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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 USD 1200 per treatment unit (tank, pH regulation apparatus, chiller, pump/filter unit). An overall accuracy of $\pm 0.05 \text{ pH}_T$ 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 buffers, thereby preventing liquid junction error. The accuracy of the system was validated through the independent calculation of pH_T (12 °C) using dissolved inorganic carbon (DIC) and total alkalinity (A_T) 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 (CO₂) produced by human activity since 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 CO₂ by the global ocean, and a subsequent increase in hydrogen ion activity caused by a series of chemical reactions initiated by the dissolution of CO₂ into

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seawater:



5 where H_2CO_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). This global reduction of ocean pH has become
10 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.

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
15 homeostasis in a more acidic environment (Pörtner 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
20 areas of naturally elevated acidity have already been reported (e.g. Gruber et al., 2012). To date, the majority of experimental research into the effects of OA has focussed 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 adap-
25 tation 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 poten-

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tial 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^{-1} , 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). 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 CO_2 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 parameters: (1) pH, notionally 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), the amount of acid required to react with all the bases in 1 kg of seawater (Dickson, 1981):

$$A_T = [\text{HCO}_3^-] + 2[\text{CO}_3^{2-}] + [\text{B(OH)}_4^-] + [\text{OH}^-] + [\text{HPO}_4^{2-}] + 2[\text{PO}_4^{3-}] + [\text{SiO(OH)}_3^-] + [\text{NH}_3] + [\text{HS}^-] - [\text{H}^+] - [\text{HSO}_4^-] - [\text{HF}] - [\text{H}_3\text{PO}_4] \quad (1)$$

(3) dissolved inorganic carbon (DIC), the combined concentrations of inorganic carbon species per kg of seawater:

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

(4) the partial pressure of atmospheric carbon dioxide in equilibrium with seawater ($p\text{CO}_2$). Of these four parameters, 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 HCO_3^- and CO_3^{2-} .

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 ($\text{CO}_2/\text{N}_2/\text{O}_2$ mix – 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 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 , Millero, 2007) compared to potentiometric techniques (± 0.002 – 0.001 , Dickson et al., 2007), and has been used to regulate highly accurate systems (± 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

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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.

2.2 Described system

2.2.1 Acidification method

The described system acidifies temperature-controlled seawater through the direct injection of 100 % CO₂ gas. pH is regulated continuously and automatically with potentiometric monitoring apparatus (TUNZE™) similar to the hobbyist grade CO₂ delivery system described in Wilcox-Freeburg et al. (2013). The direct injection of 100 % CO₂ has two key advantages over other acidification techniques. First, the addition of CO₂ gas more realistically mimics the effects of increased atmospheric CO₂ on seawater chemistry than the addition of an acid (Hurd et al., 2009; Schultz et al., 2009). Second, the “on demand” addition of 100 % CO₂ 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).

2.2.2 Potentiometric measurement of pH

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 Sect. 4 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

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convert volts to pH units; and all electrodes are subject to a certain degree of drift over time (Dickson et al., 2007).

5 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 10 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, 15 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):

$$\text{Electrode response} = \text{EMF}_{\text{AMP}} - \text{EMF}_{\text{TRIS}} / \text{pH}_{\text{TRIS}} - \text{pH}_{\text{AMP}} \quad (3)$$

where EMF refers to electromotive force, measured in Volts. Accuracy of culture tank pH was maximised through a two stage monitoring process. Seawater pH in each tank was constantly measured with electrodes connected to the CO₂ delivery system (TUNZE™, 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

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from the target pH, the TUNZE™ 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). A_T 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 MIRIANDA). 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).

2.2.3 Apparatus

The described experimental apparatus consists of three identical units (Fig. 1), each capable of independently mimicking the effects of increased atmospheric CO_2 on seawater, i.e. elevated pCO_2 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 CO_2 gas. In each tank, 80L of seawater was contained in a 120 L open top tank (870 mm (L) × 600 mm (W) × 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 $2361 \pm 10 \mu mol kg^{-1}$ ($n = 6$) and a salinity of 31.7 ± 0.6 PSU. pH in each culture tank was regulated using TUNZE™ pH/ CO_2 controller systems (glass electrodes, pH meter, solenoid switch unit, and a pressure reducer) connected to 33 kg gas cylinders containing 100 % food grade CO_2 (BOC). The TUNZE™ system automatically allowed 100 % CO_2 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

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values. CO₂ 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⁻¹. To minimise changes in seawater chemistry caused by the culture of calcifying organisms, and to maintain constant salinity, 20 L of seawater was removed from each tank every 48 h and 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.

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, pH, temperature, and salinity 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 ± 0.03; calculated: 7.45 ± 0.04, 7.64 ± 0.04), in good agreement with the accuracy goal of target pH ± 0.05 (SD) (Fig. 2). While calibration of all electrodes occurred weekly, there was very little drift in the electrodes connected to the

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CO₂ regulation apparatus. Temperature, controlled by the chiller units, was also stable across all culture tanks, while salinity showed minimal variation (Table 1). As expected, DIC (measured) and pCO₂ (calculated) increased in all aquaria after the injection of CO₂ gas (Hansen et al., 2013; Campbell and Fourquaran, 2011; Findlay et al., 2008), while A_T remained unchanged in all treatments (Table 2).

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 novaezealandensis* 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 h 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 CO₂ 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), 6.4× 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 (Fig. 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 pH in three 80 L seawater culture tanks within ± 0.05 units (SD) of target values over 90 days, while each tank held 60 live snails. pH was adjusted using CO₂ 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 400 L h⁻¹ using an aquarium pump. Seawater was replaced at a rate of 20 L/48 h to maintain uniform seawater chemistry and salinity. This system was used to culture the New Zealand mud snail, *Zeacumantus 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 USD 3600, i.e. USD 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 and repeatability of potentiometric measurement and regulation of pH in an OA simulation system by: (a) using two saltwater buffers to

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calibrate glass electrodes and report pH on the total hydrogen ion scale (pH_T , 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.

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_{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 *Marine Biology* OA special edition, 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., 2009), and are not recommended for the measurement of seawater pH (Zeebe and Gladrow, 2001; Dickson, 1984; Millero, 1986).

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 of ± 0.01 – 0.14 units in the measurement of seawater pH

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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 of potentiometrically monitored OA simulation systems, 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 (Ω_a) and calcite (Ω_c). If we apply an error of ± 0.065 pH units (the median of reported liquid junction error values) to Ω_a and Ω_c in the software program SWCO2, we generate inaccuracies of 19 % and 15 % respectively. The saturation states of aragonite and calcite are particularly vulnerable to this degree of error, as the range of these variables is 1.2–5.4 (Ω_a) and 1.9–9.2 (Ω_c) (Riebesell et al., 2010), and Ω values less than 1.0 indicate that the dissolution of these CaCO_3 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_T) is most commonly reported in published data. In the 2013 *Marine Biology* OA edition, pH_T was reported in 14 out of 32 (44 %) of experimental articles while pH_F and pH_{SWS} were not used at all. One reason for this trend is that pH_T is generated directly by pH meters calibrated with salt-water 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_T should be adopted as the default scale in OA research.

4.3 DIC and A_T analysis

Throughout the 90 day trial of this system, seawater samples were periodically taken from each culture tank and used to measure A_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_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_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_2 to seawater is expected to cause an increase in DIC but not affect A_T . However, the culture of marine organisms in OA simulation systems can alter the concentration of carbon species in seawater through photosynthesis (decreased CO_2), respiration (increased CO_2), or dissolution of calcified structures (increased CO_3^{2-}). During an earlier trial of this system, when acidified treatments were 7.1 and 7.4 pH_T (12 °C), A_T greatly exceeded the expected value of $\sim 2300 \mu\text{mol kg}^{-1}$ ($2938.04 \pm 1.29 \mu\text{mol kg}^{-1}$ (7.1 pH), $2564.16 \pm 3.50 \mu\text{mol kg}^{-1}$ (7.4 pH)), and DIC was also unusually high compared to data generated by other systems that used CO_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 CO_3^{2-} through the dissolution of calcified structures, as the snail shells had visibly dissolved, and increased the replacement rate of seawater from 20 L wk^{-1} 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

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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 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. Furthermore, additional stressors such as temperature, salinity, and UV radiation could 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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Table 1. Average values (\pm SD, $n = 64$) for pH_T , salinity, and temperature, recorded over a 90 day period in three pH treatment tanks during the culture of *Z. subcarinatus*.

	pH_T (Measured)	Temp (°C)	Salinity (PSU)
8.1 Treatment	8.09 ± 0.03	12.5 ± 0.3	31.7 ± 0.6
7.6 Treatment	7.60 ± 0.03	12.6 ± 0.6	31.9 ± 0.6
7.4 Treatment	7.40 ± 0.03	12.6 ± 0.5	31.3 ± 0.6

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Table 2. Average values (\pm SD, $n = 6$) for A_T and DIC (measured) and pH_T and pCO_2 (calculated) recorded over a 90 day period in three pH treatments during the culture of *Z. subcarinatus*.

	Alkalinity ($\mu\text{mol kg}^{-1}$)	DIC ($\mu\text{mol kg}^{-1}$)	pH_T	pCO_2 (μatm)
8.1 Treatment	2361 ± 10	2138 ± 11	8.12 ± 0.03	365 ± 30
7.6 Treatment	2389 ± 7	2351 ± 16	7.64 ± 0.04	1304 ± 115
7.4 Treatment	2375 ± 12	2397 ± 13	7.45 ± 0.04	1980 ± 110

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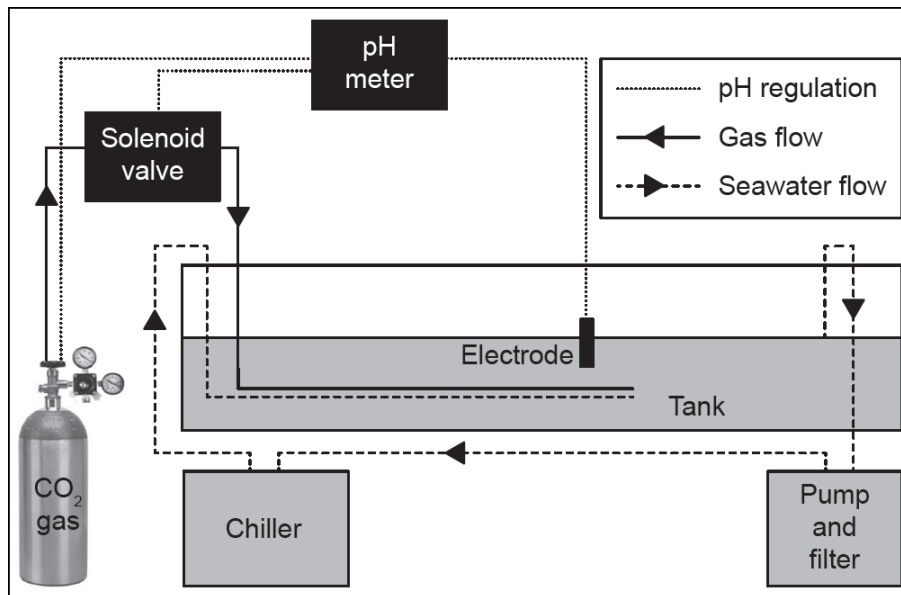


Figure 1. Schematic of one OA simulation unit. Dashed lines indicate seawater flow, solid lines indicate gas flow, and dotted lines indicate electrical connections between components of pH regulation apparatus.

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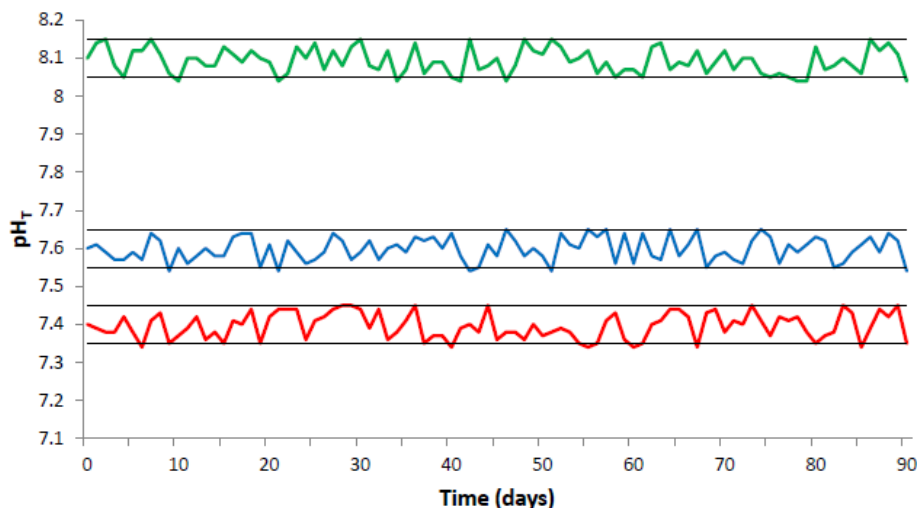


Figure 2. pH_T recorded over the course of a 90 day experiment in which snails were maintained in three culture tanks: 8.1 (green), 7.6 (blue), 7.4 (red) pH_T. Coloured lines represent pH_T data recorded on Denver AP50 hand held pH meter and black lines represent ± 0.05 error around target pH_T values.

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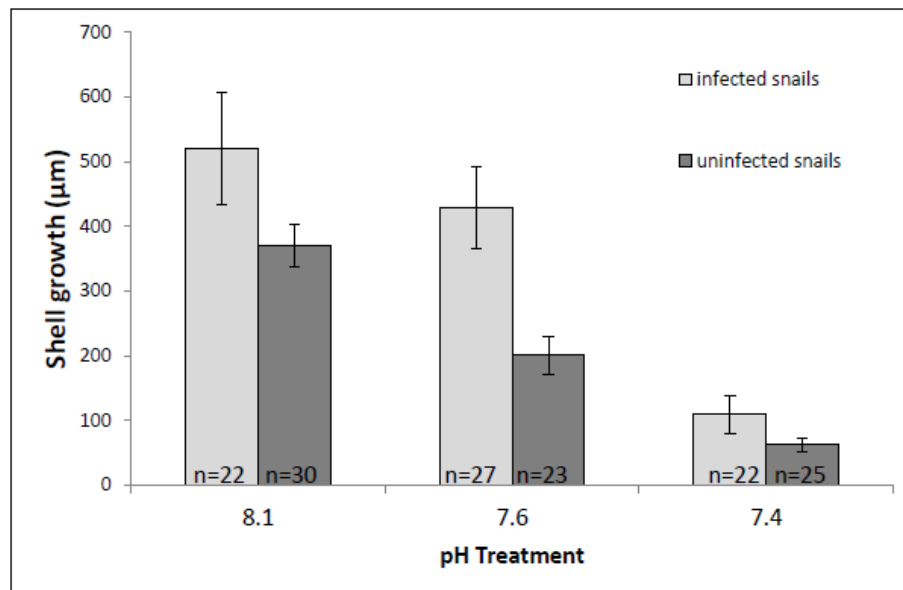


Figure 3. Average shell growth (\pm SE, sample size as indicated) of infected and uninfected snails in three pH treatments: 7.4, 7.6, 8.1.

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