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Guide to best practices for ocean acidification research and data reporting

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11 Production and export of organic matter

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11.1 Introduction

The ocean is one of the largest reservoirs of CO_2 on Earth, and one of the largest sinks for anthropogenic emissions (Sabine *et al.*, 2004). The ocean's capacity for CO_2 storage is strongly affected by biological processes (Raven & Falkowski, 1999). Organic matter production and export processes in the ocean drive CO_2 sequestration and therefore feed back to atmospheric CO_2 and global climate. The magnitude of CO_2 that is fixed each year by biological processes and exported from the surface ocean is estimated to be ~10 GTC (Boyd & Trull, 2007), and therefore about the same magnitude as the total amount of CO_2 released each year by anthropogenic activities. Thus, changes in export processes could, in principle, have a major influence on atmospheric CO_2 concentrations.

Until recently, direct effects of anthropogenic CO_2 on export processes in the ocean have largely been neglected (e.g. Broecker, 1991). It is therefore not surprising that we still find very few studies dealing with direct effects of elevated CO_2 and the related seawater acidification on biologically-mediated carbon export. Biologically driven export of organic matter in the ocean, the "biological pump" (Volk & Hoffert, 1985), is a very complex issue (Boyd & Trull, 2007, Figure 11.1). Instead of one process potentially sensitive to ocean acidification, we are dealing with a multitude of processes, such as photosynthesis, biological nitrogen fixation, microbial degradation and

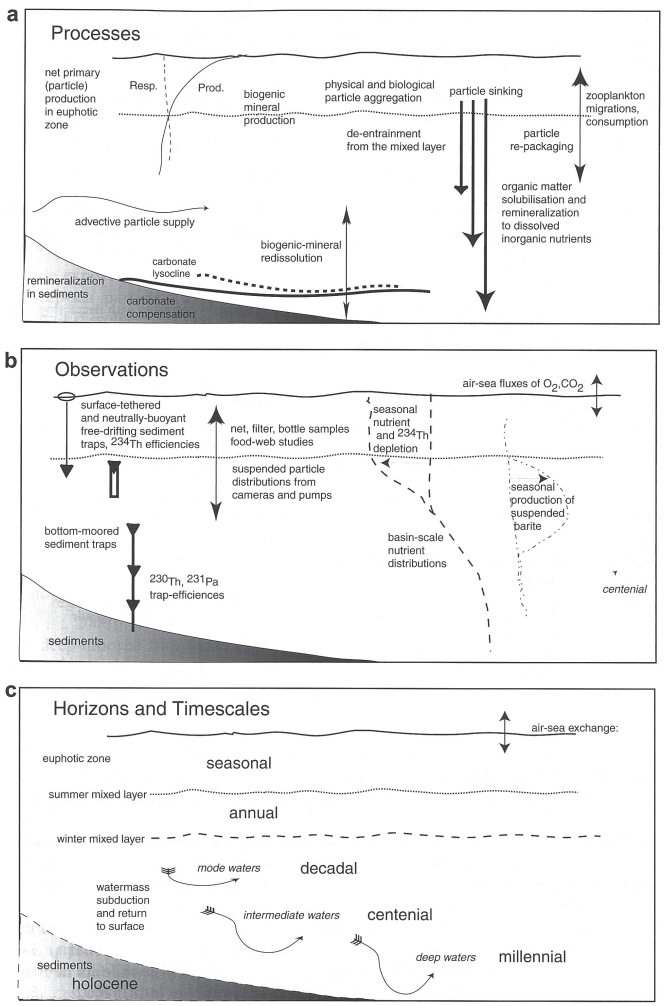

Figure 11.1 Temporal and spatial scales for processes, observations, and depth horizons important to estimate organic matter export (From Boyd & Trull, 2007, p. 279. Copyright (2007) by Elsevier).

secondary production, as well as with different modes of transport, for example particle sinking, physical mixing of dissolved and particulate compounds and vertical migration of plankton. Moreover, these processes operate on very different temporal and spatial scales, ranging from seasonal export of biogenic production from the surface ocean to its final deposition at the deep seafloor, where carbon is stored for hundreds of thousands of years. In between lies a water column that ranges from hundreds to thousands of meters, where heterotrophic processes determine the rate of organic matter remineralisation, and therefore the efficiency of export. These long temporal and large spatial scales are enormously challenging for oceanographic research.

Methodological limitations especially apply to experimental approaches in ocean acidification research. Here, CO_2 or pH perturbations of the present-day situation are usually restricted to the scale and duration of an experiment. Nevertheless, potential sensitivities of organic matter and export production to seawater acidification gather scientific and political attention due to their high potential to affect ecosystem functioning, biogeochemical cycles and global climate. This chapter will provide technical guidelines for the investigation of key processes of the biological carbon pump. Due to the complex nature and the very large variety of potential methods to be applied, only some of the most important aspects can be discussed. Nevertheless, we hope that these will be useful for considering organic and export production in ocean acidification research.

11.2 Approaches and methodologies

11.2.1 Primary production

Primary production in the sunlit ocean, i.e. the production of organic material from CO_2 and inorganic nutrients, is driven by photosynthesis. As this key process drives important elemental cycles and provides the energy for higher trophic levels, photoautotrophs like phytoplankton and cyanobacteria have been in the centre of ocean acidification research. An understanding of the sensitivity of photosynthesis to elevated CO_2 is also important when assessing other downstream processes like calcification or N_2 fixation as they depend on the energy supply from photosynthesis.

Photosynthesis involves a series of reactions that start with capturing light energy, converting it into ATP and NADPH, and using these compounds to fix CO_2 in the Calvin-Benson cycle. Several studies have focused on the process of CO_2 assimilation, which is inherently CO_2 sensitive due to the low CO_2 affinities of the primary carboxylating enzyme RuBisCO (Badger *et al.*, 1998). The effect of seawater carbonate chemistry on photosynthesis, however, depends strongly on the presence and characteristics of cellular CO_2-concentrating mechanisms (CCMs; Giordano *et al.*, 2005). In order to measure the effect of environmental factors on photosynthesis, including ocean acidification, several different methods have been developed and applied (see also Joint Global Ocean Flux Study reports 27 and 36 at http://ijgofs.whoi.edu/Publications/Report_Series).

As photosynthesis involves O_2 evolution as well as CO_2 fixation, photosynthetic rates can be inferred by monitoring the changes in concentrations of these gases over time, either continuously or by analysing discrete samples. Rates of O_2 evolution can be determined by real-time measurements using O_2 electrodes, O_2 optodes, and membrane-inlet mass spectrometry (MIMS). These approaches have been intensively used in laboratory experiments (e.g. Beardall, 1991; Nielsen, 1995; Trimborn *et al.*, 2008). Calibrations are achieved by measuring signals from known concentrations of O_2, commonly O_2-free and air-saturated (21% O_2), and therefore these three approaches yield similar results for photosynthetic O_2 evolution (as well as respiration). Rates of net O_2 evolution and/or CO_2 fixation can also be inferred by looking at the O_2 evolution or DIC drawdown in gas-tight bottles over time. O_2 concentrations are determined by Winkler titration (Winkler, 1888), and changes in DIC concentrations are measured for instance by a DIC analyser (Stoll *et al.*, 2001). Since rates are derived from two measurements (before and end of incubation), it is important to choose meaningful time intervals. The latter approach is mostly used in field studies with incubation times in the order of hours to one day. It is possible to correct for respiration by using darkened bottles. This approach is often combined with ^{14}C-DIC incubations (see below).

Another important approach to determine primary production is to look at the production of organic matter, using filtered samples at certain time intervals. This can be accomplished by measuring particulate organic carbon (for instance by CHN analyser or mass spectrometer (MS)), or by using tracers like ^{13}C-DIC or ^{14}C-DIC (Steeman Nielsen, 1951), and their respective incorporation rate over time (by means of MS and scintillation counter, respectively). Prior to the measurement of organic carbon, samples are treated with acid to remove all residual inorganic carbon from the filters. This technique can, however, easily be modified to also estimate particulate inorganic carbon production, i.e. calcification, by measuring the total particulate carbon (non-acidified samples) and using a mass balance equation. When using the ^{14}C micro diffusion technique (Paasche & Brubak, 1994), estimates for photosynthesis and calcification can be obtained from the same sample (see chapter 12 of this guide).

The photosynthetic process can also be examined using variable chlorophyll fluorescence measurements. Different techniques are in use, like Pump and Probe (PaP), Pulse Amplitude Modulation (PAM), Fast Repetition Rate Fluorescence (FRRF), Fluorescence Induction and Relaxation (FIRe) that all measure similar parameters, which can be used to elucidate single photosynthetic processes around photosystem II (e.g. Gorbunov *et al.*, 1999; Suggett *et al.*, 2003). If basic parameters, for instance the Photosynthetic Quotient (PQ) and the functional absorption cross section of photosystem II, are known, these measurements can be used to determine photosynthetic rates as well. As photosynthetic oxygen evolution occurs at photosystem II, normally the correlation between variable fluorescence-derived rates and oxygen evolution is highly linear. The techniques used are very sensitive and non-invasive and can hence be used for fieldwork.

11.2.2 Biogenic nitrogen fixation

N_2-fixing cyanobacteria (diazotrophs) are the main source of biologically available nitrogen compounds in large areas of the oligotrophic ocean (Codispoti *et al.*, 2001). Thus, they have an important role in marine ecosystems and biogeochemical cycles (Mulholland, 2007). Recent studies showed that the predominant nitrogen fixer in today's ocean, the colonial cyanobacterium *Trichodesmium spp.*, is strongly affected by increasing CO_2 concentrations (Barcelos e Ramos *et al.*, 2007; Hutchins *et al.*, 2007; Levitan *et al.*, 2007; Kranz *et al.,* 2009), as are some unicellular nitrogen-fixing cyanobacteria such as *Crocosphaera spp.* (Fu *et al.*, 2008). Both their biogeochemical role and their apparent sensitivity to increasing CO_2 concentrations highlight the importance of studying cyanobacteria in the context of ocean acidification and underline the need for accurate nitrogen fixation rate measurements.

Nitrogen fixation rates can be determined by the acetylene reduction assay (Capone, 1993 and references therein) and the ^{15}N method (Mulholland & Bernhardt, 2005). Acetylene gas is an analog of molecular N_2, and the nitrogenase enzyme catalyses reduction of the triple bond in acetylene to double-bonded ethylene. In this assay, a gas chromatograph is used to measure ethylene production after briefly incubating the N_2 fixers with added acetylene. The ratio of acetylene reduced to nitrogen fixed is then calculated using a conversion factor (Capone, 1993) and the Bunsen gas solubility coefficient (Breitbarth *et al.*, 2007). For the δ^{15}N technique, isotopically labelled N_2 gas is introduced to an incubation containing diazotrophs, followed by filter harvesting of the labelled biomass. A mass spectrometer is used to determine the ^{15}N values of the samples, which are then used to calculate rates of N_2 fixation. While the acetylene method estimates gross nitrogen fixation rate (enzyme potential), the ^{15}N method measures net nitrogen production (nitrogen fixed that stays inside the cells) (Mulholland, 2007).

Production rates of particulate organic nitrogen (PON, multiplied by growth rate) by a diazotrophic community can be used as an alternative estimate for nitrogen fixation when direct measurements are not possible. This method only works provided that additional nitrogen sources are known and quantified. In this case, a C/N elemental analyser is used to determine the PON.

11.2.3 Organic matter biogeochemistry

Because the number of direct measurements of export flux in the field is small and localised, a widely accepted approach to making large-scale estimates for export is to link export fluxes to element cycling. For instance,

carbon export is often related to nitrogen cycling based on three assumptions: (1) organic matter production follows Redfield stoichiometry (Redfield *et al.*, 1963), (2) the availability of allochthonus inorganic nitrogen, such as nitrate and fixed dinitrogen gas, constrains new biological production (Dugdale & Goering, 1967) and (3) the amount of new production determines the amount of exportable production (Eppley & Peterson, 1979). For ocean acidification research, this close link between export fluxes and nitrogen fluxes leaves, at first sight, very limited space for direct CO_2 sensitivities. However, the general applicability of the Redfield C:N:P ratio to interrelate macro-element fluxes is controversial, and there are numerous examples showing systematic deviation on the organism and species level, with the trophic status of the system, and over time and space (e.g. Banse, 1974; Geider & La Roche, 2002). Nevertheless, deviations of the C:N ratio in particulate organic matter generally are within a range of 20 to 30% (Sterner *et al.*, 2008), which is very narrow compared to terrestrial systems. A somewhat larger decoupling of C and N is observed for processes involving inorganic compounds (Banse, 1994). In order to identify potential effects of ocean acidification on element co-cycling affecting export production, researchers might particularly look for (1) systematic changes in element ratios, compared to the Redfield ratio, (2) effects on the availability of limiting nutrients, specifically nitrogen and (3) effects that lead to qualitative changes in organic matter that in turn affect export efficiencies.

In the context of biological carbon sequestration, several recent studies on the effects of ocean acidification on organic matter production reported systematic changes of element ratios (e.g. Burkhardt *et al.*, 1999; Gervais & Riebesell, 2001; Engel *et al.*, 2005; Leonardos & Geider, 2005; Barcelos e Ramos *et al.*, 2007; Hutchins *et al.*, 2007; Levitan *et al.*, 2007; Riebesell *et al.*, 2007). In these studies, element ratios were calculated from element concentrations in either organic or inorganic, dissolved or particulate form. Analysis of macro-elements include widely accepted standard techniques, such as nutrient analysis via colorimetric methods, elemental analysis (CHN-analyser and MS) after high-temperature combustion, or colorimetric analysis of elements after chemical combustion of organic components. These methods have been described in textbooks (e.g. Grasshoff *et al.*, 1999), and have been evaluated through international programs (see also JGOFS report 19 at http://ijgofs.whoi.edu/Publications/Report_Series). Results are typically reported in concentrations of either mass [g l^{-1}] or molar [mol l^{-1}] units.

Two ways of determining element ratios have to be discriminated: the calculation of concentration ratios, e.g. [C]:[N], and the derivation of reaction rate ratios, e.g. based on a linear regression model $f_C = a[N] + b$, where a is a best estimate of the reaction rate ratio Δ[C]:Δ[N] (e.g. Fanning, 1992). Here, the factor a describes the co-variation of carbon (C) and nitrogen (N), and is typically derived from the slope of a linear regression of C versus N. Concentration ratios and reaction rate ratios differ for $b \neq 0$. Results are typically given in units of mass ([g]:[g]) or as molar ratios ([mol]:[mol]). The uncertainty in determining the slope a, i.e. its standard error (σ_a), can be expressed in terms of a root mean square error (RMSE):

$$\sigma_a = \frac{\mathrm{RMSE}}{\sqrt{\sum_{i=1}^{M}(N_i - \overline{N})^2}} \quad ; \tag{11.1}$$

with

$$RMSE = \sqrt{\frac{\sum_{i=1}^{M}(f_C - C)_i^{\,2}}{(M-2)}} \quad ; \tag{11.2}$$

with M being the total number of observations. When comparing the ratio of mean concentrations, the standard deviation can simply be determined according to the product rule and becomes:

$$\frac{1}{\overline{N}^2}\sqrt{\overline{N}^2\sigma_C^2 + \overline{C}^2\sigma_N^2} \quad ; \tag{11.3}$$

where $\overline{C}$ and $\overline{N}$ are mean values, and σ_C and σ_N are the standard errors of C and N, respectively.

11.2.4 Particle export processes

Observations made during the Joint Global Ocean Flux Study (JGOFS) indicate that 50 to 80% of the vertical flux of carbon through the mesopelagic zone and into the deep ocean occurs by gravitational sinking of particles (e.g. Gardner, 2000; Baliño *et al.*, 2001; Fasham *et al.*, 2001). Sediment trap studies during JGOFS have suggested that ballast minerals influence the export of carbon from the euphotic zone. Particles sinking out of the ocean surface contain both organic matter (OM) and minerals. Minerals (opal, $CaCO_3$, and aluminosilicates) typically constitute more than half the mass of sinking particles (Ittekkot & Haake, 1990; Honjo, 1996), and this fraction increases dramatically with depth (Armstrong *et al.*, 2002; Klaas & Archer, 2002). Minerals are important for making low density organic matter sink, and may also protect it from degradation (Hedges & Oades, 1997; Nelson *et al.*, 1999), allowing it to penetrate deeper into the ocean. Armstrong *et al.* (2002) demonstrated that ratios of POC to mineral ballast converge to a nearly constant value (~3 to 7 wt% POC) at depths greater than 1800 m, and Klaas & Archer (2002) showed that variability in the OM flux data might largely be explained (r^2 = 85 to 90%) by the proportions of opal, carbonate, and aluminosilicate ballast. Calcium carbonate appears to be the most important ballast mineral in terms of organic carbon transport (François *et al.*, 2002; Klaas & Archer, 2002).

Will ocean acidification decrease $CaCO_3$ production and/or increase $CaCO_3$ dissolution, so that the ballast effect of $CaCO_3$ will decrease? A decrease in $CaCO_3$ concentration would theoretically decrease the flux of organic matter, thus decreasing the removal of carbon from the surface ocean. The resulting increase in CO_2 in the surface ocean would enhance acidification, resulting in further dissolution of $CaCO_3$, and further decrease in organic matter flux. Could such a decrease in carbon flux be measured with methods that are currently available to directly measure export, such as sediment traps and *in situ* pumps? The time scale of acidification would make the scenario just described proceed at a very slow rate. Even though we have time-series stations where sediment trap material is being collected, the error in mass flux measured is currently too large to allow such a small change in carbonate flux to be determined in the field at least in the near future. On the other hand, sediment traps might be useful in collecting samples of sinking particulate matter that would be useful in studies of dissolution of $CaCO_3$ or other minerals. Poor trapping efficiency (Yu *et al.*, 2001) of traditional sediment trap designs might bias such collections, however, since trapping efficiency is not equal across all size classes, with smaller particles being winnowed more easily than larger ones. Particle size is one of the parameters that could be used when measuring the effects of ocean acidification. Newer trap designs that avoid some of the problems with winnowing might be more effective (Peterson *et al.*, 1993, 2005; Buesseler *et al.*, 2000, Valdes & Price, 2000). Furthermore, acrylimide-filled dishes in traps are useful to preserve the three-dimensional structure of particles and enable individual size measurements and analysis (Hansen *et al.*, 1996).

In situ pumps have also been used to estimate export from surface waters. The deficit in dissolved thorium (^{234}Th) found in surface waters is an indication of how much ^{234}Th export occurs on sinking particles (Coale & Bruland, 1987; Savoye *et al.*, 2006). However, using ^{234}Th deficits to estimate particle flux is burdened with problems due to non steady-state advection (e.g. Cochran *et al.*, 2009), so that this method is also not accurate enough to measure small changes in flux over time in the field. The C/^{234}Th ratio of the particles is used to convert ^{234}Th export to C export (Buesseler *et al.*, 2006). How will acidification affect C/^{234}Th ratios? If acidification were to lower the adsorption coefficient (increase the solubility) of ^{234}Th for organic matter, C/^{234}Th ratios might increase in an acidified system. Perhaps *in situ* pumps can be used to collect samples of suspended and sinking particulate matter that would be useful in the dissolution studies mentioned above. Pumps avoid the winnowing found in many sediment trap designs.

Aggregates play a pivotal role for organic matter cycling and export, as they mediate vertical mass fluxes and element cycling in the ocean (Fowler & Knauer, 1986; Asper *et al.*, 1992). Several studies have highlighted how aggregation processes in the ocean cascade from the nano-scale up to the size of fast settling marine snow (Chin *et al.*, 1998; Engel *et al.*, 2004a; Verdugo *et al.*, 2004), primarily by the process of gel particle formation from high molecular weight organic polymers. Among the types of gel particles that have

been examined more closely are transparent exopolymer particles (TEP). TEP, and specifically the acidic polysaccharides, have been described as facilitating the coagulation of organic components, thereby affecting the partitioning between the pools of dissolved and particulate organic matter as well as organic matter export (Logan *et al.*, 1995; Engel, 2000; Passow, 2002). As photosynthesis rates increase with CO_2 concentration in several phytoplankton species (Rost *et al.*, 2003), the exudation rate of acidic polysaccharide and therefore TEP production may also rise (Engel, 2002). Recent experimental studies indicate that TEP formation is sensitive to ocean acidification (Engel, 2002; Engel *et al.*, 2004b; Mari, 2008), and this sensitivity may affect carbon sequestration in the future ocean (Arrigo, 2007). TEP are gel particles, the concentration of which is determined colorimetrically or microscopically (Passow, 2002; Engel, 2009). Although measurements of TEP are reasonably precise, concentrations of TEP in the field depend on many food chain processes, so that it might be difficult to use measurements over time to separate seasonal or interannual effects from those due to acidification. In addition, CO_2-induced changes in seawater pH may directly affect the surface charge of particles with potential implications for particle-solute and particle-particle interactions, such as adsorption and aggregation. CO_2 perturbation experiments in mesocosms should prove useful to study direct chemical, physiological or ecological effects of ocean acidification.

11.3 Strengths and weaknesses

11.3.1 Primary production

O_2 measurements in discrete samples are easy to perform, require little instrumentation, and are well suited to look at the integrated response of communities. Owing to the incubation times of hours to one day, they are not suited to look at fast responses required for most bioassays. Real-time gas measurements also are relatively easy to perform and do not, with the exception of membrane-inlet mass spectrometry (MIMS), require advanced technical instrumentation. They are useful for short to intermediate incubation times (minutes to hours). These approaches are therefore a good basis to examine the effect of acclimation conditions (e.g. CO_2/pH) in more detail, e.g. by determining the kinetics of photosynthetic O_2 evolution or C fixation as a function of light or DIC availability. Such data can provide information about underlying mechanisms (e.g. CCMs) being responsible for changes in photosynthesis.

It should be noted that rates of O_2 evolution are usually net rates, as gross O_2 evolution and uptake cannot be separated in the light when O_2 electrodes or optodes are used. MIMS has the advantage that by using $^{18}O_2$, real gross rates of photosynthesis can be obtained (Peltier & Thibault, 1985). Moreover, the $^{18}O_2$ technique yields information on light-dependent O_2 uptake such as the Mehler reaction or photorespiration, i.e. processes that alter the efficiency of net CO_2 fixation (Falkowski & Raven, 2007). By applying the photosynthetic quotient (PQ), the ratio of O_2 evolution to CO_2 fixation, rates of O_2 evolution can be converted to carbon fixation. The PQ can however change with growth conditions, for instance nutrient availability, and was found to vary between ~1.0 and 1.4 (Williams & Robertson, 1991). As MIMS can measure O_2 evolution in combination with CO_2 uptake and fixation, a detailed analysis on carbon fluxes can be obtained (e.g. Badger *et al.*, 1994; Rost *et al.*, 2007; Kranz *et al.*, 2009).

Gas exchange measurements are not ideally suited for natural phytoplankton assemblages given the small net CO_2 and O_2 fluxes, and have therefore mostly been used in laboratory experiments. In an attempt to increase signal/noise ratios high biomass is often used, which can build up high levels of O_2 (or drawdown of DIC) over the course of the measurement that inhibit photosynthesis and alter other processes. Moreover, as all these real-time gas measurements depend on stable oxygen signals, they require continuous stirring of the medium. The concomitant turbulence may damage the cells, which alters the results such as the derived kinetics. Time-course experiments under assay conditions therefore have to verify that cells are not altered by stirring.

POC production is routinely measured in laboratory experiments. Tracer approaches have the advantage that they are highly sensitive (especially ^{14}C) and thus are ideally suited for fieldwork, when there is low

photosynthetic activity. Depending on sampling intervals, which can be quite short owing to the high sensitivity, ^{14}C incubations have also been used in bioassays to obtain information on CO_2 vs. HCO_3^- uptake or DIC affinities (e.g. Elzenga *et al.*, 2000; Hutchins *et al.*, 2007; Tortell *et al.*, 2008). Short-term incubation can also provide gross rates of C fixation, whereas longer incubations tend to measure net photosynthesis. During the process of CO_2 fixation, ^{13}C and ^{14}C isotopes are discriminated by the cell, which has to be accounted for in the calculation. Estimates are moreover dependent on accurate specific activities, and it is also essential to account for blank values, especially in low productivity areas.

Estimations based on filter samples, as opposed to gas measurements in real-time or discrete samples, may be biased by absorption of labeled dissolved organic carbon (DOC) (Turnewitsch *et al.*, 2007). On the other hand, if DOC is a significant part of primary production, as is often the case under nutrient limitation, then estimates based on filtering particles underestimate total carbon fixation.

11.3.2 Biogenic nitrogen fixation

The acetylene reduction assay is extremely valuable in fieldwork, since the immediacy of the measurements allows for near real-time readjustments of sampling or experimental protocols while at sea. The major uncertainty associated with this approach is the need to assume an empirical conversion factor (typically 3 or 4:1) to convert moles of acetylene reduced into potential moles of nitrogen fixed. ^{15}N can be used to determine empirical conversion coefficients from acetylene reduction to nitrogen fixation.

The ^{15}N method uses dinitrogen gas, the actual substrate for the nitrogenase enzyme, and thus does not need a conversion factor. Another advantage of the ^{15}N tracer is that it can be used to follow the fixed nitrogen through the planktonic food web (Montoya *et al.*, 1996). Moreover, the ^{15}N method is easier to set up on field cruises and provides additional information about the carbon content, sometimes giving even simultaneous carbon fixation rates through the addition of ^{13}C-bicarbonate (Mulholland & Bernhardt, 2005; Hutchins *et al.*, 2007). However, some fraction of the fixed ^{15}N is exuded by the cell (often as dissolved organic nitrogen, and to a lesser degree as ammonium) and may be harvested on the filter as exopolymer particles or by adsorption. In this case, ^{15}N measurements will overestimate the cellular ^{15}N content. In extended incubations, re-assimilation and recycling of released ^{15}N can also be an issue (Mulholland *et al.*, 2006). Aside from these, no specific problem is associated with the ^{15}N-technique while doing experiments with CO_2 manipulation, since the samples are incubated without headspace.

The acetylene method was considered to be more sensitive (10^3 to 10^4 times; Hardy *et al.*, 1973), but recent improvements of the ^{15}N method make both methods equally sensitive (Montoya *et al.*, 1996). When working with CO_2 manipulated samples, the acetylene reduction assay may be more difficult to handle because this method requires a gas phase. This means that the CO_2 concentration of the liquid phase (manipulated CO_2) equilibrates with the CO_2 concentration of the gas phase (ambient CO_2). The effect of CO_2 on nitrogen fixation will then correspond to CO_2 concentrations that are slightly different (depending on the headspace to liquid volume ratio) from those initially adjusted in the liquid phase. The change of the CO_2 concentration may even influence the response over the short incubation period. For that reason, keeping the volume ratio of the gas phase to the liquid phase as small as possible will minimise the difference between the CO_2 levels.

The shortcoming of estimating nitrogen production rates from changes in PON is the lower sensitivity of the CHN-analyser, compared to MS and GC. Additionally, PON determination suffers from several of the same issues as the ^{15}N method, for example dissolved organic nitrogen (DON) adsorption. Due to degradation and remineralisation of PON by heterotrophic bacteria associated with diazotrophs during the incubation, only net fixation rates can be estimated. Since PON production may occur by other organisms in mixed communities, this application is limited to culture studies.

Extra care should be given to the mixing of the cultures in general, since filamentous cyanobacteria are very sensitive to high turbulence. During the incubation carried out before nitrogen fixation rate measurements, it is important to shorten the equilibration time between the gas and the liquid phase. In this case we advise the use

of a shaking plate during the incubation (low/mid velocity). Additionally, samples should be treated carefully and quickly to minimise in- or out-gassing.

11.3.3 Organic matter biogeochemistry

The instrumentation for determining biogeochemical elements like carbon, nitrogen and phosphorus in bulk dissolved organic matter (DOM) and particulate organic matter (POM), for example by nutrient autoanalyser, CHN-analyser or MS, is generally advanced, and enables high precision (<5%) measurements. Coupled to auto-sampling devices, a large number of samples can be processed in reasonable time. On-site preparation and storage of samples allows for analysis at a later time, although attention must be paid to certain storage requirements, i.e. uninterrupted cooling/freezing, and/or specific poisoning of samples (e.g. Grasshof *et al.*, 1999). Due to the relative ease of obtaining and analysing samples, determining element concentrations and ratios in particulate and dissolved matter qualifies for most kinds of experimental and field approaches. If an element is determined in its inorganic as well as its organic forms during an experiment, this approach can also be helpful to identify potential sources of errors or losses, as mass must be conserved in enclosed systems. The major limitation of this biogeochemical approach for quantifying production and export processes is that the information obtained is quite unspecific with respect to the source, turnover or quality of the material. Determining isotopic composition and fractionation in specific compounds such as fatty acids, or following the fate of stable isotopes such as ^{15}N after addition, can help to discriminate the origin of the material and its participation in autotrophic or heterotrophic processes (Dugdale & Goering, 1967; Peterson & Fry, 1987; Boschker & Middleburg, 2002; McCallister *et al.*, 2004; Veuger *et al.*, 2007). Here, care must be taken to thoroughly remove inorganic carbon prior to POC measurements as ^{13}C signatures of PIC and POC are different (Komada *et al.*, 2008). In general, attention must be paid to high background loads of organic matter especially in experimental and coastal field investigations, as these potentially decrease the ratio between the CO_2 treatment effect and the noise.

11.4 Potential pitfalls

11.4.1 Experimental set-up

CO_2 perturbation studies with plankton are often conducted using enclosures that range from a few ml batch incubations to much larger mesocosms (see chapters 5 and 6). Here, plankton organisms are usually incubated with a fixed initial amount of nutrients, while the carbonate system is perturbed by CO_2 aeration or direct pH adjustment (see chapter 2). During the course of the experiment, the build-up of organic biomass is accompanied by the drawdown of nutrients and inorganic carbon resources. Due to the resulting changes in nutrient availability, the production rates and element stoichiometry of organic matter can vary strongly over the course of experiments (Antia *et al.*, 1963; Banse, 1994; Biddanda & Benner, 1997; Engel *et al.*, 2002). Gervais & Riebesell (2001) showed that besides the absolute value, the variability of element ratios in particulate matter also increased with increasing biomass build-up during a CO_2 perturbation experiment (Figure 11.2). Besides nutrients, light availability and diurnal cycles potentially co-determine organic matter production rates and element stoichiometry in ocean acidification experiments (Burkhardt *et al.*, 1999) (Figure 11.3).

Although the consumption and reduction of nutrient resources in batch or enclosure experiments mimic natural situations in eutrophic waters, for example the “bloom” situation, they also involve the difficulty of identifying potentially small effects of ocean acidification against inherently large variations in organic C, N and P production rates. Batch experiments therefore require a sufficiently large number of replicate treatments, and high-precision measurements of co-determining factors, such as nutrients, light and temperature, to determine their variability and to identify the CO_2-effect. Moreover, initial synchronisation of cultures, the timing of sampling and of course the simultaneous sampling, are very important in comparing CO_2 treatments and have to be carefully considered during acidification studies.

A particular problem of incubation experiments may arise when cells in the ocean acidification treatments exhibit differences in length of the initial lag growth phase, and consequently in the on-set of exponential growth and drawdown of nutrients. This may, for example, be the case when cultures are not pre-adapted to the ocean acidification setting. In this case, the comparability of treatments with respect to nutrient availability may no longer be given. It is therefore important to monitor the development of the cells throughout the experiment, since a single or sporadic sampling may not capture the cell's response at the same growth stage.

Problems associated with the build-up of high biomass in batch incubations, for example significant changes in nutrient availability, self-shading, aggregation and sinking, can be alleviated in semi-continuous and dilute cultures, which require regular dilutions to keep cells exponentially growing at low densities. This is, however, laborious in long-term studies and studies conducted at naturally low nutrient concentration. For this purpose, the continuous culture (chemostat, turbidostat) is an alternative and favourable set-up (see chapter 5).

11.4.2 Transferability to the ecosystem level

As in all ecological studies, microbial processes in pelagic systems can be studied from an isolated perspective such as "what is the magnitude of microbial production?" or "what is the microbial diversity?" Alternatively, questions can be asked in a broader, more ecosystem-related, context such as "what factor in the ecosystem controls microbial production" or "what are the mechanisms maintaining microbial diversity?" At times, aquatic microbial ecology may seem overly dominated by the first type of questions. Historically this may be due to a lack of appropriate methods, only changing in the early 1980s, when appropriate methods for enumeration and activity measurements became available. The subsequent development in the field has strongly improved this situation, now allowing both types of questions to be asked, and at much more detailed levels than previously possible.

In relation to ocean acidification and its potential effects on microbial processes and the biological

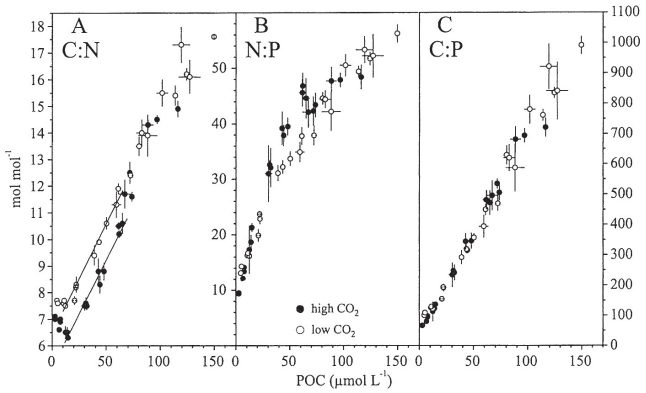

Figure 11.2 Development of element ratios during POC build-up in a batch culture experiment with the marine diatom species *S. costatum.* Perturbation of CO_2 was conducted by acid/base addition. (From Gervais & Riebesell, 2001, p. 500. Copyright (2001) by the American Society of Limnology and Oceanography, Inc.)

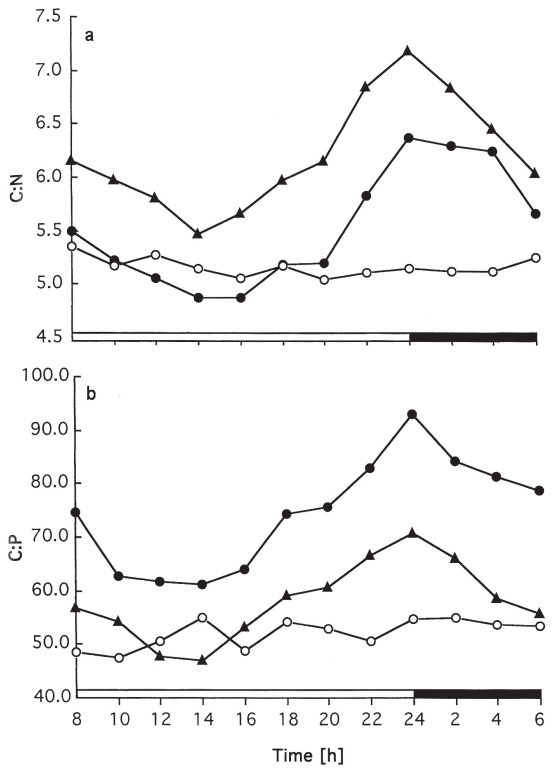

Figure 11.3 Diurnal variation of element ratios during a batch culture experiment applying low (3 µmol CO_2 kg^{-1}, triangles) and high (27 µmol CO_2 kg^{-1}, circles) CO_2 concentration. The high CO_2 experiment was conducted under continuous light (open symbols) and under a light-dark cycle (solid symbols) with the marine diatom species *S. costatum.* (From Burkhardt *et al.*, 1999, p. 687. Copyright (2001) by the American Society of Limnology and Oceanography, Inc.)

pump in natural systems, awareness of the two perspectives is of particular importance. Detection of an acidification effect on, for example, microbial production may be interesting in itself, but does not provide much insight unless it is known whether this results from a direct effect on the cell's physiology or an indirect effect caused by food web interactions, for example from increased production of labile organic matter by phytoplankton. Two direct effects of acidification on the microbial food web presently receive the most attention; the potential reduction in calcification and the change in C:N:P stoichiometry of primary production (Riebesell *et al.*, 2007; Bellerby *et al.*, 2008; Rost *et al.*, 2008).

The "model organism" for calcification within the microbial food web is *Emiliania huxleyi*, where the direct effect of acidification on the formation of coccoliths can be studied as an isolated phenomenon in laboratory cultures (De Bodt *et al.*, 2008), or in mixed natural communities in mesocosms (Delille *et al.*, 2005; Engel *et al.*, 2005; Paulino *et al.*, 2008). Transferring such observations to an understanding of the mechanisms in the microbial part of the ecosystem does however immediately expose our lack of understanding of trophic couplings in the microbial food web. When observing the success of *Emiliania huxleyi* in many mesocosm studies, a tempting conceptual model is that *E. huxleyi* is slightly better protected against microzooplankton grazing than other small flagellates, and thus slowly can outgrow these, potentially explaining the occurrence of large *E. huxleyi* blooms. The possible consequence is a bloom dominated by a single species, reaching a density sufficient for the support of species-specific lytic viruses – and thus leading to the observed viral-induced collapses of the bloom (Bratbak *et al.*, 1993, 1996). The biogeochemical consequences of such a scenario are potentially large: while grazing may lead to vertical export of the coccoliths and thus of alkalinity, viral lysis will release the non-sinking individual coccoliths in the photic zone. The implication would be a complicated feedback between acidification and vertical alkalinity distribution, mediated through the interactions of the microbial loop. However, the experimental evidence for crucial assumptions, for example that the coccoliths serve a role in predator defense, is lacking.

The observed effect of an increased C:N-stoichiometry in primary production at increased $p(CO_2)$ (Riebesell *et al.*, 2007) illustrates the potential complexity in translating an understanding of a direct microbial effect into an understanding of its consequences for the microbial ecosystem and the biological pump. If bacterial growth is limited by the availability of labile organic carbon, such extra organic input may increase bacterial consumption of mineral nutrients, with a potential reduction in mineral nutrient limited primary production as a result (Thingstad *et al.*, 2008). The resulting net changes inside the photic zone ecosystem are difficult to predict, both in terms of autotroph-heterotroph balance and community composition of heterotrophic prokaryotes. Predicting the consequences for the amount, the stoichiometric composition, and degradability of material exported via sinking particles or dissolved organic material, is equally difficult.

One lesson to be drawn from the, by now, extensive set of mesocosm experiments performed with different perturbations of the photic zone microbial ecosystem, is the need to be cautious in generalising from single experiments. The system's response to a perturbation depends not only on the nature of the perturbation, but also on the state of the system at the time of perturbation (presence of diatoms, limiting factor for bacterial growth, abundance of mesozooplankton, microbial species composition etc.). With the present level of understanding of how the food web works as a system, generalisations from single experiments thus bear the risk of, in hindsight, being naive.

Moreover, prediction of future ecosystem responses has to account for possible acclimation and/or adaptation of species to ocean acidification. Most laboratory and mesocosm studies performed so far have been restricted to a few days or weeks, excluding evolutionary changes. Evolution may be particularly rapid in microbial communities with generation times of hours to days. Different responses to acidification may occur among different strains of the same species, such as observed for *E. huxleyi* (Langer *et al.*, 2009). Strain selection and/or evolution are therefore likely to occur during real-time ocean acidification and are difficult to include in perturbation studies. Long-term studies are therefore necessary to investigate the potential of acclimation and adaptation to acidification of marine microorganisms.

11.5 Suggestions for improvements

One of the major uncertainties with regard to how changes in organic matter will affect export processes is that we do not know if the produced organic matter effectively contributes to sinking fluxes or rather stays suspended, or whether settling velocities of particles are so slow that degradation would likely occur within the upper water column. Most manipulative systems are on the scale of a few litres to cubic meters, and therefore limit the direct determination of export fluxes.

If we suppose, for example, that changes in organic matter stoichiometry primarily occur on the production side, based for example on the sensitivity of carbon and nitrogen acquisition to CO_2, we also need to know how these elements are partitioned thereafter. Several studies have indicated that unbalanced acquisition of elements results in exudation of the excess element into the dissolved phase; microbial processes and abiotic coagulation processes then determine whether these elements will be transformed into particulate organic matter, and will also determine the size distribution of particles, and therefore the settling velocity of particles. Hence, more information on the processes associated with the partitioning of and size distribution of particles can greatly improve our understanding of the responses of organic and export production to ocean acidification.

Moreover, our understanding of organic matter biogeochemistry has been deeply impacted by the microscopic inspection of the particles produced. What we directly see under the microscope are mostly plankton organisms. What we do not see unless we use compound specific stains are gel particles that especially form during unbalanced growth, such as during nutrient limitation. Carbohydrate gel particles, for example TEP, can be mostly responsible for the increase in carbon to nitrogen or phosphorus ratios during phytoplankton blooms (Engel *et al.*, 2002; Schartau *et al.*, 2007). Early studies already indicated that POC concentration is not equivalent to the carbon content of organisms, which at times contribute less than 50% to POC (Riley, 1970). This should be taken into account when deriving cellular production or cell quotas by normalising POC to cell abundance. Because there is no *a priori* constraint for element ratios in extracellular organic matter, improving our understanding of element partitioning can help to better estimate effects of ocean acidification.

11.6 Data reporting

In order to assess how acidification affects biogeochemical, physiological and ecological processes, it is recommended that data be normalised. Normalisation can be defined as "a mathematical process that adjusts for differences among data from varying sources in order to create a common basis for comparison." (www.hud.gov/offices/pih/programs/ph/phecc/definitions.cfm). What we choose to normalise to, i.e. the choice that we make for the common basis for comparison, depends on the context. For many biologists, the obvious choice is to normalise to the number of individuals, whereas for geochemists it might be more appropriate to normalise to the concentration of a particular element, for example nitrogen. Perhaps the most widely used example of normalisation in biological oceanography is the division of primary productivity (units of g C m^{-3} d^{-1}) by chlorophyll *a* concentration (units of g Chl *a* m^{-3}) to obtain the assimilation number (units of g C (g Chl *a*)$^{-1}$ d^{-1}). Much oceanographic research involves identifying controlling or limiting factors of ecological and biogeochemical processes (Arrigo, 2005). With regard to ocean acidification research, we are interested in both:

1. the effects of increased p(CO_2) in a more acidic ocean on for example CO_2-limitation of photosynthesis and N_2-fixation, and
2. the effect of acidification on the coupling of biogeochemical and ecological processes in relation to other limiting factors including light, nutrients, iron and/or other trace elements.

There are two main ways to characterise limitation, namely in terms of limitation of the yield of a process or limitation of the rate of a process (Cullen *et al.*, 1992). Yield is the biomass that is produced, with

typical units of g m^{-2} or g m^{-3}. Rate determines how long it takes to reach the yield, and is often expressed as a specific growth rate with units of d^{-1}. These two types of limitation are often referred to as Liebig's law and Blackman's law.

Liebig's law is based on limitation imposed on the yield by the availability of nutrients. It applies to situations where biomass is produced more or less in proportion to the amount of resource consumed. Strictly, Liebig's law is a special case of yield limitation in which yield is assumed to be controlled by only one limiting factor at a time (e.g., it is a threshold type of limitation and does not allow co-limitation). Co-limitation may be more prevalent than commonly assumed. Even if any given component of the community is limited by only one resource, different components may be limited by different resources leading to co-limitation at the community level. Assessment of yield limitation requires information on element composition, with the biomass yield for non-limiting elements determined by their multiples (e.g. indices A, B, C, D, Z in equation (11.4)) of the limiting element, where phosphorus is commonly taken as the basis for comparison (e.g. equation (11.4)).

$$[\text{Organic}]:[\text{Inorganic}] = [C_A H_B O_C N_D P Fe_Z)]:[CaCO_3, SiO_2] \tag{11.4}$$

Within the organic fraction, a common reference value is the Redfield ratio, but it is well documented that there can be significant deviations from the Redfield proportions of 106C:16N:1P (Geider & LaRoche, 2002). The Redfield ratio can also be extended to include trace elements such as iron (Fe) (Morel & Hudson, 1984). It is also necessary to consider the ratio of hard parts (such as $CaCO_3$ and opal) to soft tissue, which depends on the organisms present as well as the growth conditions. Thus, even when research is focused on element stoichiometry, it will be informative to have supplementary information on diversity and abundance of the species that make up the ecological community. Yield limitation can apply to both organic and inorganic components of biomass. For example, yield of $CaCO_3$ can be expressed as:

$$Y_{CaCO_3} = M_N \varepsilon_N \left(\frac{CaCO_3}{N} \right); \tag{11.5}$$

where N refers to a nutrient, which may be nitrogen or some other nutrient (e.g. Fe). Y_{CaCO_3} is the yield of $CaCO_3$ from a region of the ocean, M_N is the mass of the limiting nutrient "N" transported into that region, ε_N is the efficiency of uptake and retention of nutrient "N" by calcifiers and $\left(\frac{CaCO_3}{N} \right)$ describes the ratio of $CaCO_3$ to N in calcifiers.

The upper limit on yield is ultimately set by the supply of a limiting nutrient, M_N. However, the achieved yield depends also on the efficiency of capture of that nutrient by calcifiers and the ratio of $CaCO_3$ production to nutrient assimilation. Here the term nutrient is used in a generic sense and both dissolved and particulate nutrients, including nutrients contained in organic matter (both living and dead). Normalisation in this context involves determining the proportion of the nutrient contained in a population of interest and the ratio of $CaCO_3$ to N within this target group.

Blackman's law applies to situations when the rate is determined by the physical environment or when the limiting resource is replenished as it is consumed. Thus, Blackman limitation applies to the direct effects of light, temperature and pH, but can apply to nutrients if these are replenished (e.g. CO_2 from HCO_3^-; Fe from Fe bound to ligands (Fe-L)). Strictly, Blackman's law is a special case of rate limitation in which growth rate is determined by only one limiting factor (e.g., limitation of growth rate by one factor will be replaced by another factor once an upper limit imposed by the first factor is reached).

There are a number of different biomass variables to which productivity can be normalised to obtain a biomass-specific rate (Table 11.1). These include the production to biomass ratio (P:B), the assimilation number and the nitrogen-use efficiency.

Table 11.1 Examples of typical data normalisations in biological oceanography.

Symbol	Definition	Typical units	Notes
$P^{cell} = \frac{P}{cell}$	Cell-specific productivity	g C cell^{-1} d^{-1}	Depends on organism size: change in rate may occur due to change in cell size at constant biomass-specific rate.
$\mu = \frac{P}{B} = \frac{1}{B}\frac{dB}{dt}$	Specific growth rate (μ) = production to biomass ratio	d^{-1}	May depend on choice of biomass variable (e.g. unbalanced growth).
$P^{Chl} = \frac{P}{Chl}$	Assimilation number	g C (g Chl)$^{-1}$ d^{-1}	Requires knowledge of C:Chl to obtain growth rate.
$\frac{P}{N}$	Nitrogen use efficiency	mol C (mol N)$^{-1}$ d^{-1}	A similar approach can be applied to other elements.

The P to B ratio is used when P and B are measured in terms of the same biomass index, say C or N. It is used most often when describing growth or productivity of animal populations. However, it also applies to microbial populations. When used with microbes, the P to B ratio is often referred to as the specific growth rate, often designated μ. Other biomass-specific rates employ mixed currencies. Examples include the assimilation number, P^{Chl}, used for phytoplankton because it is easier to measure chlorophyll *a* than phytoplankton carbon. The nitrogen use efficiency was originally used with vascular plants, or plant leaves, but is also applicable to phytoplankton and other microbes. In analogy with nitrogen use efficiency, the use efficiencies for other elements, such as Fe, can also be measured (Raven, 1990). These different ways of expressing biomass-specific rates may not show the same response to ocean acidification.

Productivity is a rate per unit volume or surface area, and is obtained by multiplying the biomass by the biomass-specific rate: $P = P^B \times B$. For example, the rate of calcium carbonate production depends on the biomass of calcifiers, their growth rate and the $CaCO_3$ to biomass ratio:

$$P_{CaCO_3} = B_{calcifiers} \times \mu_{calcifiers} \times \left(\frac{CaCO_3}{B}\right); \quad (11.6)$$

where P_{CaCO_3} is the volume- or area-specific $CaCO_3$ production rate, $B_{calcifiers}$ = the biomass of calcifiers per unit volume or area, $\mu_{calcifiers}$ = the specific growth rate (P:B) of calcifiers, and $\left(\frac{CaCO_3}{B}\right)$ = the ratio of $CaCO_3$ to N in calcifiers.

Here, normalisation includes both (1) the specific growth rate and (2) the (element or mass) ratio of calcium carbon to biomass. Biomass can be expressed in terms of N, C or abundance.

As experimentalists, we often choose biomass variables for convenience or for answering one type of question. Microbiologists, for example, often normalise rates to the number of cells in a sample. Examples of normalising variables are given in Table 11.2.

Table 11.2 Specifications of data normalisation.

	Normalising to the number of individuals
Individual organisms	The individual is one of the basic hierarchical categories in biology. Individuals obtain resources from the environment, grow and reproduce. Many biological and ecological processes scale with the size of the individual. These include metabolic and growth rates, encounter rates, aggregation rates (and hence grazing and sinking rates).
Abundance (population size)	The population is one of the basic hierarchical categories in ecology. Natural selection operates on populations of organisms.
	Normalising to biomass
Biomass	Although physiological processes and population dynamics require knowledge of individuals and populations, many ecological and biogeochemical processes require normalisation of rates to some measure of biomass.
Chlorophyll *a*	Chlorophyll *a* can be measured readily and unambiguously. It is the most common measure of phytoplankton abundance. Other variables often scale with chlorophyll *a* (e.g. bacterial and zooplankton abundance or activity).
Nitrogen	Nitrogen is a key nutrient element in ocean biogeochemistry. The C:N of functional components of organisms falls within a narrow range (5 to 10 g C : g N). Primary production in much of the ocean is nitrogen limited (or N and P co-limited). In HNLC regions, N assimilation and CO_2 fixation are often limited by the same factors (Fe and/or light).
Organic carbon	Carbon is a key element in ocean and atmospheric biogeochemistry.

11.7 Recommendations for standards and guidelines

1. In order to identify the direct response of an organism to ocean acidification, potential co-effects of nutrient availability, light, diurnal cycles, temperature, and if applicable, community interactions (e.g. bacteria) need to be assessed carefully.
2. Since the response of a community depends not only on the type and strength of the perturbation, but also on ecological interactions, the impact of ocean acidification on organic and export production needs to be investigated at the ecosystem level, for example via mesocosm and field experiments. This enables the consideration of key features such as the food web structure, bottom-up control, top-down control and the type of growth limitation.
3. A better interpretation of changes in organic matter production with respect to export, can be obtained when the size and quality of organic matter (e.g. cellular material, mineral ballast, gels) is determined.

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