ were prepared by serial dilution of the stock.

9. Flavin Mononucleotide (Ribo-Flavin-5'-phosphate di-sodium salt), RFP, (C$_{17}$H$_{21}$N$_4$O$_9$P), FW 456.3 g (Sigma 98%). 15 mg P L$^{-1}$ stock was prepared by dissolving 2.5 mg in 10 mL of UPW. Working standards in the range of 50 - 100 µg P L$^{-1}$ were prepared by serial dilution of the stock.

10. Aminoethyl phosphoric acid, AMP, (C$_2$H$_6$NO$_3$P), FW 125.07 g (Sigma). 100 mg P L$^{-1}$ stock was prepared by dissolving 0.0404 g in 100 mL of UPW.
Working standards in the range 50 - 100 μg P L⁻¹ were prepared by serial dilution of the stock.

11. Pentasodium triphosphate, STP, (Na₅O₁₀P₃), FW 367.86 (Sigma 98%). 100 mg P L⁻¹ stock was prepared by 0.059 g in 100 mL of UPW. Working standards in the range 50 - 100 μg P L⁻¹ were prepared by serial dilution of the stock.

12. Potassium peroxoydisulphate, K₂S₂O₈, FW 270.33, (Sigma 99%). 0.1 M stock solution was prepared by dissolving 2.703 g in 100 mL of UPW.

13. Potassium dihydrogen phosphate, P, KH₂PO₄, FW 136.09 g (BDH 99.5%). 1000 mg P L⁻¹ stock was prepared by dissolving 0.4414 g in 100 mL of UPW. Working standards in the range 50 - 200 μg P L⁻¹ were prepared by serial dilution of the stock.

14. Ammonium molybdate (NH₄)₆Mo₇O₂₄·4H₂O). 0.1 M solution was prepared by dissolving 12.395 g in 100 mL of UPW. A 0.00081 M solution was prepared by serial dilution of the stock.

15. Tin(II) chloride, (SnCl₂), FW 225.63 g (BDH 98 - 100%). 0.02 M stock prepared by dissolving 0.451 g in 100 mL of UPW. A 0.00089 M solution was prepared from the stock solution by dilution. The stock solution was kept in the cupboard at room temperature protected from light.

16. Hydrazine sulphate, (NH₂NH₂H₂SO₄), FW 130.12 g (Fisons 98-100%). A 0.2 M stock was prepared by dissolving 2.6024 g in 100 mL of UPW. 0.015 M was prepared from this stock by dilution. The stock solution was stored in the cupboard at room temperature and protected from light.

17. Sulphuric acid, (H₂SO₄), AnalAr stock (18.4 M). A 5 M stock solution was prepared by diluting 135.9 mL of the AnalAr stock in 250 mL volumetric flask with UPW. Working standards of various concentrations were prepared from the 5 M stock by dilution.

18. A 0.63 M and 0.5 M H₂SO₄ for molybdate and tin(II) chloride reagents respectively, were made by dilution of the 5 M stock solution.

4.1.2. Autoclave procedures

Degradation (autoclaving) was performed with a Series 200 Autoclave, Boxer model Type 200/400 (Boxer Lab Equip Ltd, UK). This had "a load probe" which is a horizontally mounted chamber with a circulation section constructed from BS321 stainless steel to BS 5500. The lid was also faced with stainless steel and equipped with a closure bolt. The door closure bolt was of "Quick Action" design.
and is electrically interlocked to prove correct closure. The bolt was fitted with a thermal lock, which normally operated at 80 °C. This lock prevented the door from being opened when the chamber or load temperature exceeded the pre-set lock temperature. Also fitted was a pressure lock system, which prevented operation of the closure bolt when the chamber pressure exceeded 0.5 psi. Steam was generated within the chamber. One heater was fitted into the bottom of the chamber, which was manually filled with water to the level mark on the false bottom. A valve was fitted into the bottom of the chamber to enable the water to be drained. Air was removed by downward displacement via a solenoid-operated valve, which remained open until all air has been expelled from the chamber. Only a small amount of condensate was emitted from the air valve since the majority ran back into the bottom of the chamber and was retained. A fan was fitted into the base of the autoclave, which blows air across the chamber after the sterilising cycle was complete to speed up the cooling cycle. A 3 kW Inconel Sheathed immersion heater was fitted into the bottom of the chamber and protected by an overheat cut-out clipped to the heater [4].

In this research, digestion was achieved using a variety of different protocols as listed below.

**Method 1: Acidic peroxydisulphate**

(Method 1A) A 20 mL aliquot of each organic compound was measured into a series of autoclave bottles (100 mL capacity) with 0.15 g of potassium peroxydisulphate and 1.0 mL of 0.90 M H₂SO₄ added.

(Method 1B) A 20 mL aliquot of each organic compound was measured into a series of autoclave bottles (100 mL capacity) with 2.5 mL of 4 M H⁺ and 140 µL of 0.1 M peroxydisulphate i.e. 0.0014 M.

(Method 1C) A 50 mL aliquot of each compound was measured into a series of autoclave bottles (100 mL capacity) with 0.4 g of peroxydisulphate to give a final [H⁺] of 0.5 M. 50 mL instead of 20 mL of sample was used in this method to minimise losses during digestion.

**Method 2: Peroxydisulphate (no acid)**

(Method 2A) A 20 mL aliquot of each organic compound was measured into a series of autoclave bottles (100 mL capacity) and 800 µL of 0.1 M K₂S₂O₈, i.e. 0.004 M final concentration was added.
(Method 2B) A 50 mL aliquot of sample was taken and 2.0 mL of 0.1 M per oxydisulphate, i.e. 0.004 M final concentration, was added. Where lower volumes (concentrations) were used, details are stated in the text.

**Method 3: Hosomi reference method for freshwater samples**

2.65 g of NaOH was dissolved in 100 mL of UPW, and then 11.8 g of K$_2$S$_2$O$_8$ was added and made up to 295 mL with UPW. Eight mL of sample and 4 mL of reagent were placed in a 12 mL propylene tube. This was capped and mixed by inversion. This method (Hosomi et al. [5]) was chosen to compare the results obtained from the proposed method because final pH after autoclave digestion is pH 2.0 - 2.1.

**Method 4: Method 2 modified for seawater samples**

Seawater samples were diluted in the ratio of (1:4) i.e. 20 mL of seawater to 80 mL of UPW giving a final salinity of 7. To each 50 mL aliquot of diluted sample at salinity 7 was added 2.0 mL of 0.1 M K$_2$S$_2$O$_8$ and 20 µL of 1.0 M Na$_2$SO$_3$ and autoclaved. Where depleted seawater (DSW) was used in place of low nutrient seawater (LNS), it is always indicated in the text.

The internal temperature of the autoclave was set at 121 °C for a fixed time, 45 minutes, after which the samples were allowed to cool before being taken out of the autoclave. It was necessary to cool the samples at room temperature for at least three hours before analysis. This was to ensure that the bottles returned to normal atmospheric pressure. All samples and standards were analysed using the ascorbic acid manifold shown in Fig. 4.1.

4.1.3. Ascorbic acid FI manifold for phosphorus determination

The FI manifold with spectrophotometric detection incorporated 0.75 mm i.d. Teflon tubing, a peristaltic pump (Gilson Minipuls 2, Villiers le Bel, France) and a Rheodyne six-port switching valve. Data was collected automatically using LabVIEW™ and samples were injected manually (see Chapter Two). The four-channel flow injection FI system incorporated a thiosulphate stream for the determination of >100 µg P L$^{-1}$ and is shown in Fig. 4.1. The peristaltic pump was used to propel the carrier/thiosulphate and reagent streams at flow rates of 0.42 and 0.17 mL min$^{-1}$ respectively. The rotary injection valve was used to inject 440 µL of sample into the carrier stream. Sample and carrier were mixed at a T-piece
with the thiosulphate stream. Here As(V) was reduced to As(III). This was then mixed with the molybdate reagent in another T-piece and finally with the ascorbic acid reductant in a water bath at 30 °C and was passed into a uv/visible/nir spectrophotometer (Philips PU 8620, Cambridge England) at 790 nm equipped with a 30 μL flow cell (178.011-OS 10 mm light path, Optical Glass Windows 320 - 2500 nm, Hellma, UK). Detector output was automatically recorded using an in-house LabVIEW™ software programme.

**Fig. 4.1.** Four channel ascorbic acid FI manifold incorporating a thiosulphate stream for the determination of 20 - 100 μg P L⁻¹ in natural waters in the presence of arsenic(V) at <300 μg As L⁻¹.

A uv-visible spectrophotometer (Hewlett Packard HP84553) [7] was used to confirm the absorbance spectrum of the phosphomolybdenum blue complex as shown in Fig. 4.2. This spectrum was obtained in order to determine the wavelength of maximum absorbance for the FI manifold. Water samples from the Tamar estuary collected as grab samples were filtered in situ using a 0.45 μm filter (cellulose acetate) and stored in pre-cleaned HDPE containers. Water samples were analysed within 24 hours and, when possible, stored at 4 °C prior to analysis. If the samples were stored for longer periods before analysis, they were frozen at -20°C.

**Fig. 4.3** is a diagnostic LabVIEW display of the peaks obtained using the FI manifold shown in Fig. 4.1. It gave a clear description of the forms and shapes of peaks generated during analysis and helped to monitor the chemical reactions.
Fig. 4.2. Wavelength absorbance spectrum of the phosphomolybdenum blue complex formed with ascorbic acid reduction.

Fig. 4.3. Typical shapes of peaks obtained with the FI manifold shown in Fig. 4.1. The bottom left hand trace has an expanded time scale to show peak shape and the bottom right hand side has a compressed time scale to show a series of sample/standard replicates.

When there was a problem with the reagents or the system, the bottom left trace (Fig. 4.3) either broadened or shrank, e.g. with the Schlieren effect peaks decreased in size because of the initial negative response, which was clearly
seen. There was also a wide gap between each peak. For undiluted seawater samples, such peaks were obtained, but the addition of sodium sulphotite helped to minimise these effects. Diluted seawater that had sodium sulphotite added prior to autoclaving produced peaks similar to the type shown in Fig. 4.3.

4.2. RESULTS AND DISCUSSION

4.2.1. Digestion techniques for phosphorus

Digestion techniques for environmental samples are necessary for the determination of total phosphorus (TP) and total dissolved phosphorus (TDP). This is because phosphorus species in a sample contain P-O-P, C-O-P and C-P bonds that need to be broken down to release phosphorus as phosphate, which can then be determined using molybdenum blue chemistry [8]. The digestion technique must also be able to release phosphorus from biological material, e.g. algal cells and plant detritus and adsorbed/occluded P from sediments [9, 10] see Fig. 4.4. Traditional methods of digestion for natural water samples include fusion, dry ashing, perchloric acid, sulphuric acid-nitric acid and boiling on a hot plate, with more recent methods generally using autoclaving, UV photo-oxidation and microwave heating [9]. UV photo-oxidation can be used for organic phosphorus compounds in marine and freshwaters [11 - 13] but condensed polyphosphates present in the sample will not be broken down by UV photo-oxidation alone [14 - 17] and also need to be heated to 90 - 120 °C in the presence of acid [9]. Hydrolysis of condensed or linear polyphosphates is influenced by temperature, pH and concentration [18]. To ensure that all polyphosphates present in a sample are decomposed, either boiling with HCl or potassium peroxydisulphate after UV irradiation is recommended [19]. McKelvie et al. used an on-line UV photo-oxidation flow injection (FI) technique and found that results were comparable with a batch peroxydisulphate method [20].

Autoclaving methods are generally straightforward, give reproducible results and use sealed vessels that are less prone to contamination [9, 21 - 23]. In addition, microwave digestion combined with ICP-MS detection has been used to determine phosphorus in marine environmental samples and plant leaves with good recoveries [24 - 26]. However microwave heating for batch sample digestion and in FI systems with spectrophotometric detection for on-line TDP and TP digestion [15] is less widely used than UV photo-oxidation or autoclaving.
Fig. 4.4. Forms of phosphorus occurring in natural waters [10].

4.2.2. Peroxydisulphate chemistry
Many oxidising reagents have been used in the degradation of complex organic molecules in aqueous solution. Peroxydisulphate (S$_2$O$_8^{2-}$) is one of the most commonly used. Others are ozone (O$_3$), hydrogen peroxide, (H$_2$O$_2$), perchloric acid (HClO$_4$), nitric/sulphuric acid (HNO$_3$/H$_2$SO$_4$), permanganate (MnO$_4^-$) and TiO$_2$ [27 - 33]. For ozone, hydrogen peroxide, permanganate and peroxydisulphate, their oxidising power can be related to their redox potential (E°) for the half-cell reaction and are comparable.

\[
\begin{align*}
\text{S}_2\text{O}_8^{2-} + 2e^- & \rightarrow 2\text{SO}_4^{2-} & E^0 = 2.01 \text{ V} & 1 \\
\text{O}_3(\text{g}) + 2\text{H}^+ + 2e^- & \rightarrow \text{O}_2(\text{g}) + \text{H}_2\text{O} & E^0 = 2.07 \text{ V} & 2 \\
\text{H}_2\text{O}_2 + 2\text{H}^+ + 2e^- & \rightarrow 2\text{H}_2\text{O} & E^0 = 1.78 \text{ V} & 3 \\
\text{MnO}_4^- + 4\text{H}^+ + 3e^- & \rightarrow \text{MnO}_2(\text{g}) + 2\text{H}_2\text{O} & E^0 = 1.70 \text{ V} & 4
\end{align*}
\]
Peroxydisulphate oxidation is favoured by increased temperature which leads to the generation of highly reactive sulphate and hydroxyl radicals by photolysis in the aqueous phase [27,29-2]. Studies involving peroxydisulphate labelled with S-35 as initiator in polymerisation studies gave strong evidence that the sulphate radicals generated during heat assisted degradation by peroxydisulphate come from the peroxydisulphate itself. Also, peroxydisulphate oxidation may be initiated by impurities in the solution, e.g. dust or light, because the reaction is photosensitive. This effect has been reported by House [30]. Ions such as copper, silver and iodide have a catalytic effect on oxidation by peroxydisulphate. Peroxydisulphate degradation is also influenced by free radicals. The sulphate free radicals react with water producing hydroxyl free radicals. This initiates chains of radical disproportionating reactions, which are fundamental to the process [27, 30]. Studies reveal that the activation energy required for this radical polymerisation steps are different in alkaline, neutral and acid media and will thus have an overall influence on degradation and hence recoveries of substrates (see Table 4.1).

**Table 4.1. Energy of Activation (kcal mol⁻¹) for the thermal decomposition of peroxydisulphate [30].**

<table>
<thead>
<tr>
<th>Alkaline solution</th>
<th>Neutral solution</th>
<th>Acid solution</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>29.1</td>
<td>28.39</td>
</tr>
<tr>
<td></td>
<td>28.39</td>
<td>28.37</td>
</tr>
<tr>
<td>33.3 (pH 13)</td>
<td></td>
<td>26.0 (pH 1)</td>
</tr>
<tr>
<td>32.1 (pH 8)</td>
<td></td>
<td>24 ± 2 (pH 0.3)</td>
</tr>
<tr>
<td>32.5 (pH 9.5)</td>
<td>30.8</td>
<td>27.7 (pH 1.7)</td>
</tr>
</tbody>
</table>

The decomposition of peroxydisulphate by heat yields a considerable quantity of sulphuric acid e.g. 350 mg of peroxydisulphate produces on decomposition, 120 mg of H₂SO₄ in water [33].

Mechanisms of peroxydisulphate degradation reactions

(a) hydrogen ion independent reactions

\[
\begin{align*}
S_2O_8^{2-} & \rightarrow 2SO_4^{2-} & 5 \\
SO_4^{2-} + H_2O & \rightarrow HSO_4^- + OH^- & 6 \\
2OH^- & \rightarrow H_2O + \frac{1}{2}O_2 & 7
\end{align*}
\]
or $\rightarrow$ H$_2$O$_2$

(b) in acid solution

$\text{S}_2\text{O}_8^{2-} + \text{H}^+ \rightarrow \text{HS}_2\text{O}_8^{-1}$ \hspace{1cm} 8

$\text{HS}_2\text{O}_8^{-1} \rightarrow \text{SO}_4^{3-} + \text{H}_2\text{SO}_4^{-1}$ \hspace{1cm} 9

$\text{SO}_4^{3-} \rightarrow \text{SO}_3 + \frac{1}{2}\text{O}_2$ \hspace{1cm} 10

$\text{SO}_3 + \text{H}_2\text{O} \rightarrow \text{H}_2\text{SO}_4$ \hspace{1cm} 11

(c) in strong acid,

$\text{SO}_4^{3-} + \text{H}_2\text{O} \rightarrow \text{H}_2\text{SO}_5$ \hspace{1cm} 12

Mechanism of peroxydisulphate decomposition of organic compounds [27]

$\text{S}_2\text{O}_8^{2-} + \text{M} \rightarrow 2\text{SO}_4^{-1} + \text{M}^-$ \hspace{1cm} 13

(M represents organic compounds)

$\text{SO}_4^{-1} + \text{M} \rightarrow \text{M}^- + \text{products}$ \hspace{1cm} 14

$\text{HO}^- + \text{M} \rightarrow \text{M}^- + \text{products}$ \hspace{1cm} 15

$\text{M}^- + \text{S}_2\text{O}_8^{2-} \rightarrow \text{SO}_4^{-1} + \text{products}$ \hspace{1cm} 16

Excessive acidification (equation 12) of samples can lead to a reduction in pH of the peroxydisulphate solution to 1.0 or less. This will result to sluggish and incomplete oxidation of organic compounds [31]. Peroxydisulphate oxidation of organic molecules is slowed in samples containing significant concentrations of Cl$^-$ ions by the preference of peroxydisulphate to oxidise chloride [6, 33, 35].

4.2.3. Alkaline Peroxydisulphate

Menzel and Corwin first used autoclaving with peroxydisulphate in 1965 for the digestion of seawater samples [36]. Koroleff developed an alkaline peroxydisulphate alternative in 1969 [37], which was then slightly modified [38] and simplified by introducing a borate buffer [39, 40]. This enabled the simultaneous determination of TP and total nitrogen (TN), as nitrogen bonds are more effectively hydrolysed/oxidised in alkaline media [41]. Using borate buffer, the pH is alkaline (ca 9.7) at the start of the digestion process and becomes acidic (pH 4 - 5) as the sodium hydroxide decomposes [16, 41, 42]. Hosomi and Sudo also reported that pH change was important and in their method the pH decreased from 12.8 to 2.0 - 2.1 to ensure that even condensed polyphosphates were digested [5].

The alkaline method has also been used for particulate material but with relatively poor recoveries [43]. For example orchard leaves gave recoveries of 80-90% for
TP and TN [44]. Higher recoveries can be obtained by decreasing the ratio of sample to persulphate [5]. Alkaline digestion of model phosphorus compounds has been found to be efficient for turbid water samples [45 - 47] although the concentration of suspended particulate material needs to be diluted to <150 mg L⁻¹ and difficulties can arise when this material is of soil origin rather than biological origin, e.g. algal cells and plant detritus. The alkaline method has therefore been used to determine TP in turbid lake waters and suspensions of particulate material [47]. See Table 4.2 for some recommended digestion techniques.

Alkaline peroxydisulphate autoclaving, rather than acid peroxydisulphate, is recommended for the digestion of marine waters. This is because in the acid method, peroxydisulphate oxidises the chloride in seawater to free chlorine, thus reducing the oxidising power of the peroxydisulphate [35].

4.2.4. Acid Peroxydisulphate

An acid peroxydisulphate method developed by Gales et al. [48] has been adopted by the US Environmental Protection Agency [49]. Eisenreich et al. simplified the method [50] and various modifications of this approach are now used to digest different types of samples such as soil solutions, natural waters and river water [51 - 53]. The alkaline peroxydisulphate method for soil extracts is only appropriate if the total organic carbon is <100 mg L⁻¹ and manganese is <1 mg L⁻¹. Above this manganese concentration, coloured solutions or precipitates are formed, which interfere with the digestion step [54]. This interference is avoided when using acid peroxydisulphate and solutions are colourless after digestion [23].

Pate et al. described standard methods for the determination of TP and TDP using sulphuric acid-nitric acid and peroxydisulphate digestions [55] and recommended the use of sulphuric acid-nitric acid digestion to achieve good recoveries for most samples. However, this digestion method can be potentially dangerous if salts precipitate during digestion [56] and it is less easy to control than the peroxydisulphate method [23, 57]. Rowland and Haygarth compared a mild peroxydisulphate method to the more rigorous sulphuric acid-nitric acid method [57] for soil solutions and leachates. The latter method gave erratic recoveries and was more prone to contamination due to the open digestion vessels used [23]. Peroxydisulphate autoclaving is also safer than perchloric acid digestion [58, 59].
Table 4.2  Summary of digestion techniques used for the determination of organic phosphorus species in natural waters.

<table>
<thead>
<tr>
<th>Matrix</th>
<th>Flow type</th>
<th>Digestion method</th>
<th>Digestion time (min)</th>
<th>Digestion temp. (°C)</th>
<th>Final pH</th>
<th>Model compounds</th>
<th>Comments on recovery</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Marine and freshwater</td>
<td>SFA</td>
<td>H₂O₂-UV</td>
<td>7</td>
<td>37</td>
<td>6-9</td>
<td>AEPA, PA, GMP, PCC, PEPA, RP, GP, AMP and RMP</td>
<td>Recoveries were &gt; 97% except [8] PA after 5-6 dilution and compared with acidic and alkaline peroxydisulphate</td>
<td></td>
</tr>
<tr>
<td>Freshwater</td>
<td>SFA</td>
<td>Peroxydisulphate</td>
<td>60</td>
<td>120</td>
<td>2.0-2.2</td>
<td>NBSRF 1571 orchard leaves, (98%), NIES RF2 pepper bush (96%), NIES RF2 pond sediment, NIES RF3 chlorella 100% of all concentration; 50 mg L⁻¹ ATP-Na, 99-100%, 5'-ADP-Na₂, 98%, TSPP, 99-100%, SHMP, 94-97%, STP, 96-97%, G-P-K₂, 99-102%</td>
<td>Quantitative recovery for all [5] compounds analysed</td>
<td></td>
</tr>
<tr>
<td>Freshwater and seawater</td>
<td>SFA</td>
<td>Alkaline peroxydisulphate</td>
<td>30</td>
<td>115</td>
<td>4-5</td>
<td>2-AEP, 77,108,88%, PTA, 70,95, 100%, PC, 98,37,99,96%, 5'-GMP-Na₂, 99,93,100,94%, FMN, 99,99,100,97%, AMP,99,94,100, 93%, RP, 100,94,103,95%, PEP-3CHA,100,100,101,100%, β-GLY, 99,100,100,96%</td>
<td>Recoveries in order of reagents, [16] acidic water, acidic seawater, alkaline water, acidic/alkaline peroxydisulphate. UV compared to HTC. Alkaline peroxydisulphate is better for seawater</td>
<td></td>
</tr>
<tr>
<td>Seawater</td>
<td>5%</td>
<td>Peroxydisulphate</td>
<td>120</td>
<td>30</td>
<td>1.5-1.8</td>
<td>PFA, 96.5%, 1-AEP,85.5%, 2-AEP, 81.2%</td>
<td>Compared oxidation of nitrate with peroxydisulphate</td>
<td>[42]</td>
</tr>
</tbody>
</table>

100
<table>
<thead>
<tr>
<th>Matrix</th>
<th>Flow type</th>
<th>Digestion method</th>
<th>Digestion time (min)</th>
<th>Digestion temp. (°C)</th>
<th>Final pH</th>
<th>Model compounds</th>
<th>Comments on recovery</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Orchard leaves and aufwuchs</td>
<td>SFA</td>
<td>Peroxydisulphate</td>
<td>60</td>
<td>100-110</td>
<td>2.5-3.7</td>
<td>NBSRM 1571, orchard leaf, 86.9-88.7%, 93.6%, 101.4%</td>
<td>Quantitative recoveries with lower peroxydisulphate</td>
<td>[44]</td>
</tr>
<tr>
<td>Turbid lake and river water</td>
<td>SFA</td>
<td>Alkaline peroxydisulphate</td>
<td>60</td>
<td>120</td>
<td>2</td>
<td>NIES No 3 chlorella, 99-101% up to 100 μg P L⁻¹ and No 2 pond sediment, 98-104%, 60 μg P L⁻¹ and 88% μg P L⁻¹ at 100 μg P L⁻¹. Model compounds added to distil-led water and lake water, KHP, G-6-P, 113%, PTA, 101%, α-GLY, 108%, 2-AEP, 104%, PFA, 108%, o-PEA, 106%, SHMP, 114%, AlPO₄ 23%</td>
<td>Compared to alkaline peroxydisulphate, microwave hot-plate and Khedjahl digestion for TN and TP. Quantitative recovery but matrix could affect recovery</td>
<td>[45]</td>
</tr>
<tr>
<td>Seawater</td>
<td>SFA</td>
<td>Alkaline peroxydisulphate</td>
<td>30</td>
<td>120</td>
<td>8</td>
<td>Orthophosphate, phenylphosphorous acid, phenyl phosphorous acid</td>
<td>Quantitative recoveries of all model compounds in water</td>
<td>[40]</td>
</tr>
<tr>
<td>Estuarine waters</td>
<td>batch</td>
<td>Peroxydisulphate</td>
<td>60</td>
<td>120</td>
<td>1.5-1.8</td>
<td>KHP, PTA dodeca-sodium salt, 99% for 0.1 mg L⁻¹ and 106% for 1.0 mg L⁻¹.</td>
<td>Quantitative recovery of all model compounds at 50 μg P L⁻¹ level.</td>
<td>[57]</td>
</tr>
<tr>
<td>Soil extracts</td>
<td></td>
<td></td>
<td>30</td>
<td>110</td>
<td>2</td>
<td></td>
<td></td>
<td>[55]</td>
</tr>
</tbody>
</table>

The acid peroxydisulphate method generally gives good recoveries for model compounds and is simple and easy to use and is therefore recommended for TP and TDP determinations in none saline natural waters and particularly, soil solutions.

4.2.5. Model compounds

It is advisable to test the efficiency of any digestion method using a range of model phosphorus containing compounds that reflect different chemical bonds and stabilities and are representative of naturally occurring compounds. The majority of relevant compounds reported in the literature contain C-P bonds, which are very resistant to oxidation and hydrolysis [60].

Phosphonates are refractory organic phosphorus compounds and can be released into seawater from biological sources [16, 42, 61] and have been detected in soils [62] and soil leachate [63]. As phosphonates contain a strong C-P bond that is resistant to acid hydrolysis [61], they are useful compounds for recovery studies [16, 42, 45, 46, 52, 61]. Condensed inorganic (e.g. sodium tripolyphosphate) and organic (e.g. adenosine-5'-triphasphate) phosphates and cocarboxylase [64] have also been shown to be resistant to UV irradiation alone [17]. With acid or alkaline peroxydisulphate autoclaving, however, these compounds have been successfully broken down [44, 45, 52, 65, 66].

Inositol phosphates are an important class of naturally occurring organic phosphorus compounds [67]. Phytic acid, for example, is one of the more resistant compounds to hydrolysis and is also one of the most refractory organic phosphorus compounds in soils [10, 64, 67]. Other organic phosphorus compounds found in soil leachate and runoff are the sugar phosphorus compounds, e.g. D-glucose-1-phosphate and D-glucose-6-phosphate, which are labile [65]. Organic condensed phosphates, e.g. adenosine-5'-triphasphate and adenosine-5'-diphosphosphate are also important as they originate from all living systems, e.g. algae, bacteria, fungi, insects, plant and animal tissues [10, 63]. It is therefore recommended that model compounds selected for digestion studies should include one with a P-O-P bond (e.g. sodium tripolyphosphate), a refractory C-O-P compound (e.g. phytic acid), a labile C-O-P compound (e.g. D-glucose-1-phosphate or D-glucose-6-phosphate), a refractory C-P compound (e.g. 2-aminoethylphosphonate), and a compound containing C-O-P and P-O-P bonds.
(e.g. adenosine-5'-triphosphate). Orthophosphate (e.g. potassium dihydrogen orthophosphate) should also be used in all recovery studies as a method control [42]. One should also be aware that specific matrices may require additional model compounds. For example, acid soils and sediments may well contain phosphorus associated with iron or aluminium phases, which are relatively resistant to oxidative dissolution [9]. Structures of the model compounds used in these studies are given below.

<table>
<thead>
<tr>
<th>Name and structure</th>
<th>Abbreviation</th>
<th>Chemical formula</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adenosine triphosphate di-sodium salt</td>
<td>ATP</td>
<td>C_{10}H_{14}O_{13}P_{3}Na_{2}</td>
</tr>
<tr>
<td>Cocarboxylase</td>
<td>COC</td>
<td>C_{12}H_{15}ClN_{4}O_{7}P_{2}S</td>
</tr>
<tr>
<td>Penta-sodium triphosphate</td>
<td>STP</td>
<td>Na_{5}P_{3}O_{10}</td>
</tr>
<tr>
<td>Potassium dihydrogen orthophosphate</td>
<td>P</td>
<td>KH_{2}PO_{4}</td>
</tr>
</tbody>
</table>
Phytic acid

\[
\begin{array}{c}
\text{O(HO)}_2\text{P(OH)}_2\text{O}\text{P(OH)}_2\text{O}\text{P(OH)}_2\text{O} \\
\text{O=POO} \\
\text{(OH)}_2
\end{array}
\]

PTA \hspace{1cm} C_6H_{18}O_{24}P_6

Methyl triphenyl phosphobromide

\[
\begin{array}{c}
\text{C}_9\text{H}_6\text{PBr} \\
\text{H}_2\text{C} \quad \text{Br} \\
\text{H}_3\text{C} \quad \text{Br}
\end{array}
\]

MTP \hspace{1cm} C_{19}H_{18}Br

Aminoethyl phosphoric acid

\[
\begin{array}{c}
\text{NH}_2\text{C} \quad \text{P} \quad \text{OH} \\
\text{CH}_3 \quad \text{OH}
\end{array}
\]

AMP \hspace{1cm} C_2H_8NO_3P

p-Nitrophenyl phosphate sodium salt

\[
\begin{array}{c}
\text{O} \quad \text{P} \quad \text{ONa} \\
\text{ONa}
\end{array}
\]

p-NPP \hspace{1cm} C_6H_4NO_6PNa_2

Flavin mononucleotide (Ribo-Flavin-5'-phosphate mono-sodium salt)

\[
\begin{array}{c}
\text{H}_3\text{C} \quad \text{CH} \quad \text{CH} \quad \text{CH} \quad \text{CH}_2 \quad \text{O} \quad \text{P} \quad \text{OH} \\
\text{H}_3\text{C} \quad \text{N} \quad \text{N} \quad \text{N}
\end{array}
\]

RFP \hspace{1cm} C_{17}H_{20}N_4O_9PNa
4.2.6. Optimisation of reagents

Different types of reagents or combinations were used at the onset of the study and the effect of autoclaving time and temperature from 30-60 minutes and 90 - 121 °C, respectively was also investigated. However, each condition e.g. HNO₃/H₂SO₄ and/ or different combinations of acid/peroxydisulphate gave different recoveries. The recoveries of compounds high in carbon, e.g. MTP, were less than 30 %. This can be caused by carbonisation (see equation 12) which can result in incomplete digestion of carbon compounds [31], but despite this observation, a report by Jankovic et al. [68] showed that in wastewaters, high recoveries were possible with very high concentrations of strong oxidants.

The objectives of the experiments in this section were to investigate the factors that led to the low recovery of some model compounds in preliminary studies and to optimise an autoclave method for the quantitative recovery of DOP from natural (fresh and saline) waters. Results obtained from the preliminary studies are shown in Figs. 4.5 and 4.6. Batch autoclave digestions were carried out using method (1A) i.e., by adding 1.0 mL of 0.9 M H₂SO₄ and 0.15 g persulphate to 20 mL of sample and autoclaving at 121 °C for 45 min. The pH after autoclaving was <0.45.

In Fig. 4.5, the orthophosphate as P and STP gave quantitative recoveries. All other model compounds, i.e. PTA, COC and ATP recoveries were (78 - 85%) except for MTP. Storage of the autoclaved samples at 4 °C for one week had no significant effect on recoveries.

In Fig. 4.6, the low recovery of STP in 35 LNS on day 1 could be attributed to the effect of salinity. Chloride ion interferes in peroxydisulphate digestion due to the preference of peroxydisulphate to oxidize chloride to free chlorine in place of the analyte of interest. This step exerts an inhibitory effect on the system and could
lead to low recoveries [35]. Other effects caused by chloride ions are discussed in detail in section 4.3.13 on seawater.

![Graph](image)

**Fig. 4.5. Recovery of model compounds in UPW after autoclaving, i.e. method 1A. Error bars, σ ± 3s (n = 3).** Samples were stored for eight days and re-analysed to investigate the effects of storage on sample at 4°C.

The recoveries of compounds high in carbon, e.g. MTP, were low (see Fig. 4.5) because excessive acidification slows down the decomposition of carbon compounds [31] (see equation 12) and the excess acid can suppress phosphomolybdenum formation [69 - 71]. At high [H⁺], the blue chromophore is not stable and hence shows poor absorption. When applied to estuarine water samples, the recoveries of DOP were very low.

Therefore, batch autoclaving employing high acid/peroxydisulphate concentrations as reagents may not be suitable for the determination of DOP in unpolluted natural waters. This can lead to underestimation of DOP in such areas. Because of the observed effects of acidity (drop in pH) and chloride ions, reagent concentrations were re-optimised to make the method suitable for the determination of DOP in saline natural waters having low concentrations of DOP e.g. 0.1 µg P L⁻¹.

### 4.2.7. Peroxydisulphate concentration

Fig. 4.7, shows the recovery of phytic acid (in UPW) using digestion method 1B but varying the volume of 0.1 M per oxydisulphate concentration, i.e. 60 µL (0.00056 M), 70 µL (0.0007 M), 110 µL (0.0011 M), 140 µL (0.0014 M) and 190 µL (0.0019 M).
For each peroxydisulphate concentration, the pH after autoclaving was <0.9. However, as can be seen in Fig. 4.7, response (recovery) increased with increasing peroxydisulphate concentration due to increased oxidising power of the autoclave reagent. This shows that the concentration of peroxydisulphate must be selected with care for the autoclave based digestion of organic phosphorus compounds.

Fig. 4.6. Recovery of model organic compounds in UPW and 35 low nutrient seawater (LNS) after autoclaving. Samples were stored for up to 29 days after autoclaving. Error bars, $\sigma \pm 3s$ ($n = 3$).

Fig. 4.7. Effect of peroxydisulphate concentration on the recovery of 186 $\mu$g P L$^{-1}$ PTA as P at a constant $[H^+]$ of 0.5 M. Error bars $\sigma \pm 3s$ ($n = 3$).
4.2.8. H+ concentration

March et al. [69] reported quantitative recoveries of PTA when 0.44 M [H+] was used with microwave digestion but recoveries of 70% with 0.9 M [H+] and 30% with 1.9 M [H+]. They also reported high absorption for solutions at lower acidity. Using digestion method 1B but varying the acid concentration over the range 0.3 - 0.9 M resulted in decreased recovery at higher acid concentrations (see Fig. 4.8) but peak tailing in the FI manifold was more pronounced at the lowest acidity of 0.3 M. The 0.5 and 0.6 M [H+] gave the best response in terms of peak shape, no tailing and less suppression when monitored on the LabView display (see Fig. 4.3) and hence were chosen for more detailed investigation (see Fig. 4.9). Aminot et al. [12] reported a low recovery for phytic acid (PTA) using this method. No reason was postulated for the low recovery, but from this work, the low recovery is probably due to acidification, which can slow down the digestion of compounds high in carbon.

![Graph](image)

**Fig. 4.8.** Effect of [H+] on the recovery of 186 μg P L⁻¹ PTA as P at a constant peroxydisulphate concentration of 0.0014 M. Error bars, σ ± 3s (n = 3).

As shown in Fig. 4.9, 110 μL, 140 μL and 190 μL of 0.1 M K₂S₂O₈ were added to 20 mL of sample and 2.5 mL of 0.5 M or 3.0 mL of 0.6 M H⁺ added respectively as indicated. The pH after autoclave was <0.9. The 0.5 M H⁺ gave a better recovery dependent on peroxydisulphate concentration, while the 0.6 M [H⁺] gave a lower recovery but was less dependent of the peroxydisulphate concentration. The acid peroxydisulphate produced a final pH of <0.5 depending on the concentration of [H⁺] used and this can inhibit the phosphomolybdenum blue formation during the determination of phosphorus.
Fig. 4.9. Comparing [H+] and K$_2$S$_2$O$_8$ concentrations on the recovery of 186 µg P L$^{-1}$ PTA as P. Error bars,

The acid digestion method can suffer from underestimation as a result of pH effects. The excess acid formed not only produced a Schleiren effect but also had a serious effect on the development of the absorbing chromophore in the FI manifold and therefore affected the recovery of digested samples. As seen in Fig. 4.7, increasing the peroxydisulphate concentration resulted in an increase in oxidation efficiency of the peroxydisulphate reagent. This was further investigated by increasing the peroxydisulphate concentration to 0.4 g and keeping the acid concentration to 0.5 M H$_2$SO$_4$ to each 50 mL of sample i.e., method (1C). Recovery of model compounds were 92 - 105% when compared with an orthophosphate standard passed through similar autoclaving conditions (see Fig. 4.10) but the FI response (absorbance) was low and a significant drop in pH to 0.5 of the solution after digestion was also observed.
Fig. 4.10. Effect of increasing peroxydisulphate concentration to 0.4 g and 0.5 M H₂SO₄ added to each 50 mL of 50 µg P L⁻¹ sample i.e., method (1C). Error bars, σ ± 3s (n = 3).

The recoveries of model compounds were in agreement with those reported by e.g. Aminot et al. [12] and Ormaza et al. [16] with acid peroxydisulphate digestion techniques.

4.2.9 Recovery of model compounds with peroxydisulphate autoclaving.
Due to the problem associated with acidic peroxydisulphate autoclaving, i.e. difficulty in obtaining the appropriate pH for phosphomolybdate complex formation, a peroxydisulphate only digestion, i.e. no acid added, was also investigated.

Hosomi et al. [5], Menzel et al. [36] and Jenkins [56] used peroxydisulphate (with no acid) for the digestion of natural water samples through batch autoclaving with quantitative recoveries in seawater, river water and estuarine water respectively. In this work, to each 50 mL of sample was added only 2.0 mL of 0.1 M K₂S₂O₈ (i.e. method 2B) and this was digested at 121 °C for 45 min (i.e. method 2). The pH after digestion was 1.9 - 2.1. Increasing the concentration of peroxydisulphate during digestion lead to a drop in pH after autoclaving. The recovery of model compounds at 50 µg P L⁻¹ was quantitative (Fig. 4.11), in agreement with the literature [5, 12, 36, 45, 56], and the diagnostic LABVIEW display showed that there was no problem with the peak shape monitored during analysis of the digested samples. More PTA was recovered with the peroxydisulphate reagent than with the acid/peroxydisulphate reagent which demonstrates the effectiveness of this approach.
4.2.10. Comparison of peroxydisulphate reagent only (i.e. method 2B) and acid/peroxydisulphate reagent (i.e. methods 1B and 1C) for the determination of DOP in estuarine waters.

The results presented in Figs. 4.10 and 4.11 show that both the acid/peroxydisulphate and the peroxydisulphate only autoclaving techniques give good recoveries for a range of organic phosphorus compounds in UPW. This section investigates the suitability of both methods for application to samples from anthropogenically impacted estuarine waters using the FI manifold shown in Fig. 4.1 for detection. Water samples were collected from different locations in the Tamar Estuary as grab samples and filtered in situ through a 0.45 μm filter (Whatman cellulose acetate type). Samples were stored at 4 °C (storage kept to a minimum) until analysis. Also, prior to digestion and the addition of the various autoclave reagents, 1:4 dilutions were performed with UPW to minimise matrix interferences. This was necessary because samples from the upper part of the Estuary had a different colour (brownish) compared with samples from the seawater end, probably due to humic and fulvic substances [73] in the freshwater part of the estuary which absorb strongly in the uv and visible regions. Autoclave conditions were as stated in section 4.1.2.
Table 4.3. Results obtained after digestion using method 2 and method 1B and 1C, (n = 3).

<table>
<thead>
<tr>
<th>Location</th>
<th>Method 2</th>
<th>Method 1B</th>
<th>Method 1C</th>
<th>(FRP) Salinity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>TDP (µg P L⁻¹) ± %RSD</td>
<td>TDP (µg P L⁻¹) ± %RSD</td>
<td>TDP (µg P L⁻¹) ± %RSD</td>
<td>(µg P L⁻¹)</td>
</tr>
<tr>
<td>Weir Quay</td>
<td>42 ± 8</td>
<td>26 ± 12</td>
<td>26 ± 13</td>
<td>5 ± 5</td>
</tr>
<tr>
<td>Southward Farm</td>
<td>38 ± 11</td>
<td>30 ± 12</td>
<td>34 ± 20</td>
<td>13 ± 2</td>
</tr>
<tr>
<td>Calstock</td>
<td>79 ± 5</td>
<td>81 ± 5</td>
<td>68 ± 6</td>
<td>20 ± 1</td>
</tr>
<tr>
<td>Rumleigh Farm</td>
<td>77 ± 8</td>
<td>76 ± 11</td>
<td>75 ± 1</td>
<td>22 ± 2</td>
</tr>
<tr>
<td>Morwellham</td>
<td>58 ± 6</td>
<td>50 ± 7</td>
<td>33 ± 2</td>
<td>33 ± 4</td>
</tr>
</tbody>
</table>

TDP-total dissolved phosphorus and FRP soluble reactive phosphorus.

Table 4.3 shows the results for TDP obtained using two different acid peroxydisulphate reagents and the peroxydisulphate only reagent. SRP results from direct analysis of the samples using the FI manifold are also given for comparative purposes. The related physico-chemical parameters for these samples are shown in Table 4.4. Increasing acidity (method 1C) had a suppressing effect on the recovery of the digested samples compared with method 1B at the low salinity end.

Table 4.4. Physico-chemical parameters obtained at the sampling points for the fieldwork of 12th August, 2005. High tide was at 10.10 am and sampling was carried out on an incoming tide.

<table>
<thead>
<tr>
<th>Location</th>
<th>Grid reference</th>
<th>Water depth (m)</th>
<th>Temp °C</th>
<th>pH</th>
<th>DO (mg/L)</th>
<th>Salinity (mg/L)</th>
<th>Solids (mg/L)</th>
<th>Time</th>
<th>Weather</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>50°27'.35 N</td>
<td>3.1</td>
<td>19.4</td>
<td>8.1</td>
<td>10.7</td>
<td>25</td>
<td>8</td>
<td>8.23</td>
<td>Sunny bright</td>
</tr>
<tr>
<td></td>
<td>04°2.39 W</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B</td>
<td>50°21'.13 N</td>
<td>3.7</td>
<td>19.6</td>
<td>7.8</td>
<td>10.6</td>
<td>17.8</td>
<td>18</td>
<td>9.05</td>
<td>&quot;</td>
</tr>
<tr>
<td></td>
<td>04°13'.87 W</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>&quot;</td>
</tr>
<tr>
<td>C</td>
<td>50°29'.59 N</td>
<td>3.2</td>
<td>19.7</td>
<td>8.1</td>
<td>8.4</td>
<td>5</td>
<td>29</td>
<td>9.20</td>
<td>&quot;</td>
</tr>
<tr>
<td></td>
<td>04°12'.39 W</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>&quot;</td>
</tr>
<tr>
<td>D</td>
<td>50°27'.35 N</td>
<td>3.5</td>
<td>19.8</td>
<td>8.0</td>
<td>7.7</td>
<td>0</td>
<td>48</td>
<td>9.30</td>
<td>&quot;</td>
</tr>
<tr>
<td></td>
<td>04°12'.39 W</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>&quot;</td>
</tr>
<tr>
<td>E</td>
<td>50°30'.31 N</td>
<td>2.5</td>
<td>19.1</td>
<td>8.1</td>
<td>6.8</td>
<td>0</td>
<td>162</td>
<td>9.50</td>
<td>&quot;</td>
</tr>
<tr>
<td></td>
<td>04°11'.47 W</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>&quot;</td>
</tr>
</tbody>
</table>

Note: A = Weir Quay; B = Southward Farm; C = Calstock; D = Rumleigh Farm; and E = Morwellham.)
Furthermore, both of these methods gave low concentrations compared with the peroxydisulphate only (method 2B). The peroxydisulphate only method i.e. method 2B is therefore recommended for the determination of DOP in estuarine samples. The results also show that DOP in these samples is a significant fraction of the total dissolved phosphorus pool.

4.2.11. Application of the recommended method (method 2B) to estuarine waters

Natural water samples were collected from different locations of the Tamar Estuary as grab samples and analysed using the peroxydisulphate digestion method, i.e. method 2B, (2 mL of 0.004 M peroxydisulphate and 50 mL of sample). The samples were filtered in situ and diluted 1:4 prior to autoclaving to reduce potential interferences (samples from the upper estuary were reddish-brown in colour). The method proposed by Hosomi et al. [5], i.e. method 3, was used to compare the efficiency of the proposed method. The pH after autoclaving was noted and found to be 1.9 - 2.0 for the proposed method and 2.0 - 2.1 for the method reported by Hosomi et al. [5]. The results obtained are shown in Table 4.5. At 5% probability, there is no significant difference between the two sets of results, $t_{cal} = 0.61$ and $t_{tab} = 2.45$. The results show that the peroxydisulphate digestion method gives good recoveries when applied to estuarine environments. The proposed method is less complex, low reagent consumption and is therefore suitable for field studies. Only one oxidant (peroxydisulphate) is used.

Table 4.5. Application of the proposed method 2B to water samples from the Tamar Estuary.

<table>
<thead>
<tr>
<th>Location</th>
<th>Method 2B</th>
<th>Method 3</th>
<th>SRP (µg P L$^{-1}$) (±% RSD)</th>
<th>Salinity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>DOP (µg P L$^{-1}$) (±% RSD)</td>
<td>DOP (µg P L$^{-1}$) (±% RSD)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Weir Quay</td>
<td>61 ± 2</td>
<td>60 ± 1</td>
<td>34 ± 3</td>
<td>24</td>
</tr>
<tr>
<td>Halton Quay</td>
<td>8 ± 0</td>
<td>8 ± 0</td>
<td>34 ± 8</td>
<td>4</td>
</tr>
<tr>
<td>Southward Farm</td>
<td>24 ± 0</td>
<td>22 ± 1</td>
<td>37 ± 10</td>
<td>0</td>
</tr>
<tr>
<td>Calstock</td>
<td>32 ± 2</td>
<td>34 ± 1</td>
<td>51 ± 5</td>
<td>0</td>
</tr>
<tr>
<td>Rumleigh</td>
<td>48 ± 4</td>
<td>45 ± 1</td>
<td>51 ± 2</td>
<td>0</td>
</tr>
<tr>
<td>Morwellham</td>
<td>58 ± 2</td>
<td>60 ± 1</td>
<td>54 ± 3</td>
<td>0</td>
</tr>
</tbody>
</table>

* Hosomi et al. [5]
4.2.12. Analytical performance of the ascorbic acid FI method combined with the peroxydisulphate only digestion method (i.e. method 2B).

Table 4.6 shows the data for orthophosphate standards digested with the proposed peroxydisulphate digestion technique i.e. method 2B (see Experimental section). The calculated detection limit, defined as \( 3S_b + Y_B \) [72] was 0.1 \( \mu g \) P L\(^{-1}\) which is suitable for the determination of organic phosphorus species in non-marine natural waters.

4.2.13. DOP in seawater

In seawater, TDP and DOP concentrations in non-marine natural waters can be <0.01 \( \mu g \) P L\(^{-1}\), especially during periods of high biological productivity [71, 75]. Robust analytical techniques capable of detecting these concentrations are therefore necessary for environmental studies. The main challenge in the analysis of seawater is matrix interferences caused by high concentrations of chloride ions and also Ca\(^{2+}\) and Mg\(^{2+}\) [74]. In the determination of DOP by acid or peroxydisulphate autoclave digestion interferences can occur due to competing reactions caused by degradation of compounds present in seawater by autoclave reagents, e.g. equations (17 - 22). Seawater samples therefore often require more vigorous digestion techniques compared with estuarine samples.

Table 4.6. Summary of the analytical figures of merit obtained using the peroxydisulphate only (method B) and the FI manifold shown in Fig. 4.1.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Freshwater</th>
</tr>
</thead>
<tbody>
<tr>
<td>Linear range (( \mu g ) P L(^{-1}))</td>
<td>0.2-50.0</td>
</tr>
<tr>
<td>Calibration graph</td>
<td></td>
</tr>
<tr>
<td>Slope</td>
<td>0.0005</td>
</tr>
<tr>
<td>Intercept</td>
<td>0.00012</td>
</tr>
<tr>
<td>Correlation coefficient (( R^2 ))</td>
<td>0.9978</td>
</tr>
<tr>
<td>Limit of detection (( \mu g ) P L(^{-1}))</td>
<td>0.1</td>
</tr>
<tr>
<td>RSD % (n = 4)</td>
<td>2.5</td>
</tr>
<tr>
<td>Sampling frequency (h(^{-1}))</td>
<td>40</td>
</tr>
<tr>
<td>Detection temperature ((^\circ)C)</td>
<td>30</td>
</tr>
</tbody>
</table>

\begin{align*}
K_2S_2O_8 + H_2O & \rightarrow 2H^+ + \frac{1}{2}O_2 + 2SO_4^{2-} & 17 \\
K_2S_2O_8 + 2Cl^{-} & \rightarrow 2K^+ + Cl_2 (g) + 2SO_4^{2-} & 18
\end{align*}
Competing or side reactions

\[
\begin{align*}
2\text{Cl}^– + 2\text{H}^+ &\rightarrow 2\text{HCl} \quad 19 \\
2\text{SO}_4^{2–} + 4\text{H}^+ &\rightarrow 2\text{H}_2\text{SO}_4 \quad 20 \\
\text{Cl}^– + \text{Cl}^– &\rightarrow \text{Cl}_2 \ (\text{g}) \quad 21 \\
2\text{H}^+ + \text{O}_2 &\rightarrow \text{H}_2\text{O}_2 \quad 22
\end{align*}
\]

The reactions shown in equations 17 - 22 and particularly 18, 19 and 20 will affect the phosphomolybdenum complex by acting as a source of unquantified [H\(^+\)] while the reactions shown in equation 18 and 21 also affect the colour development due to competition for the oxidising agent. Ridal et al. [35] reported the inhibitory action of free chlorine on the oxidizing power of peroxydisulphate suggesting that alkaline rather than acid peroxydisulphate is preferred for the digestion of seawater. The production of [H\(^+\)] as side chain decomposition product will also increase the acidity of the system and cause a Schlieren effect with a suppressing effect on the response. Therefore, it is the generation of acid during peroxydisulphate decomposition and the competing reactions that are responsible for the low recoveries reported by several authors, especially in oligotrophic natural waters [6, 71], because excess acid inhibits phosphomolybdenum blue formation, which is the absorbing chromophore.

4.2.14. The effect of sulphite

Equations 18 - 22 are very significant for the autoclave digestion of seawater, as these are responsible for most reported low recoveries in the analysis of seawater [6]. The effect produced by equation (21) on the digestion of seawater can be eliminated by the addition of sodium sulphite in a batch system after autoclaving [6]. Sodium sulphite can also be added before batch autoclave digestion [75] to remove excess acid (equations 19 and 20) and chlorine (equation 21). In this work, both approaches (addition of sulphite before and after autoclaving) were investigated and the diagnostic LabVIEW display (Fig. 4.4) was used to monitor their effect. Samples to which sulphite was not added, before or after autoclave digestion, produced the highest Schlieren effect. The detector response was low and the blanks were high. The addition of sulphite after batch autoclave digestion improved the peak shape, but the Schleiren effect was still observed. The pH of the digests also increased to 2.5 - 3.0 with the addition of sulphite after or before autoclaving. The best result was obtained with samples diluted 1:4 (10 mL of seawater added to 40 mL of UPW). When 50 mL of sample was autoclaved with
sodium sulphite i.e. method 4, (see section 4.1.2), the peaks obtained were similar to the ones shown in the diagnostic display. Also there was no Schlieren effect and lower blanks were obtained, as shown in Fig. 4.12. Dilution of the samples before digestion is therefore recommended. It improves the sensitivity and reduces potential interferences posed by the seawater matrix such as the generation of free chlorine during digestion as a result of the decomposition of seawater, the inhibitory effect of bromide [12] and the combined effect of other ions such Ca$^{2+}$ and Mg$^{2+}$.

The final pH after autoclaving is significant because the formation of the blue phosphomolybdenum complex is pH dependent and appropriate adjustment of the final [H$^+$] of the molybdate reagent is necessary. Therefore the conditions for the FI manifold, with ascorbic acid reduction, were re-optimised for estuarine samples. The results (Table 4.7) show that a [H$^+$]:[molybdate] ratio of 62 gave the best results (i.e. lowest blank response and highest sensitivity) for autoclaved estuarine samples. They also show that 1:4 dilution of the samples gave the lowest blanks and higher [H$^+$] suppressed the response (slope). This was because the excess acid generated in the system during the peroxydisulphate decomposition of the samples inhibited the formation of the phosphomolybdenum complex [69, 71].

Table 4.7. Effect of [H$^+$]:[molybdate] on the spectrophotometric FI determination of digested seawater samples. Undiluted samples produced high blanks compared to diluted samples obtained with method 4.

<table>
<thead>
<tr>
<th>[molyb] (M)</th>
<th>[H$^+$] (M)</th>
<th>[H$^+$]:[molyb]</th>
<th>Slope</th>
<th>Intercept</th>
<th>R$^2$</th>
<th>Blank (n = 4)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.0159</td>
<td>1.2</td>
<td>75</td>
<td>0.0012</td>
<td>0.0195</td>
<td>0.9902</td>
<td>0.0185**</td>
</tr>
<tr>
<td>0.0129</td>
<td>1.0</td>
<td>78</td>
<td>0.0009</td>
<td>0.0122</td>
<td>0.7936</td>
<td>0.0095*</td>
</tr>
<tr>
<td>0.0129</td>
<td>0.8</td>
<td>62</td>
<td>0.0041</td>
<td>0.0119</td>
<td>0.9808</td>
<td>0.0108*</td>
</tr>
<tr>
<td>0.0129</td>
<td>0.8</td>
<td>62</td>
<td>0.0080</td>
<td>0.0086</td>
<td>0.9947</td>
<td>0.009 ***</td>
</tr>
<tr>
<td>0.0129</td>
<td>0.9</td>
<td>69</td>
<td>0.0013</td>
<td>0.0114</td>
<td>0.8993</td>
<td>0.0125*</td>
</tr>
</tbody>
</table>

* 1:4 dilution i.e. (10 mL low nutrient seawater and 40 mL UPW), ** seawater undiluted and *** nutrient depleted seawater
Fig. 4.12. Calibration of phosphate standards autoclaved in nutrient depleted seawater using method 4. Dilution (1:4) was carried out before autoclaving. Error bars, $\sigma \pm 3s$ (n = 3).

The recoveries for model organic P compounds from 1:4 diluted seawater matrices, using peroxydisulphate/sulphite digestion (method 4) are shown in Fig. 4.13. Good recoveries (92±1.2% - 108±2.3%) were obtained using this method. These results compare well with those reported by Aminot et al. for the determination of organic phosphorus compounds in seawater using an automated photo-oxidation method [12].

Fig. 4.13. Recovery of model compounds in 1:4 nutrient depleted seawater after autoclaving at 121 °C for 45 min. Error bars, $\sigma \pm 3s$ (n = 3).
4.3. CONCLUSIONS

1. pH is very important for the formation of the blue heteropoly phosphomolybdate complex. Therefore pH should be monitored after digestion because it significantly affects the recovery of P from digested samples. The optimum pH after autoclave digestion is in the range 1.5 - 2.0, with the lower end of the range most suited for samples from anthropogenically impacted waters. An orthophosphate standard should be passed through the autoclave digestion for quality control.

2. Dilution before batch autoclave digestion is recommended, particularly for seawater samples and samples with >200 mg L⁻¹ solids. It is also necessary to carry out dilution before batch autoclave for samples from anthropogenically impacted areas to reduce the concentrations of potential interferents, e.g. arsenate and manganese, which interfere in the spectrophotometric determination. 1:4 dilution is preferred and effects posed by humic and fulvic acids (coloured substances) can also be eliminated.

3. Organic P species high in carbon such as phytic acid (C6), MTP (C19) are not favourably recovered with high acid-peroxydisulphate because of carbonization caused by increased acidity. Use of peroxydisulphate yields quantitative recoveries.

4. A \([\text{H}^+]\) to [molybdate], mole ratio of 60 - 62 is recommended for the F₁ determination of autoclaved samples.

5. Quantitative recoveries of all the model compounds tested were obtained (94.0 ± 1.3 - 105.5 ± 2.9%) i.e., 92 - 108% using the peroxydisulphate only autoclave method.

6. The addition of sodium sulphite after sample dilution before autoclaving helped to eliminate the effects posed by the seawater matrix.

7. At this stage the method is best suited for the analysis of fresh and low salinity waters. Further investigation is necessary to make it suitable for seawater because of high concentrations of potential interferents e.g. Mg²⁺ and Cl⁻.
4.4. REFERENCES


[36] D. W. Menzel and N. Corwin, Limnology and Oceanography, 10 (1965) 280


CHAPTER FIVE

FLOW INJECTION SPECTROPHOTOMETRIC METHOD FOR THE DETERMINATION OF PHYTIC ACID USING IMMOBILISED PHYTASE

Main topics in this chapter

Phosphatases and phytases
Phytic acid
Experimental
Phytase immobilization procedure
Optimisation of Fl manifold
Substrate conversion
Application to Tamar Estuary samples
Conclusions
5. INTRODUCTION

5.1. Enzyme catalysis and reactions.

Enzymes are proteins and biological catalysts that can catalyse several stepwise biochemical reactions in which nutrient molecules are degraded [1]. Their catalytic activity depends on the original protein conformation. If an enzyme is denatured or dissociated into sub-units; primary, secondary, tertiary and quaternary structures, catalytic activity is usually lost. The relative molecular mass of enzymes ranges from 12,000 - 1,000,000. Most enzymes require a co-factor to function effectively. This could be a trace metal, e.g. Fe(II) or Fe(III), Mn(II) or Zn(II) or co-enzymes e.g. pyridoxal phosphate, cytochrome oxidase, catalase or peroxidase. Co-enzymes function as transient carriers of specific functional groups [1-4]. Enzymes are classified with respect to the type of reactions they catalyse and according to the EC (Enzyme Commission) nomenclature [3], the system divides enzymes into six major classes, all with sub-classes, based on the type of reaction catalysed. Each enzyme is assigned a four-digit classification number and a systematic name, which identifies the reaction it catalyses (see Table 5.1), e.g. phytase, a hydrolase catalyses the hydrolysis of phytic acid to inositol and phosphate [2 - 4].

Table 5.1. International classification of enzymes. Adapted from [3].

<table>
<thead>
<tr>
<th>No</th>
<th>Class</th>
<th>Type of Reaction Catalysed</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Oxidoreductases</td>
<td>Transfer of electrons (hydride ions or H atoms)</td>
</tr>
<tr>
<td>2</td>
<td>Transferases</td>
<td>Group transfer reactions</td>
</tr>
<tr>
<td>3</td>
<td>Hydrolases</td>
<td>Hydrolyse reactions (transfer of functional groups to water)</td>
</tr>
<tr>
<td>4</td>
<td>Lysases</td>
<td>Addition of groups to double bonds or formation of double bonds by removal of groups</td>
</tr>
<tr>
<td>5</td>
<td>Isomerases</td>
<td>Transfer of groups within molecules to yield isomeric forms.</td>
</tr>
<tr>
<td>6</td>
<td>Ligases</td>
<td>Formation of C-C, C-S, C-O and C-N bonds by condensation reactions coupled to ATP cleavage</td>
</tr>
</tbody>
</table>

Enzymes catalyse specific reactions and each reaction take place at an active site [5, 6]. Enzyme catalysis can be differentiated from inorganic catalysis by the binding energy [7]. The concentration of the substrate affects the rate of enzyme
catalysed reactions. As postulated by Michaelis-Menten, the enzyme first combines with the substrate [S] reversibly to form an enzyme-substrate complex and the second step involves the breakdown of the enzyme-substrate complex to release the product P and enzyme E [7 - 9]. Equations 1 and 2 briefly describe the Michaelis-Menten kinetic equation for an enzyme-substrate reaction, \( k_1 \) and \( k_2 \) are rate constants.

\[
\begin{align*}
\text{First step} & \quad E + S \rightleftharpoons ES \\
\text{Second step} & \quad ES \rightleftharpoons E + P
\end{align*}
\]

\( k_1 \)

\( k_2 \)


5.1.1. Phosphatases and phytases.

Phosphatases belong to the class of enzymes called hydrolases [3] and their subclasses are alkaline phosphatase (EC.3.1.3.1) and acid phosphatase (EC.3.1.3.2). They hydrolyse phosphate monoesters to produce an alcohol and orthophosphate. Phosphatases play a key role in metabolic reactions such as the synthesis of organic phosphate compounds, transphosphorylation and transport across cell membranes [10] and they have been isolated from a variety of sources. Alkaline phosphatase is the most studied phosphomonoesterase and has been isolated from, e.g. *Escherichia coli* [11, 12]. Acid phosphatases show broad selectivity towards phosphomonoesters and have also been isolated from *E. coli* [11].

Strickland and Parsons established a classical method using phosphatase for the determination of phosphate [13] but this method was susceptible to product inhibition by reactive phosphate already present in the sample. McKelvie et al. [11] immobilised *E. coli* onto CNBr-activated sepharose 4B beads in a FI system with an optimum pH of 8. The recovery of alkaline phosphatase hydrolysable phosphorus was low in natural waters but good in sediments. They also applied alkaline phosphatase to soils [14]. Acid and alkaline phosphatase and phytase have been used in combination to investigate organic phosphorus speciation in soils [15].

Phytases (EC.3.1.3.8) are members of the family of histidine acid phosphatases.
[16,17] that are found in plants and micro-organisms, which catalyse the hydrolysis of phytate (myo-inositol hexakis-phosphate 1,2,3,4,5,6) to less phosphorylated myo-inositol phosphates and free orthophosphates. Phytase from plant sources, e.g. wheat, first acts on the C₆ atom while that from microbial sources acts on the C₃ atom [18]. They are naturally occurring and well distributed in both plants and animals. They show broad substrate specificity because of differences arising from the molecular characteristics of phytase enzymes purified from diverse sources [19]. McKelvie et al. [14, 20] used a FI system with immobilised phytase for the determination of phytic acid in soils. Adenosine-5'-triphosphate was also hydrolysed but in low yields compared with phytic acid. Phytase has also been applied to the determination of phytic acid in the marine environment, but with low recoveries [20].

5.1.2. Phytic acid
Inositol phosphates are a family of naturally occurring non-toxic phosphoric esters. They contain six hexane-ring bound phosphate groups and are widely distributed in both aquatic and terrestrial environments with pKa values ranging from 1.9-9.5 [21, 22]. The cogeners exist in several isomeric forms with the myo-isomer (IP₆) being the most predominant [21, 23]. Depending on the type, they are generally present in the form (IPₓ) where x = 1-6. Inositol monophosphate (IP₁), inositol diphosphate (IP₂), inositol triphosphate (IP₃), inositol tetraphosphate (IP₄), inositol pentaphosphate (IP₅) and inositol hexaphosphate (IP₆) or phytic acid, the most common.

Phytic acid is the major form of storage of phosphate in seeds and legumes averaging 90 % of the available P. It is also a very important source of phosphate to the environment, constituting about 60 % of the total extractable soil organic-P where it is strongly adsorbed to iron and aluminium oxyhydroxides [22, 24 - 26]. The six phosphate groups of PTA (myo-isomer) impose high charge and density on the molecule. The high charge exerts electrostatic attraction on the molecule and this facilitates its interaction with the soil and adsorption onto clay particles in soils [27 - 29]. This is also responsible for the precipitation of iron and aluminium by PTA in acid soils. The large number of phosphate groups in the hexagonal ring helps to stabilise phytic acid, making it difficult to degrade by microbial hydrolases [29]. PTA has considerable stability when compared to other organo-phosphates (see Figs. 5. 1 and 5.2).
Fig. 5.1. Structure of phytic acid (PTA), also known as myo-inositol hexakis(dihydrogen phosphate).

The chemistry of PTA is complex. It binds Fe, Mg, Al and Ca [29, 31 - 33]. The pH significantly affects the stability and hydrolysis of phytic acid. The phosphate ether linkages in PTA are stable in basic media. Maximum activity during acid hydrolysis is at pH 4.5 and hydrolysis decreases at pH >1 [31] (see Fig. 5.2). This property of PTA has lead to increased attention by soil scientists who are interested in nutrient bioavailability to plants and potential sources to leaching of phosphorus species to aquatic environments [20, 34, 35], see also Fig. 5.3.

![Fig. 5.1. Structure of phytic acid (PTA), also known as myo-inositol hexakis(dihydrogen phosphate).](image)

Fig. 5.2. (a) Axial form of phytic acid (pH 5 – 12); (b) equatorial form of phytic acid (pH < 5 and pH > 12). $P_i = -\text{OP}_3^{2-}$, $i = 1, 2,...6$.

During periods of algal bloom, PTA has been isolated from toxin-producing cyanobacteria [36, 37]. This poses a potential global problem and a challenge to water quality as the cost of water treatment will increase.

The nutritional significance of PTA has been well studied [32, 38, 39] and it is known to prevent certain types of cancer [32]. In addition the sodium salt of phytic acid (dodecasodium salt) has been found to improve the separation efficiency and resolution of proteins. This is because of its polyanionic character and wide pH
range stability as this makes it possible to bind positively charged amino acid residues such as lysine and arginine on peptides and proteins [22, 40].

Fig. 5.3. Biogeochemical cycle of inositol phosphates in terrestrial systems. *Myo, sycllo, D-chiro, neo* are typical conformations. Adapted from [23].

In soil remediation and reclamation of contaminated soils, PTA has a potential application for sequestering mobile metal ions, e.g. uranyl ion, through ion exchange immobilization [41].

The aims of this chapter were: (1) to immobilize phytase onto controlled porosity glass after functionalization of the glass with amino-functional reagents e.g. aminopropyltriethoxy-silane and cross-linking with glutaraldehyde; and (2) use the immobilized phytase for the specific determination of phytase hydrolysable organic phosphorus species, e.g. phytic acid, in natural waters.

5.2. EXPERIMENTAL

5.2.1. Reagents

All reagents were of analytical grade and were prepared using ultra-pure water (UPW) supplied by Elga with a resistance of >18.2 MΩ cm⁻¹. All glassware and
HDPE (high density polyethylene) bottles used were first washed with Nutracon, a nutrient P free detergent, rinsed at least three times with UPW and soaked in 10% (v/v) HCl overnight and finally rinsed three times with UPW [42, 43]. Immobilisation of the phytase was performed following the recommendations of the working party on immobilized biocatalysts as agreed by the European Federation of Biotechnology and Enzyme Commission [44]. All stock solutions were stored at 4 °C and brought to room temperature before use unless otherwise stated.

1. Phytase (EC.3.1.3.8.) crude form *from Aspergillus ficuum* 2 - 5 units per mg solid. This was obtained as a suspension (200 units of enzyme) in (NH₄)₂SO₄ solution.

2. Glutaraldehyde 50% aqueous solution photographic grade, CPG (controlled porosity glass) 120 - 200 mesh, mean pore diameter 74 Å and pore volume 0.47 cm³ g⁻¹ with a surface area of 152.7 m² g⁻¹ and 3-aminopropyltriethoxy-silane (98%) were all products of Sigma St Louis, MO, USA. Phytase was stored desiccated at 0 °C, aminopropyltriethoxy-silane at 4 °C and the CPG and glutaraldehyde was stored at room temperature in the dark.

3. Phytic acid magnesium potassium salt, PTA-MgK, C₆H₁₅O₂₄P₅MgK, FW 720.4 g (Sigma 95 %). 1.0 mM stock was prepared by dissolving 0.036 g in 50 mL of UPW. Concentrations in the range of 10⁻⁷-10⁻⁵ M were prepared by serial dilutions of the stock with UPW. The stock was stable at 4 °C for several months.

4. Phytic acid calcium salt, PTA-Ca, C₆H₁₆O₂₄P₅Ca, FW 698.1 g (Sigma 90%). 1.0 mM stock solution was prepared by dissolving 0.035 g in 50 mL of UPW. Working standards were prepared by dilution of the stock solution with UPW. The stock was stable at 4 °C for several months.

5. Phytic acid dodecasodium salt, PTA-Na, C₆H₆O₂₄P₅.12Na, FW 923.83 g (Sigma 95%). 1.0 mM stock solution was prepared by dissolving 0.046 g in 50 mL of UPW. Working standards in the range 10⁻⁵ - 10⁻⁴ M were prepared by serial dilution of the stock solution with UPW. The stock was stable at 4 °C for several months.

6. Methyltriphenylphosphonium bromide, MTP, C₁₉H₁₉BrP, FW 357.25 g (Fluka, 98%). 1.0 mM stock solution was prepared by dissolving 0.018 g in 50 mL of UPW. Working standards in the range 10⁻⁶-10⁻⁴ M were prepared by dilution of the stock solution with UPW. The stock solution was stored at 4 °C and was stable for several months.
7. Adenosine-5'-triphosphate disodium salt, ATP, C₁₀H₁₄N₅O₁₃P₃Na₂, FW 551.15 g (BDH 98%). 1.0 mM stock solution was prepared by dissolving 0.276 g in 50 mL of UPW. Working standard of 10⁻⁵ M was prepared by dilution of the stock with UPW. The stock was stored at 4 °C and was stable for several months.

8. Cocarboxylase, COC, C₁₀H₁₀ClO₇P₂S, FW 460.05 g (Sigma 98 %). 1.0 mM stock solution was prepared by dissolving 0.023 g in 50 mL of UPW. Working standard of 10⁻⁵ or 10⁻⁴ M was prepared by dilution of the stock with UPW. The stock solution was stored at 4 °C and was stable for several months.

9. Penta-sodium triphosphate, STP, Na₅O₁₀P₃, FW 367.86 g (Fluka 98 %). 1.0 mM stock solution was prepared by dissolving 0.018 g in 50 mL of UPW. Working standard of 10⁻⁵ or 10⁻⁴ M was prepared by dilution of the stock with UPW. The stock solution was stable for several months when stored at 4 °C.

10. Glycine, C₂H₃NO₂, FW 75.07 g (Sigma 99 %), electrophoresis reagent. 0.2 M stock was prepared by dissolving 1.5 g in 100 mL of UPW. A 0.1 M buffer solution pH 2.5 or 5.5 was prepared using 0.2 M HCl and appropriate dilutions made. The buffer solution was stored at room temperature in the dark and was stable for several days.

11. Sodium acetate (anhydrous), C₂H₅O₂Na, FW 82.03 g (AnalaR BDH). A 0.2 M solution was prepared by dissolving 4.10 g in 250 mL of UPW. A 0.1 M buffer solution pH 5.5 was prepared using 0.2 M acetic acid and appropriate dilution made. The buffer solution was stable at room temperature for several days in the dark.

12. Acetic acid, CH₃COOH, FW 60.05 and specific gravity of 1.049 g L⁻¹ AnalaR (BDH 100 %). A 0.2M stock solution was prepared diluting 3 mL of the AnalaR stock in 250 mL UPW. The 0.2 M stock was stored in the dark at room temperature and was stable for several days.

13. Phosphate (K₂HPO₄). A 10 mg P L⁻¹ stock solution was prepared freshly from 1000 mg L⁻¹ stock solution. PO₄-P calibration standards in the range of 0.1 - 0.5 mg P L⁻¹ were prepared by serial dilution of the stock solution with UPW unless otherwise stated.

14. Ammonium molybdate ((NH₄)₆Mo₁₂O₄₀·4H₂O). A 0.1M stock solution was prepared by dissolving 12.358 g of ammonium heptamolybdate in a 100 mL container with UPW. A 0.0129 M working standard was prepared freshly.
from this stock for use. Both solutions were stable for several weeks. The 0.1 M stock was stored at room temperature in the dark in an HDPE container.

15. Sulphuric acid (H$_2$SO$_4$). The AnalaR stock has a specific gravity of 18.4 g dm$^{-3}$. A 2 M stock was prepared by dissolving 27.17 mL from this stock in a flask containing 200 mL UPW. This was made up to the 250 mL mark and stored at room temperature. A 0.9 M solution was made from this stock by dilution unless otherwise stated.

16. L-ascorbic acid (C$_6$H$_8$O$_6$). A 4.6 g of ascorbic acid was dissolved in a 100 mL volumetric flask with UPW and made up to the 100 mL mark. The solution was prepared daily.

19. Antimonyl potassium(+) -tartrate (K$_3$SbO$_3$.C$_4$H$_4$O$_6$). A 0.1 M stock was prepared by dissolving 3.249 g of antimony potassium tartrate in 100 mL of UPW. 0.0015 M was prepared from this stock and used as required. The stock solution was stable for several weeks.

5.2.2. Instrumentation and procedures

The FI manifold incorporating an immobilized enzyme reactor for the determination of phytic acid is shown in Fig. 5.4. A peristaltic pump (Gilson Minipuls III) was used to propel the UPW, and 0.1 M buffer glycine at pH 2.5 or 5.5 in 0.1 or 0.5 M NaCl solution at a flow rate of 0.79 mL min$^{-1}$ and ammonium molybdate and ascorbic acid at a flow rate of 0.17 mL min$^{-1}$. A rotary injection valve (Rheodyne 5020) was used to inject 250 µL of PTA sample into the UPW which was merged with the buffer stream before passing through the immobilized phytase reactor to hydrolyse the phytic acid and liberate PO$_4^{3-}$ (see Fig. 5.5) and merged at a T-piece with the molybdate stream. This merged at another T-piece with the ascorbic acid reductant stream. The sample then passed through a reactor coil in a water bath maintained at 40 °C and into a detector (Philips 5680 UV/VIS spectrophotometer) with a flow through cell of 18 µL volume.

The detector output was automatically recorded with a computer incorporating a LabVIEW® program. The immobilized enzyme packed in a glass column (see Fig. 5.5) was thermostated at 40 °C by flowing water through a water jacket around the enzyme column.
5.2.3. Enzyme immobilization procedure

A. 1.0 g of dry CPG was weighed and washed (boiled) with 5 % HNO₃ at 90 °C for 60 min followed by rinsing with UPW several times. This step was carried out to hydrate and clean the CPG or glass surface.

B. Activation of the dry CPG. Activation of the dry CPG was carried out by treatment of the hydrated CPG in step A with 20 mL of a 10 % aqueous solution of...
3-aminopropyltriethoxysilane, (i.e. 2 mL of aminopropyltriethoxy-silane stock was added to 18 mL of UPW). The pH change was highly basic (in the range 11.40-13.10). The pH was adjusted to 3.45 with 5 M and 0.1 M HCl and followed by incubation in a water bath at 80 °C for 2.5 h. The CPG was later filtered and washed several times with UPW.

**C. Derivatization of the CPG.** 0.5 g of the dry CPG was weighed and to this was added 30 mL 2.5 % glutaraldehyde i.e. 1.5 mL of the glutaraldehyde stock in 28.5 mL of 0.1 M phosphate buffer at pH 7 and incubated for 2 h at room temperature. The CPG was washed thoroughly with UPW and kept for use.

**D. Phytase immobilisation.** 100 mg (solid) was weighed and to this was added 10 mL of 0.1 M phosphate buffer at pH 6 to obtain a suspension (see page 128). This suspension was stirred continuously with a Techna magnetic stirrer at room temperature in a water bath maintained at 4 °C and later centrifuged at 4 °C for 20 min at 4.5 X 1000 rpm with a Denley BR401 refrigerated centrifuge machine.

Extraction of the enzyme was by precipitation with ammonium sulphate following the procedures reported by Yaqoob et al. [45] using 60 % (NH₄)₂SO₄ i.e. 6 g in 10 mL phosphate buffer at pH 6. This was added slowly and with continuous stirring because ammonium sulphate precipitation is favoured by slow addition and stirring. Precipitation was carried out for at least 30 min and stirring was done continuously with a magnetic stirrer at 4 °C, followed by centrifugation at 4 °C for 20 min at 4.5 X 1000 rpm. The precipitate obtained was purged with N₂ gas for 30 min to provide an inert surface, eliminating oxygen from the surface and then dissolved in 0.5 mL 0.1 M phosphate buffer pH 6.

The dissolved precipitate was added to the 0.5 g CPG and kept covered overnight at 4 °C. The activity of the enzyme before and after immobilization was measured according to Engelen et al. [46, 47]. The immobilized phytase on CPG was washed several times with 0.1 M glycine buffer at pH 2.5 or 5.5 to remove unbound enzyme and later with 0.1 M acetate buffer at pH 5.5 in 0.5 M or 0.1 M NaCl before use. The immobilization steps were as reported by [45, 48] and the protein content before and after immobilization was determined according to Lowry et al. [49] and the yields were 92% and 7.3% respectively . The immobilized
enzyme was then packed in a glass column and stored at 4 °C and was stable for at least six months without significant loss in activity.

5.3. RESULTS AND DISCUSSION

5.3.1. Enzyme immobilisation.
The following conditions should be considered before immobilisation.
(a) The stability of the enzyme under the conditions needed for reaction.
(b) Cross-linking reagents should react preferentially with chemical groups other than those at the active site.
(c) The cross-linking reagent should be as large as possible to prevent it from penetrating the active site.
(d) A suitable washing procedure to remove the unreacted or uncross-linked enzyme from the preparation which must not adversely affect the enzyme.
(e) The mechanical property (stability) of the support material.

Immobilisation can be achieved by covalent bonding, adsorption or physical entrapment within the enzyme phase [50, 51] (see Fig. 5.6 (i-iv)). Other possible modes of immobilisation are electrostatic bonding, copolymerisation and polymer entrapment encapsulation. Immobilisation can cause a measurable change in the parameters of an enzyme-catalysed reaction e.g., the maximum reaction velocity, Michealis-Menten constant, optimum temperature, optimum pH and inhibitory effects. The overall effect of these changes will depend not only on the immobilisation method, but also on the enzyme reaction [50, 51]. Choice of support for immobilization depends on the type or nature of the enzyme and the desired application. Typical support materials for immobilization are controlled porosity glass (CPG) and cellulose, e.g. sepharose, a polymer to which the enzyme can bond covalently. Cyanogen bromide (CNBr) is a common enzyme activator and a cross-linking reagent [52, 53]. The way in which the molecule reacts with e.g. cellulose depends on the environment. At high pH values, it reacts with the OH⁻ groups of polysaccharides and the derivative then reacts with free amino groups on the enzyme in mildly alkaline solution.

Cyanuric chloride (trichloro-triazine), which has three reactive C-Cl bonds, can also be used as a cross-linking reagent [54]. It has the advantage of reacting with the ionic charges of the enzyme-substrate complex, which is dependent upon the bridging molecule. Another useful bridging molecule and multifunctional reagent is
glutaraldehyde which has two aldehyde groups at either end of a \((\text{CH}_2)_3\) unit i.e. \(\text{CHO(CH}_2)_3\text{CHO}\). The aldehyde groups react at neutral pH values with free amino groups. One end of the glutaraldehyde molecule is attached to the support, the other to the enzyme. This serves as a bridging molecule and thus reduces the intermolecular distance of the enzyme molecules [55, 56].

![Diagram](image)

(i) Adsorption  
(ii) Covalent binding  
(iii) Physical entrapment  
(iv) Membrane confinement

Figs. 5.6 (i-iv) Different types of immobilized enzyme systems. Adapted from reference [50, 51].

Cellulose has some disadvantages when used in immobilisation. It is susceptible to microbial attack, it shows a high degree of non-specific adsorption of protein, the glycosidic bonds are hydrolysable in high concentration of \(\text{H}^+\), and it swells in strongly alkaline solution (30 - 50%), pyridine, and glacial acetic acid, which can lead to an increase in the amount held as amorphous moieties [57].

Functionalisation of porous silica glass (silanization) [58] was employed in this work (see Fig. 5.7). This is a chemical reaction between -OH groups on the glass surface and amino-functional trialkoxysilane, such as 3-aminopropyltriethoxy-silane. The amino group was activated for immobilisation of phytase with glutaraldehyde, a cross-linking reagent [58, 59]. Carbodiimide, thiophosgene or p-nitrobenzylchloride can also be used as activators or cross-linking agents.
After immobilisation or covalent attachment, it is necessary to block the unreacted active groups, which are used to deactivate the matrix. Glycine, a low molecular weight primary amine was used. Ethylamine, glucosamine or 2-amino-2-hydroxy methylpropane-1,3-ol [60] can also be used. Covalent binding offers several advantages e.g. strong binding force, no enzyme leakage, and low running problems, no bacterial and diffusional effects. The major disadvantages however includes high cost of immobilisation materials and time to prepare the materials for immobilisation and also the likelihood of matrix effects.

![Diagram of covalent bonding of CPG (controlled porosity glass) functionalization with 3-aminopropyltriethoxysilane and cross linking with glutaraldehyde.](image)

**Fig. 5.7.** Covalent bonding of CPG (controlled porosity glass) functionalization with 3-aminopropyltriethoxysilane and cross linking with glutaraldehyde.

The purpose of the immobilized enzyme column or reactor was to hydrolyse phytic acid and other phosphomonoesters thereby liberating orthophosphate as shown in Fig. 5.8. A previously used FI manifold (Chapter 2, Fig. 2.1) was used for this work.
by incorporating an immobilized enzyme reactor column after the merged UPW
and buffer carrier streams. In this work, the carrier buffer replaced the thiosulphate
stream. The following parameters were optimised to improve the sensitivity and
hence reduce analysis time; sample volume, enzyme reaction temperature, pH
and types of buffer (glycine, acetate, phthalate, and succinate buffers).

![Phytase Reaction Diagram](image)

**Fig. 5.8.** Hydrolysis of phytic acid (myo-inositol 1,2,3,4,5,6 hexakis dihydrogen phosphate) by microbial phytase (E.C.3.1.3.8) generating D-myoinositol 1,2,4,5,6 pentakis dihydrogen phosphate and inorganic phosphate (Pi).

### 5.3.2. Sample volume

It is important to optimise the volume of sample injected during analysis for
maximum sensitivity and hence reliability. The volumes chosen varied from 50 -
300 µL and as can be seen in Fig. 5.9, as the sample volume was increased,
response also increased [61]. However, at 300 µL, broad peaks were observed.
Therefore 250 µL, which gave the optimum response, was chosen for this work.

![Sample Volume Graph](image)

**Fig. 5.9.** Effect of sample volume on the phytase hydrolysis of phytic acid to
orthophosphate and subsequent determination spectrophotometrically at
790 nm. Error bars, σ ± 3s (n = 4).
5.3.3. Effect of temperature
Most enzymatic reactions are temperature dependent [62, 63]. It is therefore important to find the optimum temperature after immobilisation for effective activity which was at 40 °C as can be seen in Fig. 5.10. There was a gradual rise in response to the optimum at 40 °C and above this temperature, the activity declined. Therefore, the optimum temperature of 40 °C was used in all subsequent work.

![Graph showing the effect of temperature on absorbance.](image)

**Fig. 5.10.** Effect of temperature on the immobilized phytase packed in the column. Error bars, $\sigma \pm 3s (n = 4)$.

5.3.4. Effect of pH.
Every enzyme has a pH at which it is most active and a certain range of pH in which it demonstrates any reactivity. This is because the characteristics of ionizable side chains of amino acids depend on pH [20, 64]. At extreme pH, the tertiary structure of the protein may be disrupted and the protein denatured; at moderate pH the degree of ionisation of certain amino acid side chains and the pH profile of an enzyme may suggest the identity of those residues. The immobilised enzyme often has a different pH range from the range of the soluble enzyme because of its environment. For the fastest kinetics, work should be at the optimum pH [65]. The maximum rate may also depend on the ionic strength and the type of buffer used. e.g. the aerobic oxidation rate of glucose in the presence of the enzyme glucose oxidase is high in acetate buffer at pH 5.1, but in a phosphate buffer of the same pH, it is low. The carrier buffer must maintain the specific pH at which the enzyme exhibits maximum activity. The pH and different carrier buffers were investigated as shown in Fig. 5.11. The maximum activity for
phytase was at 5.5 [63, 66, 67] at 0.1 M concentrations of each carrier buffer except for phthalate which showed a maximum response at pH 6.

![Graph showing absorbance vs pH for different buffers](image)

**Fig. 5.11.** Effect of pH and carrier buffer (0.1 M) on the determination of phytic acid with phytase enzyme immobilized on CPG.

### 5.3.5. Choice of buffers

From the data shown in Fig. 5.11, all of the buffers, i.e. phthalate, succinate, acetate and glycine exhibited maximum activity at pH 5.5 or pH 6.0. Further effects in terms of buffer concentration were investigated for acetate and glycine, which are the most commonly, used buffers.

![Graph showing absorbance vs concentration of buffers](image)

**Fig. 5.12.** Choice of buffers in the enzymatic hydrolysis of phytic acid by phytase. Error bars, $\sigma \pm 3s$ ($n = 4$).
Increasing the concentration of both buffers resulted in increased sensitivity as seen in Fig. 5.12. The difference in response between the two buffers was not significant, implying that either of them could be used. In order to test the reliability of the proposed method, a test calibration was performed with the immobilized phytase in the column which was used for the optimisation procedures and orthophosphate standards were injected. The immobilized phytase did not inhibit the determination of orthophosphate as can be seen in Fig. 5.13.

![Graph showing calibration for phosphate using the FI manifold for the enzymatic determination of phytic acid (see Fig. 5.4). Orthophosphate was used for the calibration and 0.1 M glycine buffer pH 2.5 in 0.1 M NaCl was used. Error bars, σ ± 3s (n = 4).]

**Fig. 5.13.** Calibration for phosphate using the FI manifold for the enzymatic determination of phytic acid (see Fig. 5.4). Orthophosphate was used for the calibration and 0.1 M glycine buffer pH 2.5 in 0.1 M NaCl was used. Error bars, σ ± 3s (n = 4).

### 5.3.6. Activation of phytase enzyme by metals

Many enzymes require certain metals (cations) for activation in order to be catalytically active [20]. A metal ion can bind particular groups (ligands) by accepting free electron pairs forming co-ordinate bonds in specific orientations. Potassium, the most abundant intracellular cation, activates many enzymes, particularly those which catalyse phosphoryl transfer elimination reactions. Activation by alkaline earth metal cations (Ca$^{2+}$ and Mg$^{2+}$) involves oxygen in the carbonyl double bonds of the enzyme molecule [4].

Magnesium and calcium ions are known to activate phytases [20, 67]. Their effect on the immobilized system was therefore investigated over several concentrations. In Fig. 5.14, using the magnesium salt of phytic acid (PTA MgK), the difference in response between the activated and unactivated system is significant. However, as the concentration of activator was increased to 0.005 M, decrease in response
(sensitivity) was observed. The best concentrations were between 0.002 and 0.004 M MgCl₂ to obtain optimum response without affecting the sensitivity of the enzyme. Magnesium chloride was used as the source of Mg²⁺ and 10⁻⁶ M PTA MgK was used.

![Absorbance graph](image)

**Fig. 5.14. Activation of phytase with magnesium chloride (Mg²⁺) during phytase hydrolysis of phytic acid. Error bars, σ ± 3s (n = 4).**

The activating effect of calcium (Ca²⁺) was compared with Mg²⁺ and CaCl₂ and MgCl₂ were used as the source of the Ca²⁺ and Mg²⁺ respectively. Their activating effect on the system varied depending on the concentration of the activator and the type of phytate used (see Figs. 5.15 and 5.16).

![Absorbance graph](image)

**Fig. 5.15 Activating action of Mg²⁺ on phytase using two different types of phytates (salts of phytic acid), i.e. phytic acid calcium salt (PTA Ca) and magnesium salt (PTA MgK). Error bars, σ ± 3s (n = 4).**

As can be seen in Figs. 5.15 and 5.16, the hydrolysis of PTA by phytases is dependent on the concentration and type of activator and the type of salt used.
Thus the PTA-MgK is more extensively hydrolysed than PTA-Ca. The most common phytates occurring naturally are based on PTA-MgK and these are easily hydrolysed in natural waters in the presence of Mg$^{2+}$. Some calcium dependent phytases have also been identified in natural waters [68]. The data in Fig. 5.15 and 5.16 shows that it is necessary to introduce activators into the system to prolong the life of the enzyme in the column and to increase the rate of enzyme catalysed hydrolysis, e.g. Mg$^{2+}$ was used in the method discussed here to improve both enzyme catalysed hydrolysis of phytic acid by phytases and the lifetime of the enzyme.

![Graph showing activating action of Ca$^{2+}$ on phytase using two different types of phytates (salts of phytic acid), i.e. phytic acid calcium salt (PTA Ca) and magnesium salt (PTA MgK). Error bars, $\sigma \pm 3\sigma$ (n = 4).](image)

**Fig. 5.16** Activating action of Ca$^{2+}$ on phytase using two different types of phytates (salts of phytic acid), i.e. phytic acid calcium salt (PTA Ca) and magnesium salt (PTA MgK). Error bars, $\sigma \pm 3\sigma$ (n = 4).

### 5.3.7. Substrate conversion

Fig. 5.17 shows that phytases have broad selectivity towards other organic phosphorus compounds [36, 68]. Many organic phosphorus compounds are easily hydrolysed to orthophosphate during the determination of phytic acid [69] especially in methods employing high acid concentrations. Using the FI manifold in Fig. 5.4, only the phytase active fraction is measured. This is the difference between the response of the sample with the enzyme column (phytase active fraction plus inorganic phosphate) and that without the column (inorganic phosphate only).
Fig. 5.17 Conversion of substrates by phytase after immobilisation. Samples were in same medium as the carrier buffer, pH 2.5. Error bars, $\sigma \pm 3s$ ($n = 4$).

5.3.8. Acid hydrolysis of model organic compounds.
Some model organic compounds can be hydrolysed in acid media and this was investigated using the same manifold but without immobilised phytase to investigate their likely effects on the proposed method, table 5.2.

Table 5.2 Acid hydrolysis of model organic phosphorus compounds.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Model compound</th>
<th>Concentration (µg P L$^{-1}$)</th>
<th>Recovery after hydrolysis (µg P L$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>P</td>
<td>20</td>
<td>19.8</td>
</tr>
<tr>
<td>2</td>
<td>STP</td>
<td>20</td>
<td>3</td>
</tr>
<tr>
<td>3</td>
<td>ATP</td>
<td>20</td>
<td>3</td>
</tr>
<tr>
<td>4</td>
<td>PTA</td>
<td>20</td>
<td>3</td>
</tr>
<tr>
<td>5</td>
<td>COC</td>
<td>20</td>
<td>3.5</td>
</tr>
<tr>
<td>6</td>
<td>AMP</td>
<td>20</td>
<td>2.5</td>
</tr>
<tr>
<td>7</td>
<td>P-NPP</td>
<td>20</td>
<td>3</td>
</tr>
<tr>
<td>8</td>
<td>RFP</td>
<td>20</td>
<td>3</td>
</tr>
<tr>
<td>9</td>
<td>1+2</td>
<td>20+20</td>
<td>22</td>
</tr>
<tr>
<td>10</td>
<td>1+3</td>
<td>20+20</td>
<td>22</td>
</tr>
<tr>
<td>11</td>
<td>1+4</td>
<td>20+20</td>
<td>22</td>
</tr>
<tr>
<td>12</td>
<td>1+5</td>
<td>20+20</td>
<td>23</td>
</tr>
<tr>
<td>13</td>
<td>1+6</td>
<td>20+20</td>
<td>21.5</td>
</tr>
<tr>
<td>14</td>
<td>1+7</td>
<td>20+20</td>
<td>22</td>
</tr>
<tr>
<td>15</td>
<td>1+8</td>
<td>20+20</td>
<td>22</td>
</tr>
</tbody>
</table>
In Table 5.2, the following abbreviations are used, P-inorganic phosphate, STP-penta-sodium phosphate, ATP-adenosine triphosphate, PTA-phytic acid, COC-cocarboxylase, AMP-amino-ethyl phosphoric acid, RFP-ribo-flavine-5-phosphate, P-NPP-p-nitrophenyl phosphate.

In Table 5.2, various concentrations of model organic compounds were investigated, both separately and in the presence of 20 \( \mu g \) L\(^{-1} \) of orthophosphate as P and their responses noted. The acid hydrolysis of organo-P compounds was about 10%. This is the increase in response of the model compound in standard orthophosphate (9 - 15 in Table 5.2) and therefore this does not pose a serious problem in the proposed enzymatic method for the determination of phytic acid.

5.3.9. Enzyme inhibition

Inhibitors are substances that tend to decrease the rate of an enzyme-catalysed reaction. Inhibitors act on the active site of the enzyme, the substrate, and the cofactor or the binding site. Some bind in a reversible reaction. Thus dilution or dialysis can be carried out to restore the enzyme to full activity. Irreversible inhibitors may be very difficult to restore back to full enzymatic activity by dialysis or dilution. Heavy metal ions and some organic molecules exhibit an inhibitory effect; they bind to the -SH group of cysteine showing various forms of inhibition [70]. Mercury, lead and other toxic heavy metals that have affinity for –SH all exert inhibitory effects and consequently are toxic.

5.3.10. Effects of cations and anions on the immobilized phytase.

In Figs. 5.15 and 5.16, Mg\( ^{2+} \) and Ca\( ^{2+} \) were shown to have an activating effect on the immobilized phytase. A range of other ions were also investigated (at lower concentrations) to determine their effect on the system because certain metal ions, e.g. Hg(II) and Pb(II), have inhibitory effects on the enzyme [27]. The cations chosen and their concentrations were; Pb\( ^{2+} \) (10 mg L\(^{-1} \)), Fe\( ^{2+} \) and Fe\( ^{3+} \) (1.0 mg L\(^{-1} \)), Cu\( ^{2+} \) (1.0 mg L\(^{-1} \)), Al\( ^{3+} \) (10 mg L\(^{-1} \)), Zn\( ^{2+} \) (10 mg L\(^{-1} \)) and Mn\( ^{2+} \) (10 mg L\(^{-1} \)) while the anions were; NO\(_2^-\) (1.0 mg L\(^{-1} \)), NO\(_3^-\) (30 mg L\(^{-1} \)), I\(^-\) (1.0 mg L\(^{-1} \)), SO\(_4^{2-}\) (250 mg L\(^{-1} \)), AsO\(_4^{3-}\) (0.1 mg L\(^{-1} \)), PO\(_4^{3-}\) (2.0 mg L\(^{-1} \)), SiO\(_4^{3-}\) (5.0 mg L\(^{-1} \)) respectively. EDTA (1.0 mg L\(^{-1} \)) and urea (1.0 mg L\(^{-1} \)) were also investigated. These concentrations were above those likely to be found in unpolluted aquatic environments. Fig. 5.18 shows the effect of various cations on the immobilized phytase. As can be seen, the concentrations of some of the cations used were
much higher those found in unpolluted natural waters. This also confirmed the effect shown in Figs. 5.15 and 16, that increasing concentrations of cations (activators) have an inhibitory effect on the response. A 0.1 M glycine buffer at pH 2.5 in 0.1 M NaCl was used in the study. Cu(II) and Fe(III) enhanced the response. Cu(II) could complex with either the amine group in glycine or the functionalized support. The complex formed can cause an increase in absorption as can be seen in the response of Cu(II) in Fig. 5.18. Fe(III) can also complex with the amine group in acid media and form oxime-like complexes. It is likely that this is the complex responsible for the increase in absorption seen in Fig. 5.18.

![Graph showing the effect of cations on the immobilized phytase.](image1)

**Fig. 5.18** Effect of cations on the immobilized phytase. Error bars, $\sigma \pm 3s$ ($n = 4$). The dotted line shows the response for PTA in the absence of other cations.

![Graph showing the effect of anions on the immobilized phytase.](image2)

**Fig. 5.19** Effect of anions on the immobilized phytase. Error bars, $\sigma \pm 3s$ ($n = 4$).
Fig. 5.19 shows that the anions studied did not inhibit the response. Arsenate and silicate are major interferents at high concentrations due to their direct effect on the phosphate detection chemistry. However, the concentrations of both ions used in this study were significantly higher than those found in most natural waters. Also, since a differential method is used in the analysis of real samples their effect will normally be eliminated. Therefore, the proposed method can be applied for the determination of phytic acid as P in natural waters.

5.3.11. Recovery studies (standard addition method).
Samples containing orthophosphate (10 and 20 µg P L⁻¹) in UPW were spiked with 0.1 M HCl to bring the pH to 5.5 and these samples were also spiked with equal concentrations of phytic acid as P. Glycine buffer, 0.1 M pH 2.5 in 0.5 M NaCl was used as the carrier buffer and the results obtained using the manifold in Fig. 5.4 are presented in Table 5.3. Recovery of phytase hydrolysable P was 95 - 96% which suggests that the manifold can be used for the determination of phytase hydrolysable P in natural waters.

<table>
<thead>
<tr>
<th>µg P L⁻¹</th>
<th>Mean response</th>
<th>% RSD (n = 4)</th>
<th>Recovery%</th>
</tr>
</thead>
<tbody>
<tr>
<td>10 P</td>
<td>0.0055</td>
<td>8.2</td>
<td>100</td>
</tr>
<tr>
<td>20 P</td>
<td>0.010</td>
<td>7.1</td>
<td>100</td>
</tr>
<tr>
<td>10 PTA as P</td>
<td>0.00525</td>
<td>9.1</td>
<td>95</td>
</tr>
<tr>
<td>20 PTA as P</td>
<td>0.00975</td>
<td>11.0</td>
<td>96</td>
</tr>
</tbody>
</table>

5.3.12. Analytical figures of merit
Calibrations were performed with the analytes of interest, phosphate as inorganic P and phytic acid as organic P. Orthophosphate standards in UPW spiked with 0.1 M HCl (to bring the pH to 5.5) were first injected using the manifold described in Fig. 5.4. Orthophosphate standards containing the equivalent concentration of phytic acid as P were also injected. The differences in responses between the first (without phytic acid) and the second (with phytic acid) injections were noted and blanks subtracted before calibrations were made to determine the linear range and limit of detection (LOD). A typical calibration obtained for the determination of
phytase hydrolysable phosphorus using the method is shown in Fig. 5.20 and the analytical figures of merit are shown in Table 5.4.

![Graph showing calibration curves](image)

Fig. 5.20 A calibration for the determination of phytic acid as P. Acetate buffer (0.1 M) pH 5.5 in 0.5 M NaCl was used as the carrier buffer. Samples were brought to pH 5.5 with 0.1 M HCl. Error bars, $\sigma \pm 3s (n = 4)$.

Table 5.4 Analytical figures of merit.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>(µg P L$^{-1}$)</th>
<th>P + PTA-MgK (µg P L$^{-1}$)</th>
<th>PTA-MgK (µg P L$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Linear range (µg P L$^{-1}$)</td>
<td>2-50</td>
<td>2-50</td>
<td>4-50</td>
</tr>
<tr>
<td>Calibration graph</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean blank signal (n=4)</td>
<td>0.0005</td>
<td>0.0005</td>
<td>0.0005</td>
</tr>
<tr>
<td>Slope</td>
<td>0.0001</td>
<td>0.0003</td>
<td>0.0001</td>
</tr>
<tr>
<td>Intercept</td>
<td>0.0006</td>
<td>0.0007</td>
<td>0.0008</td>
</tr>
<tr>
<td>Correlation coefficient ($R^2$)</td>
<td>0.9815</td>
<td>0.993</td>
<td>0.9934</td>
</tr>
<tr>
<td>Limit of detection (µg P L$^{-1}$)</td>
<td>2</td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td>R.S.D. % (n = 4)</td>
<td>2.5</td>
<td>5</td>
<td>4</td>
</tr>
<tr>
<td>Sampling frequency (h$^{-1}$)</td>
<td>40</td>
<td>30</td>
<td>30</td>
</tr>
<tr>
<td>Detection temperature (°C)</td>
<td>40</td>
<td>40</td>
<td>40</td>
</tr>
</tbody>
</table>

5.3.13. Application to Tamar Estuary samples

Natural water samples were collected from different locations in the Tamar Estuary as grab samples and analysed for phytase hydrolysable phosphorus using the manifold shown in Fig. 5.4 and the results obtained are shown in Table 5.6. Samples were filtered in the field with 0.45 µm filter paper (Whatman cellulose...
acetate). Before injection, samples were spiked with 0.1 M HCl to bring the pH to 5.5. Samples were injected first without the column for the determination of phosphate (as P) and then with the column for the determination of phytase hydrolysable phosphorus (PHP). The results (after subtraction of phosphate to determine PHP) are shown in Table 5.6.

Table 5.5 Physico-chemical parameters obtained at the sampling points for the field work of 12th August, 2005. High tide was at 10.10 am and sampling was carried out on an incoming tide.

<table>
<thead>
<tr>
<th>Location</th>
<th>Grid reference</th>
<th>Water depth (m)</th>
<th>Temp oC</th>
<th>pH</th>
<th>DO mg/L</th>
<th>Salinity</th>
<th>Solids mg/L</th>
<th>Time</th>
<th>Weather</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>50°27'.35 N 04°2.39 W</td>
<td>3.1</td>
<td>19.4</td>
<td>8.1</td>
<td>10.7</td>
<td>25</td>
<td>8</td>
<td>8.23</td>
<td>Sunny bright</td>
</tr>
<tr>
<td>B</td>
<td>50°21'.13 N 04°13'.87 W</td>
<td>3.7</td>
<td>19.6</td>
<td>7.8</td>
<td>10.6</td>
<td>17.8</td>
<td>18</td>
<td>9.05</td>
<td>&quot;</td>
</tr>
<tr>
<td>C</td>
<td>50°29'.59 N 04°12'.39 W</td>
<td>3.2</td>
<td>19.7</td>
<td>8.1</td>
<td>8.4</td>
<td>5</td>
<td>29</td>
<td>9.20</td>
<td>&quot;</td>
</tr>
<tr>
<td>D</td>
<td>50°27'.35 N 04°12'.39 W</td>
<td>3.5</td>
<td>19.8</td>
<td>8.0</td>
<td>7.7</td>
<td>0</td>
<td>48</td>
<td>9.30</td>
<td>&quot;</td>
</tr>
<tr>
<td>E</td>
<td>50°30'.31 N 04°11'.47 W</td>
<td>2.5</td>
<td>19.1</td>
<td>8.1</td>
<td>6.8</td>
<td>0</td>
<td>162</td>
<td>9.50</td>
<td>&quot;</td>
</tr>
</tbody>
</table>

Note: A = Weir Quay; B = Southward Farm; C = Calstock; D = Rumleigh Farm; and E = Morwellham.

Table 5.6 Results obtained from the Tamar Estuary field work. Confidence interval is ±3 s.d.

<table>
<thead>
<tr>
<th>Location</th>
<th>TDP (µg P L⁻¹)</th>
<th>FRP (µg P L⁻¹)</th>
<th>TDP-FRP = DOP (µg P L⁻¹)</th>
<th>PHP (µg P L⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Weir Quay</td>
<td>42 ± 8</td>
<td>5 ± 5</td>
<td>38 ± 3</td>
<td>14 ± 5</td>
</tr>
<tr>
<td>Southward Farm</td>
<td>38 ± 11</td>
<td>13 ± 2</td>
<td>25 ± 9</td>
<td>14 ± 4</td>
</tr>
<tr>
<td>Calstock</td>
<td>79 ± 5</td>
<td>20 ± 1</td>
<td>59 ± 4</td>
<td>17 ± 4</td>
</tr>
<tr>
<td>Rumleigh Farm</td>
<td>77 ± 8</td>
<td>22 ± 2</td>
<td>55 ± 6</td>
<td>23 ± 3</td>
</tr>
<tr>
<td>Morwellham</td>
<td>58 ± 6</td>
<td>33 ± 4</td>
<td>25 ± 2</td>
<td>16 ± 2</td>
</tr>
</tbody>
</table>
The results obtained show the distribution of phytase hydrolysable P (PHP) in the Tamar Estuary at the time of sampling. The sampling points were representative of different conditions ranging from saline 25 to freshwater. There is considerable farming activity in the Southward Farm area while Calstock is well known for historical mining and mineral processing activities that sustained the economy of the region in the nineteenth century. Rumleigh Farm still has considerable farming activity and sewage treatment facilities that discharge to the estuary.

5.4. CONCLUSIONS

1. The enzyme phytase can be effectively immobilized on controlled porosity glass (CPG) functionalised with aminopropyltriethoxysilane and cross-linked with glutaraldehyde. The immobilized enzyme can be packed in a micro-column within a flow injection manifold for the determination of phytase hydrolysable phosphorus in samples from the Tamar Estuary.

2. The optimum temperature for the phytase after immobilisation was 40 °C as compared with 37 °C in solution. After immobilization, the phytase showed optimum activity at pH 2.5 and 5.5 in acetate, glycine, phthalate and succinate buffers. Glycine buffer (0.1 M) pH 2.5 was used for the Tamar sample. The immobilised phytase was stable for at least 6 months when stored at 4 °C without significant loss in activity.

3. The concentration of phytase hydrolysable P in samples from the Tamar Estuary were in the range (14 - 23 μg P L⁻¹) as compared with the dissolved organic phosphorus fraction (25-59 μg P L⁻¹) and the filterable reactive phosphorus fraction (5 - 33 μg P L⁻¹). Therefore the phytase hydrolysable P fraction was a significant component of the total dissolved P fraction at the time and locations of this sampling campaign (12th August, 2005)
5.5. REFERENCES


[50] http://www./sbu.ac.uk/biology/enztech/


CHAPTER SIX

TEMPORAL AND SPATIAL DISTRIBUTION OF DISSOLVED PHOSPHORUS IN THE TAMAR CATCHMENT

Main topics in this chapter

Introduction
Experimental
Tamar transects
Tidal cycle
Interferences
Conclusions
6. INTRODUCTION

The aim of this chapter was to study the behaviour of phosphorus in the Tamar Estuary and to investigate seasonal trends in nutrient concentrations. Samples were collected during estuarine transects and tidal cycles as grab samples and were analysed using a segmented flow analyser reference method (Skalar). This method was used in preference to FI because it was able to determine phosphorus, nitrogen and silicon simultaneously and had automated data processing capability for high throughput of samples. Arsenic was also studied because of anthropogenic inputs to the catchment and also because of its effect on the phosphomolybdenum chemistry as an interferent. The specific objectives were to:

1. Design and undertake targeted sampling campaigns in the Tamar Estuary.
2. Determine key physico-chemical parameters i.e. salinity, dissolved oxygen, pH and conductivity in situ during these sampling campaigns.
3. Determine the temporal and spatial variability of dissolved phosphorus in the Tamar catchment in association with related chemical parameters, i.e. silicon, nitrogen and arsenic, and field measurements.

6.1. EXPERIMENTAL

6.1.1. General reagents

All reagents were of analytical grade and were prepared using ultra-pure water (UPW) supplied by Elga with a resistance of >18.2 MΩ cm⁻¹. All glassware and HDPE (high density polyethylene) bottles used were first washed with Neutracon, a nutrient P free detergent, rinsed at least three times with UPW and soaked in 10% (v/v) HCl overnight and finally rinsed three times with UPW [1,2]. A segmented flow analyser (Skalar) reference method [3] was used for all analyses of nutrient species, i.e. phosphorus (as phosphate), silicon (as silicate) and nitrogen (as nitrate) unless stated otherwise.

All reagents were AnalAr grade. One hundred mL of 1000 mg L⁻¹ stock solutions in UPW were prepared by dissolving the following. Calcium, 0.367 g of CaCl₂; iodide, 0.169 g of KI₂; ammonium, 0.734 g of (NH₄)₂SO₄; sulphate, 1.38 g of (NH₄)₂SO₄; Fe(III), 0.861 g of (NH₄)Fe(SO₄)₂.12H₂O; Cu(II), 0.392 g of CuSO₄.5H₂O; Co(II), 0.529 g of Co(NO₃)₂.6H₂O; Pb(II), 0.164 g of Pb(NO₃)₂; Ni(II), 0.447 g of NiSO₄.6H₂O; Mg(II), 0.804 g of MgCl₂.6H₂O; Al(III), 1.775 g of Al(NO₃)₃.9H₂O; Mn(II), 0.256 g of MnSO₄.4H₂O; Cr(III), 0.515 g of CrCl₃.6H₂O.
Stock solutions were stored in the dark and were stable for several weeks. Humic acid was a Sigma-Aldrich product (technical grade) and a 5 mg L\(^{-1}\) solution was prepared by dilution of the standard stock with UPW.

### 6.1.2. Phosphate reagents and manifold

1. **Ammonium molybdate solution.** This was prepared by dissolving 230 mg of potassium antimony tartrate \((\text{K(SbO)}C_4\text{H}_6\text{O}_6\cdot0.5\text{H}_2\text{O})\) and 6 g of ammonium molybdate \(((\text{NH}_4)_6\text{Mo}_7\text{O}_{24}\cdot4\text{H}_2\text{O})\) in UPW in a 1000 mL flask. Then 69.4 mL of sulphuric acid \((\text{H}_2\text{SO}_4)\) 98% was added and made up to 1000 mL with UPW. Two mL of FFD6 reagent (surfactant) was added and the solution mixed by inversion.

2. **Ascorbic acid \((\text{C}_6\text{H}_8\text{O}_6)\) reagent.** Eleven g of ascorbic acid was dissolved in UPW in 1000 mL flask. 60 mL of acetone \((\text{C}_3\text{H}_6\text{O})\) was added and the contents made up with UPW to the 1000 mL mark. Two mL of FFD6 was added and mixed by inversion.

3. **Potassium dihydrogen orthophosphate \((\text{KH}_2\text{PO}_4)\).** One hundred mg P L\(^{-1}\) stock solution was prepared by dissolving 0.4394 g of potassium dihydrogen orthophosphate in 1000 mL of UPW. Working standards of 20 - 100 \(\mu\)g P L\(^{-1}\) was prepared by serial dilution of the stock solution with UPW.

---

![Flow diagram](image)

**Fig. 6.1.** Flow diagram (manifold) for the determination of phosphorus as phosphate using the segmented flow analyser.
6.1.3. Silicate reagents and manifold

1. Sulphuric acid solution. Ten mL of (H₂SO₄) 98% was dissolved in 1000 mL of UPW. This was made up to the 1000 mL with UPW, 2 mL of FFD6 was added and the contents mixed by inversion.

2. Ammonium molybdate solution. Twenty g of ammonium molybdate ([(NH₄)₆Mo₇O₂₄·4H₂O) was dissolved in 800 mL of UPW and made up to the 1000 mL with UPW. Two mL of FFD6 was added and the contents mixed.

3. Oxalic acid solution. Forty four g of oxalic acid (C₂H₂O₄·2H₂O) was dissolved in 800 mL UPW and up to the 1000 mL with UPW.

4. Ascorbic acid solution. Forty g of ascorbic acid (C₆H₈O₆) was dissolved in 800 mL of UPW and made up to the 1000 mL with UPW.

5. Sodium metasilicate (Na₂SiO₃·9H₂O). One hundred mg L⁻¹ of SiO₂ stock solution was prepared by dissolving 0.4464 g of sodium metasilicate in 800 mL UPW and made up to 1000 mL with UPW. Working standards of 0.4 - 2.0 mg SiO₂ L⁻¹ were prepared by serial dilution of the stock solution.

Fig. 6. 2. Flow diagram (manifold) for the determination of silicon as SiO₂ using the segmented flow analyser.
6.1.4. Nitrate reagents and manifold

1. **Buffer solution.** 50 g of ammonium chloride (NH₄Cl) was dissolved in 800 mL of UPW and 5 g of (NaOH) added and dissolved. The pH of the solution was adjusted with 1 mL of 25% ammonia solution (NH₄OH) and the content made up to 1000 mL with UPW. The solution was degassed using an ultrasonic bath at room temperature and 3 mL of Brij 35 (30 %) added.

2. **Colour reagent.** One hundred and fifty mL of 85% o-phosphoric acid (H₃PO₄) was measured into a 1000 mL flask and diluted with 700 mL of UPW. Ten g of sulphanilamide (C₅H₈N₂O₂S) and 0.5 g of α-naphthylethylene diamine dihydrochloride (C₁₂H₁₆Cl₂N₂) were added and made up to 1000 mL with UPW. The reagent was stored in the dark.

3. **Sodium nitrate.** 100 mg N L⁻¹ stock solution was prepared by dissolving 0.6068 g of sodium nitrate (NaNO₃) in 1000 mL of UPW. Working standards of 0.2 - 1.0 mg N L⁻¹ were made by serial dilution of the stock solution.

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**Fig. 6.3. Flow diagram (manifold) for the determination of nitrogen (as nitrate plus nitrite) using the segmented flow analyser.**
Fig. 6.4. Segmented flow analyser showing P, Si, Br and N channels.

Fig. 6.5. Segmented flow analyser showing auto-sampler, reagent tray, manifold, interface and computer monitor attached to the system.
6.1.5. Analytical figures of merit for P, N and Si.

For the results shown in Table 6.1 the samples used were orthophosphate standard for P, sodium metasilicate for Si and sodium nitrate for N. The limit of detection (LOD) was determined as described by Miller et al [4] i.e. $Y_b + 3S_b$ where $Y_b = \text{intercept and } S_b = \text{standard deviation of the blank}.$

Table 6.1. Analytical figures of merit for P, N and Si.

<table>
<thead>
<tr>
<th></th>
<th>Phosphorus (µg P L$^{-1}$)</th>
<th>Nitrogen (mg N L$^{-1}$)</th>
<th>Silicon (mg Si L$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Linearity</td>
<td>2 - 100</td>
<td>0.02 - 2.5</td>
<td>0.05 - 2.5</td>
</tr>
<tr>
<td>Slope</td>
<td>37.73</td>
<td>721.15</td>
<td>0.27</td>
</tr>
<tr>
<td>Gradient</td>
<td>14.504</td>
<td>27.23</td>
<td>0.95</td>
</tr>
<tr>
<td>RSD (%)</td>
<td>&gt;4</td>
<td>&gt;4</td>
<td>&gt;5</td>
</tr>
<tr>
<td>$R^2$</td>
<td>0.99960</td>
<td>0.99954</td>
<td>0.99985</td>
</tr>
<tr>
<td>LOD</td>
<td>&gt;2</td>
<td>0.020</td>
<td>0.05</td>
</tr>
</tbody>
</table>

6.1.6. Determination of total dissolved arsenic

Arsenic was analysed using hydride generation atomic absorption spectrophotometry for saline samples [5, 6] and ICP-MS for fresh water samples [7, 8]. For saline samples, a hydride generation system (PS Analytical Ltd, Orpington, UK) and atomic absorption spectrometer (SP9-AAS, Pye Unicam, Cambridge UK) incorporating a hollow cathode lamp (7 mA) and a digital recorder were used. The sample, reductant and sodium tetrahydroborate (NaBH$_4$; 0.5 % mass volume) streams, stabilized in base (0.1 % NaOH) and acid (1.0 M HCl) respectively, were propelled with the aid of a peristaltic pump to a gas-liquid separator to separate the arsine. Absorbance was monitored at 193 nm.

The ICP-MS (Fisons Plasma Quad PQ2+), with a forward power of 1350 W and electron multiplier detector, incorporated three gas flow streams; coolant flow 13 L min$^{-1}$, auxiliary flow 1 L min$^{-1}$ and nebuliser flow 0.8 L min$^{-1}$ respectively. All standards and samples were spiked with 100 ng indium (internal standard) into 50 mL. Sample was pumped through the nebuliser (plastic Kell-F 'Ebdon' type) with the aid of a peristaltic pump.
6.1.7. The Tamar Estuary

The Tamar Estuary lies between the borders of Devon and Cornwall in South West England, centred on latitude 50° 26' 14" N and 04° 11' 42" W and covering an area of 1955 hectares. The estuary flows into the English Channel and is fed by three major rivers, the Tamar, Lynher and Tavy. The Tamar River provides the main input of freshwater. Several years of settlement and industrial and agricultural activities have greatly impacted on this area. Despite the decline of industrial activities in the area, historical mining still impacts on water and sediment quality [9 - 11]. Agricultural activities are predominant in this area, which is naturally fertile. The freshwater end of the Tamar lies within a region of mixed arable and intensive dairy farming. It is also characterised by extensive livestock, cattle and sheep farming. The lower reaches of the river have extensive mud flats, which contain different types of faunal communities and provide rich feeding grounds for birds. The sampling points used are shown in Table 6.2 and Fig. 6.6.

Table 6.2. Location, grid reference and salinity at high tide of the sampling points [12, 13].

<table>
<thead>
<tr>
<th>Location</th>
<th>Latitude N</th>
<th>Longitude W</th>
<th>Grid Reference</th>
<th>Salinity typical at high tide</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Weir Head</td>
<td>50° 30' 11</td>
<td>04° 11' 27</td>
<td>439 696</td>
<td>0</td>
</tr>
<tr>
<td>2. Morwellham</td>
<td>50° 30' 31</td>
<td>04° 11' 47</td>
<td>446 697</td>
<td>0</td>
</tr>
<tr>
<td>3. Rumleigh Farm</td>
<td>50° 27' 35</td>
<td>04° 12' 39</td>
<td>445 685</td>
<td>0</td>
</tr>
<tr>
<td>4. Calstock</td>
<td>50° 29' 59</td>
<td>04° 12' 39</td>
<td>436 685</td>
<td>1</td>
</tr>
<tr>
<td>5. Southward Farm</td>
<td>50° 28' 85</td>
<td>04° 13' 02</td>
<td>427 766</td>
<td>5</td>
</tr>
<tr>
<td>6. Halton Quay</td>
<td>50° 28' 13</td>
<td>04° 13' 87</td>
<td>413 655</td>
<td>13</td>
</tr>
<tr>
<td>7. Weir Quay</td>
<td>50° 27' 35</td>
<td>04° 12' 44</td>
<td>433 647</td>
<td>25</td>
</tr>
<tr>
<td>8. Neal Point</td>
<td>50° 27' 35</td>
<td>04° 12' 39</td>
<td>436 626</td>
<td>34</td>
</tr>
<tr>
<td>9. Tamar Bridge</td>
<td>50° 24' 56</td>
<td>04° 12' 19</td>
<td>433 626</td>
<td>35</td>
</tr>
</tbody>
</table>
6.1.8. Sampling
Appropriate techniques for the collection and storage of samples before analysis is important [1,2]. Water samples were collected from the locations indicated on the map (Fig. 6.6) as grab samples aboard a research vessel Tealia (Fig. 6.7), filtered in situ using a 0.45 μm filter (cellulose acetate) and stored in pre-cleaned containers (HDPE type). Samples were analysed within 24 hours of collection using the segmented flow analyser (Skalar) (see Fig. 6.5) or stored at 4 °C prior to analysis. For longer storage, samples were frozen at -20 °C. Transect sampling was always carried out on an incoming tide.
Fig. 6.7. Research vessel Tealia during one of the sampling campaigns.

Fig. 6.8. Mine waste (tailings) deposited at the river side. Erosion (slide) washes this waste into the river and this can increase the arsenate load in the estuary. This location is 200 m down estuary from the Morwellham sampling point.
6.2. RESULTS AND DISCUSSION

6.2.1. Physico-chemical parameters

Sampling campaigns were carried out in the Tamar Estuary to investigate the seasonal variability of nutrient concentrations. During the campaigns, physico-chemical parameters e.g. DO, pH, suspended particulate matter, conductivity and salinity were measured in-situ. Arsenate a major potential interference in the spectrophotometric detection of phosphorus was also determined. The surface waters showed different colours from yellowish-brown to colourless and were sometimes covered with a scum during low flow periods. The colour of the surface water during summer periods varied significantly compared with other seasons. Creamy brown foam on surface waters giving a false impression of suspended solids was common at summer periods. This section summarises the physico-chemical data obtained during the various sampling campaigns. They did not show any unexpected trends and broadly agreed with data reported by Morris et al. [14].
**Dissolved oxygen (DO).** This is one of the most important water quality parameters. All living things require oxygen and hence it can influence the rate of biological production. DO concentrations in the tables are expressed as mg L\(^{-1}\) with lower values indicating poorer water quality. Waters with low dissolved oxygen are often characterised by poor odour. Temperature and salinity can affect DO concentrations directly and indirectly [11, 13, 15], because increased temperature can result in higher productivity and hence a reduction in oxygen.

As can be seen in Tables 6.3 - 6.7, DO levels are influenced by season, with the lowest values (6 - 7 mg L\(^{-1}\)) observed during summer periods, especially at the freshwater end of the estuary (see Tables 6.3 and 6.7), but values >11 mg L\(^{-1}\) were also observed at the high salinity end of the estuary during the same period, e.g. as shown in Table 6.7. Differential heating or cooling when shallow waters moves tidally over large, temporarily exposed mudflats lowers DO in the freshwater areas. The sewage discharge at Rumleigh Farm, which is between Morwellham and Calstock stations, could also be responsible for the observed low DO concentrations in these areas during summer periods [13] when flow rates were low.

**Temperature.** Temperature had a significant effect on the distribution of the nutrient species P, Si and N. Lowest concentrations of these species were observed during summer periods when the average temperature was 20 °C (see Table 6.7) whereas the highest values were at temperatures of 10-12 °C during the winter/spring as shown in Tables 6.4 and 6.5.

**Conductivity and salinity.** Conductivity values are indicative of the dissolved ion concentration and values of 35-45 mS were obtained at the lower end of the transect and the lowest value of 1 mS was observed at the freshwater end. As expected, the values showed a gradual increase with increasing salinity. (Salinity values ranged from 0 at Calstock and Southward Farm to 35 at the Tamar Bridge) and nutrient concentrations generally decreased with increasing conductivity. The low salinity concentration observed from Tamar Bridge to Halton Quay on the 30\(^{th}\) March 2004 (Table 6.4) was caused by flooding due to heavy rain and high river flow.

**pH.** The recorded pH was in the range 6 - 8 over the sampling period.
Table 6.3. Phosphorus and silicon distribution in the Tamar Estuary from a sampling campaign carried out on 17\textsuperscript{th} September 2003. High tide was at 10:01 am, high water was 4.0 m and the weather was sunny with some ground mist. Results are the mean of three replicates for P and Si. (Error bar ±3s).

<table>
<thead>
<tr>
<th>Location</th>
<th>Time (GMT)</th>
<th>Depth (m)</th>
<th>Air temp. (°C)</th>
<th>pH</th>
<th>DO (mg L(^{-1}))</th>
<th>Salinity</th>
<th>Conductivity (mS)</th>
<th>P (μg P L(^{-1}))</th>
<th>Si (mg Si L(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Weir Quay</td>
<td>09:00</td>
<td>4.5</td>
<td>18</td>
<td>7.3</td>
<td>8</td>
<td>20.4</td>
<td>24.8</td>
<td>26 ± 1</td>
<td>0.4 ± 0.1</td>
</tr>
<tr>
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<td>09:23</td>
<td>3.7</td>
<td>18</td>
<td>6.8</td>
<td>7</td>
<td>21</td>
<td>25.1</td>
<td>30 ± 1</td>
<td>1.0 ± 0.1</td>
</tr>
<tr>
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<td>18</td>
<td>7.0</td>
<td>7</td>
<td>26</td>
<td>31.5</td>
<td>43 ± 1</td>
<td>1.5 ± 0.1</td>
</tr>
<tr>
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<td>17</td>
<td>6.9</td>
<td>6</td>
<td>14</td>
<td>16.8</td>
<td>53 ± 1</td>
<td>2.6 ± 0.0</td>
</tr>
<tr>
<td>Rumleigh Farm</td>
<td>10:04</td>
<td>4.2</td>
<td>17</td>
<td>6.8</td>
<td>6</td>
<td>8</td>
<td>9.5</td>
<td>50 ± 1</td>
<td>3.1 ± 0.0</td>
</tr>
<tr>
<td>Morwellham</td>
<td>10:24</td>
<td>2.6</td>
<td>15</td>
<td>7.2</td>
<td>6</td>
<td>1</td>
<td>0.8</td>
<td>55 ± 1</td>
<td>3.5 ± 0.0</td>
</tr>
<tr>
<td>Weir Head</td>
<td>10:58</td>
<td>1.5</td>
<td>14</td>
<td>7.8</td>
<td>9</td>
<td>0</td>
<td>0.1</td>
<td>84 ± 16</td>
<td>2.9 ± 0.0</td>
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</tbody>
</table>
Table 6.4. Phosphorus and silicon distribution in the Tamar Estuary from a sampling campaign carried out on 30th March 2004. High tide was at 11:25 am, high water was 4.8 m and the weather was sunny. Results are the mean of three replicates for P and Si. (Error bar ± 3s).

<table>
<thead>
<tr>
<th>Location</th>
<th>Time (GMT)</th>
<th>Depth (m)</th>
<th>Air temp. (°C)</th>
<th>pH</th>
<th>Conductivity (mS)</th>
<th>DO (mg L(^{-1}))</th>
<th>Salinity (mg L(^{-1}))</th>
<th>SPM (mg L(^{-1}))</th>
<th>P (μg L(^{-1}))</th>
<th>Si (mg L(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Weir Quay</td>
<td>10:35</td>
<td>3.9</td>
<td>10</td>
<td>5.7</td>
<td>17</td>
<td>17</td>
<td>14</td>
<td>16</td>
<td>89 ± 2</td>
<td>1.6 ± 0.0</td>
</tr>
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<td>5.8</td>
<td>21</td>
<td>17</td>
<td>17</td>
<td>7</td>
<td>131 ± 3</td>
<td>1.3 ± 0.0</td>
</tr>
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<td>9</td>
<td>5.8</td>
<td>24</td>
<td>16</td>
<td>20</td>
<td>4</td>
<td>88 ± 4</td>
<td>1.4 ± 0.0</td>
</tr>
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<td>4.5</td>
<td>10</td>
<td>5.9</td>
<td>16</td>
<td>16</td>
<td>13</td>
<td>16</td>
<td>87 ± 4</td>
<td>1.6 ± 0.0</td>
</tr>
<tr>
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<td>10</td>
<td>6.2</td>
<td>5</td>
<td>18</td>
<td>4</td>
<td>6</td>
<td>95 ± 2</td>
<td>1.9 ± 0.0</td>
</tr>
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<td>11</td>
<td>7.2</td>
<td>0</td>
<td>17</td>
<td>0</td>
<td>4</td>
<td>104 ± 1</td>
<td>2.6 ± 0.0</td>
</tr>
<tr>
<td>Calstock</td>
<td>12:10</td>
<td>2.8</td>
<td>10</td>
<td>6.9</td>
<td>0</td>
<td>13</td>
<td>0</td>
<td>18</td>
<td>87 ± 2</td>
<td>2.3 ± 0.0</td>
</tr>
<tr>
<td>Rumleigh Farm</td>
<td>12:15</td>
<td>3.2</td>
<td>10</td>
<td>7.5</td>
<td>0</td>
<td>18</td>
<td>0</td>
<td>4</td>
<td>94 ± 1</td>
<td>2.5 ± 0.0</td>
</tr>
<tr>
<td>Morwellham</td>
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<td>9</td>
<td>7.3</td>
<td>0</td>
<td>13</td>
<td>0</td>
<td>0</td>
<td>90 ± 3</td>
<td>2.4 ± 0.0</td>
</tr>
<tr>
<td>Rumleigh Farm</td>
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<td>3.1</td>
<td>10</td>
<td>7.8</td>
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<td>15</td>
<td>0</td>
<td>0</td>
<td>80 ± 1</td>
<td>2.4 ± 0.0</td>
</tr>
<tr>
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<td>10</td>
<td>7.2</td>
<td>5</td>
<td>16</td>
<td>4</td>
<td>2</td>
<td>61 ± 1</td>
<td>2.4 ± 0.0</td>
</tr>
<tr>
<td>Southward Farm</td>
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<td>3.5</td>
<td>8</td>
<td>6.8</td>
<td>2</td>
<td>16</td>
<td>0</td>
<td>14</td>
<td>72 ± 1</td>
<td>2.2 ± 0.0</td>
</tr>
<tr>
<td>Halton Quay</td>
<td>13:20</td>
<td>2.7</td>
<td>10</td>
<td>6.2</td>
<td>7</td>
<td>15</td>
<td>6</td>
<td>9</td>
<td>65 ± 1</td>
<td>2.1 ± 0.0</td>
</tr>
<tr>
<td>Weir Quay</td>
<td>13:40</td>
<td>2.4</td>
<td>11</td>
<td>6.0</td>
<td>15</td>
<td>16</td>
<td>13</td>
<td>11</td>
<td>51 ± 1</td>
<td>1.5 ± 0.0</td>
</tr>
</tbody>
</table>
Table 6.5. Phosphorus and silicon distribution in the Tamar Estuary from a sampling campaign carried out on 29th April 2004. High tide was at 10:01 am, high water was 4.8 m and the weather was sunny, cold and windy. Results are the mean of three replicates for P and Si. (Error bar ± 3s).

<table>
<thead>
<tr>
<th>Location</th>
<th>Time (GMT)</th>
<th>Depth (m)</th>
<th>Air temp. (°C)</th>
<th>pH</th>
<th>Conductivity (mS)</th>
<th>DO (mg L⁻¹)</th>
<th>Salinity (mg L⁻¹)</th>
<th>SPM (mg L⁻¹)</th>
<th>P (µg L⁻¹)</th>
<th>Si (mg L⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Weir Quay</td>
<td>07:40</td>
<td>1.6</td>
<td>12</td>
<td>6.8</td>
<td>34</td>
<td>10</td>
<td>28</td>
<td>13</td>
<td>94 ± 3</td>
<td>0.5 ± 0.0</td>
</tr>
<tr>
<td>Neal Point</td>
<td>08:50</td>
<td>4.9</td>
<td>12</td>
<td>7.5</td>
<td>36</td>
<td>10</td>
<td>30</td>
<td>5</td>
<td>94 ± 8</td>
<td>0.4 ± 0.0</td>
</tr>
<tr>
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<td>7.8</td>
<td>12</td>
<td>7.7</td>
<td>35</td>
<td>10</td>
<td>29</td>
<td>4</td>
<td>70 ± 3</td>
<td>0.4 ± 0.0</td>
</tr>
<tr>
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<td>12</td>
<td>8.4</td>
<td>37</td>
<td>10</td>
<td>11</td>
<td>4</td>
<td>104 ± 5</td>
<td>0.6 ± 0.0</td>
</tr>
<tr>
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<td>13</td>
<td>7.3</td>
<td>17</td>
<td>10</td>
<td>14</td>
<td>10</td>
<td>105 ± 3</td>
<td>1.1 ± 0.0</td>
</tr>
<tr>
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<td>09:50</td>
<td>3.2</td>
<td>13</td>
<td>7.0</td>
<td>10</td>
<td>10</td>
<td>8</td>
<td>15</td>
<td>101 ± 3</td>
<td>1.2 ± 0.0</td>
</tr>
<tr>
<td>Calstock</td>
<td>10:10</td>
<td>2.3</td>
<td>13</td>
<td>7.4</td>
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<td>10</td>
<td>0</td>
<td>8</td>
<td>108 ± 1</td>
<td>1.3 ± 0.0</td>
</tr>
<tr>
<td>Rumleigh Farm</td>
<td>10:20</td>
<td>2.8</td>
<td>13</td>
<td>7.0</td>
<td>0</td>
<td>10</td>
<td>0</td>
<td>6</td>
<td>99 ± 2</td>
<td>1.2 ± 0.0</td>
</tr>
<tr>
<td>Morwellham</td>
<td>10:20</td>
<td>2.3</td>
<td>12</td>
<td>7.2</td>
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<td>10</td>
<td>0</td>
<td>4</td>
<td>151 ± 7</td>
<td>2.0 ± 0.0</td>
</tr>
<tr>
<td>Weir Head</td>
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<td>12</td>
<td>7.2</td>
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<td>10</td>
<td>0</td>
<td>6</td>
<td>88 ± 5</td>
<td>1.2 ± 0.0</td>
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<tr>
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<td>2.2</td>
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<td>7.1</td>
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<td>4</td>
<td>85 ± 1</td>
<td>1.2 ± 0.0</td>
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<td>10</td>
<td>94 ± 1</td>
<td>1.3 ± 0.0</td>
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<td>13</td>
<td>7.2</td>
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<td>9</td>
<td>108 ± 1</td>
<td>1.2 ± 0.0</td>
</tr>
<tr>
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<td>13</td>
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<td>10</td>
<td>19</td>
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<tr>
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<td>12</td>
<td>7.4</td>
<td>45</td>
<td>10</td>
<td>29</td>
<td>1</td>
<td>85 ± 1</td>
<td>0.5 ± 0.0</td>
</tr>
</tbody>
</table>
Table 6.6. Phosphorus, silicon and nitrate distribution in the Tamar Estuary from a sampling campaign carried out on 2\textsuperscript{nd} March 2005. High tide was at 09:09 am, high water was 4.9 m and the weather was sunny, cold and windy. Results are the mean of three replicates for P, Si and N. (Error bar ± 3s).

<table>
<thead>
<tr>
<th>Location</th>
<th>Time (GMT)</th>
<th>Depth (m)</th>
<th>Air temp. (°C)</th>
<th>pH</th>
<th>Conductivity (mS)</th>
<th>DO (mg L\textsuperscript{-1})</th>
<th>Salinity (mg L\textsuperscript{-1})</th>
<th>SPM (μg L\textsuperscript{-1})</th>
<th>P (mg L\textsuperscript{-1})</th>
<th>Si (mg L\textsuperscript{-1})</th>
<th>N (mg L\textsuperscript{-1})</th>
</tr>
</thead>
<tbody>
<tr>
<td>Weir Quay</td>
<td>10:25</td>
<td>5.3</td>
<td>7.3</td>
<td>6.4</td>
<td>35</td>
<td>7</td>
<td>29</td>
<td>25</td>
<td>34 ± 3</td>
<td>1.3 ± 0.2</td>
<td>1.0 ± 0.0</td>
</tr>
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<td>2</td>
<td>8</td>
<td>1</td>
<td>158</td>
<td>34 ± 3</td>
<td>2.6 ± 0.3</td>
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<td>7.5</td>
<td>7.1</td>
<td>1</td>
<td>8</td>
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<tr>
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<tr>
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<td>86</td>
<td>51 ± 2</td>
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</tr>
<tr>
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<td>7.1</td>
<td>0</td>
<td>8</td>
<td>0</td>
<td>98</td>
<td>54 ± 5</td>
<td>2.3 ± 0.4</td>
<td>1.7 ± 0.0</td>
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Table 6.7. Phosphorus, silicon and nitrate distribution in the Tamar Estuary from a sampling campaign carried out on 12th August 2005. High tide was at 09:41, high water was 4.6 m and the weather was sunny. Results are the mean of three replicates for P, Si and N. (Error bar ± 3s).

<table>
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<th>Depth (m)</th>
<th>Air Temp. (°C)</th>
<th>pH</th>
<th>Conductivity (mS)</th>
<th>DO (mg L⁻¹)</th>
<th>Salinity (mg L⁻¹)</th>
<th>SPM (μg L⁻¹)</th>
<th>P (mg L⁻¹)</th>
<th>Si (mg L⁻¹)</th>
<th>N (mg L⁻¹)</th>
</tr>
</thead>
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<td>7</td>
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<td>8</td>
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<td>0.01 ± 0.0</td>
<td>0.7 ± 0.0</td>
</tr>
<tr>
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<td>3.7</td>
<td>19.6</td>
<td>7.8</td>
<td>22</td>
<td>11</td>
<td>17</td>
<td>18</td>
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<td>0.01 ± 0.0</td>
<td>1.2 ± 0.0</td>
</tr>
<tr>
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<td>19.7</td>
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<td>9</td>
<td>4</td>
<td>29</td>
<td>20 ± 3</td>
<td>0.19 ± 0.4</td>
<td>1.4 ± 0.0</td>
</tr>
<tr>
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<td>19.8</td>
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<td>0.47 ± 0.4</td>
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<td>18</td>
<td>14</td>
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<td>0.07 ± 0.0</td>
<td>0.04 ± 0.01</td>
</tr>
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<td>35</td>
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</tr>
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<td>0.01 ± 0.0</td>
<td>0.03 ± 0.01</td>
</tr>
<tr>
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<td>31</td>
<td>3</td>
<td>4.7 ± 0</td>
<td>0.01 ± 0.0</td>
<td>0.5 ± 0.01</td>
</tr>
<tr>
<td>Weir Quay</td>
<td>12:05</td>
<td>4.6</td>
<td>20.2</td>
<td>8.2</td>
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<td>13</td>
<td>20</td>
<td>4</td>
<td>6.2 ± 3</td>
<td>0.01 ± 0.0</td>
<td>0.5 ± 0.01</td>
</tr>
</tbody>
</table>

168
6.2.2. Tamar transects

The trophic state of natural waters can be used to ascertain the nutrient status of that water. Some rivers and estuaries can have high nutrient concentrations due to inputs from point sources e.g. sewage treatment facilities and/or from diffuse sources e.g. agricultural runoff and erosion. The sea acts as a sink for nutrients which are transported from rivers or estuaries [11, 14] and therefore, the flow of a river can influence the distribution of nutrients in various section of that river [16]. Tables 6.3 - 6.7 show the phosphate concentrations measured on five sampling campaigns.

Results from the campaign of 17th September 2003 are shown in Table 6.3 and seven freshwater stations were sampled at different times. Conductivity, temperature, pH and DO varied from 0 - 25 mS, 14 - 18 °C, 6 - 8 mg L$^{-1}$ and 6 - 9 mg L$^{-1}$ respectively and salinity 0 - 24. The largest changes were in conductivity and temperature. Differential heating due to shallow waters moving tidally over exposed surfaces can lead to significant variations in temperature [14] which can affect P cycling. The highest concentration of P observed was 84 μg P L$^{-1}$ at a temperature of 14 °C and a conductivity of 0 mS while the lowest observed P concentration during the campaign was 26 μg P L$^{-1}$ at a temperature of 18 °C and a conductivity of 25 mS. The higher temperature favoured the uptake of nutrients by phytoplankton and consequently lowered dissolved P concentrations. This correlates with the low DO levels also recorded during the campaign because living organisms use oxygen for metabolism. The Si distribution followed a similar relationship with temperature and conductivity. The highest Si concentration observed was 3.5 mg Si L$^{-1}$ at a temperature of 15 °C and a conductivity of 1 mS and the lowest Si concentration of 0.4 mg Si L$^{-1}$ was at a temperature of 18 °C and a conductivity of 25 mS. Therefore temperature and conductivity influence the distribution of dissolved nutrients.

The results obtained during the campaign of 30th March 2004 are shown in Table 6.4. Sampling was carried out at 8 different stations although most of the stations were sampled more than once. Temperature was relatively constant at 10 °C, but pH, conductivity, DO, salinity and suspended particulate matter varied significantly from; 5.7 - 7.5, 0 - 23 mS, 13 - 18 mg L$^{-1}$, 0 - 2 and 0 - 16 mg L$^{-1}$ respectively. The salinity 0 – 20, lowest at the freshwater end and high DO were indicative of high flows (flooding was observed on the surrounding land) which caused massive
dilution of seawater by freshwater. P concentration was spatially variable with the highest P concentration of 131 µg P L⁻¹ observed when the salinity was 2, conductivity was 21 mS and suspended particulate matter concentration was 7 mg L⁻¹. However, the highest concentration of P was 94 µg P L⁻¹ at the freshwater end (Rumleigh Farm) of the estuary. The Si distribution was slightly different. The highest concentration of 2.6 mg Si L⁻¹ was observed at Southward Farm with a salinity of 0, conductivity 0.0 mS and suspended particulate matter 4.0 mg L⁻¹.

Time of sampling relative to tidal flow was the major factor which affected the distribution of P and Si during this campaign e.g. at Southward Farm at 11:55, P was 104 µg P L⁻¹ and Si was 2.6 mg L⁻¹. At 13:10 however, nutrient concentrations decreased to 72 µg P L⁻¹ and Si to 2.2 mg Si L⁻¹ whereas conductivity and suspended particulate matter increased from 0 - 2 mS and 4 - 14 mg L⁻¹ respectively. The high P and Si concentrations observed at the high salinity end can be explained by effects caused by tributaries and anthropogenic inputs.

Results from the campaign on 29th April 2004 are shown in Table 6.5. Sampling was carried out at 10 stations (sometimes more than once). Temperature and DO were constant at 12 °C and 10.0 mg L⁻¹ respectively. Conductivity, salinity, pH and suspended particulate matter varied significantly and were: 0 - 34 mS, 0 - 30, 6.8 - 8.4 and 1 - 13 mg L⁻¹ respectively. The highest P concentration of 151 µg P L⁻¹ observed during the campaign was at the freshwater end and salinity, conductivity and suspended particulate matter were 0, 0 and 4.0 mg L⁻¹ respectively. The lowest P value was 70 µg P L⁻¹ where salinity was 25, conductivity 35 mS and suspended particulate matter 4.0 mg L⁻¹. The Si concentration correlated with salinity. The highest observed concentration was 2.0 mg Si L⁻¹, where salinity was 0, conductivity 0 and suspended particulate matter 4.0 mg L⁻¹ and the lowest was 0.4 mg Si L⁻¹ where salinity was 25, conductivity 35 mS and suspended particulate matter 4.0 mg L⁻¹ respectively. As can be seen from the results in Table 6.5, nutrient concentrations are influenced by the state of the tide. The concentrations of phosphorus were highest at the freshwater end of the estuary due to agricultural inputs and sewage discharges but the impact of anthropogenic and tributary inputs was also significant at the lower end of the estuary as evidenced by the high concentration of 94 µg P L⁻¹ found at the seawater end (salinity 28 - 30) [14]. The high phosphate concentrations reported in Tables 6.4 and 6.5 could also be due to release from phosphate rich organic matter during decay e.g. during the
sedimentation of spring phytoplankton blooms or phaeocystis blooms which occurs more regularly [17].

The results obtained from a campaign carried out on 2nd March 2005 at seven locations are shown in Table 6.6. Conductivity and salinity varied widely from 0 - 35 mS and 0 - 29 respectively. Temperature, pH, DO and suspended particulate matter were in the ranges 12 - 15 °C, 6 - 7, 7 - 9 mg L\(^{-1}\) and 25-175 mg L\(^{-1}\) respectively. The highest P concentration of 54 µg P L\(^{-1}\) was at Morwellham where salinity, conductivity and suspended particulate matter were 0, 0 mS and 98 mg L\(^{-1}\) respectively. The lowest concentration was 34 µg P L\(^{-1}\) at two contrasting stations (Weir Quay and Halton Quay). When compared with a similar campaign carried out on 30th March 2004, the lower DO and higher suspended particulate matter are indicative of high biological activity. The highest Si concentration of 2.6 mg Si L\(^{-1}\) was observed when the level of suspended particulate matter was 175 mg L\(^{-1}\) and associates suspended particulate matter with increased Si concentration. As can be seen in Table 6.6, N followed a similar pattern with the lowest concentration of 1.0 mg N L\(^{-1}\) recorded at Weir Quay at 10:25 when suspended particulate matter was 25 mg L\(^{-1}\). Suspended particulate matter can affect the concentration of dissolved nutrients in an estuary since the surfaces act as adsorption sites for SRP [18]

Table 6.7 shows the results obtained from seven locations during a transect campaign carried out on 12th August 2005. Temperature and pH were constant at 20 °C and 8 respectively. Conductivity, DO, salinity and suspended particulate matter varied significantly from 1 - 35 mS, 7 - 13 mg L\(^{-1}\), 0 - 31 and 1 - 162 mg L\(^{-1}\) respectively. Conductivity reached 8 mS at Rumleigh Farm, a typical freshwater section of the estuary. Although the weather was sunny, previous rainfall may have introduced ions such Mg\(^{2+}\), Cl\(^-\) and Na\(^+\) which increased the conductivity at the freshwater end [12]. During this campaign, yellow-brown coloured slicks were observed on the surface waters, especially at the freshwater end of the estuary which may have been due to phaeocystis blooms. The P concentration varied significantly during this campaign from 4.5 - 33 µg P L\(^{-1}\) and was inversely related to salinity. The distribution of the other nutrients (10 - 890 µg Si L\(^{-1}\) and 0.5 - 1.5 mg N L\(^{-1}\)) showed a similar trend. The high temperature and low DO observed at the freshwater end and a pH of 7.8 – 8.2 at all the locations suggests high nutrient uptake and biological activity [17]. Evidence of the occurrence of phaeocystis was
also reported by the Environment Agency [19]. They are non-toxic, yellow-brown in appearance, like sewage slicks and usually start to form in late June or July.

Anomalies in the pattern of nutrient distributions such as those observed in this work have also been reported by Morris et al. and Mommaerts [14, 15, 20 - 22]. The SRP results obtained during the campaigns (see Tables 6.4 - 6.5) compared well with Environmental Agency reports [23, 24] of 70 - 100 µg P L\(^{-1}\) during high flow and up to 150 µg P L\(^{-1}\) for high flood waters. The data also compared well other historical data e.g. Morris et al. [14] and Gardolinski [12]. During the summer, the SRP concentration at the freshwater end of the estuary was typically 25 - 35 µg P L\(^{-1}\) (see Table 6.7).

The profiles in Fig. 6.10A-C show that a removal process of dissolved nutrients takes place within the estuary. In Fig. 6.10A, the relatively high concentration of P at the high salinity end is indicative of tributary and anthropogenic inputs of P in this region [14]. However, the Si distribution showed conservative behaviour due to seawater dilution. The profile in Fig. 6.10B showed similar trends for P, Si and N. Fig. 6.10C shows a more scattered distribution, possibly due to localised algal blooms [17] and other processes e.g. re-suspension, adsorption, precipitation [14, 18, 21]. As discussed above, the temperature was high (20 °C) which favours biological processes and suspended particulate matter varied significantly from 0-162 mg L\(^{-1}\). In Fig. 6.10C, the Si concentration was low (at the detection limit of 10 µg Si L\(^{-1}\)) and the distribution deviated from the usual conservative pattern observed in Figs. 10A and 10B. Therefore in any future campaigns it would be useful to measure the chlorophyll concentration as an indicator of biological activity.

6.2.3. Tidal cycle

The results from a tidal cycle campaign at Weir Quay on 31\(^{st}\) January 2005 are shown in Table 6.8. Salinity, conductivity and suspended particulate matter varied significantly with tide [20] whereas DO, pH and temperature were relatively constant at 9 - 12 mg L\(^{-1}\), 7.4 - 7.5 and 7.2 – 8.5 °C respectively. The salinity decreased with the outgoing tide, reaching a low of 5 (conductivity 7 mS). The lowest flow was from 13:30 to 16:00. During this period of low flow, suspended particulate matter was high (412 - 1067 mg L\(^{-1}\)) and indicative of the turbidity maximum. P, Si and N averaged 30 µg P L\(^{-1}\), 2.0 mg Si L\(^{-1}\) and 3.4 mg N L\(^{-1}\)
Fig. 6.10. The phosphorus-salinity, silicon-salinity and nitrogen-salinity relationships in the Tamar Estuary from various sampling campaigns; (A) 29th April 2004; (B) 2nd March 2005; (C) 12th August 2005.
Table 6.8. Tidal cycle distribution of phosphorus, silicon and nitrate monitored at Weir Quay (Tamar Estuary) from a sampling campaign carried out on 31st January 2005. High tide was at 09:03, high water was 5.0 m and the weather was cold and windy.

Results are the mean of three replicates for P, Si and N. (Error bar ± 3s).

<table>
<thead>
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<th>Time (GMT)</th>
<th>Air Temp. °C</th>
<th>pH</th>
<th>Conductivity (mS)</th>
<th>DO (mg L⁻¹)</th>
<th>Salinity</th>
<th>SPM (mg L⁻¹)</th>
<th>P (µg L⁻¹)</th>
<th>Si (mg L⁻¹)</th>
<th>N (mg L⁻¹)</th>
</tr>
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<td>30</td>
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<td>0.9 ± 0.1</td>
<td>1.4 ± 0.0</td>
</tr>
<tr>
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<td>7.5</td>
<td>22</td>
<td>10</td>
<td>18</td>
<td>14</td>
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<td>0.8 ± 0.0</td>
<td>1.4 ± 0.0</td>
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<td>7.5</td>
<td>23</td>
<td>11</td>
<td>19</td>
<td>7</td>
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<td>0.8 ± 0.0</td>
<td>1.4 ± 0.0</td>
</tr>
<tr>
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<td>21</td>
<td>10</td>
<td>18</td>
<td>27</td>
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<td>1.1 ± 0.0</td>
<td>1.7 ± 0.0</td>
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<td>15</td>
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<td>14</td>
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<td>79</td>
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<td>2.8 ± 0.0</td>
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<td>10</td>
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<td>362</td>
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<td>10</td>
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<td>382</td>
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<td>1.8 ± 0.5</td>
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<td>10</td>
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<td>1067</td>
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<td>11</td>
<td>8</td>
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<td>825</td>
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<td>11</td>
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<td>866</td>
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<td>3.0 ± 0.5</td>
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<td>7.4</td>
<td>8</td>
<td>11</td>
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<td>412</td>
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<td>2.3 ± 0.1</td>
<td>3.4 ± 0.7</td>
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<td>7</td>
<td>11</td>
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<td>124</td>
<td>31 ± 0</td>
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<td>3.6 ± 0.3</td>
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<td>7</td>
<td>12</td>
<td>5</td>
<td>26</td>
<td>25 ± 4</td>
<td>2.2 ± 0.4</td>
<td>3.5 ± 0.5</td>
</tr>
<tr>
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<td>176</td>
<td>35 ± 4</td>
<td>2.9 ± 0.4</td>
<td>3.4 ± 0.7</td>
</tr>
<tr>
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<td>11</td>
<td>11</td>
<td>9</td>
<td>155</td>
<td>63 ± 6</td>
<td>3.0 ± 0.3</td>
<td>2.9 ± 0.0</td>
</tr>
<tr>
<td>18:00</td>
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<td>7.5</td>
<td>15</td>
<td>11</td>
<td>12</td>
<td>137</td>
<td>69 ± 10</td>
<td>3.1 ± 0.5</td>
<td>2.5 ± 1.0</td>
</tr>
</tbody>
</table>
respectively during low flow. The lowest P concentration of 20 μg P L⁻¹ was observed when the level of particulate matter was highest (1067 mg L⁻¹) due to adsorption onto mineral surfaces e.g. Al, Ca [14, 18, 20, 21]. The Si concentration increased with particulate matter whereas N showed conservative behaviour with salinity.

Fig. 6.11 shows the effect of salinity on the distribution of P, N and Si at a fixed location. P (and to a lesser extent Si) was scattered but the suspended particulate matter was also high and variable which would have increased sorption onto mineral surfaces. Nitrogen showed more conservative behaviour and is less particle reactive than phosphorus. The observed distribution of P and Si (in particular P) could also be attributed to upstream anthropogenic inputs [14].

Fig. 6.11. Phosphorus-salinity, nitrogen-salinity and silicon-salinity relationships in the Tamar Estuary on 31st January 2005 derived from the profiles shown in Table 6.9.

6.2.4. Effect of diverse ions on the determination of P.
The purpose of this investigation was to determine the effect of matrix ions on the response for the phosphorus channel. The results in Table 6.9 show that As(V) is the major interferent, both on its own and in the presence of 20 μg P L⁻¹ [25 - 30]. Humic acid, representative of naturally occurring, high molecular weight organic compounds [31] also had a slight positive interference on the determination of P because it absorbs at 800 nm [32]. Many other ions had a small negative affect on the response for phosphorus, particularly Al³⁺ because it forms strong complexes.
with phosphate, making it unavailable for complexation with molybdate [5, 18, 33]. It is also important to filter samples prior to analysis because the particulate phase, particularly iron and manganese oxides and organic matter can also act as adsorption sites for phosphate [34, 35].

Table 6.9. Effects of diverse ions on the determination of 20 µg P L\(^{-1}\) using the Skalar instrumentation. Results are mean of three replicates for each ion.

<table>
<thead>
<tr>
<th>Ion</th>
<th>Conc. Without P</th>
<th>% RSD</th>
<th>With P</th>
<th>% RSD</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(mg L(^{-1}))</td>
<td>(µg P L(^{-1}))</td>
<td>(n = 3)</td>
<td>(µg P L(^{-1}))</td>
</tr>
<tr>
<td>P</td>
<td>0.02</td>
<td>20</td>
<td>2.4</td>
<td></td>
</tr>
<tr>
<td>SO(_4)(^{2-})</td>
<td>250</td>
<td>1.3</td>
<td>12</td>
<td>17</td>
</tr>
<tr>
<td>I(^-)</td>
<td>1</td>
<td>1.1</td>
<td>0.8</td>
<td>18</td>
</tr>
<tr>
<td>NO(_2)(^{-})</td>
<td>0.5</td>
<td>0.9</td>
<td>1</td>
<td>17</td>
</tr>
<tr>
<td>NO(_3)(^-)</td>
<td>3</td>
<td>1.3</td>
<td>6.2</td>
<td>20</td>
</tr>
<tr>
<td>NH(_4)(^+)</td>
<td>1</td>
<td>1.2</td>
<td>14</td>
<td>21</td>
</tr>
<tr>
<td>Ca(^{2+})</td>
<td>150</td>
<td>1</td>
<td>6.5</td>
<td>18</td>
</tr>
<tr>
<td>Mg(^{2+})</td>
<td>150</td>
<td>1.2</td>
<td>12</td>
<td>15</td>
</tr>
<tr>
<td>SiO(_2)</td>
<td>3</td>
<td>1.6</td>
<td>0.3</td>
<td>21</td>
</tr>
<tr>
<td>Co(^{2+})</td>
<td>0.04</td>
<td>1.3</td>
<td>5.4</td>
<td>17</td>
</tr>
<tr>
<td>Ni(^{2+})</td>
<td>0.04</td>
<td>1.4</td>
<td>0.3</td>
<td>18</td>
</tr>
<tr>
<td>Fe(^{3+})</td>
<td>0.2</td>
<td>1.2</td>
<td>0.4</td>
<td>20</td>
</tr>
<tr>
<td>As(V)</td>
<td>0.04</td>
<td>4</td>
<td>0.1</td>
<td>25</td>
</tr>
<tr>
<td>Al(^{3+})</td>
<td>0.2</td>
<td>1.2</td>
<td>2.6</td>
<td>13</td>
</tr>
<tr>
<td>Mn(^{2+})</td>
<td>0.04</td>
<td>1.4</td>
<td>6.3</td>
<td>18</td>
</tr>
<tr>
<td>Cu(^{2+})</td>
<td>0.04</td>
<td>1.3</td>
<td>0.0</td>
<td>19</td>
</tr>
<tr>
<td>Pb(^{2+})</td>
<td>0.04</td>
<td>1.4</td>
<td>0.0</td>
<td>18</td>
</tr>
<tr>
<td>Cr(^{3+})</td>
<td>0.04</td>
<td>1.8</td>
<td>2.0</td>
<td>18</td>
</tr>
<tr>
<td>HA</td>
<td>5</td>
<td>1.8</td>
<td>6.1</td>
<td>22</td>
</tr>
<tr>
<td>All ions</td>
<td>30</td>
<td>2.0</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

6.2.5. Trace metal concentrations in Tamar Estuary samples.

The Tamar Estuary has relatively high dissolved metal concentrations due to the underlying geology and the impact of historical mining activity. All samples from the field campaigns were therefore analysed for trace metals using ICP-AES and concentrations were; Cu < 20 µg L\(^{-1}\), Mn 20 - 100 µg L\(^{-1}\), Cr < 20 µg L\(^{-1}\), Al 20 - 200 µg L\(^{-1}\), Ni 20 - 43 µg L\(^{-1}\), Co < 10 µg L\(^{-1}\), Fe 20 - 150 µg L\(^{-1}\). By comparison of
these observed concentrations with the data shown in Table 6.9 it is clear that none of these ions will affect the spectrophotometric determination of phosphorus at 20 μg P L⁻¹ concentrations. However an IUPAC Technical Report on phosphorus speciation in water and sediments [26] shows the combined concentrations of metal ions can affect the spectrophotometric determination of phosphorus at low concentration and therefore the effect of the matrix should always be determined when analysing samples from metal impacted environments.

6.2.6. Arsenic distribution in the Tamar Estuary
Areas where minerals or ores were historically processed can impact greatly on the water column chemistry, e.g. the Tamar Estuary England [9, 10], Tejo Estuary Portugal [36] and Mandovi-Zuari catchments India [37], increasing the concentration of dissolved metals including arsenic. Oxygen-rich surface waters favour the dissolution and oxidation of arsenopyrite (AsFeS). There is significant historical mining in the Tamar Valley, evidenced by the presence of dilapidated/collapsed industrial workings (see Figs. 6.8 and 6.9) [10], particularly adjacent to the Rumleigh and Southward Farm sampling sites. During rain events, debris and mine tailings from these areas are washed into the estuary, which contributes to the loading of arsenic in the watercourse. Wind can also mobilise arsenic from soils/mine wastes and this effect has been reported at Devon Great Consols mine [38].

The arsenic concentrations found during a campaign on 17th September 2003 were 120-500 μg As L⁻¹ and were lowest at the freshwater end. Campaigns on 30th March 2004, 29th April 2004 and 2nd March 2005 found arsenic concentrations of 50-75 μg As L⁻¹ 13-38 μg As L⁻¹ and 20 μg As L⁻¹ respectively. The higher arsenic concentrations observed during the 17th September 2003 campaign can be related to a land slide caused by erosion (see Fig. 6.8) which occurred in the week before sampling. From Table 6.9 it can be concluded that on the 17th September 2003 and 30th March 2004 campaigns, As would have had a small positive bias on the P results of 26 - 84 and 51 - 89 μg P L⁻¹ respectively (see Tables 6.3 and 6.4). These data emphasise the need to monitor potential interfering species, particularly As, during the determination of phosphorus to avoid overestimation and hence provide a reliable data base for planning and management purposes.
6.3. CONCLUSIONS

1. Tidal influences affect the distribution of nutrients due to the mixing of freshwater and seawater and the variability of suspended particulate matter, dissolved oxygen and temperature. For future campaigns the determination of chlorophyll would provide more conclusive evidence of biological activity (algal blooms).

2. Anthropogenic inputs give rise to high arsenic loading in the Tamar Estuary. Arsenate concentrations > 40 µg As L⁻¹, which can occur in areas impacted by historical mining activity, can affect the determination of phosphorus at concentrations <20 µg P L⁻¹.

3. To minimise the effects caused by interferents present in sample matrices, e.g. arsenate and humic acids, a 1:4 dilution of sample is recommended prior to analysis (subject to suitable sensitivity).

4. Nutrients concentrations were generally higher in the winter months compared with the summer due to increased diffuse inputs during the winter and greater biological productivity during the summer.

6.4. REFERENCES


CHAPTER SEVEN

CONCLUSIONS AND FUTURE WORK
Conclusions and future work

The following conclusions can be drawn from the work presented in this thesis.

**Flow injection (FI) manifold for phosphate.** The FI technique is suitable for the determination of phosphorus (as orthophosphate) in natural waters and has several attractions such as ease of automation and on-line incorporation of masking agents, e.g. thiosulphate to mask arsenate. Arsenate is a potential interferent in the determination of phosphorus, particularly in areas of historical mining activity such as the Tamar catchment. The FI manifold incorporating a thiosulphate stream is suitable for the determination of phosphate in the presence of high arsenate concentrations, i.e. >300 μg As L⁻¹. The effect of silicate, another potential interferent, can be effectively eliminated using a low temperature (30 °C) and an acid to molybdate ratio of 70. Potential interfering ions in natural waters (Fe(II), Fe(III), Pb(II), Cu(II), Mg(II), Al(III), Mn(II), Zn(II), K and NO₃⁻, NO₂⁻, SO₄²⁻, I⁻, F⁻) have no effect on the phosphorous determination at environmentally relevant concentrations. The FI manifold reported in this thesis has a linear range of 2-100 μg P L⁻¹ with a precision of ± 2% (n = 3), and a practical limit of detection of 2.0 μg P L⁻¹. Results from the FI manifold were in good agreement with results from a segmented flow analyser (Skalar) standard method and therefore environmental data from both techniques can be reliably compared. The segmented flow analyser method is the standard method for the determination of phosphorus and silicate in natural waters and was used by 75% of all participants who stated their method (23 in total) in the May 2000 NOAA/NRC Intercomparison for nutrients in seawater. The other 25% used flow injection based methods.

**Flow injection (FI) manifold for silicate.** FI techniques are robust, economical and highly adaptable. The FI spectrophotometric method incorporating a thiosulphate stream on-line can be adapted for the determination of Si in natural waters in the presence of arsenate (>300 μg As L⁻¹). Phosphate, a major potential interferent, can be masked on-line by incorporating a strong anion exchange resin in a micro-column and other potential cationic interferences, e.g. iron and copper, can be eliminated by an iminodiacetate chelating resin in a micro-column. Potential interfering ions in natural waters (Cu(II), Ca(II), Mg(II), Zn(II), Al(III), Pb(II), Mn(II), Ni(II), Co(II), Fe(II and III), NH₄⁺, F⁻, Cl⁻, SO₄²⁻, HCO₃⁻, NO₂⁻, NO₃⁻ and urea) have no effect on the silicate determination. The linear range of the optimised manifold is 10-2000 μg Si L⁻¹ with a typical precision of ± 1.5% (n = 3)
and a practical limit of detection of 20 μg Si L⁻¹. Results from the Fl manifold were in good agreement with those obtained from a segmented flow analyser (Skalar) reference method. Silicomolybdenum formation is favoured relative to phosphomolybdenum formation at low acidity. Increased sensitivity can be achieved by increasing the temperature to 60 °C, but this also increases interference by arsenate. A temperature of 40 °C is therefore recommended for samples containing arsenate concentrations >300 μg As L⁻¹ (anthropogenically impacted areas) while 60 °C is recommended for samples from areas with low arsenate concentrations.

**Phosphorus speciation using autoclave digestion.** The major phosphorus species determined using the molybdenum blue reaction is orthophosphate. This fraction is often called soluble reactive phosphorus (SRP), molybdate reactive phosphorus (MRP) or filterable reactive phosphorus (FRP). In order to determine the dissolved organic phosphorus (DOP) fraction of the total dissolved phosphorus (TDP) pool, filtered samples need to be digested. For natural waters this is best achieved using an oxidizing reagent e.g. peroxydisulphate or a combination of acid/peroxydisulphate. The addition of these reagents affects the final pH, i.e. increases the acidity, and therefore affects the recovery of DOP from the sample matrix after digestion when using the pH sensitive molybdate reaction for detection. The optimum pH for samples is therefore 1.5 to 2.0 after batch autoclave digestion. Dilution of samples before digestion is recommended to minimise matrix interferences from e.g. humic acids (for freshwaters), suspended solids and chloride ions (for seawaters and estuarine waters). Organic compounds high in carbon, e.g. phytic acid (C₆) and MTP (C₁₉) are not quantitatively recovered in high acid-peroxydisulphate media because of carbonisation caused by increased acidity after autoclaving. Using peroxydisulphate only as a reagent yields quantitative recoveries. A mole ratio of [H⁺] to [MoO₄²⁻] of 60 - 62 is recommended for the Fl determination of phosphorus in autoclaved samples. The addition of sulphite to the sample in the ratio of 1:4 before autoclaving eliminates problems posed by the seawater matrix.

**Determination of phytic acid using immobilised phytase.** Phytic acid is one of the major species in the organic phosphorus fraction present in natural waters, soils and seeds. In natural waters phytases, which are naturally occurring enzymes, hydrolyse phytic acid and thus release phosphorus as orthophosphate.
to the aquatic environment. Phytase can be immobilized on a support material of controlled pore glass (CPG) after functionalisation with aminopropyltriethoxysilane cross-linked with glutaraldehyde. This can be incorporated in an FI manifold for the determination of phytase hydrolysable phosphorus in natural waters, e.g. from the Tamar Estuary. The optimum temperature for the maximum activity of phytase changed after immobilisation from 37 °C (soluble form) to 40 °C. The activity of phytase is also pH dependent and 5.5 was optimum after immobilisation, in the presence of glycine, acetate, phthalate and succinate buffers. Phytase can be activated with magnesium chloride or calcium chloride but magnesium chloride gives optimum activity under the operating conditions used. The immobilised phytase is stable for several months when packed in a micro-column, stored at 4 °C in buffer. When the method was applied to Tamar Estuary samples, concentrations of phytic acid were in the range 14 - 25 μg P L⁻¹ which constituted a significant component of the dissolved organic phosphorus fraction 20-30%.

**Phosphorus in the Tamar Estuary.** Various sampling cruises were carried out to validate the FI manifolds for application to natural waters and to observe seasonal trends in phosphate (and silicate) concentrations in the Tamar Estuary. As one would expect, the concentration of phosphate was generally higher in the winter compared with the summer due to factors such as higher run-off from agricultural land because of higher rainfall and lower biological activity from algal and microbial communities because of lower temperatures.

**The following are suggestions for future work.**

The various FI techniques described in Chapters Two and Three could be adapted in a variety of ways to improve their analytical performance and applicability to the investigation of nutrient biogeochemistry in aquatic systems.

1. **Automation of operation.** For real time monitoring of nutrient species it would be beneficial to automate the operation of the FI manifolds, including on-line data processing and incorporation in a suitable housing for remote deployment. This would overcome issues relating to sample stability during storage but would need to consider aspects such as sample conditioning, e.g. filtration, calibration and reagent stability.
2. **Additional chemistries.** In order to better understand nutrient biogeochemistry in catchments and estuaries it would be very attractive to be able to monitor several nutrient species simultaneously. In particular the determination of nitrate and ammonium to study nitrogen cycling would complement the determination of phosphate and silicate. For the silicate manifold further investigation of phosphate masking using oxalic acid is suggested.

3. **Phosphorus speciation.** Further studies on the autoclave digestion technique for estuarine/marine samples would be useful. The effect of sulphite on the digestive procedure and subsequent Fl determination of phosphate in seawater matrices is a particular area of interest. This work would also involve intercomparison with literature methods for a range of natural water samples from estuarine to open ocean environments.

The Fl manifold containing immobilised phytase could be further optimised and applied to the determination of phytic acid in grains and seeds. This would be of direct relevance to Ebonyi State in Nigeria where grain/seed production is an important contributor to the local economy. The use of soluble phytase could be investigated as a simpler alternative to the immobilised form.

4. **Enhanced performance of Fl techniques.** This thesis has described the systematic optimisation of Fl manifolds with spectrophotometric detection for the determination of nutrient species. Detection limits have been appropriate for the intended applications such as Tamar Estuary waters. However in certain environmental applications, e.g. open ocean waters and oligotrophic freshwaters, lower detection limits may be required. One possibility for such applications is the incorporation of a long path length flow cell (liquid core wave guide). In addition, there may be situations, e.g. waters during the rainy season in Ebonyi State, Nigeria, where high suspended solids require more rigorous sample conditioning prior to analysis. For this application on-line filtration techniques such as tangential flow filtration may be required.
Sampling, sample treatment and quality assurance issues for the determination of phosphorus species in natural waters and soils

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Abstract

Phosphorus is an important macronutrient and the accurate determination of phosphorus species in environmental matrices such as natural waters and soils is essential for understanding the biogeochemical cycling of the element, studying its role in ecosystem health and monitoring compliance with legislation. This paper provides a critical review of sample collection, storage and treatment procedures for the determination of phosphorus species in environmental matrices. Issues such as phosphorus speciation, the molybdenum blue method, digestion procedures for organic phosphorus species, choice of model compounds for analytical studies, quality assurance and the availability of environmental CRMs for phosphate are also discussed in detail.

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Keywords: Phosphorus; Natural waters; Soils; Sampling; Sample treatment; Sample digestion; Quality assurance

1. Introduction

The determination of phosphorus species in environmental matrices provides essential data for assessing the health of ecosystems, investigating biogeochemical processes and monitoring compliance with legislation. At the catchment scale, for example, phosphorus export from both point and diffuse sources can result in increased primary production and eutrophication, with the potential for seasonal development of toxic algal blooms, which can have a major impact on global water quality [1]. For accurate measurements, knowledge of phosphorus speciation is required as environmental behaviour is often critically dependent on its physico-chemical form. In aquatic systems, for example, phosphorus species are found in “dissolved”, “colloidal” and “particulate” fractions, as inorganic and organic compounds and in biotic and abiotic particles [2]. The common operationally defined aquatic forms of phosphorus and the various terms used to describe them are shown schematically in Fig. 1. The reliability and comparability of data for any of these fractions will depend on the operational protocols used and the accuracy of the method.

Most manual and automated methods of phosphorus determination are based on the reaction of phosphate with an acidified molybdate reagent to yield phosphomolybdate heteropolyacid, which is then reduced to an intensely coloured
blue compound and determined spectrophotometrically [3].

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

$$\text{H}_3\text{PO}_4(\text{MoO}_3)_{12} + \text{reducing agent} \rightarrow \text{phosphomolybdenum blue} \ [\text{Mo(VI)} \rightarrow \text{Mo(V)}]$$

There are many modifications of the original Murphy and Riley method [4], particularly the use of different reductants (e.g. ascorbic acid, tin(II) chloride) and acid strengths. As shown in the above reaction scheme, the phosphomolybdenum blue complex is formed in an acidic environment and its absorbance spectrum is dependent on the acidity, type of reductant and phosphate concentration. Under low acidity conditions, for example, non-linear colour development [5] and non-phosphate sensitized reduction (self-reduction of the molybdate) can occur. A variety of $[\text{H}^+]/[\text{MoO}_4^{2-}]$ ratios have been reported in the literature, with a ratio of 70 and a pH range of 0.57–0.88 suggested for optimum sensitivity (maximum rate of colour formation) [6].

Ascorbic acid and tin(II) chloride are the most commonly used reductants when determining phosphate concentrations in natural waters. Ascorbic acid acts as a 2-electron reductant [7] with the major advantages being that it is less salt sensitive and colour development is fairly independent of temperature [6]. Ascorbic acid on its own however has the major disadvantage of slow colour development [8], but the addition of antimony as a catalyst increases the rate of reduction of the complex [4]. Using tin(II) chloride generates a product with a wavelength maximum at 690–700 nm as compared with 882 nm for ascorbic acid [2]. This allows greater sensitivity when a solid state detector (using a red light emitting diode light source) is used [9]. However, disadvantages include unstable colour development, a considerable salt error, temperature dependence and unsatisfactory performance at high phosphorus concentrations [10].

Interferences in the formation of the phosphomolybdenum blue complex include arsenate, silicate, chromium, copper, nitrite, nitrate and sulphide [11]. However, arsenate interferences can be eliminated by reducing As(V) to As(III) prior to measurement [6], e.g. by the addition of sodium thiosulphate [12]. The acid/molybdate ratio can be altered to enhance the selectivity for phosphate relative to silicate [4]. In addition, use of an appropriate extraction solvent, e.g. n-butanol, is an efficient way of eliminating interference from silicate [13].

The phosphorus determined in the filtered fraction using the above reaction is defined as "molybdate reactive" phosphorus (MRP) or dissolved reactive phosphorus (DRP). It has also been called soluble reactive phosphorus (SRP) and filterable reactive phosphorus (FRP). However, this method also determines acid labile phosphorus containing compounds (organic and condensed phosphorus species) which can lead to overestimation of free phosphate [3,6]. Similar problems have been reported in the determination of total reactive (unfiltered) phosphorus (TRP) [3]. Methods have been developed to minimise this overestimation including a critical timing technique ('the 6 second method') in which the acid strength is adjusted prior to the formation of the complex [14] and complexing excess molybdate with a citrate–arsenate reagent [15]. Phosphorus containing organic compounds and con-
2. Natural waters

Phosphorus concentrations in natural waters fluctuate with changes in physico-chemical conditions and biological activity. In chalk-based catchments, for example, phosphorus is influenced by seasonal fluctuations in pH, dissolved carbon dioxide and total dissolved calcium concentrations [16]. Hydrological conditions also play an important role in aquatic phosphorus concentrations. The majority of phosphorus transport to catchments, from both diffuse and point sources occurs during short periods of increased discharge (e.g. storm events) [17,18], which demonstrates the importance of high temporal resolution monitoring during such events. Submersible or field-based instrumentation is desirable for monitoring dissolved phosphorus because it eliminates the need for sample collection and storage and, although such instrumentation is available [19,20], it is not used on a routine basis. Therefore, a comprehensive and effective sampling, sample treatment and analysis protocol must be adopted in order to minimise the physical, chemical and biological processes that can alter the physico-chemical forms of phosphorus during storage.

2.1. Sampling protocol

It is essential that the scientific objectives (e.g. determining bioavailable phosphorus, measuring seasonal phosphorus loads), safety issues and budgetary constraints are clearly identified prior to undertaking any sampling programme. Having established the scope of the exercise, an essential requirement of any sampling protocol is for the sample to be representative of the body of water from which it originates. It is therefore essential to adopt a well-organized protocol, which retains, as closely as possible, the original composition of the water body of interest. The protocol should be kept as simple as possible while minimizing the possibility of contamination or interferences. In rivers and streams, for example, samples should be collected from the water column at a series of depths and cross-sectional locations as individual grab samples or through the use of automated samplers for times series acquisition. Monitoring stations can be constructed to provide high quality supporting data (e.g. pH, dissolved oxygen, temperature, turbidity) in a judicious fashion via data acquisition/telemetry technology. It is also vital to avoid boundary areas, e.g. at the confluence of streams or rivers and below sewage treatment works, unless their impact on the system is being investigated. Point source phosphorus contributions from sewage treatment works, for example, can have a major affect on the overall water quality of freshwater systems [21]. Globally, phosphorus loading into receiving waters still occurs even though tertiary treatment measures (e.g. based on the reduction of phosphate by precipitation with iron chloride) are being implemented in some countries [22]. Other water bodies pose additional complications and these must be considered when designing a sampling protocol. In lakes and reservoirs, representative sampling is often difficult due to environmental heterogeneity, both spatial and temporal (e.g. seasonal thermal stratification). In order to study biogeochemical cycling in stratified water bodies appropriate depth profiling is required. For a complete study high spatial resolution sampling at the sediment–water interface is also essential but is not discussed further in this paper.

Location and frequency must also be considered when designing a sampling protocol. Site selection will ultimately depend on the problem to be addressed and safety and accessibility are of paramount importance. The frequency of sampling, from continuous to seasonal, will depend on the scientific objectives but will often be constrained by cost. For example, the highest phosphorus loadings in rivers and streams are generally correlated with intense, short-term discharges during autumn and winter months, while the lowest loadings occur in the summer months when discharge is low and biological activity is high [23,24]. In-water processes that affect phosphorus concentrations that must also be considered include plant, algal and bacterial turnover, anthropogenic inputs (e.g. sewage effluent), matrix considerations (e.g. water hardness) and resuspension of bottom sediments from increasing river discharge [21,25].

Prior to any sampling campaign it is essential to adopt an efficient cleaning protocol for all sampling equipment and storage bottles and continue this throughout the study. The walls of sample containers, for example, are excellent substrates for bacterial growth and therefore rigorous cleaning of all laboratory ware is necessary. For phosphate determination, it is recommended that containers be cleaned overnight with a nutrient free detergent, rinsed with ultrapure water, soaked in 10% HCl overnight, and then rinsed again with ultrapure water [26]. Containers should be rinsed at least twice with the water of interest prior to sample collection. In addition, sampling blanks should be taken to monitor and control the sampling process.

2.2. Sample preservation and storage

The overall effectiveness of any sample preservation and storage protocol depends on various factors including the nature of the sample matrix, cleaning procedures for sample containers, container material and size, temperature, chemical treatment (e.g. addition of chloroform) and physical treatment (e.g. filtration, irradiation of sample and pasteurization) [27–29].

Preliminary treatment often involves filtration which differentiates between the dissolved phase (operationally defined as that fraction which passes through a 0.45 or 0.2 μm filter) and suspended matter (that fraction collected on the filter) [30]. It is essential that filtration is carried out im-
immediately after the sample is collected to prevent short-term changes in phosphorus speciation. Polycarbonate or cellulose acetate membrane filters are recommended for dissolved constituents in natural waters [31]. Filtration with a 0.2 µm filter is preferred as it removes the majority of bacteria and plankton that would otherwise alter dissolved phosphorus concentrations during storage [30]. It should be stressed however that some bacteria, as well as viruses, will pass through a 0.2 µm filter. As with sample containers, the filtration apparatus (including individual filters) must be cleaned prior to use with a similar acid wash/ultrapure water rinse procedure. The filtration procedure can be conducted under positive pressure or vacuum. However, excessive pressure gradients should be avoided as rupture of algal cells and the subsequent release of intracellular contents into the sample could occur. In samples of high turbidity it is important to minimise the sample loading to prevent clogging of filter pores.

Table 1 shows a summary of reported storage/preservation methods for phosphorus determination. Physical (i.e. refrigeration, freezing and deep-freezing) and chemical (i.e. addition of chloroform, mercuric chloride and acidification) preservation techniques have been used to help maintain the original phosphorus concentration during storage. It should be noted however that the use of chloroform is now discouraged in some countries because of toxicological risks. In addition, a variety of sample containers have been used including quartz, borosilicate glass, polyethylene, polypropylene, high-density polyethylene (HDPE) and polytetrafluoroethylene (PTFE).

For phosphorus determinations, however, it is difficult to select a generic treatment protocol due to the different effects of specific matrix characteristics (e.g., phosphorus concentration, hardness, salinity, dissolved organic matter and bacterial nutrient uptake) of the sampling location. In chalk catchments, for example, studies have shown that freezing samples is not the best treatment due to the possibility of phosphate being coprecipitated with calcite when thawing the samples [26,46]. Fig. 2a demonstrates this effect, showing an immediate (after 1 day) and continuing (up to 250 days) decrease in DRP concentration in samples analysed for phosphate after storage at −20 °C [26]. Storage at 4 °C is therefore recommended, together with the addition of chloroform to prevent biological growth. However, chloroform should not be used in samples with high organic matter content, as the release of cellular enzymes into the samples is possible [26]. Other studies have recommended immediate analysis after sampling [47] or analysis after a short storage period at 4 °C in the dark (maximum 48 h) [48–51].

In contrast to the extensive studies on phosphate stability during storage, the stability of dissolved organic phosphorus (DOP), as operationally defined, has not been widely studied. Fig. 2b–d show the stability of DOP (strictly this includes all acid hydrolysable phosphorus because acidic digestion conditions were used) from natural water samples (salinities 0, 14 and 32, respectively) over 32 days of storage. The DRP concentration on day 0 (1.17, 1.31 and 0.54 µM for salinities 0, 14 and 32, respectively) was subtracted from all results, which were based on sampling, autoclaving of sub-samples and storage of autoclaved and non-autoclaved sub-samples for subsequent analysis. They showed that there were no significant differences in DOP concentration if the samples were stored at −20 °C, autoclaved and analysed on the same day or if they were autoclaved immediately after collection and stored until analysed. The same trend (not shown) was also observed with phytic acid spiked (1.11, 1.50 and 0.45 µM for salinities 0, 14 and 32, respectively) standards and samples. These results suggest that storage at −20 °C is suitable for DOP determination but the final result is dependent on a reliable determination of the original DRP concentration. Freezing as a method for storage of unfiltered and filtered

![Fig. 2. Changes in the concentration of phosphorus species in natural water samples stored over time. (a) An immediate sharp decrease in DRP concentration in samples stored at −20 °C, followed by a gradual decrease over 250 days of storage. (b–d) The stability of DOP in natural water samples (salinities 0, 14 and 32, respectively) over 32 days of storage at −20 °C. A Day 0 sample is autoclaved on day 0 then stored until analysis, and a Day x sample is stored without treatment then autoclaved and analysed on day x. The dotted lines in each figure (solid lines in (a)) represent ±2 s of the measured DRP/DOP concentrations on day 0 (i.e. immediately after collection).](image-url)
<table>
<thead>
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<th>Phosphorus species</th>
<th>Matrix</th>
<th>Storage method</th>
<th>Maximum storage time</th>
<th>Comments</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>FRP</strong></td>
<td>Distilled, tap and lake water</td>
<td>Refrigerator (4 °C)</td>
<td>1 day</td>
<td>Polypropylene and polycarbonate containers suitable for storage. Glass containers sorbed phosphorus within 1–6 h</td>
<td>[32]</td>
</tr>
<tr>
<td>FRP</td>
<td>Standards added to rain water</td>
<td>Room temperature with HgCl₂ (0–50 mg L⁻¹)</td>
<td>3 days</td>
<td>HgCl₂ interfered with method when ascorbic acid was used as a reducing agent</td>
<td>[33]</td>
</tr>
<tr>
<td>FRP</td>
<td>River water</td>
<td>−10, 4, 20 °C with/without thymol (0.01%), KF (0.01%), TBT (0.01%), H₂SO₄ (0.05 M) or CHCl₃ (5 mL L⁻¹)</td>
<td>14 days</td>
<td>Samples showed no decrease in FRP if chloriform added and samples stored at 4 °C</td>
<td>[34]</td>
</tr>
<tr>
<td>FRP, TP</td>
<td>Open ocean water</td>
<td>Frozen (quick and slow), cooled (2 °C) with/without HgCl₂ (120 mg L⁻¹), phenol (4 mg L⁻¹) and acid (pH 5)</td>
<td>60 days</td>
<td>No significant change in TP concentration when samples frozen with/without acid</td>
<td>[35]</td>
</tr>
<tr>
<td>FRP, TP</td>
<td>Coastal and estuarine waters</td>
<td>−10 °C, slow and quick freezing</td>
<td>365 days</td>
<td>Small change in FRP when samples were frozen. Quick freezing reduced losses</td>
<td>[36]</td>
</tr>
<tr>
<td>FRP, TP</td>
<td>Tap, lake and river waters</td>
<td>Room temperature, 4 °C, with the addition of HgCl₂ (40 mg L⁻¹), H₂SO₄ (0.05 M) and chloroform</td>
<td>16 days</td>
<td>Chloroform at 4 °C was suitable for only 8 days. No significant decreases in concentration (up to day 16) were shown in samples with HgCl₂ stored at 4 °C</td>
<td>[37]</td>
</tr>
<tr>
<td>FRP</td>
<td>Sea water</td>
<td>Frozen at −40 °C initially, then stored at −20 °C</td>
<td>147–210 days</td>
<td>FRP concentration decreased in samples stored longer than 4 months</td>
<td>[38]</td>
</tr>
<tr>
<td>TP, TDP, FRP and TRP</td>
<td>Lake water</td>
<td>Refrigerator (4 °C)</td>
<td>180 days</td>
<td>No change in TP in samples for up to 6 months</td>
<td>[39]</td>
</tr>
<tr>
<td>FRP</td>
<td>Stream water</td>
<td>Frozen at −16 °C</td>
<td>4–8 years</td>
<td>No significant change in FRP concentration</td>
<td>[40]</td>
</tr>
<tr>
<td>FRP</td>
<td>Soil leachates</td>
<td>Room temperature (5–19 °C), refrigeration (4 °C) frozen (−20 °C) with/without HgCl₂ (40–400 mg L⁻¹) and H₂SO₄ (0.05 M), chloroform</td>
<td>1–2 days</td>
<td>Changes occurred within 2 days for all samples with smallest changes in samples stored at room temperature or 4 °C</td>
<td>[41]</td>
</tr>
<tr>
<td>FRP</td>
<td>Sea water</td>
<td>Pasteurization and stored at room temperature</td>
<td>18 months</td>
<td>FRP remained constant for 1 year. NH₄ losses after 3 days</td>
<td>[42]</td>
</tr>
<tr>
<td>FRP, TP</td>
<td>Stream water</td>
<td>Refrigerator (4 °C), H₂SO₄ (0.05 M), freezing with dry ice and subsequent analysis</td>
<td>8 days</td>
<td>Minimal change observed in highly concentrated (FRP &gt; 1 mg L⁻¹) samples (1–3% loss after 8 days). 47% loss in FRP in lower concentrated samples</td>
<td>[43]</td>
</tr>
<tr>
<td>FRP</td>
<td>River water (chalk-based catchment), estuarine water (salinities of 0.5, 10 and 35)</td>
<td>Refrigerator (4 °C) with/without 0.1% (v/v) chloroform, −20 °C with/without 0.1% ( v/v) chloroform, −80 °C without chloroform</td>
<td>247 days</td>
<td>For chalk-based samples, 4 °C with 0.1% (v/v) chloroform was the best treatment. Freezing is not recommended due to coprecipitation of inorganic phosphate with calcite</td>
<td>[26]</td>
</tr>
<tr>
<td>TP</td>
<td>River and canal water</td>
<td>Room temperature, refrigerator (4 °C) treatment to a pH of &lt; 2 with H₂SO₄</td>
<td>28 days</td>
<td>No significant losses in TP concentration over the 28 day period for treated samples at 4 °C. No losses up to 7 days for room temperature (acidified) samples</td>
<td>[44]</td>
</tr>
<tr>
<td>FRP</td>
<td>Water extracts of poultry litter</td>
<td>Room temperature, freezing (−16 to −15 °C)</td>
<td>8 days</td>
<td>No significant losses in FRP concentration in samples stored at room temperature (up to 8 days). Freezing samples lowered concentration (up to 46%) for the 8 day period</td>
<td>[45]</td>
</tr>
</tbody>
</table>
samples for the determination of total and dissolved organic phosphorus has also been recommended by other workers [39,52–53].

3. Soils

Soil pre-treatment and storage can induce marked changes in the solubility of chemicals and therefore presents a critical control on subsequent analysis. This section focuses on phosphorus but it also has wider relevance for other elements. For example, water-extractable phosphorus is markedly influenced by even mild drying of soil. It has been known for some time that soil drying can render considerable concentrations of organic carbon soluble in water [54] and a similar effect was recently reported for phosphorus in a wide range of pasture soils from England and Wales [55]. In the latter study, 7 days air drying from approx. field moisture capacity at 30°C increased concentrations of water-extractable organic phosphorus by up to 1900%. Organic phosphorus accounted for up to 100% of the solubilized phosphorus. This was at least partly derived from microbial cells, because a strong correlation existed between solubilized organic phosphorus and microbial phosphorus (Fig. 3). It has been reported that rapid rehydration can kill between 17 and 58% of soil microorganisms through osmotic shock and cell rupture [56] and the contribution of microbial lysis has been subsequently confirmed by direct bacterial cell counting in rewetted Australian pasture soils [57].

In addition to microbial lysis, the physical stresses induced by soil drying also disrupt organic matter coatings on clay and mineral surfaces [58], which may contribute to the solubilisation of both inorganic and organic phosphorus. Indeed, functional classification of water-extractable organic phosphorus from dry Australian pasture soils revealed similar proportions of microbially derived phosphate diesters and phytic acid from the non-biomass soil organic matter [59]. A similar mechanism probably occurs following freezing and thawing [60]. Such processes probably explain the increases in phosphorus extractable in bicarbonate following soil drying [61] because the high ionic strength of bicarbonate solution may reduce the degree of osmotic stress and associated lysis of viable cells compared to extraction with water [62]. The hypothesis that non-biomass organic phosphorus dominates in bicarbonate extracts is supported by the speciation of phosphorus in such extracts, which is dominated by phosphate monooesters and is, therefore, similar to the whole-soil organic phosphorus extracted in strong alkaline solution [63,64].

The mechanisms by which soil drying could affect the solubility of non-biomass inorganic and organic phosphorus are poorly understood, but probably include both physical and chemical changes. Rapid rehydration of dry soils commonly causes aggregate breakdown [65], which increases the surface area for desorption by exposing surfaces and associated phosphorus protected within aggregates [66]. Such a process has been linked to increases in resin-extractable inorganic phosphorus following soil drying [67]. A more likely process is disruption of organic matter coatings on clay and mineral surfaces by the physical stresses induced during soil drying. This increases organic matter solubility and exposes formerly protected mineral surfaces, and has been attributed to increases in oxalate-extractable silica of up to 200% following drying of Swedish spodic B horizons [68]. Soil drying also increases the crystallinity of pure iron and aluminium oxides, which reduces the specific surface area and phosphorus sorption capacity of these minerals [69]. However, this is inconsistent with reports of increased sorption capacity of dried soils for phosphate and sulphate [70,71].

The effect of drying on phosphorus solubility does not appear to be consistent for all soils. In particular, phosphorus solubility in high organic matter soils may decrease following drying. For example, Schlichting and Leinweber [72] reported that phosphorus recovery from a German peat (pH 5.6) by a sequential fractionation procedure was markedly reduced by pre-treatment, including air-drying, freezing and lyophilization. The greatest reduction in phosphorus recovery followed lyophilization (phosphorus recovery was 75% when extracted fresh, compared with <50% from lyophilized samples) and even after storage at 4°C for 3 weeks detectable changes were still observed.

The importance of specific artefacts that result from soil pre-treatments will vary depending on the study objectives. For example, assessment of plant-available phosphorus for fertilizer requirements is based on analysis of air-dried soils for practical reasons although field-fresh soils are needed to obtain meaningful data. This is impractical for most purposes, although refrigeration may be an acceptable alternative [58]. In this respect, there is a clear requirement for a detailed study of changes in phosphorus solubility during cold storage for several different soil types. Environmental soil phosphorus tests that involve water extraction routinely use air-dried soils and only measure inorganic phosphorus (e.g. [73,74]). The results of these tests will clearly vary depending on the moisture status of the soil prior to extraction and on the inclusion (or not) of organic phosphorus. If organic phosphorus is included in such tests,

Fig. 3. The increase in water-soluble organic phosphorus after soil drying as a function of soil microbial phosphorus in a wide range of permanent pasture soils from England and Wales. Water-soluble phosphorus was determined by extracting soils at field moisture capacity with water in a 4:1 water:soil ratio for 1 h. Sub-samples were air-dried for 7 days at 30°C and extracted in an identical manner. Adapted from [57].
the standardization of soil moisture prior to extraction will be necessary. For details of extraction procedures for soil organic phosphorus see Turner et al. (this issue).

4. Digestion techniques

Digestion techniques for environmental samples are necessary for the determination of total phosphorus (TP) and total dissolved phosphorus (TDP). This is because many of the phosphorus species present contain P-O-P, C-O-P, and C-P bonds that need to be broken down to release phosphorus as phosphate, which can then be determined using molybdenum blue chemistry [4]. The digestion technique must also be able to release phosphorus from biological material, e.g. algal cells and plant detritus and adsorbed/occluded P from sediments [75]. Traditional methods of digestion for natural water samples include fusion, dry ashing, perchloric acid, sulphuric acid–nitric acid and boiling on a hot plate, with more recent methods generally using autoclaving, UV photolysis and microwave heating [75]. UV photo-oxidation can be used for organic phosphorus compounds in marine and freshwater samples [47, 76, 77] but condensed polyphosphates present in the sample will not be broken down by UV photolysis alone [2, 3, 78, 79] and also need to be heated to 90–120°C in the presence of acid [75]. To ensure that all polyphosphates present in the sample are decomposed, either boiling with HCl or potassium peroxydisulfate after UV irradiation is therefore recommended [80]. McKelvie et al. used an on-line UV photo-oxidation flow injection (FI) technique and found that results were comparable with a batch peroxydisulfate method [81].

Autoclaving methods are generally straightforward, give reproducible results and use sealed vessels that are less prone to contamination [75, 82–84]. The following section is therefore a summary of different autoclaving techniques, combined with peroxydisulfate in either an acidic or alkaline media, for the determination of phosphorus in natural waters, soil solutions and sediments (see Table 2). Most methods described in Table 2 are based on spectrophotometric detection but ICP-MS and ICP-AES have, in recent years, been used to determine phosphorus in agricultural runoff waters and soils and results were comparable with spectrophotometric methods [128, 129]. In addition, microwave digestion combined with ICP-MS detection has been used to determine phosphorus in marine environmental samples and plant leaves with good recoveries [130–132]. However microwave heating for batch sample digestion and in FI systems with spectrophotometric detection for on-line TDP and TP digestion [3] is less widely used than UV photo-oxidation or autoclaving.

4.1. Autoclaving

4.1.1. Alkaline peroxydisulfate

Menzel and Corwin first used autoclaving with peroxydisulfate in 1965 for the digestion of seawater samples [88]. Koroleff developed an alkaline peroxydisulfate alternative in 1969 [102], which was then slightly modified [101] and simplified by introducing a borate buffer [85]. This enabled the simultaneous determination of TP and total nitrogen (TN), as nitrogen bonds are only hydrolysed/oxidised in alkaline media [98]. Using a borate buffer, the pH is alkaline (ca. 9.7) at the start of the digestion process and becomes acidic (pH 4–5) as the sodium hydroxide decomposes [78, 90, 98]. Hosomi and Sudo also reported that pH change was important and in their method the pH decreased from 12.8 to 2.0–2.1 to ensure that even condensed polyphosphates were digested [92].

The alkaline method has also been used for particulate material but with relatively poor recoveries [133]. For example orchard leaves gave recoveries of 80–90% for TP and TN [91]. Higher recoveries can be obtained by decreasing the ratio of sample to peroxydisulfate [92]. Alkaline digestion of model phosphorus compounds has been found to be efficient for turbid water samples [125–127] although the concentration of suspended particulate material needs to be diluted to <150 mg L⁻¹ and difficulties can arise when this material is of soil origin rather than biological origin, e.g. algal cells and plant detritus. The alkaline method has therefore been used to determine TP in turbid lake waters and suspensions of particulate material [127].

Alkaline peroxydisulfate autoclaving, rather than acid peroxydisulfate, is recommended for the digestion of marine waters. This is because in the acid method, peroxydisulfate oxidises the chloride in seawater to free chlorine, thus reducing the oxidising power of the peroxydisulfate [104]. It is also recommended for the simultaneous determination of TP and TN.

4.1.2. Acid peroxydisulfate

An acid peroxydisulfate method developed by Gales et al. [134] has been adopted by the US Environmental Protection Agency [135]. Eisenreich et al. simplified the method [96] and various modifications of this approach are now used to digest different types of samples such as soil solutions, natural waters and river water [18, 97, 121]. The alkaline peroxydisulfate method for soil extracts is only appropriate if the total organic carbon concentration is <100 mg L⁻¹ and manganese is <1 mg L⁻¹. Above this manganese concentration, coloured solutions or precipitates are formed, which interfere with the digestion step [111]. This interference is avoided when using acid peroxydisulfate and solutions are colourless after digestion [84].

Pote et al. described standard methods for the determination of TP and TDP using sulphuric acid–nitric acid and peroxydisulfate digestions [136] and recommended the use of sulphuric acid–nitric acid digestion to achieve good recoveries for most samples. However this digestion method can be potentially dangerous if salts precipitate during digestion [89] and is less easy to control than the peroxydisulfate method [84, 122]. Rowland and Haygarth compared a mild peroxydisulfate method to the more rigorous sulphuric acid–nitric
Table 2
Acidic and alkaline peroxydisulfate autoclave digestion methods

<table>
<thead>
<tr>
<th>Matrix</th>
<th>Digestion reactant</th>
<th>Digestion time</th>
<th>Digestion temperature (°C)</th>
<th>pH</th>
<th>Model compounds(^a)</th>
<th>Comments</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Drainage waters</td>
<td>Digestion reagent: 5 g K_2S_2O_8 and 5 mL 4.5 M H_2SO_4 in 100 mL distilled deionised water; 4 mL reagent added to 50 mL sample</td>
<td>30 min</td>
<td>115</td>
<td>Not reported</td>
<td>Not reported</td>
<td>Same method as [85]</td>
<td>[86]</td>
</tr>
<tr>
<td>Drainage waters</td>
<td>0.15 g K_2S_2O_8 and 1 mL 0.5 M H_2SO_4 added to 20 mL sample</td>
<td>1 h</td>
<td>120</td>
<td>Not reported</td>
<td>Not reported</td>
<td>Same method as [84]</td>
<td>[87]</td>
</tr>
<tr>
<td>Estuarine waters</td>
<td>Acidic peroxydisulfate digestion reagent: 5 g K_2S_2O_8 and 5 mL 4.5 M H_2SO_4 in 100 mL distilled deionised water; 4 mL reagent added to 50 mL sample</td>
<td>1 h</td>
<td>120</td>
<td>Final pH 1.5–1.8</td>
<td>Orthophosphate, phenylphosphoric acid, phenylphosphorous acid</td>
<td>Same method as [88], but autoclaving time was increased from 30 min to 1 h. Quantitative recovery for model compounds at the 50 μg P level</td>
<td>[89]</td>
</tr>
<tr>
<td>Fresh and seawater</td>
<td>Acidic peroxydisulfate digestion reagent: 5 g K_2S_2O_8 and 5 mL 4.5 M H_2SO_4 in 100 mL distilled deionised water; 4 mL reagent added to 50 mL sample</td>
<td>30 min</td>
<td>115</td>
<td>For alkaline method, initial pH ca. 9.7, final pH 4–5</td>
<td>Model compounds added to demineralised water and seawater: S'-ATP-Na (_2) (99–100%), PTA (100, 70, 101, 95%), 5'-GMP-Na (_2) (99, 93, 100, 94%), PC (98, 37, 99, 96%), FMN (99, 99, 100, 97%), G-6-P-Na (100, 95, 101, 92%), AMP (99, 94, 100, 93%), RP (100, 94, 103, 95%), PEP-3CHA (100, 100, 101, 101%), β-GLY (99, 100, 100, 76%)</td>
<td>Recoveries in parentheses are in order: acidic demineralised water, acidic seawater, alkaline demineralised water, alkaline seawater. Acidic and alkaline peroxydisulfate methods [85] compared to continuous flow UV irradiation and high temperature combustion. Alkaline peroxydisulfate method recommended for marine waters</td>
<td>[90]</td>
</tr>
<tr>
<td>Fresh waters</td>
<td>Digestion reagent: 40 g K_2S_2O_8 and 9 g NaOH in 1 L distilled water; 5 mL reagent added to 10 mL sample</td>
<td>1 h</td>
<td>120</td>
<td>Initial pH 12.8, final pH 2.0–2.1</td>
<td>National Bureau of Standard Reference Material 1571 orchard leaves (98%), National Institute of Environmental Studies (NIES) Reference Material No. 1 pepper bush (96%), NIES Reference Material No. 2 pond sediment (100%), NIES Reference Material No. 3 chlorella (100%) all of concentration 50 mg L(^{-1}). Model compounds: S'-ATP-Na (_2) (99–100%), 5'-ADP-Na (_2) (98%), TSPP (99–100%), SHMP (94–97%), STP (96–97%), G-6-P-K (_2) (99–102%)</td>
<td>Analysed for TN and TP. Obtained higher recoveries for orchard leaves than [91]</td>
<td>[92]</td>
</tr>
<tr>
<td>Fresh waters</td>
<td>1 g K_2S_2O_8 and sufficient H_2SO_4 to make the sample 0.15 M acid</td>
<td>2 h</td>
<td>120</td>
<td>Not reported</td>
<td>Not reported</td>
<td>Compared UV digestion to autoclaving. Recoveries for lake water samples were 100% for the peroxydisulfate digestion and 97% for the UV digestion</td>
<td>[93]</td>
</tr>
<tr>
<td>Lake waters</td>
<td>&quot;Strong&quot; acid: 25 mL 18 M H_2SO_4 and 1 mL 18 M HNO(_3) in 1 L deionised water; 3 mL &quot;strong&quot; acid and 2.5 mL aqueous 4% (w/v) K_2S_2O_8 added to 25 mL sample</td>
<td>30 min</td>
<td>120</td>
<td>Not reported, however in the UV digestion, sample maintained at 85°C in the silica coil</td>
<td>Dipotassium hydrogenophosphate (100%), STP (100%), AMP (100%)</td>
<td>Compared UV digestion to autoclaving. Recoveries for lake water samples were 100% for the peroxydisulfate digestion and 97% for the UV digestion</td>
<td>[94]</td>
</tr>
</tbody>
</table>
Table 2 (Continued)

<table>
<thead>
<tr>
<th>Matrix</th>
<th>Digestion reagent</th>
<th>Digestion time</th>
<th>Digestion temperature (°C)</th>
<th>pH</th>
<th>Model compoundsa</th>
<th>Comments</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lake, river and pond waters, raw sewage</td>
<td>Digestion reagent: 55 mL H$_2$SO$_4$ and 60 g K$_2$S$_2$O$_5$ in 1 L solution. 2.5 mL reagent added to 35 mL sample</td>
<td>1 h</td>
<td>Not reported</td>
<td>Not reported</td>
<td>G-1-P-K$_2$ (97.5%), G-6-P-K$_2$ (105%), DNA (sodium salt) (115%), AMP (95%), S'ADP-Na$_2$ (102.5%), SOP (100%), β-GLY (107.5%), TSPP (62.5%), STP (110%), SHMP (100%), disodium hydrogen orthophosphate (97.5%)</td>
<td>Autoclave method was compared to the hot-plate digestion. Autoclave method gave more precise values for model compounds than the hot-plate procedure</td>
<td>[95]</td>
</tr>
<tr>
<td>Natural waters</td>
<td>Digestion reagent: 0.15 g K$_2$S$_2$O$_5$ and 1 mL 0.5 M H$_2$SO$_4$, 1 mL reagent added to 20 mL sample</td>
<td>45 min</td>
<td>121 C</td>
<td>Not reported</td>
<td>G-1-P (101.0%), G-6-P (103.1%), ATP (101.6%), NPP (101.9%), eAMP (101.8%), α-GLY (102.3%), myo-inositol 2-monophosphate (97.4%), PTA (85.6%), 2-AEP (99.2%), TSPN (99.5%), STP (97.7%), trisodium trimetaphosphate (98.8%), KHP (99.1%)</td>
<td>Method modified from</td>
<td>[97]</td>
</tr>
<tr>
<td>Natural waters</td>
<td>Acidic peroxydisulfate digestion reagent: 5 g K$_2$S$_2$O$_5$ and 5 mL 4.5 M H$_2$SO$_4$ in 100 mL distilled deionised water. 0.8 mL digestion reagent added to 10 mL sample. Alkaline peroxydisulfate digestion reagent: 50 g K$_2$S$_2$O$_5$, 30 g H$_3$BO$_3$ and 350 mL NaOH in 1 L distilled deionised water. 1.3 mL digestion reagent added to 10 mL sample</td>
<td>30 min</td>
<td>120 C</td>
<td>For alkaline method, initial pH ca. 9.7, final pH 4.5</td>
<td>Compared acidic peroxydisulfate [85] and alkaline peroxydisulfate [98] autoclaving methods with magnesium nitrate high-temperature oxidation, magnesium peroxydisulfate high-temperature oxidation, and UV oxidation. Magnesium nitrate high-temperature oxidation was found to be the best method Analyzed for TN and TP.</td>
<td>[78]</td>
<td></td>
</tr>
<tr>
<td>Orchard leaves and aufwuchs</td>
<td>Digestion reagent: 13.4 g K$_2$S$_2$O$_5$ and 6 g NaOH in 1 L to give 200 mg peroxydisulfate per 15 mL aliquot. Other levels of peroxydisulfate also used (300, 400 and 500 mg)</td>
<td>1 h</td>
<td>100-110</td>
<td>Initial pH 12.00 for orchard leaf samples, final pH 2.5. Initial pH 12.8 for aufwuchs samples, final pH 3.7</td>
<td>National Bureau of Standards reference material 1571 (orchard leaf) (86.9-88.7% using 500 mg peroxydisulfate) and aufwuchs (93.6% using 300 mg peroxydisulfate, and 101.4% using 400 mg peroxydisulfate)</td>
<td>Maximum recovery for orchard leaf when 500 mg peroxydisulfate was used, and 300 or 400 mg peroxydisulfate for aufwuchs</td>
<td>[91]</td>
</tr>
<tr>
<td>Pond water</td>
<td>Acidic peroxydisulfate digestion: 0.5 g K$_2$S$_2$O$_5$ and 1 mL H$_3$SO$_4$ solution (300 mL cone: H$_2$SO$_4$ in 1 L distilled water) added to 50 mL sample. Alkaline peroxydisulfate digestion: 5 mL 0.075 N NaOH and 0.1 mg K$_2$S$_2$O$_5$ added to 10 mL sample. After digestion, 1 mL borate buffer (61.8 g H$_2$BO$_3$ and 8 g NaOH in 1 L distilled water) added</td>
<td>30 min</td>
<td>1.0</td>
<td>Not reported</td>
<td>Water samples spiked with 0.2 mg L$^{-1}$ KHP. Recoveries for acidic method were 88-113%, and for the alkaline method 85-112%</td>
<td>Acidic and alkaline peroxydisulfate methods same as [99]</td>
<td>[100]</td>
</tr>
</tbody>
</table>
### Table 2 (Continued)

<table>
<thead>
<tr>
<th>Matrix</th>
<th>Digestion reagent: 0.15 g K₂S₂O₈ and 1 mL 0.5 M H₂SO₄, 1 mL added to 20 mL sample</th>
<th>Digestion time</th>
<th>Digestion temperature (°C)</th>
<th>pH</th>
<th>Model compounds*</th>
<th>Comments</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>River water</td>
<td></td>
<td>45 min</td>
<td>121</td>
<td>Not reported</td>
<td>Not reported</td>
<td>Method modified from [96]</td>
<td>[18]</td>
</tr>
<tr>
<td>River water</td>
<td>Digestion reagent: 20 g K₂S₂O₈ and 3 g NaOH in 1 L distilled deionised water, 5 mL reagent added to 5 mL sample</td>
<td>30 min</td>
<td>120</td>
<td>Initial pH 12.57, final pH 2.0</td>
<td>KHP (99.6%), TSPP (97.2%), β-GLY (96.5%), SHMP (97.6%), G-1-P (99.5%), AMP (100.8%), ADP (98.9%), ATP (98.1%)</td>
<td>Results from this method were an improvement on the alkaline oxidation method for TN and TP of [101], which was in turn a modified method from [102]</td>
<td>[103]</td>
</tr>
<tr>
<td>Seawater</td>
<td>Two concentrations of K₂S₂O₈ added (4 and 40 mL mg L⁻¹) to 10 mL sample acidified with sulphuric acid to pH 3</td>
<td>90 min</td>
<td>125</td>
<td>pH 3</td>
<td>Not reported</td>
<td>Three methods compared: autoclaving acidic peroxydisulfate method based on [85], UV irradiation and sequential use of both. The latter method gave the best recoveries</td>
<td>[104]</td>
</tr>
<tr>
<td>Seawater</td>
<td>8 mL of 5% K₂S₂O₈ added to 50 mL seawater</td>
<td>30 min</td>
<td>120</td>
<td>Final pH 1.5–1.8</td>
<td>PFA (96.5%), 1-AEP (85.5%), 2-AEP (81.2%)</td>
<td>Compared their nitrate oxidation method with peroxydisulfate oxidation method from [88]</td>
<td>[105]</td>
</tr>
<tr>
<td>Seawater</td>
<td>Digestion reagent: 50 g K₂S₂O₈, 30 g H₂SO₄, 350 mL 1 M NaOH in 1 L distilled deionised water, 4 mL reagent added to 30 mL sample</td>
<td>30 min</td>
<td>110–115</td>
<td>Initial pH 9.7, final pH 5–6</td>
<td>KHP (0.25–7 μM)</td>
<td>Alkaline peroxydisulfate method for TP and TN based on [85]</td>
<td>[98]</td>
</tr>
<tr>
<td>Seawater</td>
<td>8 mL of 5% K₂S₂O₈ added to 50 mL seawater</td>
<td>30 min</td>
<td>120</td>
<td>Final pH 1.5–1.8</td>
<td>lecithin (10%), PC (9%), AMP (99%), zooplankton (100%)</td>
<td>Recoveries of model compounds relative to sulphuric acid-hydrogen peroxide digestion [106]</td>
<td>[88]</td>
</tr>
<tr>
<td>Sediments and soils</td>
<td>1 mL 5.5 M H₂SO₄, 0.4 g K₂S₂O₈ and 1 mL distilled deionised water added to 10–50 mg sample</td>
<td>1 h</td>
<td>130</td>
<td>Not reported</td>
<td>Not reported</td>
<td>Acid peroxydisulfate digestion compared to perchloric acid digestion</td>
<td>[107]</td>
</tr>
<tr>
<td>Sewage</td>
<td>Digestion reagent: 9 g NaOH and 40 g K₂S₂O₈ in 1 L distilled deionised water, 2 mL digestion reagent added to 10 mL sample</td>
<td>90 min</td>
<td>120</td>
<td>Not reported, however</td>
<td>Sodium dihydrogen phosphate (93% using KCl/acetate, 0.15 M KCl/acetate, STP buffer pH 4.5 (85% using 0.4 M KCl/acetate, TSPP (96% using 0.4 M KCl/acetate)</td>
<td>Anion exchange chromatography used to separate ortho- and poly-phosphates using either 0.15 or 0.4 M KCl/acetate as the eluting buffer. No polyphosphates detected in raw sewage samples</td>
<td>[108]</td>
</tr>
<tr>
<td>Soil extracts</td>
<td>Digestion reagent: 0.39 M K₂S₂O₈ and 0.6 M NaOH, 2 mL reagent added to 8 mL sample</td>
<td>1 h</td>
<td>120</td>
<td>Not reported</td>
<td>Not reported</td>
<td>Same method (La Chat method 30-115-001-1-B) as [109]</td>
<td>[110]</td>
</tr>
<tr>
<td>Soil extracts</td>
<td>Digestion reagent: 13.4 g K₂S₂O₈ dissolved in 1 L 0.3 M NaOH, 15 mL reagent added to 10 mL sample, made up to 50 mL after autoclaving</td>
<td>30 min</td>
<td>110</td>
<td>pH 2</td>
<td>KHP, PTA dodeca sodium salt (99% for 0.1 mg L⁻¹, and 106% for 1.0 mg L⁻¹)</td>
<td>Analysed for TN and TP. PTA dissolved in different extractants: water, 0.1 M CaCl₂, and 0.2 M H₂SO₄, and recoveries were comparable. Alkaline peroxydisulfate method appropriate for soil extracts when concentration of total organic carbon &lt;100 mg L⁻¹</td>
<td>[111]</td>
</tr>
<tr>
<td>Matrix</td>
<td>Digestion reactant</td>
<td>Digestion time</td>
<td>Digestion temperature (°C)</td>
<td>pH</td>
<td>Model compounds</td>
<td>Comments</td>
<td>Ref.</td>
</tr>
<tr>
<td>--------------------------</td>
<td>--------------------</td>
<td>----------------</td>
<td>-----------------------------</td>
<td>----</td>
<td>-----------------</td>
<td>--------------------------------------------------------</td>
<td>------</td>
</tr>
<tr>
<td>Soil leachate</td>
<td>0.15 g K$_2$S$_2$O$_5$ and 1 mL 0.5 M H$_2$SO$_4$ added to 20 mL sample</td>
<td>1 h</td>
<td>120</td>
<td>Not reported</td>
<td>Not reported</td>
<td>Same method as [84]</td>
<td>[112–116]</td>
</tr>
<tr>
<td>Soil leachate</td>
<td>8 mg K$_2$S$_2$O$_5$ and 50 μL 0.5 M H$_2$SO$_4$ added to 1 mL sample</td>
<td>1 h</td>
<td>120</td>
<td>Not reported</td>
<td>KHP (101%), PTA (76%), TSP (95%), STP, 1-AEP (86%), G-6-P-Mg (84%), 5'-AMP-Mg (96%)</td>
<td>Preconcentration and separation method for trace elements using a scaled down version of [84]</td>
<td>[117]</td>
</tr>
<tr>
<td>Soil solutions</td>
<td>Digestion reagent: 0.05 M H$_2$SO$_4$ and 16 g L$^{-1}$ K$_2$S$_2$O$_5$, 1 mL reagent added to 1 mL sample</td>
<td>30 min</td>
<td>110</td>
<td>Not reported</td>
<td>Not reported</td>
<td>Acid peroxysulfate digestion compared to sulphuric-perchloric acid, nitric, and nitric-perchloric acid digestion. Better recoveries were found for PTA using peroxysulfate method as [111]</td>
<td>[118]</td>
</tr>
<tr>
<td>Soil solutions</td>
<td>Digestion reagent: 50 mg K$_2$S$_2$O$_5$ and 0.1 mL 5.5 M H$_2$SO$_4$ added to 1 mL sample. After digestion, solutions diluted to 10 mL with deionised water</td>
<td>1 h</td>
<td>120</td>
<td>Not reported</td>
<td>KHP, PTA (93.2–95.0% in concentration range 3.23–32.26 μM)</td>
<td>Same method as [111]</td>
<td>[119]</td>
</tr>
<tr>
<td>Soil solutions</td>
<td>Digestion reagent: 13.4 g K$_2$S$_2$O$_5$ dissolved in 1 L 0.3 M NaOH, 15 mL reagent added to 10 mL sample. Added 1.5 mL 0.3 M HCl and made up to 50 mL after autoclaving</td>
<td>30 min</td>
<td>110</td>
<td>pH 2</td>
<td>Not reported</td>
<td>Method modified from [96]</td>
<td>[120]</td>
</tr>
<tr>
<td>Soil solutions</td>
<td>0.15 g K$_2$S$_2$O$_5$ and 1 mL 0.5 M H$_2$SO$_4$ added to 20 mL sample</td>
<td>45 min</td>
<td>121</td>
<td>Not reported</td>
<td>Not reported</td>
<td>Acidic method compared to peroxide-Kjeldahl, and nitric acid-sulphuric acid digestion [122]. Acidic peroxysulfate method found to be the best method</td>
<td>[84]</td>
</tr>
<tr>
<td>Soil solutions</td>
<td>0.15 g K$_2$S$_2$O$_5$ and 1 mL 0.5 M H$_2$SO$_4$ added to 20 mL sample</td>
<td>1 h</td>
<td>120</td>
<td>Not reported</td>
<td>PTA (89%), G-6-P-Mg (99%), 5'-AMP-Mg (96%), AMP (96%), KHP</td>
<td>Same method as peroxysulfate method in [99]</td>
<td>[123]</td>
</tr>
<tr>
<td>Surface runoff</td>
<td>0.5 g K$_2$S$_2$O$_5$ and 1 mL H$_2$SO$_4$ solution (300 mL conc. H$_2$SO$_4$ in 1 L distilled water) added to 50 mL sample</td>
<td>30 min</td>
<td>110</td>
<td>Not reported</td>
<td>Not reported</td>
<td>Compared alkaline peroxysulfate autoclaving method to microwave and hot-plate digestion and Kjeldahl digestion for TN and TP. Results showed that both methods were suitable for turbid lake samples when suspended material is of biological origin</td>
<td>[124]</td>
</tr>
<tr>
<td>Turbid lake and river waters</td>
<td>Optimum digestion reagent: 0.27 M K$_2$S$_2$O$_5$ and 0.24 M NaOH, 2 mL reagent added to 10 mL sample</td>
<td>1 h</td>
<td>120</td>
<td>Final pH 2</td>
<td>NIES No 3 Chlorella (99–101% up to 100 μg PL$^{-1}$) and No 2 Pond sediment (98–104% up to 60 μg PL$^{-1}$, and 88% at 100 μg PL$^{-1}$). Model compounds added to distilled and lake water: KHP, G-6-P (113%), PTA (101%), α-GLY (108%), PEP (103%), 2-AEP (104%), PFA (105%), α-phosphoryl ethanolamine (100%), SHMP (114%), aluminium phosphate (23%)</td>
<td>Compared alkaline peroxysulfate autoclaving method to microwave and hot-plate digestion and Kjeldahl digestion for TN and TP. Results showed that both methods were suitable for turbid lake samples when suspended material is of biological origin</td>
<td>[125]</td>
</tr>
</tbody>
</table>
acid method [122] for soil solutions and leachates. The latter method gave erratic recoveries and was more prone to contamination due to the open digestion vessels used [84]. Peroxydisulfate autoclaving is also safer than perchloric acid digestion [107,137]. The acid peroxidisulfate method generally gives good recoveries for model compounds and is simple and easy to use and is therefore recommended for TP and TDP determinations in natural waters and, particularly, soil solutions.

4.2. Model compounds

It is advisable to test the efficiency of any digestion method using a range of model phosphorus containing compounds that reflect different chemical bonds and stabilities and are representative of naturally occurring compounds (see Table 3). The majority of relevant compounds contain C–O–P and/or P–O–P bonds. Few compounds reported in the literature contain C–P bonds, which are very resistant to oxidation and hydrolysis [138].

Phosphonates are refractory organic phosphorus compounds and can be released into seawater from biological sources [78,90,139], and have been detected in soils [140] and soil leachate [117]. As phosphonates contain a strong C–P bond that is resistant to acid hydrolysis [139], they are useful compounds for recovery studies [78,90,97,125,126,139]. Condensed inorganic (e.g. sodium tripolyphosphate) and organic (e.g. adenosine-5′-triphosphate) phosphates and cocarboxylate [141] have also been shown to be resistant to UV irradiation alone [79]. With acid or alkaline peroxydisulfate autoclaving, however, these compounds have been successfully broken down [97,103,125,126].

Inositol phosphates are an important class of naturally occurring organic phosphorus compounds [142]. Phytic acid, for example, is one of the more resistant compounds to hydrolysis and is also one of the most refractory organic phosphorus compounds found in soils [75,119,141]. Other organic phosphorus compounds found in soil leachate and runoff are the sugar phosphorus compounds, e.g. D-glucose-1-phosphate and D-glucose-6-phosphate, which are labile [117]. Organic condensed phosphates, e.g. adenosine-5′-triphosphate and adenosine-5′-diphosphate are also important as they originate from all living systems, e.g. algae, bacteria, fungi, insects, plant and animal tissues [117].

It is therefore recommended that model compounds selected for digestion studies should include one with a P–O–P bond (e.g. sodium tripolyphosphate), a refractory C–O–P compound (e.g. phytic acid), a labile C–O–P compound (e.g. D-glucose-1-phosphate or D-glucose-6-phosphate), a refractory C–P compound (e.g. 2-aminoethylphosphonate), and a compound containing C–O–P and P–O–P bonds (e.g. adenosine-5′-triphosphate). Orthophosphate (e.g. as potassium dihydrogen orthophosphate) should also be used in all recovery studies as a method control [90]. One should also be aware that specific matrices may require additional model compounds. For example, acid soils and sediments may well contain phosphorus associated with iron or alu-

---

**Table 2 (Continued)**

<table>
<thead>
<tr>
<th>Matrix</th>
<th>Digestion reactant</th>
<th>Digestion time</th>
<th>Digestion temperature (°C)</th>
<th>pH</th>
<th>Model compounds*</th>
<th>Comments</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Turbid lake and river waters</td>
<td>Optimum digestion reagent: 0.27 M K2SO4 and 0.24 M NaOH, 2 mL reagent added to 10 mL sample</td>
<td>1 h</td>
<td>120 °C</td>
<td>Final pH 2</td>
<td>NIES No 3 Chlorella (99–101% up to 100 µg P L⁻¹) and No 2 Pond sediment (98–104% up to 60 µg P L⁻¹), and 88% at 100 µg P L⁻¹). Model compounds added to distilled and lake water: KHP (93–99%), PTA (93–106%), 2-AEP (93–101%), α-GLY (94–102%), PPA (93–105%), β-phosphorylethanol (91–106%), PEP (93–117%).</td>
<td>Compared alkaline peroxydisulfate autoclave method to microwave digestion, and similar results were found</td>
<td>[112]</td>
</tr>
<tr>
<td>Turbid lake waters</td>
<td>Digestion reagent: 9 g NaOH, and 40 g K2SO4 in 1 L water, 2 mL reagent added to 10 mL sample</td>
<td>1 h</td>
<td>120 °C</td>
<td>Not reported</td>
<td>NIES No 3 Chlorella (94–107% up to 100 µg P L⁻¹), and 90% at 250 µg P L⁻¹) and No 2 Pond sediment (92–109% up to 100 µg P L⁻¹), and 88% at 250 µg P L⁻¹). Model compounds added to lake water: KHP (99%), STP (98%), AMP (94%), β-GLY (103%).</td>
<td>Not reported</td>
<td>[112]</td>
</tr>
<tr>
<td>Water (overland flow)</td>
<td>Digestion reagent: 0.39 M K2SO4 and 0.6 M NaOH, 2 mL reagent added to 8 mL sample</td>
<td>1 h</td>
<td>120 °C</td>
<td>Not reported</td>
<td>NIES No 3 Chlorella (94–107% up to 100 µg P L⁻¹), and 90% at 250 µg P L⁻¹) and No 2 Pond sediment (92–109% up to 100 µg P L⁻¹), and 88% at 250 µg P L⁻¹). Model compounds added to lake water: KHP (99%), STP (98%), AMP (94%), β-GLY (103%).</td>
<td>Not reported</td>
<td>[109]</td>
</tr>
</tbody>
</table>

* With recoveries given in parentheses when reported.
Table 3
Model compounds used in autoclave based digestion methods

<table>
<thead>
<tr>
<th>Model compound</th>
<th>Synonyms</th>
<th>Abbreviation used in text</th>
<th>Chemical formula</th>
<th>Structural formula</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adenosine-5'-monophosphate</td>
<td>Adenosine-5'-monophosphoric acid; 5-adenylic acid; adenosine phosphate; ert-adenylic acid; ergadenylic acid</td>
<td>AMP</td>
<td>C_{10}H_{14}N_{4}O_{5}P</td>
<td><img src="image" alt="Structural formula AMP" /></td>
</tr>
<tr>
<td>Adenosine-3',5'-cyclic monophosphate</td>
<td>Adenosine-3',5'-cyclophosphoric acid; cyclic AMP; 3',5'-cyclic AMP</td>
<td>cAMP</td>
<td>C_{10}H_{12}N_{4}O_{5}P</td>
<td><img src="image" alt="Structural formula cAMP" /></td>
</tr>
<tr>
<td>Adenosine-diphosphate</td>
<td></td>
<td>ADP</td>
<td>C_{10}H_{14}N_{4}O_{5}P_{2}</td>
<td><img src="image" alt="Structural formula ADP" /></td>
</tr>
<tr>
<td>Adenosine-5'-diphosphate (sodium salt)</td>
<td></td>
<td>5'-ADP-Na_{2}</td>
<td>C_{10}H_{12}N_{4}O_{5}P_{2}Na_{2}</td>
<td>Similar to ADP</td>
</tr>
<tr>
<td>Adenosine-5'-triphosphate</td>
<td></td>
<td>ATP</td>
<td>C_{10}H_{16}N_{4}O_{13}P_{3}</td>
<td><img src="image" alt="Structural formula ATP" /></td>
</tr>
<tr>
<td>Adenosine triphosphate disodium</td>
<td>Adenosine 5'-(tetrahydrogen triphosphate) disodium salt; adenosine 5'-triphosphate, disodium salt; adenosine 5'-triphosphate, disodium salt hydrate</td>
<td>5'-ATP-Na_{2}</td>
<td>C_{10}H_{14}N_{4}O_{13}P_{3}Na_{2}</td>
<td>Similar to ATP</td>
</tr>
<tr>
<td>1-Aminoethylphosphonate</td>
<td>1-Aminoethylphosphonic acid</td>
<td>1-AEP</td>
<td>C_{2}H_{6}NO_{3}P</td>
<td><img src="image" alt="Structural formula 1-AEP" /></td>
</tr>
<tr>
<td>2-Aminoethylphosphonate</td>
<td>2-Aminoethylphosphonic acid</td>
<td>2-AEP</td>
<td>C_{2}H_{6}NO_{3}P</td>
<td><img src="image" alt="Structural formula 2-AEP" /></td>
</tr>
<tr>
<td>Glucose-1-phosphate</td>
<td>Glucose-1-phosphoric acid</td>
<td>G-1-P</td>
<td>C_{6}H_{12}O_{5}P</td>
<td><img src="image" alt="Structural formula G-1-P" /></td>
</tr>
<tr>
<td>Model compound</td>
<td>Synonyms</td>
<td>Abbreviation used in text</td>
<td>Chemical formula</td>
<td>Structural formula</td>
</tr>
<tr>
<td>-----------------------------------------------------</td>
<td>-----------------------------------------------</td>
<td>----------------------------</td>
<td>------------------</td>
<td>-------------------</td>
</tr>
<tr>
<td>Glucose-1-phosphate dipotassium salt</td>
<td>Glucose-1-phosphoric acid (dipotassium salt)</td>
<td>G-1-P-K$_2$</td>
<td>C$<em>6$H$</em>{11}$O$_5$PK$_2$</td>
<td>Similar to G-1-P</td>
</tr>
<tr>
<td>Glucose-6-phosphate</td>
<td>Glucose-6-phosphoric acid</td>
<td>G-6-P</td>
<td>C$<em>6$H$</em>{11}$O$_5$P</td>
<td></td>
</tr>
<tr>
<td>Glucose-6-phosphoric acid (dipotassium salt)</td>
<td>α-4-Glucose-6-phosphoric acid dipotassium salt</td>
<td>G-6-P-K$_2$</td>
<td>C$<em>6$H$</em>{11}$O$_5$PK$_2$</td>
<td>Similar to G-6-P</td>
</tr>
<tr>
<td>Glucose-6-phosphate sodium salt</td>
<td></td>
<td>G-6-P-Na</td>
<td>C$<em>6$H$</em>{11}$O$_5$PNa</td>
<td>Similar to G-6-P</td>
</tr>
<tr>
<td>α-α-Glycerophosphate disodium salt</td>
<td>rac-Glycerol 1-phosphate disodium salt; α-α-glycerophosphate</td>
<td>α-GLY</td>
<td>C$<em>6$H$</em>{11}$O$_5$PNa</td>
<td></td>
</tr>
<tr>
<td>β-Glycerophosphate disodium salt hydrate</td>
<td>Glycerol 2-phosphate disodium salt hydrate; sodium β-glycerophosphate</td>
<td>β-GLY</td>
<td>C$<em>6$H$</em>{11}$O$_5$PNa</td>
<td></td>
</tr>
<tr>
<td>Guanosine 5'-diphosphate</td>
<td></td>
<td>5'-GDP</td>
<td>C$<em>{10}$H$</em>{15}$N$_2$O$_7$P$_2$</td>
<td></td>
</tr>
<tr>
<td>Guanosine 5'-monophosphate disodium hydrate</td>
<td></td>
<td>5'-GMP-Na$_3$</td>
<td>C$<em>{10}$H$</em>{12}$N$_3$O$_8$PNa$_2$</td>
<td></td>
</tr>
<tr>
<td>4-Nitrophenyl phosphate</td>
<td>4-Nitrophenyl phosphate</td>
<td>NPP</td>
<td>C$<em>6$H$</em>{11}$NO$_5$PNa$_2$</td>
<td></td>
</tr>
<tr>
<td>Phosphoenolpyruvate</td>
<td></td>
<td>PEP</td>
<td>C$<em>6$H$</em>{12}$O$_6$P</td>
<td></td>
</tr>
<tr>
<td>phosphoenolpyruvic acid tri(cyclohexylamine) salt</td>
<td></td>
<td>PEP-3CHA</td>
<td>C$<em>6$H$</em>{12}$O$_6$P(C$<em>6$H$</em>{11}$NH$_3$)$_3$</td>
<td></td>
</tr>
<tr>
<td>Phosphonoformate</td>
<td>Phosphonoformic acid</td>
<td>PFA</td>
<td>CH$_3$O$_2$P</td>
<td></td>
</tr>
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Table 3 (Continued)

<table>
<thead>
<tr>
<th>Model compound</th>
<th>Synonyms</th>
<th>Abbreviation used in text</th>
<th>Chemical formula</th>
<th>Structural formula</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phosphoryl choline chloride calcium salt tetrahydrate</td>
<td>Phosphocholine chloride calcium salt tetrahydrate; calcium phosphorylcholine chloride</td>
<td>PC</td>
<td>CsH5NO4PCaCl4·4H2O</td>
<td><img src="image1" alt="Structure" /></td>
</tr>
<tr>
<td>Phosphoserine</td>
<td></td>
<td>SOP</td>
<td>CsH6NO6P</td>
<td><img src="image2" alt="Structure" /></td>
</tr>
<tr>
<td>Phytic acid</td>
<td>Myo-inositol hexakis (dihydrogen phosphate); inositol hexaphosphoric acid</td>
<td>PTA</td>
<td>CsH16O25P6</td>
<td><img src="image3" alt="Structure" /></td>
</tr>
<tr>
<td>Riboflavin-5’-monophosphate sodium salt</td>
<td></td>
<td>FMN</td>
<td>C17H20N2O4PNa</td>
<td><img src="image4" alt="Structure" /></td>
</tr>
<tr>
<td>Ribose-5-phosphate disodium salt dihydrate</td>
<td></td>
<td>RP</td>
<td>C5H8O5PNa2</td>
<td><img src="image5" alt="Structure" /></td>
</tr>
<tr>
<td>Tetrasodium pyrophosphate</td>
<td>Sodium pyrophosphate; pyrophosphoric acid tetrasodium salt; diphosphoric acid, tetraysodium salt</td>
<td>TSPP</td>
<td>Na4P2</td>
<td><img src="image6" alt="Structure" /></td>
</tr>
<tr>
<td>Sodium tripolyphosphate</td>
<td>Pentasodium triply phosphate dihydrate; sodium tripophosphate; sodium polyphosphate; triphosphoric acid pentasodium anhydrous</td>
<td>STP</td>
<td>Na5P1O10</td>
<td><img src="image7" alt="Structure" /></td>
</tr>
<tr>
<td>Sodium hexametaphosphate</td>
<td>Sodium metaphosphate; metaphosphoric acid, hexasodium salt; sodium polymetaphosphate</td>
<td>SHMP</td>
<td>(NaPO3)6</td>
<td><img src="image8" alt="Structure" /></td>
</tr>
</tbody>
</table>

Minimum phases, which are relatively resistant to oxidative dissolution [75].

4.3. Recovery studies using alkaline and acidic peroxydisulfate autoclaving

Typical phosphorus recoveries for a range of model compounds, digested using alkaline and acid peroxydisulfate autoclaving, are shown in Fig. 4. The alkaline peroxydisulfate digestion method can be used for the simultaneous determination of TP and TN [85]. This was chosen because the borate buffer ensures that the pH is initially alkaline, to break down nitrogen containing bonds, and becomes acidic during the digestion process to break down phosphorus containing bonds. An amount of 5 mL of digestion reagent (5 g potassium peroxydisulfate and 3 g boric acid dissolved in 100 mL 0.375 M sodium hydroxide) was added to 50 mL sample. The samples were then autoclaved for 30 min at 121 °C. Model compounds chosen were phytic acid, sodium tripolyphosphate and adenosine-5’-triphosphate, and were therefore representative of a refractory C-O-P compound, a P-O-P compound and a C-O-P and P-O-P bond containing compound, respectively. Recoveries were 89 ± 13% for phytic acid, 100 ± 13%...
reactions such as the synthesis of organic phosphate compounds (transphosphorylation) and transport across cell membranes [145] and they have been isolated from a variety of sources. Alkaline phosphatase is the most studied phosphomonoesterase and has been isolated from, e.g. *Escherichia coli* [146,147]. Acid phosphatases show broad selectivity towards phosphomonoesters and have also been isolated from *E. coli* [146].

Strickland and Parsons established a classical method using phosphatase for the determination of phosphate [148] but this method was susceptible to product inhibition by reactive phosphate already present in the sample. McKelvie and co-workers immobilised *E. coli* onto CNBr-activated sepharose 4B beads in a F1 system with an optimum pH of 8. The recovery of alkaline phosphatase hydrolysable phosphorus was low in natural waters but good in sediments [146]. They also applied alkaline phosphatase to soils [59]. Acid and alkaline phosphatase and phytase have been used in combination to investigate organic phosphorus speciation in soils [149].

Inositol hexaphosphate forms the bulk of extractable soil organic phosphorus [146,149,50]. Phytases (EC 3.1.3.8) are members of the family of histidine acid phosphatases [150,151] that are found in plants and micro-organisms, which catalyse the hydrolysis of phytate (myo-inositol hexakis-phosphate 1, 2, 3, 4, 5, 6) to less phosphorylated myo-inositol phosphates and free orthophosphates. Phytase from plant sources, e.g. wheat, first acts on the C6 atom while that from microbial sources acts on the C3 atom. McKelvie et al. [59,152] used a F1 system with immobilised phytase for the determination of phytic acid in soils. Adenosine-5'-triphosphate was also hydrolysed but in low yields compared with phytic acid. Phytase has also been applied to the determination of phytic acid in the marine environment, but with low recoveries [152].

Enzymatic methods are important for assessing the potential biological availability of organic phosphorus but other methods are also needed for complete identification and this remains a challenging area of analysis.

5. Quality assurance and quality control

Phosphorus is a key determinant in most environmental monitoring and research programmes [153] and only accurate analytical data permits valid conclusions to be drawn about the phosphorus status of water bodies and soils. In addition to DRP it is also important to obtain accurate total phosphorus (TP) data because this parameter is used for load calculations, e.g. to determine discharges from sewage treatment works [18]. This has important implications regarding decisions on the installation (or not) of costly phosphorus removal technology. Programmes involving multi-national participation and international databanks [76] require adequate quality assurance/quality control (QA/QC) schemes to ensure the data integrity necessary for the comparison of data from various sources. Adherence to QA guidelines, participation in inter-
laboratory studies, use of reference materials (RMs) and certified reference materials (CRMs) are all means of achieving good data quality for phosphorus determinations [154,155].

5.1. Certified reference materials

A CRM is a reference material for which component values have been certified by a technically valid procedure and is accompanied by or traceable to a certificate or other document issued by a certifying body [156,157]. The use of CRMs is the most efficient way to measure and control accuracy [158] and can help produce reliable calibration and validation of measurement procedures [159]. CRMs can be either calibration CRMs, which are high purity substances or synthetically prepared mixtures, or matrix-matched CRMs, which can be natural samples or artificial samples simulating the composition of natural samples [158]. Few CRMs are commercially available for the determination of phosphorus species in environmental matrices (see Table 4), despite the need for such materials [155]. CRMs are not currently available for all environmental matrices routinely analysed for phosphorus species, such as estuarine waters, nor do they adequately span the range of phosphorus concentrations characteristic of environmental matrices. The National Research Council of Canada (NRCC) recognized the urgent need for CRMs for nutrients, including orthophosphate, for use in the marine sciences. MOOS-1, a natural seawater CRM available for the determination of nutrients in seawater, was developed in direct response to this need [159]. Analysis of MOOS-1 was carried out in 2002 by 25 expert laboratories participating in the ‘NOAA/NRC 2nd intercomparison study for nutrients in seawater’ [160]. Laboratories were predominantly selected on the basis of their previous satisfactory performance in a NOAA 2000 intercomparison study [159]. Flow and manual methods were used, all based on the spectrophotometric procedures of Strickland and Parsons [148]. Eighteen of the 25 laboratories achieved satisfactory Z-scores (see Section 5.2) for the determination of phosphate in seawater as shown in Fig. 5.

5.2. Intercomparison exercises

Inter-laboratory comparison studies are an essential feature of method development and validation [154] and play an important role in the certification of reference materials, such as described for MOOS-1 [159]. Performance in intercomparison studies undertaken by NOAA/NRCC in 2000 and 2002 [159,160] was used to assess the capabilities of international laboratories to quantify nutrients in MOOS-1, including orthophosphate. Z-scores [162] have been widely used for the statistical assessment of data in intercomparison exercises to give a comparative indication of performance with $|Z| < 2$ indicating satisfactory performance [160,163–166].

The main objectives of interlaboratory comparison studies are to determine inter-laboratory precision and accuracy and provide an impartial view of in-house quality control procedures. Participation can also identify best practise with respect to method, sample preparation, sample storage and training needs. The QUASIMEME project (Quality Assurance of Information for Marine Environmental Monitoring in Europe), now known as QUASIMEME Laboratory Performance Studies, was established to assist European Union labs in developing their QA/QC procedures to satisfy the data quality requirements of monitoring programmes in which they participated such as the International Marine Monitoring Programmes (OSPARCOM), the Helsinki Commission (HELCOM) and the MEDPOL programme [163,167]. Initially funded by the EU (1992–1996), the programme still continues by subscription of participating institutes. All institutes, worldwide, involved in chemical measurements in seawater are eligible to participate. The laboratory programmes for proficiency testing of most determinands are conducted twice per year and routinely include aqueous test materials containing orthophosphate and TP at concentrations similar to those found in estuarine, coastal and open water environments [168]. Regular testing is necessary to assure the quality of environmental data submitted since the performance of many laboratories does not remain constant [163,169].
<table>
<thead>
<tr>
<th>CRM</th>
<th>Matrix</th>
<th>Phosphorus species</th>
<th>Concentration</th>
<th>Comments</th>
<th>Supplier</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>MOOS-1</td>
<td>Seawater</td>
<td>Orthophosphate</td>
<td>1.56 ± 0.07 µmol L⁻¹</td>
<td>Natural seawater sample, of Cape Breton Island, NS, Canada at a depth of 200 m</td>
<td>NRCC</td>
<td>[159, 160]</td>
</tr>
<tr>
<td>QC RW1</td>
<td>Freshwater</td>
<td>Orthophosphate</td>
<td>100 µg L⁻¹</td>
<td>Artificial sample, distributed as an ampoule to be 100 times with pure water</td>
<td>VKI</td>
<td>[161]</td>
</tr>
<tr>
<td>QC RW2</td>
<td>Freshwater</td>
<td>Total phosphorus</td>
<td>200 µg L⁻¹</td>
<td>Artificial sample, distributed as an ampoule to be 100 times with pure water</td>
<td>VKI</td>
<td>[161]</td>
</tr>
<tr>
<td>Australian</td>
<td>Natural water/freshwater</td>
<td>Orthophosphate</td>
<td>27 ± 0.8 µg L⁻¹</td>
<td>Natural water sample obtained from Cape Breton Island, Canada at a depth of 200 m</td>
<td>Queensland Health Scientific Services</td>
<td></td>
</tr>
<tr>
<td>Natural water CRM</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BCR-616</td>
<td>Groundwater</td>
<td>Total dissolved phosphorus</td>
<td>37 ± 1.2 µg L⁻¹</td>
<td>Artificial groundwater sample, prepared from ultrapure water, to which required salts were added; stabilized by autoclaving</td>
<td>BCR</td>
<td>www irmmm jrc be</td>
</tr>
<tr>
<td>SRM®-2702</td>
<td>Marine sediment</td>
<td>Total phosphorus</td>
<td>0.1552 ± 0.00066%</td>
<td>Material for SRM® was collected from Chesapeake Bay, USA, freeze-dried, sieved at 70 µm (100% passing) and cone blended, then radiation sterilized and bottled</td>
<td>NIST</td>
<td>www nist gov</td>
</tr>
<tr>
<td>SRM®-1646a</td>
<td>Estuarine sediment</td>
<td>Total phosphorus</td>
<td>0.027 ± 0.0001%</td>
<td>Material for SRM® was collected from Chesapeake Bay, USA, freeze-dried, lightly deagglomerated and &lt;1 mm fraction ball milled and the &lt;75 µm blended and bottled</td>
<td>NIST</td>
<td>www nist gov</td>
</tr>
<tr>
<td>BCR-684</td>
<td>River sediment</td>
<td>NaOH-extractable P</td>
<td>500 ± 21 mg kg⁻¹</td>
<td>Material for the CRM was collected from the lower reaches of the River Po, Italy, then sieved and the &lt;2 mm fraction was dried, lightly deagglomerated, crushed and hammer-milled and &lt;90 µm blended and bottled</td>
<td>BCR</td>
<td>www irmmm jrc be</td>
</tr>
<tr>
<td></td>
<td></td>
<td>HCl-extractable</td>
<td>536 ± 28 mg kg⁻¹</td>
<td></td>
<td></td>
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<tr>
<td></td>
<td></td>
<td>Inorganic P</td>
<td>1113 ± 24 mg kg⁻¹</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td>Organic P</td>
<td>290 ± 9 mg kg⁻¹</td>
<td></td>
<td></td>
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<tr>
<td></td>
<td></td>
<td>Conc. HCl-extract P</td>
<td>1373 ± 35 mg kg⁻¹</td>
<td></td>
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<td></td>
</tr>
</tbody>
</table>

Assessment of the quality of data must be made at the time that the environmental samples are analysed. Such exercises provide vital information for improving the quality and performance of laboratories and a structure for developing robust analytical techniques. To this end, the QUASIMEME Laboratory Performance Study was designed to follow the IUPAC/ISO/AOAC international protocol for international testing [162, 170]. All laboratories that submit data to the UK National Marine Monitoring Programme (NMMP) routinely participate in QUASIMEME as a means of external QA/QC of the data collected, including orthophosphate [171]. There have been several other national and international intercomparison exercises including the series of International Council for the Exploration of the Seas (ICES) exercises [164] and the Australian National Low level Nutrient (ANLLN) exercise.

5.3. Databases

Environmental monitoring and research programmes generate large amounts of information and can provide valuable databases of analytical information if appropriate QA/QC measures are used to preserve data quality. For example, databases have been generated from the NMMP and the
‘Winter Monitoring of the Western Irish Sea’ programme [165] and both incorporated QA/QC schemes to ensure data integrity. Legislation such as the EU Water Framework Directive outlines an approach for managing water quality in the member states of the European Union which will require monitoring and environmental quality data (including P data) to be collected by member states and presented at the EU level. There is therefore the potential to add to the repository of data already held by the European Environmental Agency, and adherence to QA/QC practices such as intercomparison studies in conjunction with routine in-house use of RMs and CRMs is essential if such data are to be of practical use. Phosphorus data are also incorporated within larger assessment exercises dealing with broader issues such as water quality and eutrophication, e.g. the National Estuarine Eutrophication assessment in the United States [172].

6. Conclusions

Accurate determination of P species in environmental matrices is an important pre-requisite for understanding the biogeochemical cycling of the element. This in turn is essential for investigating the impact of phosphorus on ecosystem health. Key aspects of the analytical process for obtaining high quality phosphorus data are robust sampling and sample treatment protocols (see also Maher and Woo [75]). These cannot be universal due to the variability in behaviour of different matrices but nonetheless guidelines can be provided for aspects such as filtration, chemical treatment and storage conditions. For soils, wetting and drying have a considerable affect on phosphorus solubility.

In addition, for the determination of different phosphorus fractions and individual phosphorus containing compounds, particular attention needs to be given to the digestion process. Autoclaving (typically with peroxysulfate in acid or alkaline media) is a widely used method that gives good recoveries but it is important to quantify this using a range of environmentally relevant model phosphorus containing compounds. Selective enzymatic degradation (typically using phosphatases) is a useful additional approach for the quantification of individual phosphorus containing compounds (or classes of compounds).

A critical aspect of the overall analytical process for any laboratory is participation in intercomparison exercises. This is particularly important for phosphorus determination due to the lability of the element in biologically active environmental matrices. To supplement such exercises the availability of more environmental certified reference materials is an important requirement. Finally, co-operation between analytical scientists and environmental scientists is fundamental to the generation of high quality, publicly available databases on the spatial and temporal variability of phosphorus species in aquatic and terrestrial ecosystems.

References

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