2 Approaches and tools to manipulate the carbonate chemistry

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2.1 Introduction

Although the chemistry of ocean acidification is very well understood (see chapter 1), its impact on marine organisms and ecosystems remains poorly known. The biological response to ocean acidification is a recent field of research, the first purposeful experiments have only been carried out as late as the 1980s (Agegian, 1985) and most were not performed until the late 1990s. The potentially dire consequences of ocean acidification have attracted the interest of scientists and students with a limited knowledge of the carbonate chemistry and its experimental manipulation. Perturbation experiments are one of the key approaches used to investigate the biological response to elevated $p(CO_2)$. Such experiments are based on measurements of physiological or metabolic processes in organisms and communities exposed to seawater with normal and altered carbonate chemistry. The basics of the carbonate chemistry must be understood to perform meaningful CO_2 perturbation experiments (see chapter 1).

Briefly, the marine carbonate system considers $CO_2^*(aq)$ [the sum of CO_2 and H_2CO_3], HCO_3^-, CO_3^{2-}, H^+, OH^-, and several weak acid-base systems of which borate-boric acid ($B(OH)_4^-$, $B(OH)_3$) is the most important. As discussed by Dickson (chapter 1), if two components of the carbonate chemistry are known, all the other components can be calculated for seawater with typical nutrient concentrations at given temperature, salinity, and pressure. One of the possible pairs is of particular interest because both components can be measured with precision, accuracy, and are conservative in the sense that their concentrations do not change with temperature or pressure. Dissolved inorganic carbon (DIC) is the sum of all dissolved inorganic carbon species while total alkalinity (A_T) equals $[HCO_3^-] + 2[CO_3^{2-}] + [B(OH)_4^-] + [OH^-] - [H^+]$ + minor components, and reflects the excess of proton acceptors over proton donors with respect to a zero level of protons (see chapter 1 for a detailed definition). A_T is determined by the titration of seawater with a strong acid and thus can also be regarded as a measure of the buffering capacity. Any changes in any single component of the carbonate system will lead to changes in several, if not all, other components. In other words, it is not possible to vary a single component of the carbonate system while keeping all other components constant. This interdependency in the carbonate system is important to consider when performing CO_2 perturbation experiments.

To adjust seawater to different $p(CO_2)$ levels, the carbonate system can be manipulated in various ways that usually involve changes in A_T or DIC. The goal of this chapter is (1) to examine the benefits and drawbacks of various manipulation methods used to date and (2) to provide a simple software package to assist the design of perturbation experiments.

2.2 Approaches and methodologies

Seawater chemistry can be manipulated in various ways that alter the carbonate system differently. The following sections examine the five techniques that are most useful in the context of ocean acidification. To illustrate the discussions, each section below is followed by a numerical example. The R package seacarb was used to calculate the carbonate chemistry parameters (Lavigne & Gattuso, 2010) and the syntax used for

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each example is available in Gattuso & Lavigne (2009). Note that other packages are available (see chapter 1). Calculations were carried out using the first and second dissociation constants of carbonic acid given by Lueker *et al.* (2000). In systems open to the atmosphere, it is assumed that the seawater and atmosphere are in equilibrium with respect to CO_2 and the target $p(CO_2)$ is the projected value for the year 2100 (Gattuso & Lavigne, 2009; Table 2.1).

Table 2.1 Seawater carbonate chemistry in 2007, 2100, and after perturbations intended to simulate year 2100 carbonate chemistry (from Gattuso & Lavigne, 2009). Total alkalinity (A_T), partial pressure of CO_2 in seawater ($p(CO_2)$), salinity and temperature were used to derive all other parameters using the seacarb package (Lavigne & Gattuso, 2010) except for manipulations of the calcium concentration for which DIC was used rather than $p(CO_2)$. Temperature (18.9°C) and salinity (34.9) were assumed to remain constant, the concentrations of total phosphate and silicate were set to 0 and the seawater $p(CO_2)$ was set at 384 µatm in 2007 and 793 µatm in 2100. (a): $\times 10^{-9}$ mol kg^{-1}, (b): $\times 10^{-6}$ mol kg^{-1}.

	$pCO_{2\,sw}$ (μatm)	pH_T (−)	$[H^+]$ (a)	TA (b)	DIC (b)	$[CO_2]$ (b)	$[HCO_3^-]$ (b)	$[CO_3^{2-}]$ (b)	Ω_c (−)	Ω_a (−)
Year 2007	384	8.065	8.6	2325	2065	12.8	1865	187	4.5	2.9
Year 2100	793	7.793	16.1	2325	2191	26.4	2055	110	2.6	1.7
Gas bubbling	793	7.793	16.1	2325	2191	26.4	2055	110	2.6	1.7
Addition of high-CO_2 seawater	792	7.793	16.1	2325	2191	26.4	2055	110	2.6	1.7
Addition of CO_3^{2-} and HCO_3^-; closed sys.	793	7.942	11.4	3406	3146	26.4	2901	218	5.2	3.4
Addition of CO_3^{2-} and HCO_3^-; open sys.	384	8.207	6.2	3406	2950	12.8	2580	357	8.5	5.5
Acid addition; closed sys.	793	7.768	17.1	2184	2065	26.4	1940	98	2.3	1.5
Acid addition; open sys.	384	8.042	9.1	2184	194	12.8	1767	167	4	2.6
Addition of:										
$\quad CO_3^{2-}$ and HCO_3^-; closed sys.	400	8.073	8.4	2467	2191	13.3	1977	201	4.8	3.1
$\quad$ followed by acid addition; closed sys.	793	7.793	16.1	2325	2191	26.4	2055	110	2.6	1.7
Manipulation of $[Ca^{2+}]$	384	8.065	8.6	2325	2065	12.8	1866	187	2.6	1.7

It must be pointed out that the methods described below enable one to set the carbonate chemistry at the beginning of a perturbation experiment. The impact of biological (e.g. photosynthesis, respiration and calcification) and physical processes (e.g. air-seawater exchange of CO_2 and temperature changes) on the carbonate chemistry can distort the initial values and must be taken into account in the experimental design (see section 2.4.2).

There are several experimental approaches to adjust seawater CO_2 by either changing DIC at constant A_T (e.g. aeration with air at target $p(CO_2)$, injections of CO_2 saturated seawater and combined additions of $NaHCO_3$ or Na_2CO_3 and HCl) or changing A_T at constant DIC (NaOH and/or HCl additions). Each experiment has different requirements depending on organisms, experimental duration, incubation volumes or sampling intervals and hence one of the possible carbonate chemistry manipulations will probably be favoured.

2.2.1 Changing DIC at constant A_T

Aeration at target p(CO_2)

Bubbling seawater with gases is a very efficient way to manipulate its carbonate chemistry. The seacarb function *pgas* estimates the changes in the carbonate chemistry resulting from changes generated by bubbling gases.

Example*:* seawater with $p(CO_2)$ of 384 µatm and an A_T of 2325 µmol kg^{-1} can be bubbled with a mixture of CO_2 and air with a $p(CO_2)$ of 793 µatm. Salinity is 34.9, temperature is 18.9°C and calculations are done for surface waters. This approach exactly reproduces the values of all parameters of the carbonate system expected in the year 2100 (Table 2.1).

Two aeration techniques have been used in ocean acidification studies: pH-stat and bubbling with premixed gases. In pH-stat systems, pH is monitored continuously and a controller opens or closes the valves that deliver the gases when pH goes above or below a set value. Gases are then delivered until pH reaches the target value again. Different combinations of gases have been used: (1) air and pure CO_2, (2) CO_2-free air and pure CO_2 and (3) air, CO_2-free air and pure CO_2. CO_2-free air can be produced easily using either molecular sieves or CO_2 scrubbers such as soda lime or NaOH and $Ca(OH)_2$ (C. Hintz, pers. comm.). The pH threshold is calculated using the desired $p(CO_2)$ and total alkalinity which is either assumed to be constant or frequently measured. This method has the potential to compensate for changes in the carbonate chemistry due to photosynthesis and respiration or, in the case of open culture systems, to changes in the chemistry of the source water. However, the air-water gas exchange and CO_2 hydration is relatively slow and the system may not reach equilibrium when there is high biological activity (high biomass to volume ratio). Like with other approaches, it does not compensate for changes in total alkalinity resulting from the precipitation and dissolution of $CaCO_3$ that occur between measurements of total alkalinity.

Overall, the carbonate chemistry can be maintained with good efficiency in the culture vessel, for example $p(CO_2)$ can be controlled usually better than ±10 µatm. The main drawback of this technique is that the pH electrode must be frequently calibrated in order to correct for drift. Hence, the technique that involves bubbling with premixed gases may be attractive. Air with the desired $p(CO_2)$ can be produced using gas mixing pumps or purchased. Another technique would be to maintain atmospheric $p(CO_2)$ to the desired level in the laboratory or in the growth cabinets in which the experiments are carried out (such cabinets for maintaining terrestrial plant are commercially available). A simple air pump can then be used to bubble the experimental seawater. To the best of our knowledge, this technique has not yet been used in the context of ocean acidification.

Aeration of seawater should be used with care for two reasons. First, bubbling may enhance the surface coagulation of organic matter (Engel *et al.*, 2004). This may be critical for studies investigating the response of microbial communities since their metabolism depends on the respective abundance of dissolved and particulate organic matter. This drawback may be avoided by enclosing the community in a dialysis bag maintained in a container bubbled with a gas of the desired $p(CO_2)$ (M. G. Weinbauer, pers. comm.). Such bags are permeable to gases and small molecules but impermeable to larger molecules and particles. It is highly recommended to check that the membranes are chemically neutral as some materials leak unwarranted chemical compounds. Second, some species of phytoplankton, for instance dinoflagellates, are known to be negatively affected by turbulence and especially by continuous bubbling (Shi *et al.*, 2009). For those cases, it is useful to equilibrate the media to the desired $p(CO_2)$ level prior to the inoculation or use other means to achieve a carbonate system close to reality, i.e. where DIC varies and A_T remains constant.

Addition of high-CO₂ seawater

DIC and A_T are conservative quantities with respect to mixing (Wolf-Gladrow *et al.*, 2007). Hence, when two water parcels are mixed, the amount of a solute in the mixture equals the sum of the amounts of this solute in the two initial water parcels. The seacarb function *pmix* estimates the carbonate chemistry after mixing of two water samples.

Example: one can mix, in a closed system, 0.99623 kg of seawater having an A_T of 2325 µmol kg⁻¹, and $p(CO_2)$ of 384 µatm with 0.00377 kg of seawater having an A_T of 2325 µmol kg⁻¹ and saturated with CO_2 ($p(CO_2) = 1 \times 10^6$ µatm). The weight fraction of the high-CO_2 seawater relative to the final weight is 3.76 $\times 10^{-3}$. Salinity is 34.9, temperature is 18.9°C and calculations are made for surface waters. This produces seawater with a final $p(CO_2)$ of 793 µatm and all parameters of the projected carbonate chemistry in 2100 are perfectly reproduced.

To the best of our knowledge, this approach has been used only twice. To create a $p(CO_2)$ range from 200 to 1300 µatm, Schulz *et al.* (unpubl.) added about 20 to 200 dm³ of seawater enriched in CO_2 to 60 m³ mesocosms (Figure 2.1). C. McGraw (pers. comm., 2009) used this technique in laboratory experiments. As this approach uses water with very high $p(CO_2)$, caution has to be taken to avoid gas exchange during mixing and handling.

Figure 2.1 Aeration system for seawater carbon dioxide (CO_2) enrichment consisting of a bottle of pure CO_2 gas and two 250 l seawater containers (photo credit: K. Schulz).

Addition of strong acid as well as CO_3^{2-} and/or HCO_3^-

As will be outlined below, the addition of acid alone does not fully mimic the changes in carbonate chemistry expected during the present century. The addition of CO_3^{2-} and/or HCO_3^- followed by acid circumvents this problem. The first addition elevates DIC to the desired level and the acid addition (at constant DIC) precisely cancels the increase in A_T resulting from the addition of CO_3^{2-} and/or HCO_3^-.

Example: HCO_3^- (111.2 µmol kg⁻¹ of NaHCO₃) and CO_3^{2-} (15.3 µmol kg⁻¹ of Na₂CO₃) can be added to seawater for which p(CO_2) (384 µatm) and A_T (2325 µmol kg⁻¹) are known, salinity is 34.9, temperature is 18.9°C and calculations are done for surface waters. Then, 14.18 ml kg⁻¹ of 0.01 N HCl is added. The first addition raises DIC to the desired level of 2191 µmol kg⁻¹ but increases A_T to a value higher than target (2467 vs. 2325 µmol kg⁻¹; Table 2.1). The subsequent addition of HCl, in a closed system to prevent gas exchange, restores A_T to the desired value without affecting DIC. All carbonate parameters after both additions reach the target values.

2.2.2 Addition of strong acids and bases

The addition of a strong acid, such as HCl, or base, such as NaOH, in a system closed to the atmosphere does not alter the concentration of dissolved inorganic carbon but modifies total alkalinity. A_T decreases following addition of an acid whereas it increases following addition of a base. The change in total alkalinity after addition of a strong acid or base in a system open to the atmosphere is identical to that described above for a closed system. However, the concentration of DIC is modified through CO_2 exchange at the air-water interface. The seacarb function *ppH* estimates the changes in the carbonate chemistry during pH manipulations. The change in salinity due to the addition of acid or base is minor and can therefore be neglected.

Example: a volume of 14.08 ml of 0.01 N HCl is added to 1 kg of seawater with known p(CO_2) (384 µatm) and A_T (2325 µmol kg⁻¹); the atmospheric p(CO_2) is 384 µatm, salinity is 34.9, temperature is 18.9°C and calculations are done for surface waters. The target p(CO_2) of 793 µatm is reached in a closed system (Table 2.1) but the pH is lower than the value expected in 2100 (7.768 vs 7.793, corresponding to a 2.9 % increase in [H⁺] that results from the decrease in total alkalinity generated by acid addition). This is an undesirable effect of the direct manipulation of pH, as A_T will not change significantly during the course of this century. As a result, DIC, HCO_3^-, CO_3^{2-} and the CaCO₃ saturation states are lower than their target values. However, it is possible to restore A_T to its initial level by adding CO_3^{2-} and HCO_3^-, an approach that is described above.

2.2.3 Addition of CO_3^{2-} and/or HCO_3^-

DIC and A_T can be increased by adding CO_3^{2-} in the form of Na_2CO_3 and/or by adding HCO_3^- in the form of $NaHCO_3$. In closed systems, the change in DIC generated by these additions is proportional to the changes in concentration: $1 \times \Delta[CO_3^{2-}]$ and $1 \times \Delta[HCO_3^-]$. The contribution of these anions to A_T is proportional to the product of their charge and concentration. Thus, A_T increases by $2 \times \Delta[CO_3^{2-}]$ and $1 \times \Delta[HCO_3^-]$. The changes in the carbonate chemistry generated by manipulations of total alkalinity therefore depend on the proportion of CO_3^{2-} and HCO_3^- added. This approach can be used to hold pH constant or be combined with acid addition to maintain A_T constant (see section 2.2.1).

The seacarb function *pTA* estimates the changes in the carbonate chemistry following addition of CO_3^{2-} and/ or HCO_3^-. In an open system, the carbonate system re-equilibrates through air-sea CO_2 gas exchange after the addition of chemicals but A_T remains at a level higher than the target value.

Example: HCO_3^- (1081 µmol kg^{-1} of $NaHCO_3$) is added to seawater for which p(CO_2) (384 µatm) and A_T (2325 µmol kg^{-1}) are known. No CO_3^{2-} is added, the atmospheric p(CO_2) is 384 µatm, salinity is 34.9, temperature is 18.9°C and calculations are done for surface waters. Results are shown in Table 2.1. In a closed system, the target p(CO_2) of 793 µatm is reached but all other parameters of the carbonate system are very different from their values expected in 2100. pH is lower than it should be (7.942 vs. 7.993) and A_T, DIC, [CO_3^{2-}] as well as the saturation states of aragonite (Ω_a) and calcite (Ω_c) are higher than the target values and are even higher than the values of the initial seawater. Differences are magnified in open systems.

2.2.4 Manipulation of the Ca^{2+} concentration

Although manipulating the calcium concentration is not technically altering the carbonate chemistry *per se*, this approach has been used in the context of ocean acidification. The reason is that some calcifying organisms, such as corals, respond to the calcium carbonate saturation state of seawater Ω which is expressed as:

$$\Omega = \frac{[Ca^{2+}][CO_3^{2-}]}{K_{sp}^*}, \tag{2.1}$$

where $[Ca^{2+}]$ and $[CO_3^{2-}]$ are the concentrations of calcium and carbonate ions in seawater, respectively, and K_{sp}^* is the solubility product at the *in situ* conditions of temperature, salinity and pressure (Zeebe & Wolf-Gladrow, 2001). It can readily be appreciated that the changes in Ω resulting from a decrease in [CO_3^{2-}] driven by ocean acidification can be mimicked by altering [Ca^{2+}]. Uncoupling Ω from the carbonate chemistry can also be useful, for instance, to examine a possible dependence of photosynthesis on calcification (Gattuso *et al.*, 2000; Trimborn *et al.*, 2007). It is also useful to replenish calcium when its concentration decreases below its natural levels during long-term experiments with calcifiers (Langdon *et al.*, 2000).

The seacarb function *pCa* estimates the changes in Ω_c and Ω_a resulting from the manipulation of the concentration of $Ca^{2+}{}_{sw}$. It is recommended to use the simplified recipe for synthetic seawater based on DOE (1994) described by Gattuso *et al.* (1998) because it is the basis of the synthetic seawater that has been used to determine a variety of equilibrium constants for use in seawater. Note that the effect of the changes in the calcium concentration on the dissociation constants of carbonic acid and on the solubility product of $CaCO_3$ may have to be considered (Ben-Yaakov & Goldhaber, 1973).

Example: artificial seawater with a known A_T (2325 µmol kg^{-1}) and (2064 µmol kg^{-1}) and with a calcium concentration set to 6.03 µmol kg^{-1} reproduces well the saturation states of aragonite and calcite expected in 2100 without affecting any of the other parameters of the carbonate system which remain at their 2007 values (Table 2.1).

2.3 Strengths and weaknesses

The seacarb function *oa* (Lavigne & Gattuso, 2010) describes the various approaches that can be used to alter the seawater carbonate system. It provides precise guidelines on how the target carbonate chemistry can be reached as well as a plot showing, in the A_T vs. DIC space, the changes generated by the five main perturbation techniques.

It can readily be seen from Figure 2.2 and Table 2.1 that three approaches closely mimic the on-going and future changes in the seawater carbonate chemistry: gas bubbling, addition of high-CO_2 seawater, and combined additions of acid and bicarbonate and/or carbonate. All three methods increase dissolved inorganic carbon at constant total alkalinity, a situation that closely resembles the changes in the carbonate chemistry that occurred during the past 200 years and are expected to continue in the next few hundreds years. All three approaches therefore allow precise control of all carbonate parameters to reach target values at the beginning of a perturbation experiment. Gas bubbling is the easiest to implement and can be used to maintain constant conditions over long periods of time. Note that, in all approaches, biological processes (e.g. photosynthesis, respiration, calcification, dissolution of $CaCO_3$, nutrient uptake and release) can significantly distort the target carbonate chemistry by changing DIC and A_T when experiments are run with high biomass (Rost et $al.$, 2008). Gas bubbling can maintain the concentration of DIC constant, provided that the dissolution of CO_2 is faster than its biological uptake, but does not compensate the drift in A_T. Also, in all three methods, in addition to A_T, calcification can also deplete the concentration of Ca^{2+} when the organism to volume ratio is high or the incubation time is long.

The approach that adds CO_3^{2-} and/or HCO_3^- appears of limited practical value because most carbonate chemistry variables deviate from target values. Obviously, due to gas exchange at the air-water interface, only gas bubbling can successfully be used to adjust $p(CO_2)$ and other parameters of the carbonate chemistry in an open system.

Several studies have used acid addition to manipulate the carbonate chemistry. Although this technique enables to precisely control $p(CO_2)$ in systems closed to the atmosphere, it also alters A_T which results in carbonate parameters which are different from those expected in the future: pH is lower than its target value, DIC remains unchanged (whereas it increases under natural ocean acidification), and the concentrations of bicarbonate and carbonate as well as $CaCO_3$ saturation states are lower than expected. Gattuso & Lavigne (2009) and Schulz et $al.$ (2009) provide a detailed analysis of the similarities and differences between acid addition and DIC manipulations. It is recommended to combine the addition of bicarbonate and/or carbonate, to increase DIC, with acid addition to avoid this drawback.

Iglesias-Rodriguez et $al.$ (2008a) recently reported that, in contrast to all previous reports, calcification of coccolithophorids increases at elevated $p(CO_2)$. They argued that this is due to the approaches used to manipulate the carbonate chemistry and that the approach of gas bubbling is superiour to that of acid addition (Iglesias-Rodriguez et $al.$, 2008a and 2008b). The argument is that acid addition does not reproduce the increase of the HCO_3^- concentration generated by natural ocean acidification whereas gas bubbling does. The authors claim that since HCO_3^- may stimulate photosynthesis and, in turn, calcification, experiments that used acid addition and demonstrated that calcification declines at lower pH, confounded the issue. This statement is misleading, although there is no doubt that gas bubbling better mimics the future carbonate chemistry (Table 2.1). Several previous perturbation experiments were carried out with gas bubbling and also reported lower rates of calcification of coccolithophores at lower pH or higher $p(CO_2)$ (e.g. Sciandra et $al.$, 2003; Delille et $al.$, 2005; Feng et $al.$, 2008). It should also be pointed out that perturbation experiments carried out using gas bubbling can, like all other approaches, also lead to poor control of the carbonate chemistry, for example when the duration of the experiments is too long, when experiments are run at high biomass, or when the DIC uptake is larger than the dissolution of CO_2 (see above). It therefore seems that the different responses of coccolithophores reported in the literature do not originate from the approach used to manipulate the carbonate chemistry. The situation is clearer in reef-building corals as Schneider & Erez (2006) measured the rate of calcification under constant DIC, constant pH and constant $p(CO_2)$ and showed that calcification is controlled by the concentration of CO_3^{2-}, the future value of which is relatively well mimicked by acid addition.

2.4   Potential pitfalls and suggestions for improvements

2.4.1   Seawater filtration and autoclaving

As filtration can significantly shift the carbonate chemistry of seawater, the carbonate system should be manipulated after filtration. If this cannot be done, samples for carbonate chemistry determination must be
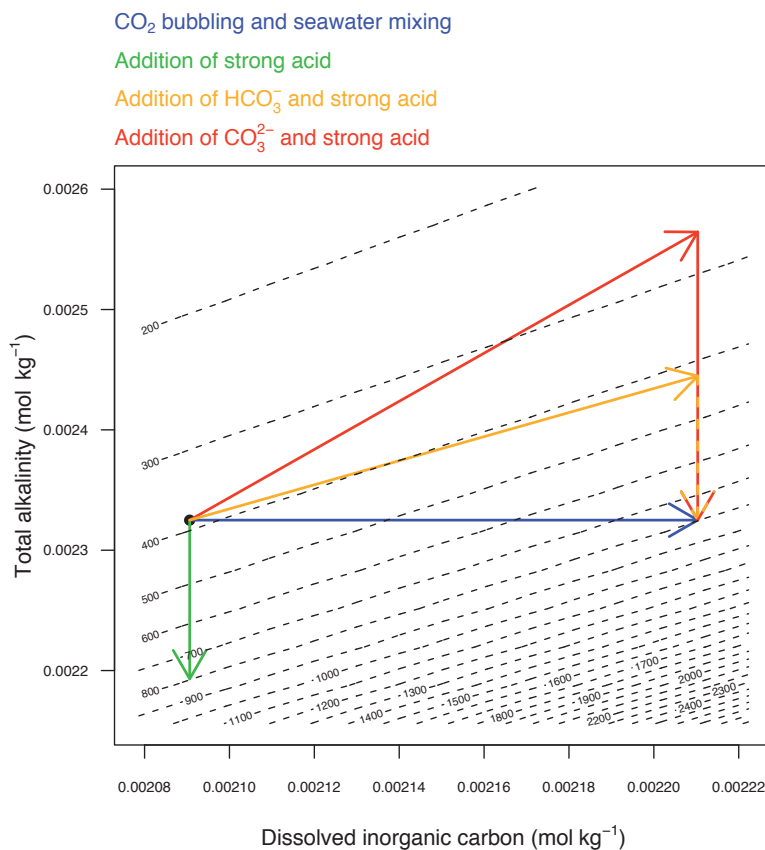
Figure 2.2 Changes in the carbonate chemistry generated by various perturbation techniques, in the A_T vs. DIC space. The plot was generated using the seacarb function *oa*.

taken after filtration to account for any gas exchange. Gentle pressure filtration through a cartridge should be performed because vacuum filtration or strong pressure filtration alter p(CO_2) and DIC (see Grasshoff *et al.*, 1999) and could lead to cell rupture, which in turn increases total alkalinity.

Seawater autoclaving should also be performed prior to manipulating the carbonate chemistry. It can severely change the carbonate chemistry as boiling seawater strips off gases and most of the DIC is lost. A_T has been reported to change as well: it increases, together with salinity, due to water evaporation but can also decrease due to precipitation of carbonate. Overall, the p(CO_2) of autoclaved water is initially relatively low and pH quite high. When the seawater cools, some of the DIC is likely to re-dissolve from the headspace into the water phase. When autoclaving natural seawater, it is recommended to sample for DIC and A_T determinations before and after autoclaving in order to ascertain the impact of the operating procedure on the carbonate chemistry. In case of artificial seawater, autoclaving will not change the carbonate chemistry if it is carried out before the addition of $NaHCO_3$ or Na_2CO_3.

2.4.2   Reaching and maintaining target values

When seawater is manipulated via bubbling with gases of different p(CO_2), one must ascertain that equilibrium has been reached before starting an experiment. The required time to reach equilibrium depends on several factors such as the biomass to volume ratio, p(CO_2), gas flow rate, bubble size, volume and shape of the flask and temperature, and may require several days. Since both bottled gas mixtures and CO_2-free air from generators do not contain any water vapour, it is important to humidify the dry air

before bubbling in order to avoid evaporation that would increase salinity, itself leading to changes in the parameters of the carbonate system.

Once the carbonate chemistry has reached a target level, it is critical to avoid any process leading to gas exchange between seawater and the atmosphere such as autoclaving, vacuum filtration or temperature changes. Unless the experimental set-up is open and bubbling is running continuously, gas-tight bottles filled without headspace should be used.

When working with high cell densities, processes such as photosynthesis and calcification can severely shift the carbonate chemistry. This problem is most pronounced in closed systems but must also be considered in open systems subject to continuous bubbling because biologically-driven changes can exceed the capability of the regulation system and thus cause a departure from the desired carbonate chemistry. This problem is more pronounced when investigating calcifying systems because calcification decreases A_T. The drawdown of Ca^{2+} associated to the decline of A_T might also need to be considered as it could also impact on the $CaCO_3$ saturation state.

2.4.3 Contributions of dissolved organic matter, dissolved inorganic nutrients and pH buffers to total alkalinity

During photosynthesis, phytoplankton release dissolved organic compounds containing basic functional groups that readily react with protons during seawater titration, and thereby contribute to total alkalinity. The magnitude of the contribution of dissolved organic compounds to A_T depends on the species and on the age of the culture, suggesting that individual phytoplankton species exude dissolved organic compounds with unique proton accepting capacities (Kim & Lee, 2009). This contribution could be significant in perturbation experiments carried out at relatively high biomass:volume ratios. In that case, which must be avoided in perturbation experiments, one might consider the use of calculated carbonate parameters (e.g. total alkalinity derived from pH and DIC or from $p(CO_2)$ and DIC) rather than the measured total alkalinity.

Some salts, such as inorganic nutrients, must be considered in experiments manipulating the carbonate chemistry as they contribute to A_T. The use of pH buffers causes large deviations from the natural carbonate chemistry as they increase A_T to values too high for accurate measurements and therefore precludes the calculation of the carbonate system using A_T. DIC and pH or $p(CO_2)$ then have to be used instead. Depending on chemical form and concentration, inorganic nutrient addition can change A_T and should be included in carbonate chemistry calculations. Furthermore, possible changes of experimental inorganic nutrient concentrations and speciation, and their impact on A_T (for details see Brewer & Goldman (1976); Wolf-Gladrow et al. (2007)) highlight the importance of concomitant sampling for nutrients such as phosphate, ammonium and silicate, together with those for carbonate chemistry determination.

Phosphate (PO_4^{3-}) is usually added to seawater as the sodium salt $NaH_2PO_4 \cdot H_2O$ (see Guillard & Ryther, 1962). Its addition does not alter A_T as the immediate dissociation products Na^+ and $H_2PO_4^-$ do not contribute to A_T (see equation 1.50). In fact, since the other three phosphate species (H_3PO_4, HPO_4^{2-}, PO_4^{3-}; equation 1.50) are included in A_T, the addition of $NaH_2PO_4 \cdot H_2O$ do increase the alkalinity contribution by phosphate but concomitantly reduces the contribution of other A_T components by the same amount (equation 1.43). However, if phosphate is added as phosphoric acid (H_3PO_4), total alkalinity is reduced by one mole per mole of phosphoric acid added. Hence, when calculating carbonate system speciation from measured A_T, the phosphate contribution should be taken into account. However, the error made by ignoring the contribution of phosphate to A_T is negligible at concentrations below 1 μmol kg^{-1}.

Nitrate (NO_3^-) is usually added to seawater as the sodium salt $NaNO_3$ which does not alter A_T. Furthermore, as nitrate has no A_T component, it does not need to be considered when calculating the carbonate system from A_T measurements. However, the addition of nitric acid (HNO_3) decreases A_T. Ammonia is usually added as ammonium chloride (NH_4), which does not change A_T. Nevertheless, since NH_3 contributes to A_T, it must be considered in carbonate chemistry calculations. In practice it can probably be ignored in most cases because of its relatively low concentration.

Silica in the form of H_4SiO_4 is generally added as the sodium salt $Na_2SiO_3 \cdot 9H_2O$. This changes A_T as SiO_3^{2-} combines with H_2O to form $H_2SiO_4^{2-}$, which quickly converts to $H_3SiO_4^-$ by consuming a proton. At seawater pH, most of the $H_3SiO_4^-$ further converts to H_4SiO_4, consuming another proton. Therefore, A_T increases by two moles for each mole of Na_2SiO_3 added. Additions of silica to either natural seawater or artificial seawater can be relatively high, in the 100 μmol kg^{-1} range. In these cases it might be advisable to counterbalance the otherwise relatively large increase of A_T by additions of HCl. As $H_3SiO_4^-$ contributes to A_T, it should be included in the carbonate system calculations, although in many cases it can safely be ignored due to is relatively low concentrations at typical seawater pH (see Zeebe & Wolf-Gladrow, 2001).

2.4.4 Isotopic labelling of dissolved inorganic carbon

Labelling of the dissolved inorganic carbon (DIC) pool with ^{13}C or ^{14}C requires the same precautions during preparation and handling as described above for the carbonate chemistry. In fact, it is a carbonate chemistry manipulation in itself and hence should be the last step in the preparation of an experiment. Any headspace should be avoided as seawater-atmosphere CO_2 gas exchange reduces the concentration of the label.

Even if seawater is close to ambient $p(CO_2)$, any ^{13}C or ^{14}C added would outgas with time, driven by the difference in seawater and atmosphere [13]CO_2 or [14]CO_2 as their respective atmospheric partial pressure are close to 0: about 4 μatm for [13]CO_2 and 1 x 10^{-13} μatm for [14]CO_2 (calculated according to Coplen *et al.*(2002) and Nydal & Lövseth (1996)). Therefore, aeration of seawater with air at target CO_2 would increase ^{13}C or ^{14}C outgassing even though $p(CO_2)$ would be kept constant. Furthermore, depending on the amount of ^{13}C or ^{14}C sodium salts added, DIC and A_T and hence $p(CO_2)$ can change significantly.

2.4.5 Sampling of carbonate chemistry parameters

Measuring and reporting of at least two quantities of the carbonate system prior to, after and ideally during experiments will ensure constant conditions or reveal possible shifts. Discrete samples for determination of DIC or pH should be taken with care because CO_2 gas exchange between sample water and atmosphere could otherwise compromise the measurements. Sampling for A_T measurements, however, is not critically influenced by changes in DIC or pH related to gas exchange. Even if the water were stripped of any dissolved inorganic carbon, for example by warming the sample, A_T would stay constant provided that there is no evaporation and that salinity remains the same.

Samples should be poisoned for storage (see Grasshoff *et al.* (1999) and Dickson *et al.* (2007) for details) and kept at low temperatures before analysis. Headspaces within DIC or pH sample vials must be avoided. Depending on organism and experimental setup, DIC and A_T measurements should be performed on filtered seawater. For instance, phytoplankton cells grown to relatively high densities in comparison to oceanic waters, can disintegrate during A_T or DIC measurements because of necessary acid additions. This could release A_T or DIC components from the particulate to the dissolved phase and compromise analysis. Furthermore, negatively charged groups in cellular plasma membranes can absorb protons added during A_T titration, thereby compromising the measurement (Kim *et al.*, 2006). Similarly, the study of calcifying organisms can also make DIC and A_T filtration necessary because $CaCO_3$ dissolves during measurements due to acid additions, which artificially increases both DIC and A_T. Filtration of DIC samples must be carried out with care to avoid water-atmosphere CO_2 gas exchange.

2.4.6 Headspace and storage

Seawater in which the carbonate system has been manipulated and that is to be used in experiments should be handled very carefully. Wherever possible, headspace should be avoided as concentrations and speciation will otherwise change through water-atmosphere CO_2 gas exchange if seawater $p(CO_2)$ differs from atmospheric $p(CO_2)$ (current atmospheric values are about 390 μatm while those inside closed rooms are usually higher). An exception is seawater that is constantly aerated at target $p(CO_2)$ throughout the experiment. Nevertheless, frequent sampling for at least two carbonate chemistry parameters is necessary for quality control.

Likewise, when storing manipulated seawater prior to the experiment, there should be no headspace. In this respect, it is important to keep in mind that temperature variations will change the carbonate chemistry. For instance, cooling seawater will initially decrease $p(CO_2)$, while increasing pH. If there is no headspace and the seawater is afterwards adjusted to intended temperatures, the carbonate system will shift back to pre-set conditions. This would not be the case if the water were stored with a headspace. The same applies for seawater that was aerated at target CO_2. If aeration is carried out at different temperatures than experimental incubation, $p(CO_2)$ and hence carbonate chemistry speciation will change.

2.5 Data reporting

It is essential to report not only on the results obtained, but also on the methods used. The metadata should be included in databases (see chapter 15) in order to enable comparisons of different studies and meta-analysis. Table 2.2 provides a checklist of the information on the manipulation of the carbonate chemistry that should be reported when describing a perturbation experiment

Table 2.2 Checklist of the information on the manipulation of the carbonate chemistry that should be reported when describing a perturbation experiment.

Method used to manipulate the carbonate chemistry	Which of the methods below was used: – Aeration with air at target $p(CO_2)$ (indicate $p(CO_2)$ level and flow rate). – Addition of high-CO_2 seawater (indicate $p(CO_2)$ and mixing ratio). – Addition of strong acid as well as CO_3^{2-} and/or HCO_3^- (indicate volume and normality of acid added as well as the quantity of inorganic carbon added). – Addition of strong acids and bases (indicate volume and normality). – Manipulation of the Ca^{2+} concentration (indicate the recipe of artificial seawater used).
Type of manipulation	– Once before the experiment. – Continuous control during the experiment.
Parameters of the carbonate chemistry	– Values at the beginning and end of the experiment. If available, values during the experiment should also be provided. – At least two parameters of the carbonate system should be reported together with temperature and salinity. – The pH scale must be indicated.
History of organisms investigated	– Describe the conditions under which the organisms were maintained prior to the experiment. Were they pre-acclimated? If so, indicate the environmental conditions and the duration of the pre-acclimation phase.

2.6 Recommendations for standards and guidelines

Several factors can collectively affect the success of CO_2 manipulation experiments. Here we make recommendations regarding the method of seawater CO_2 manipulation; the choice of which can be critical in obtaining significant and reproducible results in CO_2 perturbation experiments.

The method of manipulating carbonate chemistry in seawater is of utmost importance. Bubbling seawater with CO_2 enriched air may be the first choice because it is a very efficient way to manipulate seawater carbonate chemistry and, more importantly, it exactly mimics carbonate chemistry changes occurring in the years to come; the future scenario for ocean carbon chemistry being an increase in $p(CO_2)$ and DIC (decrease in pH) without alteration of A_T. However, seawater aeration by bubbling may lead to difficulties in phytoplankton cultures (Shi *et al.*, 2009). Bubbling must therefore be sufficiently gentle to cause minimal impact on the phytoplankton assemblage or the cells should be separated from the bubbles by a gas-permeable membrane. Alternative but equally effective methods, when species are highly sensitive to bubbling, is to equilibrate the culture media with air at target $p(CO_2)$ or to mix it with high-CO_2 seawater prior to cell inoculation. Although the later method has yet to be widely tested, it also exactly mimics changes in carbonate chemistry in the future ocean just as the method of bubbling with high-CO_2 gases. The third method, equally reliable, is the combined addition of acid and bicarbonate and/or carbonate (increase in $p(CO_2)$ and DIC, but decrease in pH and A_T) and then addition of Na_2CO_3 and/or $NaHCO_3$ (to restore A_T). This method also yields the conditions predicted to occur in the future ocean. Other methods (manipulation of A_T and Ca^{2+}) can be useful in the context of specific process studies (such as calcification).

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