The effect of quick freezing and fast thawing on stored seawater nutrient concentrations of dissolved inorganic Phosphate, Nitrate and Ammonium

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Abstract

In an attempt to improve the quality of preserving seawater nutrients for delayed analyses, a sample of unfiltered and aged nutrient-depleted seawater was obtained from Marine and Coastal Management (M&CM) and spiked with small different volumes of standard nutrient solutions. These were prepared into 100 mL glass bottles to constitute the lowest, intermediate and highest levels of dissolved inorganic phosphate (0.5, 1.5, 3.0 μM), nitrate (5, 15 and 30 μM) and ammonium (0.5, 1.5, 2.5 μM) that are found in the southern Benguela region.

After spiking, the samples were analysed to determine their "true" initial concentrations and immediately quick frozen using liquid nitrogen. A time series analysis (up to seven weeks) was performed by first fast-thawing the samples using a microwave oven and fixing up the nutrients within 20 minutes. Variations in nutrient concentrations did not exceed 7 % for phosphate, 4.0 % for nitrate at all levels and 8 % for ammonium at the intermediate and highest levels, but varied by 19.2 % at the lowest level.

Statistical tests for significance of the means were made at $\alpha = 0.05$ level and no difference was found at any level for the nitrate, and the lowest level phosphate. The higher levels of phosphate, and all ammonium were found to be significantly different. Hence, the experimental conditions employed in this paper would not be recommended for the all ammonium and the higher levels of phosphate concentrations. These variations were however not large or regular in pattern but erratic, which suggested that they were not due to microorganisms and optimizing those conditions may well to yield improved results.

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Chapter 1: Introduction and Research focus

1.1 Background

In most oceanographic expeditions, accurate determination of dissolved inorganic nutrients (NO_2^--N, NO_3^--N, PO_4^{3-}-P, silicate, NH_4^+-N and rarely urea) has always been among the priorities because of their vital role and applications in marine systems.

The availability of these inorganic nutrients is essential in regulating the dynamics of plankton and the extent of algal blooms. Moreover, this direct link provides the basis for the marine food chain, for both fish and marine mammals' biomass (where the chain culminates). Therefore, nutrient determination is crucial in ocean management because biological researchers use this data when estimating primary production over a certain area in a particular time. Phytoplankton normally satisfy their nutritional requirements by utilising dissolved inorganic forms: Phosphorus is assimilated in a form of ortho-phosphate, for the use in photosynthesis and other physiological processes. The nitrogen source is nitrate, but is easily assimilated as ammonium (the less energetic form) for photosynthesis and for the synthesis of amino acids. In nutrient-depleted surface waters e.g. of the tropical and subtropical Pacific, ammonium becomes the dominant source of nitrogen [Thomas (1970)]. Silicon in soluble form as orthosilicic acid is mainly needed for the growth of organisms having siliceous frustules [Riley (1971), Millero (1996) and Hattori (1982)].

Quality nutrient analysis has been instrumental in investigating the various sources, mixing and distribution of oceanic water masses. Broecker (1974) published a paper on the usefulness of using conservative water mass tracers "NO" and "PO" as $9[NO_3^-] + O_2$

and 135[PO_4^{3-}] + O_2 ratio respectively, which link the consumption and production of dissolved oxygen with nutrient utilization and generation in subsurface water. This technique is a useful tool when temperature and salinity, which are traditionally used for characterizing water masses, are inadequately informative, as seen especially in studies of intermediate and deep-water sources, where geothermal heating may introduce a temperature error. Boundary layers for water masses of Atlantic and Pacific origins have so far been successfully defined on the basis of basin-to-basin differences of conservative tracers [Lee et al, 1999].

A third useful application of well-determined nutrient concentrations is the monitoring of water quality in estuaries or semi-closed basins because of excessive input of nutrients from sewers or catchments that span over highly fertilized agricultural areas.

When collected seawater samples are not preserved, their nutrient concentrations are subject to alteration through chemical, physical and biological processes that also govern their rate of alteration. Therefore, sample analyses must be performed immediately or samples must be stored in a manner that preserves original concentrations in an unchanged and reliable way until a suitable environment for analysis has been found. However, immediate or inshore determinations are not always possible, especially in marine research, where bad weather, inadequate laboratory space, time constraint with regard to the usual high number of samples collected, poor electrical power conditioning and high contamination potential may preclude satisfactory analyses. It may also be more cost-effective if payment of the chemists aboard the research vessel is avoided. Some problems can be solved, but if chances of introducing errors when performing shipboard

analysis are high compared to storing or preserving samples, then the latter option is favoured [Dore et al, 1996; Kattner, 1999 and Venrick and Hayward, 1985].

Various marine studies require a particular nutrient concentration level of accuracy of the initial sample concentrations for a particular preservation and storage method to be considered effective and reliable. In addition, the method should be simple, easy to perform and minimum steps to avoid superfluous handling with all analytes of interest in one sample container to avoid space constraints [Dore et al, 1996].

The approach to meet the above-mentioned criteria for "well-preserved" nutrient concentrations has however been a daunting task to the researchers for more than 50 years [Clementson and Wayte 1992], following Harvey's initial examination of the problem in 1948.

A number of different storage and preservation techniques have been conducted and assessed for their effectiveness. Freezing samples in polyethylene containers has so far been a popular method in preserving nutrients. But conclusions drawn from Morse et al's (1982) findings showed that all (excluding ammonium studies) nutrient concentrations were not satisfactorily preserved when samples were kept frozen. Differences were more pronounced for near-surface seawater, where concentrations are low. Large discrepancies were particularly observed with phosphate and silicate. Venrick and Hayward (1985) found that the phosphate concentration in stored samples tended to increase with time, accompanied by loss of both accuracy and precision. This tendency however could not prove the findings by other researchers that differential adsorption by plastic containers

had an effect on the phosphate concentration. Silicate stored samples also lost accuracy with increased storage time, associated with a decrease in concentrations.

Poisoning of seawater using mercury chloride and chloroform [Kattner, 1999; Degobbis, 1973 and Jenkins, 1968] has also received attention as a form of storing and preserving dissolved inorganic nutrients. The use of biocides has an ability to prevent the consumption of nutrients as they kill biological organisms that tend to alter concentrations, thus making freezing apparatus superfluous, and circumventing the precipitation problem of small particles likely to occur in frozen samples. However, an increase in the concentrations of ammonium was observed within a few hours when samples were treated with chloroform [Degobbis, 1973]. Results from Kattner (1999) on the use of mercuric chloride as nutrient preservative indicated variability in concentrations especially in phosphates. Although the technique seemed to be an alternative to freezing, different concentrations of mercuric chloride that were needed to preserve specific nutrients' concentrations was the tricky part and also decreased the lifetime of the copperised cadmium reductor needed for the determination of total dissolved inorganic nitrogen. Another concern highlighted about the use of mercuric chloride is the toxicity of mercury derivatives to humans and therefore its use requires proper handling and appropriate disposal [Sadler, 1997].

The third technique that has been tested for its effectiveness in preserving nutrient concentrations is heat treatment that included pasteurization (single heating) and tyndalization (multiple heating) [Aminot and Kérouel, 1997], and like all other methods

this could as well be achieved through inhibiting microbial activities. This method was thought to be appropriate as it does not involve any preservation additives and samples could be kept at room temperature for a reasonable time. Both poisoning and heat treatment techniques assume that nutrient concentrations are not altered by chemicals to be added and by heating respectively. A study by Aminot and Kérouel (1991) found significant alterations in nutrient concentrations for both heating methods, which was attributed to partial hydrolysis of nutrients at high temperatures in samples and they subsequently carried out a similar heat treatment study (Aminot and Kérouel 1997), but at reduced temperatures in both glass and plastic bottles. Results obtained from this study indicated that nitrate and nitrite were reasonably preserved in both bottle types. Ammonium was detectably reduced, while phosphate showed an increase in samples stored in glass bottles. Silicate was not considered in this technique for reasons not mentioned.

As an attempt to find a rational solution to these contradicting results, different preservation methods were evaluated [Morse et al, 1982; Venrick and Hayward, 1985]. Some of the source problems identified include different needs of the studies, different biological, chemical and physical constituents of sample type and unreasonably long storage periods (as storage should not be a substitute but an alternative when immediate analyses are not feasible or reliable), that caused different interpretations on the significance of the results. In summary, all storage and preservation techniques are aimed at instantly halting or eliminating possible processes (see **Fig. 1**) that alter nutrient concentrations upon sample collection and storage until analysis.

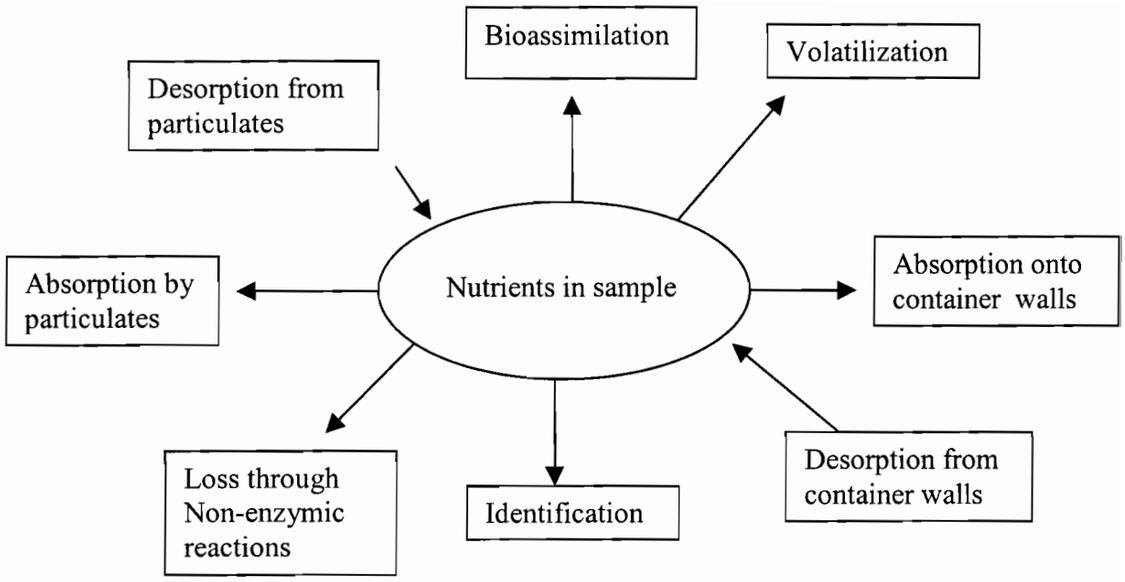


Fig. 1: Processes that affect the recovery of nutrients from sample container [taken from Sadler, 1997].

It is therefore necessary that the container type and particulate matter in addition to microorganisms, mainly responsible for the variation in nutrient concentrations [Hattori, 1982], in the stored sample be considered.

1.2 Research focus

Nutrient concentrations in upwelling regions are subject to variation, both in space and time. In the southern Benguela region, nitrate ranges between 0 and 30 μM, phosphate varies between 0.1 and 3 μM, ammonium is below 3 μM, and nitrite below 1 μM [Chapman and Mostert, 1990]. So, this project was envisaged as an ideal storage technique assessment for delayed analyses in the region or any area with a similar marine environment.

Hence, the focus of this research was on storage and preservation of dissolved inorganic nutrients (phosphate, nitrate, and ammonium) in artificially fertilized seawater (as full ranges could not easily be obtained for real samples) through combined quick-freezing and fast thawing over the above-mentioned ranges. Silicate was not considered in this study as fast thawing defeats its recovery process in frozen samples and a further error could be introduced into the samples stored in silica-made glass bottles [Grasshoff et al, 1983], needed for the microwave oven. The suggestion made by Kremling and Wenck (1986) that phosphate could be taken up by proliferating micro-organisms during freezing and thawing periods has led to an assumption that the combination of the two (quick-freezing and fast thawing) would halt the consumption process. Since other nutrients, especially nitrogen, are equally important to micro-organisms, it is assumed that other nutrients would also be assimilated during those periods.

Chapman and Mostert (1990) suggested that variations in nutrient concentrations taken from the open ocean, where particulate matter is rare, might be due to differences in

thawing periods. Their analyses were carried out with samples that were thawed in hot water of up to 70°C. Therefore, this study aimed at improving the storage of nutrient concentration by quick-freezing and shortening the thawing periods using the microwave oven to avoid giving microorganisms enough time to adapt to changing environments. The mean concentrations were then tested statistically to determine if there would significant differences over the storage time.

Chapter 2 gives details of the materials used in this study and the methods used to determine the concentrations of the nutrients. The reagents are also provided.

Chapter 3 gives details of the results for each nutrient in turn and provides a statistical analysis of their variations.

The thesis ends with the discussion and conclusions presented in **Chapters 4 and 5** respectively.

Chapter 2: Materials and Methods

A 2 L sample of ageing, unfiltered nutrient depleted seawater was obtained from M&CM (Marine and Coastal Management) and analysed to confirm its true concentrations, which were found to be 0.10; 0.07; 0.29 and 0.78 μM for phosphate, nitrate, nitrite and ammonium respectively, while the pH was 7.93. Based on that analysis, a series of increasing concentrations was set up by introducing small standard concentration volumes to seawater samples so that the following approximate values are available e.g. Nitrate (5, 15 and 30 μM); ammonium (0.5, 1.5, 2.5 μM) and phosphate (0.5, 1.5, 3.0 μM) and were prepared as follows:

0.50 μM Phosphate: Pipette 0.017 mL of 6.00 mM $K_2H_2PO_4$ standard stock into a 200 mL volumetric flask and make up to the mark with seawater sample.

1.50 μM Phosphate: Pipette 0.050 mL of 6.00 mM $K_2H_2PO_4$ standard stock into a 200 mL volumetric flask and make up to the mark with seawater sample.

3.00 μM Phosphate: Pipette 0.093 mL of 6.00 mM $K_2H_2PO_4$ standard stock into a 200 mL volumetric flask and make up to the mark with seawater sample.

5.00 μM Nitrate: Pipette 1.40 mL of 7.14 x 10^{-4} μmol/L N as NO_3^- into a 200 mL volumetric flask and make up to the mark with seawater sample.

15.00 μM Nitrate: Pipette 4.20 mL of 7.14 x 10^{-4} μmol/L N as NO_3^- into a 200 mL volumetric flask and make up to the mark with seawater sample.

28.00 μM Nitrate: Pipette 7.84 mL of 7.14 x 10^{-4} μmol/L N as NO_3^- into a 200 mL volumetric flask and make up to the mark with seawater sample.

1.50 µM Ammonium: In a 200 mL volumetric flask pipette in 1.44 mL of 100 µM ammonium chloride dilute stock. Dissolve and make up to the mark with seawater sample.

3.00 µM Ammonium: In a 200 mL volumetric flask pipette in 4.44 mL of 100 µM ammonium chloride dilute standard. Dissolve and make up to the mark with seawater sample. Note that there was no spiking for the lowest amount of ammonium, as initial value in the sample was **0.78 µM**.

These values represent the lowest, intermediate and the highest seawater nutrient concentrations that exist in the southern Benguela upwelling region at different locations and times. Immediate analyses were carried out to determine the "true" concentrations after spiking, where 0.57 µM (93.0%) of the theoretical amount (0.60 µM), 1.48 (92.5%) µM of the theoretical value (1.60 µM) and 2.79 (96.5%) of the theoretical amount (2.89 µM) were recovered for phosphate.

The lowest concentration for nitrate was 5.036 µM (99.2%) recovery of 5.074 µM theoretical amount; the intermediate was 14.540 µM (96.2%) of 15.075 µM theoretical value, while the highest level was 27.074 µM (96.4) recovery of 28.074 µM theoretical yield.

The two prepared ammonium concentrations were recovered as follows: 1.58 µM (5.3% increase) of the 1.50 µM theoretical amount, and 3.15 µM (4.0% increase) of the 3.03 theoretical value were found.

Ammonium and phosphate were spectrophotometrically analysed using the colorimetric manual method, while nitrate was be analysed by the *QuickChem FIA +, 8000 Series; Reagent pump RP – 100 Series* autoanalyser. Nitrite analysis was omitted due to the

paucity of nutrient depleted seawater sample (only 2 L could be obtained) that allows a controllable study. Both samples and standards were analysed in four replicates. For the manual method the same cuvette was used for each analysis, as it is necessary to have a uniform pathway for the light.

Each sample representative was poured (~2/3 full) into four different new 100ml Media blue screw cap glass bottles (rinsed twice with the same sample) and quick frozen using liquid nitrogen and kept frozen in a deep freezer. Subsequent analyses of samples by the same procedure were then again done by first thawing them using the microwave oven (thawing time was 3-5 min) and letting samples stand (plus proper mixing) for about 15 minutes prior to analysis to allow them to attain room temperature. These subsequent analyses were done after 2, 21, 35, 49 days, as a seven-week freezing period would be a usual duration in many cruise expeditions. The in-between measurements are crucial in determining possible fluctuations of nutrient concentrations during the freezing period. The "after 2 days" analysis is necessary for the detection of possible immediate reaction or behaviour of nutrients when quickly subjected to that environment. Similar samples were kept in the laboratory at ambient conditions and analysed after a month to assess the extent of alteration and to validate our assumption that microorganisms would utilize those artificially introduced nutrients.

Note that no samples were subjected to repeat thaw/freeze cycles, as four sample representatives were prepared to cater for the three time subsequent experimental analysis and one set for the control analysis.

2.1. **Principles of the methods and reagents for phosphate determination**

-Based on Grasshoff (1983)-

2.1.1. Phosphate (PO_4^{3-}-P)

All methods used for the determination of dissolved inorganic phosphate rely on the formation of a phospho-molybdate complex obtained by interaction between ammonium molybdate and orthophosphate in the presence of antimony. This complex is then reduced with ascorbic acid to form a blue compound, which has a maximum absorbency at 885nm. This method has a detection value of 0.01 μM and the relationship between absorbances and concentrations is linearly proportional up to 28 μM.

2.1.2. Reagents for phosphate determination

(a) Ammonium molybdate solution: Dissolve 7.5g of analytical grade ammonium paramolybdate ($(NH_4)_6Mo_7O_{24}$. $4H_2O$ in 250ml volumetric flask and make up to the mark with distilled water. Store in a plastic bottle away from direct sunlight. It is stable.

(b) Sulphuric acid (H_2SO_4): Add 70ml of concentrated analytical reagent quality sulphuric acid to 450ml of distilled water. Cool down the solution and store it in a glass bottle.

(c) Ascorbic acid ($C_6H_8O_6$): Dissolve 10.8g of ascorbic acid in 200ml of distilled water. Store in a glass bottle in a refrigerator. It is stable for at least a week at room temperature and can be used, as long it as remains colourless.

(d) Potassium antimonyl-tartrate solution [PAT], ($K(SbO)C_4H_4O_6$): Dissolve 0.34g of PAT in 250ml of distilled water, warming if necessary. Store in glass bottle. The solution is stable for many months.

(e) Combined colour reagent: Mix the four reagents above in the following order: ammonium molybdate; sulphuric acid; ascorbic acid; and potassium antimonyl-tartrate in ratio 2 : 5 : 2 : 1 respectively. Swirl to mix after addition of each reagent. It is best when freshly prepared before each analysis.

(f) Standard Stock reagent: Dissolve 0.818g of anhydrous Potassium dihydrogen phosphate (KH_2PO_4) in distilled water. Dilute the solution to 1000ml with distilled water. This has a concentration of 6.00mmol.L^{-1} (mM). It is stable for many months.

(g) Working standards were prepared by mixing and making up the 6.00mM KH_2PO_4 stock standard with deionised water in 200mL volumetric flasks as indicated below: The required volumes were dispensed using the "Eppendorf" pipettes capable of dispensing as little as 0.5µL (5.00×10^{-4}mL). These standards were prepared daily with each analysis.

Standard	1	2	3	4
Conc. of PO_4^{3-} (µM)	**3.00**	**2.00**	**0.99**	**0.51**
Vol. of 6.00mM KH_2PO_4	100µL (0.10mL)	60µL	33µL	17µL

2.1.3. Determination procedure

Prepare 5ml triplicates of distilled water blank, standards and seawater sample in different test tubes.

Pipette 0.50ml of combined colour reagent into the blanks, standards and samples and mix thoroughly using a 'Vortex Mixer'.

Allow a minimum of 5 minutes and a maximum of 2-3 hours for colour development and then read the absorbances spectrophotometrically at 885nm.

2.1.4. Nitrate (NO_3^- - N)

- based on Grasshoff (1983) and Smith (2001)

There is no direct chemical method for measuring nitrate-nitrogen concentration in seawater. Therefore the sample to be analyzed for nitrate is passed through a column packed with granules of copper –coated cadmium metal that reduce it to nitrite. Hence when a sample is passed through the reductor (column) it is then the combination of nitrite, originally present, and the reduced nitrate that is obtained. The sample is then analysed off-column and the difference between nitrogen concentrations obtained through the column and off the column gives the nitrate-nitrogen in the sample.

In this method, nitrite reacts with sulphanilamide forming the diazonium ion, which is subsequently coupled with N-(1-napthyl)-ethylenediamine dihydrochloride to form an azo dye that is colorimetrically measured at 540 nm. The applicable range is 5 to 400 μg N/L (or 0.36 to 28.6 μM N).

2.1.5. Reagents for nitrate determination

(a) 15 N Sodium hydroxide: Carefully dissolve 150 g NaOH in 250 mL deionised water. Cool down and store in a plastic bottle.

(b) Ammonium chloride buffer: Dissolve 85.0 g ammonium chloride (NH_4Cl) and 1.0 g disodium ethylenediamine tetra acetic acid dihydrate ($Na_2EDTA \cdot 2H_2O$) in about 800 mL deionised water in a 1 L volumetric flask. Adjust the pH to 8.5 with 15 N sodium hydroxide. Dilute to the litre mark and invert to mix.

(c) Sulfanilamide colour reagent: Add about 600 mL deionised water to a 1 L volumetric flask followed by 100 mL 85% phosphoric acid (H_3PO_4), 40.0 g sulfanilamide and 1.0 g N-(naphthyl) ethylenediamine dihydrochloride (NED). Stir to dissolve for about 30 min. Dilute to the mark and invert to mix properly. Store in a dark bottle and is stable for at least one month or until a pink colour develops.

(d) Nitrate stock standard solution (7.13×10^{-2} M $NaNO_3$) - **A**: Dissolve 6.04 g of sodium nitrate ($NaNO_3$) in approximately 900 mL deionised water in a 1 L volumetric flask. Dilute to the mark and invert to mix.

(e) Intermediate stock standard (714.3 µM N as NO_3^-) - **B**: Pipette out 10 mL of standard **A** into a 1 L volumetric flask. Dilute to the mark and invert to mix.

(f) Working stock standard (71.43 µM N as NO_3^-) – **C**: Pipette out 100 mL of standard **B** and proceed as under (e).

Working standards were prepared on day of analyses as follows using deionised water:

Standard	**1**	**2**	**3**	**4**	**5**
Conc. µM N as NO_3^-	**28.57**	**14.28**	**7.14**	**1.78**	**0.36**
Dilution with deionised water	200 mL of working stock standard (f) to 500 mL	50 mL of standard **1** to 100 mL	25 mL of standard **1** to 100 mL	25 mL of standard **3** to 100 mL	25 mL of standard **3** to 100 mL

2.1.6. Determination procedure for nitrate

The sample is carried through by distilled water and all the solutions, except the standards, are degassed using helium gas.

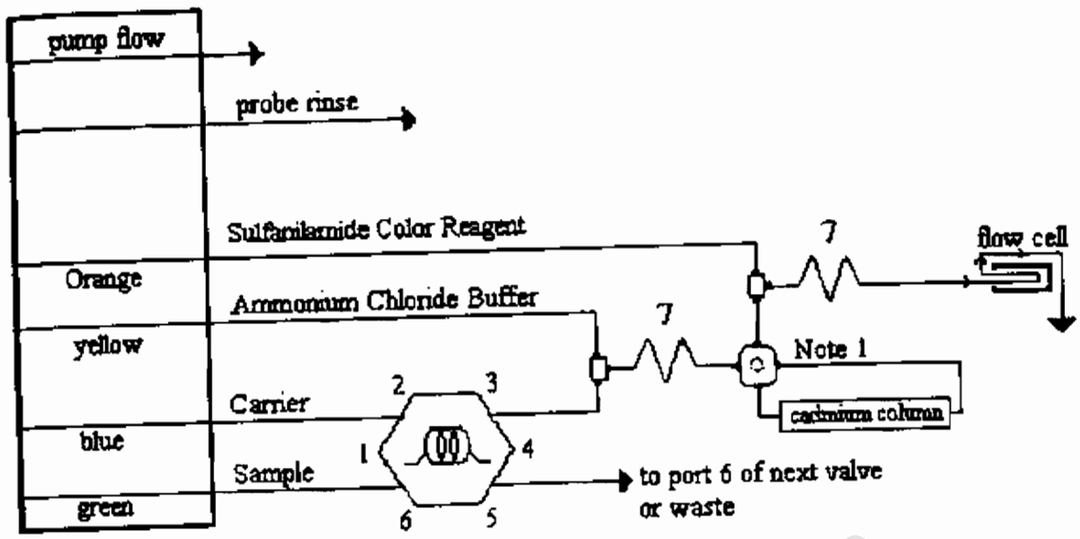
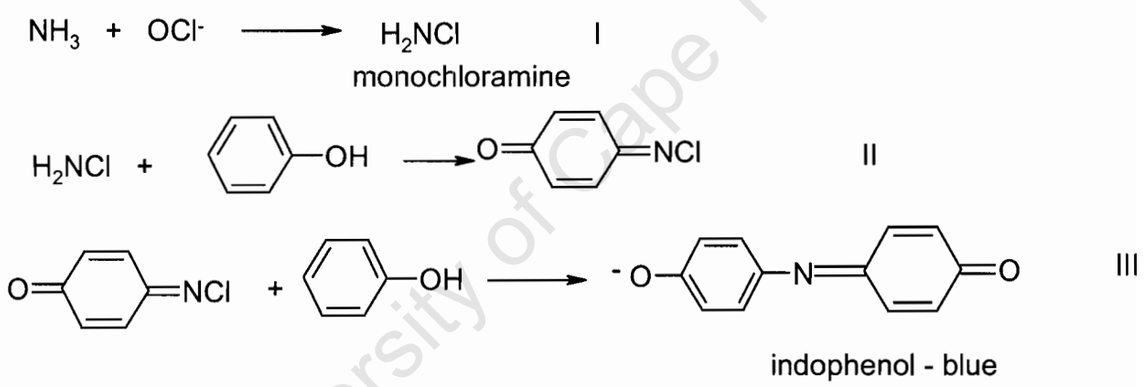


Fig 2: Flow diagram for the nitrate analysis using the Autoanalyser [Taken from the user's manual written by P. Smith and K. Bogren, 2001: Lachat Instruments]

2.1.7. Ammonium (NH_4^+-N)

The two forms (NH_4^+ and NH_3) of nitrogen are pH dependent as a conjugate acid-base pair and during determination the sum of the two is recorded in all the methods. The names (ammonia or ammonium) if interchangeably used in the text are of the same meaning. The commonly used [Amino, Kirkwood and Kerouel, 1997] method is based on the formation of indophenol-blue (IPB) first reported by Berthelot. Under alkaline conditions, ammonia and hypochlorite firstly form a monochloramine, which then reacts successively with two phenol molecules. Nitroprusside (NP) acts as a catalyst and also enhances the sensitivity of the method, but little is known about its actual function. Citrate is used as a complexing reagent for Mg^{2+} to prevent hydroxide precipitation.

The Berthelot reaction is reckoned to proceed as follows according to Patton and Crouch (1977)



This indophenol blue complex forms completely after about 8 hours and is stable for up to 24 hours at room temperature, however can be speeded up by increasing reactants' concentration, by elevating the pH to over 12 and by increasing reaction temperatures up to 80°C. It has a maximum absorption at a wavelength of 630 nm with a detection limit

of 0.05 μM and Beer-Lambert's law (which relates concentration to its absorption) is obeyed up to a concentration of 40 μM.

2.1.8. Reagents for Ammonium (NH_4^+-N) determination

(a) NH_4Cl Standard Solution (10μmol/ml): Take 0.535g of NH_4Cl and make up to 1 litre with milli-q water.

(b) Phenol (C_6H_5OH) / Nitroprusside ($Na2Fe(CN)_5NO \cdot 2H_2O$): Take 6.79g of phenol and 0.0718g of disodium nitroprusside dihydrate. Make up to 250ml with milli-q water. The reagent is stable for months.

(c) Citrate: Take 342.8g of tri-sodium citrate dihydrate ($C_6H_5Na_3O_7 \cdot 2H_2O$), make up to more than 1000ml with milli-q water in a Scott bottle. Make alkaline with the addition of 10mmol. NaOH. Boil it down in the fume cupboard and using a magnetic stirrer to close to the litre mark. This drives off NH_3. Allow the solution to cool completely and make up to 1 litre with small volume of milli-q water. Store in a well-stoppered polyethylene bottle. The solution is stable.

(d) Hypochlorite (Trione): Take 0.1833g of Trione (Dichloroisocyanuric acid). Make up to 100ml with 0.36M NaOH. Store cold in an amber lab glass bottle. The reagent is stable for at least three weeks.

(e) 0.36M NaOH: Take 14.4g of NaOH and make up to more than one litre with milli-q water in a Scott bottle. Boil it down in the fume cupboard and using a magnetic stirrer to close to the litre mark. This drives off NH_3. Allow the solution to cool completely and make up to 1 litre mark with small volume of milli-q water.

(f) Ammonium chloride stock standard (NH_4Cl) ($10\mu mol.ml^{-1}$): Dissolve 0.536 g NH_4Cl in a 1 L volumetric flask and dilute to the mark with milli-q water.

(g) Dilute stock (100 μM NH_4Cl): In a 100 mL volumetric flask, add 1.00 mL of standard stock ($10\ \mu mol.ml^{-1}$ NH_4Cl). Dilute to the mark with milli-q water.

Working standards were prepared in milli-q water as follows, all in 100 mL volumetric flasks:

Standard	1	2	3	4
Concentration in μM	**0.50**	**1.00**	**3.00**	**6.00**
Vol. in mL of $100\mu mol.L^{-1}$ NH_4Cl	0.50	1.00	3.00	6.00

2.1.9. Determination procedure

Pipette four replicates of 5ml standards, blank (milli-q water) and seawater samples in different test tubes.

To all 5ml replicates add 0.20ml citrate followed by 0.20ml phenol/nitroprusside. Mix properly by using the vortex.

Add 0.20ml hypochlorite (trione) and vortex again. Cover the test tubes with laboratory parafilm foil and allow colour to develop for at least 8 hours and a maximum of 24 hours. Read the absorbance of blank, standards and samples on the spectrophotometer at 630nm. Concentrations of ammonia in the samples were obtained by interpolating from the calibration curve based on their absorbances.

Chapter 3: Results

3.1. Phosphate

The calibration results are presented in **Table 1** with least-squares regression line equations and coefficient of determination, r^2, below each table. Reproducibility in absorbances for the standards was 100 per cent in most measurements. There were only small standard deviations or correlation coefficients of 4.4 % and 7.1%, n = 4 for 0.50 µM standards during initial and after 21 days respectively. The coefficient of determination ranges from 0.991 to 0.999 with residuals ranging from -3.50×10^{-3} to 2.40 $\times 10^{-3}$. There was only a slight difference in slopes, as the range was 0.0232 to 0.0236 and the absorbance axis (y-intercept) was consistently intercepted slightly below zero.

a. initial

Concentration in µM (x)	**Absorbance (y)**	**Residual**
0.0 (blank)	--	
0.50	0.013 (5.8×10^{-4})	1.65×10^{-3}
1.00	0.022	-9.00×10^{-4}
2.00	0.043	-3.00×10^{-3}
3.00	0.071	1.90×10^{-3}

$y = 0.0231x - 1.90 \times 10^{-4}$ $r^2 = 0.995$

b. 2 days

Concentration in µM (x)	Absorbance (y)	Residual
0.0	--	
0.50	0.012	6.00×10^{-4}
1.00	0.021	-2.20×10^{-3}
2.00	0.048	1.20×10^{-3}
3.00	0.070	-4.00×10^{-3}

$y = 0.0236x - 4.40 \times 10^{-4}$ $r^2 = 0.998$

c. 21 days

Concentration in µM (x)	Absorbance (y)	Residual
0.0	--	
0.50	0.010 (7.1×10^{-4})	-5.50×10^{-4}
1.00	0.023	8.00×10^{-4}
2.00	0.042	-3.50×10^{-3}
3.00	0.071	2.20×10^{-3}

$y = 0.0233x - 1.10 \times 10^{-3}$ $r^2 = 0.994$

d. 49 days

Concentration in µM (x)	Absorbance (y)	Residual
0.0	--	
0.50	0.013	2.15×10^{-3}
1.00	0.020	-2.60×10^{-3}
2.00	0.043	-3.10×10^{-3}
3.00	0.072	2.40×10^{-3}

$y = 0.0235x - 9.05 \times10^{-4}$ $r^2 = 0.991$

Table 1: Calibration and statistics data for phosphate analysis

	Lowest	Intermediate	Highest
Initial mean concentrations in μM PO_4^{3-}	0.57(0.033)	1.48(0.0)	2.79(0.020)
After 2 days	0.59 (0.038)	1.43(0.020)	2.75(0.023)
21 days	0.61(0.052)	1.54(0.020)	2.83(0.0)
49 days	0.57(0.023)	1.49(0.0)	2.78(0.023)

Table 2: Comparison of mean phosphate analysis of samples for different levels. Standard deviations are given in parenthesis.

The results for phosphate analysis are shown in **Table 2** and graphically represented in **Figure 3** for all three concentration levels studied. In the lowest category, there was an increase of 0.02 μM (3.5 %) and 0.04 μM (7.0 %) after 2 and 21 days respectively in relation to the initial concentration. However, after 49 days there was no observed difference, but there was variability within a group of replicates as manifested in their standard deviations, initial day (0.033 μM) and 49 days (0.023 μM).

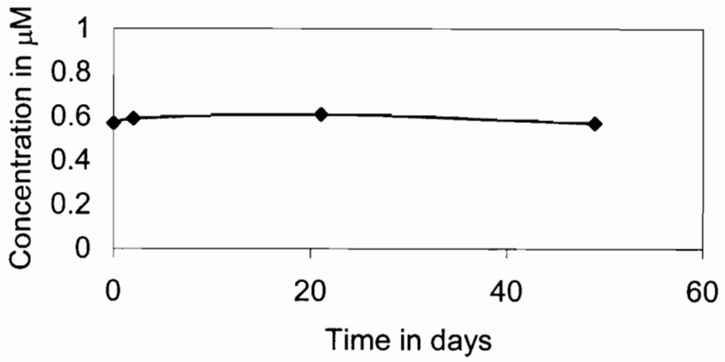
Analysis of the variance (ANOVA), and the F-test using STATISTICA software, found no significant difference in the concentration means of the stored samples [F = 0.209; degrees of freedom (d.f) = 3,12, probability (α) = 0.05; Zar, 1984] in the lowest level.

There was an improvement in the reproducibility of intermediate concentrations with standard deviations of only 0.02 μM observed in days 2 and 21. There was a decrease of 0.05 μM (3.4 %) after 2 days, but the concentration increased again by 0.06 μM (4.1 %) after 21 days. After 49 days the concentration was just marginally (0.01 μM) above the

initial amount, but the variability range in between days of analysis was 1.43 to 1.54 μM (i.e. 7.7 % difference). The ANOVA test showed a significant difference ($F = 40.67$; d.f = 3,12 and $\alpha = 0.05$).

The highest concentration level also decreased by 0.04 μM (1.4 %) to the initial concentration after two days, but once again increased by the same margin after 21 days. At the end of analysis (after seven weeks), the range in the highest concentration category was 2.75 to 2.83 μM. Analysis of variance showed a significant difference ($F = 13.36$; d.f = 3,12 and $\alpha = 0.05$) in the concentration of stored samples with time.

Unpreserved samples serving as confirmatory tests on the microorganisms' ability to utilize the artificially introduced nutrients found that the lowest level (0.57 μM) was reduced to below detection level, the intermediate (1.48 μM) was reduced to 0.84 (±0.022) μM (43.2 % loss) and the highest (2.79 μM) was reduced to 2.03 (± 0.041) μM (27.2 % loss) after seven weeks' storage period.



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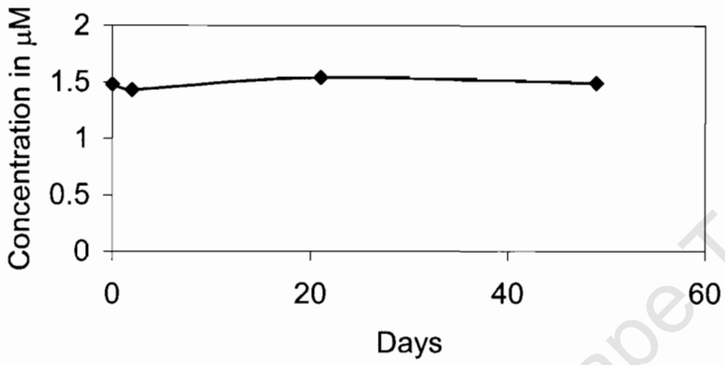

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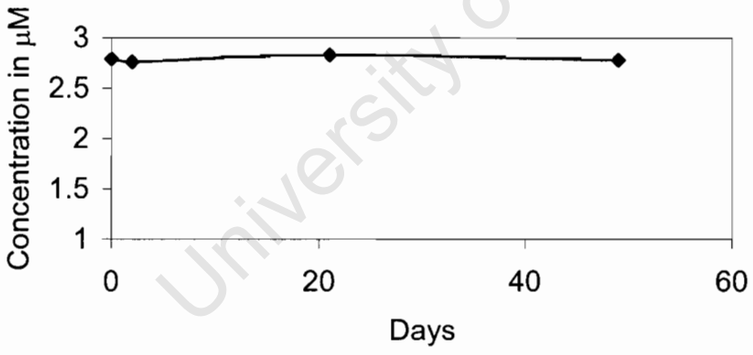


c

Fig. 3: Variation in concentration of dissolved phosphates with storage time, **a:** lowest; **b:** intermediate and **c:** highest

3.2 Nitrate

Calibration data for the nitrate determination are given in **Table 3** as obtained from the Flow Injection Analyser (FIA). The least square regression line equations and coefficient of determination, r^2, for each day of analysis are given underneath each table. Data points were obtained from single measurements. There is only a slight variation in the slope of the lines ranging from 0.0789 to 0.0847 unit area/concentration. Residuals analysis showed a range of -0.1056 to 0.5434 unit area on initial day that is a slight shift to the positive intercept. The residuals for the remainder of the analysis showed slight negative y-intercepts.

a. initial

Concentration in μM (x)	Area (y)	Residual
0.0	-0.048121	
0.36	0.629901	-1.20×10^{-3}
1.78	2.219519	-0.1056
7.14	9.221003	0.5434
14.28	17.063600	-0.084
28.57	34.025468	-0.0621

$y = 0.0847x + 0.2076$ $r^2 = 0.9996$

b. 2 days

Concentration in μM (x)	Area (y)	Residual
0.0	0.028266	
0.36	0.569542	0.1980
1.78	1.361598	-0.6239
7.14	8.420829	0.3829
14.28	16.175229	0.0672
28.57	32.135862	-0.1121

$y = 0.0807x - 0.0323$ $r^2 = 0.9992$

c. 21 days

Concentration in μM (x)	Area (y)	Residual
0.0	0.092944	
0.36	0.53322	0.1504
1.78	2.19933	0.2085
7.14	7.91918	-0.1016
14.27	15.31431	-0.7465
28.57	32.54164	0.4008

$y = 0.0804x - 0.0192$ $r^2 = 0.9990$

d. 49 days

Concentration in $\mu g.L^{-1}$ (x)	Area (y)	Residual
0.0	0.039967	
0.36	0.546762	0.17496
1.78	1.285345	-0.66446
7.14	8.369321	0.50202
14.27	15.77701	0.01971
28.57	31.43803	-0.09927

$y = 0.0789x - 0.0227$, $r^2 = 0.9990$

Table 3: Calibration and statistics data for nitrate analysis

Initial mean concentrations in μM NO_3^-	5.036(0.070)	14.540(0.068)	27.072(0.123)
After 2 days	5.030(0.021)	15.079(0.039)	26.629(0.895)
21 days	5.048(0.110)	14.417(0.333)	26.920(0.184)
49 days	5.020(0.089)	14.551(0.056)	27.884(0.082)

Table 4: Comparison of mean nitrates analysis of samples for different levels. Standard deviations are given in parentheses.

The variation of nitrate mean concentrations with storage period are shown in **Table 4** and presented graphically in **Figure 4.** There was only a slight decrease (0.006 μM) in the lowest category after two days, but an increase of 0.012 μM (0.2%) was detected after 21 days. After 49 days (seven weeks) the concentration was 5.020 μM (99.7 % of the initial amount). There was little variation in the reproducibility of sample replicates with the coefficient of variation, CV = [(standard deviation/mean) *100 %] ranging from 0.4 to 2.2 %. The concentration range over the entire storing period is 5.020 μM (49 days) to 5.048 μM (21 days). The statistical analysis (ANOVA) showed no significant difference ($F = 0.037$; d.f = 3,12 and $\alpha = 0.05$).

The intermediate concentration showed contrary results to the lowest after 2 days as it increased by 0.539 μM (3.7 %). However it decreased by 0.123 μM after 21 days but slightly increased by 0.011 μM after 49 days. The overall variation in this category

ranges from 15.079 μM (after 2 days) to 14.551 μM (after 49 days). The coefficient of variation among the replicates ranged from 0.3 % to 2.3 %. Analysis of variance showed a significant difference ($F = 49.68$; d.f = 3,12 and $\alpha = 0.05$).

In the highest concentration category, there were decreases of 0.443 μM (1.6 %) and 0.152 μM (0.6 %) after 2 and 21 days respectively. After 49 days an increase of 0.812 μM (3.0 %) was detected. Only two replicates were averaged, as the other two were anomalously high. The variation in concentration ranged from 26.629 μM to 27.884 μM for the duration of storage. The coefficient of variation was highest in the "after 2 days" analysis (3.4 %) and lowest in the samples analysed after 49 days (0.3 %), although only two replicates were considered. There was no significant difference in the mean concentrations between storage times ($F = 2.81$; d.f = 3,10 and $\alpha = 0.05$).

After seven weeks of storage, unpreserved samples' concentrations were 2.444 (± 0.199) μM, 9.095 (± 0.124) μM and 16.119 (± 0.111) μM for the lowest (5.036 μM), intermediate (14.540 μM) and highest (27.072 μM) levels respectively. These were losses of 51.2, 37.4 and 40.2 % respectively.

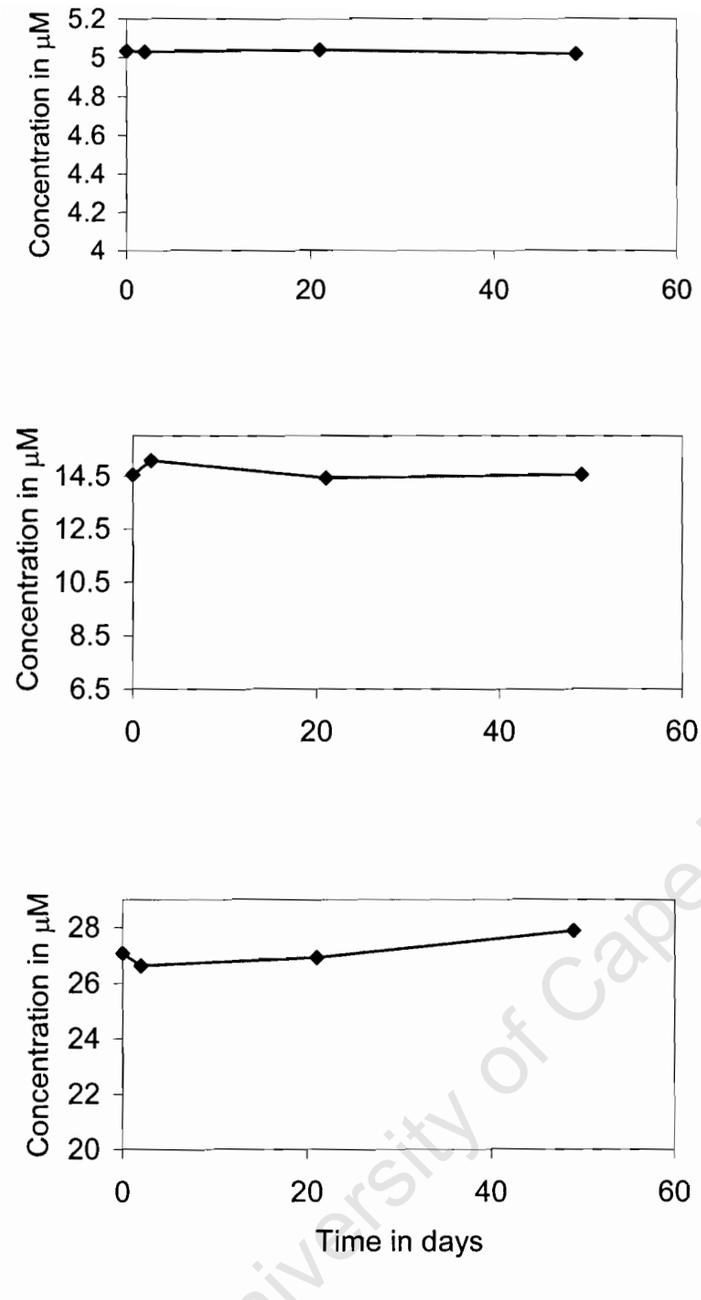


Fig. 4: Variation in concentration of dissolved nitrates with storage time, **a:** lowest; **b:** intermediate and **c:** highest

3.3. Ammonium

The calibration and statistical data for the ammonium determination are given in **Table 5** together with regression line equations and coefficients of determination (r^2) below each table. All the points are averages of four replicates (standard deviations are given in parentheses). There are only slight differences between line slopes, with the largest being 0.0019 absorbance/concentration after 49 days, which is 12.0 % more than the initial day slope. Coefficients of variations for the replicates have all been under 10 % with the exception of 16.1% detected after 2 days at 3.00 μM.

Coefficients of determination ranged from 0.965 to 0.999, and all the residuals showed a slight shift towards the negative absorbance axis.

a. initial

Concentration in μM (x)	Absorbance (y)	Residual
0.0	--	
0.5	0.005(0.002)	2.40×10^{-3}
1.00	0.008(0.001)	-2.50×10^{-3}
3.00	0.031(0.003)	-1.11×10^{-2}
6.00	0.095(0.006)	5.50×10^{-3}

$y = 0.0158x - 5.30\times10^{-3}$ $r^2 = 0.9688$

b. 2 days

Concentration in μM (x)	Absorbance (y)	Residual
0.0	--	
0.5	0.004(0.001)	1.70×10^{-3}
1.00	0.009(0.002)	-1.50×10^{-3}
3.00	0.031(0.005)	-1.23×10^{-2}
6.00	0.099(0.003)	6.50×10^{-3}

$y = 0.0164x - 5.90\times10^{-3}$ $r^2 = 0.9655$

c. 21 days

Concentration in μM (x)	Absorbance (y)	Residual
0.0	--	
0.5	0.008(0.001)	2.10×10^{-3}
1.00	0.012(0.002)	-2.40×10^{-3}
3.00	0.043(0.002)	-5.40×10^{-3}
6.00	0.102(0.002)	2.60×10^{-3}

$y = 0.0170x - 2.60\times10^{-3}$ $r^2 = 0.9925$

d. 49 days

Concentration in μM (x)	Absorbance (y)	Residual
0.0	--	
0.5	0.007(0.002)	-2.50×10^{-3}
1.00	0.016(0.003)	-1.00×10^{-4}
3.00	0.049(0.002)	-2.50×10^{-3}
6.00	0.106(0.003)	1.40×10^{-3}

$y = 0.0177x - 1.60\times10^{-3}$ $r^2 = 0.9988$

Table 5: Calibration and statistics data for ammonium analysis

Initial mean concentrations in μM NH_4^+	0.78(0.052)	1.58(0.032)	3.15(0.036)
After 2 days	0.73(0.050)	1.46(0.050)	3.13(0.126)
21 days	0.63(0.048)	1.50(0.048)	3.21(0.074)
49 days	0.75(0.071)	1.56(0.046)	2.94(0.098)

Table 6: Comparison of mean ammonium analysis of samples for different levels. Standard deviations are given in parentheses.

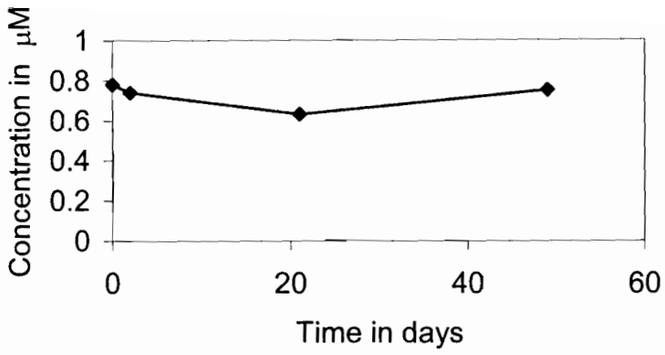
The results for ammonium are shown in **Table 6** and presented as graphs in **Figure 5.** Theses are also average values (n = 4) and their corresponding standard deviations are given in parentheses. The lowest concentration category showed a decrease of 0.05 μM (6.4 %) after two days and decreased further by 0.15 μM (19.2 %) after 21 days. However after 49 days the concentration was only 0.03 μM (3.8 %) lower than the initial amount. The overall variation range in concentrations is 0.63 to 0.78 μM. The highest variation in the replicates in this category was observed after 49 days where the coefficient of variation was 9.5 % while the lowest of 6.7 % was for the initial day.

Analysis of variance showed a slight significant difference ($F = 5.45$; d.f = 3,12 and $\alpha = 0.05$) in the concentration storage time.

The intermediate concentrations showed a decrease of 0.12 μM (7.5 %) after two days, but only a decrease of 0.08 μM (5.1 %) after 21 days. At the end of storage time (49 days), the concentration was 1.56 μM, which is 98.7 % of the initial concentration. The range of variation was 1.46 to 1.58 μM. and the standard deviations among the replicates were highest after 2 days analysis where CV was 3.4 %, while the lowest variation was 2.0 % for the initial analysis. Statistical test (ANOVA) showed a significant difference ($F = 6.61$; d.f = 3,12 and $\alpha = 0.05$) in the mean concentrations.

The highest concentration category showed a marginal decrease of 0.02 μM (0.6 %) after 2 days but showed an increase of 0.06 μM (1.9 %) after 21 days. It then decreased further by 0.21 μM (6.7 %) after 49 days. The range of variation over the storage period was 2.92 to 3.21 μM. Reproducibility among replicates were again highest after 2 days (CV = 4.0 %) and lowest for the initial analysis (CV = 1.1 %). The mean concentrations were once again found to be significantly different ($F = 6.54$; d.f = 3,12 and $\alpha = 0.05$).

The unpreserved samples serving as a confirmatory test on the extent of artificial nutrients uptake by the microorganisms found 0.20 μM (25.6 %), 0.60 μM (38.0 %) and 0.99 μM (31.6 %) of the initial lowest, intermediate and highest category respectively.



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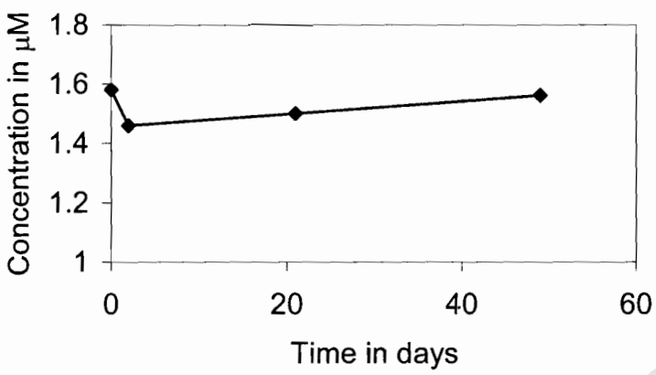

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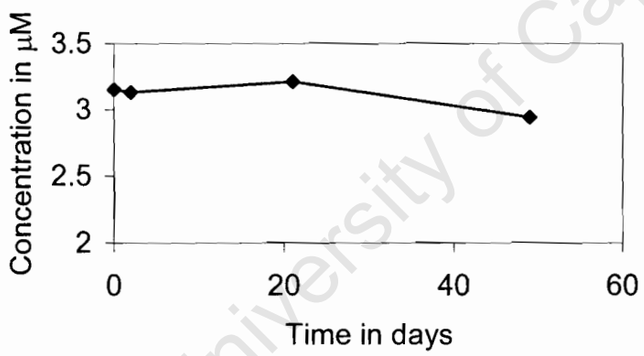

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Fig. 5: Variation in concentration of dissolved ammonium with storage time, **a:** lowest; **b:** intermediate and **c:** highest

Chapter 4: Discussion

The nutrient concentrations in the samples showed mixed results in their variations after a seven weeks' storage period after they had been quick frozen and fast thawed. The lowest level concentration (0.57 μM) showed no significant difference in the means for phosphate. This is in agreement with the previous findings (Dore et al, 1996) and there is no evidence of decreasing as was found by Gilmartin, 1967. The highest deviation from the initial concentration was only 0.04 μM (7.0 %).

Although the results for the intermediate concentration (1.48 μM) showed a substantial significant difference ($F = 40.67$; d.f = 3,12 and $\alpha = 0.05$) in the means over the storage time, there was no clear-cut trend whether the sample concentration eventually increased with time, as there was only an increase of 0.7 % after 49 days. The fact that the average concentration from four sets (initial, 2, 21 and 49 days) was 1.49 (stdev = 0.045) μM might suggest an overall increase.

Since such an increase was not observed in the lowest concentration level, breaking down by microorganisms and cell lysis (both of which tend to increase the phosphate concentration in samples, during quick freezing (Chapman P. and Mostert S. A., 1990)) could not be the source. The highest level (2.79 μM) was 0.4 % lower than the initial after 49 days, but the mean concentrations analysed on different days were found to be significantly different ($F = 13.36$; d.f = 3,12 and $\alpha = 0.05$). This was due to equal (1.4%) but opposite deviations encountered after 2 and 21 days. Since there was no linear trend

in the variation of concentrations, this slight increase in phosphate concentration might be attributed to the analytical procedures rather than to microbial activities.

The concentration for the lowest level (5.036 μM) of nitrate studied showed that it could be preserved to 99.7 % after seven weeks and the variability during storage period did not exceed 0.2 % when samples were subjected to quick freezing and fast thawing. This was in agreement with Chapman P. and Mostert S. A., 1990, where they found variability less than 10 per cent in low nitrate samples. The intermediate level showed the means that were significantly different ($F = 49.68$; d.f = 3,12 and $\alpha = 0.05$), but when the data obtained after two days was removed from the analysis, the remaining three sets (initial, 21 and 49 days), were found to be not significantly different ($F = 0.55$, d.f = 2,9 and $\alpha = 0.05$). This suggested that an analytical error might have caused such an aberrant value, which coincided with a poor coefficient of determination ($r^2 = 0.965$) for the calibration standards on that day. Thus after the anomalous data was removed, the intermediate level was only 0.011 μM (< 0.1 %) higher than the initial concentration after seven weeks.
There was no significant difference in the means ($F = 2.81$; d.f = 3,10 and $\alpha = 0.05$) for the highest level with storage time and the concentration was 0.812 μM (3.0 %) higher at the end of the storage period. These small increases were also encountered by Aminot and Kerouel (1998), which they attributed to the mineralisation of nitrogeneous compounds and oxidation into nitrate.

Ammonium results were more erratic than those for phosphates and nitrates. All the levels studied in this work were found to be significantly different in their mean

concentrations (see **Fig. 5**). At the end of the storage period all levels were lower than the initial concentration with the highest level at 6.7 % lower than the initial concentration, but the trend in the coefficients of variation (CV) suggested that reproducibility improved as concentrations increased. This contradicted the findings of Degobbis, (1973) who found slight increases after a few days for samples stored in glass bottles. The decrease encountered in all concentration levels after two days might be due to excessive heating during the thawing with the microwave oven, which could have driven off ammonium. Contaminations, calibrations and different analysis conditions were more likely to cause these erratic variations as opposed to biological effects that would result in a regular trend.

Chapter 5: Conclusions

In conclusion, it appeared that quick-freezing using liquid nitrogen combined with fast thawing using a microwave oven could be a reliable method for preserving nutrients, in particular nitrate, at all levels. Although phosphate showed statistically different results at higher levels, those changes were small. Ammonium seemed to be the most difficult nutrient to preserve, and therefore the experimental approach used in this paper is not recommended. However, the results suggested that optimising fast thawing in conjunction with proper handling to minimize contamination would be vital to stabilising the ammonium.

Although alterations and recoveries of nutrients in a sample could be due to many factors (see **Fig. 1**), the substantial changes in unpreserved samples demonstrated that microorganisms (main cause of changes) were well capable of utilizing these artificially introduced micronutrients under normal conditions. So, this was a yardstick for the potential of quick-freezing and fast-thawing to preserve dissolved nutrients.

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