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MEDITERRANEAN ACTION PLAN

Meeting of the MED POL National Coordinators

Sangemini, Italy, 27 - 30 May 2003

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REGIONAL SEAS

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IN

SEAWATER

Reference Methods for Marine Pollution Studies
(Draft)

2003

Within the Regional Seas Programme of UNEP, many scientists are concerned about eutrophication problems and there is therefore an increasing demand for the reliable analysis of both nutrients and phytoplankton pigments in seawater.

This manual is based on the Manuals prepared by Dr. Ron Johnstone of the Zoology Institute, University of Stockholm, Sweden, and Dr. Martin Preston of the Oceanography Laboratories, University of Liverpool, United Kingdom for IOC (Manuals and Guide No 28) and for the MOOPAM (third edition, 1999) of ROPME.

This is a version updated in January 2003 by Dr. M. Preston to take into account the slight changes in procedure and standardization of the units that occurred since these Manuals were edited. Some basic procedures for phytoplankton pigment analysis have now also been included though advanced instrumental techniques for identification and quantitation of pigments are outside the scope of this manual.

This Manual was compiled by Dr. Jean-Pierre Villeneuve, IAEA-Marine Environmental Laboratory, Monaco

Table of contents: **Page:**

I General safety matters

All laboratories should have a properly formulated safety policy, which complies with all national health and safety legislation. Clearly, this policy must be constructed with the aim of providing a working environment that is both safe and appropriate for the procedures that are to be conducted within it. It should include things such as appropriate procedures for the handling of chemicals, a spatial plan for the laboratory with items such as emergency exits and fire extinguishers highlighted, as well as an integrated presentation of duties and prescribed responses under different situations in line with the specific laboratory facility. Our experience has been that many laboratories have, at best, a poorly structured laboratory policy and rarely have a policy in which is customized to the particular facility. The adoption of a general policy from an external or foreign system may be good first step but this is of limited use unless it is modified so that it is appropriate for the local situation.

All laboratory exercises involve some hazards and it is vital to ensure that as much as possible is done to understand and minimize them before commencing work. Some of the chemicals described in this document are highly damaging to human health if improperly used. Chemical companies will now normally provide documentation with each chemical purchased that identifies the specific dangers involved. This information should be assimilated and applied appropriately.

The information and procedures outlined in this manual are supplied in good faith but must be carefully evaluated by the potential user and procedures should only be conducted when all appropriate risk and chemical hazard assessments have been satisfactorily completed.

II Sample collection

Water samples from specified depth are normally collected and analyzed for a variety of physical, chemical and biological parameters as well as for pollutant analysis. A number of monitoring programs rely only on the collection of surface water samples. In such cases investigations should establish that the water column is homogeneous with no nutrient concentration variations between surface and deep waters and therefore that a surface sample is representative of the whole water column.

Water samplers of various designs have been used and there are numerous designs, which are commercially available. The non-reversing type sampler - such as Niskin or van Dorn type - usually consists of a PVC cylinder with top and bottom caps. The top and bottom caps are held open by a clamp or hook against the tension of a spring or a rubber band connecting them through the bottle.

The action of a messenger releases the clamp, and the caps are pulled into position, closing off the top and bottom of the bottle. When the bottles are used in a series, the closure of one bottle releases a messenger below it, which travels down the

wire and trips the next bottle. The samplers, which are lowered to depths with both caps open, are adequately flushed during lowering.

Niskin bottles may also be conveniently mounted on a rosette (which may also carry other instrumentation such as a CTD). In this case bottles can be closed at will through commands issued from the surface and transmitted to the unit through a conductive cable.

Another type of non-reversing water sampler recommended for this sampling is the “Go-Flo” type water sampler. This type of sampler was designed for use in pollution studies to collect uncontaminated water samples from specified depths. The sampler enters the water closed, to avoid surface film contamination, is opened by a pressure actuated valve and is re-closed by a messenger at the proper depth (or via an electronic signal if mounted in a rosette). Since this type of sampler has no internal closing springs, which contact the sample, they are highly recommended for both general water samples and pollutant samples though they are less well suited to waters with a high suspended sediment load because sediment particles may attach to the bottle seals in such a way as to prevent the bottles from closing properly.

Water sampling bottles should be held in a rack, which enables the sub-samples to be collected. Below the bottles, there should be a compartmentalised sub-sample bottle rack with one compartment for sub-sample bottles under each water sampler. The rack should be designed to accommodate the sampling bottles. If an inside wet laboratory is not available for the rack to be installed, canvas screens must be rigged to protect it from sunlight, salt spray and wind.

General procedures for handling samples

Although extremely simple, the process of rinsing and filling sub-sample bottles must follow a strict routine. The general procedures are as follow:

a) Empty previous contents of bottle, if any.
b) Fill bottle one third full taking care to keep fingers clear of the water. ***Note that the fingers of people who smoke may be contaminated with potassium nitrate and it is therefore particularly important that there is no contact with samples for nutrient analysis. In general there should be a no smoking policy and plastic gloves should be worn during sample handling***
c) Replace cap loosely and shake vigorously.
d) Pour out.
e) Repeat b, c and d - repeat twice, if bottles are used for the first time during the cruise.
f) Fill bottle to just above the shoulders of the bottle.
g) Wipe the screw thread on the outside of the top of the bottle and the inside of the cap with a new tissue paper and then discard the paper.
h) Screw on the cap gently but firmly.

The above procedures outline the general considerations of water sampling and should be treated as guidelines for the following detailed collection methods.

III Procedures for the determination of chlorophyll

Note: for a comprehensive review of the analysis of phytoplankton pigments the book "Phytoplankton pigments in Oceanography", S.W. Jeffrey, R.F.C. Mantoura and S.W. Wright (1997) UNESCO Publishing ISBN 92-3-103275-5 should be consulted.

A) Sampling and sample handling

The general guidelines outlined in Section IIA above represent good practice in sample handling. It is further recommended that sample should be drawn into a polyethylene or other plastic container and should be filtered as soon as possible after collection

B) Determination of Chlorophyll using spectrophotometer

The procedure agreed to by a SCOR/UNESCO working group and published by UNESCO in Monographs on Oceanographic Methodology, No 1, is adopted for the determination of chlorophyll pigments. It is also possible to determine chlorophyll by using in-situ fluorometer or by HPLC (the details of these procedures are not given in this manual but can be found in Jeffrey et al. 1997).

i) Equipment

a) Filtering apparatus consisting of the following items:
   - Vacuum pump
   - Manifold (3 or 6 place)
   - Funnel assembly (3 or 6)
   - Vacuum hose

b) Polyethylene measuring cylinder (1 L)
c) Polyethylene bottle (2 L)
d) Nylon net (0.3 mm mesh size)
e) Filters (0.45 µm in pore size: glassfibre filter, Whatman GF/F)
f) Spectrophotometer with cell having a path length of 4 – 10 cm
g) Swing out type centrifuge
h) Stoppered graduated centrifuge tubes of 15 ml capacity having both glass and polyethylene stoppers

ii) Reagents

a) Silica gel

b) Magnesium carbonate suspension.
Add approximately 1 g of fine magnesium carbonate to 100 ml of distilled water in a stoppered Erlenmeyer flask. Shake vigorously to suspend the powder immediately before use.

c) Acetone 90 % solution:
Distill reagent grade acetone over about 1 % of its weight of both anhydrous sodium carbonate and anhydrous sodium sulfate. Collect the fraction boiling at a constant temperature near 56.6 ºC. Put 100 ml of distilled water into a one liter volumetric flask and add acetone to make the volume exactly 1000 ml. The re-distilled acetone should be stored in a tightly stoppered dark glass bottle and the 90 % reagent prepared in moderately small amounts for use. If good quality acetone is available, it should be shaken with a little granular anhydrous sodium carbonate and decanted directly for use.

iii) Filtration of the sample

It is recommended that the sample drawn from the water sampler should be filtered immediately on board. However, samples may be stored for short periods in the dark and at ~4^{o}C (for longer storage see note below). The volume of sample required depends on the amount of phytoplankton present; with ocean water, about four to five liters should be used but with coastal and bay waters, sometimes one tenth of this amount is sufficient.

Set up the filtering system on board or in the laboratory. Place the filter on the base of the filter holder, install funnel and clamp together. Add about 10 mg $MgCO_3/cm^2$ of filter surface as a suspension in filtered seawater.

The required volume of a sample should be measured in a polyethylene measuring cylinder, transferred to a polyethylene bottle and shaken vigorously, before filtration.

Invert the sample bottle into the funnel through a small piece of clean 0.3 mm mesh nylon netting to remove the larger zooplankton and then commence filtration with no more than two thirds of full vacuum.

Drain the filter thoroughly under suction before removing it from the filtration equipment but do not suck large quantities of air through it. The filter is ready for chlorophyll measurement.

Note: It is preferable to extract the damp filter immediately and make photometric measurements without delay, however, the filter can be stored in the dark at freezer 18 ºC or less for up to two months.

iv) Extraction and measurement procedure

Fold the filter (sample inside) and place it in a small (5 – 15 ml) glass, pestle type homogenizer.

Add 2 - 3 ml 90 % acetone. Grind it for one minute. Transfer the mixture to a stoppered centrifuge tube and wash the pestle and homogenizer two or three times with 90 % acetone so that the total volume is 10 - 15 ml. Keep it for 10 minutes in the dark at room temperature. During the extraction period pigments are very photosensitive and neither extracts nor the un-extracted sample should be exposed to strong sunlight or else chlorophyll values will be reduced to a small fraction of their initial level.

Centrifuge for 10 minutes at 4000 - 5000 rpm, but note that the efficiency of this step should be tested with each instrument used. It is recommended that a swing-out type centrifuge is utilized, because it gives better separation than an angle centrifuge. The liquid should be decanted and transferred into a calibrated flask and made up to volume. Then, the liquid is carefully poured or pipetted into the spectrophotometer cell.

If turbid, try to clear by adding a little 100 % acetone or by centrifuging again. If necessary dilute to a convenient volume. This depends on the spectrophotometer cell used. Dilute with 90 % acetone if the extinction is greater than 0.8.

As part of the laboratory Quality Assurance procedures it is desirable to check the wavelength calibration of the spectrophotometer from time to time using rare earth salt solutions (holmium or didymium) or optical filters.

For pigment analysis use a spectrophotometer with a band width of 3 nm or less, and cells with a light path of 4 – 10 cm. Read the extinctions at 750, 663, 645 and 630 nm against a 90 % acetone blank. Correct the extinctions at each wavelength against blank value. If the 750 nm is greater than 0.005/cm light path, reduce the turbidity as described above. Note that it is important to check the cell blanks and zero settings at all wavelengths.

v) Calculations

Subtract the extinction at 750 nm from the extinction at 663, 645 and 630 nm. Divide the answers by the light path of the cells in centimeters. If these corrected extinctions are E_{660}, E_{645} and E_{630} the concentration of chlorophyll a in the 90 % acetone extract as µg/ml is given by the following equation:

$$\text{Chlorophyll } \underline{a} = 11.64\ E_{663} - 2.16\ E_{645} + 0.10\ E_{630}$$

Note: the Jeffrey et al. (1997) SCOR/UNESCO book suggests that the original SCOR-UNESCO (1966) equations are good only for Chlorophyll a. For a, b and (c_1 + c_2) the equations below are recommended for mixed phytoplankton communities.

Note also that slightly different wavelengths are used for the absorbance measurements:

$$\text{Chlorophyll a} = 11.85\ E_{664} - 1.54\ E_{647} + 0.08\ E_{630}$$
$$\text{Chlorophyll b} = -5.43\ E_{644} + 21.03\ E_{647} - 2.66\ E_{630}$$
$$\text{Chlorophyll } c_1+c_2 = -1.67\ E_{664} - 7.60\ E_{647} + 24.52\ E_{660}$$

If the values are multiplied by the volume of the extract in milliliters and divided by the volume of the seawater sample in liters, the concentration of the chlorophyll in seawater is obtained as µg/l (= mg/m^3).

Note: Determination of Chlorophyll and their degradation products using HPLC: Again, the Jeffrey et al. (1997) book has very detailed information about HPLC procedures and the readers should be referred to it.

IV Nutrients - Collecting, handling and preservation

A) General comments

Nutrient concentrations in surface water are often very low. This is particularly true for tropical/subtropical waters, open ocean (blue water) environments and during the summer months in temperate waters. Such low concentrations present a significant challenge to the analyst. This situation is further complicated by the high reactivity of nutrient species which renders samples relatively unstable. As a result of these considerations, a number of general principles can be recommended:

1) The time between sample collection and analysis should be as short as possible.
2) To gain maximum sensitivity, the optical cells utilized for spectrometric analysis should be as long as possible. 10 cm cells are recommended and cells shorter than this should be regarded as being suitable only for higher concentration samples.
3) The concentration of standards should always bracket the concentrations of samples. This is particularly important at the extremes of the concentration ranges where non-linear effects can be highly significant.
4) All spectrophotometric measurements should be referenced against distilled water. The practice of using blank solutions in the reference cell is unsatisfactory because it can introduce additional and unnecessary uncertainty into the measurement.
5) To avoid salinity effects, the use of Low Nutrient Sea Water (LNSW) for the final dilution stages of sample preparation is recommended.

6) All apparatus must be carefully cleaned and dedicated to the task.
7) Certain analyses should be conducted in different laboratories. For example Nitrate analysis (using NH_4Cl) should not be conducted in the same laboratory as ammonia analysis. Nitric acid should not be used anywhere in nutrient analysis.
8) Many of the chemicals utilized in nutrient analyses have significant toxicities. All laboratories should conduct proper risk and hazard assessments in accordance with their local regulations before commencing work.

B) Blanks

It is essential to make adequate assessment of analytical blanks. In the case of nutrient analysis these will include reagent blanks and cell blanks. Reagent blanks can be measured by treating an aliquot of distilled water as a sample and carrying out the full analysis. Cell blanks are assessed by filling spectrophotometer cells with distilled water and measuring the difference between sample and reference cells. In both cases the blank values must be subtracted from standard and sample readings before any plots/calculations are made.

C) Quality control

The requirement for adequate control of the quality of the data produced by a laboratory is paramount both if the data is to be useful to the scientists producing the data and the wider scientific community. It is impossible to compare differences in nutrient concentrations at different times or at different places if the errors associated with the analysis are greater than the differences or, worse, the errors are unknown.

Internal Reference Samples can be prepared by the laboratories for their own use according to the method of Aminot and Kerouel (1995). A know quantity of nutrients is added to a nutrient depleted seawater (NDSW - also known as Low Nutrient Sea Water LNSW). This NDSW is gravity-filtered through a 10 µm Gelman polypropylene membrane fitted in an on-line Millipore filter-holder (47 mm in diameter). After mixing, the seawater is bottled and autoclaved in a benchtop autoclave at 120 °C for 30 minutes.

Laboratories are also encouraged to participate in regional or international Quality Control schemes such as those operated by IAEA or QUASIMEME

D) General sampling and sample handling

Since water samples collected for nutrient analysis must often be filtered prior to analysis, it is recommended that aliquots of the water sample which is filtered for suspended particulate matter determination be used for nutrient analysis in order to minimize differences between regional sampling programs.

E) Filtration

Note: Filtration is not recommended for ammonia analysis (see below).

In general it is to be recommended that samples be filtered if there is visible turbidity but not if the water is clear. It is more likely to be necessary to filter waters from close to the land than those from samples obtained from "blue" waters off the continental shelf. If filtration is necessary then this should be performed as soon as possible after collection and the exposure of the sample to the air must be minimized. Dispensing of samples in a way that generates bubbles, for example, should be actively avoided.

The easiest and most effective filtration units for nutrient analysis are those which may be directly attached to a syringe containing the sample. Suitable units are made by a number of manufacturers but the unit made by Millipore may be taken as a reference point. Filter units should be properly cleaned before use and, filters once installed, should be pre-cleaned by passing distilled de-ionized water (ddW) through them. At least two sample volumes of distilled water should be used for cleaning purposes. Also, check that the filter is not ruptured during the installation process. This may easily be done with syringe/filter combinations by gently trying to push air through the wetted filter. If when you release the plunger, it springs back, then the filter is in good order, if however, the plunger does not do this, then it is likely that the filter is either not correctly sealed or it is ruptured. We have obtained good results with 0.4 µm Nuclepore filters (cleaned in cold 6 mol/l HCl for 3 days and rinsed with distilled water) or, if silicate analysis is not to be performed, with glass fibre filters from Sartorius (Type S 13400, cleaned for a maximum of 4 hours in 0.1 M HCl). Filtration can be carried out by suction or with a pressure filtration system (an all-plastic filtration apparatus, for example, is available from Sartorius (Type SM 16510)). In any case, however, the applied filtration procedures should be examined carefully and independently for each nutrient component of interest. Furthermore, when reporting analytical results from filtered samples the "filtration blanks" should also be quoted.

F) Specific details of sample collection and preservation

In this section only sample collection, preservation and storage will be discussed while detailed analytical methodology will be presented in the next section.

i) Phosphate - P

Water samples for phosphate analysis should be collected in stoppered glass bottles of 50 to 100 ml volume directly from the outlet tube of the in line filter used to collect suspended particulates. The samples are stored in a cool dark place until the analysis can

be performed. For phosphate, the analysis should be commenced as soon as possible, preferably within half an hour, certainly before 2 hours and only glass bottles should be used for intermediate storage of the samples. The samples should not be stored in polyethylene or polyvinylchloride containers since phosphate has been shown to disappear rapidly in these containers. Other plastic e.g. polycarbonate may be satisfactory but should be thoroughly tested before use. Once collected, samples should be stored out of the light in a refrigerator until required for the analysis.

The addition of acid to unfiltered samples cannot be recommended since this cause hydrolysis of any polyphosphates and release of phosphate from plankton and bacteria. The addition of all of the reagents of the analytical procedure to the sample and postponement of the photometric measurement is also not possible, since arsenic and silicate will also react and cause erroneous phosphate readings.

Summarizing, it can be stated that storage of samples for the analysis of dissolved phosphate for more than one hour should be avoided.

ii) Ammoniacal - N

Samples for ammonia analysis should only be taken and stored in tightly sealed seawater-aged glass or polyethylene bottles, which should only be used for the analysis of ammonia. Filtration of samples should also be avoided, if possible, because it is nearly impossible to obtain filters free of ammonia. In addition, vacuum filtration will cause erroneously low ammonia values to be determined because of the “pumping out” of ammonia gas during filtration. Waters with high turbidity frequently contain high concentrations of ammonia and may therefore be diluted before the analysis (the residual turbidity may then be compensated by subtraction of the absorbance of the appropriately diluted sample without addition of reagents).

Ammonia is a nutrient compound, which rapidly undergoes biological conversion, i.e., oxidation into nitrite and nitrate and fixation as amino-bound nitrogen in organisms. The analysis of ammonia should be commenced without delay after sub-sampling. Chemical methods for preservation have been proved unsatisfactory because of the fact that organisms rapidly release ammonia. It is therefore strongly recommended that the ammonia reagents be added within one hour after sampling.

iii) Nitrite - N

Nitrite is an intermediate compound, which occurs if ammonia is oxidized or nitrate is reduced. The presence of higher amounts of nitrite (> 1.5 µM of NO_2) signifies the presence of high bacterial activity in the seawater sample. Storage of samples for nitrite analysis can therefore not be recommended. Chemical preservation (e.g. addition of chloroform) also seems to be unsatisfactory. In turbid waters a filtration step is necessary. Therefore, collect the sub-sample for nitrite determination directly from the

outlet of the in-line filter described above in a 100 - 150 ml glass container. The nitrite reagents should, if possible, be added to the sample within one hour. Intermediate storage of the sample in glass bottles in a refrigerator for up to 3 hours causes, in most cases, no significant changes in the nitrite content, if the original ammonia level is low (< 0.07 µgM). Samples should be stored in tightly sealed glass or polyethylene bottles only.

iv) Nitrate - N

Nitrate is the final oxidation product of nitrogen compounds. Changes of the original nitrate content of a seawater sample can, therefore, only result from oxidation of ammonia and of nitrite or from adsorption of nitrate to the material of the sample container. Adsorption of nitrate into particles seems to be insignificant since the analytical procedure liberates any nitrate, which may be adsorbed. For reasons yet unknown, the nitrate content of a sample decreases rapidly if stored in polyethylene bottles, and at a level of 1.4 µM NO_3 about half of the nitrate disappears within seven days after storage at room temperature. This indicates that only glass or suitable plastic (***other than*** polyethylene) bottles with tight screw caps (preferably with Teflon liners) should be used.

If larger plastic bottles are used for sub-sampling for all nutrient analysis, the amount needed for nitrate should be transferred into a glass bottle within one hour after the sampling. The analysis should not be delayed for more than 5 hours. In this case the samples should be stored in a refrigerator.

v) Silicate - Si

It is obvious that glass bottles should not be used for storage and analysis of seawater samples for reactive silicate. The sub-sampling for silicate analysis should be performed with plastic bottles (made of polyethylene or polypropylene). A few days storage of the sample in the dark in a refrigerator does not lead to significant changes in the silicate content. Polymerization of orthosilicate during storage of frozen samples has been reported from fresh water samples but does not occur in seawater.

The best procedure for storage and preservation seems to be the acidification of the seawater sample with sulfuric acid to a pH of 2.5 and storage in tightly sealed, seawater-aged, high density polyethylene bottles in the dark at about 4 ºC. However, as with all nutrients immediate analysis of the sample is the preferred option

V Nutrient analysis - Analytical procedures

The following procedures assume the samples have been filtered using 0.45µm membrane filter.

Calibration techniques applied in the following procedures are based on the traditional way of calibration using a series of different concentrations of calibration standards chosen so that they bracket all sample concentrations. Absorbance readings are blank corrected, plotted against concentration and a best fit calibration line determined.

Calculations based on molar absorbtivities can also be used though these are regarded as less satisfactory by most analysts.

In the case of any nutrient the calibration standards are prepared by diluting the standard stock solution. A blank and at least four calibration standards in graduated amounts in the appropriate range should be prepared. Modern procedures use low nutrient seawater (LNSW) for the final dilution stages. If this is not available then to avoid the complexity of the sample matrix the best way is to use the method of standard addition in which aliquots of standard are added to a series of sub-samples. The use of distilled water for making up the final dilutions of standards can in some circumstances leas to salt errors.

Generally, in the method of standard addition, equal volumes of sample are added to the de-ionized distilled water blank and at least to three standards containing different known amounts of the determinant. The volume of the blank and the standards must be the same. The absorbance of each solution is determined and then plotted on the vertical axis of a graph, with the concentrations of the known standards plotted on the horizontal axis. When the resulting line is extrapolated back to zero absorbance, the point of interception of the abscissa is the concentration of the unknown. The abscissa on the left of the ordinate is scaled at the same as on the right side, but in the opposite direction from the ordinate. The slope of the plot can be considered as the slope of the calibration curve.

A) Determination of Phosphate - P

Scope and field of application:

The present methods in the analysis of inorganic phosphate in seawater follow essentially the colorimetric method by Murphy and Riley (1962) which is based on the formation of a highly coloured blue phosphomolybdate complex. The modified procedure described here mainly follows the method outlined by Koroleff (1983).

The method shows no measurable effects from salinity but interference may be observed with relatively high concentrations of silicate, arsenate or hydrogen sulfide. The absorbances are proportional to the phosphate concentrations up to about 28 µmole/l when measured in a 1 cm cell.

Principle:

The phosphate ions in the sample react in acidic solution with ammonium molybdate to yield a phosphomolybdate complex. This heteropoly acid is reduced by

ascorbic acid with trivalent antimony ions as catalyst to a blue-colored complex (with molar absorbtivity of about 22,700), the absorbance of which is then measured in a spectro or filter photometer at 882 nm. In order to obtain a rapid colour development and to depress the interference to silicate, it is important that the final reaction pH is less than 1, and that the ratio between sulfuric acid (in mol/l) and molybdate is kept between 2 and 3 percent.

Reagents:

a) Sulfuric acid (4.5 M)

Add 250 ml concentrated acid (d=1.84 g.ml) slowly, under cooling and mixing, to about 700 ml distilled water. Finally, adjust the volume to 1000 ml with distilled water.

b) Ammonium heptamolybdate solution

Dissolve 9.0 g $(NH_4)_6Mo_7O_{24}.4H_2O$ in about 90 ml distilled water and dilute to 100 ml.

The solution should be stored in a polyethylene bottle and should be renewed if any precipitation occurs.

c) Potassium antimonyl tartrate solution

Dissolve 3.25 g $K(SbO)C_4H_4O_6$ in distilled water and dilute to 100 ml.

The solution should be renewed if any precipitation occurs.

d) Mixed reagent

Mix 200 ml sulfuric acid (as above) under continuous stirring with 45 ml molybdate solution. Then add 5 ml tartrate solution.

If the reagent is stored in cool conditions, it is stable for several months.

e) Ascorbic acid solution

Dissolve 7.0 g $C_6H_8O_6$ in distilled water and dilute to 100 ml. Note that solid ascorbic acid is not stable indefinitely and should be rejected if it gives rise to colored solutions.

The solution is stable for at least a week (as long as it remains colorless) if stored in a dark bottle and in a refrigerator.

f) Phosphate stock solution (10 mM PO_4) (A)

Potassium dihydrogen-phosphate (KH_2PO_4, a.g.) is dried in an oven at 110 ºC. Then, exactly 1.3609 g of KH_2PO_4 are dissolved in distilled water, 2 ml of 4.5 M H_2SO_4 is added and the mixture diluted to 1000 ml with distilled water in a volumetric flask.

The solution is stable for at least several months.

g) Phosphate working solution (10 µM PO_4) (B)

Dilute 10 ml of the stock solution with distilled water to 1000 ml in a volumetric flask.

This solution should be prepared daily.

Apparatus:

a) Glass stoppered bottles (ca. 50 ml) or other suitable containers (e.g. polypropylene bottles, Nalgene type No. 2105-002) for sub-sampling.
b) Graduated cylinder (glass or plastic made) for the quick sub-sampling of 50 ml nutrient sample portions (with a hole as sample overflow at 50 ml, see Figure 1).
c) Automatic syringe pipettes of 1 ml or 2 ml volume for reagent additions.
d) Spectro- or filter photometer with filter at or close to 882 nm and cells of 10 cm length (Shorter cells e.g. 5 cm may be used but will give proportionally higher detection limits).

Note: All glass and plastic ware to be used must be cleaned and should be reserved solely for phosphate analysis. The procedure used for cleaning must be tested for phosphate contamination (Common detergents also usually contain phosphates, so that care must be taken when choosing cleaning chemicals). This is also necessary for the distilled or de-ionized water used in the cleaning and analytical procedure described here. It is also recommended to store the sample bottles, when not in use, in 0.1 % v/v HCl solution (after the previous major cleaning) and to rinse them, before sub-sampling, three times with the sample solution.

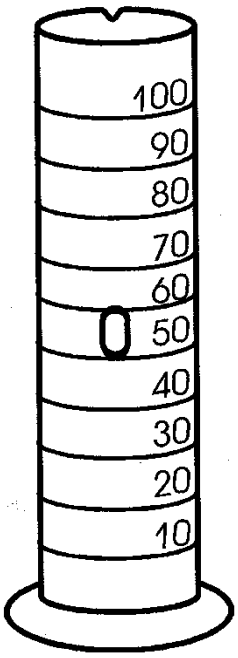

Figure 1: Special type of graduated cylinder (glass or plastic made) for the quick sub-sampling of 50 ml portions in nutrient analysis (with the hole as sample overflow).

Method:

a) Calibration

Prepare a series of working standards from the phosphate working solution (B). To 100 ml volumetric flasks add (by means of pipette or burette) the following volumes and fill up with low nutrient seawater (distilled water may also be used, but salt effects should be considered) to the 100 ml mark. Then the resulting standard concentrations are:

0.5 ml of working solution = 0.5µM PO_4^{3-}
1.0 ml of working solution = 1.0 µM PO_4^{3-}
2.0 ml of working solution = 2.0 µM PO_4^{3-}
3.0 ml of working solution = 3.0 µM PO_4^{3-}
4.0 ml of working solution = 4.0 µM PO_4^{3-}
5.0 ml of working solution = 5.0 µM PO_4^{3-}

To 50 ml portions of these working standards add the reagents and follow the same procedure as described below for analysis of the sample. Prepare a reagent blank from the same volume of distilled water and reagents. Plot the standard concentrations as abscissa versus the absorbances (corrected for the reagent blank). The calibration curve should be linear over the range of concentration.

b) Analysis of samples

Transfer two 50 ml portions of the sample to two reaction flasks by means of the graduated cylinder. One of the portions is regarded as the sample, the other one as the turbidity blank. To each of the portions add 1.5 ml of the mixed reagent and 1.5 ml of the mixed reagent ascorbic acid solution. Mix well between the additions. After 10 minutes (but within half an hour) measure the absorbance of the sample and the turbidity blank at 882 nm against distilled water as reference (the turbidity blank may be negligible especially if filtered samples are employed, and can therefore be omitted at high phosphate concentrations and insignificant turbidity).

c) Interferences

There are some other ions occurring in seawater which potentially may interfere with the formation of the blue phosphomolybdic complex. The major interferences are briefly considered in the following paragraph.

It is well know that silicate and arsenate ions gradually form similar blue heteropoly acid complexes with molybdate ions. As a general rule, however, it may be stated that if the color is measured after 10 minutes, there are practically no interferences caused by silicate (up to 200 µmole/l) or arsenate (at "normal" total arsenic seawater concentration of around 25 µmole/l). If measurements are performed after 30 minutes for example, 200 nmole/l silicate gives - according to Koroleff (1983) - a net but almost negligible increase in absorbance of 0.003 in a 10 cm cell.

The analysis of phosphate may also be influenced by high concentrations of hydrogen sulfide. It has been found that sulfide concentrations up to about 60 µmole/l do not interfere with the phosphate determination. At higher concentrations antimony sulfide is formed (with greenish color) when the acid molybdate reagent is added to these waters. Since high sulfide concentrations are mostly associated with elevated phosphate concentrations, the effect of sulfide can be simply eliminated by diluting the sample with distilled water. If this step is not possible, the sulfide ions should be oxidized by adding bromine water (0.9 ml of bromine in 100 ml of water) drop by drop to an acidified sample (add 0.2 ml of 4.5 mol/l acid to 100 ml sample). The excess bromine is then removed by passing a stream of air or nitrogen through the sample (for about 15 minutes) before commencing the phosphate determination.

Calculation of Results:

Note that some modern spectrophotometers calculate the concentrations automatically from the absorbances of the standards, however, much software cannot deal with two separate blanks (e.g. a reagent and a turbidity blank). In such cases, alternate methods of calculation are preferred.

The concentration of phosphate is determined from the standard curve as described in the calibration section. Calculate the slope b of the calibration curve (for the individual cell length used) which follows the equation:

$$\text{Absorbance} = b \times \text{concentration}$$

Then the phosphate concentration of the sample is obtained from:

$$C\ (\mu g/l) = (A_s - A_{bl} - A_t) / b$$

Where A_s, A_{bl} and A_t are the absorbances of the sample, the reagent blank and the turbidity blank, respectively (Note: A_t is normally very close to 0 for filtered samples).

Estimation of Precision and Accuracy:

Systematic errors in the phosphate analysis mainly originate from an improper cleaning of the glassware, from difficulties during the sub-sampling and from prolonged storage of the untreated samples. The precision of the method can be considered as being between ± 2 % at a relatively high concentration level (about 3 µM) and ± 15 % at the low level of around 0.22 µM.

B) Determination of Ammoniacal - N

The method is specific for ammonia and is based on the formation of the blue colored indophenol by phenol and hypochlorite in the presence of the NH_4^+ and NH_3 species. The reaction requires an elevated temperature or a catalyst. The color is measured at 630 nm and is stable for at least 30 hours.

The procedure outlined here, mainly follows the methods described by Grasshoff and Johansen (1973) and by Koroleff (1983) as described by Hansen and Koroleff (1999). The detection limit of the method is about 0.05 µg/l (in a 10 cm cell), and the Lambert-Beers's Law is followed up to an ammonia concentration of about 40 µg/l.

Principle:

In moderately alkaline solution ammonia reacts with hypochlorite to form monochloramine which, in the presence of phenol, catalytic amount of nitroprusside ions and excess hypochlorite, gives indophenol blue (with a molar absorbtivity of about 20,000). The reaction is quantitative in the pH range between 10.8 and 11.4. The reaction mechanism, however, is complicated and not yet fully understood.

The mentioned salt effect depends on the fact that the final reaction pH is a function of the sample salinity (i.e. increasing salinity with increasing buffer capacity

decrease the final reaction pH). The precipitation of magnesium and calcium hydroxides in the sample solution (occurring at a pH higher than 9.6) is avoided by the addition of a complexing reagent (citrate) which keeps the Mg and Ca ions in solution.

Reagents:

a) "Ammonia-free" water

There is no standard procedure for the preparation of water with very low ammonia content. De-ionized water may sometimes be used without subsequent distillation, but it must be noticed that ion exchange resins potentially bleed out organic substances and ammonia. In case the ammonia blank concentrations are higher than 0.3 µmol/l., the water should be subjected to subsequent distillation. In this second step, 0.3 g NaOH and 1 g $K_2S_2O_2$ are added to 1000 ml of water (in a 2 L flask). The solution should be boiled for 10 minutes to remove ammonia (without the condenser) and then distilled until a residue of about 150 ml. The distilled water should be stored in a tightly sealed container, preferably made of glass. The method of preparation of ammonia-free water should be regularly checked and appropriate blanks must be analyzed with every batch of samples

b) Buffer solution

Dissolve 240 g tri-sodium citrate dihydrate ($C_6H_5Na_3O_7.2H_2O$), 20 g of disodium EDTA and 0.4 g NaOH in about 600 ml distilled water. The solution is boiled (to remove ammonia) until the volume is below 500 ml. It is then cooled and diluted to 500 ml with "ammonia-free" water.

The solution is stable and should be stored in a well-stoppered polyethylene bottle.

c) Phenol reagent

Dissolve 80 g colorless phenol (C_6H_5OH) in 300 ml of ethanol, add 600 ml of distilled water and 600 mg sodium nitroprusside dihydrate [$Na_2Fe(CN)_5NO.2H_2O$] in "ammonia-free" water and dilute to 1000 ml. When stored in a tightly closed dark bottle and in a refrigerator, the solution should be stable for several months.

WARNING: Phenol is a particularly toxic compound and safety glasses and gloves should be worn and all handling conducted in a fume cupboard.

d) Hypochlorite reagent

Dissolve 1 g "Trione", the commercial name for the sodium salt of dichloroisocyanuric acid [dichloro-s-triazine-2, 3, 6 (1H, 3H, 5H)-trione] and 8 g NaOH in "ammonia-free" water and dilute to 500 ml. The reagent "Trione" is employed as a hypochlorite donor (in comparison to generally used commercial hypochlorite solutions) has the advantage of being a stable solid, and that it is easy to prepare.

The solution should be stored in a dark bottle in a refrigerator and is stable for at least a week.

e) Ammonia stock solution (A) (10 mM NH_3)

Ammonium chloride (NH_4Cl) is dried at 100 ºC to constant weight. Then dissolve 0.0535 g NH_4Cl in "ammonia-free" water and dilute to 100 ml in a volumetric flask.

When kept in a glass bottle (protected from sunlight) and in a refrigerator, the solution should be stable for at least several weeks.

f) Ammonia working solution (B) (100 µM NH_3)

Exactly 10.0 ml of the stock solution is diluted with "ammonia-free" water to a final volume of 1000 ml in a volumetric flask made of glass.

This solution must be prepared daily.

Apparatus:

a) Stoppered 50 ml flasks (of glass, reserved solely for this determination and stored closed and filled with "ammonia-free" water between analysis).
b) Automatic syringe pipettes of 2 ml volume for reagent additions.
c) Spectro- or filter photometer with a filter having maximum transmission at 630 nm, and cells of 1 cm, 5 cm and 10 cm length as required.

Note: All flasks and tubes to be used should be cleaned with acid, rinsed well with "ammonia-free" water and kept closed between analyses. The analysis should be performed in a well-ventilated room with no ammoniacal solutions stored (Note this should include any cleaning agents containing ammonia and used by laboratory cleaning staff during or outside normal working hours). This includes the NH_4Cl reagent used for nitrate analysis. **Smoking should be forbidden**.

Method:

a) Calibration

Prepare a series of working standards from the ammonia working solution (B). To 100 ml volumetric flasks add (by means of pipette or burette) the following volumes and fill up with "ammonia-free" water to the 100 ml mark. In this instance, it is probably best not to use low nutrient seawater unless it is known to have a suitably low ammonia concentration. Then the resulting standard concentrations are:

0.5 ml of working solution = 0.50 µM NH_3
1.0 ml of working solution = 1.00 µM NH_3
2.0 ml of working solution = 2.00 µM NH_3
3.0 ml of working solution = 3.00 µM NH_3
5.0 ml of working solution = 5.00 µM NH_3
7.0 ml of working solution = 7.00 µM NH_3
10.0 ml of working solution = 10.00 µM NH_3

To 50 ml portions of these working standards add the reagents and follow the procedure outlined below for analysis of the sample. In addition, prepare a "blank sample" from the same volumes of the distilled water used and the reagents. Use a cuvette of similar length (preferably at least 5 cm) filled with distilled water as reference. Plot the measured absorbances (corrected for the blank) versus the standard concentrations.

b) Analysis of samples

Measure 50 ml of the sample with a graduated cylinder and transfer it into the reaction flasks. Add 2 ml phenol reagent, 1 ml buffer solution and 2 ml hypochlorite reagent. Mix well by swirling between the additions. Close the reaction bottles properly and keep them in a dark place during the reaction time which is at least 6 hours at room temperature but which is reduced to 30 minutes if samples are incubated in a water bath at 37 ºC ± 1 ºC.

Measure the absorbance after 6 hours in a cell of suitable length at 630 nm, and use a cuvette of similar length filled with distilled water as reference. (In ocean waters the reaction requires about 5 hours at room temperature; since the color of the indophenol blue is stable for at least 30 hours, it is convenient, in routine analysis, to let the sample react overnight).

With detectable traces of ammonia in the re-distilled water, the blank from the reagents added is determined in the following way: First, measure the absorbance (A_b) by carrying out the above procedure using 50 ml of distilled water. Repeat the determination by adding, however, 3 ml phenol reagent, 1.5 ml buffer solution and 3 ml hypochlorite reagent to only 47.5 ml of the same distilled water. Measure the absorbance ($A_{1.5b}$). Then the blank caused by the reagent only is calculated from:

$$A_{rb} = 2\ (A_{1.5b} - A_b)$$

Determine the reagent blank at regular intervals (and for each new batch of solution), because the reagents may absorb ammonia from the air.

c) Interferences

Interferences from amino acids and urea (at seawater levels) can be neglected but may be significant in estuarine or brackish waters, especially where these are contaminated with urban waste. Hydrogen sulfide can be tolerated up to about 60 µmol/l. Samples with higher H_2S concentrations should be diluted. The blue color of the indophenol, however, is influenced by salinity, which has to be compensated by the application of a salt factor (see below).

Calculation of Results:

Calculate the slope b of the calibration curve described above (for the length used) which follows the equation:

$$\text{Absorbance} = b \times \text{Concentration}$$

As already mentioned, for any given concentration of ammonia the blue color produced in seawater is less intensive than in distilled water. Thus, for each sample a correction has to be made with respect to its salinity and the resulting pH. The salt effect factor (SF) can be taken from the following table:

Salinity	0	5	10	15	20	25	30	35
Ca.pH	11.0	10.7	10.5	10.4	10.2	10.1	9.9	9.8
SF	1.0	1.03	1.06	1.09	1.14	1.18	1.22	1.25

Thus, the ammonia concentration of the sample is obtained from:

$$C\ (\mu g/l) = (A_s - A_{rb} - A_t) \times SF / b$$

Where A_s, A_{rb} and A_t are the absorbances of the sample, the reagent blank and the turbidity blank (if any), respectively.

In many circumstances a simpler correction (Grasshoff et al. 2002) may be used. In this case, the correction is given by:

$NH_{3(corr)} = [1 + 0.0073S_s]NH_{3(uncorr)}$, where S_s is the salinity of the sample.

Estimation of Precision and Accuracy:

Good accuracy and precision in the analysis of ammonia is difficult to obtain, and highly dependent on how successfully the contamination control is achieved during cleaning procedures, sample handling and analytical steps. The main pitfalls are caused by contamination from airborne ammonia (especially tobacco smoke) as well as from the reagents and glassware.

The precision of the method (under ideal circumstances) can be considered as being between 2 and 5 % at a concentration level of about 5 µM.

C) Determination of Nitrite - N

Scope and Field of Application:

The method is specific for nitrite ions (NO_2) and is based on the formation of a highly colored azo dye which is measured colorimetrically at 540 nm. The procedure outlined here is applicable to all types of marine waters and follows the methods by Bendscheider and Robinson (1952) and Grasshoff (1983). It shows a detection limit of 0.25 µg/l and a linearity between the amount of the azo dye formed and the initial concentration of nitrite over a wide range of concentration (0 - 100 µg/l).

The reaction is widely free from interferences of compounds normally present in ocean or in inshore waters. Salinity does not affect the absorbance significantly. If hydrogen sulfide is suspected to be present in a sample (nitrite and sulfide cannot co-exist for long periods in natural seawater), the gas must be expelled with nitrogen after addition of the acid sulfanilamide reagent.

Principle:

The determination of nitrite is based on the reaction of nitrite with an aromatic amine (sulfanilamide) which leads (at pH 1.5 - 2.0) to the formation of a diazonium compound. This diazo compound then couples with a second aromatic amine N-(1-naphthyl)-ethylenediamine to form the azo dye with a molar absorbtivity of about 46,000.

Reagents:

a) Sulfanilamide reagent

Dissolve 10 g crystalline sulfanilamide ($NH_2C_6H_4SO_2NH_2$) in a mixture of 100 ml concentrated hydrochloric acid (HCl, a.g.) and about 500 ml distilled water. (Moderate heating accelerates the dissolution). After cooling, the solution is diluted to 1000 ml with distilled water.

The reagent is stable for several months.

b) N-(1-naphthyl)-ethylenediamine solution

Dissolve 0.5 g of N-(1-naphthyl)-ethylenediamine dihydrochloride [$C_{10}H_7NH(CH_2)2NH_2.2HCl$] in distilled water and dilute to 500 ml.

The solution should be stored in a dark glass bottle in a refrigerator, and must be renewed as soon as it develops a brown color (usually stable for 1 month).

c) Nitrite stock solution (A) (10 mM)

Anhydrous sodium nitrite ($NaNO_2$, a.g.) is dried at 100 ºC (for about 1 hour) to constant weight. Then 0.690 g of the dry salt is dissolved in distilled water and diluted to 1000 ml in a volumetric flask.

The solution should be stored in a glass bottle and is stable for at least several months.

Note: Aged solid reagents, even of analytical grade, may contain less than 100 % $NaNO_2$ and should, therefore, not be used for the preparation of the nitrite standard solution. For calibration purposes (in distilled water) silver nitrite may be preferred instead because of its higher stability.

d) Nitrite working solution (B) (100 µM)

Transfer exactly 10.0 ml of the nitrite solution to a volumetric flask and dilute to 1000 ml with distilled water.

This solution must be renewed daily.

Apparatus:

a) Stoppered glass bottles with a capacity of about 100 ml
b) Reagent dispensers (automatic syringe pipette or piston pipettes)
c) Spectro- or filter photometer, with filter at or close to 540 nm, and cuvettes of 10 cm.

Method:

a) Calibration

Prepare a series of working standards from the nitrate working solution (B). To 100 ml volumetric flasks add (by means of micropipettes) the following volumes and fill up with low nutrient seawater (or distilled water) to the 100 ml mark. Then the resulting standard concentrations are:

0.10 ml of working solution = 0.1 µM NO_2
0.20 ml of working solution = 0.2 µM NO_2
0.40 ml of working solution = 0.4 µM NO_2
0.60 ml of working solution = 0.6 µM NO_2
0.80 ml of working solution = 0.8 µM NO_2
1.20 ml of working solution = 1.2 µM NO_2

To 50 ml of these working standards add the reagents and follow the procedure outlined below for analysis of the sample. In addition, prepare a "blank sample" from the same volumes of distilled water and the reagents. Measure the absorbances in (at least) a 5 cm cuvette. Plot the measured absorbances (corrected for the blank) versus the standard concentrations.

b) Analysis of the sample

Transfer 50 ml of the sample with a graduated cylinder into the reaction bottle and add 1 ml of the sulfanilamide reagent. Then mix well. After reaction time of about 1 minute, add 1 ml of the diamine solution. Shake the flask and allow the azo dye to develop for at least 20-30 minutes. Measure the absorbance in a cell of suitable length at 540 nm against distilled water as reference. The color intensity is constant for about two hours. (The dye should not be exposed to bright daylight).

For precise measurements of low nitrite concentrations, any turbidity in the sample must be compensated by a "turbidity blank". For this reason add 1 ml of the sulfanilamide reagent to 50 ml of the sample and measure the absorbance against distilled water as reference. (The addition of acid to a sample usually changes its turbidity; Therefore, it is important that the acidic sulfanilamide reagent is added not only to the sample, but also to the "turbidity blank" sample).

c) Calculation of results

Calculate the slope b of the calibration curve described above (for the individual cell length used) from the equation:

$$\text{Absorbance} = b \times \text{Concentration}$$

The concentration of nitrite is then calculated according to:

$$C\ (\mu g/l) = (A_s - A_b - A_t) / b$$

Where A_s, A_b and A_t are the absorbance values from the sample, the "blank sample" and the "turbidity blank" sample respectively.

d) Estimation of precision

The precision of the method is ± 0.02 µM though an increase of precision to ± 3 nM is possible with a well setup continuous flow system.

D) Determination of Nitrate - N

Note: It is self evident that glassware must not come into contact with nitric acid in this procedure, but analysts should be aware that cigarette tobacco may contain potassium nitrate and that great care must be taken to avoid problems with this during both sample collection and analysis.

The method is generally applied for the determination of nitrate (NO_3) is based on its reduction to nitrite, which is then determined colorimetrically via the formation of an azo dye. The method outlined here is based on a heterogeneous reaction with copper-coated cadmium granules and follows mainly the procedure by Grasshoff (1983). It has proved to be reliable and useful for work at sea and is widely free from interferences in nearshore and oceanic waters.

The method determines the sum of nitrite and nitrate, therefore, a separate determination of nitrite must be conducted, and concentration subtracted from that obtained with this method. At concentration levels higher than about 20 µmol/l, calibration curves for a low and high range must be established.

Principle:

Nitrate is reduced to nitrite in a reduction column filled with copper-coated cadmium granules. The yield of the reduction depends on the pH of the solution and on the activity of the metal surface. The conditions of the reduction described in the method are adjusted to a pH of about 8.5, so that nitrate is converted to nitrite almost quantitatively (90-95 %) and not reduced further. Ammonium chloride buffer is used to control the pH and to complex the liberated cadmium ions.

The nitrite formed is then determined colorimetrically (at 540 nm).

Reagents:

a) Ammonium chloride buffer

Dissolve 10 g ammonium chloride (NH_4Cl, a.g.) in distilled water and dilute to 1000 ml. The pH is adjusted to 8.5 by adding about 1.6 ml of concentrated ammonium hydroxide (NH_4OH).

b) Sulfanilamide reagent (same reagent as for nitrite determination)

Dissolve 10 g crystalline sulfanilamide ($NH_2C_6SO_2NH_2$) in a mixture of 100 ml concentrated hydrochloric acid (HCl, a.g.) and about 500 ml distilled water and make up to 1000 ml with distilled water.

The reagent is stable for several months.

c) N-(1-naphthyl)-ethylenediamine: (same reagent as for nitrite determination).

Dissolve 0.5 g of N-(naphthyl)-ethylenediamine dihydrochloride [$C_{10}H_7NH(CH_2)_2NH_2.2HCl$] in distilled water and dilute to 500 ml.

The solution should be stored cool in a dark glass bottle and should be renewed as soon as it develops a brown color (usually stable for 1 month).

d) Filling the reduction column

Commercially available granulated cadmium (e.g. coarse powder for reductors grade-BDH) is sieved and the fraction between 40 and 60 mesh (i.e. around 0.25 and 0.42 mm) is retained and used.

WARNING: Cadmium can be poisonous. It should, therefore, be handled with great care. Never inhale the dust. Perform all operations on the dry metal in a fume hood.

e) Copper sulfate solution

Dissolve 10 g copper sulfate pentahydrate ($CuSO_4.5H_2O$) in about 1000 ml distilled water.

f) Nitrate stock solution (A) (10 mM)

Dissolve 1.011 g dry potassium nitrate (KNO_3, a.g.) in distilled water and dilute to 1000 ml in a volumetric flask.

The solution is stable for at least several months.

g) Nitrate stock solution (B) (1 mM)

Transfer 10 ml of the nitrate stock solution (A) to a volumetric flask and dilute to 100 ml with distilled water.

This solution must be renewed daily.

h) Nitrate working solution (C) (10 µM)

Transfer 10 ml of nitrate stock solution (B) to a volumetric flask and dilute to 1000 ml with low nutrient sea water (or distilled water).

This solution must be renewed daily.

Apparatus:

a) Reduction column (see Figure 2).

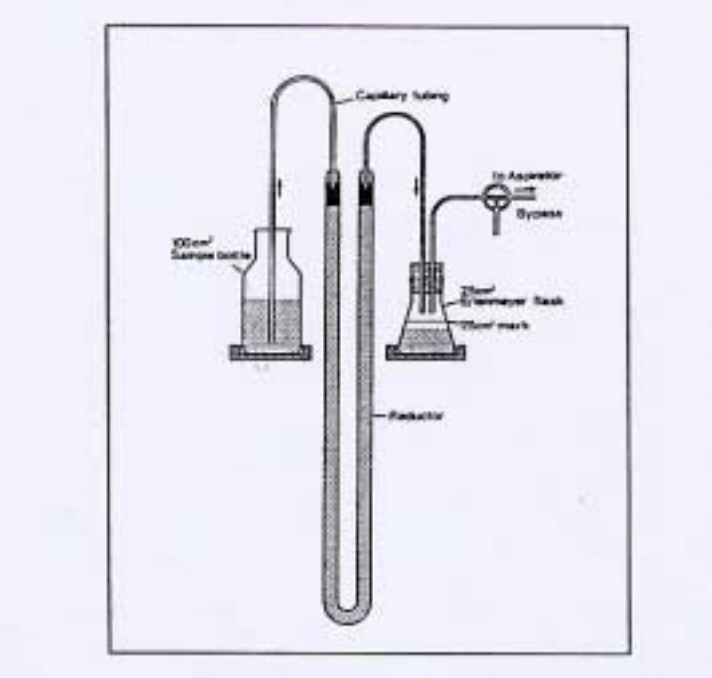

Figure 2: Reduction column for the analysis of nitrate.

The major part of the reduction column consists of a U-shaped glass tube with a total length of about 10-25 cm and an inner diameter of 3 mm. Connections to the 100 ml sample bottle and the 25 ml (marked) Erlenmeyer flask are made from flexible capillary tubing (tygon). The sample is drawn through the column by an aspirator or by a small peristaltic pump (with control of the flow rate by means of a three-way stopcock with a bypass). For practical purpose, the whole set-up can be mounted in a box. Suitable flow rates should be determined by experimentation.

b) Stoppered glass bottles, reagent dispensers, and a spectro- or filter photometer (with at least 5 cm cuvettes) as described for the analysis of nitrite are required.

Method:

a) Preparation of the reduction column

Free the sieved cadmium granules from oxides by washing them in 2 M hydrochloric acid. Then shake the granules in a 200 ml beaker vigorously (for about 3 minutes) with 100 ml of the copper sulfate solution. Afterwards rinse the copperized cadmium granules under gentle shaking, decant the water and continue washing until the water is free from finely dispersed copper.

Then pour the copperized granules into the reduction column (with the aid of distilled water and a funnel). Encourage effective packing by gently tapping the column

with a pencil. When one arm is filled, connect the funnel to the other arm and repeat the procedure. Leave some space in both side arms in order to pack in some glass wool.

Activate the metal by passing through about 250 ml buffer solution (ammonium chloride) containing about 100 µmol/l nitrate. Then rinse thoroughly with buffer solution before the reducer is used for analysis.

Check the reduction efficiency of the reduction column by analyzing a nitrate standard solution of suitable concentration (e.g. equimolar). Compare the determined absorbance with that of a nitrite solution of the same concentration (e.g. if A_{NO3} = 0.200, A_{NO2} = 0.210, the reduction efficiency would be (0.200 x 100) / 0.210 = 95.2 %.

Note: Repeat the activation procedure if the reduction column has not been used for several days, or, if the column has accidentally been filled up with air bubbles. When not in use, keep the column brimful with the ammonium chloride buffer solution. If the efficiency cannot be brought back to above 90 % of the theoretical value, it is preferable to refill the column. If frequently used, however, it should last for several months.

b) Calibration (low and high concentrations)

Prepare, a series of working standards from the nitrate working solution (C). To 100 ml volumetric flasks add the following volumes of the nitrate standard solutions respectively and fill up with low nutrient seawater (or distilled water) to the 100 ml mark. Then the resulting standard concentrations are:

Stock solution	C	B µM
0.1 ml of working solution =	0.01	1
0.25 ml of working solution =	0.025	2.5
0.5 ml of working solution =	0.05	5
1.0 ml of working solution =	0.1	10
2.5 ml of working solution =	0.25	25
5.0 ml of working solution =	0.5	50

To 25 ml of the working standard add 25 ml of the buffer solution. In addition, prepare a “blank sample” from 25 ml distilled water and the same volume of buffer solution. Analyze the standard and blank solutions in the same way as described below for the analysis of the sample. Plot the measured absorbance (corrected for the blank value) versus the standard concentrations. Both curves should be linear over the entire range of concentrations.

c) Analysis of the sample

Transfer 25 ml of the sample into the 100 ml reaction flask, add 25 ml of the buffer solution (ammonium chloride buffer) and mix well. If nitrate concentrations of more than about 15 µM are expected, 25 ml of the sample must be diluted with 75 ml of the buffer solution.

Pass about 20 ml of the mixture through the reduction column in order to rinse the system and to adjust the time of passing (3-5 minutes). Discard this fraction. Then pass (at unchanged speed) another fraction through the column until the level in the Erlemeyer flask has reached the 25 ml mark.

Stop the collection of the reduced sample and add 0.5 ml of the sulfanilamide reagent and 0.5 ml of the diamine solution in the same way as described for the analysis of nitrite. Determine the azo dye color within about 1 hour (as 540 nm) in 1 cm or 5 cm cells against distilled water as reference.

Calculation of results:

Calculate the slope b of the calibration curves described above (separately for the low and high concentration levels and for the individual cell lengths used) from the equation:

$$\text{Absorbance} = b \times \text{Concentration}$$

The concentration of nitrate is then calculated according to:

$$C\ (\mu g/l) = (A_s - A_b) / b \quad - \quad C_{NO_2^-}$$

Where A_s and A_b are the absorbances of the sample and of the "blank sample", and $C_{NO_2^-}$ is the nitrite concentration (in µg/l) observed in the same sample.

Estimation of precision and accuracy:

In routine analysis the precision (standard deviation) of the method with one and the same reduction column is about ± 0.05 µmol/l for nitrate concentration of 5 µmol/l, ± 0.2 µmol/l in the range 5 - 10 µmol/l and about ± 0.5 µmol/l in the higher concentration range. If different reduction columns are used, the deviation of results depends, of course, strongly on the reduction efficiency of the columns.

The accuracy depends on the reliability of the standard used for the calibration procedure, on the quality of sampling, and/or, if necessary, on the procedure of storage.

E) Determination of Silicate - Si

Scope and field of application:

The determination of dissolved silicon compounds is based on the formulation of a heteropoly acid when the sample is treated with a molybdate solution. This silicomolybdic acid (occurring in two isomeric forms) is then reduced to an intensely blue-colored complex by adding ascorbic acid as a reductant. The color is formed within 30 minutes determined at 810 nm, and is stable for several hours.

The method outlined here mainly follows a procedure described by Koroleff (1983). It has a concentration range up to 800 µg/l (samples with higher concentrations are diluted with distilled water), and a detection limit of about 1.0 µg/l (in a 5 cm cell). However, only silicic acid and its dimer react with molybdate; therefore, the method gives only the amount of "reactive" silicate.

Principle:

Similar to phosphate ions, "reactive" silicate forms, in acid solution, a heteropoly acid when treated with molybdate ions. This silicomolybdic complex exists in two isomeric forms (α- and β- isomer) depending on the pH at formation. The isomers have different stabilities, are both yellow-colored, showing however, only low molar absorptivities in the range of about 1200 and 3300 absorbance units/mole for the α- and β- isomer, respectively. Major analytical efforts were therefore dedicated to the development of methods in which the heteropoly acids are reduced to intensely blue-colored complexes. The method outlined here involves the addition of oxalic acid (to avoid the reduction of any excess molybdate reagent and to eliminate the influence of any phosphate present), and the use of ascorbic acid as the reductant. The blue complex shows a molar absorptivity in ocean waters of about 19000 absorbance units/mole, with stability of the color for at least several hours.

Reagents:

All solutions should be prepared from reagents of known analytical grade only, using high purity de-ionized water. The silicate content of this water should be checked (as a precaution) at frequent intervals according to the procedure described below, and the solutions must be stored in plastic bottles.

a) Sulfuric acid (3.6 M)

Add slowly 200 ml concentrated acid (d = 1.84 g/ml). Under cooling and mixing, to about 700 ml distilled water. Finally the volume is adjusted to 1000 ml with distilled water. The solution should be stored in a polyethylene bottle.

b) Ammonium heptamolybdate solution

Dissolve 20 g $(NH_4)_6MO_7O_{24}.4H_2O$ in about 80 ml distilled water (in a plastic beaker) by moderate heating and dilute to 100 ml.

c) Mixed reagent

A measured volume of the molybdate solution is added to an equal amount of the sulfuric acid under mixing. **DO NOT** add acid to the molybdate solution.

The solution should be stored in a polyethylene bottle protected from sunlight and should be stable for several months.

d) Oxalic acid solution

Dissolve 10 g $(COOH)_2.2H_2O$ in 100 ml distilled water.

The saturated solution is stored in a plastic bottle and is stable indefinitely.

e) Ascorbic acid solution

Dissolve 1.75 g $C_6H_8O_6$ in 100 ml distilled water. The solution is stored in an amber glass bottle in a refrigerator.

The reagent is effective as long as it remains colorless.

f) Silicate stock solution (A) (10 mM)

Disodium hexafluoro silicate, $NaSiF_6$, is dried at 105 ºC to constant weight. Then 1.8806 g of the salt is dissolved in distilled water (in a plastic beaker) and diluted to 1000 ml in a volumetric flask. The solution is immediately transferred to a polyethylene bottle.

Note that the silica content of this material may vary slightly and that the manufacturer's assay should be consulted and the weight of standard weighed out adjusted as necessary.

g) Silicate working solution (B) (500 µM)

Dilute 4 ml of the stock solution with distilled water to a final volume of 100 ml in a volumetric flask, preferably made of plastic material.

This solution must be renewed daily.

Apparatus:

a) Stoppered bottles made of plastic (e.g. polyethylene bottles of ca. 100 ml).
b) Automatic syringe pipettes of 2 ml volume for reagent additions.
c) Spectro- or filter photometer with filter at or close to 810 nm and cells of 1 cm, 5 cm and 10 cm length as required.

Method:

a) Calibration

Prepare a series of working standards for the silicate working solution (B). To 100 ml plastic volumetric flasks add (by means of pipette) the following volumes and fill up with low nutrient seawater (or distilled water) to the 100 ml mark. Then the resulting standard concentrations are:

0.25 ml of working solution	= 1.25 µM Si
0.5 ml of working solution	= 2.5 µM Si
1.0 ml of working solution	= 5 µM Si
2.0 ml of working solution	= 10 µM Si
5.0 ml of working solution	= 25 µM Si
10 ml of working solution	= 50 µM Si

To 50 ml portions of these working standards add the reagents and follows the same procedures outlined below for analysis of the sample. In addition, prepare a blank sample from the same volumes of distilled water and reagents. The blank sample compensates for the silicate content in the reagents as well as in the distilled water used for the preparation of the standard solutions. Plot the measured absorbances versus the

standard concentrations (corrected for the reagent blank). The calibration curve should be linear over the range of concentration.

b) Analysis of samples

Measure 50 ml of the sample with a graduated cylinder, and transfer it into the plastic reaction bottle. Add 1.5 ml of the mixed reagent and mix well. After 10-20 minutes add 1 ml oxalic acid immediately followed by 1 ml ascorbic acid. Mix well between the additions. Measure the absorbance after 30-40 minutes in a cell of suitable length at 810 nm against distilled water as reference.

As distilled water usually contains detectable amounts of silicate (of the order of 2 µmol/l Si), the reagent blank for analysis of the sample must be determined in a different way than described for the calibration procedure. For this reason the blank is best prepared by carrying out the above procedure using 50 ml of distilled water. The absorbance is denoted $A_{1.5}$. Repeat the determination, but add only 1.0 ml of the mixed reagent. Measure the absorbance ($A_{1.0}$). The absorbance caused by the reagent (A_{rb})only is calculated from:

$$A_{rb} = 3\ (A_{1.5} - A_{1.0})$$

Determine the reagent blank for each new batch of mixed reagent.

c) Interferences

Interferences are observed from salinity which reduces the final color intensity to some extent. Hydrogen sulfide can be tolerated up to about 150 µmol/l without problems.

For precise estimates of low silicate concentrations (using a 10 cm cell), measure a reference absorbance for every sample to compensate for its natural turbidity. For this reason add 3 ml sulfuric acid (0.25 mol/l) to 50 ml of the sample and measure the absorbance against distilled water as reference. (If the sample has a visible turbidity centrifuge or filter it, before analysis, through a well-rinsed 0.4 µ Nucleopore filter).

Samples containing more than 150 µmol/l sulfide must be treated with bromine water in the same way as already described (before) for phosphate.

Calculation of results:

Calculate the slope b of the calibration curve described above (for the individual cell length used) which follows the equation:

Absorbance = b x Concentration

Then the "reactive" silicate concentration of the sample is obtained from:

$$C\ (\mu g/l) = (A_s - A_{rb} - A_t) \times SF / b$$

Where A_s, A_{rb} and A_t are the absorbances of the sample, the reagent blank, and the turbidity blank, respectively.

Estimation of precision and accuracy:

Several national and international intercalibration studies in the past have proven the blue silicamolybdic acid method as a very accurate procedure with a rather low tendency to systematic errors. The precision of the method can be considered as being between ± 2.5 and ± 4 % at concentration levels of about 50 µM and 5 µM respectively.

VI Analysis of nutrients using continuous flow or other technologies

A) Continuous flow methods

The principle used by the continuous flow analyzers is recognized as the most reliable and accurate method for determination of nutrients. Different systems are available and can be configured to meet the standard methods such as ISO, EPA, ASTM, etc… Wherever possible it is strongly recommended that such analyzers are used because of the considerable increase in precision and sample throughput that they offer. Ideally such analyzers can be used in laboratories on board a research vessel allowing problems of sample deterioration during storage to be circumvented.

Note: 1. System reagents and configurations should be available with the operation manual of the instrument

2. Analytical quality control should be considered with each batch of samples to check the performance of the instrument

B) Other technologies

A number of *in situ* multi-channel nutrient analyzers are now available on the market and are suitable for deployment for extended periods at depths down to a few hundred meters. Flow injection systems are an alternative to the larger scale continuous flow analyzers. Some high sensitivity techniques using e.g. chemiluminescence are

available but these are largely within the domain of research as opposed to monitoring programs.

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