



Vrije Universiteit Brussel



Syllabus

Practical exercises and Fieldwork



Master of Ecological Marine Management

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This syllabus gives you a brief outline on some basic methods used in marine ecological research. It is beyond the scope of this course to give a complete methodological overview. It might be a help in your experimental set-up and carrying out basic experiments.

At present, the text is oriented towards practical exercises focusing on the C-cycling in pelagic ecosystems. This syllabus should be extended in the course of future practical exercises and excursions, offering research facilities on different subjects and under other circumstances (e.g. benthic research, population dynamics, in situ measurements).

All personal contributions and remarks are of course very welcome (please send them to the EcoMaMa coordinators).

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List of abbreviations

p.a.	Pro analysis, to be used for analysis
POC	Particulate organic Carbon
TPM	Total Particulate Matter
Chl a/b/c	Chlorophyll a, b or c

1 Introduction

1.1 Energy and living organisms: the carbon cycle

Living organisms basically take up and transform energy from the environment. The **autotrophs** (plants) take up **inorganic components** from the environment: mostly CO_2 , H_2O and nutrients such as P, N, Si which are necessary to build up **organic material**. Organic, contrary to inorganic material, consists mainly of **carbon**. Plants contain **chlorophyll**, a molecule specially constructed to capture the energy provided by the sunlight. Using the sunlight as energy source, plants build up their own organic matter (carbohydrates, proteins, fatty acids) through **primary production**.

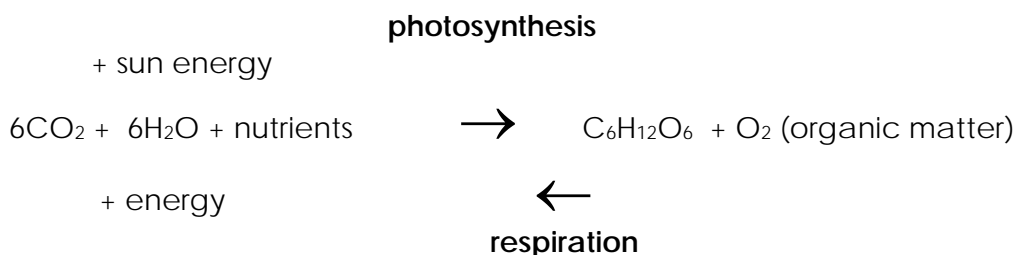
Heterotrophs (animals) cannot convert inorganic matter into organic matter, but feed on the organic matter produced by primary production, either directly by eating plants, or indirectly eating other animals or dead plants or animals. They take up organic matter as food (**ingestion, I**), digest it into simple molecules (e.g. proteins into amino acids), and transform this partly into their own biomass (**production, P**). Another part of the ingested food is oxidized (**respiration, R**), to produce energy and CO_2 . This energy is needed to perform the digestion and transformation of food into their own biomass, and to perform other live functions (locomotion, transfer of electric signals in nervous systems etc). A certain part of the food cannot be used and is excreted (**excretion, E**).

$$I = P + R + E$$

When a living organism dies, its biomass is reconverted into inorganic compounds (nutrients) by the heterotrophic activity of bacteria. These nutrients can again be used by primary producers.

Not only the heterotrophs, but also the autotrophic organisms respire and excrete some of the organic matter they produce. In fact, the energy captured by the plants from the sunlight is transformed into **chemical energy** on the one hand, which is "stored" in the body mass, and into other, more immediately used energy forms, such as the energy needed for locomotion or for electronic nerve signals.

The reactions below show that photosynthesis and respiration are in fact reverse processes: photosynthesis constructs organic matter out of inorganic matter using sun energy, respiration breaks down organic matter into inorganic matter and frees energy. The first process takes place only in plants, the latter in both plants and animals.



The ratio between energy stored as biomass (B) and respired (R) decreases with the size of the organism and generally follows the relationship: $R = kB^{2/3}$ (k is a constant). This means that, the bigger an organism the more energy it stocks under the form of biomass, relative to what it respire.

Another characteristic that varies with the size of organisms is their **abundance** (= concentration, also called density): in sea water bacteria occur in abundances of $10^9 - 10^{12}$ litre⁻¹, while the abundance of e.g. copepods is maximally 1 liter⁻¹. The fact that bacteria are so abundant and convert such relative high amounts of energy allows them, through their **heterotrophic activity**, to recycle the organic matter of dead plants and animals into inorganic components. Just as the heterotrophic animals, they take up organic matter, and respire a (relatively large) part of it, while the rest is stocked as biomass.

Within each ecosystem, the co-occurrence of primary production on the one hand and heterotrophic activity on the other hand leads to the cycling of energy and organic matter in the ecosystem. Because carbon is the main element in this cycling, it is generally called the **carbon cycle**.

1.2 The carbon cycle in marine systems

In the water column of aquatic systems, the main actors in this transformation of energy are plankton organisms. Plankton organisms are defined as those organisms which live in the water column, and cannot actively swim against the water currents. Plankton comprises both vegetal algal species, the **phytoplankton**, and animal species, the **zooplankton**. Phytoplankton species in marine ecosystems mostly belong to two major groups: the **diatoms**, which are generally large (5 – 100 µm) cells, having a silica shell, and the **flagellates**, which are generally smaller than diatoms (2 – 40 µm), and have one or more flagella, but no silica shell. The most frequently studied zooplankton organisms in marine ecosystems are the mesozooplankton (organisms between 100 – 1000µm in size), because of their important biomass encountered in many marine systems. Within the mesozooplankton, **copepodes** are usually dominating. To understand the structure and functioning of pelagic systems, it is necessary to quantify the abundance of various planktonic components, as well as their activity. The phytoplankton performs primary production, while the zooplankton feeds on the organic matter produced by the phytoplankton, and in turn serves as food for higher trophic levels: fish.

These in turn serve as prey to top predators such as seabirds. When phytoplankton and zooplankton die, the dead organic matter (**detritus**) is further decomposed into dissolved components by the action of microplanktonic organisms (see Figure 1) yeasts and bacteria, and further recycled to nutrients by the heterotrophic activity of the bacteria.

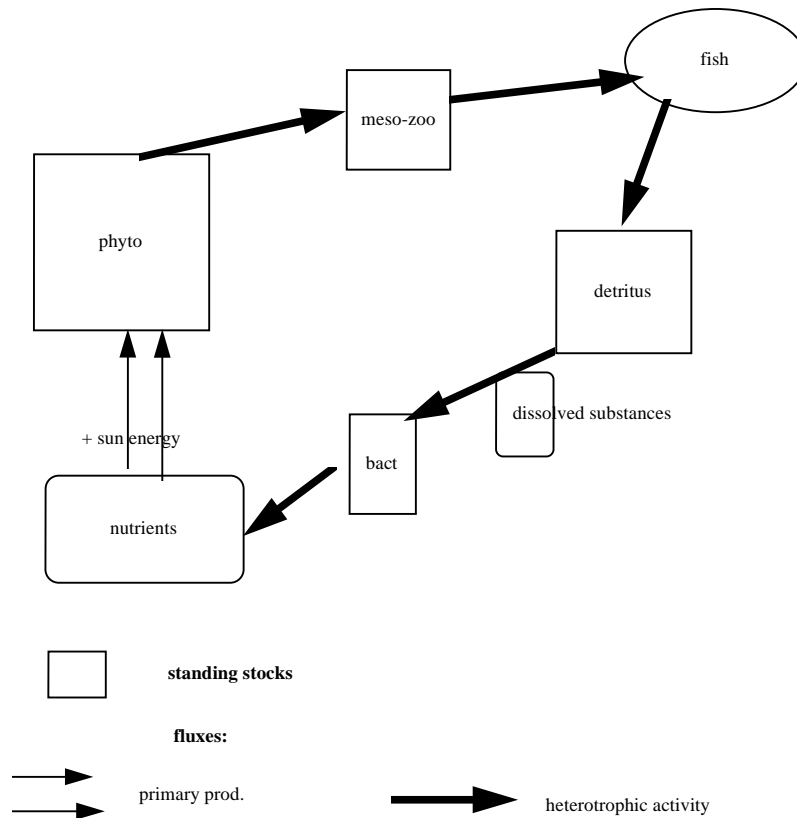


Figure 1. Transfer of energy in an aquatic ecosystem: the Carbon-cycle

The various groups of organisms and components acting in the transformation of energy within a system are called the **compartments**, and their (bio)mass present in the system is the **standing stock** (represented by the boxes in Figure 1).

To obtain a quantitative measure of the energy flow through the various compartments of an ecosystem, we have to quantify these standing stocks and fluxes. Because carbon is the basic element of this energy flow, and to have a uniform measure throughout the different components, this quantification is usually done in carbon. Standing stocks are expressed in carbon weight per unit water volume (e.g. mgC m^{-3}); fluxes are expressed in carbon weight per unit water volume per unit time (e.g. $\text{mgC m}^{-3} \text{d}^{-1}$).

Because both the rate at which chemical reactions take place, and the activity of living organisms are influenced by the environmental conditions in which they occur, we will also need to quantify a number of physio-chemical variables, such as temperature (T) and salinity (S).

The final aim of these quantifications is usually not only to get an idea of how energy runs through a given system during the time of the study, but to be able to deduce mathematical relationships describing the processes between the stocks, fluxes, and the factors influencing them.

For example, as shown in Figure 2 the ingestion rate (I) of mesozooplankton organisms can vary with the concentration of phytoplankton (C) following a saturation type curve, which can be mathematically described as a Michaelis-Menten curve:

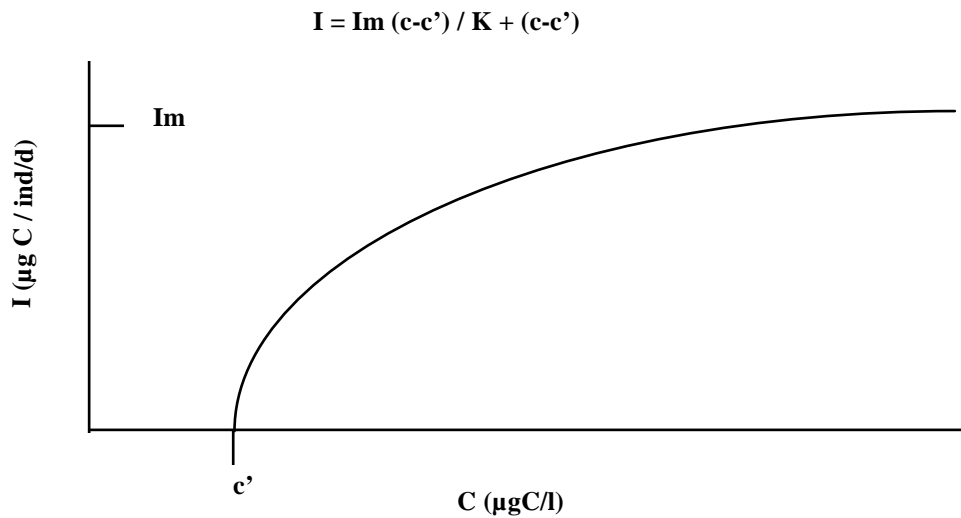


Figure 2. Ingestion rate of mesozooplankton organisms as a function of phytoplankton concentration C (I_m : maximum ingestion rate, K : half saturation constant, c' : concentration at which the ingestion stops)

These relationships can then be combined in an ecosystem model, which allows to make predictions on developments in the ecosystem (or one of its components) as a function of factors influencing the system. These factors can be changes in salinity caused by rainy and dry seasons, or man- induced changes such as increasing loads of detritus input caused by deforestation and subsequent increase in erosion. Such models can be used as tools in the management of ecosystems, as they allow to test the outcome likely to be obtained from various management scenarios.

1.3 Trophic webs

The above description of the carbon cycle in marine systems is an oversimplification. Several other components and processes act in the carbon cycling in marine systems, the most important are described in the following sections.

1.3.1 Mixotrophic organisms

In many marine ecosystems, **mixotrophic** organisms, which can both perform primary production and feed heterotrophically on organic matter are abundantly present. These organisms are mainly planktonic, such as cyanobacteria and dinoflagellates.

1.3.2 Microbial web

In many cases the phytoplankton is not only eaten by (meso) zooplankton, but also by micro (zoo)plankton organisms. The microplankton consists of organisms such as ciliates, tintinnids, but also mixotrophic organisms like some dinoflagellates.

1.3.3 Trophic pathway

The degree in which mesozooplankton on the one hand and / or microplankton on the other hand develop determines the way in which the ecosystem functions, in other words through which pathways the carbon (and energy) flow is passing through the system. At the basis, this is influenced by the **turbulence** of the water, and the **nutrient concentrations** and ratio's between these nutrients. In general terms, small phytoplankton cells can take up nutrients faster than large phytoplankton cells because they have a large surface / volume ratio. In less turbulent water, where nutrient import from outside the system by turbulence is low, nutrient concentrations are generally low, and large phytoplankton species can not compete with small phytoplankton species in nutrient uptake. This leads to the development of small phytoplankton species, which are inefficiently fed upon by mesozooplankton organism. Microplankters, on the contrary, do feed efficiently on small phytoplankton and develop large populations. Some mesozooplankton species do feed on microzooplankton, however, and as such a (limited) transfer to the higher trophic levels also occurs. The bulk of the primary production is used by micro-organisms, or becomes detritus and is finally recycled by bacteria. This type of passway for carbon cycling is called the **microbial loop or web** (see Figure 4b).

In turbulent systems, the import of nutrients by turbulence is higher, and in those conditions, large phytoplankton species (mostly diatoms) can compete with small species for nutrient uptake. So in these systems, large phytoplankton species develop, and can serve directly as food for the mesozooplankton.

The resulting development of mesozooplankton populations also allows the development of higher trophic levels. These are the type of systems that can be exploited as fishing grounds. This trophic pathway is called the **herbivorous food web** (see Figure 4a). In reality, a wide range of 'types' of ecosystem functioning exists, of which the 'herbivorous food web' and the 'microbial loop' or the two extremes.

1.3.4 Seston

The above only considers phytoplankton as the food for the mesozooplankton and micro(zoo)plankton. In many cases however, the zooplankton has a broader choice of food items. Both micro- and mesozooplankters are **suspension feeders**, which means they feed on particles suspended in the water. Besides phytoplankton cells, also dead plant fragments (phytoplankton and benthic algae), remains of dead animals (zooplankton and higher organisms) as well as bacteria are present in the water. The term **seston** stands for all particulate matter suspended in water. **Particulate** means that the particles are $> 1\mu\text{m}$ (as opposed to the materials in solution which passes through a $1\mu\text{m}$ filter). **Suspended** means that these particles cannot actively maintain their position against a current.

So the seston is composed of:

- **organic components:**
 - living plankton (phytoplankton, zooplankton and bacterioplankton)
 - dead particles: detritus
- **inorganic particles:** e.g. carbonates, sand, minerals

Sometimes, particles which were present (settled) on the bottom of the ecosystem are resuspended into the water column. These **resuspended sediments** are usually a mix of both organic and inorganic components.

In practice, bacteria and zooplankton are not often considered under seston studies, which concentrate mainly on phytoplankton, detritus and inorganic particles (e.g. sand).

Besides counting the seston components, which is a labour intensive method, a number of chemical measurements can also be used to quantify the (bio)mass of seston components (see Table 1).

Table 1. Overview of the methods used to quantify the biomass of different seston components of the marine system

Analysis	Quantified seston component
Dry weight	Total particulate matter (TPM): <ul style="list-style-type: none"> • organic matter • inorganic matter
POC (Carbon Analyzer or wet oxidation method)	Amount of organic matter: <ul style="list-style-type: none"> • living phytoplankton • detritus
Chlorophyll by spectrophotometric method or HPLC	Living phytoplankton

The (relative) abundance of the different types of particles present in the seston depends on the ecosystem, and is strongly influenced by the location (depth) of the system (see Figure 3).

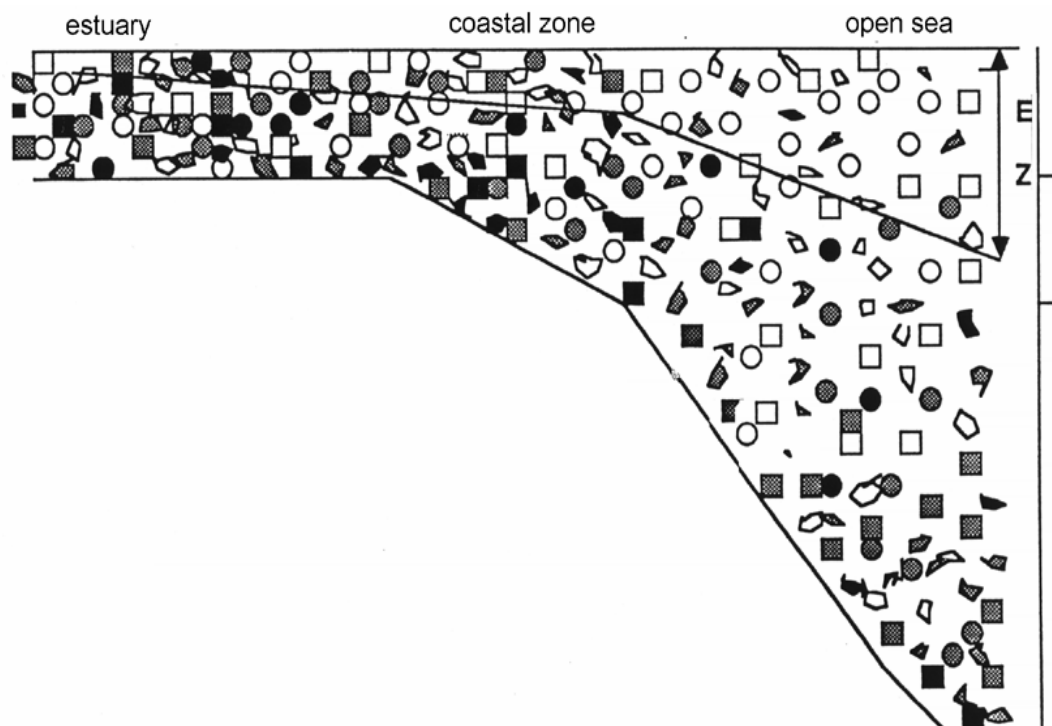


Figure 3. Abundance of various types of particles on a transect going from an estuary through the coastal zone towards the deep sea.

In the **deep (open) sea systems**, the phytoplankton receives enough light for primary production only in the upper water layers. When the cells die, they rapidly sink to the bottom, because turbulence is low. Particles on the bottom (detritus, sediments) are not strongly resuspended, so that the seston in the upper water layers consists mainly of phytoplankton (and zooplankton).

In the **coastal zone**, the tidal movements and wind action creates a higher turbulence because of the limited depth here, detritus and sediments are resuspended regularly in the water column, and are more abundant than in the open sea. There is also a substantial import of material from terrestrial origin, such as plant detritus, eroded soil etc. The seston in coastal zones is a mixture of phytoplankton, detritus and resuspended sediments.

In **estuaries**, the action of the tidal currents is very strong because of the limited depth and space through which the water has to pass at each tidal cycle. Here a lot of detritus and sediments are kept in suspension, and total particulate matter concentration is very high. The high import of particulate matter from terrestrial origin by the river inflow contributes extra to the high particle concentration. Because of this high particle concentration, **turbidity** is high (light penetrates only through a very limited depth into the water), and primary production is light limited. As a consequence, phytoplankton abundance is relatively low, and the majority of the seston in estuaries consist of detritus.

So in most systems, the particulate matter available to the zooplankton as food consist of various types of particles. Depending on the (relative) concentration of these various seston components, the zooplankton can feed on the various particles with varying intensity (cf. Zooplankton course).

Figure 4 gives a more complete picture of the carbon cycling in marine ecosystem, indicating the various seston components and interacting components from outside the system. In the case of the herbivorous food web the major part of the primary production goes to the development of zooplankton and higher trophic levels. In the case of microbial web most of the primary production is used by bacteria and microplankton, while mesozooplankton and higher level development is limited.

1.3.5 **Benthos**

Because these practical exercises are focused on the C-cycling in the pelagic zone, the above explains mainly about the role of planktonic organisms. It should be realised however, that the benthic components also play a very important role in the C-cycling of aquatic ecosystems. Biogeochemical processes taking place in and on the bottom can provide an important interaction between the benthic and the pelagic compartments (e.g. nutrient release from the bottom into the water, consumption of phytoplankton by filter feeding benthic organisms).

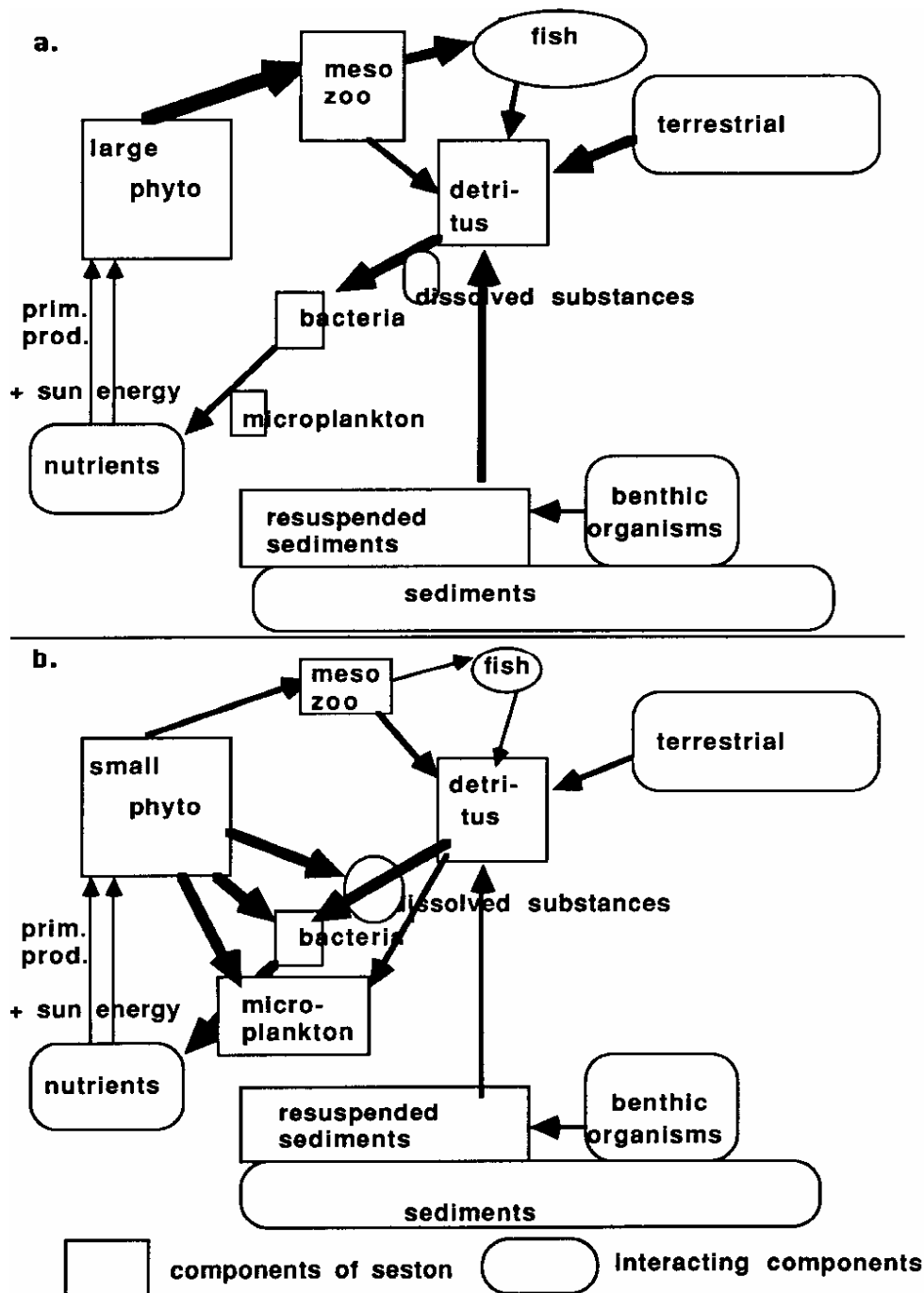


Figure 4. Interactions between different components in the herbivorous (a) and microbial (b) food web

1.4 Sample size and treatment

1.4.1 Sample size

To make adequate measurements of any stock or process in an ecosystem, one has to rely on samples, so each time research on a specific topic in an ecosystem is started, the question arises: "what is the appropriate sample size?"

An important phenomenon in this regard in aquatic ecosystems is the fact that the abundance of organisms (and particles in general) decreases with their size. For

example, there are millions of bacteria in 1 ml of seawater, but there are generally only a few copepods per litre of seawater. Because of this adequate sample size is dependant on the size of the particles you want to study. As can be seen from Figure 5 it is feasible to obtain a representative sample of flagellate cells (pictured in grey) in 1 l sample, but this is not sufficient for sampling copepods (in black). This requires at least 50 l of water.

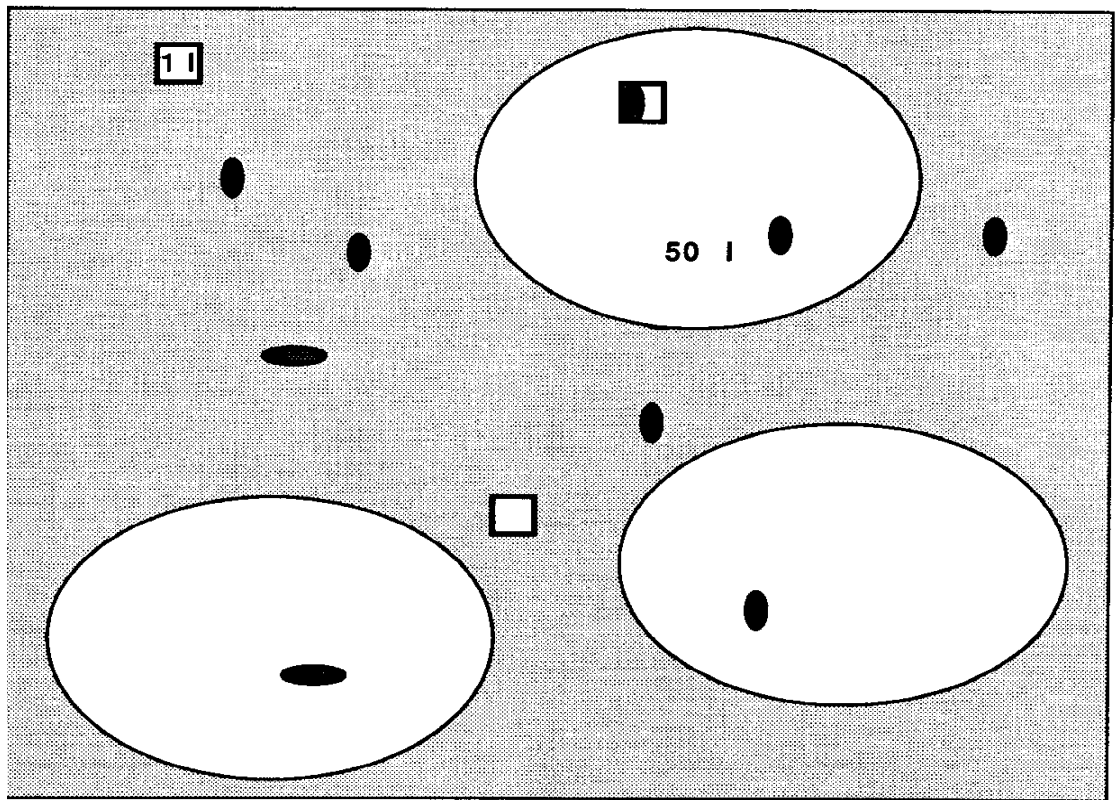


Figure 5. Representation of abundance of various particle sizes in natural samples

This example illustrates that, the larger the sample size, the better the chance to make a correct estimate of the abundance of a component. We can test what is the minimum sample size needed for a specific component by taking a number of replicate samples and analyzing them. Figure 6 shows an example of tintinnid abundance counted from a sample taken in a marine lagoon, ranging from 1 to 10 litres in size (average of 3 replicates). We see that the average obtained fluctuates strongly for small samples, but stabilizes around 16 ind L⁻¹ from 7 litres onwards.

In this example, we would conclude that 7 litres is the minimum sample size needed to quantify the abundance of tintinnids, in this specific ecosystem, correctly. We also notice that the deviation of the measurements (bars in Figure 6) from the mean, becomes smaller as sample size increases (this is represented by standard deviation). So as sample size increases, the standard deviation (σ) will decrease and the mean (m) obtained will be closer to the real value. A simple way to quantify the standard deviation in relation to the mean is the **coefficient of variation**:

$$c.v. = \frac{\sigma * 100}{m}$$

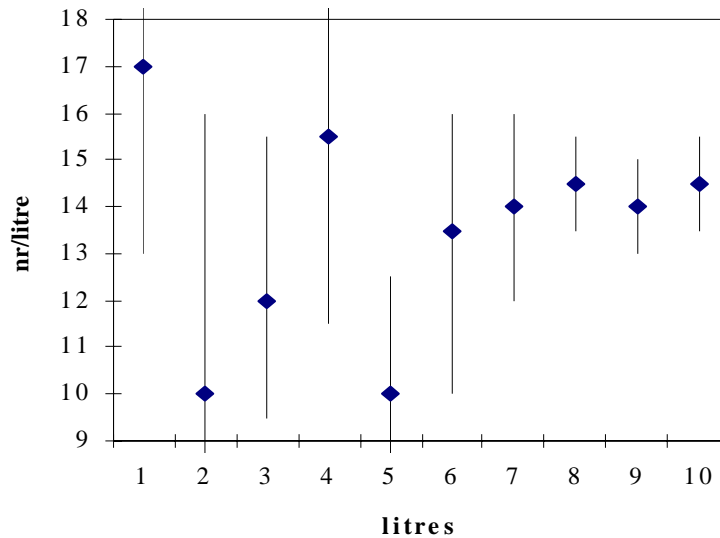


Figure 6. Estimates of the abundance of tintinnids obtained from counts of various sized samples

In principle, one should make a series as shown in Figure 6 each time one starts to study a new ecosystem. In practice however, one knows from experience which are 'safe' sample sizes to work with in most cases. The equipment used for sampling for specific seston components is adjusted to these sample sizes.

For the smaller components, bacteria and phytoplankton, water is usually collected with a Niskin bottle, which has a volume of 10 - 50 litres. From this volume smaller sub samples are usually taken for different specific analysis, such as bacteria abundance counts (a few ml is sufficient here) or microscopic phytoplankton cell counts (0.1-2l) or analysis of chemical components representing specific components (e.g. chlorophyll a; 0.3-2l).

For mesozooplankton, a volume of 50-200l needs to be sampled, and this is done by filtering this volume of water through a 50µm net, and collecting the zooplankton trapped in the net. If we want to quantify the abundance of large zooplankton organisms, such as fish larvae, we need to sample a few thousands of litres, and this can be done by dragging a large net (with mesh size e.g. 300 µm) behind a ship.

In the process of the sample analysis, sub samples are often taken such as e.g. samples for different analysis from the Niskin bottle. This again poses the question: how big do the sub samples have to be? In fact, the question of sample size will return several times in the process of the different steps of sample analysis. At each step, the same principles explained above for the field sample, hold. The specific applications will be outlined with each specific analysis below.

1.4.2 Sample treatment

Depending on the specific objectives, the samples will be treated differently:

- For microscopic phytoplankton counting and identification samples will be fixed with lugol's solution

- For zooplankton counting and identification samples will be fixed with 4% formaline
- For chemical analysis sample water is filtered on glassfibre or membrane filters (GF/C) and stored in a deep freezer.

Detailed explanations on sample treatment is given in the sections concerning the different components.

2 Abiotic factors

2.1 Temperature

The in situ temperature is usually measured directly or on samples taken with a water sampler (at depth) (e.g. Niskin bottles, Van Dorn Sampler) or a bucket (at surface). Usually a mercury thermometer is used. Take care to wait for stabilization before reading. In situ temperature at great depths are measured with a reversible thermometer, mounted on a sampler (e.g. Niskin bottle).

2.2 Salinity

2.2.1 Definitions

1. **Chlorinity** (Cl ‰) is defined as 0.3285234 times the weight of the silver precipitated as silver halides (halides are inorganic bounds of a metal element with fluorine (F), chlorine (Cl), bromine (Br) or iodine (I)) from 1 kg of sea water, all weighings being in vacuum.
2. **Chlorosity** (g Cl/l) is the quantity determined by volumetric methods and is defined as the weight of the silver precipitated as silver halides from 1l of sea water at a fixed temperature.
3. **Salinity** (S, in ‰ or PSU): the content of dissolved salts in sea water is usually expressed as salinity (pro-mill or PSU, Practical Salinity Units). In practice, the salinity is defined in terms of chlorosity by the Knudsen equation:

$$S (\text{‰}) = 0.030 + 1.8050 \text{ Cl (g Cl/l)} \times P^{-1}, \text{ where } P = \text{density of sea water at that chlorosity}$$

2.2.2 Sampling

Determination of the salinity of a water sample can be done in different ways:

1. Using a refractometer: A few drops of sea water are sufficient for salinity readings with a refractometer.
2. Using the Mohr-Knudsen method: A volume of 10 ml of sea water is required for the Mohr-Knudsen titration. Standard medicine bottles are ideal for collecting, handling and storage of samples for salinity determinations. After rinsing the bottles thoroughly with the sample, fill them up to the shoulder. They are stoppered using a waxed cork or a cork covered with Parafilm (plastic sheet) under these circumstances no changes in salinity are expected over a period of many years. Once the bottles opened, analysis should be performed within the hour. An outline of the method is given below.

2.2.3 Determination of salinity

1. Standardization of AgNO₃

- 10 ml of standard sea water is brought into a 100 ml beaker
- dilute with a few ml aq. Dest
- add 5 drops of indicator solution
- add AgNO₃-solution from the burette drop per drop until you see a change in color: first orange, then red
- note the volume of AgNO₃-solution added = A

2. Titration of sea water sample

- 10 ml of sample is titrated (same procedure as in 1.)
- note the volume of AgNO₃ added = a (gCl/l)

Calculations

1. Chlorosity Chls (Cl/l) = 10 x a x A⁻¹
2. Salinity S (‰) = 0.030 + (1.8050 x Chls x P⁻¹), where P is the density of sea water at chlorosity Chls

Conversion table: see in annex.

P.S.: It is assumed that the temperature of samples and reagents is the same and approximately 20° C at the time of the titration. If this is not the case, you need to involve a correction factor for the temperature (see Strickland & Parsons).

2.2.3.2 Refractometer

The refraction index of a liquid increases with its salinity. This instrument allows determination of the salinity (S ‰) on a 0.02 ml sample. Precision is 1 ‰. Adjustment for temperature is provided.

2.2.4 Artificial seawater

Artificial seawater can be obtained by dissolving sea-salt in de-ionized water. Strong air-bubbling can be used to facilitate the dissolving of the salt. This also causes a number of elements in the salt to react with each other and to form precipitates. These can be removed by filtration. Dissolving 42 g salt/l gives a salinity of 35 ‰. Check for each brand.

To perform experiments at different salinities:

- Salinities lower than the available seawater: dilute filtered seawater with aqua dest or prepare with sea-salt and deionized water.
- Salinities higher than the available seawater:
 - evaporate natural seawater: time consuming, unpractical
 - prepare from sea-salt & deionized water

2.3 Light intensity

The quantity and quality of light at the sea fluctuates depending on the tide, weather conditions and angular incidence and has a major influence on many biological processes (e.g. primary productivity). Quantitative measurements of light intensity can be obtained in different ways:

- expressed as an illumination (only visible part of spectrum (300 nm 760 nm)), as a luminous flux per unit area: LUX
- expressed as an energy unit (whole spectrum including UV and infra red (IR)): per unit area: cal/cm²
- expressed as a quantum: micro-Einstein per second per m²

$\mu\text{E s}^{-1} \text{m}^{-2}$ recently most used

2.3.1 Light intensity as a function of water depth

The absorption and scattering (and subsequently the penetration) of light in the water column depends on the amount of particles that are suspended in the water column (suspended matter) and on the depth.

The amount of light that penetrates the water column at a certain depth d , depends on the light intensity of the incoming light (I_0) and a vertical extinction coefficient k :

$$I_d = I_0 e^{-kd}$$

with : I_0 incoming light intensity
 I_d incoming light intensity at depth d
 k varies with wave length

In the lab the different light intensities that occur at different depths are simulated using bags or bottles with different transparency filters (see section on primary production).

2.4 pH

2.4.1 Sampling

pH can be measured on small (sub)samples of ± 20 ml

2.4.2 Colorimetric method

Put one or a few drops of the fluid to be analyzed on the indicator-paper. After 1 min. the color is compared with the reference - scale. Precision is dependent on the interval-magnitude of this scale.

2.4.3 pH meter

By means of a glass-electrode in combination with a reference electrode, pH values can be read. The reference electrode is usually built-in in the glass-electrode.

2.4.3.1 Use of pH meter

At each series of measurements first measure the temperature of the water and adjust the pH meter to this temperature.

Calibration & measurement

- Ideally the temperature of the calibrating solution should be the same as that of the water to be analyzed.
- Adjust temperature setting of pH meter to the temperature of the fluid to be measured.
- Bring electrode in calibrating solution with pH = 7 and turn button till readout indicates 7.
- Rinse electrode with aqua dest.
- Rinse electrode with aqua dest.
- Dip calibrated and temperature adjusted electrode in water to be analyzed. Stir gently till readout stabilizes.

Important:

After each series of measurements, rinse the electrode with aqua dest and keep it in 3M KCl solution. This has to be done even if the electrode is not used for even a few minutes !

Remember the glass membrane is easily damaged.

2.5 Total Alkalinity

The aim of this section is give a brief overview of the principles of the most widely used methods for the determination of seawater alkalinity.

2.5.1 Definition

The alkalinity may be defined as the excess of anions of weak acids in seawater. In other words, alkalinity is a quantitative measure of the ability to react with H⁺ ions: the higher the alkalinity of a solution, the greater its capacity to react with H⁺.

In most natural waters, carbonate species are the predominant form of alkalinity, but other weak acids and a wide variety of dissolved or suspended materials may also contribute to alkalinity. Therefore, Total Alkalinity (TALK) is defined as:

$$\text{TALK} = [\text{HCO}_3^-] + 2[\text{CO}_3^{2-}] + [\text{B(OH)}_4^-] + [\text{OH}^-] - [\text{H}^+] + [\text{other strong bases}]$$

The alkalinity can be titrated and therefore total alkalinity or titration alkalinity corresponds to the amount of strong acid in milli-equivalents (or millimoles) required to neutralize 1 kg of seawater.

2.5.2 Sampling

The samples are taken from an ordinary hydrocast and transferred to neutral glass bottles with rubber stoppers. The bottles should preferably be aged with hydrochloric acid for several months. Input or release of carbon dioxide has no effect on the total alkalinity, but has an effect on the total carbonate concentration. Biological activity may change the total alkalinity and therefore it is preferable that the analyses be

carried out on board ship, if conditions permit. In the case of samples brought back to the laboratory, it is advisable to add the exact amount of acid and sample into the bottles immediately on board.

2.5.3 Methods

2.5.3.1 The back titration method

The original method by Gripenberg has been modified by different workers. However, the principle of the method involves acidification of the water sample with strong hydrochloric acid to a pH of about 3.5. Carbon dioxide is driven off by boiling. The solution is then back titrated with sodium hydroxide to pH 6 using bromothymol blue as an indicator. Carbon dioxide free air is bubbled through the sample during the titration. Only negligible amounts of boric acid are back titrated and thus

$$\text{TALK} = [\text{H}^+]_{\text{added}} - [\text{OH}^-]_{\text{added}}$$

2.5.3.2 The pH method

This method is essentially a single point potentiometric titration of seawater. The sample is acidified to about pH 3.5. The pH is measured with a high precision instrument and the alkalinity can be calculated from difference between the amount of acid added and the excess acid present. The latter is obtained from already determined empirical activity coefficient values.

2.5.3.3 The potentiometric titration method

This method has been widely used and adapted to ship board conditions. From the potentiometric titration of seawater with hydrochloric acid, TALK, CO₂ and pH can be evaluated. The calculations are rather complicated and usually a small computer is employed.

2.6 Oxygen

2.6.1 Sampling

Concentration of oxygen can be measured on small (sub)samples (50 ml) taken in situ. When sampling for oxygen concentration measurements, care has to be taken to avoid air-bubbles. To keep turbulence minimal, most sampling devices (e.g. Niskin bottles) are equipped with special taps.

When bringing samples into BOD-bottles, use a plastic tube. To open bottles without getting air-bubbles inside: remove cork very carefully, drop cork on top for closing.

2.6.2 Winkler Method

2.6.2.1 Outline

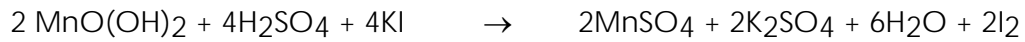
Mn(OH)₂ is formed in the water to be analyzed:



Mn(OH)₂ uses the free O₂ in the water to form MnO(OH)₂



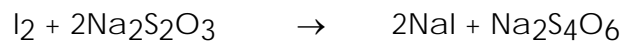
In the presence of KI, addition of a strong acid (H₂SO₂) liberates I₂



The amount of liberated I₂ is equivalent with the dissolved O₂ in the water : 2 molecules I₂ are formed for each molecule of O₂ present

I₂ colors the solution brown

I₂ is titrated with a standardized solution of Na₂S₂O₃



End of titration is made visible by adding a little bit of starch which colors the I₂ blue.

2.6.2.2 Equipment

- glass stoppered BOD bottles
- glass 50 ml pipet
- glass erlenmeyer 100 ml
- glass buret (precision 0.1 or 0.05 ml)
- spatula

2.6.2.3 Reagents

- MnSO₄ solution: 48 g MnSO₄ · 4H₂O in 100 ml dest
- alkaline iodide:
 - 30 g KI in 50 ml aqua dest
 - +
 - 50 g NaOH in 50 ml aqua dest
- Na₂S₂O₃: Dilute from stock solution to a 0.01 N solution
- Starch
- concentrated H₂SO₄

2.6.2.4 Procedure

1. Fill 50 or 100 ml Winkler bottles with the water to be analyzed (use a tube, very gently, avoiding air bubbles)
2. Add 0.4 ml MnSO₄ for every 100 ml sample
3. Add 0.4 ml alkaline KI for every 100 ml sample
4. Close bottle, shake well
5. Let the precipitate settle (± 1/2 hour)

If not analyzed immediately, bottles can be conserved for 1-2 days this way (preferably in fridge).

6. Add 0.2 ml H₂SO₄, shake till all the precipitate is dissolved and titrate within half an hour!

Titration

1. Shake bottle, bring 50 ml into an erlenmeyer with a pipette
2. Add Na₂S₂O₃ (diluted 0.01N solution) drop by drop till color is light-yellow
3. Add a little bit of starch with a spatula, then the colour becomes dark blue
4. Carefully continue to add drops of Na₂S₂O₃ till water becomes colorless

2.6.2.5 Calculation of O₂ content

The concentration of O₂ can be calculated as follows :

$$\begin{aligned}
 (\text{O}_2) \text{ mg l}^{-1} &= \\
 & \frac{\text{Normality Na}_2\text{S}_2\text{O}_3 \times 0.25 \times 32 \times 1000 \times \text{volume Na}_2\text{S}_2\text{O}_3 \text{ added (ml)}}{\text{volume titrated.}} \\
 &= \frac{0.01 \times .25 \times 32 \times 1000 \times \text{vol. Na}_2\text{S}_2\text{O}_3 \text{ added (ml)}}{50} \\
 &= \text{Vol. Na}_2\text{S}_2\text{O}_3 \text{ added (ml)} \times 1.6
 \end{aligned}$$

When measuring fluxes in a carbon cycle analysis, it is usually necessary to express the final result in C in stead of O₂.

To convert mg O₂.l⁻¹ to mg C.l⁻¹ =

$$\frac{\text{mg O}_2}{l} \times \frac{\text{MW C}}{\text{MW O}_2} = \frac{\text{mg O}_2}{l} \times \frac{12}{32} = \frac{\text{mg O}_2}{l} \times 0.375$$

To convert to ml l⁻¹ : 1 mole of a gas has a volume of 22,4 l at 1 atm and a temperature of 4° C

$$\rightarrow \frac{\text{mg O}_2}{l} \times \frac{1}{32} \times 22,41 = \frac{\text{mg O}_2}{l} \times 0.7 = \frac{\text{ml O}_2}{l}$$

2.6.2.6 Standardization on Na₂S₂O₃ (0.01N-solution)

$$\rightarrow \frac{\text{mg O}_2}{l} \times 0,7 = \frac{\text{ml O}_2}{l}$$

Reagents

- KIO₃ 0.1N
- KI 10 %
- H₂SO₄ conc.

Method

1. Dry KIO₃ at 105° C for 1 hour
2. Prepare a stock solution of KIO₃ 0.1N by weighing 0,3567 gr KIO₃ and dissolving it in 100 ml
3. Dilute the KIO₃ 0.1N-solution 10 times to get a 0.01N-solution
4. With a analytical pypette bring 10 ml KIO₃ 0.01N into an erlenmeyer add 10 ml of a 10 % KI-solution and a few drops of conc H₂SO₄ add about 50 ml distilled water and with Na₂S₂O₃ 0.01N like discribed earlier.

Result

The normality of Na₂S₂O₃ 0.01N is :

$$\frac{\text{normality of KIO}_3}{\text{normality of Na}_2\text{S}_2\text{O}_3} = \frac{\text{ml KIO}_3 \text{ 0.01 used}}{\text{ml Na}_2\text{S}_2\text{O}_3 \text{ 0.01N used}}$$

$$\frac{0.01 \text{ N}}{x} = \frac{10 \text{ ml}}{\text{ml Na}_2\text{S}_2\text{O}_3}$$

$$x = \frac{0.01\text{N} \times \text{ml Na}_2\text{S}_2\text{O}_3}{10 \text{ L}}$$

2.7 Nutrients

2.7.1 Sampling

Sea water samples are taken with Niskin bottles and sub-samples are brought into clean polyethylene vials, which are stored in the refrigerator. Ammonium should be analyzed immediately after sampling. The samples for nitrate and silicate analyses can be kept in the fridge for several hours before concentration changes occur. For nitrites and phosphates, the analysis should be carried out within two hours after sampling.

In case immediate analysis is impossible, samples should be filtered on 0.45 µm porosity membrane filter or GF/C filters and deep frozen. Nitrates, silicates and phosphates can be correctly measured on such samples, whereas ammonium and nitrites can no longer be quantified on these preserved samples.

In the following sections the methods for phosphate, nitrate and silicate are described.

2.7.2 Phosphate

The method relies on the formation of a phosphorus-molybdate complex and its subsequent reduction to highly colored compounds that can be quantified with a spectrophotometer at a specific wavelength.

2.7.2.1 Outline

The sea water sample is allowed to react with a composite reagent containing molybdic acid, ascorbic acid, and trivalent antimony.

The resulting complex heteropoly acid is reduced to give a blue solution (phosphomolybdate complex), of which the absorption is measured at 885 nm.

2.7.2.2 Equipment

- Polyethylene bottles of 130 ml volume, with a mark at the 100 ml level
- Spectrophotometer
- thermostated water bath (only in case the room temperature is below 15° C or above 30° C).

2.7.2.3 Reagents

- Ammonium molybdate solution
 - Dissolve 15 g of ammonium paramolybdate ((NH₄)₆ Mo₇O₂₄ 4H₂O p.a) in 500 ml aq. dest.
 - Store in a plastic bottle out of direct sunlight
 - This solution is stable indefinitely, even at room temperature
- Sulphuric acid solution (serves to obtain a pH < 1)
 - Add 140 ml of concentrated sulphuric acid (H₂SO₄ p.a.) little by little to 900 ml aq. dest.
 - Allow the solution to cool
 - Store in a glass bottle at room temperature
- Ascorbic acid solution (is reductor)
 - Dissolve 27 g of ascorbic acid (C₆ H₈ O₆ p.a.) in 500 ml aq. dest. in deep freeze !
 - Store the solution in a plastic bottle in the deep-freezer.
 - Thaw for use and refreeze at once.
 - The solution is then stable for many months, but should not be kept at room temperature for more than a week.
- Potassium antimonyl-tartrate solution (is katalysator)
 - Dissolve 0.34 g of potassium antimonyl-tartrate (C₈ H₄ K₂ O₁₂ Sb₂.3H₂O) in 250 ml aq. dest, warm if necessary.
 - Store in a glass or plastic bottle.
 - The solution is stable for many months, even at room temperature.
- Mixed reagent: mix together the following
 - 100 ml ammonium molybdate solution
 - 250 ml sulphuric acid solution
 - 100 ml ascorbic acid solution

- 50 ml potassium antimonyl-tartrate solution
- Remarks: Prepare this reagent in a quantity sufficient for the samples for use at hand. The quantity mentioned above is for about 50 samples. Do not store this solution for more than 6 hours.

2.7.2.4 Procedure

Determination of the calibration curve:

- Dissolve 0.816 g of anhydrous potassium dihydrogen phosphate (KH_2PO_4 p.a.) (MW = 136) in 1000 ml aq. dest. Store in a dark bottle with 1 ml of chloroform (CHCl_3), the solution is stable for many months. This solution thus contains 6 μmol $\text{KH}_2\text{PO}_4/\text{ml}$ or 6.0 μg - at P (microgram - atoms of phosphate phosphorus)/ml
- Dilute 10 ml of this solution to 1000 ml with aq. dest. Store in a dark bottle with 1 ml of chloroform added, the solution is stable for about a month.

$$1 \text{ ml} = 6.0 \times 10^{-2} \mu\text{g-at P}$$
- Prepare five standards, using between 0,25ml and 10 ml (0,25 - 0,5 - 1 - 2 - 4 - 8 - 10) of the diluted phosphate solution. Make each of them up to 100 ml with aq.dest.
- Transfer the solution to dry bottles and fill two more bottles with 100 ml of aq. dest to serve as blanks.
- Add 10 ml of the mixed reagent solution and mix immediately.
- After at least 5 minutes and within the first 2-3 hours, measure the extinction of the solution in a spectrophotometer against aq. dest. at a wavelength of 885 nm.
- Using the standards' concentrations (μg at P/l) and the corrected extinction values obtained from the spectrophotometer (corrected extinction = extinction standard - extinction reagent blanks), make a calibration plot.
- Calculate the calibration curve through regression analysis:

$$\text{Extinction} = a \cdot \text{concentration} (\mu\text{g at P/l}) + b$$

Determination of P-concentration in the samples:

- Allow the samples to come to room temperature
- To 100 ml of sample, add 10.0 ml of mixed reagent solution and mix at once
- Calculate the phosphate concentration in microgram-atoms of phosphate phosphorus (μg - at P/l) from the expression:

$$[\text{P}] = \frac{\text{corrected extinction} - b}{a}$$

where a and b are obtained from the calibration curve

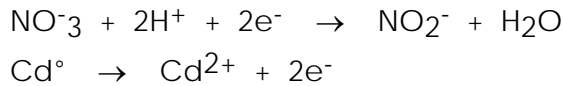
Remarks:

1. To exclude effects of turbidity, it is best to filter the samples on 0.45 μm porosity membrane filters or GF/C filters before analysis.
2. If the obtained samples' E falls beyond the range of the standard Extinction, include additional standards in the calibration curve.

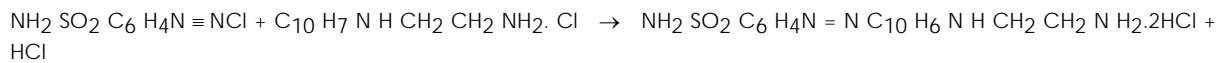
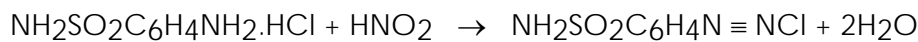
2.7.3 Nitrate

2.7.3.1 *Outline of the method*

The nitrate in sea water is reduced almost quantitatively to nitrite when a sample is run through a column containing cadmium (Cd) filings loosely coated with metallic copper (Cu).



The nitrite thus produced is quantified by diazoting with sulfanilamide and coupling with N-(1-naphthyl) ethylenediamine to form a highly colored azo dye



The extinction of the colored complex is measured spectrophotometrically at 543 nm.

2.7.3.2 *Equipment*

- Glass-tube to prepare the Cd-Cu column
- 125 ml erlenmeyer flasks
- A spectrophotometer
- 50 ml graduated cylinders

2.7.3.3 *Reagents*

- Concentrated ammonium chloride solution:
 - dissolve 125 g of ammonium chloride (NH₄Cl p.a.) in 500 ml aq. dest
 - store in a glass or plastic bottle.
- Diluted ammonium chloride solution:
 - dilute 50 ml of the concentrated solution to 2000 ml with aq. dest
 - store in a glass or plastic bottle.
- Cadmium-copper filings:
 - Stir about 100 g of cadmium filings (enough for 2 columns) with 500 ml of a 2 % W/V solution of copper sulphate pentahydrate (CuSO₄·5H₂O), until the blue color has left the solution and semi-colloidal copper particles begin to enter the supernatant liquid.
 - Roll very fine copper turnings between fingers and thumb to make a small plug and push this in the bottom of a reductor column, (glass or quartz wool can be used if you can not obtain very fine copper "wool" turnings).
- Fill the column with diluted ammonium chloride solution, or the supernatant liquor from the preparation of Cd-Cu turnings above, and pour in sufficient Cd-Cu mixture to produce a column of about 30 cm of length.

- Add the filings little at a time, tapping the column hard after each addition to make sure that the filings are well settled.
- Wash the column thoroughly with diluted ammonium chloride solution.
- The flow rate must be such that 100 ml of solution takes 8 to 12 minutes to flow completely through the column.
 - If flow time is less than 8 minutes: pack more Cu or glass wool at the outlet of the column
 - If flow time is more than 12 minutes: loosen the packing at the base of the column
- Finally add a small plug of copper or glass wool to the top of the column to prevent cadmium filings being washed into the top chamber when solutions are added to the column.
- When not in use, columns must be left with the metal filings completely covered with diluted ammonium chloride solution.
- To regenerate a column that has lost efficiency of reduction:
 - empty the column into a beaker
 - wash the filings with 100 ml of 5 % (weight per. volume) hydrochloric acid (HCl)
 - decant the acid
 - repeat the procedure once more
 - wash the metal with 100 ml portions of aq. dest until the pH of the wash exceeds the value 5
 - decant the liquid to leave the metal as dry as possible
 - retreat the metal with copper sulphate solution as described above.
- Sulphanilamide solution:
 - dissolve 5 g of sulphanilamide ($C_6H_6N_2O_2S$) in a mixture of 50 ml of concentrated hydrochloric acid (HCl) and about 300 ml of aq. dest
 - Dilute to 500 ml with aq. dest. This solution is stable for months.
- N- (1-naphtyl)-ethylenediamine dihydrochloride solution:
 - dissolve 0.50 g of the dihydrochloride in 500 ml aq. dest.
 - Store the solution in a dark bottle, it should be renewed monthly.

2.7.3.4 Procedure

Determination of the calibration curve:

This should be carried out using synthetic sea water or natural sea water with a nitrate concentration less than $1 \mu\text{g-at N/l}$.

The concentration-extinction relationship is strictly linear and the factor need therefore be obtained at only one level of nitrate concentration.

- Synthetic sea water: dissolve 310 g of sodium chloride (NaCl p.a.), 100 g of magnesium sulfate ($MgSO_4 \cdot 7H_2O$ p.a.) and 0.50 g of sodium bicarbonate ($NaHCO_3 \cdot H_2O$ p.a.) in 10 l aq. dest.
- Standard nitrate solution: dissolve 1.02 g of potassium nitrate (KNO_3 p.a.) in 1000 ml aq. dest. The solution is stable indefinitely in the absence of evaporation. $1 \text{ ml} = 10.0 \mu\text{g-at N}$
- Dilute 4.00 ml of this solution to 2000 ml with synthetic sea water. This solution should be stored in a dark bottle and prepared freshly immediately before use.

concentration = 20 µg- at N/l

- As a reagent blank: pour 110 ml aq. dest in a 125 ml erlenmeyer
- Pour about 110 ml of the diluted nitrate standard solution into a clean dry 125 ml erlenmeyer flask, do this in triplo
- Add 1 ml of naphthylethylenediamine solution, mix immediately
- After 10 minutes to 2 hours, measure the extinction of the solution against aq. dest at a wavelength of 543 nm.
- Correct the mean of the three extinctions by the blank extinction
- Calculate the factor F from the expression

$$F = \frac{20.0}{E}$$

where E is the mean extinction of the three values corrected for a blank.

Determination of NO₃-concentration in the samples:

- bring 50.0 ml of sea water (filtered on a 0.45 µm porosity membrane filter or GF/C filter if turbidity is too high) in a 125 ml erlenmeyer flask.
- Add 2.0 ml of concentrated ammonium chloride to the sample in the Erlenmeyer flask.
- Mix the solution and pour about 5 ml on top of the column and allow it to pass through.
- Add the remainder of the sample to the column and place the drained Erlenmeyer flask under the collection tube.
- When 40 ml has passed through the column, drain the collection tube into the flask, rinse the flask with this effluent, drain it and replace beneath the collection tube.
- Collect a further 50 ml in the collection tube and rapidly empty it into the Erlenmeyer flask. The column will not be quite empty and should then be allowed to drain until flow ceases.
- Add 1.0 ml of sulphaniamide solution, mix and allow the reagents to react for 2-8 minutes.
- Add 1 ml of naphthylethylenediamine solution, mix immediately.
- After 10 minutes to 2 hours, measure the extinction of the solution against aq. dest at a wavelength of 543 nm.
- Correct the measured extinction for the reagent blank.
- Calculate the nitrate-nitrogen concentration in microgram-atoms of nitrogen per liter (µg-at N/l) as:

$$\mu\text{g-at N/l} = \text{corrected extinction} \times F$$

where the corrected extinction = measured extinction - extinction of reagent blank

2.7.4 Silicate

2.7.4.1 Outline

The sea water sample is stored in polyethylene bottles in the dark for maximum a day. If longer storage is planned deep freezing of the samples is better.

The sample is allowed to react with molybdate under acid conditions ($1 < \text{pH} < 2$) to form the silicomolybdate, phosphomolybdate and arsenomolybdate complexes.

A reducing solution, containing metol and oxalic acid, is then added to give a blue reduction compound and to simultaneously decompose the arsenomolybdate and phosphomolybdate complexes. In this way, interference of arsenate and phosphate is eliminated. The extinction of the resulting solution is measured at 810 nm.

2.7.4.2 Equipment

- 50 ml graduated glass measuring cylinders (Cleaning: fill them with chromic-sulphuric acid cleaning mixture, rinse thoroughly with aq. dest just before use)
- polyethylene bottles
- spectrophotometer

2.7.4.3 Reagents

Molybdate reagents:

- Dissolve 4.0 g of ammonium molybdate ($(\text{NH}_4)_6\text{MO}_7\text{O}_{24} \cdot 4\text{H}_2\text{O}$ p.a.) in about 300 ml aq. dest.
- Add 12.0 ml of concentrated hydrochloric acid (HCl p.a.), mix and make the volume to 500 ml with aq. dest.
- Store this solution in a polyethylene bottle, in which it is stable for many months if not exposed to direct sunlight.

Metol-sulphite solution:

- Dissolve 6 g of anhydrous sodium sulphite (Na_2SO_3 p.a.) in 500 ml aq. dest
- Add 10 g of metol (p-methylaminophenol sulphate) ($(\text{HOC}_6\text{H}_4\text{NHCH}_3)_2 \cdot \text{H}_2\text{SO}_4$)
- When the metol has dissolved, filter the solution through a Whatman N° 1 filter paper and store it in a glass bottle which is tightly stoppered.
- This solution should be prepared fresh monthly.

Oxalic acid solution:

- Prepare a saturated oxalic acid by shaking 50 g oxalic acid dihydrate ($(\text{COOH})_2 \cdot 2\text{H}_2\text{O}$ p.a.) with 500 ml aq. dest.
- Decant the solution from the crystals for use
- This solution can be kept indefinitely in a glass bottle

Sulphuric acid solution 50 % V/V:

- Add 250 ml of concentrated sulphuric acid (H_2SO_4 p.a.) little by little into 250 ml aq. dest.
- Allow to cool to room temperature and make the volume to 500 ml with a little extra aq. Dest.

Reducing reagent:

- Mix 10 ml of metol-sulphite solution with 60 ml of oxalic acid solution
- Add slowly, with mixing, 60 ml of the 50 % V/V sulphuric acid solution.
- Make the mixture to a volume of 300 ml with aq. dest.

- This solution should be prepared for immediate use.

2.7.4.4 Procedure

Determination of the calibration curve:

- Standard silicate solution : 1g/1000ml
 - Weigh 0.960 g of fine powdered ammonium silicofluoride ((NH₄)₂SiF₆), crush any lumps if present.
 - Dissolve the salt by stirring it with 50-100 ml aq. dest in a plastic beaker using a nickel spatula.
 - Transfer the solution to a 1000 ml measuring flask, rinse the beaker thoroughly and make the volume to the mark with aq. dest.
 - Mix and transfer the solution to a polyethylene bottle for storage as soon as possible (while the solution rapidly picks up silica from glass)
 - The solution is stable indefinitely
1 ml = 5 µg-at Si (microgram-atoms of silicate silicon)
- Dilute 10 ml of this solution to 500 ml with synthetic sea water, this diluted solution should be used within a few hours.
1 ml = 0.1 µg- at Si
- Synthetic sea water:
 - Dissolve 25 g sodium chloride (NaCl) and 8 g of magnesium sulphate heptahydrate (MgSO₄.7H₂O) in 1000 ml aq. dest.
 - This water is equivalent to sea water with salinity 28 ‰.
 - This solution must be stored in polyethylene bottles.
- Add 10 ml of molybdate solution to 7 dry 50-ml measuring cylinder
- As reagent blanks, add 25.0 ml of synthetic sea water to two of the measuring cylinder, restopper and mix, allow to stand for 10 minutes.

For the standards, add 5.0 - 25.0 ml of the diluted silicon standard to five of the 50 ml measuring cylinders. Make up each of these to 25.0 ml with aq. dest.

Rapidly add the reducing reagent (15 ml) so as to make the volume exactly to 50 ml and mix immediately.

Allow the solution to stand for 2-3 hours to complete the reduction of the silicomolybdate complex.

Measure the extinctions of the blanks and the standards against aq. dest at a wavelength of 810 nm

Make a calibration plot: (corrected extinction in function of the Standard concentration (µg - at Si/l)).

Calculate the calibration curve. Extinction = a . concentration (µg at Si/l) +b

Determination of the Si-concentration in the samples:

- Add 10 ml of molybdate solution to a dry 50 ml measuring cylinder.

- Add 25.0 ml of the sea water (can be filtered on a 0.45 µm porosity membrane filter or a GF/C filter in case turbidity is too high), stopper the cylinder, mix and allow the solution to stand for 10 minutes.
- Follow the previous (treatment of standards)
- Correct the measured extinction by subtracting the mean of the two reagent blanks.
- Calculate the silicate concentration in microgram-atoms of silicate silicon per liter from the expression

$$\mu\text{g-at Si/l} = \frac{\text{corrected extinction} - b}{a}$$

(a and b obtained from the calibration curve)

2.7.5 Ammonium

2.7.5.1 Outline

In alkaline medium the dissolved NH₃ reacts with hypochlorite (HClO) and form a monochloramine. This will form a blue indofenol in oxydising medium, and in the presence of a fenol. At 20°C and with nitroprussaat (Na(Fe(CN)₅NO).2H₂O) as a caterlyst, this reaction will take 12 hours.

Precipitation of Ca and Mg in basic medium is avoided by complexation with sodiumcitraatdihydraat.

2.7.5.2 Equipment

- 100 ml polyethylene bottles
- spectrophotometer

If natural samples are to be measured, cuvettes with an optical path of 10 cm should be used as ammonium concentrations may be very low in natural seawater.

2.7.5.3 Reagents

Reagent 1:

- Dissolve subsequently 17.5 g of phenol (p.a.) and 0.2 g of sodiumnitroprusside ((Na₂(Fe(CN)₅NO).2H₂O) in MilliQ water and dilute to 500 ml with MilliQ water
- Transfer the solution to a dispenser and label it as 'Reagent 1' (R1)
- Store in a refrigerator until use.

Reagent 2:

- Dissolve subsequently 140 g of tri-sodiumcitraate-dihydrate (p.a.) and 11 g NaOH (p.a.) in MilliQ water
- When complete dissolution is reached, add 20 ml of sodium hypochlorite (Javel 10° BE)
- Dilute the solution to 500 ml with MilliQ water
- Transfer to a dispenser and store in a refrigerator until use.

Note: do not store this reagent for more than 14 days!!!

2.7.5.4 Procedure

Determination of the calibration curve:

- Prepare the standard solutions of ammonium as follows:
 - Dissolve 0.05349g of ammonium chloride (NH_4Cl) in 1000ml highly purified (or distilled) water. This solution contains 14mg N per litre (14mg/L or 14000 $\mu\text{g/L}$).
 - Make a series of dilutions of the stock solution to obtain concentrations in the range from 5 – 500 $\mu\text{g/L}$. Some suggested concentrations: 0 (blank)-5-10-25-50-100-150-200-500 $\mu\text{g/L}$
 - Use either freshly made synthetic seawater (see Section 2.7.4.4).
 - Then add the two reagents as explained below
- Add 3 ml of Reagent 1 to 100 ml of your standard solutions.
- Shake well and then add another 3 ml of Reagent 2 and shake thoroughly.
- Store in a dark place for a minimum of 12h but not more than 48h (preferably overnight)
- Measure the absorbance of your standard solutions at 630 nm wavelength
- Use highly purified water as a blank.

Remark: For better results prepare your standard solutions in duplicate, so that you finally work with a mean value.

Determination of the NH_3 -concentration in the samples:

- Measure 100 ml of your seawater sample
- Treat it exactly the same as you did for the standard solutions
- Several replicates are generally advised

3 Biotic factors

3.1 Seston

3.1.1 Definitions

Seston is the term used for **all particulate matter in suspension** in the water.

So seston is composed of all particles $> 1 \mu\text{m}$ (= particulate) which cannot maintain their position against a current (= in suspension). It includes all kinds of particles and living organisms from bacteria to zooplankton. In practice, bacteria and zooplankton are not often considered in seston studies, which concentrate mainly on **phytoplankton, detritus and inorganic particles** (e.g. sand).

A general quantification of the composition of a seston sample can be obtained from:

Analysis	Quantified seston component
Dry weight	Total particulate matter (TPM): <ul style="list-style-type: none">• organic matter• inorganic matter
POC (Carbon Analyzer or wet oxidation method)	Amount of organic matter: <ul style="list-style-type: none">• living phytoplankton• detritus
Chlorophyll by spectrophotometric method or HPLC	Living phytoplankton

3.1.2 Sampling

Water samples (usually 5-20 l) are taken with a water sampler (e.g. Niskin bottle) at the desired depth. Division in sub-samples is done fractionally (divide a number of small volumes over each of the sub-samples).

3.1.3 Seston Counting method

3.1.3.1 *Sample treatment*

For microscopical counting and analysis, samples are preserved with lugol's solution.

Preparation of lugol's solution (in 1l distilled water):

- 50 g I₂
- 100 g KI
- 100 g acetic acid (CH₃COOH)

The lugol's solution should be kept in a dark in a well closed glass bottle.

Samples of 0,25-1l are put into glass bottles. Add 2-3 ml of lugol's solution. Keep the bottles closed and in the dark (preferably in the fridge). After at least 2-3 days, the seston is sedimented to the bottom. The supernatant can be removed by suction.

Preferably use a plastic tube that is not being used for other purposes, as lugol's solution is absorbed by plastic. Hold the tube end just below the water surface, and reduce the volume to somewhat less than 100 ml.

Shake the bottle to free the seston from the bottom, and pour the content into a 100 ml cylinder and adjust the volume to 100 ml with tap water. The cylinder is covered with aluminum foil, and kept standing (in the fridge) for minimum two days, to allow settling. Then, the volume can be reduced to 10 ml by the same procedure as described above (see Figure 7).

These samples can be kept in closed glass containers and stored in the dark (preferably in the fridge). If the samples are stored for a long period (months), formaline should be added (1 ml of 40 % formaline per 10 ml of sample). For shorter periods, addition of some extra lugol's solution should be done every two weeks, so that the color is kept dark brown.

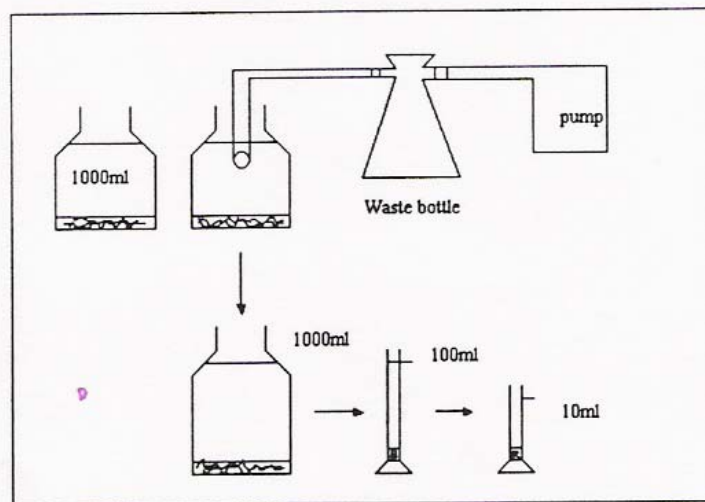


Figure 7. Schematic overview for the concentration of seston samples

3.1.3.2 Microscopic analysis

There are different types of microscopes:

1. Reversed microscope:
 - a. Bring a few ml's of the concentrated sample into the counting chamber (depending on the type of counting chamber used).
 - b. Allow to sediment for + 15 min. before analysis.
2. Normal microscope:
 - a. Bring a drop of the concentrated 10 ml sample on a glass microscope slide. Cover with a cover glass.
 - b. Put the slide on the microscope table under the clamps.

Analysis: (both type of microscopes)

1. Use the smallest magnification (shortest objective) to get an overview of the sample.

2. Focus with the control knob. When the slide's contents are focused under low magnification, switch to a higher magnification.
3. Only use the fine adjustment knob when focusing under high power magnification.
4. For a good recognition of the phytoplankton cells, an objective 40 x should be used.

Qualitative observations

In a first phase, seston analysis can be limited to the identification of the dominant phytoplankton types and the determination of the proportion of phytoplankton cells to other particles. This is done by looking at a certain number of viewing fields (e.g. 20). In each viewing field, the number of phytoplankton cells and the number of other particles (mostly detritus) are counted. From the total counts of all the fields, the proportion phytoplankton - detritus is calculated.

Quantitative observation

Counts (concentration in sample), using a reversed microscope
 At a given magnification M_i a number of viewing field (N_v) are analyzed.
 In each viewing field, the number of particles of interest (n) are counted.

Calculations

$$N = \frac{n \cdot x}{N_v} \cdot \frac{1}{v_2} \cdot \frac{1}{cf}$$

With N: concentration of particles in the original sample (particles/ml)
 x: nr. of viewing fields in the cuvette at magnification

$$M_i = \frac{\text{surface cuvette } (S_C)}{\text{surface viewing field } (S_v)}$$

v_2 : volume of concentrated sample in the cuvette (ml)
 n : nr of particles counted in N_v viewing fields
 N_v : nr of viewing fields counted
 cf : concentration factor

to determine x:

1. Measure surface of viewing field (S_v), see Figure 8

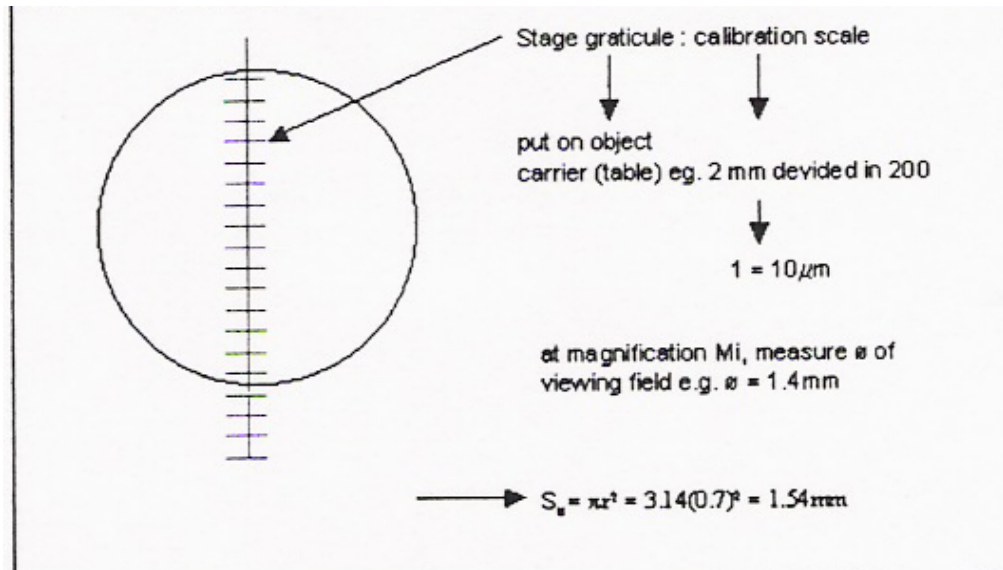
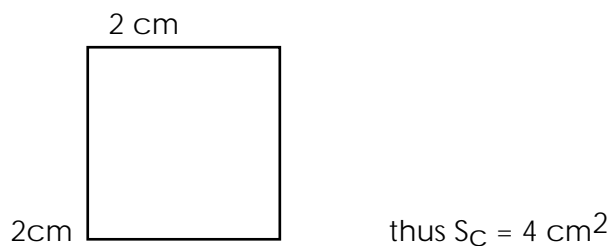


Figure 8. How to measure the surface of the viewing field of a microscope

2. Measure dimensions of cuvette (S_C)

e.g.

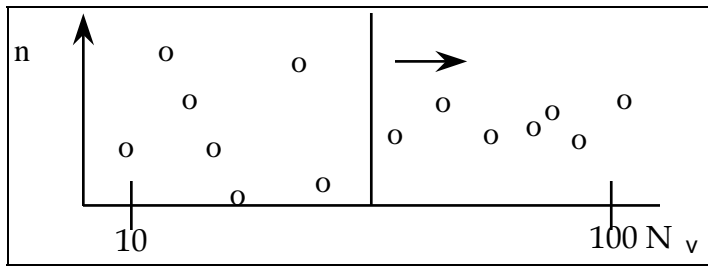


400

And thus $x = \frac{400}{1.54} = 260$

Remarks:

- cf, v_2 : can be adjusted to concentration of particles of interest in original sample.
- Sometimes different combinations necessary for different types of particles in one sample
- Magnification: M as particle size
- N_V : in principle, increase till $n \gg ct$, in practice $n \gg 100$ (at least > 20), depends on "feasibility"



Measuring the size of particles

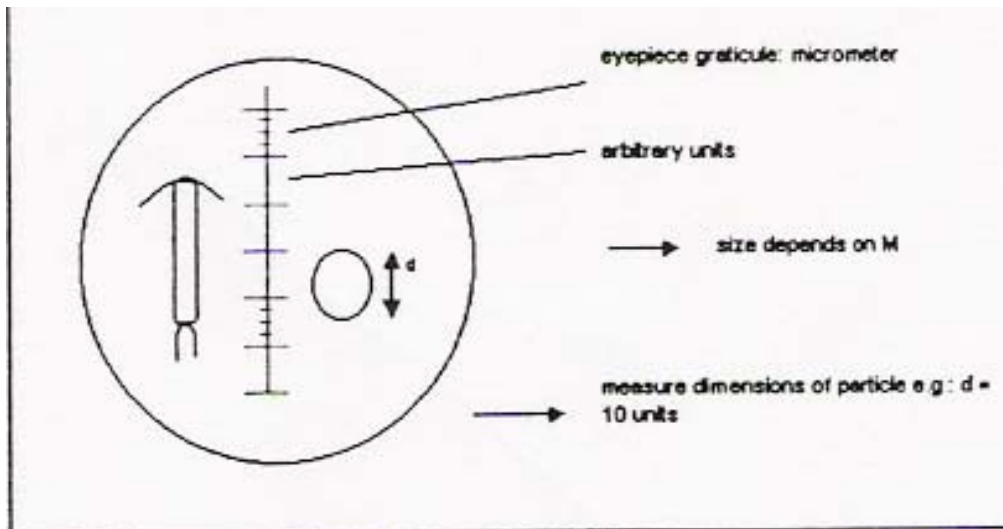


Figure 9. How to measure the size of particles under the microscope

And calibration:

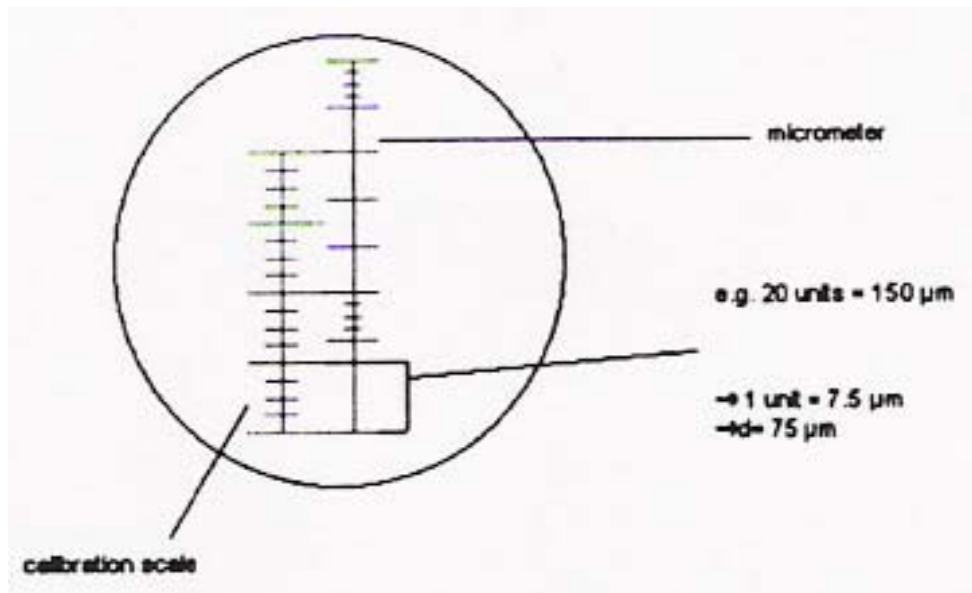


Figure 10. Calibration to determine the size of particles under the microscope

3.1.3.3 Electronical counting

Electronical particle counters count particles and divide them in a number of size classes (channels) according to their spheric equivalent diameter (S.E.D.). The particle concentration can be plotted as volume distribution, by multiplying the concentrations (in number of particles ml^{-1}) by the spheric equivalent volume (S.E.V.) of each size class (e.g. fig. II.1.2). For details see specific instructions of the apparatus at hand.

With e.g. Coulter Counter, Elzone

Analysis of a sample (with reference to Figure 11):

1. Before analysis, a count is made with filtered seawater to check the background (electrical noise).
2. A beaker (4) with ± 200 ml of the sample to be analyzed is put under the measuring tube.
3. The aperture tube and electrode (1,2) are emerged in a beaker with ± 200 ml of the sample to be analyzed (4). The aperture tube (opening of the orifice) (3), is chosen in function of the largest particles present in the samples (to be determined experimentally). Sometimes multiple tube analysis is necessary to cover the complete range of particle sizes. The volume of water sucked through the orifice can be adapted to each type of tube by the manometer (5). after each count, the results are either printed by the coulter-population count accessory (PCA) unit (6), or stored in the computer for further data processing.

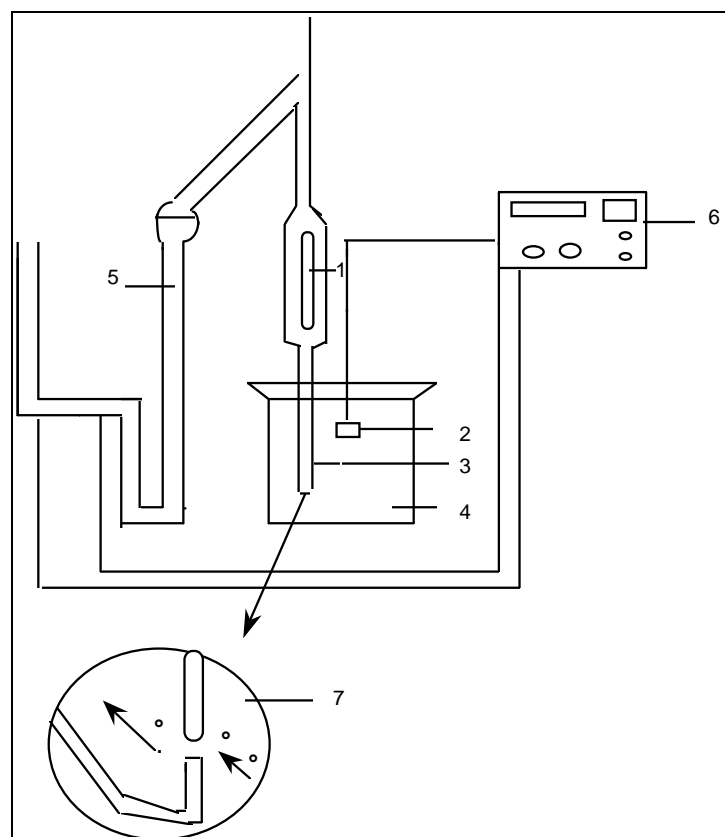


Figure 11. Schematic overview of an electronic particle counter

3.1.4 Total Particulate Matter (TPM)

The amount of total particulate matter present in a sample can be quantified as dry weight.

Glass-fiber filters (Whatman GF/C 0.47 mm) are numbered and predried at 60 °C during > 6 hours. The blank weight of each filter is determined on a electrobalance (see specifications for each model) after the filter has cooled for a few minutes in a dessiator.

Samples are filtered onto a glass-fiber filter (Whatman GF/C 0.47 mm) (keep suction low < 10 cm Hq vacuum), and dried at 60° C for 6 hours. Usually, 1 l samples are filtered, but the volume can be adjusted to the seston concentration at hand. As a rule of thumb, material should be clearly visible on the filter, but the filter should not clog (filtration must be fluent).

Dried filters are cooled to room temperature in a descicator and weighed on a micro-balans

$$\frac{(\text{DW sample} - \text{DW blank}) \times 1000}{\text{volume of sample filtered (ml)}} = \text{g/l} \quad \text{if the reading of the micro-balans is in g}$$

3.1.5 Organic Matter/Particulate Organic Carbon (POC)

Particulate Organic Carbon is a measure of the amount of organic material (phytoplankton + detritus) in a seston sample.

3.1.5.1 Sampling and methods

Sea water is filtered on a GF/C-filter (47 mm of diameter) under low suction.

Take care not to touch the filters with your hands when handling them: always use a pincette!

Standard, 1 l of sea water is filtered. Sample size can of course be adjusted to the concentration of seston in the samples.

For storage, filters can be deep-frozen after packing them in a clean special filter box or in aluminium foil.

POC can be determined with:

- a) a carbon analyzer
- b) the wet oxidation method

As a C-analyzer is not always available, only the principle of this method is given. The wet oxidation method is described completely.

3.1.5.2 The C-analyzer

The function of this instrument is based on an automatic coulometric titration. Carbon is transformed to carbon dioxide (CO₂), which is absorbed in an alkaline barium perchlorate (Ba(ClO₄)₂) solution. This causes a decrease of pH of the solution. With barium hydroxide (Ba(OH)₂) formed by electrolysis, an automatic back titration to the initial pH takes place. A built-in electronic unit transforms the measured amount of electricity used for the back-titration into counts displayed, of which 1 count represents 2 x 10⁻⁷ g C.

- Total Particulate Carbon (T.P.C.): all of the carbon present is oxidized to CO₂ in an oxygen stream while the filter is heated to 900° C in a quartz tube.
- Particulate Inorganic Carbon (P.I.C.): all of the inorganic carbon present is converted to CO₂ in an acid solution (8.5 % orthophosphoric acid (ortho-H₃PO₄)).
- Particulate Organic Carbon (P.O.C.)
P.O.C. = T.P.C. - P.I.C.

3.1.5.3 The wet oxidation method

Carbon is determined by "wet ashing" with a mixture of potassium dichromate (K₂Cr₂O₇) and concentrated sulphuric acid (H₂SO₄). The decrease in extinction of the yellow dichromate solution after it has been reduced by the organic matter is measured.

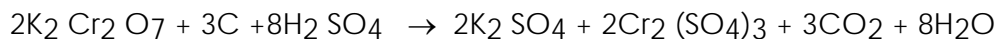


The following text describes the practical handlings for performing this method.

This method uses strong acids. Be careful !! Wear a lab-jacket !

Principle

C is oxidized by acid dichromate



The reaction is quantified by measuring the decrease in adsorption of the K₂Cr₂O₇ solution spectrophotometrically.

Equipment

- GF/C filters (47 mm of diameter), filtration apparatus
- an oven to reach 100-110°C
- a spectrophotometer
- ideally stoppered graduated measuring cylinders.
- 50 ml Pyrex glass beakers fitted with cover-glasses.

Cleaning of the glassware: the beakers should be cleaned in hot chromic-sulphuric acid cleaning mixture and stored in a desiccator or another container where dust

contamination is impossible. It is also possible to just clean the beakers thoroughly with alkaline soap and hot water, rinsing them thoroughly with aq. dest, rinse with diluted nitric acid (HNO_3) and rinse them again with aq. dest.

Reagents

1. Sulphuric acid-dichromate oxidant.
 - a. Dissolve 4.84 g of potassium dichromate $\text{K}_2\text{Cr}_2\text{O}_7$ in 20 ml of distilled water.
 - b. Add this solution little by little to about 500 ml of concentrated analytical quality sulphuric acid (H_2SO_4 p.a.) in a 1000 ml volumetric flask.
 - c. Cool the mixture to room temperature and make to volume with more concentrated acid.
 - d. Store the solution in a glass stoppered bottle. This solution stays stable indefinitely.
2. Phosphoric acid : use an analytical reagent grade of 70 % H_3PO_4
3. Sodium sulfate solution: Dissolve 45 g of best quality anhydrous sodium sulfate Na_2SO_4 in 1000 ml aq. Dest

Procedure

a) sampling

- filter a convenient volume of sea water or algal culture in triplate on a GF/C-filter under low suction, allow the filter to be sucked dry.
- add 2.0 ml of the sodium sulphate solution, and immediately suck the filter dry; repeat this once more.
- fold the filter double in aluminium foil, put in fridge or deep-freeze while preparing calibration and measurements reagents (if you're not performing the analysis immediately)
- proceed together with calibration series, see next page

b) Calibration and measurement

- prepare a standard glucose solution: dissolve 7.50 g of pure glucose ($\text{C}_6\text{H}_{12}\text{O}_6$) in 100 ml aq. dest. Keep this solution in the refrigerator (if you plan to store longtime, add a few crystals of mercury (II) chloride (HgCl_2)).
- diluted glucose solution for calibration curve:
 - dilute 10.0 ml of the glucose stock solution to 1000 ml with aq. dest.
 - make a new diluted solution daily.
- take 13 GF/C filters
- take 17 beakers of 50 ml: 3 blanks, 4 samples + 5 x 2 for 5 different concentrations of glucose solution
 - in 13 of them: put a filter moistured with 1 ml phosphoric acid, cover with Al. foil.
 - in the 4 remaining beakers: put the filters with your samples and moister with 1 ml phosphoric acid
- heat the beakers for 30 minutes in the oven at 100-110° C

- to three of the beakers: add 4.0 ml aq. dest and 10.0 ml of the sulphuric acid-dichromate oxidant = blanks
- to 5 x 2 beakers: add 10 ml oxidant solution and an increasing number of mls of the diluted glucose solution (e.g. 0.5-1-2-3-4 ml), if necessary adjusting to 14 ml with aq. dest.
- replace the Al. foil on the beakers and reheat them all for 60 minutes in an oven at 100-110° C
- let the mixture cool to room temperature
- transfer the mixture with filter pulp to a volumetric flask of 50 ml, washing the sides of the beaker thoroughly with aq. Dest
- bring to volume with aq. dest and mix
- allow most of the filter pulp to settle
- decant 10 ml into a centrifugation tube
- centrifuge for about 5 minutes at 2000 rpm
- adjust the wavelength of the spectrometer to 440 nm.
- zero the spectrometer with a cuvette filled with aq. Dest
- measure the extinction of each blank solution
- measure the extinction of the calibration samples and the phytoplankton samples.

Calculation

a) the calibration curve

E: mean extinction of blanks mean - extinction of calibration samples

The molecular weight of glucose (C₆ H₁₂ O₆) is 180, of which 40 % is carbon weight.

The glucose stock solution (being 7.50 g in 100 ml) contains 40 % or 3.00 g carbon/100 ml.

The stock solution is diluted 100 times (10 ml stock diluted to 1000 ml). The diluted glucose solution contains

$$\frac{3 \text{ g}/100 \text{ ml}}{100} = 30 \text{ mg C}/100 \text{ ml} = .3 \text{ mg/ml} = 300 \text{ } \mu\text{g/ml}$$

Thus: 1 ml diluted glucose solution corresponds to 300 μg of carbon

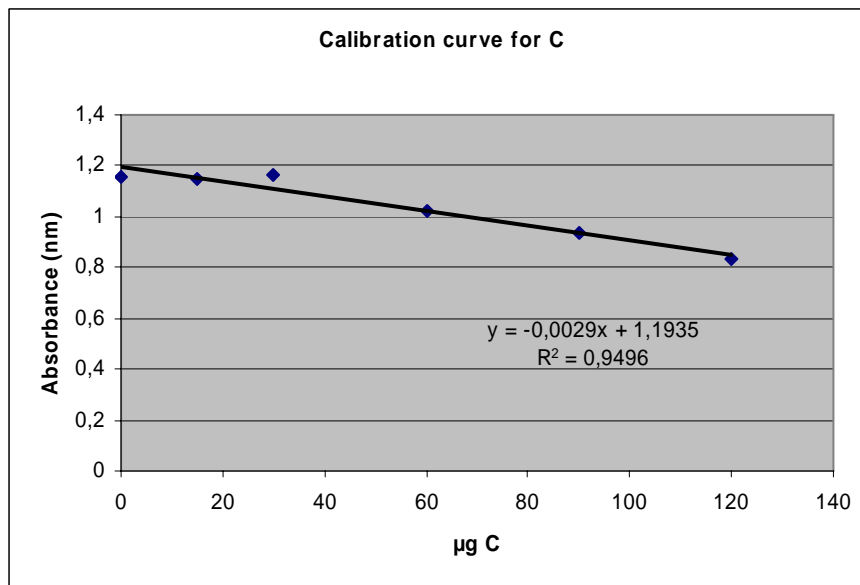


Figure 12. Example of a calibration curve for POC

b) natural samples

Calculate, from the extinction values measured in the calibration, the linear regression between extinction and amount of carbon.

$$E = aC + b$$

Whereout: $C = \frac{E-b}{a}$ µg carbon in the solution measured

Thus: $C = \frac{E-b}{aV}$ µg C/l for V liter of sea water filtered

Important remark: The **ratio P.O.C./Chl a** gives information about the **phytoplankton part in the organic matter**.

3.2 Phytoplankton

3.2.1 Standing stock quantification

The only rapid chemical method known for estimating living plant matter in the particulate organic matter of sea water is to determine the characteristic plant pigments: the chlorophylls, carotenes and anthophylls.

The methods described determine the three chlorophylls commonly found in planktonic algae: chlorophyll a, b and c.

3.2.1.1 Spectrophotometric quantification of chlorophyll pigments

Sampling

In the standard procedure, 1 l of sea water is filtered on a GF/C-filter under low suction. Sample size can of course be adjusted to the phytoplankton concentration in the sample.

If immediate measurement is impossible, the filters can be stored in a deep-freezer for some weeks after packing them in a clean special filter box or aluminium foil.

Equipment

- GF/C-filters, filtration apparatus
- centrifuge tubes of 15 ml capacity, with glass or polyethylene stoppers
- centrifuge
- spectrophotometer

Reagents

90 % acetone (CH_3COCH_3) in aq. dest. The acetone is especially purified for chromatographic and spectrophotometric purposes.

Procedure

- The filters are brought into 10 ml of 90 % acetone in a centrifuge tube and immediately placed in the fridge, as chlorophyll is very unstable in light.
- During a period of at least 1 hour and maximum 24 hours in the refrigerator, the chlorophyll pigments are extracted out of the phytoplankton by the acetone.
- The samples are centrifuged for 10 minutes at 3000 rpm

If not measured immediately, the samples are put back in the fridge or at least in the dark until measurement in the spectrophotometer.

The basis of quantification is the difference in extinction of light between the pigments and a blank at 665, 645 and 630 nm.

- Fill two cuvettes with 90 % acetone (blanks)
- Adjust the wavelength to 750.
- First zero the spectrometer by putting the blank cuvette in position.
- Measure the extinction after 2nd blank (correction for possible differences in optical properties of cuvettes).
- fill the second cuvette with the supernatant of the centrifuged sample (be careful to avoid stirring up of filterparticles)
- Measure the extinction
- Switch to 665 nm wavelength, repeat zeroing and measurement
- Repeat at 645 and 630 nm

Calculation

The chlorophyll values are estimated following the Parsons-Strickland formulas.

Subtract reading at 750 nm from all other readings.

units : $\mu\text{g/ml}$ if a 1 cm cuvette is used

$$\begin{aligned} C(\text{Chl a}) &= 11.6 E_{665} - 1.31 E_{645} - 0.14 E_{630} \\ C(\text{Chl b}) &= 20.7 E_{645} - 4.42 E_{630} - 4.34 E_{665} \\ C(\text{Chl c}) &= 55 E_{630} - 4.64 E_{665} - 16.3 E_{645} \end{aligned}$$

The results obtained from these formulas are multiplied by a factor f , in order to obtain the chlorophyll concentrations in mg/m^3

$$f = \frac{v}{l \times V}$$

where l = length of cuvette (in cm) this means the length of the path through which the light passes the cuvette

v = ml of acetone used for extraction

V = volume of sea water filtered (in l)

3.2.1.2 Measurement with H.P.L.C. (High Pressure Liquid Chromatograph demonstration)

The working of a HPLC is shown in Figure 13. Calculation of the Chlorophyll contents are based on standard curves.

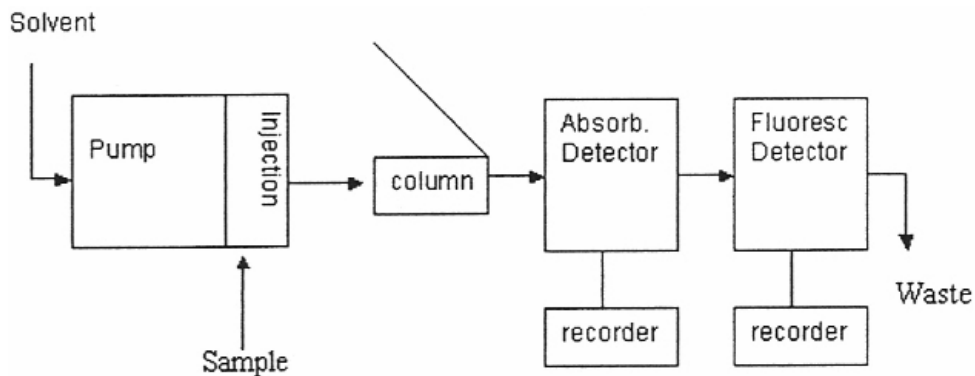


Figure 13. High Pressure Liquid Chromatograph (HPLC)

3.2.2 Primary production/Respiration

The phytoplankton population growth depends on **growth coefficient (b)** and biomass (B)

$$\frac{dB}{dt} = b \times B \quad \frac{\text{mg}}{\text{m}^3 \times \text{t}}$$

The growth coefficient (b) is determined by

- k = photosynthesis rate
- r = respiration rate
- e = excretion rate
- m = mortality rate
- p = predation rate

Therefore: $\frac{dB}{dt} = (k-r-e-m-p) B = \text{net production}$.

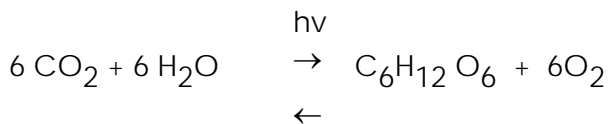
Gross production = net production + respiration + excretion + mortality + predation

Thus:

- population increase if: $k > r+m+e+p$
- population decrease if: $k < r+m+e+p$

In lab experiments with cultures 'r' (respiration rate) is the most important loss-factor and thus $r \gg m + e + p$

Remember the photosynthesis reaction:



Photosynthesis thus leads

- to an increase in the O_2 concentration in the medium
- to an incorporation of inorganic carbon into the cells and a formation of organic carbon in the cells

The photosynthesis rate can therefore be measured as

- an increase in O_2 concentration in the medium (in a closed system) during a period of time unit (e.g. 1 hour). The O_2 concentrations can be measured with the Winkler method (see section 2.6.2).
- the amount of "newly formed organic matter", incorporated into the cells, during a period of time.

3.2.2.1 Radioactive tracers

To distinguish existing and newly formed organic matter, a radioactive tracer $\text{HC}^{14}\text{O}_3^-$ is introduced in the medium (at t_0) and the amount of C^{14} incorporated in the cells after a period of time is measured. C^{14} in the cells can be measured in a

scintillator counter (as desintegration part per minute (DPM)) after filtration of the samples on a GFC filter.

The principle of using tracers:

It is hypothesized that the tracer is incorporated to the same relative extend as the natural compound.

We therefore determine, in the case of photosynthesis, the fraction of the tracer ($\text{HC}^{14}\text{O}_3^-$) incorporated in the cells (filtered on a GFC filter) during a time period and we multiply this by the total amount of natural HCO_3^- ($\text{HC}^{12}\text{O}_3^-$) in the medium (mg/m^3). This gives the total amount of HCO_3^- that is incorporated per time unit and thus the rate of photosynthesis.

Excretion rate: e

Excretion(exudation) of organic matter leads to a loss of organic matter from the cells and to an addition of dissolved organic matter in the medium.

The excretion rate can be measured considering this "new" dissolved organic matter. The excretion rate is therefore measured as the increase in radioactive organic material (after elimination of the inorganic radioactive material (evaporation)) in the solution (filtrate) during a period of time.

Respiration rate: r

A respiration rate can be measured as

- a decrease in oxygen in the medium (closed system) after a period of time. This gives a measure of the total sample respiration rate (including respiration by phytoplankton, bacteria and zooplankton).
- a loss of organic matter from the cells, (filtered on a GFC filter) during a period of time after pre - introducing $\text{HC}^{14}\text{O}_3^-$ in the medium for a period of time, to allow the cells to incorporate C^{14} before the experiment starts. This value also includes loss due to excretion. One can distinguish light (= photo-respiration) and dark respiration.

3.2.2.2 O₂-method

Primary production and respiration of algae (micro or macro) can be measured by determining the O_2 produced and respired in closed bottles during different incubation. Due to the production or respiration of the organism studied, the O_2 concentration in the water will change.

Outline

Fill 3 bottles (100 ml) with seawater or culture (using a plastic tube) and fix immediately with MnSO_4 and alkaline KI to determine O_2 concentration at time 0 (C_{t_0}) (see section 2.6.2).

Fill n 100ml bottles with (seawater) culture where n is determined by the number of samplings x 3 replicate; *i.e.* 4 x 3 bottles for 4 samplings.

Incubate for different periods of time (e.g. 30, 60, 120, 180 minutes) in the light incubator (light bottles, LB) and in the dark (DB), at constant water temperature.

Determine O_2 concentration in all bottles with the Winkler method described in detail in section 2.6.2. The changes in O_2 concentrations in the **light bottles** are due to **photosynthesis and respiration**. The changes in O_2 concentrations in the **dark bottles** are due to **respiration** only.

When oxygen concentration is calculated (mg/l), primary production (NP and GP) and **respiration** (R) can be calculated as follows:

$$R [\text{mgO}_2\text{l}^{-1}\text{hr}^{-1}] = C_{t_0} - C_{\text{DB}}$$

Where

- C_{t_0} = oxygen concentration at time zero
- C_{DB} = oxygen concentration in the dark bottle
- T = incubation time (hr)

Net production (NP) is calculated from the equation:

$$NP [\text{mg l}^{-1}\text{hr}^{-1}] = C_{\text{LB}} - C_{t_0}$$

Where

- C_{LB} = oxygen concentration in the light bottles at the end of the incubation

Gross production (GP) is calculated as follows:

$$GP [\text{mgO}_2\text{l}^{-1}\text{hr}^{-1}] = NP + R = C_{\text{LB}} - C_{\text{DB}}$$

When working with phytoplankton, the cells are considered to be **homogeneously distributed** over the incubation bottle and the volume sampled is considered as a measure of 'biomass'. Production and Respiration can thus be directly converted into 'in situ' rates (e.g. $\text{mgO}_2\text{l}^{-1}\text{hr}^{-1}$)

When working with organisms which are not homogeneously distributed in the water (e.g. seaweeds, zooplankton) one measures the effect of the production and/or respiration of these organisms on the oxygen concentration of the water only. To calculate the production or respiration of the organisms, the amount of oxygen which has been produced (respired during the incubation) has to be calculated.

Example: Primary Production experiment with a piece of macroalgae with a wet weight of 3g, incubated in 100ml of water. Incubation time : 6 hours.

$$C_{t_0} = 0.7 \text{ mg l}^{-1} \pm 0.1 \text{ mg l}^{-1}$$

$$C_{LB} = 1.0 \text{ mg l}^{-1} \pm 0.15 \text{ mg l}^{-1}$$

Difference in oxygen concentration due to primary production by the leaf

$$\Delta O = 1.0 \text{ mg l}^{-1} - 0.7 \text{ mg l}^{-1} = 0.3 \text{ mg l}^{-1} = \text{Net amount of oxygen produced in 6 hours}$$

$$NP = 0.3 \text{ mg l}^{-1} / 6 \text{ hr} = 0.05 \text{ mg O}_2 \text{ hr}^{-1} \quad \text{by 3g of algae}$$

$$= \frac{0.05 \text{ mg O}_2 \text{ hr}^{-1}}{3 \text{ g}} \quad \text{per g wetweight}$$

For incorporation in the C-cycle, production and respiration can be converted into $\text{mg Cl}^{-1} \text{ hr}^{-1}$ (see Section 2.6.2.5)

From the different O_2 concentrations measured we can therefore calculate in $\text{mg O}_2 \text{ l}^{-1} \text{ hr}^{-1}$:

$$\begin{aligned} R &= C_{t_0} - C_{DB} \\ NP &= C_{LB} - C_{t_0} \\ GP &= NP + R = C_{LB} - C_{DB} \end{aligned}$$

3.2.2.3 C-14 Method

Important:

- Wear plastic gloves at all times when working with radio-activity!
- Do not inhale scintillation liquid vapors!

Measuring Primary Production (PP) as a function of time: kinetics

The primary production can be measured by determining the amount of C^{14} incorporated in the cells as organic matter after addition of a known quantity of $\text{NaH}^{14}\text{CO}_3$. The following time series will show a linear uptake indicating that recalculation to hourly rates does not introduce errors.

The fraction of tracer incorporated into the cells per time unit (= $\text{DPM counted} / \text{DPM added} \times t$) is thus determined in a time series.

By multiplying this fraction with the total amount of dissolved inorganic C in natural marine waters ($25\,000 \text{ mg/m}^3$) one obtains the total amount of C incorporated into the cells per time unit.

Procedure for C^{14} incorporation in function of time:

- Fill 2 bottles of $\pm 400 \text{ ml}$ with a phytoplankton culture or seawater, add $20 \mu\text{Ci}$ of $\text{NaH}^{14}\text{CO}_3$ to each bottle (see remark about DPM added), shake well.

Incubate 1 bottle under light condition (incubator) and 1 bottle in the dark, at constant temperature.

- Filter immediately (t_0) and after different time intervals (cfr. O₂ method) 3 x 10 mls of samples from the light and dark bottles on glass-fiber (GF/C.45 μm) filters (\varnothing 25 mm). Wash the filters with an excess of seawater.
- Put filters (+ 1 blank: either a dry filter, or a filtered on which non radioactive culture has been filtered) in scintillation-bottle. Let the filter dry, and add a few drops of H₂SO₄ (10 %) let evaporate (to CO₂). Add 10 ml of scintillation liquid. Mix thoroughly and put in the scintillation counter for analysis after at least 2 hours.
- Use of scintillation counter: see instructions on apparatus at hand.
- With a modern apparatus, counts per minute (CPM) are automatically corrected for noise and the readout gives desintegrations per minute (DPM).
- Take 3 x 10 ml samples out of each bottle at time intervals : 30, 60, 120, 180 minutes
- DPM values of blank filters have to be subtracted from these.

Calculation :

$$PP = \frac{\text{DPM}/10 \text{ ml} \times 25000}{\text{DPM added}/10 \text{ ml} \times t} \quad (\text{mg C}/\text{m}^3/\text{hr})$$

Where:

DPM : readout of the scintillation counter, corrected for blank value
 t : incubation time
 25000 : concentration of dissolved carbon in sea water (mg m^{-3})

Remark : Be careful on the units used !

DPM: Radio-active NaH¹⁴CO₃ is obtained in glass containers which usually contain (1 mCi) of RA liquid. The usual procedure to divide this amount into smaller amounts of e.g. 50 μCi , is to dilute the 1 ml to 20 ml with distilled water. So a stock is obtained in which 0,4 ml ^a 20 μCi . The exact amount of radioactivity added has to be determined for each set of measurements. To keep the read out of the undiluted trace measurement within the range of the machine, put a small amount (eg 0,2 ml \sim 10 μCi) in a scintillation bottle). Add 10 ml of scintillation liquid. Analyse in scintillation counter after at least two hours.

3.2.3 Primary Production (PP) at various light intensities

The PP at different light intensities (necessary to integrate PP over the water column, different depths and thus different light intensities) can be measured by incubating a series of bottles (200 ml), covered with light filters (0-100 % transparency) over a period of time (usually 3 hours). The transparency of the bottles can be measured beforehand with a lux meter.

To determine PP in the water column in situ incubation can be done at several depths, as shown in Figure 14. All bottles are attached to a floating body. Light and

dark bottles are incubated at the surface, while light bottles are incubated at several depths.

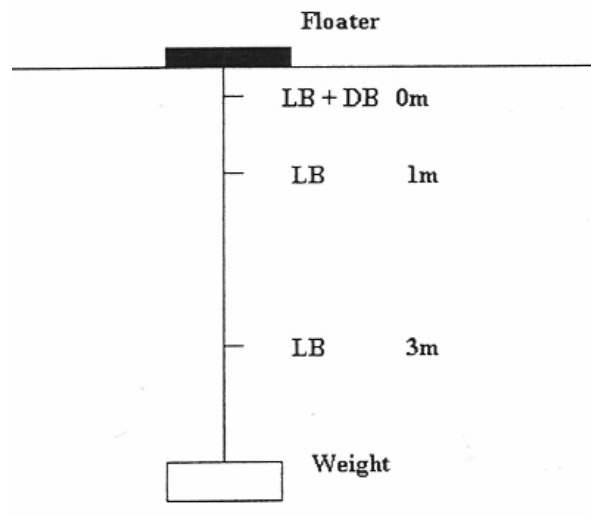


Figure 14. Experimental set-up for determination of primary production in the water column

As an example, Total production of the water column can then be calculated as:
Example (see Figure 15):

PP measured at surface : 183 mg C m⁻³ hr⁻¹
 PP measured at 1 m depth : 75 mg C m⁻³ hr⁻¹
 PP measured at 3 m depth : 0 mg C m⁻³ hr⁻¹

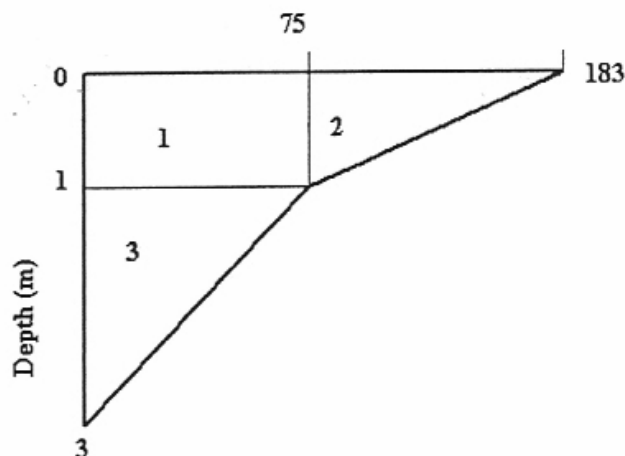


Figure 15. Example of calculation of total primary productivity in the water column

Total PP over the water column:

$$1) 75.00 \times 1 = 75$$

$$2) \frac{(183 - 75) \times 1}{2} = 54$$

$$2$$

$$3) \frac{75.00 \times 2}{2} = 75$$

$$\text{Total} = 203.91 \text{ mg C m}^{-3} \text{ hr}^{-1}$$

$$\text{Average: } \frac{203.91}{3} = 67.97 \text{ mg C m}^{-3} \text{ hr}^{-1}$$

3.3 Zooplankton

3.3.1 Standing stock

3.3.1.1 Sampling

For zooplankton sampling a known volume of water (50-200 l) is filtered through a 50 or 65 μm net. The concentrated zooplankton is rinsed into a small container, and preserved with formaline. Final concentration 4 %; so for example 10 ml formaline (40 %) is added to 90 ml of sample. The concentration of formaline has to be exact, if the samples will later be used for weight determinations, because dry weight of organisms are affected by formaline.

For sampling of larger zooplankton organisms, which have a lower in situ abundance larger volumes of water have to be filtered. This can be done with a high-speed sampler, pulled behind the ship. The volume of water sampled is then measured by means of a flow-meter, attached in the mouth of the net. The read-out of the meter is noted before and after the tow.

Calculation

The opening area of the plankton net must be known or has to be calculated. The water volume passed through the plankton net (V) is determined as follows:

$$V \text{ (l)} = n \times 0,3 \times S \times 1000$$

Where

n= indicated number of revolutions

S= net opening area in $\text{m}^2 = \pi \times r^2$

Example

The Plankton Net Model 438 030 has a diameter of 40 cm, i.e. the opening area is $0,125 \text{ m}^2$. If the number of revolutions associated with a tow is 266 (noted from the flow-meter counter), the water volume passed through the plankton net is $V = 266 \times 0,3 \times 0,125 \times 1000 = 9\,975 \text{ l}$

3.3.1.2 Counting

Countings can be done to determine the density of individuals.

1. Samples are concentrated to a small volume over a 50 μm net, and all organisms are counted on a grid-petri plate under binocular.
2. If the samples contain too many organisms (takes too long to count), the sample is brought in a beaker and the volume is adjusted to a certain value (e.g. 600 ml). The sample is mixed with a stirrer on a magnetic turning table, and a subsample of e.g. 10ml can be taken and counted on a petri-dish under binoculars.
3. The volume of sample in the beaker and the sub-sample taken can be adjusted according to the concentration of animals in the sample. Sometimes different dilutions or sub-samples have to be taken for different stages (e.g. nauplii/adults of copepods) which can differ greatly in abundance within one sample.

3.3.1.3 Dry weight determination

Use of the ovens and balances: see specific instructions.

- When determining dry weight of marine organisms care has to be taken to remove salt particles adhering to the organisms. Rinse in fresh water.
- The weight of the "recipient" in which the organisms are weighed (e.g. aluminum foil), must be as low as possible to allow an accurate weighing. If filters are used, a series of blanks must undergo the same treatment as the samples (e.g. rinsing with fresh water, drying) to determine an average blank-value.
- To obtain a reliable weight measurement for a certaintype of organism, one has to obtain a sample that is "representative" of the population in question.
 - If the organism is large enough to measure its weight individually, several specimens can be weighed one by one.
 - If the organisms are too small, a sample containing a (large) number of individuals has to be weighed.

Sample size

To determine what is the minimum size of the sample to be representative, series of different sized samples (e.g. 10 animals; 50 animals, 100 animals sample⁻¹) can be analyzed in replicates. Sample size is sufficient if the average weight measured obtains a constant value. Figure 16 shows average weight of one copepod (Y-axis) as a function of sample size (X-axis). It is shown that from sample size 40 onwards, the average weight stabilizes.

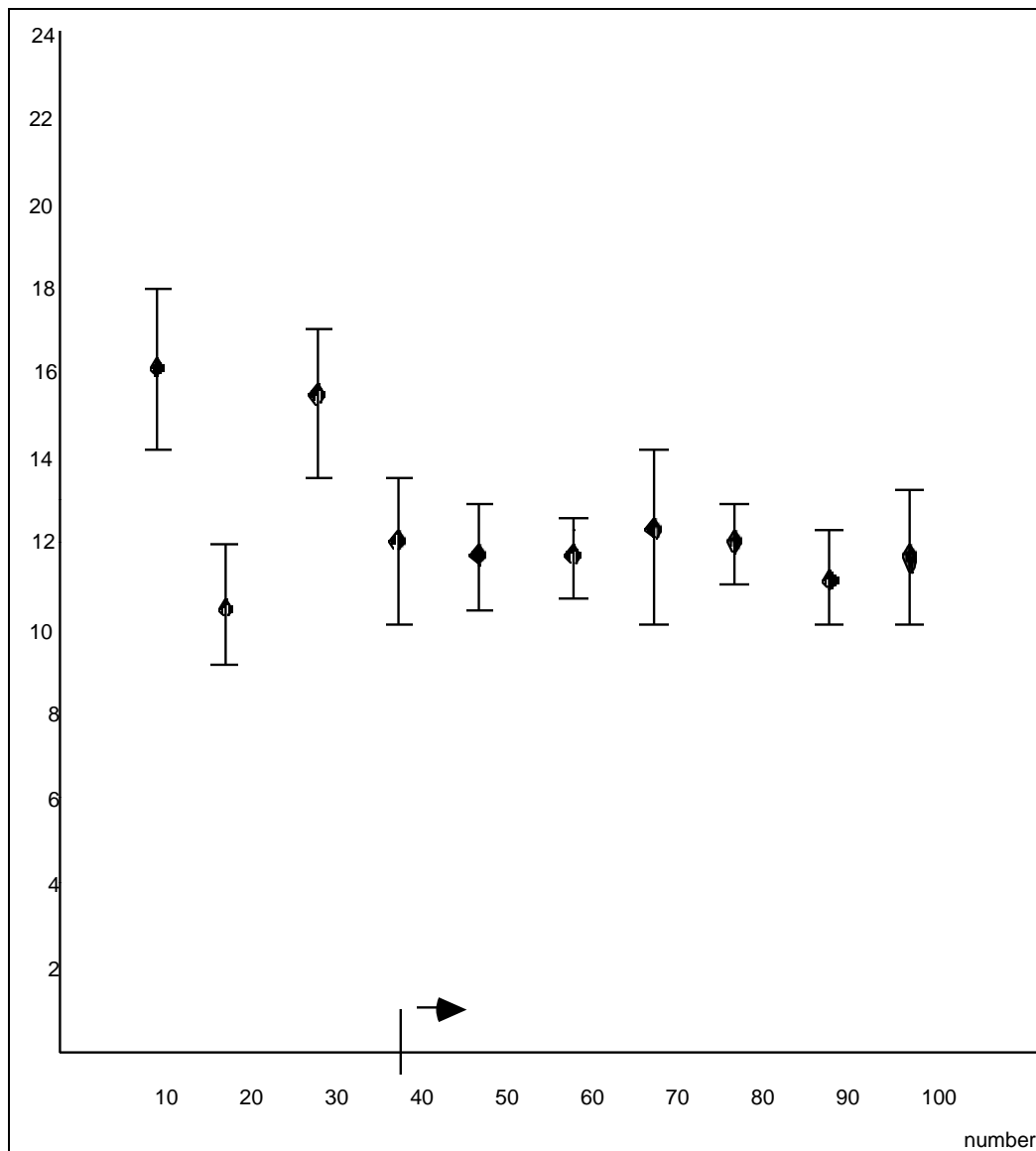


Figure 16. Determination of appropriate sample size for copepod weight from the North Sea (Source: Van Gijsegem, 1979)

Remarks

1. When samples are stored prior to dry weight analysis a preservative is added. For crustaceans a formaline solution of 4 % is used. The addition of this preservative however causes a certain loss of dry weight.
2. A correction factor fresh-dry weight versus preserved-dry weight has to be determined in these cases. This factor is also influenced by the time the samples are kept in formaline solution.

3.3.2 Feeding (Grazing)

3.3.2.1 C-14 method

The herbivorous activity of an animal is quantified by measuring the uptake of radioactive plant material.

In plankton experiments the phytoplankton (culture or natural sample) can either be pre-labeled (A) or labeled during the experiment itself (B, direct labelling).

Important:

- Wear plastic gloves at all times when working with radio-activity
- Do not inhale scintillation liquid vapors

A) Pre-labeling

- To 1100 ml of algal suspension, add 50 μCi of $\text{NaH}^{14}\text{CO}_3$. Incubate for 12-24 hours under light conditions.
- Filter 2 x 50 ml of the pre-labeled culture onto GFC ($.45\mu\text{m}$) filters. Rinse filters with a few ml's of filtered seawater, and put them in a scintillation bottle.
- Add zooplankton (e.g. ± 25 adult *Artemia* l^{-1}).
- Incubate for 0.5 hrs.
- Remove zooplankton (filter over a net ($50\mu\text{m}$)).
- Filter 2 x 50 ml's of the filtrate on GFC filter. Rinse with a few ml's of filtered sea water.
- Put filter in scintillation bottle.
- Isolate the animals in e.g. 5 sets of 5 individuals. Put in scintillation bottles with as little water as possible. If animals have to be stored prior to analysis, put them in deep freezer (e.g. in a plastic container with a little bit of filtered sea water), or preserve with formalin (4 % concentration). Also put one blank filter.
- To all of the scintillation containers add 10 ml of scintillation liquid. Count in scintillation counter after 24 hours.

For use of the scintillation counter: see instructions on the apparatus at hand.

B) Direct labeling

- Bring 200 μCi l^{-1} of $\text{Na}_2^{14}\text{CO}_3$ into the algal suspension on which grazing activity is to be measured.
- Bring zooplankton into the grazing bottle.
- Animals can either be brought in individually (after isolating them under binocular) or concentrated on a 50-100 μm net. If natural zooplankton concentrations are high, concentration is not necessary. As a rule of thumb ± 25 adult copepods are necessary for measuring radio-activity. Incubate for 1 hour under light conditions.

Further procedures as in A).

Calculations

A) Pre-labeling

$$F = \frac{\text{DPM}_{\text{zoo}} \text{ at time } t}{\frac{\text{DPM}_{\text{phyto } t_0} + \text{DPM}_{\text{phyto } t}}{2}} \cdot \frac{1}{t} \quad (\text{ml animal hour}^{-1})$$

with : F : clearance rate.

DPM_{zoo} : desintegrations per minute in zooplankton (DPM animal^{-1})

$\text{DPM}_{\text{phyto}}$: desintegrations per minute in phytoplankton (DPM ml^{-1}).

t : incubation time (h)

n : number of animals used

B) Direct labeling

$$F = \frac{\text{DPM}_{\text{zoo}} \text{ at time } t}{\frac{\text{DPM}_{\text{phyto } t}}{2}} \cdot \frac{1}{t} \quad (\text{ml animal hour}^{-1})$$

3.3.2.2 Counting method

The counting method measures the feeding activity of an animal on suspended particulate matter by quantifying the difference in particle concentration between a grazing bottle (with animals) and a control bottle (without animals). Determination of the particle concentration can be done by microscope (see Section 3.1.3.2) or by an electrical counter (Coulter Counter).

The procedure is as follows:

- Fill 9 glass bottles with the particle suspension on which grazing is to be measured to about halfway the bottle volume (usually 200 ml bottles are used). When working with natural samples, these have to be pre-filtered through a 100-150 μm net to remove zooplankton. Take care to fill the bottles gradually (dividing sub-samples of water over the different bottles).
- Isolate 3 x N(**) Artemia in a small volume of algal suspension (e.g. 20 ml). Add the Artemia to 3 of the bottles (grazing bottles).
- Fill all bottles upto 200 ml exactly with suspension, close them and put them in a rotating incubator (2 rpm.), preferably in the dark. Keep 3 samples for time 0 (t_0) sampling.
- From each t_0 bottle take subsamples for the following analysis. Shake bottle well to homogenize before sampling
 - 50 ml + lugol → microscopic count
 - 50 ml → Coulter Count
 - 2 x 25 ml → Chl a
- At the end of the experiment, (t ~ 6 h) take sub-samples from each bottle for analysis as explained above for t_0 .

* N depends on the size of Artemia sp. (e.g. 10 adults, 50 nauplii), see also remark on the density of experimental animals below

Calculations

$$C_t = C_o \cdot e^{kt} \quad C_{zt} = C_o \cdot e^{(k-g)t}$$

$$k = \frac{1}{t} \ln \frac{C_t}{C_o} \quad g = \frac{1}{t} \ln \frac{C_t}{C_{zt}}$$

With k : growth constant (hr^{-1})
g : grazing constant (hr^{-1})
Co : particle concentration (in control-and grazing bottles) at time o
Ct : particle concentration in control bottle at time t
t : incubation time (hr)
Czt : particle concentration in grazing bottle at time t.

$$F = \frac{V \cdot g}{n}$$

With: F : clearance rate ($\text{ml animal}^{-1} \text{ hr}^{-1}$)
V : volume of water used (ml)
n : numbers of animals in the grazing bottle

$$I = \frac{F \cdot C_o (e^{(k-g)t} - 1)}{(k-g) t} \text{ (particles animal}^{-1} \text{ h}^{-1}\text{)}$$

With: I = ingestion rate.

When counting particle concentration with an electronical counter, these formulae are applied to each of the size classes. Total ingestion rate is calculated by summing over all size classes.

3.3.3 Respiration

3.3.3.1 O₂ method

The respiration (R) of aquatic animals can be measured by enclosing them in a (dark) BOD bottle (50-100 ml) and measuring the difference in oxygen concentration between control bottles without animals and bottles with animals after incubation.

It is important that the **exact volume** of the respiration bottle is known: use calibrated BOD bottles or determine the exact volume by filling the bottle completely with tap water, closing it with a given glass-stopper, and afterwards pouring the water into a measuring cylinder.

- Fill 6 bottles of 100 ml with algal suspension (or filtered sea water), using a plastic tube to avoid air bubbles.
- Fill 3 bottles halfway. Isolate 3 x N(*) Artemia in a small volume of algal suspension (e.g. 20 ml) with a pipette. Add N animals to each of the halfway filled bottles. Fill bottles completely with algal suspension, close all bottles.

- Note the time at the beginning of the incubation (t_0). Fix 3 t_0 bottles (with algal suspension only) to determine O_2 concentration at t_0 (see Section 2.6.2.4), C_0 .
- Incubate 3 control (with algal suspension only) and 3 respiration bottles (with *Artemia*) at room temperature during 4-6 hours.
- Note the time at the end of the incubation (t_e), to determine the exact incubation time
- fix all bottles and determine the O_2 concentration at t_0 (mean), t_e control (mean) and t_e (respiration) for each bottle with the Winkler method described in Section 2.6.2.

Calculation of respiration rate :

For each respiration bottle, calculate:

$$\Delta O_2 = C_c - C_r$$

Where

C_c = mean concentration of O_2 in the control bottles at t_e (after incubation)

C_r = concentration of O_2 in the respiration bottle at t_e (after incubation)

$$\text{Total amount of } O_2 \text{ used} = \frac{\Delta O_2 \times v}{1000} \quad \text{mg } O_2$$

Where

v = volume of water used (ml)

The respiration rate is calculated as follows:

$$R = \frac{\Delta O_2 \times v}{1000 \times t \times n} \quad \text{mg } O_2 \text{ animal}^{-1} \text{ hour}^{-1}$$

Where

t = incubation time (hours) = $t_e - t_0$

n = number of animals used in each bottle.

Remark

$C_0 - C_c = O_2$ produced or consumed by algae and bacteria in the control bottles

Density of experimental animals

In experiments where a process is quantified by measuring differences in concentration of a variable (e.g. difference in O_2 concentration in a respiration experiment) care has to be taken to some methodological conditions:

- To measure a decline in O_2 concentration, the experiment has to be carried out in a closed system, so there is only a given amount of oxygen present ($O_2 = \{O_2\} \times \text{volume flask}$).

- To be able to measure respiration, one has to put enough animals in the flask so that the difference in $\{O_2\}$ is large enough to be detected with the measuring device used (Winkler method, O_2 electrode).
- On the other hand, one cannot put too many animals in one flask, because their respiration will be influenced by stress, and by the available oxygen.
- The factor incubation time can influence the results in a similar way as the concentration of test animals.

3.4 Bacteria

3.4.1 Standing stock

3.4.1.1 Sampling

Water is usually collected by means of a water sampler (cf abiotic factors, seston) and brought into sterile glass bottles before further treatment.

3.4.1.2 Plate count

Marine bacteria can be grown and the colonies visually counted on petri dishes:

- 22,4g marine agar powder is diluted in 400 ml distilled water using a 500 ml jar. The bottle is covered with cotton and Aluminum foil and sterilized for 20 minutes in an autoclave. The air pressure of the autoclave is dropped to take out the bottle and ± 15 ml agar is poured onto the petri dishes (don't put the cover on the table and sterilize the bottle mouth between filling 2 petri dishes), leave for 24 hours.
- 0.05 ml seawater is added to the petri dishes using sterile pipets (3 hours, in oven at $180^\circ C$), sterilizing the mouth of the pipet holder after taking a pipet.
- The seawater drop is spread on the dishes with a bacterial spreader (sterilized with alcohol and heating (above the flame)).

The dish walls may not be touched !

- The same routine is applied with serial dilution series of 10^{-2} , 10^{-4} and 10^{-6} ($10^{-2} = 0,1$ ml in 10 ml sterile seawater). The sterile seawater is made by putting 10 ml of seawater in test tubes, closing them with metal covers and sterilizing in the autoclave for 20 minutes.
- Two dishes per dilution is usually taken as a minimum.
- The petri dishes are left (reversed) for x days and counted (10 days for marine bacteria).

Considering the number of colonies counted, the dilution factor and the volume of sea water added to the petridish one can then calculate the estimated average number of colonies/ m^3 .

In a similar way, abundance of bacteria such as coli's (e.g. *E. coli*) or streptococs can be measured on special agartypes after incubation at specific temperatures. In these cases, dilution of the sample is usually not necessary.

3.4.1.3 Acridine Orange Direct Count (AODC)

Materials

- Black polycarbonate nuclepore filters ($\text{Ø}=25$ mm, pore size= 0.22 μm)
- 0.1% Acridine Orange (filtered through 0.22 μm GFC filter)
- Non-fluorescent immersion oil
- glass slides
- glass cover slips

Procedure

- Samples of ± 20 ml are fixed with 40 % formalin (2 % final concentration) and stored at 5 to 10° C in the dark.
- Place a black polycarbonate nuclepore filter on a filterstand. If only white filters are available, these can be put in irgalan black solution for a few hours and rinsed with deionized water before use.
- Place 2 ml of water sample into the filtering apparatus. Add 0.2 ml acridine orange (final concentration 0.01%). Filter after 3 minutes. Use low suction (<20 cm Hg)
- If the bacterial numbers per viewing field are too high, dilutions can be made. If bacterial numbers are too low, the volume to be filtered can be increased up to 10 ml, but with the proportionate amount of acridine orange (final conc. 0.01%)
- Put a drop of oil on a glass slide. Transfer filter from the filter stand to the glass slide. Place another drop of oil on top of the filter and cover with a cover slip. Add one more drop of oil on top of the slip.
- Counting is done under an epifluorescence microscope using blue light excitation (470 nm wavelength) at 1250 x magnification.
- Bacterial cells will fluoresce bright green in a black background. Count fluorescing bacteria in 10 viewing fields. Measure the diameter of a viewing field using a calibration slide.
- Prepared slides must be kept in the dark (use provided slide tray) and can be stored at 4°C (fridge) for a few days.

NOTE : When using the microscope, please do not change any settings except for focusing purposes. Clean the objectives after use.

Microscopic Calculation

number of bacteria/ml =

$$\frac{N \times a}{c \times b \times d}$$

With N : number of bacteria counted in c viewing surfaces
a : area of the filter
b : area of the viewing-field
c : number of viewing fields counted
d : volume of sample filtered.

Biomass calculations

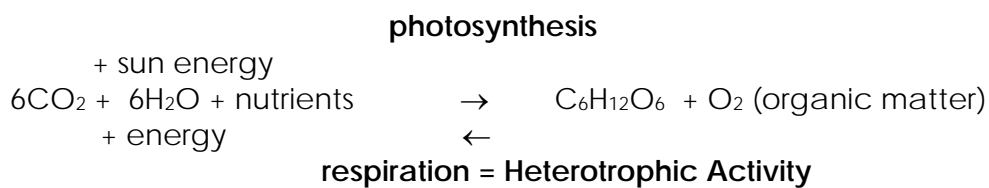
Biomass is expressed in mgC/m^3 and is calculated by :

number of bacteria/ml $\times 20 \times 10^{-15}$ gC/cell (Lee and Fuhrman, '87)

If it is possible to measure length and width of a bacterial cell, the conversion factor of 5.6×10^{-13} $\text{gC}/\mu\text{m}^3$ can be used (Bratbak, '85).

3.4.1.4 *Heterotrophic Activity*

Principle



O₂ consumption and B.O.D.₅

The planktonic respiration or the amount of O₂ consumed can be calculated after dark incubation (see section 3.2.2.2).

The amount of "easily degradable" organic material can be measured by the biological oxygen demand method (B.O.D.₅): B.O.D. for 5 days: (B.O.D.₅) at 18° C is often taken as a standard setup.

50 ml BOD bottles are filled completely (with a tube), closed (no air !) and kept at constant water t° in dark. The O₂ is fixed at 0, 30, 60, 90, 120, 180 hours and 5 days (see primary production) (use triplicates).

3.4.1.5 *Incorporation and metabolization of radioactive glucose C¹⁴*

Principle

The uptake rate of a substrate (glucose (vg)) can be determined by the use of a radioactive tracer (C¹⁴-glucose)

Theoretically as:

$$\frac{\text{C}^{14} \text{ taken up by bacteria}}{\text{C}^{14} \text{ added}} \quad (\text{concentration of lglucose (natural + C}^{14} \text{ glucose added)}) \quad t$$

$$\text{Vg} = \frac{\text{dpm filter (natural glucose concentration (g C/l) + g C}^{14}\text{/l added)}}{\text{dpm added} \times t}$$

$$= \frac{\text{g C}}{\text{l hour}}$$

As the uptake of glucose is regulated by an active transport system, the uptake rate (V_g) increases with the water glucose concentration until saturation (v_{max}).

As the glucose incorporated into the bacterial cells is respired after a certain time, the C^{14} glucose on the filter (A) in function of time is only linear for a certain period. The initial uptake rate from the filter is therefore calculated as dpm/h or % ^{14}C glucose incorporated/hour.

-As the natural substrate concentration is often not known, the bacterial activity is expressed as a turnover rate $T = \frac{\text{Radio-activity added (dpm)}}{\text{initial uptake rate (dpm/h)}}$

The uptake rate can also be calculated from the addition of the ^{14}C cells (A) and the $^{14}CO_2$ respired back into solution and measured in the filtrate (B)

Procedure

250 ml of seawater is poured into a sterile 250 jar, 0.1 μCi glucose C^{14} is added and the solution is mixed. The sample is kept at water temperature, in dark. A sub-sample (10 or 20 ml) is taken at time 0, 20, 40, 60, 120, 180 minutes on a 0.2 μm filter, (under vacuum).

A) The filter is washed with 5 ml saline water (under vacuum) and dropped into a scintillator flask. Add 10 ml scintillation liquid to the flask and measure the DPMS after 2 hours in a scintillation counter.

The curve of radioactivity (C^{14}) on the filters in function of time allows one to calculate the initial rate of uptake of C^{14} glucose (DPM/h). The turnover time of glucose (t) is then:

$$t(h) = \frac{\text{Radioactivity added (DPM)}}{\text{initial uptake rate (DPM/h)}}$$

B) the filtrate is collected and the respired CO_2 in the filtrate immediately bubbled into a 10 ml scintillation liquid with addition of 0.2 ml H_2SO_4 .

The radioactive CO_2 can directly be measured in the scintillator (Don't forget blanks).

The respiration curve: * $^{14}CO_2$ in function of time can be determined. The curve has a lag. time (the original, non radioactive CO_2 is eliminated).