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Guide to best practices for ocean acidification research and data reporting

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5 Bioassays, batch culture and chemostat experimentation

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5.1 Introduction

This section describes some general guidelines and considerations to be observed when carrying out experiments that manipulate the CO_2 levels in laboratory cultures of microorganisms, especially for phytoplankton. The main premise for the establishment of these guidelines is that the experiments should represent the conditions of the future ocean as accurately as possible with respect to the carbonate system. Other manipulations mimicking for example the low CO_2 levels of glacial times or excessive CO_2 enrichments are also valid, for example to test for tipping points or to improve our understanding of certain processes. Regardless of whether one is working with mixed natural populations of microorganisms, or with monospecific cultures, there are two basic approaches that can be used to carry out manipulations of the carbonate system in small volume experiments: (1) batch and dilute batch cultures in the laboratory, alternatively called grow outs or bioassays in the field and (2) continuous cultures or chemostats and their variations such as turbidostats and cyclostats. A third alternative is to use semi-continuous cultures which are periodically diluted with fresh culture media in order to keep cells in the exponential growth phase. Batch, dilute batch and continuous cultures are best suited to answer different types of questions and have associated advantages and drawbacks that are discussed below. These types of approaches have been used for monospecific cultures in the laboratory and mixed microbial populations in the field (Hutchins *et al.*, 2003; Sciandra *et al.*, 2003; Cullen & Sherrell, 2005; Leonardos & Geider, 2005).

The acceptable methods for manipulating the carbonate chemistry have been described elsewhere in this guide to ocean acidification research (chapter 2) and are only briefly summarised here. The most representative ways to adjust the CO_2 levels so that they accurately reflect the conditions in the future ocean are (1) to aerate the culture media and growing cultures with an air-CO_2 gas mixture containing the desired CO_2 levels (Rost *et al.*, 2008), (2) to add equimolar amounts of HCO_3^- and HCl or (3) to add CO_2-saturated seawater to a known volume of culture media (Schulz *et al.*, 2009). All of these methods are indistinguishable in terms of their effects on the carbonate chemistry. The method of choice for culture experiments, either an open aerated system or a closed system without headspace (Figure 5.1), will depend on the questions addressed, and on the phytoplankton species that are under investigation. In general, large or fragile phytoplankton, such as for example *Trichodesmium erythraeum* and dinoflagellate species, might be affected by the turbulence created by aeration. Thus, the effect of aeration and bubble size on growth rate and the general physiology of a phytoplankton species should be assessed in preliminary experiments. The goal of culturing phytoplankton in either batch cultures or chemostats is often to optimise cell yield. This is reflected by the frequent use of nutrient-rich culture media and high cell density at harvest. Ocean acidification research has a different goal; rather to accurately represent the present and future

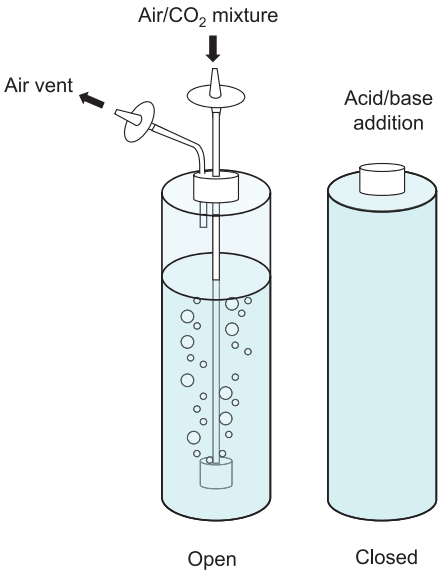

Figure 5.1 Representation of open and closed vessels to manipulate carbonate chemistry in laboratory cultures.The closed system does not have a headspace.

carbonate chemistry in the ocean. In all types of experiments involving culturing techniques, this will require working at low cell density to avoid undesired change in the carbonate chemistry during the experiment, as recommended in chapter 2 of this guide.

5.2 Approaches and methodologies

5.2.1 Culture media

Culturing phytoplankton requires the preparation of a sterile, nutrient-enriched seawater medium. The variable nutritional requirements of the diverse phytoplankton species and the particular questions addressed by different investigators have led to the formulation of diverse culture media. The detailed description and composition of several commonly used culture media can be found on homepages of various culture collections (e.g. https://ccmp.bigelow.org/node/73) and only basic principles are discussed here. Seawater sterilisation techniques include autoclaving, microwaving, UV irradiating or filtering through a 0.22 µm filter. The effect of some of these sterilisation techniques on the carbonate chemistry have been discussed in chapter 2. While autoclaving is the most effective method of sterilisation, a drawback of this approach is the resulting degassing of the medium, which significantly affects the carbonate chemistry. It is important to re-equilibrate the autoclaved medium with sterile air or an appropriate air/CO_2 mixture.

Although many researchers use natural seawater for the preparation of enriched culture media, artificial seawater-based media have been developed for experimental work that requires a completely defined seawater composition, for example to avoid uncharacterised dissolved organic matter found in natural seawater. Additionally, an artificial seawater culture medium is useful when access to clean, oceanic water is restricted or when seasonal variations in the seawater composition affect reproducibility of culture conditions. The classical formulation of nutrient amendment of f/2 (882 µmol kg^{-1} $NaNO_3$, 36.2 µmol kg^{-1} NaH_2PO_4, 106 µmol kg^{-1} Na_2SiO_3) is specifically targeted for growing coastal diatoms (Guillard, 1975). Dilutions of the f/2 nutrient amendment, for example f/50, are often used when working with phytoplankton species that prefer lower nutrient concentrations such as, for example, *Emiliania huxeyi*. K medium (Keller & Guillard, 1985; Keller *et al.*, 1987) designed especially for the growth of oceanic phytoplankton, includes many nutrients not present in the classical f/2 media derivatives, such as NH_4Cl and Na_2 b-glycerophosphate, and selenium. The 10 times higher chelation of trace metals by ethylenediaminetetraacetic acid (EDTA) in K medium helps achieve good growth of oceanic species that are generally more sensitive to trace metals then their coastal counterparts. Similarly, there exist several formulations of artificial seawater media but the most widely used are ASW (Goldman & McCarthy, 1978), ESAW (Harrison *et al.*, 1980; Berges *et al.*, 2001), and AQUIL (Morel *et al.*, 1979; Price *et al.*, 1989), the latter being a specific medium for the study of trace metal speciation. Marine photosynthetic diazotrophs such as *Trichodesmium erythraeum* are grown in YBCII, an artificial seawater medium free of fixed nitrogen species (Chen *et al.*, 1996). SN medium for marine cyanobacteria (Waterbury *et al.*, 1986) prescribes the addition of 100 µmol kg^{-1} Na_2CO_3 to the natural seawater. In summary, no universal culture medium suitable for the growth of all phytoplankton exists, because specific groups of phytoplankton require specialised media. It is therefore very important to report precisely the constituents of the culture medium, when possible using the appropriate citation, and when applicable, describing any modifications made to the original formulation. For natural seawater media, the collection point, the collection date and background nutrient concentrations of the water should be reported, as well as any treatment that could have modified the chemistry of the seawater.

5.2.2 Selection of phytoplankton strains

The choice of phytoplankton species, strains and clonal isolates can have a significant effect on the outcome of the experimental work. Definitions and concepts related to phytoplankton strains have been reviewed by Lakeman *et al.* (2009). It is important to acknowledge that the first selection step in a microalgal culture is the isolation of the targeted organism in culture. Isolation methods, such as single cell picking, sorting by flow cytometry or enrichment cultures followed by end-point dilution all introduce some bias in selection pressures. Following isolation, the establishment of the culture will also lead to selection of organisms that are best suited

for the culture conditions, which may not necessarily reflect the natural conditions. The establishment of a new culture after the initial isolation process will eventually lead to genetic adaptation of the strain, as selection processes drive the phenotype of the culture to a new optimum. Maintenance of cultures will vary between laboratories and this can lead to divergence of the initial strain into several distinct phenotypes, potentially leading to variation in experimental results between investigators. The coccolithophorid *Emiliania huxleyi* is particularly prone to changes in phenotype during long-term culturing of stock cultures, sometimes leading to the loss of calcifying ability with time. Media rich in nutrients leading to high cell density, as well as prolonged periods of time in the stationary phase, may cause this shift in phenotype (Paasche, 2001; Lakeman *et al.*, 2009). Alternatively, it can also be argued that the culturing environment is less variable than the natural environment, and may lead to the accumulation of conditionally neutral mutations that are purged only under more stressful conditions. Stress tolerance may therefore be modified or lost in long-term culturing of a strain.

Readers are referred to the review of Lakeman *et al.* (2009) for detailed recommendations to address the effects of various evolutionary processes that can occur when working with cultures. Some general recommendations are listed below:

1. The use of cryopreserved phytoplankton strains whenever possible would reduce the impact of mutations. Most commercially available phytoplankton strains from major culture collections are cryopreserved and would allow comparisons of experiments conducted several years apart with the same starting genetic material.
2. The effect of recombination can be minimised by avoiding sexual reproduction.
3. Small population bottleneck causing genetic drift can be avoided by frequent transfers of large innocula.
4. Culture conditions should mimic the natural conditions of the isolates as closely as possible.
5. Culture conditions, strain description and source should be described as completely as possible. A small sample of the cells should be collected and frozen at the time of the experiments to allow for future verification of the strain identity and its genetic makeup. This may be important in cases where different investigators are obtaining divergent results with a presumed common strain.

Although cultures obtained from culture collection are monoalgal, they are not always axenic, i.e. free of contaminating bacteria. Recent phytoplankton isolates require time to become firmly established in culture and cannot immediately be cultured in axenic conditions. The process of rendering a culture axenic is tedious, but is usually an attainable goal if a methodical approach is followed (e.g. Berg *et al.*, 2002). In some cases, the phytoplankton species may grow better when cultivated with the bacterial contaminants. While it can be argued that axenic cultures are not representative of the natural environment, contaminating bacteria introduce an additional level of complexity to culture experiments that cannot always be controlled, as for example in the study of dissolved organic matter. Bacterial contaminants may vary between phytoplankton strains and may no longer have a relevance to the natural environment from which the strain was isolated. Whenever possible, experiments should be carried out with axenic phytoplankton strains, which can be obtained from most major culture collections and are best maintained as stock cultures in autoclaved media. Establishing that a culture is axenic requires microscopic observation with an epifluorescent microscope after staining a filtered culture aliquot with DAPI or acridine orange. Simple observations of a culture with a light microscope are not sufficient to ascertain that a culture is axenic. The addition of 1 ml of marine broth (e.g. Difco™ Marine Broth 2216) to a 2 ml culture aliquot, together with appropriate controls (e.g. sterile culture medium) incubated for several days and examined by epifluorescence microscopy also provides additional evidence of sterility when bacteria are in low abundance in the phytoplankton culture (Berg *et al.*, 2002). Stock cultures and transfer media should be tested for bacterial contamination with every maintenance transfer. When a strain cannot be obtained as an axenic culture or if recent isolates contain bacterial contaminants, it may be important to identify the phylogenetic affiliation of the contaminants using molecular biological methods such as ribotyping (e.g. sequencing of the 16S rRNA gene).

5.2.3 Batch cultures

Batch cultures are generally used when studying growth in the presence of excess nutrients. Predetermined nutrient concentrations are added to the culture medium, with the essential macro- and micronutrients provided in a fixed ratio, which is usually a variation of the classical nutrient-rich f/2 medium (Guillard, 1975). Classical batch cultures in rich nutrient medium have the potential to reach very high biomass and are prone to large shifts in carbonate chemistry. After a short lag phase, growth is exponential and represents the maximum growth rate achievable under the selected light and temperature conditions. After a period of exponential growth, a stationary phase is reached, often because one of the nutrients is exhausted. Alternatively, cells may reach their upper pH limits or become CO_2 limited long before nutrients become limited in nutrient-rich medium (e.g. Hinga, 2002; Hansen *et al.*, 2007). The initial amount of pre-adapted cells that is inoculated to initiate an experiment will have an effect on the length of the time lag preceding exponential growth. A large inoculum can shorten the time lag to a minimum, but under these initial conditions, some cultures will quickly reach high cell densities, which may change the growth conditions and the carbonate chemistry. The need to keep the carbonate chemistry as constant as possible in ocean acidification research has led to the development of dilute batch culturing of phytoplankton. Under dilute growth conditions, the phytoplankton biomass at the time of harvest should have drawn down less than 5% of the total dissolved inorganic carbon (DIC) in the culture medium (Zondervan *et al.*, 2002). The dilute batch cultures are usually carried out at lower nutrient concentrations than the classical f/2 medium with a N:P ratio of 24. In contrast, typical nutrient concentrations used in dilute batch cultures are around 100µmol kg^{-1} nitrate and 6.25 µmol kg^{-1} phosphate (Zondervan *et al.*, 2002; Langer *et al.*, 2009), with an N:P ratio of 16.

In all types of cultures, it is important to ensure good pre-conditioning of the microorganisms. The pre-culture should be grown at the same experimental conditions (light, temperature and nutrients) as the experimental cultures for more than five generations. Pre-culturing of the cell inoculum can be done in a small volume as long as the cultures are kept at low cell density and in the exponential phase until the onset of the experiment. After inoculation with the pre-culture, the experimental culture should be grown for 5 to 10 generations in the exponential growth phase before harvesting. The maximum cell density that can be sustained at the time of harvest, without introducing large changes in the DIC (<5%) will decrease with increase in species cell size, and can be estimated from the cell carbon quota of a given phytoplankton species. Empirical relationships between cell volume and cell carbon quota can provide a first order approximation of the targeted cell density when planning experiments (Mullin *et al.*, 1966). For coccolithophores such as *E. huxleyi*, a typical inoculum and final cell concentration at the time of harvest are in the range of 30 to 100 cells ml^{-1}, and 50,000 to 60,000 cells ml^{-1}, respectively. The size of the inoculum as well as the final cell density of the culture that can be reached without significant changes in the carbonate chemistry are thus prescribed by the cellular carbon quota.

For all culture types, it is important to carefully choose the time of sampling and keep this routine throughout the experiment because cell division can be synchronised by the light/dark cycle prevailing in the laboratory incubations or in the field (van Bleijswijk *et al.*, 1994; Zondervan *et al.*, 2002). If cultures are grown under continuous light, i.e. rather artificial conditions for most phytoplankton, the responses can differ strongly from those observed under more realistic light conditions (e.g. Rost *et al.*, 2006). Similarly, it is important to mix cultures at least daily to prevent settling of cells. This is more important for cultures that are not continuously aerated with an air/CO_2 mixture and can be done manually by gentle inversion of the culture bottles, continuous rotation, or by a stirring rod. Stir bars should be avoided whenever possible because they can lead to damage in larger cells, such as dinoflagellates.

5.2.4 Grow outs or bioassay experiments

Grow outs or bioassay experiments are a type of batch cultures carried out with natural microbial communities, for example on shipboard field experiments. They are often combined with nutrient additions and serve a special purpose in identifying the nutrient that is limiting primary production or other biological processes. Classical examples of successful applications of bioassay experiments in oceanographic research are those of Ryther & Dustan (1971) and

Martin & Fitzwater (1988). The latter provided the first evidence that iron was a limiting nutrient in the high-nutrient, low-chlorophyll (HNLC) regions. Bioassay experiments are usually incubated for one to several days depending on the oligotrophy level of the water with which they are performed, and their main purpose is to determine the short-term physiological response of the microbial community to a treatment. Short-term incubations while minimising bottle effects do not allow the assessment of the full acclimation potential of the natural microbial community. In bioassay experiments, the nutrient enrichments are small compared to the concentrations added to culture media, and are normally of the order of 1 to 2 µmol kg^{-1} of nitrate or ammonium, and 0.1 to 0.2 µmol kg^{-1} phosphate. Compared to experiments conducted with monoalgal cultures, larger volumes are required to obtain enough biomass to carry out physiological rate measurements such as carbon and nitrogen fixation. Grow outs or bioassays are a simple way to carry out the manipulation in the field or at sea. However, the confinement of natural populations in a small bottle of 1 to 5 l may lead to bottle effects and heterotrophic bacterial growth, and grazing may be a problem in certain areas. The effect of grazers could be monitored by control incubations with and without grazers (Landry *et al.*, 1995), and shifts in microbial community structure can be monitored using molecular biological techniques. As for monoalgal culture experiments, equilibration to reach the desired CO_2 levels can be carried our either by HCl/ HCO_3^- addition in equimolar ratio by aerating with an air-CO_2 gas mixture, or by the addition of a known volume of sterile-filtered water with saturated CO_2 concentration (see chapter 2). As for culture experiments, measurements of at least two parameters of the carbonate system are needed to confirm that the targeted CO_2 levels have been achieved. Grow out experiments and bioassay experiments are usually carried out over a period of a few days, and are generally too short to assess the effect of a particular treatment on microbial species succession. In oligotrophic areas, the incubated water samples will rapidly become nutrient-limited without the addition of nutrients. Short-term bioassay experiments, lasting a few days only, assess the physiological state and physiological acclimation of the microbial population present at the time of sampling.

Owing to the sluggish air-water gas exchange and reaction kinetics involved in CO_2/ HCO_3^- interconversion, the equilibrium of certain carbonate species establishes very slowly. Depending on temperature, target $p(CO_2)$, water volume and the aeration rate, the pre-conditioning of the seawater to the target $p(CO_2)$, may take place over the whole duration of the experiment. It is therefore important to document the experimental conditions with adequate measurements of the carbonate system in order to allow the calculation of the $p(CO_2)$ levels at the start and the end of such experiments. Some of these problems can however be circumvented by diluting/ exchanging with filtered pre-acclimated seawater at given time intervals as discussed in the next section on semi-continuous batch incubations (e.g. Tortell *et al.*, 2002, 2008).

5.2.5 Semi-continuous cultures

Semi-continuous cultures are different from traditional batch cultures in that the organisms can be maintained in exponential growth for long periods of time. Semi-continuous cultures have been very useful for studying the effect of trace metal limitation in phytoplankton (Sunda & Huntsman, 1995) and are currently applied to ocean acidification research (e.g. Kranz *et al.*, 2009). Logistically, these are easier to conduct than continuous cultures because the cultures can be diluted daily or once every few days, depending on the growth rate of the species under the experimental conditions. They do not require pumps and sophisticated instrumentation. However, prior information on growth rate is required in order to adjust the dilution frequency of the cultures every day in order to keep the cells in the exponential growth phase and the biomass to an optically thin cell density.

The difference in the cell density between semi-continuous and batch cultures is seen in Figure 5.2. As for the batch culture, the achieved growth rate is not determined by nutrients but rather depends on the light and temperature conditions. In such cultures, growth rates are not fixed by the dilution rate, and can be measured from the increase in cell density between a time interval. As for batch cultures, sampling should be done at roughly the same time every day to avoid diel variations in the physiological conditions of the cells.

Variations on the semi-continuous culture design have also been used in shipboard field experiments with natural microbial communities (Tortell *et al.*, 2002, 2008). These can be carried out longer than grow out

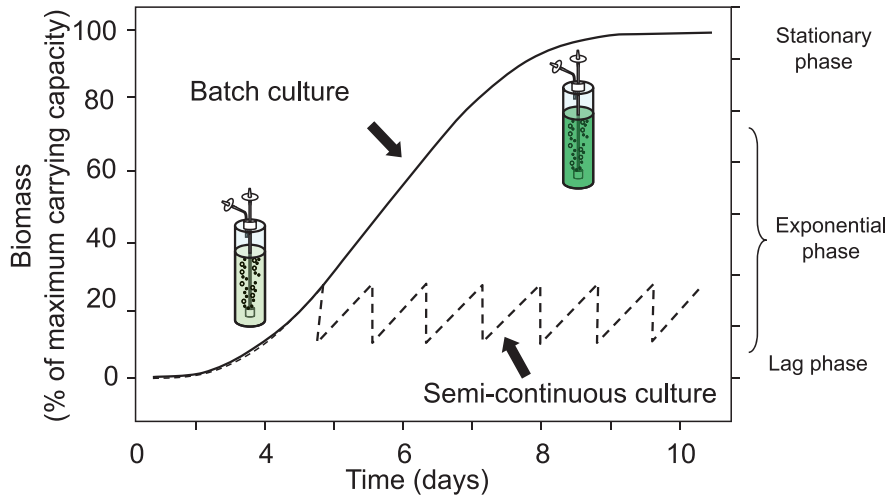

Figure 5.2 Differences in the biomass levels between batch and semi-continuous cultures. In semi-continuous cultures, the biomass is maintained at a low level for the duration of the culturing period in order to reduce the change in growth conditions. The time period between the dilutions of the culture will vary as a function of the growth rate and the desired biomass (redrawn from Hutchins *et al.*, 2003).

experiments because the incubation water is periodically exchanged with fresh filtered seawater pre-acclimated to the desired CO_2 level. Such semi-continuous batch incubations can be carried out for several days and are useful in assessing the effect of CO_2 on growth rate of natural microbial populations and on short-term changes in community structure (e.g. Tortell *et al.*, 2008).

5.2.6 Chemostats

A chemostat is a bioreactor that has reached equilibrium such that the chemical properties (pH, nutrients, cell density) remain constant with time (Novick & Szilard, 1950). In these systems, the input and output flow rates are the same and the experimentalist can define the growth rate of the organisms as a function of the dilution rate of a limiting nutrient (Figure 5.3). Chemostats are thus particularly useful to study the physiological response of microorganisms under nutrient-limited conditions, as observed in oligotrophic oceans. In order to be meaningful, it is important that the chemostat culture reaches steady-state. A chemostat can deem to be at steady-state when measured variables, such as for example cell density, or particulate organic carbon (POC), remain constant for 3 to 4 consecutive days. This is usually achieved after the cells have grown for 10 to 15 generations, but can sometimes be reached faster, depending on the physiological status of the inoculum.

Depending on the size of the incubator and the dilution rate, large volumes of media may be required to run chemostats. It is important to pre-equilibrate the culture media to the required CO_2 concentration by aerating the media reservoir for a few days before pumping it in the culture vessel or by employing another of the methods recommended in chapter 2. Once the chemostat is started, the culture vessel should continue to be aerated with the desired CO_2 concentration for the duration of the experiment. Pre-equilibration of the culture medium gives the carbonate chemistry a chance to equilibrate in the seawater, without the effect of biological CO_2 drawdown. In cases where aeration has a negative effect on the cultured phytoplankton species, one can choose to purposely manipulate the $p(CO_2)$ of the medium and chemostat to be higher than the target value in order to compensate for the expected biologically-induced shift in carbonate chemistry. Whether the culture is aerated or not, it is important to measure the carbonate chemistry in the chemostat culture at the beginning of the experiment and at the time of harvest in order to determine the $p(CO_2)$ value to which the phytoplankton was exposed.

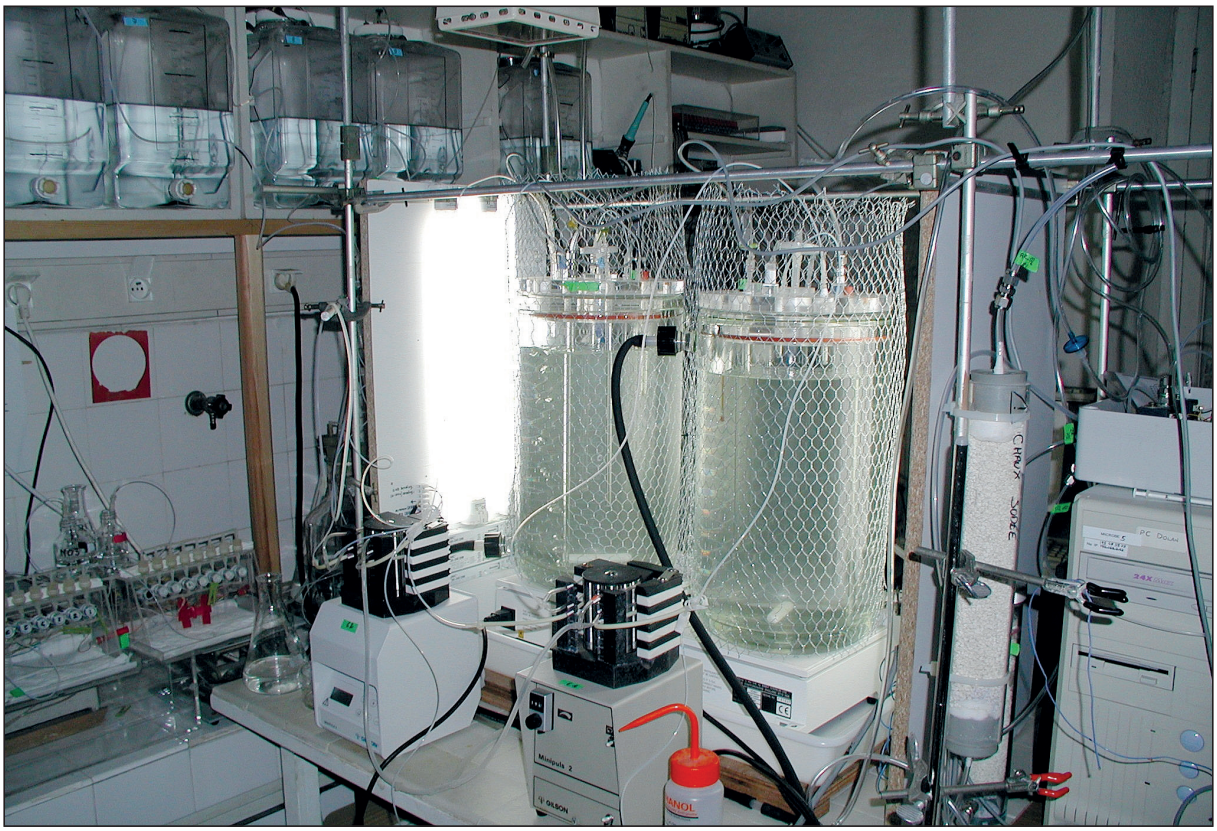

Figure 5.3 Chemostat used by Sciandra *et al.* (2003) to investigate the response of *Emiliana huxleyi* to elevated $p(CO_2)$ (photo credit: J.-P. Gattuso).

At steady-state, the biomass (x) and growth rates (μ, d^{-1}) of the algal species in the chemostat are usually determined by the concentration of the limiting nutrient (S_n) and the dilution rate (D, d^{-1}), respectively according to the following equations:

$$x = \frac{S_{n_0} - S_n}{q}; \tag{5.1}$$

$$\mu = D; \tag{5.2}$$

where x is the cell abundance (cell l^{-1}), S_{n_0} the concentration of the limiting nutrient in the medium reservoir (mol kg^{-1}), S_n is the concentration of the nutrient inside the incubator (mol kg^{-1}), q is the cell quota (mol $cell^{-1}$). More detailed mathematical descriptions of the chemostat can be found in Monod (1950), Droop (1974), Janasch (1974) and Burmaster (1979).

In chemostats, the limiting nutrient is set by reducing its concentration relative to the other essential nutrients. In the case of a nitrate-limited or phosphate-limited chemostat, this would require supplying N:P at a ratio much below or much above the Redfield N:P ratio of 16, respectively. Since large amounts of media are required for chemostat cultures, some valuable time and media can be saved if the inoculum is pre-adapted to the experimental conditions first, as for a batch culture. Some algae can store significant amounts of excess nutrients, for example iron and phosphorus, and reducing the cell quota of the pre-inoculation culture to a limiting level will help reach steady-state more rapidly, for example after 5 to 6 generations. Most chemostats are therefore initially started as batch cultures, which grow at the maximum growth rate. Once biomass has reached the desired cell density, the flow rate of the input media is set to the desired rate and the outflow from the culture vessel removes the excess media at the same rate. After 5 to 15 generations, the phytoplankton growth rate should have adjusted to steady-state. To achieve steady-state growth rates, the constancy of growth

conditions must be maintained (flow rate, temperature, $p(CO_2)$, nutrient concentration and composition). Bacterial contamination is often a problem for chemostat cultures because bacteria tend to thrive in nutrient-limited phytoplankton cultures. In order to prevent contamination of the sterile medium reservoir, it is important to interrupt the liquid path by one or several air breaks in the tubing because bacteria do not travel upstream as easily through air as through liquid media (Figure 5.4). In addition, continuous mixing of cultures in the chemostat is necessary to avoid sedimentation of cells and to minimise wall growth.

Continuous light does not mimic natural conditions in most regions and most experiments are carried out with a light-dark cycle (Leonardos & Geider, 2005). Such culture systems are called *cyclostats* because a cyclic behaviour in the growth rates and other physiological rates is imposed by the light-dark regime (Gotham & Rhee, 1982). In continuous culture systems, the microorganisms can be grown over a range of low to high growth rates that reflect the degree of nutrient limitation. However, once the dilution rate is approaching the maximum growth rate, the culture will start washing out rapidly. For scientific questions that require high growth rates, a system like a semi-continuous culture or a batch culturing system is more appropriate.

A few chemostat experiments have been conducted at sea, to study the effect of a constant low supply of the limiting nutrient Fe (iron) on phytoplankton growth in HNLC regions (Hutchins *et al.*, 2003). This is logistically difficult to conduct; requiring large volumes of amended seawater media, the need to acid wash the whole chemostat apparatus and incubating large culture volumes (2.7 l) in replicates for each growth condition. It is more difficult, if not impossible, to achieve steady-state of the chemostats with complex field populations, which are usually grown under the ambient fluctuating light intensities (Sommer, 1985). In contrast to grow outs or bioassay experiments which assess the nutrient that limits primary production, shipboard chemostat experiments have the added advantage to allow the study of microbial communities at low nutrient input, more representative of the natural conditions. These experiments can be maintained for several days at a constant dilution rate.

Daily sampling of chemostat cultures should be limited to less than 10 to 15% of the culture volume to avoid significant perturbation of the steady-state. If possible, carbon dioxide in the inlet of the culture should be continuously monitored by an infrared gas analyser in line with the culture vessel. However, constant monitoring by infrared gas analysers can become quite expensive when several chemostats with different CO_2 concentrations are run in parallel. Continuous measurements of pH and temperature of the seawater with pH/T-probes inserted directly into the culture vessel could be used as a more economical alternative. However, the lower precision of pH electrodes requires occasional measurements of DIC and A_T to precisely characterise the state of the carbonate system in the culture media. For cyclostats, an operational definition of steady-state

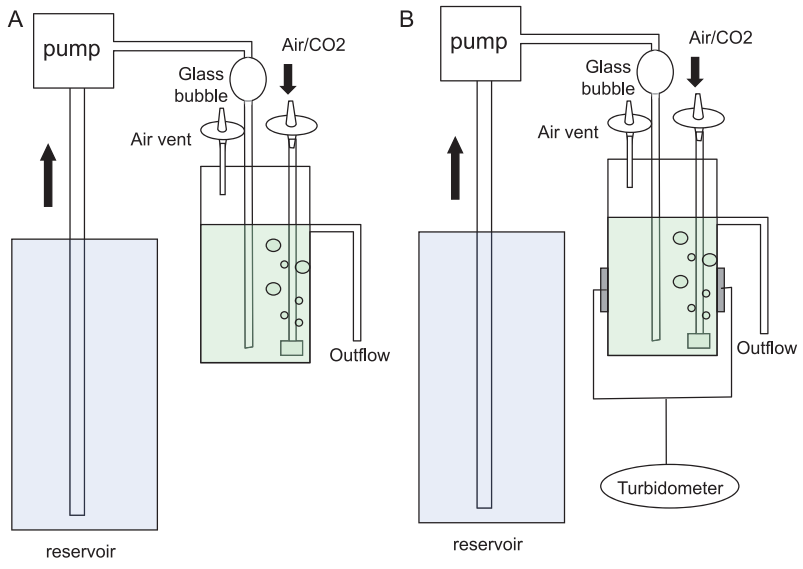

Figure 5.4 Experimental set-up for a a) chemostat and b) turbidostat showing the hardware that is necessary for conducting such experiments. The nutrient reservoir should be pre-equilibrated with the same air/CO_2 mixture that is used for the culture vessel.

should be established. Other investigators have used less than 10% variation in biomass, determined either from cell density or chlorophyll, for at least three days as a measure of steady-state (Leonardos & Geider, 2005). As for other types of cultures, sampling should always be carried out at the same time of the day.

Additional concerns arise in the use of chemostat cultures for ocean acidification research. It is well established that in nutrient-limited chemostats, and in particular nitrogen-limited chemostats, the C:N molar ratio of the phytoplankton can greatly exceed the Redfield ratio of 6.6 (Goldman *et al.*, 1979). In fact, the C:N ratio varies largely as a function of the dilution rate, being highest at low dilution rates. This means that photosynthetic drawdown of DIC will not necessarily be in balance with the set growth rate and at low dilution rates, biological drawdown of CO_2 may quickly exceed CO_2 delivery by bubbling at a constant rate, because of an increased retention time of the cells in the culture vessel. Thus, care should be taken to assess at least two parameters of the carbonate chemistry (pH, DIC, A_T) at all dilution rates as it may vary significantly between chemostats run at different dilution rates. One would expect the strongest shift in carbonate chemistry at the lowest dilution rates, especially when the cell abundance is high.

Special caution is required when working with calcifying algae in culture systems, because of the effect of calcification on the carbonate chemistry of seawater. Due to the drawdown of carbonates by the cells, the alkalinity in the incubator becomes lower than that in the medium reservoir, and the carbonate chemistry of seawater, and thereby pH, deviates strongly from target chemistry despite continuous aeration (see chapter 2 for explanations). In principle, there are two ways to minimise effects of calcifying algae on the carbonate system in a chemostat: a) to reduce cell abundance, and therewith the amount of alkalinity drawdown within the incubator and, b) to increase the daily addition of alkalinity from the reservoir, for example by increase of alkalinity in the reservoir (Borchard *et al.*, subm.). The maximum abundance of calcifying algae in a chemostat that can be sustained by a given total alkalinity can be estimated from:

$$n = \frac{1}{c} D(A_{T_m} - A_{T_i}); \tag{5.3}$$

where n is the maximum sustainable abundance of cells (cells l^{-1}), D is the dilution rate (d^{-1}), c is the cell specific change in alkalinity due to calcification (µmol $cell^{-1}$ d^{-1}), and A_{T_m} and A_{T_i} are the total alkalinity (µmol l^{-1}) within the medium reservoir and the target alkalinity in the incubator, respectively. Because calcification rate may change with growth rate, for example for *E. huxleyi*, c should be known for each dilution rate, or conservatively estimated in order to avoid significant reduction of alkalinity or even carbonate undersaturation.

The choice of method to minimise biological effects on carbonate chemistry in a chemostat will depend on the purpose of the experiment but ideally, cell abundance should be kept as low as possible. Thus, assays to study ocean acidification with chemostats clearly differ from classical chemostat studies that opt for high biomass yield. In summary, the strong drift in carbonate chemistry, which can vary in magnitude for different dilution rates, may complicate the analysis of the data and certainly requires caution when designing experiments with chemostats. Similarly, the $p(CO_2)$ in the aerating gas is not necessarily equal to the $p(CO_2)$ in the medium, and it is important to measure two parameters of the carbonate system in the culture itself rather than in the inflowing medium or gas mixture.

5.2.7 Turbidostats

A turbidostat is a continuous culturing method where the dilution with fresh culture medium does not take place continuously but only to restore the turbidity of the culture, thus keeping the biomass constant. This is achieved by measuring turbidity, or chlorophyll fluorescence through sensors placed on the outside of the culture vessel. It is very similar in principle to the semi-continuous culture approach but more complex to set up instrumentally. Logistically, turbidostats and chemostats are much more difficult to set up than batch cultures because they require peristaltic pumps and large volumes of media. In addition, the increased complexity makes them susceptible to bacterial contamination, cell wall growth and creeping of the cultured cells into the reservoir of fresh medium.

5.3 Strengths and weaknesses

5.3.1 Batch cultures

Strengths

Batch cultures are easy to set up, conduct and replicate. They require minimal equipment compared to other culture types. If the starting culture is axenic, it is easy to maintain the batch cultures free of contaminating bacteria by following good standard microbiological techniques. The exponential growth phase should represent the maximum growth rate under a given light and temperature regime. They are suitable for questions that require nutrient replete conditions or bloom conditions.

Weaknesses

The growth conditions within the classical batch culture are changing rapidly, because the organisms are almost never in balanced growth, while those of the dilute batch culture are more stable. Dilute and classical batch cultures are of fixed volume, which means that the number of samples that can be removed is limited. They are poor models for oligotrophic regions. The results obtained from classical batch cultures are more difficult to reproduce than those from chemostats. This is because of the rapid changes that occur as biomass increases and starts modifying first the carbonate system, then the nutrients and the light environment. This means that they are also not particularly well suited for global gene expression studies and all of the associated -omics methods where reproducibility is very important for detecting small changes in the metabolic status. In general, dilute batch cultures are more appropriate for ocean acidification work than the classical batch cultures. However, the low biomass yield means that large volumes of dilute cultures are sometimes required to carry out the analyses. The results of the batch culture will be very dependent on the physiological status of the cell inoculum. It should be stressed here that pre-acclimation of the inoculum to the set experimental conditions can greatly minimise or eliminate the lag-phase.

5.3.2 Bioassays and grow outs

Strengths

The advantages are similar to those of batch cultures, except that mixed microbial populations are used for the incubations. They are simpler to conduct at sea than continuous cultures which require more sophisticated equipment. In combination with nutrient additions, they can uncover the nutrient that is most likely to limit productivity in a given environment.

Weaknesses

In coastal areas where nutrients are high, grow outs will be subject to the same disadvantage as the batch cultures. In oligotrophic areas, large volumes need to be incubated in order to get accurate measurements, as is the case for nitrogen fixation measurements using the $^{15}N_2$ gas technique. Except for bloom conditions, where nutrients are injected through mixing in the euphotic zone, the mode of pulsed nutrient delivery used in bioassay experiments is unrealistic for most of the ocean. Variability in the initial microbial populations, initial environmental conditions and the pre-conditioning of the microbial populations cannot be controlled in bioassay experiments, making this approach much more difficult than batch culture experiments with monospecific cultures.

5.3.3 Semi-continuous cultures

Strengths

The growth of semi-continuous cultures is maintained in exponential phase, and it is therefore easier to sample the culture at the same physiological state in repeated experiments than in batch cultures. It is an appropriate type of culture for determining the maximum growth rate achievable under a set of experimental conditions, because repeat measures can be made on the same culture. They are simpler to conduct than true continuous cultures such as chemostats and turbidostats. They can be used to study micronutrient limitation, for example

Fe limitation when used in combination with high concentrations of chelators in the culture media. However, care should be taken with chelators such as EDTA because they impede calculations of the carbonate system via A_T (see section 2.4.3 of this guide).

Weaknesses

Depending on the level of automation in the experimental set-up, semi-continuous cultures may require daily attention to maintain the cell density.

5.3.4 Chemostat cultures

Strengths

Chemostat cultures are especially useful when looking at nutrient-limited growth but in addition they offer reproducibility of growth conditions. The mathematical description of cellular growth in a chemostat is a definite advantage for transferring the gained knowledge into mathematical models. Recently, there has been resurgence in popularity of chemostats because of the reproducibility that they offer for studies involving transcriptomics, proteomics and metabolomics. Although a cell culture pre-adapted to the growth conditions will reach steady-state faster once the chemostat mode is started, the physiological state of the inoculum culture will not affect the results once the culture has reached steady-state and the growth rate is equal to the dilution rate. Chemostats are particularly useful when studying dissolved organic matter (DOM), since the release of DOM by the autotrophic cell is often associated with nutrient limitation.

Weaknesses

Chemostats are easily contaminated with bacteria, even when practicing good microbiological sterile techniques, and they require large amounts of culture media and sophisticated equipment. Establishing steady-state in a chemostat requires the microorganisms to grow for up to 15 generations. After several weeks, wall growth may be a problem as well as growth in the lines leading to the medium reservoir.

5.4 Potential pitfalls

For all of the culture methods, there is a potential for the biomass to be high enough to significantly deplete the dissolved inorganic carbon in the culture media, followed by additional changes in the growth environment such as self-shading. In batch cultures, nutrient depletion can be a problem when low nutrient concentrations are used to maintain the biomass low. Some phytoplankton species may not like the turbulence caused by aeration and might grow at suboptimal growth rates under these conditions. Aeration should therefore be gentle and applied only as much as necessary.

In order to homogenise the supply of cells with respect to light, CO_2 and nutrients, and also to avoid accumulations of cells and of metabolic waste products, the cultures need to be well mixed. Besides aeration, mixing can be accomplished by means of agitation with static stirrers. If magnetic stirring is applied, the direct contact between the magnetic stirring bar and the vessel bottom should be avoided as this may cause cell damage, DOM release and foam generation.

Care should be taken to minimise temperature fluctuations during incubations, because CO_2 dissolution in seawater and the rates of the majority of enzymatic reactions varies as a function of temperature. This can best be achieved by placing smaller culture vessels, for example batch cultures, in a water bath or by having temperature controlled incubators or facilities where the cultures can be set up.

5.5 Suggestions for improvements

Under all growth conditions, the cultures should be kept at optically thin cell densities in order to limit the depletion of DIC to less than 5% of the total. Batch cultures should be monitored regularly and be harvested before the onset of changes in carbonate chemistry, unless the goal of the study is to simulate bloom conditions. A simple measurement

of the phytoplankton variable fluorescence (F_v/F_m) can be used to screen for nutrient limitation. Test for axenicity (bacterial contamination) should be conducted in each culture at harvest as described in section 5.2. In order to ascertain the identity of phytoplankton strains and to anticipate potential issues of genetic drift due to long-term cultivation, a small amount of the culture should be harvested and stored frozen at -80°C for future references. Some cultures that do not grow well axenically may contain variable bacterial contaminants. The frozen culture sample can also be used for identifying the bacterial contaminants associated with the phytoplankton strains.

5.6 Data reporting

For batch cultures, it is important to report basic information characterising the physiological state of the initial inoculum. Records should include full details on the origin of the strain used in the experiment, cell density and detailed growth conditions of the pre-acclimated culture, number of cells inoculated, the Chl cell, light intensity, temperature and composition of the initial culture media. Additionally, a measure such as F_v/F_m would give information on whether the pre-acclimated culture was in exponentially growing phase. In addition to the specific experimental data collected by each investigator, it is important that the growth and two parameters of the carbonate system be reported for the main cultures when carrying out experiments at different CO_2 levels (see chapters 1 and 2). The carbonate system should be measured at the beginning and the end of the experiment, as well as in the culture medium. The investigators should report initial and final measurements of the carbonate system parameters, and should clearly indicate in the data analyses whether initial, final or mean values were used. The investigators should also report whether or not the cultures were axenic. As a suggestion, it may be useful to freeze a small amount of each culture for future nucleic acid extractions. This may prove important to resolve issues about possible heterogeneity in the physiology of different strains of a given species.

5.7 Recommendations for standards and guidelines

1. Keep biomass low to avoid depletion of DIC, self-shading and undesired nutrient limitation.
2. Drawdown of DIC by biological carbon fixation in any type of cultures should be less than 5% of the total DIC.
3. Axenic strains of phytoplankton should be used whenever possible.
4. Nucleic acid samples from culture strains should be collected on a 0.2 µm filter for future reference.
5. For batch culture experiments, the pre-culture should be acclimated to the same conditions as applied in the experiments and dilute batch cultures should be used.
6. Cultures should be run in replicates (triplicates) and the entire experiment should be replicated at least twice with different batches of culture media.
7. The DIC system should be characterised by at least two independent measurements at the beginning of the experiment and at the time of culture harvest in order to assess any drift in the carbonate chemistry. Depending on the questions addressed in the experiment, replicates that are not within the acceptable range of variation in DIC should be clearly reported and possibly left out of the further data analyses.
8. Chemostats should be used when studying the effect of CO_2 levels on growth under nutrient-limited conditions.
9. For chemostat cultures, the effluent volume and the cell density should be measured every day.

5.8 References

Berg G. M., Repeta D. J. & LaRoche J., 2002. Dissolved organic nitrogen hydrolysis rates in axenic cultures of *Aureococcus anophagefferens* (Pelagophyceae): comparison with heterotrophic bacteria. *Applied and Environmental Microbiology* 68:401-404.

Berges J. A., Franklin D. J. & Harrison P. J., 2001. Evolution of an artificial seawater medium: improvements in enriched seawater, artificial water over the last two decades. *Journal of Phycology* 37:1138–1145.

Burmaster D. E., 1979. The continuous culture of phytoplankton: mathematical equivalence among three steady state models. *American Naturalist* 113:123-134.

Chen Y. B., Zehr J. P. & Mellon M., 1996. Growth and nitrogen fixation of the diazotrophic filamentous nonheterocystous cyanobacterium *Trichodesmium sp.* IMS 101 in defined media: evidence for a circadian rhythm. *Journal of Phycology* 32:916-923.

Cullen J. T. & Sherrell R. M., 2005. Effects of dissolved carbon dioxide, zinc, and manganese on the cadmium to phosphorus ratio in natural phytoplankton assemblages. *Limnology and Oceanography* 50:1193-1204.

Droop M. R., 1974. The nutrient status of algal cells in continuous culture. *Journal of the Marine Biological Association of the UK* 54:825-855.

Goldman J. C., McCarthy J. J. & Peavey D. G., 1979. Growth rate influence on the chemical composition of phytoplankton in oceanic waters. *Nature* 279:210-215.

Goldman J. C. & McCarthy J. J., 1978. Steady-state growth and ammonium uptake of a fast-growing marine diatom. *Limnology and Oceanography* 23:695–703.

Gotham I. J. & Rhee G.-Y., 1982. Effects of nitrate and phosphate limitation on cyclostat growth of two freshwater diatoms. *Journal of General Microbiology* 128:199-205.

Guillard R. R. L., 1975. Culture of phytoplankton for feeding marine invertebrates. In: Smith W. L. & Chanley M. H. (Eds.), *Culture of marine invertebrate animals*, pp. 26-60. New York: Plenum Publishing Corporation.

Hansen P. J., Lundholm N. & Rost B., 2007. Growth limitation in marine red-tide dinoflagellates: effects of pH versus inorganic carbon availability. *Marine Ecology Progress Series* 334:63-71.

Harrison P. J., Waters R. E. & Taylor F. J. R., 1980. A broad spectrum artificial seawater medium for coastal and open ocean phytoplankton. *Journal of Phycology* 16:28-35.

Hinga K. R., 2002. Effects of pH on coastal marine phytoplankton. *Marine Ecology Progress Series* 238:281-300.

Hutchins D. A., Pustizzi F., Hare C. E. & DiTullio G. R., 2003. A shipboard natural community continuous culture system for ecologically relevant low-level nutrient enrichment experiments. *Limnology and Oceanography Methods* 1:82-91.

Janasch H. W., 1974. Steady state and the chemostat in ecology. *Limnology and Oceanography* 19:716-720.

Keller M. D. & Guillard R. R. L., 1985. Factors significant to marine diatom culture. In: Anderson D. M., White A. W. & Baden D. G. (Eds.), *Toxic dinoflagellates*, pp. 113-116. New York: Elsevier.

Keller M. D., Selvin R. C., Claus W. & Guillard R. R. L., 1987. Media for the culture of oceanic ultraphytoplankton. *Journal of Phycology* 23:633-638.

Kranz S. A., Sültemeyer D., Richter K. U. & Rost B., 2009. Carbon acquisition in *Trichodesmium*: diurnal variation and effect of pCO_2. *Limnology and Oceanography* 54:548-559.

Lakeman M. B., von Dassow P. & Cattolico A., 2009. The strain concept in phytoplankton ecology. *Harmful Algae* 8:746-758.

Landry M. R., Kirshtein J. & Constantinou J., 1995. A refined dilution technique for measuring the community grazing impact of microzooplankton, with experimental test in the central equatorial Pacific. *Marine Ecology Progress Series* 120:53-63.

Langer G., Nehrke G., Probert I., Ly J. & Ziveri P., 2009. Strain-specific responses of *Emiliania huxleyi* to changing seawater carbonate chemistry. *Biogeosciences* 6:2637-2646.

Leonardos N. & Geider R. J., 2005. Elemental and biochemical composition of *Rhinomonas reticulata* (Cryptophyta) in relation to light and nitrate-to-phosphate supply ratios. *Journal of Phycology* 41:567-576.

Martin J. H. & Fitzwater S. E., 1988. Iron deficiency limits phytoplankton growth in the north-east Pacific subarctic. *Nature* 331:341-343.

Monod J., 1950. La technique de culture continue théorie et applications. *Annales de l'Institut Pasteur* 79:390-410.

Morel F. M. M., Rueter U. J. G., Anderson D. M. & Guillard R. R. L., 1979. Aquil: a chemically defined phytoplankton culture medium for trace metal studies. *Journal of Phycology* 15:135-141.

Mullin M. M., Sloan P. R. & Eppley R. W., 1966. Relationship between carbon content, cell volume, and area in phytoplankton. *Limnology and Oceanography* 11:307-311.

Novick A. & Szilard L., 1950. Description of the chemostat. *Science* 112:715-716.

Paasche E., 2001. A review of the coccolithophorid *Emiliania huxleyi* (Prymnesiophyceae), with particular reference to growth, coccolith formation, and calcification-photosynthesis interactions. *Phycologia* 40:503-529.

Price N. M., Harrison G. I., Hering J. G., Hudson R. J., Nirel P. M. V., Palenik B. & Morel F. M. M., 1988. Preparation and chemistry of the artificial algal culture medium Aquil.*Biological Oceanography* 6:443-461.

Rost B., Zondervan I. & Wolf-Gladrow D., 2008. Sensitivity of phytoplankton to future changes in ocean carbonate chemistry: current knowledge, contradictions and research directions. *Marine Ecology Progress Series* 373:227-237.

Rost B., Riebesell U. & Sültemeyer D., 2006. Carbon acquisition of marine phytoplankton: effect of photoperiod length. *Limnology and Oceanography* 51:12-20.

Ryther J. H. & Dunstan W. M., 1971. Nitrogen, phosphorus, and eutrophication in the coastal marine environment. *Science* 171:1008-1013.

Schulz K. G., Barcelos e Ramos J., Zeebe R. E. & Riebesell U., 2009. CO_2 perturbation experiments: similarities and differences between dissolved inorganic carbon and total alkalinity manipulations. *Biogeosciences* 6:2145-2153.

Sciandra A., Harlay J., Lefèvre D., Lemée R., Rimmelin P., Denis M. & Gattuso J.-P., 2003. Response of coccolithophorid *Emiliania huxleyi* to elevated partial pressure of CO_2 under nitrogen limitation. *Marine Ecology Progress Series* 261:111-122.

Sommer U., 1985. Comparison between steady state and non-steady state competition: experiments with natural phytoplankton. *Limnology and Oceanography* 30:335-346.

Sunda W. G. & Huntsman S. A., 1995. Cobalt and zinc interreplacement in marine phytoplankton: biological and geochemical implications. *Limnology and Oceanography* 40:1404-1417.

Tortell P. D., DiTullio G. R., Sigman D. M. & Morel F. M. M., 2002. CO_2 effects on taxonomic composition and nutrient utilization in an Equatorial Pacific phytoplankton assemblage. *Marine Ecology Progress Series* 236:37-43.

Tortell P. D., Payne C. D., Li Y. Y., Trimborn S., Rost B., Smith W. O., Riesselman C., Dunbar R. B., Sedwick P. & DiTullio G. R., 2008. CO_2 sensitivity of Southern Ocean phytoplankton. *Geophysical Research Letters* 35, L04605. doi:10.1029/2007GL032583.

Van Bleijswijk J. D. L., Kempers R. S., Veldhuis M. J. & Westbroek P., 1994. Cell and growth characteristics of types A and B of *Emiliania huxleyi* (Prymnesiophyceae) as determined by flow cytometry and chemical analyses. *Journal of Phycology* 30:230-241.

Waterbury J. B., Watson S. W., Valois F. W. & Franks D. G., 1986. Biological and ecological characterization of the marine unicellular cyanobacterium *Synechococcus*. In: Platt T. & Li W. K. I. (Eds.), *Photosynthetic picoplankton*, pp. 71-120. Ottawa: Department of Fisheries and Oceans.

Zondervan I., Rost B. & Riebesell U., 2002. Effect of CO_2 concentration on the PIC/POC ratio in the coccolithophore *Emiliania huxleyi* grown under light-limiting conditions and different daylengths. *Journal of Experimental Marine Biology and Ecology* 272:55-70.