Technical Note: Maximizing accuracy and minimizing cost of a potentiometrically regulated ocean acidification simulation system

C.D. MacLeod, H.L. Doyle, & K.I. Currie

**Please note that all page and line numbers apply to the marked up version of the manuscript**

Andrew Dickson

Comments:

1) This is a useful manuscript which I would recommend publishing after some further editing. It shows that it is practical to maintain an ocean acidification simulation system within ±0.05 pH units of a target value, even when using relatively economical pH measuring and control systems, provided that the pH electrodes used are tested to ensure that they exhibit an approximately Nernstian response, and as long as care is taken to adjust for electrode calibration drift on a regular basis. Nevertheless, I feel that the current version is not as carefully written as it could be; it seemed somewhat disorganized and repetitive to me.

Authors’ response: we agree that the structure of the article should be reviewed.

Authors’ proposed change: the order of sections 2.2.2 and 2.2.3 have been reversed to improve the logical flow of the description of the system. Two redundant descriptions of the TUNZE apparatus have been removed. Small changes have been made throughout the manuscript to improve its readability.

2) The key initial point is to explicitly recognize that adequate control of CO₂ chemistry in seawater media requires that one control as many variables as would be needed to fully describe the CO₂ chemistry.

Authors’ response: we agree that this point should be made more clearly.

Authors’ proposed change: this point has been added to the section that reviews OA simulation systems on page 5, lines 1-10.

3) In the case of the system described here, pH and T are actively controlled, while S and Aₜ are more passively controlled by the choice of water replacement rate. This is never stated clearly, and indeed it is not until the discussion that the importance of water replacement rate is clearly acknowledged.

Authors’ response: we agree that the passive control of salinity and alkalinity should be discussed earlier in the article.
Authors’ proposed change: passive control of salinity and alkalinity are now described on page 7, lines 4-6.

See also page 7, lines 29-32 for details of seawater replacement rate.

4) Indeed, there are real advantages in controlling pH rather than p(CO₂) as there is less sensitivity to temperature fluctuations.

Authors’ response: we believe that this point was made on page 7, lines 1-4.

Authors’ proposed change: The section describing the benefits of pH control has been rewritten to more clearly state the advantages of this approach. See page 6, lines 28-31 and page 7, lines 1-4.

5) I also feel that the authors should explicitly discuss what they feel would be adequate tolerances for these parameters, given the goal of achieving an ocean acidification simulation system. The only tolerance that is even explicitly mentioned is ±0.05 in pH, and – as far as I can – the justification for this seems to be that it can be met. I feel it would be useful to look at the sensitivity to changes in the other parameters and point out that they are indeed adequately controlled in this system. (For this too, the discussion of A_T control comes too late, I believe.)

Authors’ response: we agree with the reviewer that the reasons for choosing 0.05 pH should be expanded upon, and that we should look at sensitivity of pH to changes in other parameters.

Authors’ proposed change: we have inserted some discussion of these points, both in the context of what tolerance is realistically achievable for the majority of OA researchers, and with a view to standardising tolerances of potentiometric systems. See page 3, lines 19-26.

A section discussing the regulation and reporting of temperature, salinity and A_T, has been added to the manuscript. See page 6, lines 3-21 and page 12, lines 18-21.

A table has also been added to the manuscript that illustrates the sensitivity of calculated carbonate parameters to offsets in temperature, salinity, and A_T (Table 3, page 23).

6) Finally, I feel uneasy with the use of the word “accuracy”; pH measurements are seldom “accurate”, insofar as they accurately provide values for the parameter that is specified – –log a(H⁺) – or even a hydrogen ion concentration.

Authors’ response: we agree that the term ‘accuracy’ should be used more carefully.
Authors’ proposed change: the term ‘accuracy’ has been replaced throughout the manuscript with ‘tolerance’ (with a definition given on page 3, line 22) or other, more appropriate, terms.

7) The calibration buffers used here (Tris and AMP) are assigned pH values using spectrophotometry (using “pure” metacresol purple which is, as yet, not widely available together with the calibration of Liu et al.); however, no comment is made as to the possible uncertainties in this approach except to note that the buffers are not fully matched to the seawater being used. The discrepancy between the pH measured directly in the test seawater, and that calculated from measurements of $A_T$ and $C_T$ is about −0.04 at all 3 pH values, yet this is not discussed as a possible indication of overall uncertainty but rather as an indication that the estimate ±0.05 is correct. I recommend the authors rethink how they discuss this so as to make clear the primary sources of uncertainty and their implications, for other parameters such as saturation index, etc. (For example, the uncertainty in $S$, $T$, or $A_T$).

Authors’ response: we agree that the issue of uncertainties inherent in all techniques should be appropriately discussed.

Authors’ proposed change: we have added a section directly discussing sources of uncertainty in all measured parameters. See page 10, lines 12-25. See also Table 3, page 23.

Other comments

8) The introduction seems poorly put together. As the level of CO$_2$ in the atmosphere continues to rise, the canonical values of 0.1 and 30% (a mismatched pair) get more and more outdated.

Authors’ response: we agree that the general description of changes to atmospheric CO$_2$ and oceanic pH should be updated based on the IPCC 2014 report.

Authors’ proposed change: the introductory section has been changed to incorporate more recent data. See page 2, lines 2-5.

9) The equations R2, R3 don’t balance chemically (they don’t need the H$_2$O); also, strictly, the process of acidification involves the excess hydrogen ion generated by carbonic acid dissociation going on to react with carbonate ion: it would be clearer for the reader if this was pointed out.

Authors’ response: the authors agree with the suggestions regarding equations R2 and R3.

Authors’ proposed change: $H_2$O has been removed from equations R2 and R3, balancing them chemically. An additional equation (R4) has been added to show the reaction of carbonate ions and excess hydrogen ions. See page 2, lines 12-15.
10) Finally the discussions of future predictions are also somewhat dated (c.f. the recent AR5 reports), but my larger criticism is that it is not clear just what the postulated decreases in pH by 2100 (or 2300) are to be compared to: is it the current pH, or that at the start of the industrial revolution?

Authors’ response: we agree with the reviewer’s comments.

Authors’ proposed change: this section has been replaced with references to the IPCC 2014 report and future changes to oceanic pH have been re-worded. See page 2, lines 2-4.

11) The discussion of carbonate chemistry too is not particularly rigorous: the statement that only 2 parameters need to be measured is an over-simplification. Of course one needs S, T, and (if alkalinity is used) information about other acid-base systems in addition to CO₂.

Authors’ response: we agree with the reviewer’s comments.

Authors’ proposed change: the reviewer’s comments have been incorporated into the description of carbonate chemistry on page 4, line 4 and page 5, lines 1-5.

12) The AIRICA DIC analyzer is made by MARIANDA (not MIRIANDA)

Authors’ response: we agree.

Authors’ proposed change: The correction has been made on page 9, line 16.

13) Practical salinity does not have “units” (strictly it has unit 1) and “PSU” is meaningless.

Authors’ response: we agree with the reviewer.

Authors’ proposed change: the practical salinity scale has been described at the beginning of the manuscript, and no units for salinity have been used in the text.

14) I feel there should be some discussion as to why the salinity appears to have a significantly larger relative variability (~2%) than the alkalinity does (<0.5%). This seems odd.

Authors’ response: we agree that this discrepancy should be discussed.

Authors’ proposed change: this discrepancy is discussed on page 10, lines 5-8.
15) Despite these many criticisms, I do believe that this is potentially a valuable paper. But it needs significant editing to improve its readability and to address the points I note here. (I am not sure whether to refer to these as minor or major revisions; I feel the manuscript will benefit from substantial rewriting, but will not materially change its main points.)

Authors’ response: we agree with the reviewer.

Authors’ proposed change: we feel the manuscript has been improved by the editing suggested by the reviewer.

Anonymous Referee #2

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This paper makes a valuable contribution in enabling our ability to understand the impact of changing seawater chemistry on marine biota. This study not only contributes to the community by providing a less expensive alternative to conduct these important studies but it highlights key aspects in experimental design which challenge cross study comparisons. The researchers have done a great job in surveying the users’ reliance on electrodes instead of spectrophotometry for pH measurements by examining a special issue on the biological effects of ocean acidification. They model the best technique for CO2 manipulation (CO2 injection) in their study and they effectively advise users on sound practices of electrode calibration (i.e. matching salinity and ionic strength to reduce liquid junction potential errors; two point calibration to determine slope of the electrode; and use of the recommended total pH scale). They have effectively assessed their user base priorities (common practices) and have provided insight into experimental design which will greatly enhance the quality and reproducibility of experiments. The experimental design was well-implemented. It was effective to incorporate the use of DIC/TA checks to confirm the validity of the pH data. This is an important and timely document providing a wealth of detail on how to effectively conduct well-executed and cost efficient experiments on changing ocean chemistry and temperature.

Minor revisions:

1) Atomically balance equations R1-R3 (hydrogens and carbons) – could use (aq) as a subscript to imply the water in the system. (page 7661)

Authors’ response: we agree.

Authors’ proposed change - the equations on page 2, lines 12-15 are now balanced by the removal of H2O

2) Place a space between 80 and L (page 7666, line 17).

Authors’ response: we agree.

Authors’ proposed change: a space has now been added between 80 and L on page 7, line 12.
Technical note: maximising accuracy and minimising cost of a potentiometrically regulated ocean acidification simulation system

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Abstract

This article describes a potentiometric ocean acidification simulation system which automatically regulates pH through the injection of 100% CO₂ gas into temperature-controlled seawater. The system is ideally suited to long-term experimental studies of the effect of acidification on biological processes involving small-bodied (10-20 mm) calcifying or non-calcifying organisms. Using hobbyist grade equipment, the system was constructed for approximately US$1200 per treatment unit (tank, pH regulation apparatus, chiller, pump/filter unit). An overall accuracy tolerance of ± 0.05 pH₇ units (SD) was achieved over 90 days in two acidified treatments (7.60 and 7.40) at 12 °C using glass electrodes calibrated with salt water synthetic seawater buffers, thereby preventing liquid junction error. The accuracy performance of the system was validated through the independent calculation of pH₇ (12 °C) using dissolved inorganic carbon and total alkalinity data taken from discrete acidified seawater samples. The system was used to compare the shell growth of the marine gastropod Zeacumantus subcarinatus infected with the trematode parasite Maritrema novaezealandensis with that of uninfected snails, at pH levels of 7.4, 7.6, and 8.1.
1 Introduction

The carbon dioxide (CO$_2$) produced by human activity since 1850 has reduced average surface oceanic pH from approximately 8.2 to 8.1, while current CO$_2$ emission projections predict that oceanic pH will reach 8.06-7.77 by 2100, and approximately 7.41 by 2300 (IPCC, 2014). The beginning of the Industrial Revolution (c. 1790) has caused a decrease in ocean pH of approximately 0.1 units, equivalent to a 30% increase in hydrogen ion (H$^+$) concentration in seawater (Raven et al., 2005). The mechanism responsible for this process is the sequestration of atmospheric CO$_2$ by the global ocean, and a subsequent increase in hydrogen ion activity caused by a series of chemical reactions initiated by the dissolution of CO$_2$ into seawater:

\[
\begin{align*}
\text{CO}_2(aq) + \text{H}_2\text{O}(l) & \rightleftharpoons \text{H}_2\text{CO}_3(aq) & (1) \\
\text{H}_2\text{CO}_3(aq) & \rightleftharpoons \text{HCO}_3^-(aq) + \text{H}^+(aq) & (2) \\
\text{HCO}_3^-(aq) & \rightleftharpoons \text{CO}_3^{2-}(aq) + \text{H}^+(aq) & (3) \\
\text{CO}_3^{2-}(aq) + \text{H}^+(aq) & \rightleftharpoons \text{HCO}_3^-(aq) & (4)
\end{align*}
\]

where H$_2$CO$_3$ is carbonic acid, and HCO$_3^-$ and CO$_3^{2-}$ are the bicarbonate and carbonate ions, respectively. Predictive models based on the range of CO$_2$ emission scenarios outlined in the IPCC report (2007) have estimated that ocean pH will drop 0.3-0.5 units by 2100 and 0.8-1.4 units by 2300 (Caldeira and Wickett, 2003; Caldeira and Wickett, 2005; Montenegro et al., 2007). The global reduction of ocean pH has become known as ocean acidification (OA), although the term also refers to changes in the concentration of carbonic acid, bicarbonate and carbonate ions, in addition to increased hydrogen ion activity (Equations 1-4).

The altered chemical speciation of seawater caused by OA poses a variety of challenges to all marine species, e.g. maintenance of intra- and extra-cellular acid-base homeostasis in a more acidic environment (Portner et al., 2004), or synthesis and dissolution of calcium carbonate (CaCO$_3$) structures in seawater undersaturated with regard to component ions (Weiner and Dove, 2003). A meta-analysis conducted by Kroeker et al. (2013) showed that OA will likely have a varied yet negative effect on many marine organisms in future, while negative effects on calcifying species found in areas of naturally elevated acidity have already been reported.
To date, the majority of experimental research into the effects of OA has focused on single marine species in an attempt to identify those with or without the ability to adapt to acidified conditions within a single generation. The identification of such phenotypic plasticity in response to stressors associated with OA is vital, as evolutionary adaptation may not occur at a sufficient rate to protect some species from changing marine conditions (Bell and Collins, 2008). However, it is now accepted that OA research must move beyond single species experiments and begin investigating the effects of combined abiotic factors, such as pH and temperature (Boyd, 2011), and the potential effects of OA on biological interactions such as competition (Hoffman et al, 2012), predation (Dixon et al 2010; Allan et al., 2013), and parasitism (MacLeod and Poulin, 2012). This paradigm does not negate the importance of single-species/single-factor experiments, but rather broadens the scope of OA research. A thorough investigation of a species’ response to novel abiotic stressors should begin with single factor manipulations and then introduce increasing levels of complexity to fully document potential synergistic reactions between parameters. Given the current rate of ocean acidification (~0.0018 pH units/yr, Feely et al., 2009) the identification of species and species’ interactions that are vulnerable to OA, alone or in combination with other abiotic factors, should be urgently addressed; lab-based simulations will play an important role in achieving this goal (Widdecombe et al., 2010).

This article provides a detailed description of a low-cost, easy set-up, OA simulation system that reliably mimics the effects of elevated atmospheric CO₂ on seawater chemistry by controlling temperature, salinity, pH, and total alkalinity (Aₜ). In addition, we suggest goal tolerances, i.e. the variability around target parameter values expressed as standard deviations, for control of these parameters: temperature (± 0.5 °C), salinity (± 0.6), pH (± 0.05), and Aₜ (±10 μmol kg⁻¹). We believe these tolerance values represent realistic and achievable goals for OA simulation systems, as they can be met with relatively inexpensive apparatus, and cause minimal changes to calculated carbonate parameters (Table 3).

Consequently, this article provides a detailed description of a low-cost, easy set-up, OA simulation system which accurately mimics the effects of elevated atmospheric CO₂ on seawater chemistry, and may allow greater access to an experimental field which can be prohibitively expensive (Wilcox-Freeburg, 2013).
2 OA simulation systems

2.1 Review

OA simulation systems must be able to reliably manipulate the carbonate chemistry of seawater, which is characterised by the measurement of four-seven parameters: 1. Temperature (°C); 2. Salinity (reported on the Practical Salinity Scale); 3. Depth (metres); 4. pH:

\[ \text{pH} = -\log[H^+] \]

— notationally defined as the negative log of hydrogen ion activity, although there are multiple pH scales currently in use (Marion et al., 2011); 2. Total alkalinity (\(A_T\)-μmol kg\(^{-1}\)):

the amount of acid required to react with all the bases in 1 kg of seawater (Dickson, 1981):

\[ A_T = [\text{HCO}_3^-] + 2[\text{CO}_3^{2-}] + [\text{B(OH)}_4^-] + [\text{OH}^-] + [\text{HPO}_4^{2-}] + 2[\text{PO}_4^{3-}] + [\text{SiO(OH)}_3^-] + [\text{NH}_3] + [\text{HS}^-] - [\text{H}^+] - [\text{HSO}_4^-] - [\text{HF}] - [\text{H}_2\text{PO}_4] \ldots \] (5)

6. Dissolved inorganic carbon concentration (DIC- μmol kg\(^{-1}\)):

the combined concentrations of inorganic carbon species per kg of seawater:

\[ \text{DIC} = [\text{CO}_2] + [\text{H}_2\text{CO}_3] + [\text{HCO}_3^-] + [\text{CO}_3^{2-}] \] (6)

7. Partial pressure of atmospheric carbon dioxide seawater CO\(_2\) (p\(\text{CO}_2\)-μatm):

in equilibrium with seawater (p\(\text{CO}_2\)):

\[ p(\text{CO}_2) = x(\text{CO}_2)P \] (7)

where \(x(\text{CO}_2)\) represents the mole fraction of CO\(_2\) in the gas phase in equilibrium with seawater, and P represents the total pressure. For detailed definitions of the analytical parameters used to characterise seawater carbonate chemistry, please see Dickson et al.
(2007). Of the seven variables listed above, temperature, salinity, depth (if applicable), and two of the four analytical parameters must be known, in addition to appropriate equilibrium constants, to fully characterise the carbonate chemistry of the modified seawater and quantify variables central to the effects of OA, e.g. saturation states of calcium carbonate polymorphs or concentrations of $\text{HCO}_3^-$ and $\text{CO}_3^{2-}$. Only two need to be measured to calculate the remaining two, along with other important characteristics relevant to the study of OA, e.g. saturation states of calcium carbonate polymorphs or concentrations of $\text{HCO}_3^-$ and $\text{CO}_3^{2-}$. Accordingly, one must control salinity, temperature, and two of the four analytical parameters described above to manipulate the carbonate chemistry of seawater in experimental OA simulation systems.

Riebesell et al. (2010) compiled a detailed guide for the standardisation of methodology used in the manipulation and measurement of carbonate chemistry (The Guide to Best Practises for Ocean Acidification Research and Data Reporting). Since publication of the guide, there have been several published descriptions of OA simulation systems which use a variety of techniques to acidify seawater: gas injection (CO$_2$/air mix/O$_2$/N$_2$ - Bockmon et al., 2013; 100% CO$_2$ - Wilcox-Freeburg et al., 2013), the addition of CO$_2$ enriched seawater (McGraw et al., 2010), and the addition of HCl and NaOH (Riebesell et al., 2000). Despite the many differences between experimental approaches, almost all simulation systems are regulated through the measurement of pH as a master variable.

The current gold standard for monitoring pH in an OA simulation system is by the automated spectrophotometric analysis of seawater samples integrated into a software-based regulation system (e.g. McGraw et al., 2010). Spectrophotometric analysis of pH provides a high degree of precision (±0.0004, Carter et al., 2013; Clayton and Byrne, 2013 Millero, 2007) compared to potentiometric techniques (±0.002-0.001, Dickson et al., 2007), and has been used to regulate OA simulation systems with minimal variation around target pH values of ± 0.02, McGraw et al., 2010). However, spectrophotometric pH regulation can prove extremely expensive, as these systems must be custom-designed (Wilcox-Freeburg et al., 2013). Despite the reduced degree of precision, potentiometric measurement of pH is the central component of most OA simulation systems designed to explore the effects of reduced pH on biological organisms (Easley and Byrne, 2012). Indeed, in the 2013 special OA issue of the journal Marine Biology (August, Volume 160, Issue 8)),
31 out of 32 (97%) of experimental articles used manipulation techniques controlled by, or monitored through, the potentiometric measurement of pH.

The regulation of temperature, salinity, and $A_T$, is often not discussed in detail in the OA literature, despite the central role of these variables in the control of carbonate chemistry. Temperature is typically controlled by actively heating or cooling the acidified seawater to a target value using a variety of commonly available lab equipment, e.g. chiller units, temperature controlled rooms, or heating coils. Salinity is often monitored but not controlled, as many simulation systems are supplied with seawater from a large reservoir or permanent connection to the ocean, or passively controlled through the regular replacement of seawater.

The $A_T$ of an OA simulation system can be altered by the biological activity of experimental organisms. Consequently, $A_T$ is often also regulated through the replacement of seawater or with a flow through system. Possibly as a consequence of the commonplace (temperature) or passive (salinity and $A_T$) methods of regulation, tolerances of these parameters are often not reported in OA literature. In the 2013 special OA issue of the journal *Marine Biology*, 14 studies used temperature, salinity, pH, and $A_T$ to control and describe seawater carbonate chemistry. Six of these studies reported no measure of temperature variance, 8 reported no salinity variance, and 5 reported no $A_T$ variance. In addition, some articles gave parameter tolerances as standard error (SE), with or without the corresponding sample size, making comparisons of tolerance levels between studies difficult. As the measurement of pH is subject to many sources of uncertainty, the tolerances of temperature, salinity, and $A_T$ should be stated explicitly and clearly in the description of OA simulation systems.

### 2.2 Described system

#### 2.2.1 Acidification method Overview

The described system manipulates the carbonate chemistry of acidifies temperature-controlled seawater through the direct pH-controlled injection of 100% CO$_2$ gas. pH is regulated continuously and automatically with potentiometric monitoring apparatus (TUNZETM) similar to the hobbyist grade CO$_2$ delivery system described in Wilcox-Freeburg et al (2013). The direct injection of 100% CO$_2$—The use of pH as a controlling variable and CO$_2$ gas as an acidifying agent has two key advantages over other acidification techniques. First, the addition of CO$_2$ gas more realistically mimics the effects of increased atmospheric CO$_2$ on seawater chemistry than the addition of an acid (Hurd et al 2009, Schultz et al 2009). Second,
the “on-demand” pH-controlled addition of 100% CO2 gas reduces pH variation when compared to the injection of gas/air mixes at a fixed rate; the latter can result in unwanted fluctuations in pH caused by biological activity, changes in temperature, or increases in ambient atmospheric CO2 (Wilcox-Freeburg et al., 2013). In this system, seawater temperature was actively maintained at 12.6 ± 0.5 °C, while salinity (31.6 ± 0.6) and AT (2375 ± 10 μmol kg\(^{-1}\)) were passively controlled through the regular replacement of seawater.

### 2.2.2 Apparatus

The described experimental apparatus consists of three identical units (Figure 1), each capable of independently mimicking the effects of increased atmospheric CO2 on seawater, i.e. elevated pCO2 and DIC, and reduced pH. The pH of culture tank seawater was constantly monitored potentiometrically, and automatically regulated through the injection of 100% food grade CO2 gas. In each tank, 80 L of seawater was contained in a 120 L open top tank (870 mm (L) x 600 mm (W) x 295 mm (H), Food Grade - Low Density Polyethylene, Stowers Containment Solutions, NZ). Unamended seawater was supplied by the Portobello Marine Research Station, Dunedin, New Zealand, and was high pressure-filtered through sand prior to use. The unamended seawater had a total alkalinity of 2354 ± 10 μmol kg\(^{-1}\) (n=6) and a salinity of 31.5 ± 0.5 PSU. pH in each culture tank was regulated using TUNZE™ pH/CO2 controller systems (glass electrodes, pH meter, solenoid switch unit, and a pressure reducer) connected to 33 kg gas cylinders containing 100% food grade CO2 (BOC). The TUNZE™ system automatically allowed 100% CO2 gas to flow from the pressurised cylinders through the solenoid switch unit into the culture tank when the pH of acidified seawater rose above target values. Carbon dioxide gas diffused into the acidified seawater through a perforated 4 mm plastic tube which was wrapped around the water inflow pipe. This allowed for a maximum rate of dispersal of dissolved gas through the culture tank, minimising any pH gradient relative to the gas input point. To ensure that ambient temperature variations did not alter pH (TUNZE™ pH meters have no automatic temperature compensation function), seawater was pumped through a 1/5 hp refrigeration unit (Hailea HC-150A) using an aquarium pump/filter system (Aqua One®, Aquis700) at a rate of approximately 400 L/h. To minimise changes in seawater chemistry salinity and AT caused by the culture of calcifying organisms evaporation, calcification, shell dissolution, or respiration, and to maintain constant salinity 20 L of seawater was removed from each tank every 48 hours and gradually (30 L/hr) replaced with unamended seawater. Each culture tank was also aerated with ambient
air by an aquarium bubbler (AquaOne 9500), and oxygen saturation (measured daily with a
YSI ProODO) was greater than 95% for the duration of the experimental period.

2.2.3 Measurement of analytical parameters

As noted in Easley and Byrne (2012), there are a number of challenges inherent in the
potentiometric measurement of pH: calibration buffers must be of similar ionic strength to
samples to avoid liquid junction error (see the Discussion for a complete description of liquid
junction error)(Millero et al., 1993; Waters, 2012); preparing saltwater buffers in the lab can
lead to pH variation due to human error; post-preparation, the pH of buffers can be altered
through contact with ambient atmospheric CO₂; electrode function can degrade over time and
result in a deviation from the ideal Nernstian slope required to convert volts to pH units; and
all electrodes are subject to a certain degree of drift over time (Dickson et al., 2007).

In the described system, pH meters were calibrated using homemade saltwater buffers (2-
amino-2-hydroxy-1,3-propanediol (TRIS) and 2-aminopyridine (AMP)) prepared in
accordance with Dickson et al. (2007). Buffer salinity was slightly higher than that of
seawater in the culture tanks (35 vs. ~32); however, the consequent error was assumed to be
less than 0.005 pH units (Dickson et al., 2007). In case of small deviations of buffer pH
caused by human error during preparation, buffers were analysed with an Agilent 8453
spectrophotometer using pure meta-Cresol Purple (mCP) (provided by the laboratory of
Professor Robert H. Byrne, University of South Florida) at 25 °C, and pH_T calculated from a
measured mCP spectrum using the calibration of Liu et al. (2011). After preparation,
saltwater buffers were aliquoted into 100 mL borosilicate Schott bottles in front of an air
pump modified to produce CO₂-depleted air, thus minimising the effect of ambient CO₂ on
buffer pH. With appropriate storage protocols, saltwater buffers prepared in this way have
proved stable for up to a year, and subsequent degradation is approximately 0.0005 pH units
per year (Nemzer and Dickson, 2005). In addition to frequent calibration of pH electrodes to
compensate for drift, TRIS and AMP buffers were used to ensure that all electrode responses
were within 0.2-0.3% of the ideal Nernst value (0.05916 V) at 25 °C (Dickson et al., 2007;
Millero et al., 1993):

Electrode response = EMF_{AMP} – EMF_{TRIS}/pH_{TRIS} – pH_{AMP}  

(7)
where EMF refers to electromotive force, measured in Volts. Variability in culture tank pH was minimised through a two stage monitoring process. Seawater pH in each tank was constantly measured with electrodes connected to the CO$_2$ delivery system (TUNZETM, 2 point calibration, ± 0.01 pH units). As individual electrodes are prone to drift even with frequent calibration (Dickson et al., 2007), an independent, hand-held pH meter (Denver Instrument Company AP50, 2 point calibration, ± 0.002 pH units) was also used to measure culture tank pH daily. If the Denver pH meter detected deviations from the target pH, the TUNZETM apparatus was adjusted, allowing for centralized control of pH using the most precise meter available.

The performance of the potentiometric apparatus was also validated with the calculation of pH$_T$ (12 °C) based on A$_T$ and DIC data taken from culture tank seawater, using SWCO2 Software (Hunter, 2007) and the dissociation constants of Mehrbach et al (1973) refit by Dickson and Millero (1987). Total alkalinity was measured with closed-cell potentiometric apparatus, based on the system described by Dickson et al. (2007), while DIC was measured using infra-red analyses of CO$_2$ evolved from an acidified sample (AIRICA DIC analyser, by MARIANDA). Measurements of A$_T$ and DIC were calibrated using certified reference materials (CRM) from the lab of Professor Andrew Dickson, University of California San Diego. Seawater taken from culture tanks was stored in 1000 ml borosilicate Schott bottles and fixed with a saturated solution of mercuric chloride prior to A$_T$ and DIC analysis (per recommendations of Riebesell et al. (2010)).

### 3 Assessment

#### 3.1 Carbonate parameters

Carbonate parameters were monitored throughout a 90 day experiment to culture the New Zealand mud snail (*Zeacumantus subcarinatus*), collected from Otago Harbour, Dunedin, New Zealand. During the experimental period, temperature, salinity, and pH were measured daily (Table 1), while A$_T$ and DIC were analysed from samples taken approximately every 18 days (Table 2). Table 2 also lists other relevant carbonate parameters calculated using DIC and A$_T$ as measured variables.

pH$_T$ (12 °C), measured both potentiometrically and calculated from DIC and A$_T$ data, varied by ± 0.03-0.04 units (SD) in all three culture tanks over the 90 day period (measured: 7.40 ±
0.03, 7.60 ± 0.04; calculated: 7.45 ± 0.04, 7.64 ± 0.04) in good agreement with the accuracy goal of target pH ±0.05 (SD) (Figure 2). While calibration of all electrodes occurred weekly, there was very little drift in the electrodes connected to the CO₂ regulation apparatus. Temperature, controlled by the chiller units, was also stable across all culture tanks, while salinity and A_T showed minimal variation (Table 1). However, there was a greater relative uncertainty in salinity (approximately 2%) than A_T (<0.5%) over the experimental period. We assume that this was due to a greater variability in salinity over the entire 90 day period, detected by more frequent sampling (n=64) compared to A_T (n=6). As expected, DIC (measured) and pCO₂ (calculated) increased in all culture tanks after the injection of CO₂ gas (Hansen et al., 2013; Campbell and Fourqueran, 2011; Findlay et al., 2008), while A_T remained unchanged in all treatments (Table 2).

Sources of error in our measurement of pH include: spectrophotometric measurement of buffer pH (± 0.004, Carter et al., 2013); differences between buffer salinity and seawater salinity (<0.005, Dickson et al., 2007); and the potentiometric measurement of seawater pH (± 0.01-0.002, pH meter specifications).

In addition, while the variability of temperature, salinity and A_T was relatively minor, measurement errors or incorrect calibrations (“offsets”) in these parameters will result in offsets in the calculated parameters central to the study of the effects of OA on marine organisms. Table 3 contains examples of the offsets in calculated carbonate parameters caused by values of uncertainty found in this study. The uncertainty in calculated pH resulting from uncertainties in measured A_T (10 μmol kg⁻¹) and DIC (10 μmol kg⁻¹), and uncertainty in the dissociation constants (pK) of H₂CO₃ (0.01) and HCO⁻₃ (0.02), gives an uncertainty in calculated pHₚ of approximately 0.05 pH (Dickson 1978). Thus, this error estimate in pH is in good agreement with the difference between our measured and calculated values for seawater pH; measured pH was between 0.03 and 0.05 lower than calculated pH in all pH treatments.

3.2 Culture of biological organisms

To investigate the potential interaction of infection stress and stressors associated with OA on the growth of Z. subcarinatus, 180 snails (average length, 14.4 ± 1.3 mm; average mass, 0.22 ± 0.05 g) were distributed evenly between three pH treatments: 8.1, 7.6, and 7.4. Of the 60 snails in each treatment, 30 were infected with the marine trematode parasite Maritrema
*novaezelandensis* and 30 had no parasitic infection. Each group of thirty snails was further subdivided into groups of 5 and placed in mesh chambers which allowed the flow-through of seawater. Prior to exposure to acidified seawater, all snails were soaked for 24 hours in a saltwater solution of calcein, a soluble fluorochrome which is incorporated into growing calcified structures and produces a fluorescent band which can be treated as a baseline for subsequent growth (Riascos et al., 2007). The snails were maintained in the three pH treatments for a total of 90 days, although during that time each tank was assigned a particular pH for only 30 days. During reassignment of tank pH, snails from the control (8.1 pH) culture tank were first removed and placed in a second aerated container. The now vacant tank was then acidified to 7.6 pH and snails transferred from the tank previously assigned that treatment. This process was repeated for the snails in the 7.4 pH treatment, and the tank originally assigned 7.4 pH was allowed to re-equilibrate with atmospheric CO$_2$ before the ‘control’ snails were replaced. This stepwise changeover removed the potential for tank effect to bias experimental data, and reduced any variation in pH conditions experienced by the snails.

After 90 days, all snails were removed from the culture tanks and the growing edge of their shell imaged under UV light (Leica camera (DFC320) and dissecting scope (MZFL11), 6.4x magnification). New shell growth, visible beyond the fluorescent band, was measured with ImageJ software and these data were analysed with a 2-Factor ANOVA to test the effects of pH and infection on shell growth. Analysis of variance showed that there was significantly reduced growth under acidified conditions in infected and uninfected snails (Figure 3), and that infected snails grew more than uninfected individuals in all pH treatments. The complete details of this study and the biological interpretations of the findings will be published elsewhere.

### 4 Discussion and recommendations

#### 4.1 Overview

This article describes a potentiometrically regulated OA simulation system that maintained temperature, salinity, pH, and A$_T$ within goal tolerances in three 80 L seawater culture tanks over 90 days. within ±0.05 units (SD) of target values over 90 days, days, while each tank held 60 live snails. pH was adjusted using CO$_2$-regulation apparatus which injected 100%
CO₂ gas into each culture tank until target pH was achieved. Subsequently, CO₂ gas was added automatically whenever pH rose above pre-set, target values. To avoid fluctuations in pH caused by changes in ambient temperature, seawater in each culture tank was maintained at 12.0°C with a 1/5 hp water chiller, and circulated at 400l/h using an aquarium pump. Seawater was replaced at a rate of 20L/48h to maintain uniform seawater chemistry and salinity. The system was used to culture the New Zealand mud snail, *Zeacumantis subcarinatus*, over a 90-day period to investigate the effects of reduced pH on individuals infected with the marine trematode *M. novaezealandensis* relative to uninfected conspecifics. All apparatus used in the construction of the described system was purchased through aquarium suppliers at a cost of approximately $3600US, i.e. US$1200 per unit.

The design of OA simulation systems is under constant development and review (e.g. Findlay et al., 2008; McGraw et al., 2010; Wilcox-Freeburg et al., 2013). The system described here improves the accuracy, tolerance and repeatability of potentiometric measurement and regulation of pH in an OA simulation system by: a) using two saltwater synthetic seawater buffers to calibrate glass electrodes and report pH on the total hydrogen ion scale (pHT, Hanson, 1973) and b) measuring two additional, non-pH, carbonate parameters to independently validate pH, and monitor changes to seawater chemistry caused by the culture of calcifying organisms. This article also includes an evaluation of offsets in calculated carbonate parameters caused by potential offsets and calibration errors in our measurement of temperature, salinity, pHT, and AT (Table 3). We recommend that this type of assessment is carried out by all researchers working with OA simulation systems.

### 4.2 Calibration buffers

To date, the most commonly used buffers for the calibration of electrodes used in OA simulation systems are defined by the National Bureau of Standards (NBS), now known as the National Institute of Standards and Technology (NIST), and report pH on the NBS scale (pH\textsubscript{NBS}). NBS buffers are inexpensive, commonly available in most labs, and have pH values which are typically pre-programmed into pH meters to facilitate ease of electrode calibration. In the 2013 special OA issue of the journal *Marine Biology*, 18 out of 32 (56%) experimental articles used these buffers and reported pH on the NBS scale. However, NBS/NIST buffers have a low ionic strength compared to seawater (0.1 M vs. 0.7 M, Waters, 2012; Hurd et al.,
When measuring pH with potentiometric apparatus, the use of calibration buffers with a different ionic strength from sampled media leads to an error based on a fundamental assumption of potentiometric theory, i.e. that the difference in electric potential between the electrode solution and buffer solution is the same as that between the electrode solution and sample solution (Covington, 1985). This error is referred to as liquid junction error, and has been discussed in several articles describing the potentiometric measurement of pH (Dickson et al., 2007; Illingworth, 1981; Easley and Byrne, 2012). The pH scale is essentially a quantification of the difference in electric potential between an ion-selective electrode and a sample solution. If the difference in ionic strength between the calibration buffer and sample is great, the electrode will not accurately report the difference in electric potential, or provide repeatable measurements (Zeebe and Gladrow, 2001; Weburg et al., 2009). Liquid junction error has been reported to cause inaccuracies uncertainties of ± 0.01-0.14 units in the measurement of seawater pH when using electrodes calibrated with low ionic strength buffers (Dickson, 1993; Easley and Byrne, 2012). The use of NBS buffers not only compromises the accuracy repeatability of potentiometrically regulated OA simulation experiments, this error is also propagated through calculations of other important seawater characteristics commonly reported in the OA literature, e.g. the saturation states of aragonite (Ωa) and calcite (Ωc). If we apply an error of ± 0.065 pH units (the median of reported liquid junction error values) to Ωa and Ωc in the software program SWCO2, we generate inaccuracies errors of 19% and 15% respectively (Table. 3). The saturation states of aragonite and calcite are particularly vulnerable to this degree of error, as the current range of these variables is 1.2-5.4 (Ωa) and 1.9-9.2 (Ωc) (Riebesell et al., 2010), and Ω values less than 1.0, commonly achieved in OA simulation systems, indicate that the dissolution of these CaCO3 polymorphs is thermodynamically favoured (Andersson et al., 2007). This type of error could prevent the correct interpretation of data sets generated in OA experimental studies, as they may indicate dissolution of calcified structures at saturation states greater than 1.0.

An additional consideration when reporting data generated by an OA simulation system is the choice of pH scale. Measurement of seawater pH can be reported accurately on three scales: the free proton scale (pH_F), the total hydrogen ion scale (pH_T), and the seawater scale (pH_SWS). There has been considerable debate over which scale is the most appropriate for...
reporting seawater pH in OA experiments (e.g. Waters and Millero, 2013), although the total hydrogen ion scale (pH\textsubscript{T}) is most commonly reported in published data. In the 2013 special OA issue of the journal *Marine Biology*, pH\textsubscript{T} was reported in 14 out of 32 (44\%) of experimental articles while pH\textsubscript{F} and pH\textsubscript{SWS} were not used at all. One reason for this trend is that pH\textsubscript{T} is generated directly by pH meters calibrated with saltwater buffers without additional calculation or conversion, as with the free proton and seawater scales. With the increasing availability of these buffers, and the importance of establishing comparability between data sets, it seems appropriate that pH\textsubscript{T} should be adopted as the default scale in OA research.

4.3 DIC and A\textsubscript{T} analysis

Throughout the 90 day trial of this system, seawater samples were periodically taken from each culture tank and used to measure A\textsubscript{T} and DIC. The primary purpose of this analysis was to validate the performance of the described system, with respect to regulation of pH, by using DIC and A\textsubscript{T} data to independently calculate the pH of culture tank seawater using the SWCO2 software. As previously discussed, the calculated pH was in good agreement with the potentiometrically measured pH, and it is advisable that this additional validation process should be standard procedure after the initial construction of a potentiometrically regulated OA simulation system. A secondary function of measuring A\textsubscript{T} and DIC is the identification of alterations to seawater chemistry caused by the culture of calcifying organisms in acidified seawater. As discussed in Hurd et al. (2009), the addition of 100\% CO\textsubscript{2} to seawater is expected to cause an increase in DIC but not affect A\textsubscript{T}. However, the culture of marine organisms in OA simulation systems can alter the concentration of carbon species in seawater through photosynthesis (decreased CO\textsubscript{2}), respiration (increased CO\textsubscript{2}), or dissolution of calcified structures (increased HCO\textsubscript{3}\textsuperscript{-}). During an earlier trial of this system, when acidified treatments were 7.1 and 7.4 pH\textsubscript{T} (12 \textdegree\textsuperscript{C}), A\textsubscript{T} greatly exceeded the expected value of \(~2300\) \(\mu\text{mol kg}^{-1}\) (2938.04 \(\pm\) 1.29 \(\mu\text{mol kg}^{-1}\) (7.1pH), 2564.16 \(\pm\) 3.50 \(\mu\text{mol kg}^{-1}\) (7.4 pH)), and DIC was also unusually high compared to data generated by other systems that used CO\textsubscript{2} gas to reduce pH (3098.54 \(\pm\) 5.14 \(\mu\text{mol kg}^{-1}\) (7.1 pH) and 2614.34 \(\pm\) 2.61 \(\mu\text{mol kg}^{-1}\) (7.4 pH)). We assumed that the observed changes in seawater chemistry were caused by the release of HCO\textsubscript{3}\textsuperscript{-} through the dissolution of calcified structures, as the snail shells had visibly dissolved,
and therefore we increased the replacement rate of seawater from 20 L/wk. to 20 L/48 h. As reported earlier in this paper, further analysis of $A_T$ and DIC showed that these parameters had returned to expected levels, supporting the assumption that the dissolution of calcified structures had altered seawater chemistry. It is important to note that the replacement rate of seawater used in this simulation system may be specific to the size and number of snails in culture, and the volume of culture tanks. These observations illustrate the importance of measuring both $A_T$ and DIC during the culture of calcifying organisms in acidified seawater, especially in closed or partially closed systems. If only DIC had been measured, and $A_T$ assumed to be constant, elevated DIC could have been solely attributed to an increase in dissolved the addition of CO$_2$ (the carbon species responsible for elevated DIC in CO$_2$ enriched seawater), and resulted in the introduction of an unknown, additional abiotic factor to the experimental design.

5 Conclusion

The described system increases the accessibility of reliable OA simulation apparatus by using relatively inexpensive equipment that is readily available from aquarium suppliers. With careful calibration and the use of appropriate buffers, it is possible to generate high quality and repeatable data. Incorporating DIC and $A_T$ analysis in the validation of this system also provides a greater degree of reliability with regard to pH manipulation, and a more complete understanding of the complex nature of seawater chemistry. Additional stressors such as temperature, salinity, and UV radiation could also be easily incorporated into experimental design due to the modular design of this system. Consequently, this system will facilitate the increase in research effort required to identify species, and species’ interactions, vulnerable to novel stressors associated with OA, alone or in combination with other abiotic factors.

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http://neon.otago.ac.nz/research/mfc/people/keith_hunter/software/swco2


Table 1 Average values (±SD, n=64) for pH<sub>T</sub>, temperature, and salinity, recorded over a 90 day period in three pH treatment tanks during the culture of <i>Z. subcarinatus</i>.

<table>
<thead>
<tr>
<th>pH&lt;sub&gt;T&lt;/sub&gt; (Measured)</th>
<th>Temp. (°C)</th>
<th>Salinity (PSU)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>8.1 Treatment</strong></td>
<td>8.09 ± 0.03</td>
<td>12.5 ± 0.3</td>
</tr>
<tr>
<td><strong>7.6 Treatment</strong></td>
<td>7.60 ± 0.03</td>
<td>12.6 ± 0.6</td>
</tr>
<tr>
<td><strong>7.4 Treatment</strong></td>
<td>7.40 ± 0.03</td>
<td>12.6 ± 0.5</td>
</tr>
</tbody>
</table>

Table 2 Average values (±SD, n=6) for A<sub>T</sub> and DIC (measured) and pH<sub>T</sub> and pCO<sub>2</sub> (calculated) recorded over a 90 day period in three pH<sub>T</sub> treatments during the culture of <i>Z. subcarinatus</i>.

<table>
<thead>
<tr>
<th>Alkalinity (µmol kg&lt;sup&gt;-1&lt;/sup&gt;)</th>
<th>DIC (µmol kg&lt;sup&gt;-1&lt;/sup&gt;)</th>
<th>pH&lt;sub&gt;T&lt;/sub&gt; (calculated)</th>
<th>pCO&lt;sub&gt;2&lt;/sub&gt; (calculated)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>8.1 Treatment</strong></td>
<td>2361 ± 10</td>
<td>8.12 ± 0.03</td>
<td>365 ± 30</td>
</tr>
<tr>
<td><strong>7.6 Treatment</strong></td>
<td>2389 ± 7</td>
<td>7.64 ± 0.04</td>
<td>1304 ± 115</td>
</tr>
<tr>
<td><strong>7.4 Treatment</strong></td>
<td>2375 ± 12</td>
<td>7.45 ± 0.04</td>
<td>1980 ± 110</td>
</tr>
</tbody>
</table>
Table 3. A comparison of the offsets resulting in calculated carbonate parameters by offsets or calibration errors in measured variables. The top line shows calculated values for DIC, pCO$_2$, Ωa, and Ωc calculated based on the average oceanic values for temperature, salinity, pH, and A$_T$ reported in Riebesell et al. (2010). Text in bold indicates the parameter that was varied.

<table>
<thead>
<tr>
<th>Measured parameters</th>
<th>Temperature ($^\circ$C)</th>
<th>Salinity</th>
<th>pH$_T$</th>
<th>A$_T$ (μmol kg$^{-1}$)</th>
<th>DIC (μmol kg$^{-1}$)</th>
<th>pCO$_2$ (μatm)</th>
<th>Ωa</th>
<th>Ωc</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oceanic average (2010)</td>
<td>18.7</td>
<td>34.8</td>
<td>8.062</td>
<td>2305</td>
<td>2050</td>
<td>384</td>
<td>2.83</td>
<td>4.38</td>
</tr>
<tr>
<td>Temperature (± 0.5 °C)</td>
<td>18.2-19.2</td>
<td>34.8</td>
<td>8.062</td>
<td>2305</td>
<td>2054-2045</td>
<td>384-384</td>
<td>2.79-2.88</td>
<td>4.31-4.45</td>
</tr>
<tr>
<td>Salinity (± 0.6)</td>
<td>18.7</td>
<td>34.2-35.4</td>
<td>8.062</td>
<td>2305</td>
<td>2054-2046</td>
<td>386-382</td>
<td>2.81-2.86</td>
<td>4.35-4.41</td>
</tr>
<tr>
<td>pH$_T$ (± 0.05)</td>
<td>18.7</td>
<td>34.8</td>
<td><strong>8.012-8.112</strong></td>
<td>2305</td>
<td>2075-2022</td>
<td>440-334</td>
<td>2.58-3.11</td>
<td>3.99-4.80</td>
</tr>
<tr>
<td>A$_T$ (± 10 μmol/kg)</td>
<td>18.7</td>
<td>34.8</td>
<td>8.062</td>
<td><strong>2295-2315</strong></td>
<td>2040-2058</td>
<td>381-384</td>
<td>2.83-2.85</td>
<td>4.37-4.41</td>
</tr>
<tr>
<td>Temp. &amp; salinity</td>
<td>18.2-19.2</td>
<td>34.2-35.4</td>
<td>8.062</td>
<td>2305</td>
<td>2057-2041</td>
<td>385-381</td>
<td>2.77-2.91</td>
<td>4.29-4.48</td>
</tr>
<tr>
<td>Temp., salinity &amp; A$_T$</td>
<td>18.2-19.2</td>
<td>34.2-35.4</td>
<td>8.062</td>
<td><strong>2295-2315</strong></td>
<td>2048-2050</td>
<td>383-382</td>
<td>2.76-2.92</td>
<td>4.27-4.50</td>
</tr>
<tr>
<td>Temp., salinity, A$_T$, &amp; pH$_T$</td>
<td>18.2-19.2</td>
<td>34.2-35.4</td>
<td>8.012-8.112</td>
<td><strong>2295-2315</strong></td>
<td>2074-2023</td>
<td>440-334</td>
<td>2.51-3.19</td>
<td>3.88-4.92</td>
</tr>
<tr>
<td>Liquid junction error (±0.065 pH)</td>
<td>18.7</td>
<td>34.8</td>
<td><strong>7.997-8.127</strong></td>
<td>2305</td>
<td>2083-2014</td>
<td>458-320</td>
<td>2.51-3.19</td>
<td>3.88-4.93</td>
</tr>
</tbody>
</table>
Figure 1Schematic of one OA simulation unit. Dashed lines indicate gas flow, solid lines indicate seawater flow, and dotted lines indicate electrical connections between components of pH regulation apparatus.
Figure 2 pH$_T$ recorded over the course of a 90-day experiment in which snails were maintained in three culture tanks: 8.1 (green), 7.6 (blue), 7.4 (red) pH$_T$. Coloured lines represent pH$_T$ data recorded on Denver AP50 hand held pH meter and black lines represent ±0.05 error around target pH$_T$ values.
Figure 3 Average shell growth (± SE, sample size as indicated) of infected and uninfected snails in three pH treatment: 7.4, 7.6, 8.1.