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MEDITERRANEAN ACTION PLAN

MED POL

SAMPLING AND ANALYSIS TECHNIQUES
FOR THE EUTROPHICATION MONITORING STRATEGY OF MED POL

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- Curbing Pollution
- Safeguarding Natural and Cultural Resources
- Managing Coastal Areas
- Integrating the Environment and Development

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Introduction

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 as well as phytoplankton determinations. The manual presented here is designed to support the Short-term Eutrophication Monitoring Strategy (UNEP(DEC)/MED WG.231/14, 2003) of MED POL (UNEP/MAP) for the Mediterranean Sea. According to the short-term monitoring strategy, some physical (temperature, pH, salinity, transparency, dissolved oxygen: saturated and measured), chemical (ortophosphate, total phosphorus, ammonium, nitrite, nitrate and silicate) and biological (chlorophyll-a, phytoplankton abundance and bloom dominance) variables were considered as mandatory monitoring parameters.

The first draft of this manual was produced in 2003, later revised and completed by a group of experts in 2004 and finalized in 2005. Chapter I of this manual is based on common literature (Grasshoff et al.,1983, 1999 and APHA-AWWA-WPWF, 1992), Chapter II is based on an IOC publication (Manuals and Guide No 28, 1993) and the MOOPAM (third edition, 1999) of ROPME and Chapter III is based on the Italian National Monitoring Guidelines for 2001-2003 produced by ICRAM.

I   On-board measurements of hydrographical and optical parameters and Dissolved Oxygen

I.1   Determination of salinity with salinometer

The majority of present-day determinations of salinity are carried out with salinometers. These instruments are specially designed to measure the conductivity of seawater relative to reference standard seawater. In general terms, the conductivity is a measure of the total ion concentration of the water. Therefore, conductivity gives a more reliable estimate of the density of the water especially if small variations of the relative ionic proportions occur in waters having salinities different from average ocean water. The conductivity of an electrolyte solution is usually determined by means of a bridge circuit where by the resistance of the cell containing the electrolyte is compared with the resistance of a standard solution. The technical specifications of the commercial salinometers are evaluated extensively in *Grasshoff et al.,* (1999).

Sub-sampling and storage: The glass type salinity bottle is rinsed twice with about 15 to 20 cm^3 of the seawater. The bottle is then filled with the sample just short of the top and locked, and may be stored for several weeks. Before measuring, the samples are taken to where the salinometer is and allowed to warm up to room temperature within ± 1 $^{\circ}$C. The temperature of the room should not vary more than ± 1 $^{\circ}$C. At least 2 h before measuring, the bottles should be shaken vigorously and the stopper removed quickly in order to release any pressure head and, thus, reduce the risk of outgassing of the sample in the salinometer cell.

Standardization of the salinometer: The primary standardization of the salinometer is carried out with Standard Seawater that has been equilibrated to room temperature. The salinometers have a built-in aspirator for filling the cell. If the reading is unstable, it is usually an indication of the presence of gas bubbles in the cell. In this case the filling procedure must be repeated.

Measurement of the samples: If the salinity of samples deviates more than ±5 x 10^{-3} from the salinity of the Standard Seawater or the substandard, the cell should be rinsed with an "extra sample". The temperature of the sample should be within the range in which the built-in compensating circuit is able to balance the change in conductivity. After switching to "conductivity ratio", the indicator instrument is set to zero by means of the conductivity ratio dials. Any unstable reading of the instrument indicates either outgassing in the cell (warming) or cooling of the sample. In the case of outgassing the cell contents must be drained into the sample bottle and the cell refilled. The time of the reading and the temperature are noted in the records together with the conductivity ratio. After about half an hour or at the end series of measurements the instrument drift is controlled by measuring a standard or preferably a substandard. The conductivity ratio is converted to a salinity value by the use of the formulae given in Grasshoff et al. (1983). On the basis of these equations and the algorithm of practical salinity formulated, new International Oceanographic Tables were recently issued by UNESCO (1981c) which replaces the "old" Tables form 1966.

Precision and accuracy: Most manufacturers of the bench-type inductive salinometer claim a reproducibility of ±0.003 to ±0.002 x 10^{-3} S. This precision can be obtained, if the measurements are performed carefully (see *Grasshoff* et al., 1983, 1999, for further details of the method).

I.2   Salinity and Temperature from *in situ* measurements: CTD profilers

Oceanographers were always keen to have visible and available *in situ* salinity and temperature on-line. The most effective method to achieve this goal was to construct an instrument that measures three basic thermodynamic parameters simultaneously with high precision from a research vessel, transfer the data *via* a one-conductor cable to the deck,

calculate salinity from the three basic parameters, make them visible and store them. The parameters easiest to measure electrically, are pressure P, temperature T and the specific electrical conductivity of sea water C relative to a fixed reference. With the introduction of the practical salinity scale of 1978 (*Grasshoff* et al.,1999 and references cited therein), a unique procedure was prescribed to calculate salinity S from these three basic measurements.

CTD-rosette system should be used by following the manufacturer's recommendations and make sure by intensive rinsing with fresh water that all mechanical parts of the rosette work properly. CTD sensors respond best when lowered at continuous speed with no upward movement in between that may be induced by the ship's pitch and roll. Accuracies achievable for measurements in the ocean are 0.002 mK for T, 0.05 % for P and 0.002 for S. The sensors should be sent to the manufacturer periodically for the basic calibration.

I.3 Dissolved Oxygen

Using the best available sets of empirically determined data of oxygen concentrations in seawater of different salinities and different temperatures and using exact thermodynamical equations, *Weis* established an equation for the solubility of oxygen in seawater (Grasshoff *et al*., 1983 and related references cited therein). The present-day tables of oxygen saturation values in Volume II of the International Oceanographic Tables (UNESCO, 1973) are based on this equation.

The *Weiss* equation:
$$\ln C_s = A_1 + A_2 (10^2 \cdot T^{-1}) + A_3 \ln (T \cdot 10^{-2}) + A_4 (T \cdot 10^{-2}) + S (B_1 + B_2 T \cdot 10^{-2} + B_3 T^2 \cdot 10^{-4})$$

where A_1, A_2,… are constants, S is the salinity of the seawater ($\times 10^3$), T is the absolute temperature

A_1 = -173.4292 and B_1 = -0.033069
A_2 = 249.6339 B_2 = 0.014259
A_3 = 143.3483 B_3 = -0.0017000
A_4 = -21. 8492

and C_s is the saturation concentration of oxygen in mL/L ($cm^3 \cdot dm^{-3}$).

$$\%O_2 \text{ (measured)} = C_m \text{ (measured)}/C_s \text{ (saturated)} \cdot 100$$

A) Determination of Oxygen by Winkler Titration Method

Chemical determination of oxygen concentrations in seawater was first proposed by *Winkler* (1888). The *Winkler* method is based on the following principle: The physically dissolved oxygen in a measured amount of water is chemically bound by manganese (II) hydroxide in a strongly alkaline medium. The manganese (II) is oxidized to manganese (III), and not to manganese (IV) as often stated, because of the large surplus of manganese (II) hydroxide present. This is a heterogeneous reaction, i.e. the precipitated manganese (II) hydroxide reacts with the dissolved oxygen. Because of the instability of manganese (II) in an alkaline medium, the hydroxide is oxidized easily and the reaction is quantitative in the analytical sense. After complete fixation of the oxygen and precipitation and mixed manganese (II) and (III) hydroxides the sample is acidified to a pH less than 2.5 but not less than about pH 1. The precipitated hydroxides dissolve and manganese (III) ions are liberated. Manganese (III) is a strong oxidizing agent in acidic media and reacts with iodide ions previously added to the water sample together with the potassium hydroxide. The iodide ions are oxidized to iodine, which in turn forms a complex with the surplus iodide. The

complex has a low iodine vapor pressure but decomposes readily if iodine is removed from the system.

In the third step of the analysis, the iodine is titrated with thiosulphate. The iodine is reduced to iodine and the thiosulphate is in turn oxidized to the tetrathionate ion. The concentration of the thiosuphate solution used for the titration must be known precisely. The endpoint of the redox titration is indicated by a starch indicator. The endpoint of the titration is clearly marked the change from blue to colourless. The stoichiometric equations for the reactions and possible sources of contamination are given and discussed extensively in Grasshoff et al. (1983, 1999).

<u>Reagents:</u>
Distilled (pure) water is used to prepare reagents and standards.
1- Manganese(II) chloride: 60g of $MnCl_2.4H_2O$ are dissolved and made up to 100 mL with pure water.
2- Alkaline iodide: 60g of KI and 30g KOH are dissolved separately in a minimum amount of water and combined. The solution is made up to 100 mL with pure water. If the solution displays a yellowish –brown color, discard and prepare again with fresh reagents.
3- Sulfuric acid: 50 ml of concentrated sulfuric acid are added carefully to 50 ml of pure water.
4. a- Sodium thiosulphate, 0.2 mol/L:49.5 g of $Na_2S_2O_3.5H_2O$ is dissolved and made up to 1 L with pure water. 2.5 g of sodium borate ($Na_2B_4O_7$, analytical grade) may be added as a preservative. This stock solution is stored in a refrigerator.
4. b- Sodium thiosulphate, 0.02 mol/L working solution is a 1:10 dilution of 4a.
5- Starch solution: 1 g of soluble starch is dispersed in 100 mL of pure water. The solution is quickly heated to boiling point. (The starch solution should not be stored longer than one week and may be stabilized with 1 ml of phenol). Instead of soluble starch, a commercial zinc starch compound may be used, which dissolves readily in water.
6- Iodide standard: Exactly 325 mg (0,833 mmol) of $KH(IO_3)_2$ or 356,7 mg (1,667 mmol) of KIO_3 are dissolved carefully and made up to 1000 ml with pure water. The solution has an oxidation concentration of 0,010 mol/L of electrons.

<u>Standardization of the thiosulphate solution:</u> About 50 mL of distilled is placed in a 100mL titration beaker and 1 mL of the 50% sulfuric acid solution, 1 mL alkaline iodide solution and 1mL manganese(II) chloride reagent are added separately. After each reagent addition the solution is thoroughly mixed to avoid any precipitation of manganese hydroxides. Then 10 mL of the iodide standard solution are added with the calibrated pipette. The liberated iodine is titrated to a light yellow color; then 1 mL starch indicator solution is added and the titration is continued until the blue color then disappears. Near the endpoint the solution becomes 'cloudy' directly after addition of the thiosulphate solution. The endpoint is reached when this 'cloudy' effect can no longer be seen. Diffuse illumination from below and moderate ambient light facilitate detection of the endpoint.

<u>Subsampling and fixation of dissolved oxygen:</u> As a rule, sub sampling for the determination of dissolved oxygen should be carried out as soon as possible after the samples have been recovered. Sample bottles (100 mL capacity) are unstoppered and after flushing the nozzle of the hydro cast sampler with sample water, the end of the nozzle tube is inserted into the sample bottle almost to the bottom. The nozzle should be transparent and sufficiently in the tube but wide enough to fill the bottle rapidly. In order to flush the bottles, about twice the volume of the bottle should be allowed to flow trough the oxygen bottle until it is finally filled. The sample stream should not generate too much turbulence in the bottle to avoid intrusion of atmospheric oxygen. When the final stage of filling is reached, the nozzle is slowly withdrawn. If an air bubble has nevertheless been trapped on the walls of the bottle, it should be driven off by tapping the bottle gently. The bottle is now filled to the brim. Without intermediate stoppering, 1mL each of manganese (II) chloride (reagent 1) and alkaline iodide (reagent 2) are added, preferably with an automated twin-dispenser or semi-automated dispensers. The pipette tips are inserted almost to the bottom of the flask and slowly

withdrawn while the reagents are added. Because of the bottle and do not mix with the sample .The stopper is then inserted, which thus replaces the top of the water sample which might have been contaminated by atmospheric oxygen. The bottles are shaken vigorously for about 1 min to bring each molecule of dissolved oxygen into contact with manganese (II) hydroxide. After fixation of the oxygen, the precipitate is allowed to settle (which takes 10-20 minutes, depending upon the sample salinity).

Storage: The oxygen samples may be stored for a few hours after addition of the reagents and after complete fixation. The bottles should be kept in the dark, and any change of temperature should be avoided because of the risk of 'breathing ', i.e., the volume of the sample may expand and contract and thus aspirate atmospheric oxygen. The necks of the bottles should be sealed with seawater or the stoppers are fixed with clamps and the bottles submerged in seawater.

Titration: Before titration, the precipitated hydroxides are dissolved with sulfuric acid. For this, 1 mL of sulfuric acid (reagent 3) is pipetted into the sample bottle immediately after unstoppering. The tip of the pipette is inserted almost to the level of the precipitate and then slowly withdrawn without disturbing the precipitated hydroxides. A small magnetic stirring bar is carefully deployed in the bottle. The rotation of the stirrer is slowly accelerated. No hydroxide precipitate is to be whirled up beyond about half of the bottle height, i.e., it should be kept well away from the surface. In case of sample transfer and titration in a separate beaker, the bottle is stoppered again, care being taken that no air bubbles are trapped. The hydroxides are dissolved by shaking, the stopper is removed the film of sample on the stopper now containing liberated iodine is washed down carefully into a titration beaker of about twice the size of the sample volume and the contents of the bottle are quantitatively transferred into the beaker. The bottle is rinsed twice with a few mL of pure water, and wash water is combined with the bulk of the sample. The titration is carried out immediately as described in the thiosulphate standardization.

Determination of the reagent blank: The reagents added to the seawater sample contain oxygen. This amount of oxygen is minimized by using reagents that are almost saturated with respect to their salt content. According to Murray et. al (1968) 1 mL of the reagents contains approximately 0.0017 mL (0.0759 µmol) oxygen. If no total reagent blank is determined (see below), the oxygen concentration of the sample may be corrected by subtraction of a blank concentration DO_R

$$DO_R=1000 \cdot V_R \cdot F_R/V_S$$

V_S and V_R are the sample and the reagent volumes (mL) respectively, and F_R is the oxygen concentration of 1 mL of reagent. As the ratio of reagent addition and sample volume (V_r/V_s) is constant (2:100 in nearly all Modifications of the Winkler method), DO_R is 0.034 ml/L or 1.52 µmol/L. In addition to small amounts of oxygen, the reagents may contain impurities, e.g., higher oxidation states of manganese or traces of iodine which result in elevated blank values. Before the start of the oxygen determinations the reagent blank has to be checked.

In order to determine the "overall" blank of the method the following procedure is proposed:
Three oxygen bottles are filled carefully with tap water or any seawater sample (the same water must be used for all three bottles). Extreme care must be taken that the oxygen concentration in the three bottles is identical. Sampling is carried out as the sample. To the first bottle, one set of fixation reagents is added; to the second, two sets; to the third, three sets. The samples are then treated further as described in the thiosulphate standardization. The apparent oxygen concentration in each bottle is calculated as described below; but a correction is applied for the additional volume of the double and triple sets of reagents. The results are plotted on o graph (apparent concentration against number of sets or reagents). If there is no significant reagent blank, a straight line parallel to the abscissa should result. If there is a reagent blank, the slope of the resulting straight line gives the reagent blank. If the

reagent blank corresponds to more than 0.1 ml/L oxygen, the reagents should be discarded and prepared afresh. This procedure to determine the reagent blank takes into consideration the possibility of contamination of all reagents, whereas determination by means of the reversed addition of reagents gives the contribution of oxidized iodide.

Calculation of the result

The thiosulphate concentration, C, is determined from the titration with the iodate primary standard as

$$C = \frac{10.00 * 0.1667 * 10^{-2} * 6}{V} = \frac{0.1000}{V} \text{ mmol/L}$$

where V mL is the volume of thiosulphate used. For the ideal case, 1 ml of 0.02 mol/L thiosulphate solution corresponds to 0.160 mg oxygen or 0.112 mL (cm^3) oxygen. If the amount of thiosulphate solution required for the standardizations is V mL, the factor f, with which this must be multiplied to get the corresponding amount of 0.02 mol/L (M) solution is

Including the standardization of the thiosulphate solution, the sample concentration of oxygen is calculated as:

$$C_{OX} = \frac{(a - a_R) * V_{STD} * C_{STD} * E_{OX}}{(a_{STD} - a_R) * (V_b - V_R)} - D_{OR}$$

Where
a, a_R, a_{STD} are thiosulphate titration volumes in mL of the sample, the reagent blank and the iodide standard, respectively;
V_{STD}, V_b, V_R are volumes in mL of the iodide standard, sample (bottle) and fixing reagents (manganese-II chloride plus alkaline iodide), respectively;
C_{STD} is the molar concentration of the iodide standard.
C_{OX} is the sample concentration of oxygen in mL/L or μmol/L depending on the dimension of the oxygen equivalent E_{OX}; D_{OR} is the correction term for traces of oxygen contained in the fixation reagents. For routine analyses it may be advantageous to simplify the calculation by combining the predetermined variables as described in Grasshoff et al. (1983, 1999).

Accuracy: It is extremely difficult to estimate exactly the accuracy of the determination since the major contribution to the systematic error has its source in the sampling procedure itself. A convenient check for the exclusion of errors during the subsampling procedure is possible if water with zero oxygen content can be sampled and zero oxygen is really found after the application of the correction for the reagent blank.

B) In situ Determination of Oxygen

Various modifications of the iodometric method have been developed to eliminate or minimize effects of interferences; nevertheless, the method still is inapplicable to a variety of industrial and domestic wastewaters. Moreover, the iodometric method is not suited for field testing and cannot be adapted easily for continuous monitoring or for dissolved oxygen determinations *in situ*.

Membrane electrodes of the polarographic as well as the galvanic type have been used for oxygen measurements in lakes, estuarine and oceanographic studies. Being completely submersible, membrane electrodes are suited for analysis *in situ*. Their portability and ease of operation and maintenance make them particularly convenient for field applications. Membrane electrodes are commercially available in some variety (e.g. Interocean Sytems Inc., San Diego, USA and Meerestechnik Elektonik GmbH, Germany). These electrodes exhibit a relatively high temperature coefficient largely due to changes in the membrane permeability. Temperature compensation also can be made automatically by using

thermistors in the electrode circuit. Salinity correction is also needed for effect of salting-out on electrode sensitivity. The sensor should be kept in a 100% relative humidity environment when not in use. In order to obtain guaranteed precision and accuracy, the manufacturer's calibration procedure should be flowed exactly. Preferably calibrate with samples of water under test. The field calibration generally is a two point calibration which assumes constant zero reading. If required, the zero value may be checked with a freshly prepared sodium sulphite solution (add excess sodium sulfite, Na_2SO_3, and a trace of cobalt chloride, $CoCl_2$, to bring oxygen to zero in water). The second calibration point is a defined oxygen saturation value close to 100 % saturation. The preparation of a calibration sample with a well defined oxygen content is rather difficult and time consuming and not practicable at sea. The only recommended method is the calibration of the sensor against a *Winkler*-titration of samples.

I.4   *In situ* determination of pH

The potentiometric determination of pH by means of glass electrodes as described in Grasshoff *et al.* (1999) and the corresponding calibration procedures can be used for *in situ* determinations in surface water and water column. One-rod pH electrodes may be combined with standard CTD (conductivity temperature depth) probes used for *in situ* registrations within the limits of their specified pressure stabilities. The response times of common glass electrodes (usually > 3 s) in *in situ* applications are generally sufficient with respect to the pH changes. However, in cases of rapid environmental temperature changes, the rather high heat capacities of standard pH electrodes with considerable electrode masses cause a temperature gradient between environmental seawater and inner electrode buffer. Precision measurements of the *in situ* pH below a depth of about 400 m require separate glass and reference electrodes with electrolyte junction and pressure compensation. Some commercial CTD probes (*e.g.*, 'Aquatrace' by Chelsea Instruments and Sea-Bird) are provided with pH sensors which can operate down to 4000 m and provide accuracies of < 0.05 pH units. The pH electrode should be kept in its soaker bottle at all times when not in actual use or undergoing calibration. As the soaker solution has a pH of about 4, it is important to rinse the sensor thoroughly with distilled or demonized water before attempting calibrations.

I.5   *In situ* determination of turbidity

The Seapoint model turbidity meter (Seapoint Sensons inc., Kingston, USA) attached to the Sea-Bird CTD Rozette system is a sensor that measures turbidity by detected scattered light from suspended particles in water. Its small size, very low power consumption, high sensitivity allow this sensor to be used in most applications where turbidity or suspended particle concentrations are to be measured. The sensor is also insensitive to ambient light when under water and has a very low temperature coefficient. The Seapoint turbidity meter senses scattered light from a small volume within 5 centimeters of the sensor windows. Confining the sensing volume allows the sensor to be calibrated in relatively small water containers without errors from surface and wall reflections. It also allows the sensor to be used in tight spaces such as crowded instrumentation packages, pipes, and shallow streams. Sensitivities of 2, 10, 40 and 200 mV/FTU are possible. Each sensor is factory adjusted for consistent response to Formazin Turbidity Standard measured in Formazin Turbidity Units (FTU). The user may also calibrate the sensor with particles of interest to measure their suspended concentrations. The sensors can also be calibrated by the user to measure suspended particle concentrations. Like all optical instruments, this calibration must be performed using a sample from the measurement site. Although these sensors are electronically stable with time, starching or fouling of the windows will result in reduced sensitivity.

I.6 *In situ* determination of PAR

The LI-193SA Spherical Quantum Sensor (LI-COR, Nebraska, USA) attached on the Sea-Bird CTD system can be used for measuring Photosynthetically Active Radiation (PAR) in aquatic environments and specifically the Photosynthetic Photon Flux Fluence Rate (PPFFR). The LI-193SA gives an added dimension to underwater PAR measurements in that it measures PAR from all directions. The LI-193SA Sensor can also be used in air. Because PPFFR can be defined as those photons having a wavelength between 400 and 700 nm that are indecent per unit time on the surface of a sphere divided by the cross-sectional area of the sphere, the LI-193SA Spherical Quantum Sensor is designed to respond equally to photons between 400 and 700 nm. Because the energy of photon is inversely proportional to its wavelength, a sensor which responds equally to photons will have a linear energy response with wavelength. Therefore, an ideal PPFFR sensor would have a slope of 1 % per 7 nanometers (nm) if it were normalized to 100% at 700 nm.

I.7 *In situ* determination of fluorescence

An *in situ* fluorometer (e.g. WET Labs, Philomath, USA and Sea Tech Inc., Corravalis, USA) allows the user to measure relative chlorophyll concentrations by directly measuring the amount of fluorescence emission from a given sample of water. The sample media is pumped through a quartz tube mounted through the long axis of the instrument. Chlorophyll, when excited by the presence of an external light source, absorbs light in certain regions of the visible spectrum and re-emits a small portion of this light as fluorescence at longer wavelengths. The WETStar fluorometer uses two bright blue LEDs (centered at approximately 470 nm and modulated at 1 kHz) to provide the excitation. Blue interference filters are used to reject the small amount of red light emitted by the LEDs. A detector, positioned at 90 degrees to the axis of the LED mounts, measures the emitted light from the sample volume. The approximately 0.25 cm^3 sample volume is defined by the intersection of the excitation light with the field of view of the detector, within the quartz flow tube. A red interference filter is used to discriminate against the scattered blue excitation light. The red fluorescence emitted at 90 degrees is synchronously detected at 1 kHz by a silicon photodiode. The amplified and demodulated voltage output of the photodiode is provided to the user for connection to a digital voltöeter or an a-d converter. Blue excitation light (470 nm) is absorbed by phytoplankton, which contains chlorophyll. A portion of this energy is re-emitted as red light at approximately 685 nm. Scattered blue light is blocked by the red interference filter. The red fluoresced light passes through the interference filter and is detected by the photodiode, which in turn passes the analog signal as a DC output voltage that can be measured and recorded.

I.8 Determination of Secchi Disk depth

The [Secchi disk] is named after Pietro Secchi, a Jesuit scientist, who studied the Mediterranean Sea in the mid-1800's. The Secchi disc is a simple scientific instrument used to measure water transparency. The Secchi disk is an eight-inch disk painted with alternating black and white quadrants. The disk is lowered into the water until it can no longer be seen by the volunteer. The disk is then raised until it reappears. The depth of the water where the disk vanishes and reappears is the Secchi disk reading. The Secchi disc depth indicates the water transparency and provides a rough estimate of light penetration in the water column. It is used primarily for its simplicity. A more accurate measurement of underwater irradiance can be made by the use of photometer.

II   Nutrient and Phytoplankton Pigment Analysis in Seawater

II.1   General Safety Matters

All laboratories should have a properly formulated safety policy, and safe working practices, 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 <u>before</u> 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.

- Before handling any chemical, the documentation referred to in the manufacturer's documentation must be read and appropriate precautions adopted.
- Chemicals should be stored according to the manufacturer's instructions.
- All personal safety precautions must be observed and personal contact with chemicals must be prevented through the use of gloves, safety glasses, laboratory coats, etc.
- All chemical wastes should be stored in appropriate and labeled containers and an inventory of the contents should be maintained.
- Great care must be taken to avoid placing mutually reactive wastes in the same container.
- Solvent wastes must be stored separately and chlorinated and non-chlorinated solvents must not be mixed.
- The cadmium waste generated, as part of the nitrate procedure, must be handled and disposed of according to local regulations.

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.2   Sample collection

Water samples from specified depths 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. If this is the case, then, this information would be for a spatial survey, for example a river survey, but it should be remembered that deep water is rarely, if ever, homogenous and so the surface survey would in the case of an offshore or coastal water column only yield limited

information. It is both better and more informative if a depth survey is carried out during a particular water sampling exercise.

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), and lowered down through the water column by the ship's winch. In this case, the sample bottles can be closed at will through commands issued from the surface deck-unit, and transmitted to the rosette through a conductive cable.

Collecting samples by pumping, using an underwater pump and flexible plastic tubing, may also be used when a continuous horizontal record of distributions is required. It is less suitable for vertical profiling unless great care is taken to take into account of the tube dead-volume when relating a water sample to a specific depth. However, this approach is well suited to the sampling throughout a transect (e.g. from shore to open waters as suggested in JAMP/OSPAR guidelines for eutrophication/nutrients).

Water sampling bottles should be held in a rack, which enables the sub-samples to be collected. Ideally, there should be a compartmentalized sub-sample bottle rack below the bottles with one compartment for sub-sample bottles under each water sampler. However, these are uncommon and samples are commonly removed directly to a separate storage container (e.g. a divided crate). 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. Whatever the system use great care must be taken to avoid contamination and careful labeling of samples is essential to remove the possibility of confusion over sample identities.

Any source of contamination from the sampling equipment, ship and on-board activities should be avoided while sampling is undertaken. For example, discharges from ships are significant sources of organic nitrogen, ammonia and phosphate, therefore, all such activities generating these contaminants have to be stopped (or controlled) during sampling. Sampling bottles should be kept closed when not in use and touching the inside of the bottles should be avoided. When the Niskin bottles are kept dry for a long time, it is recommended to wash the bottles with dilute HCl and rinse with water before use.

Samples are collected in high-density polyethylene bottles. Collection bottles are always acid washed (3 x 1 M HCl), rinsed with de-ionized distilled water and dried before the sample collection.

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 3 times.
f)   Fill bottle to just above the shoulders of the bottle.
g)   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.

II.3   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 II 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 (1966) forms the basis of the method adopted for the determination of chlorophyll pigments.

It is also possible to determine chlorophyll by using in-situ fluorometer, spectrofluorometry at the laboratory 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 (Instead, it is also recommended to use forceps to remove zooplankton since larger size of phytoplankton can also be removed with mesh).
e)   Filters (0.7 µm in pore size: glassfiber filter, Whatman GF/F).
f)   Spectrophotometer with cell having a path length of 4 - 10 cm. Although path lengths longer than 5 cm are unusual, there are useful gains in the signal to noise ratio to be obtained with longer cells. When purchasing new spectrophotometers for analysis of low concentrations, it is worth considering whether long path lengths are available. Note that low volume (thick-wall) cuvettes may produce erroneous data in spectrophotometers having very converging beams.
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) Acetone 90 % solution:
Good quality acetone should be shaken with a little granular anhydrous sodium carbonate and decanted directly for use.
If good quality acetone is not available, 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 ^{o}C. 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.

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°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.

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 ^{o}C or less for up to two months.

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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 at least 10 minutes in the dark at room temperature or, alternatively, overnight at 4 °C then allowing samples to return to room temperature before centrifugation. 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 2 nm or less, and cells with a light path of a minimum of 4 cm. Read the extinctions at 750, 663, 645 and 630 nm against a 90 % acetone blank. Correct the extinctions at each wavelength for the blank value. If the 750 nm reading 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 $\underline{a}$, $\underline{b}$ and ($\underline{c_1 + c_2}$) in the 90 % acetone extract as µg/ml is given by the following equations recommended for mixed phytoplankton communities:

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

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

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

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.

II.4   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, and in many cases, the concentrations are below the detection limits of conventional colorimetric analyses. 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 (washing with dilute acid (see note 7 below) is recommended for glassware, followed by copious washing in distilled water) and dedicated to the task.
7)   Certain analyses should ideally 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. Good, clean techniques will avoid many problems and regular checks for contamination are mandatory.
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 national and 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.

It is also recommended to obtain full procedural blanks which includes sample bottle blank filled with low nutrient sea water (LNSW) and treated as samples. A statistically significant number of bottles should be preserved with each set of samples during sampling (e.g. 3-5 bottles filled with LNSW for a group of sampling stations).

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 known 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, bottles are tightly capped, and autoclaved in a bench-top autoclave at 120 °C for 30 minutes.

As another internal quality monitor, replicate samples of 5 can be obtained both from low and high nutrient waters (e.g. reference and polluted sites) to ensure the repeatability of sampling and analysis.

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

It is also recommended that chemical laboratories be certified according to the ELOT EN ISO / IEC 17025 procedure.

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 better if samples are not filtered, but it is recommended that samples be filtered <u>only</u> if there is visible turbidity in the sample. 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 (Swinnex units) 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 <u>gently</u> 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. In general it is best that samples are analyzed as quickly as possible after collection (i.e. within a few hours not exceeding 24). If samples are to be preserved for longer periods, then samples may be frozen (after filtration if visibly turbid) using e.g. a solid CO_2/methanol mixture slurry. "Aged" high density polyethylene bottles are suitable for this purpose though, as always, analysts should ensure that their own techniques are satisfactory.

NOTES:
- It is not possible to preserve samples satisfactorily for ammonium analysis, so, rapid determination of this parameter is mandatory.
- Freezing of samples may cause silicate species to polymerize in high concentration samples. Samples must be thawed for ~24 hours before analysis to allow for depolymerization.

i) Phosphate - P

Water samples for phosphate analysis should be collected in stoppered glass or "aged" polyethylene 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 new polyethylene or polyvinylchloride containers since phosphate has been shown to disappear rapidly in these containers. Therefore, aged high-density polyethylene bottles or other plastic e.g. polycarbonate may be satisfactory but all sample containers 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) Ammonium - N

Samples for ammonium analysis should only be taken and stored in tightly sealed seawater-aged glass or high-density 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 ammonium. 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).

Ammonium 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 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 µmol/l 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 µmol/l). Samples should be stored in tightly sealed glass or polyethylene bottles only. Sulphide ions have been reported to interfere with the determination of nitrite and, thus, when hydrogen sulphide is suspected to be present in a sample, the gas should be expelled with nitrogen after the addition of the acid sulphanilamide reagent (Grasshoff et al., 1983).

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 µmol/l NO_3 about half of the nitrate disappears within seven days after storage at room temperature. This indicates that only glass or "aged" high-density 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 or "aged" high-density polyethylene 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. If longer storage is unavoidable, the sample should be quickly frozen to -20 °C after the addition of the ammonium chloride buffer solution (Grasshoff et al., 1983).

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. However, during seasons of high productivity, do not store them for longer than a day. Polymerization of orthosilicate during storage of frozen samples has been reported from fresh water samples but does not occur in seawater. If kept frozen, it is recommended to thaw the sample gradually at room temperature for at least 24 hours. However, as with all nutrients immediate analysis of sample is the preferred option.

The best procedure for storage and preservation of fresh-water samples 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 ^{o}C. However, as with all nutrients immediate analysis of the sample is the preferred option.

II.5   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*

Principle:

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 colored 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 µmol/l when measured in a 1 cm cell.

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 color 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 mol/l)

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.

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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.

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f)   Phosphate stock solution (10 mmol/l PO_4) (A)

Potassium dihydrogen-phosphate (KH_2PO_4, a.g.) is dried in an oven at 110 °C. Then, exactly 1.3609 g of KH_2PO_4 are dissolved in distilled water, 2 ml of 4.5 mol/l 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.

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g)   Phosphate working solution (10 µmol/l 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.

<u>Apparatus:</u>

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 more important 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.
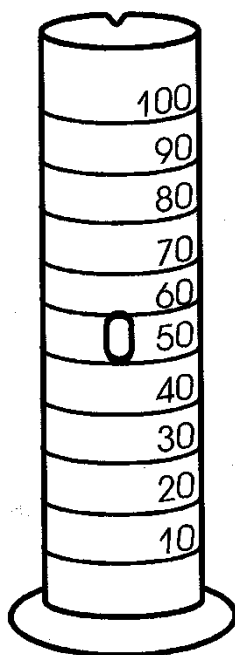
Figure 1: Special type of graduated cylinder (glass or plastic made) for the quick sub-sampling of 50 ml portions in nutrient analysis (with the hole as sample overflow). These devices are less accurate than say a 50mL pipette but better than multiple uses of a smaller pipette and safer to use on a small boat than large glass pipettes.

<u>Method:</u>

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:

$$0.5 \text{ ml of working solution} = 0.5 \mu mol/l \ PO_4^{3-}$$
$$1.0 \text{ ml of working solution} = 1.0 \ \mu mol/l \ PO_4^{3-}$$
$$2.0 \text{ ml of working solution} = 2.0 \ \mu mol/l \ PO_4^{3-}$$
$$3.0 \text{ ml of working solution} = 3.0 \ \mu mol/l \ PO_4^{3-}$$
$$4.0 \text{ ml of working solution} = 4.0 \ \mu mol/l \ PO_4^{3-}$$
$$5.0 \text{ ml of working solution} = 5.0 \ \mu mol/l \ PO_4^{3-}$$

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 acid-molybdate solution (mixed reagent) and afterwards 1.5 ml of the ascorbic acid solution is added only to the sample. Mix well after each addition to both portions. 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 µmol/l) or arsenate (at "normal" total arsenic seawater concentration of around 25 µmol/l). If measurements are performed after 30 minutes for example, 200 nmol/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.

<u>Calculation of Results:</u>

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 <u>b</u> of the calibration curve (for the individual cell length used), which follows the equation:

$$Absorbance = b \times concentration$$

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).

<u>Estimation of Precision and Accuracy:</u>

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 µmol/l) and ± 15 % at the low level of around 0.22 µmol/l.

<u>Recommendation</u>: Dissolved inorganic phosphate concentrations for some surface waters in the Eastern Mediterranean may be lower than 0.1 µmol/l and in order to apply sensitive and reproducible techniques for these type of waters, once can refer to MAGIC (magnesium-induced co-precipitation) method (Karl and Tien, 1992).

B)   *Determination of Ammonium - N*

<u>Principle:</u>

The method is specific for ammonium and is based on the formation of the blue colored indophenol complex, 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 µmol/l (in a 10 cm cell), and the Lambert-Beers's Law is followed up to an ammonia concentration of about 40 µmol/l.

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). Therefore the pH of the samples from estuaries should be measured after the addition of reagents. 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 $K_2S_2O_8$ 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. As an alternative, "open sea surface water" can be used as "ammonia-free" water.

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 mmol/l NH$_3$)

Ammonium chloride (NH$_4$Cl) is dried at 100 °C to constant weight. Then dissolve 0.0535 g NH$_4$Cl 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 µmol/l 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.
d)

> **Note**: All flasks and tubes to be used should be cleaned with hot HCl, 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$_4$Cl reagent used for nitrate analysis. **Smoking should be forbidden**.
>
> Alternative: Before use, all flasks should be treated by performing the reaction in them with the addition of chemical reagents to the "ammonia-free water" or "open sea surface water". The reaction should proceed at least for 6 hours and the flasks should be shaken time to time during the reaction period. Later, the flask should be rinsed with ammonia-free water and kept stoppered when not in use. The flasks should not be washed between the analysis of different sets of samples/standards, but just rinsed with ammonia-free water and kept closed.

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 or "open sea surface 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 µmol/l NH$_3$
1.0 ml of working solution = 1.00 µmol/l NH$_3$
2.0 ml of working solution = 2.00 µmol/l NH$_3$

3.0 ml of working solution = 3.00 µmol/l NH_3
5.0 ml of working solution = 5.00 µmol/l NH_3
7.0 ml of working solution = 7.00 µmol/l NH_3
10.0 ml of working solution = 10.00 µmol/l 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 or "open sea surface 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. Note that standards and samples of the same series must be treated simultaneously, and in the same way.

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 or "open sea surface 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 or "open sea surface 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).

<u>Calculation of Results:</u>

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

Absorbance = b x Concentration

As already mentioned, for any given concentration of ammonium 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. In many circumstances a simple correction (Grasshoff et al. 2002) may be used where 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 µmol/l.

C) *Determination of Nitrite - N*

Principle:

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 0.02 µmol/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 - 10 µmol/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.

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 mmol/l)

Anhydrous sodium nitrite ($NaNO_2$, a.g.) is dried at 100 ^{o}C (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 µmol/l)

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 µmol/l NO_2
0.20 ml of working solution = 0.2 µmol/l NO_2
0.40 ml of working solution = 0.4 µmol/l NO_2
0.60 ml of working solution = 0.6 µmol/l NO_2

0.80 ml of working solution = 0.8 µmol/l NO_2
1.20 ml of working solution = 1.2 µmol/l 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:

$$Absorbance = b \times Concentration$$

The concentration of nitrite is then calculated according to:

$$C\ (µg/l) = (A_s - A_b - A_t) / b$$

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 µmol/l though an increase of precision to ± 3 nmol/l 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.

<u>Principle:</u>

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.

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).

<u>Reagents:</u>

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).

> Note: the preparation ideally should be done in another laboratory to avoid contamination of the samples by the atmosphere.

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 is 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 mmol/l)

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 mmol/l)

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 µmol/l)

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).

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.
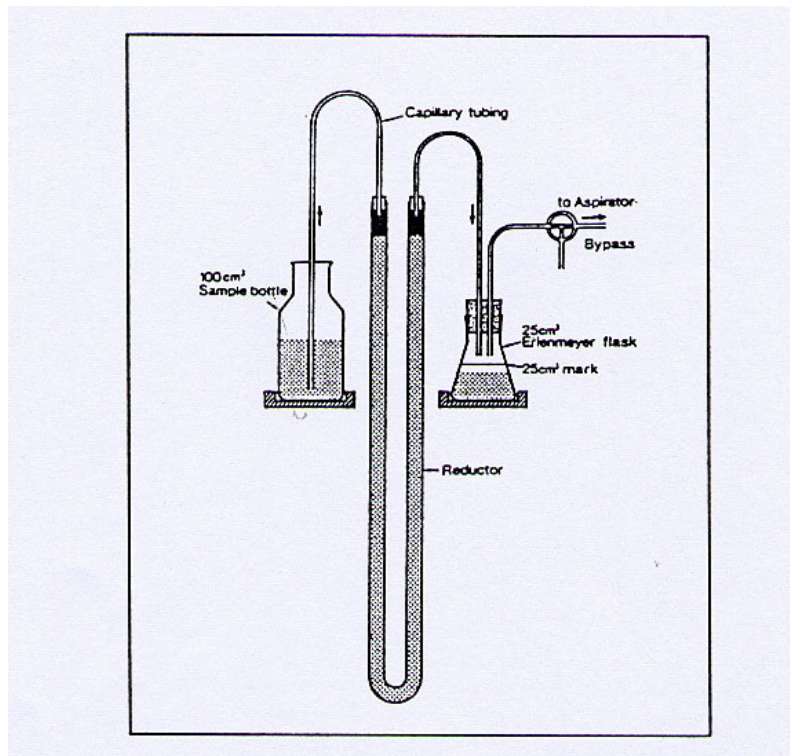
Figure 2: Reduction column for the analysis of nitrate.

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 mol/l 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 stock solution (B) and from the working solution (C) (section: Reagents g) and h) page 28). 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 µmol/l	B µmol/l
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 µmol/l 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 <u>b</u> 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:

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

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 µmol/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

Principle:

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 30 µmol/l (samples with higher concentrations are diluted with distilled water), and a detection limit of about 0.04 µmol/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.

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 19,000 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 mol/l)

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 mmol/l)

Disodium hexafluoro silicate, Na_2SiF_6, is dried at 105 °C 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 µmol/l)

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.

<u>Apparatus:</u>

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.

<u>Method:</u>

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 µmol/l Si
0.5 ml of working solution	= 2.5 µmol/l Si
1.0 ml of working solution	= 5 µmol/l Si
2.0 ml of working solution	= 10 µmol/l Si
5.0 ml of working solution	= 25 µmol/l Si
10 ml of working solution	= 50 µmol/l 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 0.45 µm 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:

$$Absorbance = b \times Concentration$$

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

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

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 µmol/l and 5 µmol/l respectively.

F)   *Determination of Total Phosphorus*

Methods given in Grasshoff *et al.* (1983, 1999) and APHA-AWWA-WPWF (1992) can be used.

Reagents

An oxidizing reagent is required in addition to the reagents listed for phosphate:
Potassium persulphate solution. Dilute 5 mL sulphuric acid (4.5 mol/L) with water to 100 cm^3. Add and dissolve 5 g potassium peroxodisulphate, $K_2S_2O_8$. Store this saturated solution at room temperature in a polyethylene bottle protected from direct sunlight. The reagent is stable for about a week.
Potassium persulphate. $K_2S_2O_8$, solid
Sodium Hydroxide (1.0 mol/L): Dissolve 40.0 grams of NaOH in 1.0 liter of pure water.

Sampling and storage

The analysis should be commended as soon as possible, preferably within 2 h of sampling. When immediate analysis is not possible, the samples are acidified with 0.4 cm^3 of

4.5 mol/L sulphuric acid per 100 mL and stored cold in polyethylene bottles in the dark. Because of the observed uptake of phosphorus by glass, glass flasks should not be used for long storage. Deep-freezing in polyethylene bottles can also be recommended.

<u>Sample and Blank Measurements</u>

To 50 mL of the sample in the oxidation bottle is added 4 mL peroxodisulphate solution. Alternatively add 0.2 mL sulphuric acid (4.5 mol/L) and 220 ± 20 mg solid persulphate. If the sample is preserved with acid, no further acid is added. Dissolve the reagent by swirling. Close the bottles and place them in the autoclave, which has been filled with about 200 mL water. Autoclave the samples for 30 min. After cooling to room temperature, check that the stoppers are tight, then open the bottles and transfer their contents quantitatively to the phosphate flasks. The volume of the samples should remain unchanged. Neutralize the digested samples with NaOH. Then add 1 mL ascorbic acid, mix, wait for about half a minute and then add 1 mL of the mixed reagent prepared for the phosphate determination. Measure the absorbance at 880 nm after 10-30 minutes using 10 cm cell (or the longest available cell).
Free chlorine formed is reduced by first adding ascorbic acid in the subsequent phosphate analysis. The flow-analysis manifold for phosphate may be modified accordingly, *i.e.*, ascorbic acid solution is introduced as the first reagent, or 0.5 ml of ascorbic acid is added to the sample and mixed before the flow-analysis. Then transfer the required volumes into the reaction flasks for phosphate determination or into sample containers for the flow-analyser.

Take at least three 50 mL potions of distilled de-ionized water (free of phosphorus) and pour into the oxidation bottles. Add 4 cm^3 peroxodisulphate solution. Alternatively add 0.2 mL sulphuric acid (4.5 mol/L) and 220 ± 20 mg solid persulphate. Dissolve the reagent by swirling. The blanks are then treated exactly as described for the samples. In near-shore waters the determination of turbidity blank may be necessary and is then made on a second portion of the sample, by omitting the reagents after the oxidation step. The phosphorus concentrations calculated after blank corrections represent all forms of phosphorus in unfiltered samples. When the analysis is performed using a filtered sample, the result includes all the dissolved forms of the element.

Alternatively, samples are digested on a preheated hot plate for 30 to 40 min or until a final volume of 10 mL is reached. Then, cool the sample and blank, dilute to 30 mL with P-free water, add 0.05 mL (1 drop) phenolphthalein indicator solution, and neutralize to a faint pink color with NaOH. Make up to 50 mL with distilled water and measure phosphorus concentration by adding ascorbic acid and mixed reagent. In some samples a precipitate may from at this stage, but do not filter. For any subsequent subdividing of the sample, shake well. The precipitate (which is possibly a calcium phosphate) redissolves under the acid conditions of the colorimetric reactive phosphorus measurement.

The flow-analysis manifold for phosphate may be modified accordingly, *i.e.*, ascorbic acid solution is introduced as the first reagent, or 0.5 ml of ascorbic acid (Section 10.2.5.4; reagent 4) is added to the sample and mixed before the flow-analysis. Transfer the required volumes into the reaction flasks for phosphate determination or into sample containers for the flow-analyser.

G) *Simultaneous determination of Total Nitrogen and Total Phosphorus*

For the determination of total nitrogen (TN) and total phosphorus (TP), the methods in Grasshoff et al. (1999) can be used.

<u>Reagents:</u>

a) Oxidation mixture: Dissolve 50 g potassium peroxysulphate and 30 g of boric acid in 1 liter of 0.375 mol/l NaOH and store this saturated solution in a well-closed polyethylene bottle wrapped with aluminium foil at room temperature. This mixture is stable for several weeks. Since TN is determined all reagents must be very low in nitrogen.

In the simultaneous oxidation of samples, the reaction starts at about pH 9.7 and ends at 4-5. These conditions are established using a boric acid-NaOH system.

<u>Sampling and storage</u>

Samples can be stored for a long time in the oxidation bottle without adding any preservative if a sufficient number of bottles are available.

Preservation with acid should be avoided. Deep-freezing of the samples in polyethylene bottles is also recommended.

<u>Analysis</u>

To 50 ml of sample, 5 ml of the oxidizing agent is added. Close the flask and autoclave for at least half an hour. Then, open the autoclave and allow the flasks to cool to about room temperature. With a bulb pipette take from the flask 5 ml of the digested sample, on which TN as nitrate can be determined separately. Transfer the remaining 50 ml of sample to a phosphate reaction flask and add 1 ml of ascorbic acid and mixed reagent solutions. Mix swirling between additions. Measure the absorbance at 880 nm after 10-30 minutes using 10 cm cell (or the longest available cell).

II.6 Analysis of nutrients using continuous flow or other technologies

A) Continuous flow methods

The principle used by the continuous segmented-flow auto-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.

Many different analytical techniques have been developed for these analysers and can be found in the scientific literature.

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

IMPORTANT OPERATING NOTES FOR AUTOMATED MEASUREMENTS OF NUTRIENTS:

1. Ammonia:

Sodium salicylate has replaced phenol in the latest automated method of BANN+LUBBE.

i) For seawater analysis, use a sampler wash solution containing sodium hydrogen carbonate, magnesium chloride, magnesium sulphate and calcium chloride or "ammonia-free open sea surface water". If the salinity of seawater samples is markedly lower than in the artificial seawater formulation, the dilution of the wash water is required because of the different refractive index. If the concentration of calcium and magnesium ions is markedly lower than in the artificial seawater formulation, artificial seawater containing only sodium chloride (35 g/l) and sodium hydrogen carbonate (0.2 g/l) can be used.

ii) For rain, river and waste water analysis, use only distilled-de-ionized water wash solution.

iii) The diluent for the standards must have the same matrix as the sample and the sampler wash solution. Therefore, use artificial seawater or low-nutrient seawater for seawater analysis. To avoid errors from ammonia content in the inorganic salts used for artificial seawater, using a zero calibration standard of low-nutrient seawater of known low concentration is recommended.

iv) Final pH of (sample + reagent)

For optimum results, the pH of the final reaction must lie within certain limits. Therefore, collect the solution from the flowcell waste line to check the pH.
Final pH (with salicylate reagents): ph 12.8-13.1
Final pH (with phenate reagents): pH 11.5-11.9
If the final pH is too low, increase the sodium hydroxide concentration.

v) **WARNING for seawater analysis!** Without salicylate and phenol, the hydroxides of magnesium and calcium precipitate after the addition of dichloro isocyanuric acid reagent. Therefore add salicylate and phenol first or remove it last when starting up or shutting down the system

During the day, it may occur that a slight precipitation occurs in the 5-turn coil after DIC addition. This does not affect performance. If it occurs, during the shutdown procedure clean the system with 1 N HCl.

vi) Ammonia is a common contaminant in the atmosphere and general environment. Take extra precautions to avoid contamination of the reagents. Do not touch any surfaces which will be in contact with reagents or samples. Check the reagent absorbance each time fresh reagents are made. Rinse sample cups (pre-washed with 1 N HCl) with samples before filling them. Fill and place the sample cups just before analysis in order to minimize contamination risk from atmosphere. Check sample cup blanks occasionally.

2. Phosphate

i) For seawater analysis, use sampler wash solution containing sodium chloride and sodium hydrogen carbonate or "phosphate-low seawater". For water and waste water analysis use only DDW as sampler wash solution.

ii) The standard diluent must have the same matrix as the samples and the sampler wash solution. Therefore, use artificial seawater or low-nutrient seawater for seawater analysis. To avoid errors from phosphate content in the inorganic salt used for artificial seawater, analysis of a zero calibration standard of low-nutrient seawater of known low concentration is recommended.

3. Silicate

i) Prepare all reagents in polyethylene containers. Use plastic flasks to prepare working standards. If mixed standards (including nitrate, phosphate and ammonia) are prepared, use the same flasks.

ii) For seawater analysis, use sampler wash solution containing sodium chloride and sodium hydrogen carbonate. For water and wastewater analysis, use only DI water.

iii) For the most accurate results, the standard diluent should have the same matrix as the samples. Therefore, use artificial seawater or low-nutrient seawater for seawater analysis.

4. Nitrate and Nitrite

i) For seawater analysis, use a sampler wash solution containing sodium chloride and sodium hydrogen carbonate. For water and wastewater analysis, use only DI water.

ii) For the most accurate results, the standard dilutes should have the same matrix as the samples. Therefore, use artificial seawater or low-nutrient seawater for seawater analysis.

iii) The nitrite value of samples can be determined by eliminating the reduction column and standardizing with an appropriate nitrite solution. In order to determine the nitrate values, the nitrite alone must be subtracted from the total (nitrite + nitrate). Check the efficiency of the reduction (cadmium) column daily by analyzing equi-molar nitrate and nitrite standards. If the reduction efficiency of the column is less than 90 %, activate the column or replace it by a new one.

B) Other technologies

A number of *in situ* multi-channel nutrient analyzers are now available on the commercial market and are being developed for deployment for extended periods at depths down to a few hundred meters. Flow injection systems are also an alternative to the larger scale continuous flow analyzers. Some high sensitivity techniques have been developed, for example using chemiluminescence technology to analyse nanomolar concentrations of nitrate and nitrite. More recently ammonium has been analysed using a combination of Teflon-membrane gas diffusion coupled with fluorescence detection, this again allows nanomolecular detection limits. Most recently there are developments using the classical colorimetric analytical chemistries similar to those described above but with long path-length flow cells up to 2 metres in lengths called liquid waveguide capillary cells. These systems are now regularly used in some laboratories in their oceanic monitoring and research programmes.

II.7 Recommendations for Water Sampling, Sample Processing and Chemical Analysis

Contamination sources: Contamination from the sampling equipments, ship and on-board activities should be avoided while sampling is undertaken. Sampling bottles should be cleaned with dilute HCL acid and washed with pure water and always be kept closed when not in use.

Sub-sampling protocol: Firstly, take DO and pH samples using tygon tubing; then in the order of nitrite, other nutrients, chlorophyll and phytoplankton samples. Water samples for dissolved inorganic and total nutrients are taken in high density polyethylene bottles. The bottle quality should be checked before their use by filling both Low Nutrient Sea Water (LNSW) and nutrient-spiked SW.

Sample Handling on board: It is recommended to analyze the samples as quickly as possible after collection and filtration (if visibly turbid). If immediate analysis is not possible, freeze the samples in "aged" and "tested" polyethylene bottles. Water samples for simultaneous analysis of total nitrogen and phosphorus can be stored for a long time in the oxidation bottle. Preservation with acid should be avoided. Samples taken for only total-P is acidified and kept cool until analysis.

Chemical Measurement: All spectrophotometric measurements should be referenced against distilled water. To avoid salinity effect, use Low Nutrient Sea Water (LNSW) for standard preparations and as wash-water in the automated method. It is also recommended to obtain full "procedural blanks" which includes sample bottle blank filled with low nutrient sea water (LNSW) and treated as samples. Pay attention to provide nutrient-free chemicals. Nitrate analysis (using NH_4Cl) should not be conducted in the same laboratory as ammonia analysis. Check Nitrate reduction column efficiency (>90%) before analysis. The colorimetric reactions of the nutrient measurements are quantitative only within certain pH ranges. Therefore, check the final pH for samples during analysis.

Quality Control: Replicate samples of 5 can be obtained both from low and high nutrient waters (e.g. reference and polluted sites) to ensure the repeatability of sampling and analysis. Laboratories are also encouraged to participate in regional or international Quality Control schemes such as those operated by IAEA/MEL or QUASIMEME.

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III   Phytoplankton Determinations

III.1   The Analysis of Phytoplankton

This part was modified from Magaletti *et al.*, 2001.

A) Sampling

Collect surface waters (at approximately 0.5 m depth) using a Niskin bottle or, if this is not available, a bucket. If a subsurface chlorophyll *a* maximum is present, an additional sample has to be taken at this depth, always using a Niskin bottle.

Due to its substantial seasonal variability, phytoplankton should be sampled at least once a month, fortnightly in summer.

In presence of an oligotrophic monitoring area, nets are useful in filtering large volumes of water and could be employed to provide material for qualitative purposes.
Transfer the sample in 250, 500 or 1000 ml dark glass bottles with screw cap. Do not fill up the bottle completely to allow a gentle mixing of the sample when in the lab. Keep the bottles in a dark, cool place.

B) Preservation and storage of samples

(Sournia, 1978)

The most commonly used fixatives are formaldehyde and Lugol's solution, but it is good practice to observe an alive subsample first. This would allow a better characterization of cells morphology, colour and motility.

Lugol
Acidic Lugol's solution is recommended, since it is less toxic than formaldehyde. Lugol's solution is adequate for the preservation of dinoflagellates, diatoms and delicate species, such as small flagellates. It is less indicated for the preservation of coccolithophorids, because it can dissolve the $CaCO_3$ (if storage time is longer than one month).
The use of Lugol's solution excludes the possibility to use epifluorescence microscopy and SEM.
Add 0,5-1 ml of Lugol's solution to 250 ml of sample. If the cells become too dark it is possible to obtain a lighter colour by adding sodium thiosulfate.
Store the fixed samples at room temperature, in the dark. Samples can be stored for up to 12 months, but it is recommended to check the colour of the solution, that tends to become lighter with time due to the oxidation of iodine, thus reducing fixation.

Acidic Lugol's solution:
• 20 g potassium iodine (KI)
• 200 ml distilled water
• 10 g iodine (I_2)
• 20 ml acetic acid (CH_3COOH)

Formaldehyde
Formaldehyde can be used as an alternative to Lugol's solution. The advantage is that it allows further investigation by epifluorescence microscopy or SEM.

Formaldehyde solution:

• use 40% formaldehyde and add an equal volume of distilled water (20% final concentration)

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100 g/L of hexamethylenetetramine to keep the final pH neutral or slightly basic.
Filter the solution before use. Store the solution at 5-6°C and add 20-40 ml of solution per litre of sample.
For a neutralized (weakly alkaline) solution add sodium borate or sodium carbonate.

C) Analysis

Perform a quali-quantitative analysis of the subsample using the inverted microscope method described by Utermöhl (1958) (Zingone *et al*., 1990).
On the basis of the observed chlorophyll *a* values, use 10, 25, 50 or 100 ml sedimentation chambers. Gently shake up and down the glass bottle for about 1 min (or about 100 times) before adding the subsample to the sedimentation chamber.

Counting must be performed when all the cells have settled to the bottom. A sedimentation time of 3 hours per centimetre chamber height commonly results in a complete sedimentation of all the organisms, but is suggested to use longer times especially for those samples containing many small species.

Cell counts are to be performed on casual fields or on transects for abundant species, and on the whole bottom area for less abundant species.
- *casual fields*: the number of fields must be at least 1/100 of the surface area of the sedimentation chamber, according to the magnification used. If the total number of cells counted is less than 200, increase the number of fields. Use a magnification of about 400x.
- *transects*: choose two perpendicular transects. If the total number of cells counted is less than 200, increase the number of transects. Use a magnification of about 400x.

Use the following equations for estimating cell concentrations:

a) cell counts performed on fields
$$C = (N \cdot 1000 \cdot A) / (n \cdot v \cdot a)$$
where:
C = cells concentration, expressed as cells/L
N = total number of cells counted on all fields
A = surface area of the bottom of the sedimentation chamber (in mm^2)
n = number of fields used for cell counts
v = volume (in ml) of the sample
a = area of the visual field (in mm^2)

b) cell counts performed on transects
$$C = (N \cdot \pi \cdot r \cdot 1000) / (2 \cdot h \cdot v \cdot n)$$
where:
C = cells concentration, expressed as cells/L
N = total number of cells counted on all transects
r = radius (in mm) of the sedimentation chamber
h = height (in mm) of the transect (diameter of the visual field)
v = volume (in ml) of the sample
n = number of transects used for cell counts

D) Recommendations

When studying eutrophicated environments it is very important to give an estimation of the phytoplankton biomass which is involved into the process. For this purpose it is recommended to transform cell abundance data into a more useful form, cell volume, by morphometric measurements and geometric formulae.

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III.2 Direct Counting of Nanoplankton by Epiluorescence Microscopy

A) Sampling and preservation

1. Water sampling by Niskin bottles

2. Preservation
  subsamples (100 ml) in plastic bottles are preserved with prefiltered (0.22 µm) glutaraldehyde (final concentration 1%) and stored in the dark at +4 °C until slides are prepared (within few days from sampling).

B) Direct counting

The analysis is performed using the Sherr method (Sherr *et al.*,1983), which involves the observation by epifluorescence microscopy of 20 ml of sample. This volume of water is prefiltered (low vacuum, 48 kPa) onto black policarbonate filters (2 µm pore size, 25 mm diameter). A Millipore filter (0.45 µm) was placed underneath the Isopore black filter to promote even dispersion of sample on the black filter. When 2 ml are left to filter, 2 µL of a DAPI solution (1 mg/mL) are added to the sample, which is finally left for 15 min at total darkness without vacuum. This allows the formation of a DNA-DAPI complex. The filter is then preserved at -20° (total darkness) until observation by epifluorescence microscopy. The filter is mounted on a glass slide and it is observed using a final magnification of 1000x. Heterotrophs are counted using an UV-filter set (BP 340-380 exitation filter, RKP 400 dichroic mirror, LP 430 barrier filter). Autotrophs are counted using a Blue-filter set (BP 450-490 excitation filter, RKP 510 dichroic mirror, LP 510 barrier filter). The number of observed fields (20-30) depends on sample concentration (minimum cell number: 200).

Mounting of the filter on the microscope glass slide (in Maugeri T.L. *et al.*, 1990)

Nanoplankton calculation:

$$cell/mL = N. (A_r/A_c) . 1 /V$$

where: N = average number of cells counted in the field
  A_f = filtration area
  A_c = field area
  V = volume of sample filtered (in mL)

III.3 Direct Counting of Picoplankton by Epifluorescence Microscopy
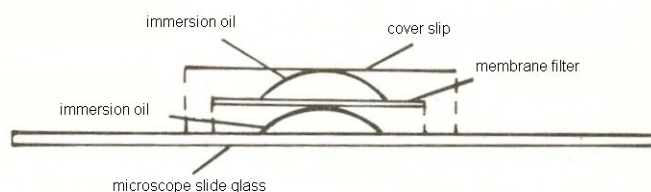
A) Sampling and preservation

1. Water sampling by Niskin bottles

2. Preservation
  subsamples (100 ml) in plastic bottles are preserved with prefiltered (0.22 µm) formaldehyde (final concentration 2 %) and stored in the dark at +4 °C until slides are prepared (within few days from sampling).

B) Direct counting

The analysis is performed using the Porter & Feig method (Porter & Feig, 1980), which involves the observation by epifluorescence microscopy of 2-3 mL of sample. 2-3 µL of a DAPI solution (1mg/ml) are added to this volume of water, which is then stored in total darkness for 15 min. Subsequently the sample is filtered (low vacuum, 48 kPa) onto a black policarbonate filter (2 µm pore size, 25 mm diameter). A 0.45 µm Millipore filter was placed underneath it to promote even dispersion of sample on the black filter. The filter is then preserved at -20° (total darkness) until observation by epifluorescence microscopy. The filter is mounted on a glass slide and it is observed using a final magnification of 1000x. Heterotrophs are counted using an UV-filter set (BP 340-380 exitation filter, RKP 400 dichroic mirror, LP 430 barrier filter). Autotrophs are counted using a Blue-filter set (BP 450-490 excitation filter, RKP 510 dichroic mirror, LP 510 barrier filter). The number of observed fields (20-30) depends on sample concentration (minimum cell number: 200).
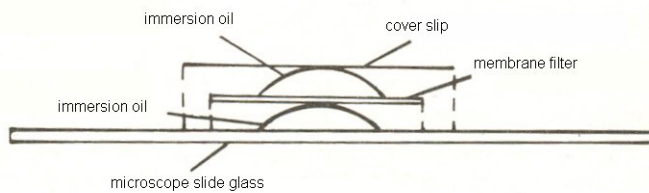
Mounting of the filter on the microscope glass slide (in Maugeri T.L. *et al*., 1990)

Picoplankton calculation:

$$\text{cell/mL} = N. (A_f/A_c) . 1 /V$$

where: N = average number of cells counted in the field
A_f = filtration area
A_c = field area
V = volume of sample filtered (in mL)

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Medidas comunes adoptadas por las Partes Contratantes en el convenio para la Proteccion del Mar Mediterraneo contra la Contaminacion.

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