

Lab 3: Inorganic Plant Nutrients: Nitrogen, Phosphorus, Silicate

Introduction

Compounds of nitrogen and phosphorus are major cellular components of organisms. Since the availability of these elements may be less than the biological demand, environmental resources can regulate or limit the productivity of organisms in aquatic ecosystems (“nutrient limitation”). Other elements such as iron and sulfur are essential cellular constituents as well but are required in much lower concentrations (nevertheless, iron is discussed as a major limiting factor for phytoplankton production in the Antarctic Ocean and the North Pacific Ocean). The major base cations (calcium, magnesium, sodium, potassium) are generally in sufficient supply in seawater. Silicate can play a regulating role in the growth of such organisms that carry shells of silicate. Most important are diatoms, which may form phytoplankton blooms under conditions of sufficient silicate in the water, and silicate is often depleted after the diatom spring bloom in temperate regions.

Certain nutrients such as magnesium and sodium are relatively conservative in their concentrations. Due to their high concentrations relative to the biological demand, they are relatively unaffected by biological processes. Concentrations of nitrogen and phosphorus, on the other hand, are highly dynamic because they may be utilized, stored, transformed and excreted rapidly and repeatedly by the various aquatic organisms. The biogeochemical cycle of nitrogen is further complicated by the different forms in which nitrogen can occur dissolved in the water: nitrate, nitrite, ammonium, urea, amino acids, complex organic nitrogen compounds. Whereas the dissolved organic nitrogen (DON; amino acids, urea, other compounds) plays a major role as substrates for bacteria, phytoplankton predominantly rely on inorganic nitrogen forms for their nutrition. Nevertheless, some phytoplankton can make use of dissolved amino acids and urea as nitrogen sources, and of course bacteria will compete for ammonium in nitrogen-poor waters.

In terms of which nutrient limits the growth of phytoplankton, it is not necessarily the nutrient that occurs at lowest concentrations. Organisms require nutrients in different quantities. The elemental ratio of healthy phytoplankton, the **Redfield Ratio** (named after an author who published these results), of carbon to nitrogen to phosphorus (C:N:P) is 106:16:1. This means that for each phosphorus atom, healthy organisms in balanced growth would need 16 nitrogen atoms. If only 14 nitrogen atoms are available, the organism cannot make use of the 1 phosphorus atom. In more general terms: nitrogen will be the limiting nutrient if the N:P ratio of nutrient concentrations in the water sinks below 16:1. Remember that these ratios are elemental ratios; thus, they refer to nutrient concentrations in molar units ($\mu\text{mol l}^{-1}$), not in units of mass ($\mu\text{g l}^{-1}$). For balanced growth, the molar concentration of nitrogen in the water has to be 16 times the concentration on phosphorus. For example, if the nitrogen concentration is $3.0 \mu\text{mol l}^{-1}$ and the phosphorus concentration is $0.2 \mu\text{mol l}^{-1}$, nitrogen would be the limiting nutrient (N:P < 16). Most marine systems are nitrogen-limited (N:P < 16), whereas most freshwater systems are phosphorus-limited. Note in the previous example that nitrogen is the limiting nutrient although its concentration ($3.0 \mu\text{mol l}^{-1}$) is much higher than the phosphorus concentration ($0.2 \mu\text{mol l}^{-1}$)!

In marine sciences, the concentrations of nutrients are usually given in molar units, $\mu\text{mol l}^{-1}$ (micromole per liter). One micromole (μmol) is a 10^{-6} moles. One mole of a substance is 6.0221367×10^{23} molecules. The correct unit is either “ $\mu\text{mol l}^{-1}$ ” or “ μM ”; thus, “M” is equivalent to “ mol l^{-1} ”. Never give nutrient concentrations as “ $\mu\text{M l}^{-1}$ ” – this unit does not exist! In limnology, nutrient concentrations are still cited in mass units, $\mu\text{g l}^{-1}$. To convert mass units into molar units, the molecular weight (formular weight) of the respective substance has to be used.

Nutrients that are highly dynamic, may become growth-limiting, and are required in high concentrations by organisms are referred to as **macronutrients** (N, P, Si). Essential nutrients required by organisms in low concentrations and that usually not limiting are referred to as **micronutrients** (Fe, Se, Cu, Co, Mn). This lab class will concentrate on the measurement of macronutrients only. We will compare the macronutrient concentrations at different stations sampled during the field trip. In the next class, we will analyze the phytoplankton community of the sampled stations and relate the differences in biomass and community structure of phytoplankton to differences in nutrient concentrations and the *Redfield Ratio*.

The inorganic macronutrients of phytoplankton

Ammonium Nitrogen: Except under very alkaline conditions ($\text{pH} > 9.0$), most of the ammonia (NH_3) in water exists in the ionic form for ammonium (NH_4^+). Ammonium is an important source of nitrogen for bacteria, algae and larger plants (seagrass, macroalgae) in freshwater and marine systems. Ammonium originates from the bacterial degradation of organic material and from the excretion of animals. Its concentration is highly variable but usually low because ammonium is the preferred nitrogen source for most algae. The reason for this preference is that algae can incorporate (assimilate) ammonium directly into amino acids; other nitrogen forms such as nitrate and nitrite have to be converted enzymatically into NH_4^+ to be utilized, which costs energy. Because of the general preference of NH_4^+ over nitrate, ammonium concentrations are usually below $1 \mu\text{M}$ and ammonium is taken up by phytoplankton as it is produced by bacteria and animals. The turnover of ammonium is extremely fast.

Ammonium in water is measured by a phenol-hypochlorite method using sodium nitroprusside as a catalyst. Ammonium reacts with phenol and hypochlorite under alkaline conditions to form indophenol blue. The color development is proportional to the NH_4^+ concentration. The blue color can be measured in a photometer. Since phenol is a very hazardous material, all samples for NH_4^+ analyses must be handled with gloves.

Nitrite Nitrogen: Nitrite occurs usually in low concentrations of less than $0.4 \mu\text{M}$. Higher concentrations can be found in oxygen-poor water and where bacterial nitrification occurs. Nitrification is the process of transforming NH_4^+ into nitrate (NO_3^-) with nitrite (NO_2^-) as an intermediate product. The two steps of nitrification are performed by different bacterial species, the ammonium oxidizers (convert NH_4^+ to NO_2^-), and the nitrate oxidizers (convert NO_2^- to NO_3^-). Under low oxygen concentrations or anoxic conditions, certain bacteria utilize the oxygen of the nitrate, thereby producing nitrite. Nitrite can become toxic at higher concentrations.

Nitrite is measured by a sensitive diazotization method, during which a stable pink azo dye is formed. The pink color can be measured in a photometer and is proportional to the NO_2^- concentration. The primary reason to measure NO_2^- is the correction of nitrate measurements (see below). Nitrate cannot be measured directly but has to be reduced to nitrite, which is then measured by the above method. To calculate nitrate concentrations, nitrite concentrations (without reduction) have to be subtracted from the nitrate measured as nitrite.

Nitrate Nitrogen: Nitrate is by far the most important nitrogen source for phytoplankton blooms. Whereas phytoplankton can grow on ammonium as the sole nitrogen source, NH_4^+ concentrations of usually $<1 \mu\text{M}$ cannot sustain mass developments of phytoplankton. It is only where substantial amounts of nitrate are introduced into the surface layers (by vertical mixing, storms, upwelling, riverine input) that phytoplankton blooms occur. Since marine phytoplankton is mostly nitrogen rather than phosphorus limited (see above), the exhaustion of the nitrate pool marks the decline of the phytoplankton bloom.

Base nitrate concentrations prior to bloom formation may range up to $20 - 50 \mu\text{M}$. At the end of the bloom, as well as in tropical, subtropical, and temperate summer systems, nitrate concentrations are usually $<1 \mu\text{M}$ and often below the limit of detection by the here applied technique. Utilization of nitrate by algae requires a reduction of NO_3^- into NO_2^- within the cell by an enzyme called nitrate reductase; nitrite is further reduced to NH_4^+ by nitrite reductase before the nitrogen can be assimilated into amino acids. Both reduction steps require energy, and nitrate reductase is usually the speed-limiting process. Ammonium in concentrations above ca. $0.5 \mu\text{M}$ inhibits the function and synthesis of nitrate reductase, thereby preventing NO_3^- utilization.

The best method to measure nitrate is the reduction of NO_3^- to NO_2^- in an alkaline buffer solution. Ammonium chloride acts as the buffer, and the seawater sample with added buffer is passed through a column of cadmium grains that were pre-treated with copper. A fresh cadmium column should have an efficiency of 80-95%, i.e. 80-95% of the nitrate in the seawater sample is reduced to nitrite. The nitrite is measured by the diazotization technique as described above. Sample throughput is about eight samples per hour. Air bubbles and letting the cadmium column run dry should be avoided because it reduces the column efficiency.

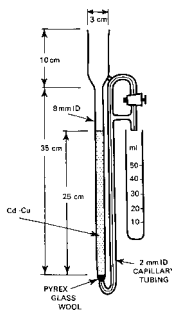


Fig. 1: Cadmium reduction column for nitrate analysis

Phosphorus: Among the major macronutrients, phosphorus is usually least abundant in concentration ($<0.5 \mu\text{M}$). Because nitrate and ammonium concentrations in numerous marine systems are low as well, and organisms require much more nitrogen than phosphorus (see *Redfield Ratio*), phosphorus is usually not the limiting nutrient in the oceans. The cycling of phosphorus is complex, and most of the phosphorus in the pelagic system is in the particulate phase (organisms, detritus). Labile phosphorus compounds are excreted by organisms as a high-molecular weight colloidal phase. Part of this colloidal phase is lost from the productive (euphotic) surface layer by sedimentation, and part is hydrolyzed to soluble orthophosphate. The latter is rapidly assimilated by organisms again.

The phosphorus usually measured as inorganic nutrient is the dissolved orthophosphate, also referred to as “soluble reactive phosphorus”. The seawater sample is allowed to react with a composite reagent of molybdate, ascorbic acid, and trivalent antimony. The molybdic acids formed are reduced to a blue-colored complex, which can be measured in a photometer. The method cannot discriminate between phosphate (PO_4^{3-}) and arsenate (AsO_4^{3-}). Although arsenate occurs in concentrations much below phosphate in most natural waters, arsenate is a common contaminant from pesticide treatment of terrestrial vegetation. The method is specific for PO_4^{3-} , but it can hydrolyze labile organic compounds containing phosphorus and hence overestimates the biologically available phosphorus.

Dissolved Silica: Dissolved silica (SiO_2) occurs essentially unionized and is relatively unreactive chemically. Nevertheless, diatoms and some other algal groups and radiolarians assimilate silica in large quantities in the synthesis of their cell walls or frustules. Since diatoms are prominent algal components in phytoplankton blooms, they can modify greatly the concentration and flux rates of dissolved silica in both marine and freshwater systems. The availability of dissolved silica can have a marked influence on the productivity and species succession of phytoplankton communities. Below a concentration of about $5 \mu\text{M SiO}_2$, most diatoms cannot compete effectively with non-siliceous algae.

Silicon in solution as silicic acid (H_4SiO_4) or silicate (SiO_3^{2-}) reacts with acidic ammonium molybdate to form a yellow silicomolybdate complex. This complex is reduced by sodium sulfite to form the molybdate blue color that can be measured in a photometer. When water samples are frozen, the dissolved silica may be converted to an unreactive form. However, if thawed samples are allowed to stand for a period of time, the silicon will become reactive again. The reactions are dependent on the water salinity, and salinity corrections shall be applied by a correction factor of $F = 1 + 0.0045 \times S\%$.

Lab Work

1. Work Hypothesis

From your knowledge on nutrients in marine pelagic environments, develop a hypothesis for potential differences in the nutrient concentrations among the sampled field stations. Note your hypothesis in your lab journal and use it for your discussion in your final lab report.

2. General Instructions

As part of the pelagic field study, we will analyze the nutrient concentrations for the four sampled field stations and assess differences in the nutrient regime among the stations. In a later lab, we will analyze the phytoplankton biomass and community structure (species composition), and we will try to relate the differences in the phytoplankton communities to the nutrient regime. For the final lab report, each group of four students will be assigned to one field station and will be responsible to document all results for “their” station. For the nutrient analyses in this class, however, each group will assume responsibility for one nutrient and analyze all samples for this nutrient.

Nutrient analyses (except nitrate, see special instructions below) are performed in 12 ml plastic tubes. For each set of analyses, a standard curve has to be prepared. We will prepare a standard curve of 7 known nutrient concentrations (see Table 1), each tube containing a final volume of 10 ml. Additional tubes will be filled with 10 ml of water sample. Prepare 3 replicate tubes for each water sample and one tube for each known nutrient concentration.

The standards will be mixed using distilled water and a nutrient standard solution of $10 \mu\text{M}$ concentration. Before you start your preparations, prepare a mixing plan on how to establish the required nutrient

standard concentration, i.e. how to dilute the 10 μM standard stock with distilled water for a final volume of 10 ml.

After all standards are prepared and the water samples are filled into the testing tubes, chemicals are added according to the specific instructions for each nutrient given below. After the incubation time specific for each nutrient, the samples will be measured in the provided Genesis UV10 spectrophotometer. In contrast to usual spectrophotometers, the Genesis UV10 is equipped with 50 mm pathlength cuvettes. The low concentration of nutrients in marine samples require the use of longer cuvettes to increase the sensitivity of the measurements. Your instructor will be present for the measurements and explain the use of the photometer and the sample handling. Do not operate the Genesis UV10 without the instructor.

Table 1: Protocol scheme for photometric nutrient measurements

Sample	0.0 μM	0.1 μM	0.2 μM	0.5 μM	1.0 μM	2.0 μM	5.0 μM	Sample 1	Sample 2	Sample X
Extinction											

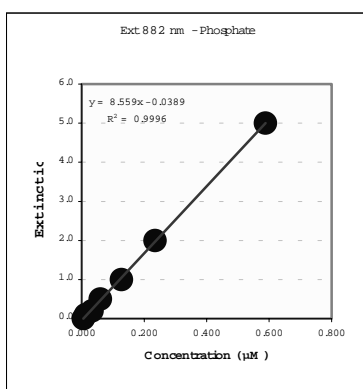


Fig. 2: Standard curve for PO_4^{3-}

Start your measurements with the standard curve, beginning with the lowest concentration (0.0 μM ; only distilled water). The photometer holds one blank cuvette (filled with distilled water all the time to set the baseline for the instrument) and two sample cuvettes. Fill both sample cuvettes, take the reading of the extinction (photometer reading), and note in your protocol table according to Tab. 1. Empty the sample cuvettes by the provided vacuum system and fill with the next sample. Wash the sample cuvettes between the last standard (5.0 μM) and the first field sample (washing between field samples is usually not required unless one field sample exhibits deep color).

When you have all measurements completed, type your standard curve and field sample data into the provided spreadsheet on the central lab computer (Fig. 2). The program will automatically draw a standard curve in the graph window and will list the regression line for your standards. The regression coefficient R^2 for your regression should be >0.99 (poorer regressions will reveal your pipetting errors). Use the regression equation provided by the program in the graph window to calculate your nutrient concentrations from your photometer measurements. Calculate the average and standard deviation of the triplicate concentrations for each field station and write these results into the field stations result table on the blackboard. At the end of the class, copy the blackboard table into your lab journal (you will need it later to discuss the field study!).

3. Nutrient-specific Instructions

Make sure you use the right chemicals for your analyses. To prevent confusion, chemicals for each nutrient are labeled by color code.

Silicate

1. add 300 μl Si mix reagent
2. wait 10 – 20 min
3. add 200 μl oxalic acid
4. immediately add 200 μl Si ascorbic acid (do not confuse with PO_4 ascorbic acid!)
5. incubate at room temperature for 30 min
6. measure at 810 nm wavelength

Phosphate

1. add 300 μl PO_4 mix reagent
2. add 300 μl PO_4 ascorbic acid (do not confuse with Si ascorbic acid!)
3. incubate at room temperature for 10 min to 3 hrs
4. measure at 882 nm

Ammonium (handle all samples and chemicals while wearing protective gloves!)

1. add 200 μ l buffer
2. add 200 μ l Reagent A
3. add 500 μ l Reagent B
4. incubate at room temperature for 1 hr (stable for 24 hrs)
5. measure at 630 nm

Nitrite

1. add 200 μ l SA reagent (sulfanile amide)
2. wait 2 – 3 min
3. add 200 μ l NED reagent
4. incubate at room temperature for 20 min
5. measure at 542 nm

Nitrate

1. the Cd reductor column has been activated by washing with NH_4Cl solution prior to class
2. add 10 ml sample and 10 ml NH_4Cl buffer in 25 ml test tube
3. run sample/ NH_4Cl mix through Cd reductor column (peristaltic pump)
4. discard the first 8-9 ml that run out of the Cd column
5. collect the next 10.0 ml from the Cd column in a 10 ml measuring cylinder
6. transfer 10 ml from measuring cylinder into 12 ml plastic test tube
7. process next sample in the same manner; when all samples are reduced, proceed with #8.
8. add 200 μ l SA reagent (sulfanile amide)
9. wait 2 – 3 min
10. add 200 μ l NED reagent
11. incubate at room temperature for 20 min
12. measure at 542 nm

For the final nitrate concentration, subtract the nitrite concentration (from above Nitrite procedure) from the here measured nitrate concentration (remember: all nitrate is reduced to nitrite by the Cd column).

4. Data analysis

Remember to correct the final concentrations of dissolved silica (SiO_2) as derived from the regression of the standard curve for the salinity of your station (refer to your lab journal of the field trip) by a correction factor of $F = 1 + 0.0045 \times S\%$. Write only this corrected concentration to the blackboard table.

After all nutrient concentrations are collected on the blackboard and you have copied the table from the blackboard, for your final lab report discuss the results and the differences of nutrient concentrations among the sampled stations. Do the results support your working hypothesis?

Calculate the *Redfield Ratio* of nitrogen and phosphorus. Is your ratio higher or lower than the *Redfield Ratio* of N:P = 16:1? From your established N:P ratio, discuss which nutrient is growth limiting for phytoplankton, and are there differences in nutrient limitation among the sampled stations? Remember that you have to add the concentrations of NH_4^+ , NO_2^- , and NO_3^- for the total inorganic nitrogen concentration you need to calculate the N:P ratio!

From the background information given in the Introduction, discuss if the measured concentrations of dissolved silica are sufficient to sustain competitive growth by diatoms. Keep this discussion in mind when we will analyze the phytoplankton community structure at the sampled stations and re-visit this question when discussing the results of the phytoplankton community structure analyses.