Determination of nitrate and phosphate in seawater at nanomolar concentrations

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Over much of the world's surface oceans, nitrate and phosphate concentrations are below the limit of detection (LOD) of conventional techniques of analysis. However, these nutrients play a controlling role in primary productivity and carbon sequestration in these waters. In recent years, techniques have been developed to address this challenge, and methods are now available for the shipboard analysis of nanomolar (nM) nitrate and phosphate concentrations with a high sample throughput.

This article provides an overview of the methods for nM nitrate and phosphate analysis in seawater. We outline in detail a system comprising liquid waveguide capillary cells connected to a conventional segmented-flow autoanalyser and using miniaturised spectrophotometers. This approach is suitable for routine field measurements of nitrate and phosphate and achieves LODs of 0.8 nM phosphate and 1.5 nM nitrate.

Keywords: Liquid-waveguide-capillary cell; Nanomolar concentration; Nitrate; Nutrient; Ocean; Phosphate; Seawater; Segmented-flow autoanalyser; Spectrophotometer

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1. Introduction

1.1. Nitrate and phosphate in marine waters

All living organisms require the nutrients nitrogen and phosphorus for their growth, metabolism and reproduction. Nitrogen is a component of amino acids, nucleic acids and other cell components, while phosphorus is found primarily in nucleic acids, phospholipids and adenosine triphosphate (ATP). Research has demonstrated that phytoplankton productivity in the surface ocean is often limited by the amount of available fixed inorganic nitrogen (i.e. dissolved forms other than molecular nitrogen) [1] and, in some cases, available phosphorus [2,3].

Nitrogen is present in the marine environment in various forms. Nitrate is the principal form of fixed dissolved inorganic nitrogen assimilated by organisms, although certain organisms can utilise nitrite, ammonium or even dissolved molecular nitrogen. Orthophosphate (predominantly HPO_4^{2-}) is considered the most important phosphorus species in seawater that is immediately biologically available. Dissolved inorganic nutrients are usually the preferred substrates for phytoplankton, since organic sources of nitrogen and phosphorus generally require enzymatic remineralisation. However, some photosynthetic organisms can access dissolved organic nutrients, and there is growing interest in dissolved organic nitrogen (DON) and dissolved organic phosphorus (DOP) cycling in marine ecosystems. DON (or DOP) concentrations are determined indirectly as the difference between total dissolved nitrogen (or phosphorus) and inorganic dissolved nitrogen (or phosphorus). Since DON and DOP measurements contain the errors of two or more analytical measurements, accurate and precise measurements of nitrate and phosphate are essential [4,5].

Large temporal and spatial variations in nutrient concentrations exist in the oceans because of physical and biological processes. In surface waters, biological uptake depletes nitrate and phosphate. In highly stratified oligotrophic surface waters, with low nutrient inputs, nitrate and phosphate are typically at nanomolar (nM) concentrations. Approximately 40% of the world's oceans fall into this category. Nitrate and phosphate concentrations increase to micromolar concentrations with depth, as remineralisation of sinking particulate matter returns

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dissolved nutrients to the water column. Fig. 1 shows an example of a vertical profile of nitrate and phosphate concentrations in the North Atlantic Ocean.

The high spatio-temporal variability of nitrate and phosphate in oceanic surface waters, combined with severe problems associated with storage of samples containing nM nutrient concentrations, poses a need for ship-based measurements. This puts further demands on the method, which needs to be rugged, portable, easy to operate, and with a high sample throughput [6].

1.2. Traditional techniques

A variety of methods has been used to determine nitrate and phosphate in seawater. These methods can be divided into three broad categories:

(1) manual methods, where each sample is treated individually;
(2) automated methods, which are usually based on flow analysis; and,
(3) sensors, which, upon contact with the seawater, monitor a signal that is indicative of the analyte concentration [7].

Sensors would represent the ideal way to quantify nutrients in the marine environment, but do not yet show sufficient sensitivity or precision and often suffer interference from the high concentrations of ions present in seawater. For example, sensors are available that measure in-situ nitrate concentrations directly by monitoring UV absorbance at 220 nm [8,9]. They can provide instantaneous, near-continuous in-situ measurements in the oceans. However, the lowest reported limit of detection (LOD) is 0.21 μM [8], which limits its use in many surface waters. Interferences from organic matter and other anions, such as bromide and carbonate, are also a problem.

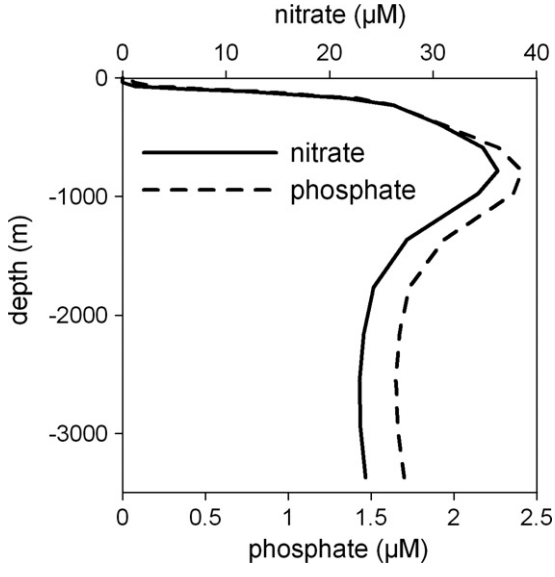

Figure 1. Vertical profile of nitrate and phosphate in the tropical North-East Atlantic at 17°N, 24°W, determined on 9 February 2006 during a research cruise aboard the *FS Poseidon*. Analysis was carried out using a conventional segmented-flow autoanalyser.

Flow analysis is a common technique used to automate chemical analyses. Typically, peristaltic pumps precisely mix sample with reagents in flow-through tubes or capillaries, while reaction products are continuously monitored using a flow-through detector. Various forms of flow analysis exist, including segmented continuous flow analysis (SCFA), flow-injection analysis (FIA) and sequential injection analysis (SIA). Automation, together with high sample throughput, high analytical precision and a reduced risk of sample contamination, has resulted in the widespread use of flow analysis for nutrient measurements in natural waters. Several recent reviews provided comprehensive overviews of the use of flow analysis for nitrate and phosphate [10–13].

The most widely used method for the analysis of nitrate involves reduction of nitrate to nitrite, usually using a copperised cadmium column. Nitrite is then determined spectrophotometrically (at 540 nm) following formation of a highly coloured dye through diazotisation with sulphanilamide and coupling with *N*-(1-naphthyl)-ethylenediamine dihydrochloride (NED) [7]. This analytical method determines the sum of the nitrate (NO_3^-) and nitrite (NO_2^-) concentrations; to calculate the nitrate concentration, it is necessary to measure nitrite separately in the sample (by omitting the reduction step) and subtract it from the combined $NO_2^- + NO_3^-$ measurement. The technique is robust, sensitive and suffers from no known interferences in oxygenated seawater [7,14].

For the analysis of phosphate, Murphy and Riley's molybdenum blue (MB) method [15] forms the basis for most methods. It involves reaction of the orthophosphate with ammonium molybdate under acidic conditions to form 12-molybdophosphate, a yellow-coloured complex. This complex is reduced by either ascorbic acid or stannous chloride in the presence of antimony to give a phosphor-MB complex, which is determined at 660–880 nm, depending on reaction conditions. Antimony is not essential for the formation of the phosphor-MB complex, but its inclusion results in faster formation of the final product, which incorporates the element in a 1:2 P:Sb ratio [16]. Unfortunately, this reaction is not completely specific for orthophosphate; silicic acid (SiO_4^{4-}) and arsenate (AsO_4^{3-}) also form MB complexes, although formation of the former can be minimised with optimised reaction conditions [17]. Arsenate interference can be eliminated by reduction to arsenite (AsO_3^{3-}) [18,19], but the precipitation of colloidal sulphur limits the usefulness of the procedure [20], so field measurements are very rarely corrected for arsenate. Furthermore, the acidic reaction conditions employed in the method hydrolyse

Table 1. Overview of reported methods for nanomolar phosphate analysis in seawater

Detection	Chemistry	Technique	Figures of Merit	Comments	Ref.
Colorimetry	Phospho-molybdenum blue	SCFA 2-m LWCC used as flow-cell	LOD: 0.8 nM P: 4.8% (at 10 nM) R: 0.8–600	+ Adapted from established standard technique + Automated and requires no sample pre-treatment + Simultaneous parallel measurement of nitrite/nitrate ~ 4 min per analytical cycle	this study
Colorimetry	Phospho-molybdenum blue - cetyltrimethylammonium bromide (PMB-CTAB)	FIA PMB-CTAB ion-pair complex pre-concentrated onto a C18 SPE cartridge 2-cm flow-cell	LOD: 1.6 nM P: 4.5% (at 32.4 nM) R: 3.2–48.5 nM	+ Automated system + Accurate measurement of sample volume required; reagents are added directly to sample bottle by FIA system + Slow formation of ion-association complex ~ 30 min per analytical cycle	[26]
Chemiluminescence	12-molybdophosphate–cetyltrimethylammonium bromide (MP-CTAB)	FIA MP-CTAB ion-pair complex pre-concentrated onto a C18 SPE cartridge	LOD: 2 nM P: 4.7% (at 97 nM) R: 5–194 nM	+ Accurate measurement of sample volume required, since reagents are added directly to sample bottle + 2-step rinse of SPE cartridge required to remove all traces of sample matrix, which interferes with CL reaction ~10 min per analytical cycle	[28]
Colorimetry	12-molybdophosphate - malachite green, surfactant	Manual sample preparation 10-cm quartz cell	LOD: 8 nM P: 3.4% (at 50 nM) R: 10–400 nM	+ Uses less acidic reaction conditions than previous MG methods, although pH still lower than for PMB methods + 40 min to develop colour	[21]
Colorimetry	Phospho-molybdenum blue	Manual sample preparation MAGIC 25 x pre-concentration factor 10-cm cell	LOD: 0.8 nM P: 102% (at 2 nM) R: 0.8–200 nM	+ Improved version of the MAGIC procedure with reduced analysis time (~60 min) + Samples pre-filtered to reduce turbidity	[53]
Colorimetry	Phospho-molybdenum blue	Manual sample preparation HPLC analysis with C8 column	LOD: 1 nM P: 5.6% (at 1 nM) R: 3–300 nM	+ Purification of reagents necessary for concentrations below 10 nm ~15 min HPLC injection interval	[27]
Colorimetry	Phospho-molybdenum blue	SCFA 2-m LWCC used as flow-cell	LOD: 0.5 nM P: 2% (at 10 nM) R: 0.5–200	+ Adapted from established standard technique + Automated and requires no sample pre-treatment ~2 min per analytical cycle	[34]
Chemiluminescence	Vanadomolybdophosphate - dodecylpyridinium bromide (VMP-DDPB)	Manual sample preparation VMP-DDPB ion-pair complex extracted onto paper filters and measured in a CL photometer	LOD: 0.6 nM P: 14% (at 0.97 μM) R: 2–55 nM	+ Does not require organic solvents unlike other filter pre-concentration methods ~25 min per sample	[54]

Table 1 (*continued*)

Detection	Chemistry	Technique	Figures of Merit	Comments	Ref.
Colorimetry	Phospho-molybdenum blue	Manual sample preparation $Mg(OH)_2$–induced co-precipitation to concentrate PO_4^{3-} (MAGIC) 100 x pre-conc. factor 10-cm cell	LOD: 0.2 nM P: 10% (at 2 nM) R: NR	+ Requires only a centrifuge + Reagents added after pre-concentration step so blanks lower + Long procedure (~90 min) with multiple steps	[3]
Colorimetry	Phospho-molybdenum blue	Manual sample preparation n-hexanol liquid-liquid extraction 10-cm cell	LOD: 4 nM P: NR R: 0–300 nM	+ Requires no specialised equipment + Contains several steps and uses significant volumes of organic solvents ~20 min sample preparation	[7]
Colorimetry	12-molybdophosphate-malachite green	Manual sample preparation MP-MG ion-pair complex concentrated by extracting onto a cellulose nitrate filter	LOD: 2 nM P: 0.57% (at 97 nM) R: 2–600	~20 min per analytical cycle	[25]
Colorimetry	Phospho-molybdenum blue	Manual sample preparation 60-cm capillary cell with standard LED source and photodiode detector	LOD: 1 nM P: 6% (at 8 nM) R: 1–500 nM	+ Standard LED and photodiode detection + Non-linearity and attenuation of light in cell ~ 40 min per analytical cycle	[51]
Colorimetry	Phospho-molybdenum blue - dodecyltrimethylammonium bromide (PMB-DTAB)	Manual sample preparation PMB-DTAB ion-pair complex concentrated onto a 25-mm, 0.45-μm cellulose nitrate filter, followed by dissolution in DMF	LOD: 0.6 nM P: 2.2% (at 34 nM) R: 32–4500 nM	+ Uses less solvent than liquid-liquid extraction ~ 25 min sample preparation	[24]
Colorimetry	12-molybdophosphate - malachite green	Manual sample preparation toluene/methylpentan-2-one liquid-liquid extraction 10-cm cell	LOD: 3 nM P: 1.1% (at 139 nM) R: NR	+ Requires no specialised equipment + Contains several steps and uses significant volumes of organic solvents	[23]
Colorimetry	Phospho-molybdenum blue	Manual sample preparation 1-m capillary cell	LOD: 0.2 nM P: 5% (at 1.6 nM) R: 0.2–323 nM	+ Non-linearity and attenuation of light in cell + Specialised optics and laser light source used ~30 min per analytical cycle	[31]
TL colorimetry	Phospho-molybdenum blue	Manual sample preparation 1-cm cell with high-powered laser and specialised optics	LOD: 0.2 nM P: 11.6% (at 3 nM) R:0.2–16 nM	+ Complex and bulky equipment required ~ 30 min per analytical cycle	[30]

Methods are listed in order of the year they appeared in the literature, with the most recent listed first. CL, Chemiluminescence; FIA, Flow-injection analysis; SPE, Solid-phase extraction; TL, Thermal lensing; LED, Light-emitting diode; SCFA, Segmented continuous flow analysis; LWCC, Liquid-waveguide-capillary cell; LOD, Limit of detection; P, Precision (RSD); NR, Not reported; R, Range of concentrations for which method is reported to be suitable.

pyrophosphate ($P_2O_7^{4-}$) and selected organic-P compounds, resulting in the overestimation of orthophosphate concentrations. For this reason, the fraction measured by the MB procedure is termed soluble reactive phosphate (SRP).

These well-established techniques provide analyses with a precision of around 1% RSD and are relatively simple to perform. Their main limitation is that the LOD is approximately 0.1 μM nitrate and 0.03 μM phosphate, which means that variations in nM nitrate and phosphate concentrations will pass unobserved in oligotrophic ocean regions where these nutrients control primary production. In recent decades, researchers have developed a range of methods to determine nitrate and phosphate in seawater at nM concentrations. This article outlines the various approaches, with particular emphasis put on the analytical challenges associated with the methods and their suitability for field analysis.

2. Nanomolar phosphate methods

There are, in principle, three ways to lower the LOD of a chemical analysis:

(1) optimise the chemistry so that, for example, the reaction produces a more easily detected product;
(2) pre-concentrate the analyte prior to analysis; or,
(3) use a more sensitive instrument to detect the reaction product.

Most methods centre on pre-concentration and/or detector sensitivity. Table 1 shows an overview of the reported methods, their LODs, precision, and concentration range for the analysis of phosphate in seawater at nM concentrations.

2.1. Optimising the chemistry

There are only limited options to improve the LOD of phosphate analysis by altering the chemistry. The MB method has been in use since the 1920s and numerous improvements have been made over the years [5]. Colour development is rapid and pH and reagent concentrations have been optimised to increase specificity for orthophosphate. It seems unlikely that further significant improvements will be made with this method. Using a more highly coloured chromophore is another option but, in general, the molar absorptivities of dyes are of the same order of magnitude. Malachite green, a cationic dye, is one alternative that has received significant attention. When combined with 12-molybdophosphate, the dye forms a highly coloured ion-pair complex with a molar absorptivity coefficient around five times that of the phosphor-MB complex. Historically, malachite green methods have suffered from poor reagent stability, chromophore stability and poor selectivity, the last being due to acidic reaction conditions resulting in more hydrolysis of organic phosphorus compounds compared with phosphor-MB. There has been some recent work on this method, which has addressed the principal limitations [21]. However, colour development takes around 40 min, which limits its suitability for automated analysis.

2.2. Pre-concentration approaches

Perhaps the most widely used method for determining nM concentrations of phosphate is the magnesium-induced co-precipitation (MAGIC) method, developed by Karl and Tien [22]. It involves addition of sodium hydroxide to the water sample to induce precipitation of brucite ($Mg(OH)_2$). Orthophosphate is quantitatively removed from solution by adsorption to the precipitate, which is collected by centrifugation and dissolved in a small volume of dilute acid. Phosphate is then determined using the standard MB protocol. Unlike other pre-concentration techniques, most of the reagents are added after the concentration step, resulting in low blank values. The pre-concentration factor (the ratio between the volume of the initial sample and the redissolved precipitate) can be altered to allow the determination of different concentration ranges, and LODs as low as 0.2 nM PO_4^{3-} have been reported [3]. Low LODs and high precision, combined with a requirement for only basic laboratory instrumentation, have resulted in the widespread adoption of the technique. Nonetheless, the MAGIC procedure comprises several manual steps and is therefore susceptible to contamination, time consuming and inconvenient for the analyses of large numbers of samples at sea. It also requires relatively large sample volumes (up to 250 ml) in order to achieve a high pre-concentration factor.

It is also possible to concentrate the analyte after formation of the chromophore. One approach is to use an immiscible organic solvent, such as hexane, to extract and concentrate the MB [7] or 12-molybdophosphate-malachite-green ion-pair complex [23]. Alternatively, the coloured compound can be concentrated by extraction onto an acetate or cellulose nitrate filter, followed by dissolution of the filter in a small volume of organic solvent prior to spectrophotometric analysis [24,25]. While LODs as low as 0.6 nM have been reported [24], all of these methods involve several manual steps and require the use of organic solvents. More recent efforts have included an automated FIA system, which concentrates an ion-pair complex of phosphor-MB and cetyltrimethylammonium bromide (CTAB), a cationic surfactant, onto a C18 SPE cartridge [26]. An LOD of 1.6 nM was reported, but slow ion-pair formation resulted in low sample throughput.

Analogously, in a reported HPLC method, the phosphor-MB complex is concentrated onto a C8 column [27]. This method uses manual sample derivatisation prior to HPLC analysis, so it is more labour intensive than the FIA approaches.

A general disadvantage of all the methods in which the chromophore is concentrated is that the reagents are also concentrated, resulting in increased blank values. In many cases, it is necessary to purify the reagents prior to use, or to purchase very pure reagents.

2.3. Enhancing the detection technique

Another way to determine nM phosphate concentrations is to use a method of detection that is more sensitive than conventional spectrophotometry. Chemiluminescence offers superior sensitivity to spectrophotometry, because the signal is determined against a low background, so it is often applied in trace analysis. The oxidation of luminol (3-aminophthalhydrazide) results in the chemiluminescent emission of blue light ($\lambda \sim 440$ nm) and is the basis of several methods for phosphate analysis. Since Mg^{2+}, Ca^{2+} and other metal cations present in seawater can also facilitate luminol oxidation, the technique is combined with a pre-concentration step, which removes the sample matrix and concentrates the analyte. A recent example is a luminol-based FIA system, in which the ion-pair complex between CTAB and 12-molybdophosphate is extracted onto a C18 SPE cartridge [28]. In this approach, the cartridge required rinsing with both water and ethanol to remove traces of sea-salt matrix, and this, along with the need to buffer the luminol reaction at high pH, made the injection programme somewhat complicated. The approach provided an LOD of 2 nM PO_4^{3-}, but the precision (12% RSD at 42 nM PO_4^{3-}) was low in comparison with other available methods.

Electrochemical methods for the analysis of phosphate are also available. Orthophosphate is electrochemically inactive and therefore requires derivatisation in order to be detectable. Many reported electrochemical techniques for phosphate analysis rely on the reduction of 12-molybdophosphate or the oxidation of phosphor-MB. Electrochemical techniques have advantages over spectrophotometric methods:

(1) they suffer less interference from dissolved silicon or turbidity; and,
(2) they do not suffer from refractive index (Schlieren) effects in high-salinity samples.

However, the LOD of these techniques is typically of the order of 0.1–5 μM [29], so it is necessary to combine them with analyte pre-concentration. To date, there have been no reports of an electrochemical method suitable for the determination of nM phosphate in seawater.

In absorbance spectrophotometry, lower LODs can be achieved with thermal lensing colorimetry. This uses high-power lasers to increase the signal-to-noise ratio. This has been applied to molybdenum phosphate analysis, giving an LOD of 0.2 nM [30], but it requires complex, expensive and bulky equipment and is not amenable to field applications.

A simpler way to improve the sensitivity of spectrophotometry is to increase the optical path length of the measurement cell. Initially, glass capillaries were coated with aluminium paint or foil to make them internally reflective, but these suffered from non-linearity [31] and attenuation of the light source [31,32].

More recently, coiled quartz capillaries coated with fluoropolymer Teflon AF have been developed [33], allowing the total internal reflection of light within the capillary and creating a long absorbance cell. These liquid-waveguide-capillary cells (LWCCs) are compact, available in various lengths up to 5 m, and do not suffer from the same attenuation or the non-linearity problems associated with early glass capillaries. One of the biggest advantages of using LWCCs is that the simplicity of standard spectrophotometric analysis is maintained, while achieving very low LODs. It is also possible to combine an LWCC with a standard SCFA to create an automated system capable of measuring nM phosphate concentrations with high sample throughput [34]. LODs are of the order of 0.5–1 nM, and data from a number of field studies using this type of approach have been published [2,34].

2.4. Alternative approaches

With the chemical techniques discussed above, sufficiently low LODs are achieved to allow the determination of phosphate concentrations in the majority of surface ocean waters. However, as already mentioned, the reaction conditions employed for SRP measurements result in an overestimation of the true orthophosphate concentration. This overestimation is particularly significant in waters where orthophosphate concentrations are very low. Biological radiolabelled phosphate uptake assays use the ambient microbial community in seawater to determine the true bio-available phosphate concentration. In a bioassay approach used in marine waters, very precise measurements of phosphate concentrations as low as 1 nM have been reported in conjunction with SRP measurements made by long-path-length LWCC photometry [35]. Comparison of the two techniques revealed that the bioassay measurements gave values 7–55% of the SRP determinations. An alternative method has been used successfully in freshwaters to determine phosphate down to concentrations of several 10s of pM, but the technique has not yet been applied to marine samples [36]. While these techniques provide the most specific measure of nutrient concentrations, they are laborious and unsuitable for routine analysis.

3. Nanomolar nitrate methods

Almost all available methods for the analysis of nitrate in seawater rely on its reduction to the more reactive nitrite

Table 2. An overview of reported methods for nanomolar nitrate analysis in seawater

Detection	Chemistry	Technique	Figures of Merit	Comments	Ref.
Colorimetry	Sulphanilamide-NEDD	SCFA 2-m LWCC used as flow cell	LOD: 1.5 nM P: 1.7% (at 20 nM) R: 1.5–600 nM	+ Adapted from established standard technique + Automated and requires no sample pre-treatment + Simultaneous parallel measurement of phosphate ~4 min per analytical cycle	This study
UV abs	None	Ion exchange chromatography	LOD: 40 nM NO_3^- P: 0.6% (at 60 µM NO_3^-) R: 1–60 µM NO_3^-	+ Direct NO_3^- detection + Adversely aected by ship's motion ~40 min per analytical cycle	[43]
FLQ	Tetra-substituted amino aluminium phthalocyanine	Manual sample preparation followed by analysis in a standard fluorometer. Cu-Cd column used to measure nitrate	LOD: 7 nM NO_2^- P: 3.2% (at 350 nM NO_2^-) R: 21–840 nM NO_2^-	Method performance in seawater only assessed for nitrite ~15 min sample preparation and analysis	[55]
Fluorescence	Aniline	rFIA system λex/λem = 610 nm/686 nm	LOD: 6.9 nM NO_3^- P: 50% (at 6.9 nM NO_3^-) R: NR	+ Fully automated system + Corrections made for background fluorescence and reagent blank ~3 min per analytical cycle	[39]
Colorimetry	Sulphanilamide-NEDD	SCFA 2-m LWCC used as flow cell	LOD: 2 nM P: 2.9% (at 10 nM) R: 2–250 nM	+ Adapted from established standard technique + Automated and requires no sample pre-treatment ~2 min per analytical cycle	[46]
UV abs	None	Ion exchange chromatography	LOD: 8 nM NO_3^- P: < 1.2% (conc. NR) R: NR	+ Direct NO_3^- detection + Adversely aected by ship's motion ~40 min per analytical cycle	[42]
Colorimetry	Sulphanilamide-NEDD	Manual sample preparation 4.5-m LWCC	LOD: 1.5 nM NO_3^- P: 8% (at 10 nM NO_3^-) R: 1.5–50 nM NO_3^-	~20 min sample preparation and analysis	[45]

Table 2 (*continued*)

Detection	Chemistry	Technique	Figures of Merit	Comments	Ref.
Chemiluminescence	Ti^{iii}, I^-, O_3	Manual sample preparation followed by analysis in a CL analyser	LOD: 80 nM NO_3^- P: 5% (conc. NR) R: 80–4 000 nM NO_3^-	+ Complex and bulky equipment required + Difficult to control NO_3^- reduction + 5-ml sample volume; a smaller volume can be used to analyse higher concentration samples ~5 min sample preparation and analysis	[56]
Colorimetry	2,4-dnph	NO_2^- method manual sample preparation HPLC analysis with C1 column	LOD: 0.1 nM NO_2^- P: 4% (conc. NR) R: 0.5–1 000 nM NO_2^-	+ Purification of reagents is required ~10 min HPLC injection interval	[38]
TL colorimetry	Sulphanilamide-NEDD	Manual sample preparation	LOD: 0.2 nM NO_2^- P: 0.2% (conc. NR) R: 0.2–50 nM NO_2^-	+ Complex and bulky equipment required + Method could, in principle, be applied to NO_3^- analysis	[44]
Chemiluminescence	Fe^{ii}/moo_4^{2-} , O_3	Manual sample preparation followed by He purging of sample and NO released fed into a CL analyser	LOD: 2 nM nM NO_3 P: 1% (full scale) R: 2–20 000 nM NO_3	+ Varied sample volume for different sample concentrations + Complex equipment + Difficult to maintain temperature of sample for no production ~5–6 min per analytical cycle	[40]
Chemiluminescence	Ti^{iii}, I^-, O_3	FIA system	LOD: 10 nM NO_3^- P: 6.7% (at 10 nM NO_3^-) R: 100–10 000 nM NO_3^-	+ Complex and bulky equipment required + Difficult to control NO_3^- reduction ~3 min per analytical cycle	[41]
Colorimetry	Sulphanilamide-NEDD	NO_2^- method Manual sample preparation Anion exchange pre-concentration 5-cm cell used	LOD: 1–2 nM NO_2^- P: 6.6% (at 4 nM NO_2^-) R: 1–100 nM NO_2^-	+ 500–1000 ml sample required + Multiple steps involved + Sensitive to atmospheric contamination ~40 min per sample	[37]

A number of nitrite methods are included, since these form the basis of most methods for analysis. Where methods are described for both nitrite and nitrate, figures of merit for nitrate only are listed. Methods are listed in order of the year they appeared in the literature, with the most recent listed first. 2,4-DNPH, 2,4-dinitrophenylhydrazine; NEDD, N-naphthylethylenediamine dihydrochloride; FLQ, Fluorescence quenching; UV Abs, UV absorption spectrophotometry; CL, Chemiluminescence; TL , Thermal lensing; rFIA, Reverse flow-injection analysis; SCFA, Segmented continuous flow analysis; LWCC, Liquid-waveguide-capillary cell; LOD, Limit of detection; P, Precision (RSD); NR, Not reported; R, Range of concentrations for which method is reported to be suitable.

anion prior to determination. For this reason, methods for the analysis of nitrate and nitrite must be considered together. Table 2 shows an overview of the reported methods, their LODs, precision, and concentration range for the analysis of nitrate/nitrite in seawater at nM concentrations.

3.1. Pre-concentration approaches

In contrast with trace phosphate, there have been few reports detailing analyte pre-concentration for nitrate analysis. One example uses concentration of the azo-dye product of the standard sulphanilamide and NED procedure on an anion-exchange resin prior to spectrophotometric analysis [37]. This approach yielded an LOD of 1–2 nM NO_2^-; however, the method was sensitive to atmospheric contamination, and large sample volumes (up to 1000 ml) were required. Combined with a prior reduction step, required for nitrate analysis, the procedure would become extremely lengthy and vulnerable to sample contamination.

HPLC has also been applied to determine low concentrations of nitrite. The method relies on the reaction of nitrite with 2,4-dinitrophenylhydrazine to form an azide, which is chromatographically separated from interfering compounds and quantified by light absorption at 307 nm. Concentrations as low as 0.1 nM NO_2^- can be detected [38]. Although highly sensitive, the method is labour intensive, and requires extremely pure reagents. Any adaptation of this method for nitrate analysis would increase its complexity and the risk of sample contamination, making it unsuitable for shipboard analysis.

3.2. Enhancing the detection technique

A number of fluorometric methods are available for the trace analysis of nitrate and nitrite, but interferences from other ions and background fluorescence from dissolved organic matter (DOM) hamper their application to seawater. A recently reported fluorescence approach utilises reverse FIA (rFIA) to reduce interferences [36]. In rFIA, the sample acts as the carrier solution, while a fixed volume of reagent is injected into the sample stream. A background fluorescence reading is determined prior to the fluorescence peak, which results from the reagent addition. The reported rFIA technique is capable of simultaneous analysis of NO_2^- and $NO_2^- + NO_3^-$ (using an in-line copper-cadmium column) using diazotisation of nitrite with aniline [39]. Using data correction for background fluorescence and reagent fluorescence, the approach yielded low LODs (6.9 nM NO_3^-) and generated results in good agreement with a chemiluminescence-based reference technique. The analytical method is capable of 18 analytical measurements per hour, and was applied successfully at sea.

Chemiluminescence-based approaches for nitrate determination have been reported, but, as with fluorometric methods, other ions present in seawater often interfere. Gas-phase chemiluminescence based on the reaction between NO and ozone offers a convenient way of removing matrix effects, and gives a high sensitivity [40]. The reduction of nitrite with acidified KI liberates gaseous NO from the solution, which is subsequently channelled into an NO analyser, where it is reacted with ozone. This reaction produces nitrogen dioxide in an excited state, which decays via the emission of photons. A strong reductant, typically Ti(III), can be used for simultaneous determination of nitrate and nitrite. The main drawbacks of this approach include its technical complexity and the high temperatures (600°C) required to sustain NO concentrations prior to NO reaction with ozone. In addition, the precision of NO_3^- at low levels is impaired, because of the difficulty of precisely controlling the reduction of NO_3^- to NO [41]. The LOD for the method is ca. 10 nM NO_3^-, with a precision of 6.7% at this concentration.

Several groups have developed sensitive ion-exchange chromatographic (IEC) methods for nitrate in seawater [42,43]. With direct UV detection, it is a simple procedure and one of the few techniques that measures nitrate separately from nitrite. Generally, LODs are relatively high, due to the poor shape of the nitrate peak and the tendency of nitrate to co-elute with bromide, and long elution times of 30 min or more are required. LODs as low as 8 nM NO_3^- have been reported [42], but, during sea trials, Maruo et al. obtained a lower sensitivity than in the laboratory due to motion of the ship [43].

As with phosphate, thermal lensing has been applied to the standard colorimetric analysis of nitrite [44], but this approach has not been widely used due to the complexity and cost of the equipment.

Again, in parallel with phosphate analysis, LWCCs have been used to enhance the sensitivity of the standard colorimetric nitrate (and nitrite) analysis. At first, sample and reagents were mixed manually prior to introduction into the flow cell [45].

More recently, Zhang combined a segmented continuous flow autoanalyser with a 2-m LWCC to produce an automated instrument capable of detecting 0.1 nM NO_2^- and 2 nM NO_3^- with a throughput of 30 samples per hour [46]. The inherent simplicity of long-path-length spectrophotometry has enabled its successful application to nitrate analysis at sea [2,46,47].

3.3. SCFA combined with LWCCs

An analytical instrument capable of simultaneous analysis of nitrate (plus nitrite) and phosphate at nM concentrations has been constructed in our laboratory. The system is capable of measuring 15 samples per hour with high precision, and has an LOD ($3 \times \sigma$ of blank) of 0.8 nM PO_4^{3-} and 1.5 nM NO_2^- plus NO_3^-. The instrument comprises a purpose-built, 2-channel SCFA system

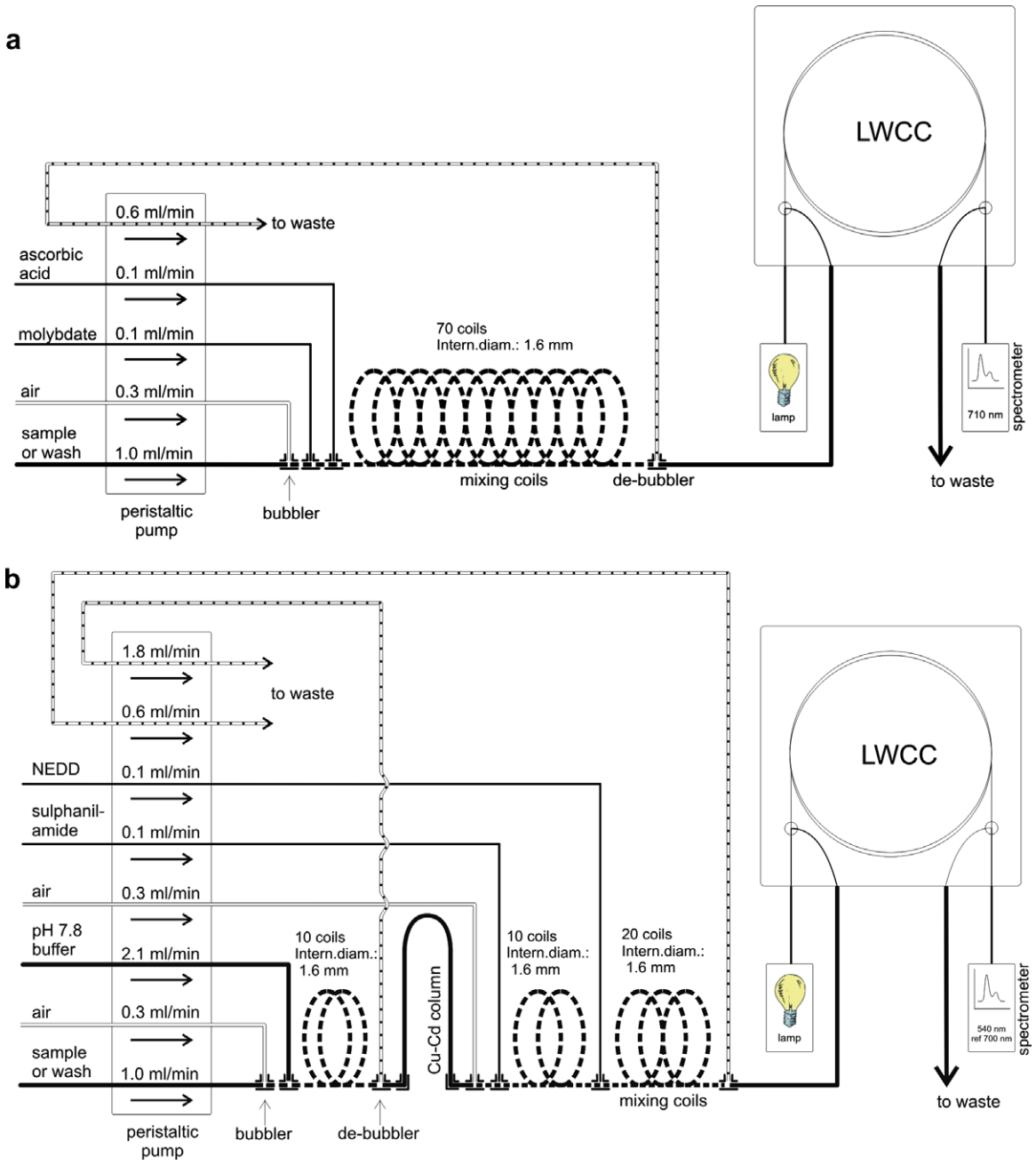

Figure 2. Phosphate and nitrate+nitrite SCFA-LWCC systems in use in our laboratory. The system design is based on Zhang [34,46]. The glass coils used are 1.6-mm ID and larger than the 1-mm ID components used by Zhang, which may account for the lower analytical throughput achieved with the systems in our laboratory. a) Phosphate SCFA-LWCC system showing flow rates in ml/min. b) Nitrate+nitrite SCFA-LWCC system, showing flow rates in ml/min.

connected to two 2-m LWCCs (WPI Inc, USA) (Figs. 2a and b). A peristaltic pump continuously mixes the reagents with the sample stream, and the coloured products form in the glass mixing coils. Two tungsten-halogen light sources (LS1-LL, Ocean Optics Inc., USA) are used and two miniaturised USB spectrophotometers with fibre-optic connections (USB2000 VIS-NIR, Ocean Optics Inc., USA) continuously monitor the absorbance at appropriate wavelengths in the LWCC flow-cells. Four SMA-terminated fibre-optic cables (Ocean Optics Inc., USA) transmit light to and from the LWCCs. Samples are introduced into the instrument manually or using an autosampler. Analytical reagent-grade chemicals are used throughout, with the exception of the nitrate standard, which is prepared from high-purity KNO_3. Reagents and stock standard solutions are prepared in de-ionised water (Milli-Q, Millipore; resistivity >18.2 MΩ/cm).

Refractive index changes (Schlieren effect) caused by differences in salinity between samples, standards and wash solution can cause baseline instability and lead to errors in peak-height determination. For this reason, it is important to match the salinity of wash solution and standards to the salinity of the sample [7]. Low-nutrient surface seawater is ideal for this purpose. In the case of phosphate analysis, it is possible to prepare phosphate-free seawater: 1M NaOH is added to seawater at a ratio 1:40 v/v, the phosphate-containing precipitate is allowed to settle overnight and the overlying solution is siphoned off [22,34]. However, there is no convenient way to remove nitrate from seawater, so it is necessary to analyse nitrate standards prepared in deionised water for seawater-sample calibration. Fortunately, with the nitrate method, the dilution of the sample with the buffer solution (in our case it is diluted ~3-fold with a 0.06 M imidazole buffer at pH 7.8) usually results in minimal ionic strength differences between seawater sample and standards in the final mixture [7,46].

The instrument uses the sulphanilamide/NED reaction for nitrate analysis (incorporating a copperised cadmium column for reduction of NO_3^- to NO_2^-) and the MB reaction for phosphate [34,46]. For the nitrate chemistry, the detection wavelength is 540 nm. A reference wavelength of 700 nm is used to compensate for light-intensity fluctuations resulting from various sources including variations in lamp intensity, micro-bubbles within the flow cell, or Schlieren effect, and this approach hence enhances the signal-to-noise ratio.

The standard wavelength for the phospho-MB procedure is 880 nm or 885 nm. However, the transmission of light of these wavelengths in a 2-m LWCC is negligible, due to the absorption of far-red wavelengths by water. This phenomenon precludes the use of long-path-length LWCC spectrophotometry with aqueous solutions at wavelengths greater than approximately 750 nm. For this reason, phospho-MB is determined using a slightly less intense absorption wavelength of 710 nm. Another general limitation of the phospho-MB flow-analysis techniques is the lack of a suitable reference wavelength to correct for intensity fluctuations. The analysis of phosphate is therefore more strongly affected by the formation of micro-bubbles within the flow cell and the Schlieren effect, resulting in a lower signal-to-noise ratio compared with the nitrate system, for which a suitable reference wavelength exists. However, broadly similar LODs are obtained for both analytical nutrient techniques since the nitrate method requires dilution of the sample with a buffer solution. The influence of the Schlieren effect on the phosphate analysis also means that it is important to match the salinity of wash solution to that of standard and sample solutions.

Figs. 3a and b show the output of the phosphate instrument during calibration and the corresponding calibration curve. With SCFA, analyte contamination in the reagent solutions does not contribute proportionally to the analytical blanks. This is because the reagents are continuously pumped through the analyser and the resulting baseline signal is usually set to zero. However, it is still desirable to use reagents containing minimal concentrations of the analyte of interest, since this will lower the baseline, give an improved LOD and increase the linear dynamic range of the method.

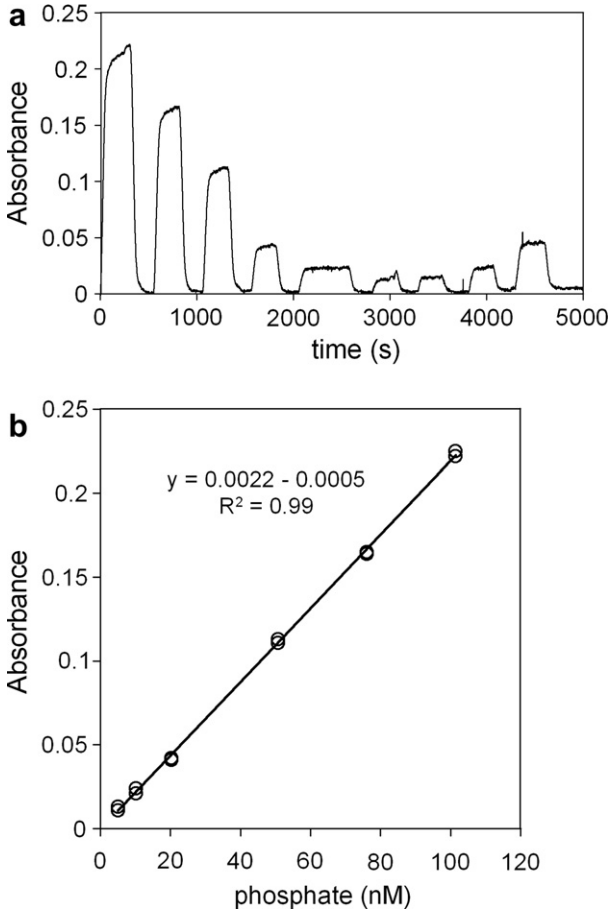

Figure 3. (a) Example of phosphate instrument output during a calibration. Samples were introduced manually, rather than using the autosampler, resulting in varying peak widths. The peaks represent (in chronological order) 100, 75, 50, 20, 10, 5, 5, 10 and 20-nM PO_4^{3-}. (b) Linear regression resulting from the instrumental trace shown in Fig. 3 (a). Additional 50, 75 and 100-nM standard peaks included later in the analytical run have been included in the plot, but are not shown in Fig. 3 (a).

3.4. Analytical challenges

Sample contamination is a major issue when determining nM nutrient concentrations. For this reason, it is preferable to use bottles, volumetric flasks and other apparatus made from plastics that are easy to clean, such as polyethylene or polypropylene. All vessels and instruments that make contact with sample or standard solutions should be thoroughly cleaned in acid. Soaking equipment in 1M HCl overnight, followed by three rinses

with deionised water is sufficient to remove traces of nutrients. Cleaning protocols involving nitric acid, common in trace-metal analysis, risk introducing nitrate contamination and are unsuitable for trace-nitrate analysis.

Atmospheric contamination forms a risk during nitrate analysis. Yao et al. noted that sample blanks left open to the atmosphere overnight, developed nitrite concentrations of between 73 and 170 nM [45]. Zafiriou et al. noted a similar effect with their nM nitrite measurements [48].

Sample and standard stability forms another potential challenge. Our approach is to dilute working standards immediately prior to analysis. Samples are stored in low-density polyethylene (LDPE) bottles in a refrigerator and analysed as soon as is practicable and preferably within 3 hours of sampling. However, there are no reported studies of the stability of seawater samples containing nM nutrient concentrations. One comparison of results from the analysis of frozen samples with samples that were analysed immediately after sampling demonstrated that the samples containing lower concentrations of nutrients were poorly preserved [2].

Contamination of the wash solution is common. Since concentrations are calculated from the height of the sample peak above the baseline, any contamination of the wash solution will increase the absorbance of the baseline and may lead to an underestimation of sample concentrations. This is more likely to occur when samples are introduced manually by moving the sample line between sample or standard solutions and the wash solution. Refreshing the wash solution minimises the contamination risks, and analysis of one or two standards at regular intervals during sample runs will help to spot any irregularities.

Micro-bubbles, which can form from dissolved air in the sample and reagent lines within the instrument can also pose a major challenge. These micro-bubbles have a tendency to attach to the internal surfaces of the LWCC, resulting in erroneously high and fluctuating absorbance readings. The large internal surface-area-to-volume ratio of the LWCC makes this much more of a problem compared with smaller conventional flow-cells. One solution is to de-gas reagent and sample solutions prior to their introduction into the instrument. Vacuum de-gassing or sparging with a low-solubility gas, such as helium, is commonly used in FIA, but is not easily applied to SCFA, since this approach involves the deliberate introduction of bubbles into the flow stream. In-line degassers with very small internal volumes are now commercially available. As with LWCCs, they contain Teflon AF capillaries, but here use is made of the exceptionally high gas permeability of Teflon AF rather than its special optical properties. The use of such a degasser inserted between a nitrate FIA system and an LWCC has recently been reported [49], and significant improvements in signal-to-noise ratio were demonstrated. However, Teflon AF degassers are relatively expensive and not currently in widespread use. Alternatively, the tendency of micro-bubbles to attach to the walls of the LWCC can be reduced by maintaining the

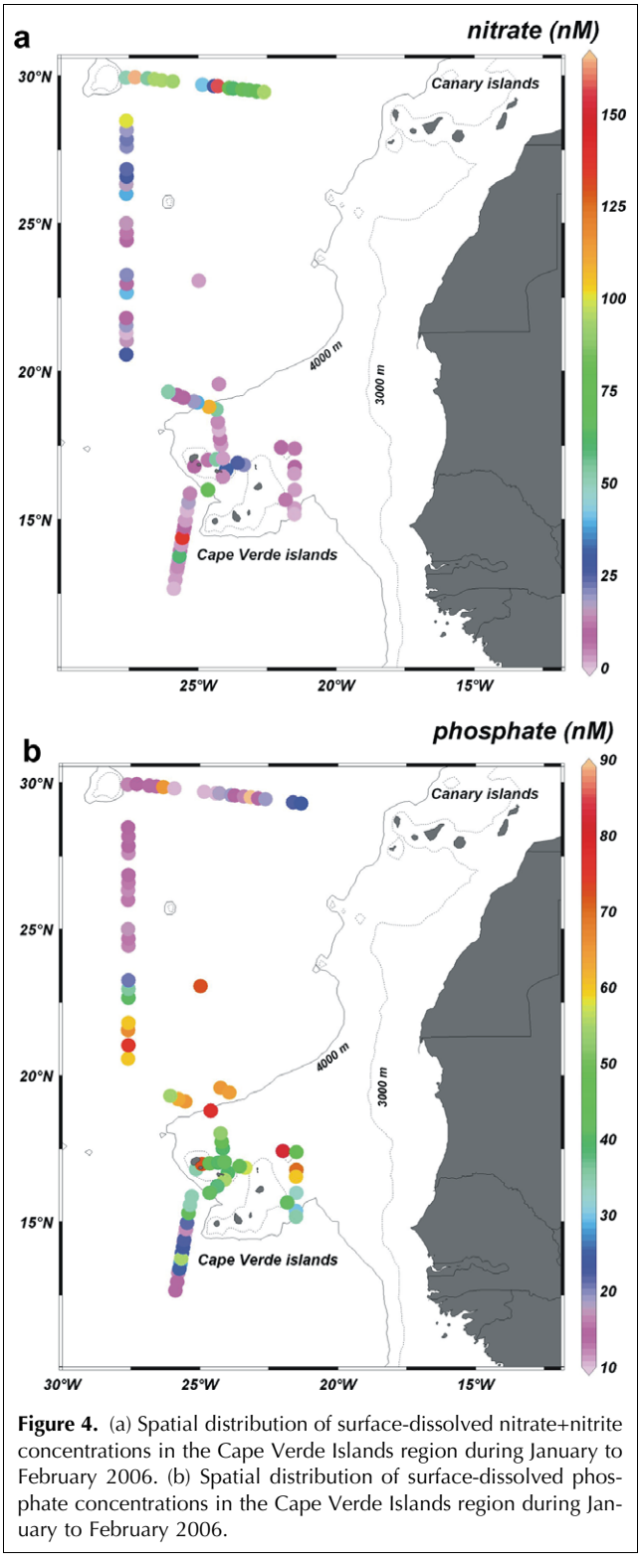

Figure 4. (a) Spatial distribution of surface-dissolved nitrate+nitrite concentrations in the Cape Verde Islands region during January to February 2006. (b) Spatial distribution of surface-dissolved phosphate concentrations in the Cape Verde Islands region during January to February 2006.

cell in a clean state. In our laboratory, the LWCC is cleaned thoroughly before and after use according to the manufacturer's instructions, and this approach has been found to be effective.

3.5. Field study

The SCFA-LWCC instrument was deployed during a research cruise on the *FS Poseidon* (PS332) to the tropical and sub-tropical North-East Atlantic Ocean in January–February 2006. Surface samples (ca. 3 m depth) were collected using a towed fish, which allowed contamination-free sampling within a clean laboratory container. Over a 4-week period, 170 samples were collected and analysed for nitrate (+ nitrite) and phosphate using the instrument. Figs. 4a and b show the spatial distributions of nitrate (+ nitrite) and phosphate, respectively, in the study region. The observed nutrient concentrations were low because of active biological uptake. Surface nitrate (+ nitrite) concentrations in surface waters were in the range <1.5–165 nM, with the lowest concentrations in thermally stratified waters (mixed layer depth ca. 60 m) in the western and southern areas of the study region, and higher concentrations in the northern areas with deeper mixed layers (ca. 120 m). Surface-phosphate concentrations were in the range 10–90 nM, with the lowest concentrations in thermally stratified waters in the western and southern areas of the study region. The highest phosphate concentrations were observed in the area between 18°N and 22°N as a result of upwelling of nutrient-rich deep waters and horizontal advection of nutrient-rich waters from the Northwest African upwelling region. These results revealed low nutrient concentrations, which strongly influence primary productivity in the study region and are undetectable with conventional methods of analysis.

4. Conclusions

A large number of techniques exist to determine nM concentrations of nitrate and phosphate in seawater and other natural waters. Of these, only the MAGIC method for phosphate has achieved widespread acceptance, but this method is labour intensive and not amenable to automation. More recently, techniques employing LWCC flow cells have become more popular. These cells can be added to existing flow-analysis-based instruments with relative ease to give nM LODs. Flow-analysis systems are generally rugged, portable, automated and capable of high sample throughput with minimised risk of sample contamination, which makes them ideally suited for shipboard analysis.

4.1. Future directions

Various challenges still need to be addressed in nM nutrient analysis which is still a relatively new field.

As mentioned previously, corrections for interference from arsenate are rarely applied to phosphate measurements. Measurements of relatively high concentrations of phosphate contain only a small error, due to the low concentrations of arsenate present in seawater (10–30 nM [7,18,50]). However, now that it is possible to analyse phosphate concentrations of several nM, the interference from arsenate may be significant. There is a pressing need for a new method for the elimination of arsenate interference that is compatible with flow analysis.

Another challenge is the lack of any certified seawater standards containing zero or very low nutrient concentrations. It is possible to prepare standards by dilution from higher concentration solutions into low-nutrient seawater, but corrections are often required for salinity differences and low concentrations of nutrients in the standard matrix. Furthermore, the lack of any common standards makes verification of results from different studies difficult. Since solutions containing nM nutrient concentrations are unstable and easily contaminated, it is currently not feasible to produce such standards. Very few studies have investigated the stability of solutions containing nM concentrations of nutrients, yet such studies are essential in order to move forwards in this area.

At present, there is a drive to automate and to miniaturise analytical systems with a view to make them submersible [11,51]. Such in-situ analysers could potentially be attached to frames lowered from research vessels or left on moorings for months at a time. A submersible nitrite system containing an LWCC has been reported [52]. Such an approach will eliminate sample-handling and storage issues and provide data at much higher resolution than is possible with traditional sampling methodologies.

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