

## A laboratory-based, experimental system for the study of ocean acidification effects on marine invertebrate larvae

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### Abstract

Ocean acidification, a reduction in ocean pH due to the uptake of anthropogenic carbon dioxide (CO<sub>2</sub>) by surface waters, has recently emerged as a research theme in marine biology due to an expected deleterious effect of altered seawater chemistry on calcification. Owing to the importance of larval survival and dispersal for the maintenance of adult populations, early life history stages of calcifying marine invertebrates have been a central focus of this research. Here, we present an experimental system that unites the culturing needs of larval invertebrates with methods for careful control, monitoring, and manipulation of seawater carbonate chemistry necessary to conduct laboratory-based studies of ocean acidification. Using a series of mass flow controllers, the system produces three unique *p*CO<sub>2</sub> levels, which are bubbled into gas-mixing reservoirs for equilibration with filtered seawater. This seawater is then delivered to larval culturing vessels providing the larvae with a continual supply of clean seawater consistent with optimal culturing methodologies. In this flow-through system, *p*CO<sub>2</sub> levels are determined at 3 points: the inflowing seawater, the gas-mixing reservoirs, and the larval culture vessels. The delivered gas *p*CO<sub>2</sub> values are adjusted to achieve the desired stable-state relationship for each experimental *p*CO<sub>2</sub> treatment. We evaluated the performance of this system in terms of 1) the stability of the parameters of the inorganic carbonate system in all experimental vessels and 2) our ability to successfully rear larvae using these methodologies. Our results indicate the suitability of this design for successful manipulation of *p*CO<sub>2</sub> for ocean acidification experiments on larvae.

Ocean acidification, a decrease in oceanic pH due to the absorption of atmospheric CO<sub>2</sub> by surface waters (Caldeira and

Wickett 2003), has emerged as a major research focus in the study of global change biology in the world ocean (see Pörtner et al. 2005; Hoegh-Guldberg et al. 2007; Fabry et al. 2008; Doney et al. 2009; Hofmann et al. 2010; and articles in *Oceanography's* 2009 special issue on the future of ocean biogeochemistry in a high-CO<sub>2</sub> world [Doney et al. 2009]). One major research activity has been to explore the effects of elevated CO<sub>2</sub>, lowered pH, and/or altered carbonate chemistry on the physiological performance of marine organisms in manipulative laboratory experiments (e.g., Green et al. 2004; Metzger et al. 2007; Anthony et al. 2008; Dupont et al. 2008; Kurihara 2008; Comeau et al. 2009; O'Donnell et al. 2009; Ries 2009; Todgham and Hofmann 2009). The need for these manipulative experiments is clear: they begin to reveal the current physiological plasticity possessed by marine organisms, which may allow them to tolerate and/or compensate for the altered seawater chemistry (e.g., Fabry et al. 2008; Widdicombe and Spicer 2008; Hofmann and Todgham 2010). This information

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is critical because the rate of change in the physical environment as a result of anthropogenic influence will likely occur faster than biological adaptation or microevolution can occur (see Gienapp et al. 2008; Visser 2008; Bradshaw and Holzapfel 2010). Thus, evolutionary rescue for some species may not be an expected outcome (see Bell and Collins 2008).

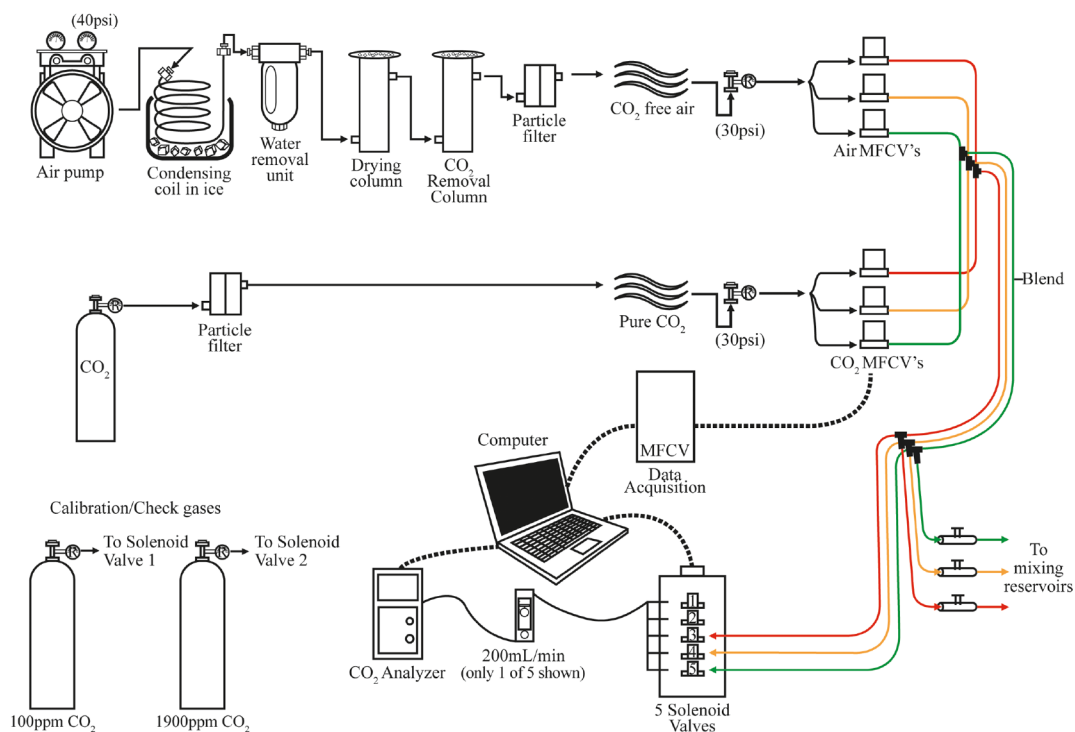
In this article, we present a laboratory-based, experimental system to test the response of marine invertebrate embryos and larvae to CO<sub>2</sub>-acidified seawater. Early life history stages have been a focus of much of the first wave of ocean acidification research, given the central role these stages play in maintenance of adult populations via dispersal and recruitment processes (see Kurihara 2008 for a review). The main concern here is that embryonic larval stages may be highly vulnerable and that sensitivity of these stages to physiological stress induced by conditions of ocean acidification could be amplified into deleterious effects on marine communities. Indeed, accumulating evidence suggests that events such as fertilization (Kurihara and Shirayama 2004; Havenhand et al. 2008; but see also Havenhand and Schlegel 2009; Byrne et al. 2010), early embryonic stages, and larval forms (e.g., Kurihara 2008; Dupont and Thorndyke 2009; Egilisdottir et al. 2009; Ellis et al. 2009; Talmage and Gobler 2009; Todgham and Hofmann 2009; O'Donnell et al. 2010) are all sensitive to ocean acidification stress. Additionally, other studies highlight the potential of interacting stressors in the complex and changing marine environment (Egilisdottir et al. 2009; O'Donnell et al. 2009; Findlay et al. 2010). In combination with evidence that ocean-warming affects the composition of the meroplankton (Kirby et al. 2007, 2008) and has a strong role in determining planktonic larval duration (O'Connor et al. 2007), understanding the response of early life history stages to rapidly changing oceanic conditions is indeed a leading research priority for organismal biologists working in marine ecosystems.

A variety of experimental approaches have been employed to rear embryos or larvae under low pH and/or elevated CO<sub>2</sub> conditions ranging from the direct addition of acid (e.g., Kurihara and Shirayama 2004; Parker et al. 2009), the use of premixed commercial gases (e.g., Miller et al. 2009; O'Donnell et al. 2009; Todgham and Hofmann 2009), or studies that mix pure CO<sub>2</sub> with ambient air to achieve the desired *p*CO<sub>2</sub> levels (e.g., Dupont et al. 2008; Byrne et al. 2009; Talmage and Gobler 2009). Other systems have been described for large-scale mesocosms (e.g., Kim et al. 2008) and for the study of intertidal organisms (Findlay et al. 2008) or benthic invertebrates (Talmage and Gobler 2009). The motivation for the design of the experimental system described here was to raise swimming echinopluteus larvae under tightly controlled water chemistry conditions and at culturing densities high enough to generate the necessary quantity of tissue for molecular biology assays (a goal of many biologists interested in the molecular response of organisms to ocean acidification), at the same time doing so in a manner that is consistent with the established best practices for successful larval culturing (Strathmann 1987).

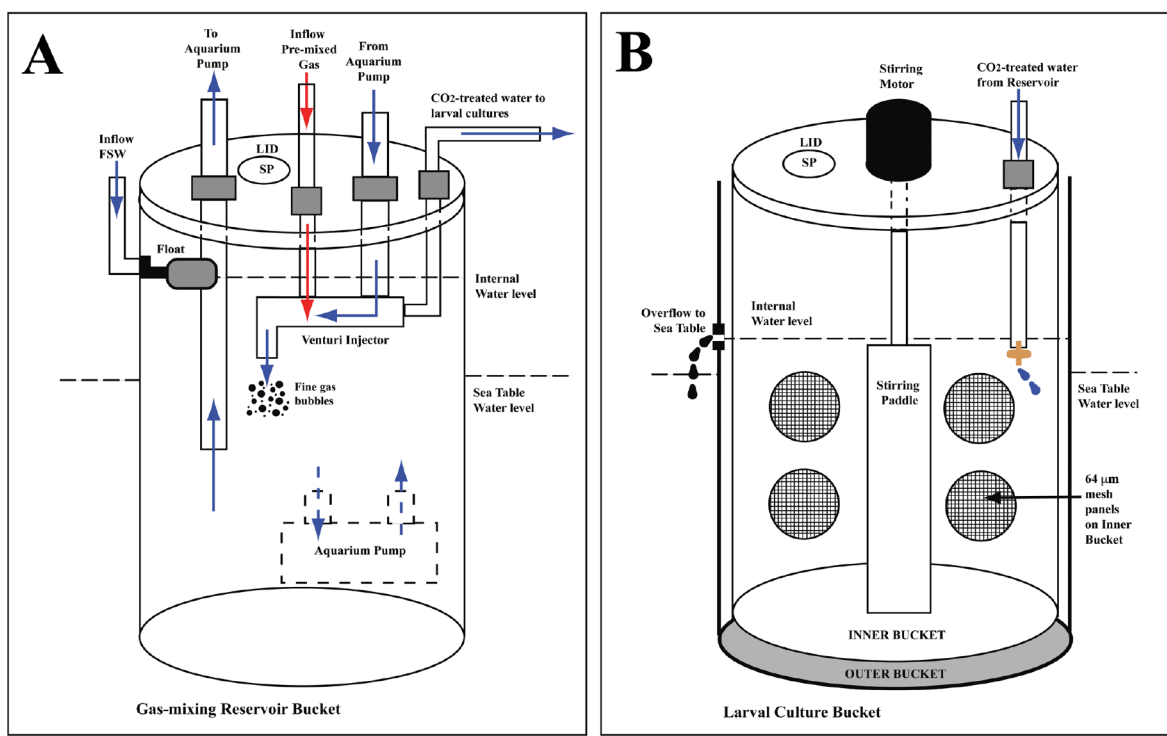
## Materials and procedures

**Procedural overview**—We describe a system whereby we blend dry, CO<sub>2</sub>-free atmospheric air with pure CO<sub>2</sub> to produce three *p*CO<sub>2</sub> levels using mass flow controllers (MFC) (Fig. 1). A portion of each blended gas is periodically sampled and analyzed for CO<sub>2</sub> content using a calibrated infrared CO<sub>2</sub> analyzer and customized monitoring computer software. The remaining gas for each mixture is continually delivered to one of three gas-mixing reservoirs for equilibration with experimental seawater to the desired *p*CO<sub>2</sub> level (Fig. 2A). Each of the gas-mixing reservoirs then supplies CO<sub>2</sub>-equilibrated seawater to experimental larval culture buckets using lawn irrigation drippers (Fig. 2B). In this system, filtered seawater is continually introduced for the duration of the experiment for two main reasons: (1) invertebrate larvae require a continuous supply of clean seawater for normal development, and (2) our experiments require a large water volume that exceeds our ability to store a single seawater sample for the entire experiment. Because of the necessity to use a flow-through seawater system, we never achieve perfect equilibrium between our gas *p*CO<sub>2</sub> levels and that of the experimental seawater. Instead, we regularly determine *p*CO<sub>2</sub> levels at 3 points in the system: the inflowing seawater, the gas-mixing reservoirs, and the larval culture vessels, and adjust our delivered gas *p*CO<sub>2</sub> values to achieve the desired stable-state relationship for each experimental *p*CO<sub>2</sub> treatment. The larval culture buckets described here have been modified from Russell (2000) such that there is a continual inflow of new treatment water (rather than culturing under stagnant conditions) and so that the bubbling of CO<sub>2</sub> gas does not interfere with the normal development of invertebrate larvae (Hinegardner 1969). Each of the reservoir and culture buckets has a tight-fitting lid with a sampling port so that water samples for chemical analyses can be drawn frequently during the experiment. In keeping with the *Guide to Best Practices for Ocean Acidification Research and Data Reporting* (Riebesell et al. 2010), we describe our use and modification of cost-effective, analytical methods to rapidly characterize the parameters of the marine inorganic carbon system in our experimental vessels. As supplemental material, we have provided a detailed parts list to aid users in system design along with a copy of our Excel spreadsheet used to calculate Total Alkalinity (A<sub>T</sub>) from the raw titration data.

**Description of the *p*CO<sub>2</sub> generation system**—The *p*CO<sub>2</sub> regulation system (Fig. 1) was designed to generate three unique *p*CO<sub>2</sub> levels across a wide range of CO<sub>2</sub> concentrations including *p*CO<sub>2</sub> levels below the 2009 global average atmospheric CO<sub>2</sub> concentration of ~388 ppm (Peter Tans, NOAA/ESRL, [www.esrl.noaa.gov/gmd/ccgg/trends](http://www.esrl.noaa.gov/gmd/ccgg/trends)). For each of the target *p*CO<sub>2</sub> treatments, we blend dry, CO<sub>2</sub>-free air with pure CO<sub>2</sub> to the desired concentration using an air MFC (Smart-Trak 100C, 0–10 L min<sup>-1</sup>, Sierra Instruments) and a CO<sub>2</sub> MFC (Micro-Trak 101, 0–2.95 mL min<sup>-1</sup>, Sierra Instruments). In total, we use 6 MFC valves (Fig. 1) mounted on a board for horizontal gas



**Fig. 1.** Diagram of the  $p\text{CO}_2$  generation system. The upper portion of the figure shows how we blend dry, CO<sub>2</sub>-free air with pure CO<sub>2</sub> to 1 of 3 desired  $p\text{CO}_2$  levels. The bottom portion of the figure traces the path of the blended gases to either the computer-monitoring system or to the gas-mixing reservoirs. Dotted lines indicate electrical connections whereas solid lines represent gas flow tubes. We use solid red, yellow, and green lines to trace the ultimate blending of high, mid, and low CO<sub>2</sub> level gases, respectively.



**Fig. 2.** Schematic of a single gas-mixing reservoir (A) and larval culture vessel (B). Blue arrows indicate water flows into and out of the gas-mixing reservoir (A) for eventual entry into a representative larval culture bucket through an irrigation dripper (B). Red arrows indicate the flow of a CO<sub>2</sub> treatment gas into the Venturi injector resulting in very fine gas bubble production.

flow with metering set-points controlled by a pilot module (100-RDO, Sierra Instruments). Dry, CO<sub>2</sub>-free air is generated by first compressing atmospheric air using an oil-free, diaphragm pump (DOA P704 AA, Gast Manufacturing Corp.), removing moisture by passing this pump-heated air through an ice-cooled, stainless steel condensing coil, a water removal unit (20 SCFM Eliminator, Reading Technologies), and a 0.5 L desiccant column (106-C, W.H. Hammond Drierite Co., Xenia, OH), and then through a 0.5 L CO<sub>2</sub>-removal column (26800, W.H. Hammond Drierite Co.) filled with Soda Lime (Sodasorb®-HP, 4-8 mesh, Amron International). The air passes through a particle filter (DS0330-4000 Nalgene®, 5 µm) before entering the air MFCs. Removal of moisture and particulates upstream of the MFC valves is important to avoid MFC malfunction. Pressure in the air line is maintained at 40 psi using the air pump's regulator and stepped down to the set pressure of 30 psi before entering the MFC valves using a standard air regulator. Similarly, ultra-pure (99.999%) CO<sub>2</sub> is regulated at 40 and then 30 psi using CO<sub>2</sub> regulators, passes through a 5-µm particle filter and enters the CO<sub>2</sub> MFCs. Using the dual pressure regulation scheme for each of our source gas lines provided the most stable pressure into the MFCs. In this experiment, each air MFC delivered air at a flow rate of 2.63 L min<sup>-1</sup> whereas the CO<sub>2</sub> MFCs were set to deliver varying flow rates between ~0.800 to 2.60 mL min<sup>-1</sup> depending on the desired CO<sub>2</sub> mixture. At these gas flow rates, the Sodasorb media was replaced approximately daily. For each treatment gas, backflow prevention valves (7933K32, McMaster-Carr) as well as pressure gauges (4089K13, McMaster-Carr) were installed downstream of the MFCs to protect them from possible seawater backflow and to monitor the backpressure on the MFCs, respectively.

*Description of the gas blend pCO<sub>2</sub> monitoring system*—Using customized computer software written in LabView (version 7.1, National Instruments), we continually monitored and recorded the function of our CO<sub>2</sub> delivery system. Once the treatment gases are blended, a portion of each gas is routed through a series of computer-controlled solenoid valves (Fig. 1, Solenoids 3-5) that send each gas to an infrared CO<sub>2</sub> analyzer (S151, Qubit Systems) for measurement of the CO<sub>2</sub> content in the gas mix. In addition to sampling our treatment gas blends, we also measured two primary reference gas mixtures (Praxair, Santa Barbara, 100 and 1900 ppm CO<sub>2</sub> ± 1% balance air, solenoids 1-2), which bracket our experimental CO<sub>2</sub> levels for monitoring of CO<sub>2</sub> analyzer function during our experiments. The flow rate of each gas through the CO<sub>2</sub> analyzer is held constant at 200 mL min<sup>-1</sup>, equivalent to the calibration flow rate, using rotameters (scaled at 250 cc/min air, Aalborg Instruments). Each treatment gas is sampled by the CO<sub>2</sub> analyzer for 10 min of each hour with CO<sub>2</sub> content measures recorded every 10 s. These data are then averaged over the last 3 min of the 10-min period (to ensure the CO<sub>2</sub> analyzer reading has stabilized) and are used to characterize each gas's CO<sub>2</sub> content. In total, each gas blend is sampled 24 times per day

and the reference gas standards are checked once per day. This information is used to confirm the accuracy of our CO<sub>2</sub> mixtures and to diagnose any system malfunctions. The CO<sub>2</sub> concentrations are recorded along with the gas delivery characteristics of each MFC, and the information is posted in real-time online for ease of system monitoring, even remotely. The software also has an alert function such that users can be emailed in the case of a system malfunction or if CO<sub>2</sub> levels deviate from the desired range.

*Description of the seawater mixing reservoirs*—Once our experimental treatment gases are produced, each gas is mixed with filtered seawater (FSW) (0.35 micron filtered, ultraviolet sterilized) in a gas-mixing reservoir bucket (Fig. 2A) rather than bubbling these gases into our larval culture vessels directly. Each reservoir consists of a 19-L food-grade plastic bucket and lid (High-density polyethylene, U.S. Plastics) with a submersible aquarium pump (Mag Drive 72, Aquatic Ecosystems, Apopka, FL) that provides vigorous circulation of the reservoir as well as the water pressure needed to pump CO<sub>2</sub>-treated FSW to the larval cultures, and a float valve (5741K28, McMaster-Carr) to keep the reservoir filled with FSW at all times. In combination with the aquarium pump, a Venturi injector (MK-484, Mazzei Injector Company LLC) mixes each CO<sub>2</sub> treatment gas with FSW by directing the water through a small orifice creating negative pressure and drawing in the gas mixture as a very fine stream of bubbles for efficient mixing. The aquarium pumps and reservoir buckets are submerged in a temperature-regulated sea table (set point 15.5 ± 0.5°C) to ensure that experimental water temperatures are tightly controlled.

*Description of the larval culture vessels*—To unite the culturing needs of developing invertebrate larvae with careful control of the marine inorganic carbonate system necessary for ocean acidification experiments, we developed a unique larval culturing methodology. Each culture vessel consisted of two 19-L food grade buckets (High-density polyethylene, U.S. Plastics) nested inside one another (Fig. 2B). The inner bucket has 12 55-mm diameter holes covered with 64-µm Nitex mesh (Aquatic Ecosystems). These holes allow for exchange of water between the inner and outer buckets while keeping the larvae contained in the inner bucket. Water flow rates from the mixing reservoirs into each culture vessel were metered by irrigation drippers (W221G Button dripper, DIG Irrigation Products) at a flow rate of 1 L h<sup>-1</sup>. Though the drippers operated below their specified delivery pressure of 15-25 psi, testing showed that these particular drippers provided stable and reliable output flows under our operating conditions. However, other brands tested did not provide accurate flow metering, and dripper rates should always be carefully monitored. The water level in the culture vessel was set by an overflow hole in the outer bucket and the total water volume in the culture vessel was 12 L. The larval culture buckets were submerged in a temperature-regulated sea table (set point 15.5 ± 0.5°C) for careful control of experimental water tem-

peratures. Gentle stirring of each culture was achieved using a DC gear motor (Anaheim Automation) mounted to the bucket lid rotating a Plexiglas paddle ( $1.9 \times 7.6 \times 10.2$  cm) at 12 RPMs. These design features are particularly relevant for ocean acidification studies involving the culture of marine larvae: 1) where continual replacement of CO<sub>2</sub>-acidified water at a flow rate high enough to wash out any biological effects on the carbonate system due to the presence of the larvae is desired, 2) for species where it is desirable to separate the gas/seawater mixing system from the larval culture system so that bubbles do not damage fragile larval forms (Hinegardner 1969), and 3) when large numbers of larvae are reared for relatively long time periods (days to weeks) making daily water changes on stagnant cultures inconvenient and leading to inconsistent water quality.

**Description of larval culture**—Adult purple sea urchins, *Strongylocentrotus purpuratus*, were collected by SCUBA from Goleta Pier (Goleta, CA, USA) during the spawning season (March 2009) and maintained in flow-through sea tables at 10°–12°C. Urchin spawning and culturing followed the methods described in Todgham and Hofmann (2009); however, we used only two females and a single male to generate the cultures for each of our two biological test runs. Fertilized embryos from a single mother were split into 3 equal batches of 400,000 and introduced into 1 of 3 larval culture buckets per CO<sub>2</sub> treatment. High densities of larvae per culture bucket (ca 33/mL), whereas not always desirable, are required to obtain adequate tissue for many molecular or physiological assays. We simulated a typical experiment (e.g., Todgham and Hofmann 2009) by sampling 60,000 larvae per culture at 3 time points (hatched blastula, gastrula, and early 4-arm pluteus) during each experimental series. Careful attention must be paid to densities of larvae during development for two main reasons: 1) biological activity can affect experimental seawater chemistry and water flows must be sufficiently high to wash out any of these effects, and 2) high larval densities can result in abnormal developmental patterns (Wray et al. 2004). We assessed the quality of the larvae during the experiment by continuously monitoring the hallmarks of normal development including cleavage patterns, regularity of cell surfaces, and symmetry of larval form.

**Description of the analytical water chemistry analysis**—To describe the dissolved inorganic carbon chemistry in our experimental seawater treatments, we measured temperature (°C), salinity, total alkalinity ( $A_T$ ,  $\mu\text{mol kg}^{-1}$ ), and pH following the procedures outlined in the *Guide to Best Practices for Ocean CO<sub>2</sub> Measurements* (Dickson et al. 2007) with some modifications. From these data, we calculated the  $p\text{CO}_2$  (ppm) and dissolved inorganic carbon ( $C_T$ ,  $\mu\text{mol kg}^{-1}$ ) using CO2sys (Pierrot et al. 2006) with the carbonic acid dissociation constants of Mehrbach et al. (1973) and refitted in different functional forms by Dickson and Millero (1987).

**Determination of total alkalinity**—To determine total alkalinity ( $A_T$ ) in seawater samples, we performed a potentiometric

titration procedure (SOP 3b, Dickson et al. 2007) using a commercially available titration unit (T50, Mettler Toledo) and certified acid titrant (0.1M HCl, 0.6M NaCl; A. Dickson, Scripps Institution of Oceanography). Samples of seawater were collected from reservoir and larval culture buckets as well as from the incoming FSW at least daily using a silicone siphon tube (0.3 cm I.D., 0.6 cm O.D., Fisher Scientific) into 125 mL borosilicate glass-stoppered bottles (1500-125, Corning). Water samples were not poisoned with mercuric chloride, as this step is not necessary if measurements are done promptly (A. Dickson pers. comm.). All alkalinity measurements were made within 8 h of sampling. The mass of each sample to be titrated was recorded to 0.001 g, and the temperature of the acid titrant and the sample were kept within 0.1°C of one another using a jacketed beaker and circulating water bath set to 25°C. We adapted the titration procedure described in SOP3b to run on the Mettler T50 LabX software. Briefly, we programmed the titrator to perform an equivalence point titration (EQP) and 1) dispense enough hydrochloric acid to bring the sample to a pH just above 3.6 (modified from a pH of 3.5 as described in SOP 3b, A. Dickson pers. comm.), 2) stir the acidified sample for 6 min to allow for CO<sub>2</sub> degassing, and 3) titrate the sample using 0.05 cm<sup>3</sup> increments of HCl to a final pH of 3.0. We bubbled air into each sample during the titration using an aquarium pump and tubing, at a gas flow rate of 100 mL min<sup>-1</sup>. The equivalence point of each titration is determined using a nonlinear, least-squares fit of the results calculated from the data points in the pH region of 3.0 to 3.6. Following SOP3b, total dissolved inorganic carbon is assumed to be zero, and corrections are made for hydrogen sulfate and hydrogen fluoride formation. All calculations were performed in Excel (Microsoft 2007) and total alkalinity ( $A_T$ ) is expressed in  $\mu\text{mol kg}^{-1}$ .

**Determination of pH**—We determined pH using a spectrophotometer (Shimadzu BioSpec 1601) and the indicator dye m-cresol purple (Sigma-Aldrich) following SOP 6b (Dickson et al. 2007). Samples of seawater for pH determination were collected from all experimental vessels at least daily. Water samples were collected in 20 mL glass scintillation vials leaving no head space and vials were placed in a dark, 25°C water bath for temperature equilibration. Preliminary experiments confirmed no deterioration in measured pH values in the first 3 h after sampling, and pH was always measured within 2 h of sample collection. To make each pH measurement, 3 mL of each sample was carefully pipetted into a quartz cuvette with a 1 cm pathlength (modification of the 10 cm pathlength in SOP 6b), the cuvette was sealed with a Teflon cover, and the temperature of the sample was held at 25°C in the temperature control chamber of the spectrophotometer. We measured absorbance at 730 nm, 578nm ( $A_1$ ), and 434 nm ( $A_2$ ) before dye addition and then added 50  $\mu\text{L}$  m-cresol purple to the cuvette, inverted the cuvette 3 times to mix, and remeasured absorbance at the three wavelengths (SOP 6b). We used equations in section 8.2 and 8.3 of SOP 6b

to correct  $A_1/A_2$  for the addition of dye and to calculate the pH of our seawater samples.

**Assessment of total alkalinity and pH measures**—We assessed the quality (precision and accuracy) of our pH and alkalinity measurements by the continual measurement of certified reference materials (CRM). A sample with a known  $A_T$  (Reference Material for Oceanic CO<sub>2</sub> Measurement, Batch 97,  $A_T = 2211.21 \mu\text{mol kg}^{-1}$ , A. Dickson, Scripps Institution of Oceanography) was titrated at the beginning and end of each day's measurements. The results from this series of measurements on Batch 97 were  $A_T = 2210.7 \pm 2.4 \mu\text{mol kg}^{-1}$  (mean  $\pm$ SD) ( $n = 26$  measures). Similarly, we took 13 successive daily pH measurements (never more than one per day) on certified reference material (Tris Buffer in synthetic seawater, Batch 2, Bottle 56, pH = 8.0929, A. Dickson, Scripps Institution of Oceanography) to confirm the stability of our pH measurement. The results of this series of measurements were pH =  $8.0931 \pm 0.0010$  (mean  $\pm$ SD).

**Oxygen, salinity, and temperature**—Oxygen levels in the larval culture buckets were monitored using an oxygen probe (YSI 556, Yellow Springs Instruments) at least once daily. Salinity ( $\pm 0.1$ ) was determined from each alkalinity sample using a salinity/conductivity instrument (3100, Yellow Springs Instruments) calibrated following manufacturer's instructions. A Thermolyne thermocouple (PM20700, series 1218, Omega Engineering) was used to measure culture bucket water temperatures to within 0.1°C. Careful measurement of salinity and temperature are necessary for both pH determination as well as for CO<sub>2</sub>sys calculations (Pierrot et al. 2006) for parameters of the marine carbonate system, and these measurements were made at each water sampling time point.

**Generation of target CO<sub>2</sub> concentrations**—Low, mid, and high seawater  $p\text{CO}_2$  target values for this experiment were 388, 550, and 970 ppm, respectively. These values were chosen to approximate the 2009 global average atmospheric CO<sub>2</sub> as well as 2 scenarios for atmospheric CO<sub>2</sub> increase by the year 2100 by the Intergovernmental Panel on Climate Change (IPCC), B1 and A1FI, respectively (Meehl et al. 2007). To initiate the experiment, the MFCs were set to produce 388, 550, and 970 ppm CO<sub>2</sub>, and gas was delivered to a gas-mixing reservoir bucket at a flow rate of  $\sim 2.63 \text{ L/min}$ . During this initial mixing phase, the gas-mixing reservoir buckets were continually

exchanging with FSW at a rate of  $2 \text{ L h}^{-1}$  (equivalent to the supply of experimental seawater needed for delivery to the duplicate larval culture buckets for each CO<sub>2</sub> treatment level). Our mixing reservoir  $p\text{CO}_2$ 's did not perfectly represent those of our delivered gas  $p\text{CO}_2$ 's as this relationship is dictated by FSW residence time and gas equilibration time. Through continual measurement of the parameters of the carbonate system in the gas-mixing reservoir buckets (samples drawn approximately every 2 hours), we were able to monitor the stabilization of  $p\text{CO}_2$  values in the reservoir buckets. Stabilization of  $p\text{CO}_2$  values happened relatively quickly, in approximately 2–4 h (data not shown), but we ran our reservoir buckets for at least a full day to allow for slight adjustments to the MFC set points. Once consistent  $p\text{CO}_2$  levels in the reservoirs had been recorded for at least 8 h, we then began filling each larval culture bucket at a rate of  $1 \text{ L h}^{-1}$ .

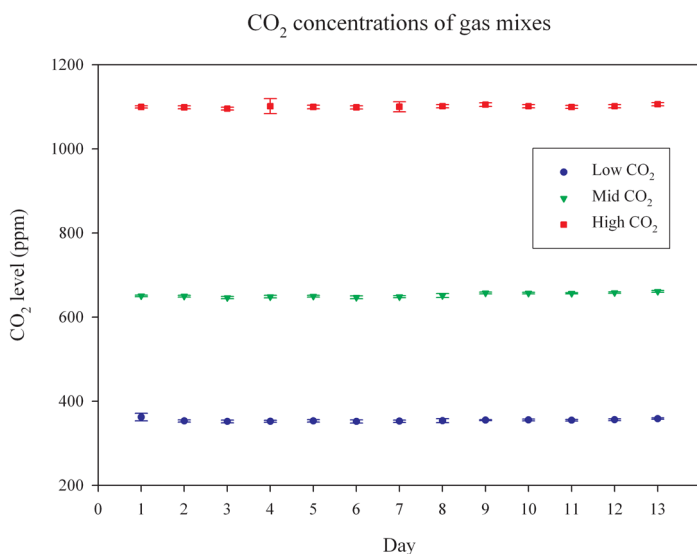
## Assessment

**Evaluation of the CO<sub>2</sub> system**—Once the initial CO<sub>2</sub> delivery system set-up was complete, and the water chemistry parameters (in the reservoir and larval culture buckets) had stabilized for a minimum of two consecutive days (data not shown), we assessed the performance of our CO<sub>2</sub> delivery system by determining: 1) the stability of the CO<sub>2</sub> levels in the gas blends, 2) how well our gas-mixing reservoirs equilibrated our FSW with our gas blends, and 3) the relationship between reservoir  $p\text{CO}_2$  and larval culture buckets with and without larvae present.

We monitored the temperature and salinity of the incoming FSW and of our gas-mixing reservoir buckets over the duration of the 13-d experiment (Table 1). Whereas incoming FSW fluctuated from 17.1°C to 18.3°C during the experiment, the temperatures of our reservoir buckets were very stable at  $\sim 16.0^\circ\text{C}$ . Temperatures in the larval culture buckets were approximately 0.5°C lower than those in the gas-mixing reservoirs because the circulating water pump added a small amount of heat to the reservoir water. Salinity was also very consistent, ranging from 33.2–33.5 in the incoming FSW and all vessels, for the duration of the experiment (Table 1). For both temperature and salinity, replicate larval culture buckets were always within 0.1°C and 0.1, respectively, of one another. Oxygen saturation never fell below 95% at any time in any vessel over the duration of the experiment.

**Table 1.** Temperature (°C) and salinity values for the incoming filtered seawater (FSW), each gas-mixing reservoir, and duplicate larval culture buckets. Data are expressed as mean  $\pm$ SD ( $n = 13$  for incoming FSW and reservoir buckets, and  $n = 26$  for larval culture vessels).

	Incoming FSW	Gas-mixing reservoirs			Culture buckets		
		Low CO <sub>2</sub>	Mid CO <sub>2</sub>	High CO <sub>2</sub>	Low CO <sub>2</sub>	Mid CO <sub>2</sub>	High CO <sub>2</sub>
Temperature (°C)							
Mean $\pm$ SD	17.7 $\pm$ 0.43	16.0 $\pm$ 0.27	15.9 $\pm$ 0.30	16.0 $\pm$ 0.29	15.5 $\pm$ 0.27	15.4 $\pm$ 0.28	15.4 $\pm$ 0.24
Salinity							
Mean $\pm$ SD	33.3 $\pm$ 0.08	33.3 $\pm$ 0.08	33.3 $\pm$ 0.10	33.3 $\pm$ 0.09	33.3 $\pm$ 0.08	33.3 $\pm$ 0.08	33.3 $\pm$ 0.09



**Fig. 3.** CO<sub>2</sub> levels (ppm) of the low (blue), mid (green), and high (red) CO<sub>2</sub> experimental gas mixes measured for the duration of our experiment. Stable CO<sub>2</sub> levels of each gas are recorded for 3 min of each hour, and an average is calculated. The overall mean ( $\pm$ SD) of the 24 data points collected for each gas per day are plotted.

The CO<sub>2</sub> levels (ppm) in all 3 gas mixes were very consistent for the duration of the experiment presented here:  $355.3 \pm 3.10$ ,  $654.1 \pm 5.74$ , and  $1101.9 \pm 3.73$  (mean  $\pm$ SD,  $n = 312$  measures for each gas, Fig. 3) confirming the stability and accuracy of blending gas using MFCs. The resultant seawater chemistry in our gas-mixing reservoirs was also very stable for the duration of the 13-d experiment (Fig. 4). Introduction of the gas mixes above resulted in gas-mixing reservoir  $p\text{CO}_2$  values of  $368.5 \pm 8.20$ ,  $610.9 \pm 11.66$ , and  $938.1 \pm 26.87$  for the duration of the experiment (mean  $\pm$ SD,  $n = 13$ , low to high CO<sub>2</sub> levels, respectively; Fig. 4C). These changes in  $p\text{CO}_2$  dictated the changes in pH and total dissolved inorganic carbon ( $C_T$ ) of the reservoir buckets (Fig. 4A and 4D) with each of our three CO<sub>2</sub> treatment levels remaining clearly distinct for the duration of the experiment. As expected, the  $A_T$  in the gas-mixing reservoirs remained relatively stable over time (ranging from 2218–2235  $\mu\text{mol kg}^{-1}$ ) and fluctuated in concert with fluctuations in the  $A_T$  of the incoming FSW (Fig. 4B).

To demonstrate our ability to successfully rear sea urchin larvae in our custom larval-culturing vessels under different levels of  $p\text{CO}_2$ , we characterized the seawater carbonate parameters in our larval culture buckets both with and without larvae developing in the system. Figure 5 shows the relationship between each of our 3 gas-mixing reservoir buckets and the resultant seawater parameters in our larval culture buckets. The  $p\text{CO}_2$  value in our mid-CO<sub>2</sub> gas-mixing reservoir closely matched that in the mid-CO<sub>2</sub> larval culture buckets (Fig. 5A). However, there was a consistent difference between our low- and high-CO<sub>2</sub> gas-mixing reservoirs and the larval culture buckets. The

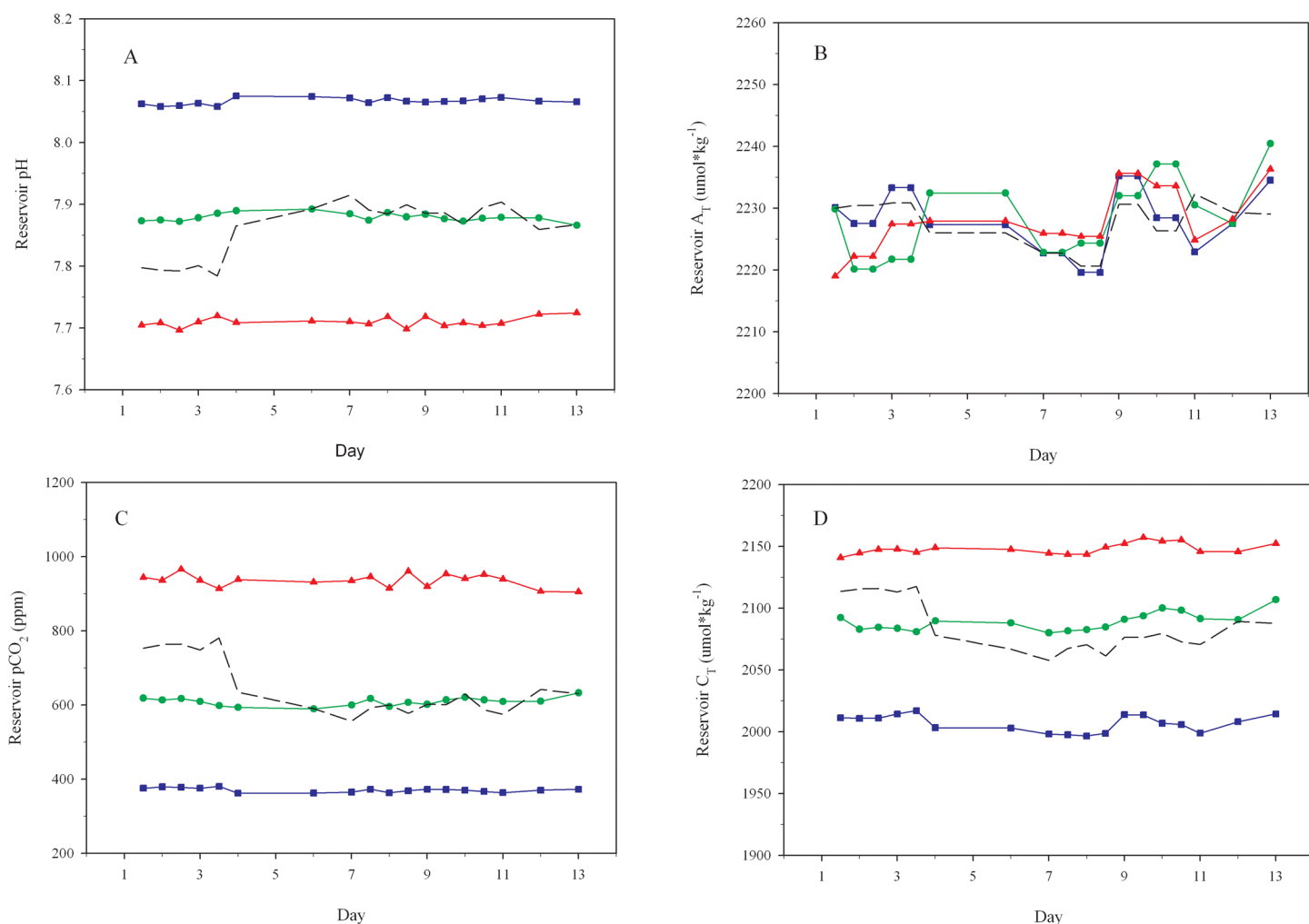
$p\text{CO}_2$  values in our low-CO<sub>2</sub> larval culture buckets were  $\sim 386$  ppm, roughly 18 ppm higher than the low-CO<sub>2</sub> mixing reservoir, and the  $p\text{CO}_2$ 's of the high-CO<sub>2</sub> larval culture buckets were  $\sim 840$  ppm, nearly 100 ppm lower than high-CO<sub>2</sub> reservoir bucket. These differences are probably due to the gradient between ambient CO<sub>2</sub> levels and CO<sub>2</sub> levels in our treatment water because of our choice not to control CO<sub>2</sub> levels in the headspaces of our culture buckets. In our experience, CO<sub>2</sub> levels are variable inside our experimental seawater room, often exceeding 450 ppm in this poorly ventilated space.

Manipulation of the larval culture bucket  $p\text{CO}_2$  values resulted in pH values of  $8.052 \pm 0.01$ ,  $7.888 \pm 0.001$ , and  $7.752 \pm 0.01$ , and  $C_T$  values of  $2018.0 \pm 4.82$ ,  $2088.6 \pm 4.79$ , and  $2134.2 \pm 9.00$  for the duration of the experiment (mean  $\pm$ SD,  $n = 26$  per treatment, low to high CO<sub>2</sub> levels, respectively) (Fig. 5A and 5D). Similar to the  $A_T$  values for the reservoir buckets, culture bucket  $A_T$  changed little over time between larval culture buckets with values of  $2229.2 \pm 5.14$ ,  $2229.5 \pm 4.90$ , and  $2226.1 \pm 8.24$  (mean  $\pm$ SD,  $n = 26$  per treatment, low to high CO<sub>2</sub> levels, respectively) (Fig. 5B) contributing very little (2–5 ppm) to the overall variation in  $p\text{CO}_2$  levels in this experiment within an experimental treatment. We were able to demonstrate our ability to deliver consistent  $p\text{CO}_2$  exposures to replicate larval culture buckets. In general, replicate larval culture buckets tracked each other closely, maintaining  $p\text{CO}_2$  levels within 5–15 ppm of one another for the duration of the experiment, regardless of larval presence/absence (Fig. 5C). The variation between replicate buckets was most likely attributable to subtle differences inherent to each vessel such as slight differences in the flow rates of incoming treatment water as well as variation in gas exchange with the ambient atmosphere. We also showed that a flow rate of 1 L h<sup>-1</sup> into each larval culture bucket was sufficient to wash out any effects on seawater chemistry due to biological activity (Fig. 5, compare presence and absence of larvae data). Finally, in all larval culturing buckets in both of our larval rearing trials, all purple urchin larvae developed synchronously and demonstrated the hallmarks of normal development.

## Discussion

Larval stages of many marine invertebrates may be particularly sensitive to the effects of ocean acidification (Dupont et al. 2008; Kurihara 2008; Dupont and Thorndyke 2009; Todgham and Hofmann 2009; O'Donnell et al. 2010). Traditional larval culture techniques, however, are difficult to apply while maintaining controlled seawater conditions necessary for ocean acidification research. To unite the needs of larval invertebrates with tightly controlled and monitored seawater chemistry, we report the design of a culturing system for laboratory-based experiments to support the raising of marine invertebrate embryos and larvae under different levels of CO<sub>2</sub>. Our CO<sub>2</sub> mixing system delivered gas with a CO<sub>2</sub> content ranging from 355–1101 ppm CO<sub>2</sub>, and has been used to reach higher levels of 1600 ppm CO<sub>2</sub>, which are consistent with





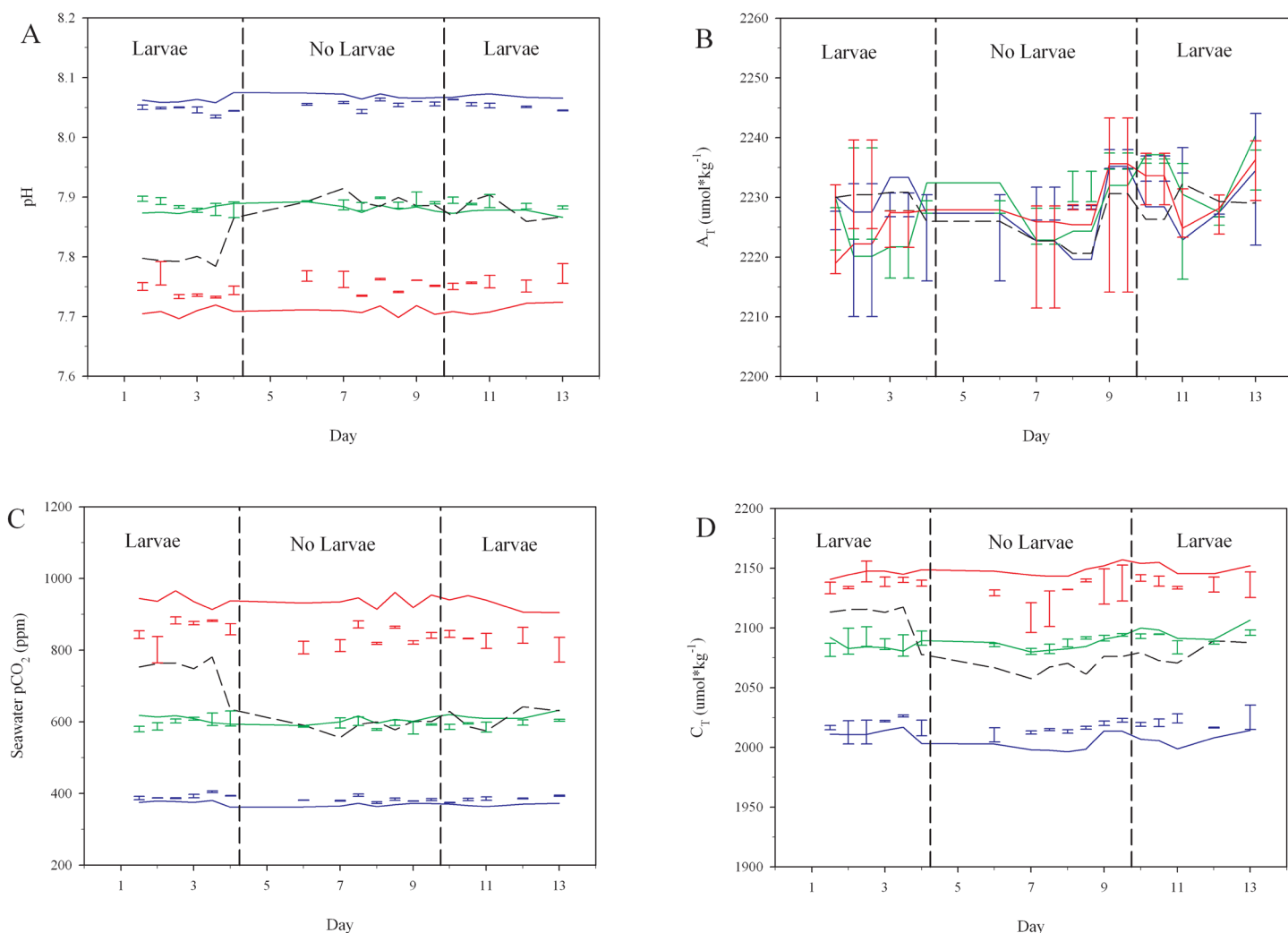
**Fig. 4.** Values of (A) pH, (B) total alkalinity ( $A_T$ ), (C)  $p\text{CO}_2$ , and (D) total dissolved inorganic carbon ( $C_T$ ) in the low (blue), mid (green), and high (red) CO<sub>2</sub> gas-mixing reservoirs over the 13-d experimental period. Values for the incoming filtered seawater are also shown (black dashed line). Symbols indicate measurement sampling points.

$p\text{CO}_2$ 's found in upwelled water (Feely et al. 2008). The CO<sub>2</sub> delivery system is very stable through time. Although this article reports data for 13 consecutive days, we have since run the MFCs for greater than 4 consecutive months achieving very stable CO<sub>2</sub> mixtures and seawater carbonate chemistry. The stability of the CO<sub>2</sub> delivery system as well as the continual replenishment of fresh seawater to the culturing vessels allows marine invertebrates to be reared for extended developmental periods to accommodate the study of other biological processes such as metamorphosis and the induction of settlement in larvae, as well as prolonged growth studies that would necessitate the ability to introduce feed to the larvae. The continuous flow of water through the system allows for the introduction of food without requiring frequent and complete water changes. Urchin larvae (*S. purpuratus*) have been fed successfully in this system for several weeks (M. O'Donnell, unpubl. data). In addition, the system has been used to successfully raise a variety of

sea urchin species including the painted urchin (*Lytechinus pictus*, O'Donnell et al. 2010), the purple urchin (*S. purpuratus*, this study; Todgham and Hofmann 2009), the green urchin (*S. droebachiensis*, P. Matson unpubl. data), and the crowned urchin (*Centrostephanus coronatus*, N. Fangue unpubl. data), and larval development has proceeded normally.

In the CO<sub>2</sub>-delivery system described here, we were not able to achieve a perfect relationship between gas blend  $p\text{CO}_2$ , reservoir  $p\text{CO}_2$ , and larval culture bucket  $p\text{CO}_2$ . This was due to several factors, including the continual inflow of FSW into the system, as well as the loss or gain of CO<sub>2</sub> from the atmosphere (we did not control the CO<sub>2</sub> levels in the headspace of the culture buckets). This has implications for other experimental designs, which purchase pre-mixed gas and assume that it is completely equilibrated with experimental seawater (e.g. O'Donnell et al. 2009). Therefore, we adjusted the CO<sub>2</sub> levels in our gas blends to those that would allow us to achieve





**Fig. 5.** Values of (A) pH, (B) total alkalinity ( $A_T$ ), (C)  $p\text{CO}_2$ , and (D) total dissolved inorganic carbon ( $C_T$ ) in replicate larval culture buckets (low, mid, and high  $p\text{CO}_2$  treatments shown with blue, green, and red data points, respectively) over the 13-d experimental period. Error bars represent the mean  $\pm$  SD of the replicate larval culture buckets. Data collected when no larvae were present in the system are shown within the black dotted lines. For comparison, solid lines show data from the three gas-mixing reservoirs and from the incoming filtered seawater (black dashed line) over the 13-d experimental period (data from Fig. 4 with sampling points removed).

stable  $p\text{CO}_2$ 's in our experimental larval culture buckets very near our desired levels. We have recently improved our system design by filling each of our culture buckets' headspace with a portion of our CO<sub>2</sub> treatment gases at a flowrate of 150 mL min<sup>-1</sup>. With headspaces filled with gas, we showed an even closer correspondence between  $p\text{CO}_2$  levels in our low and high CO<sub>2</sub> reservoir and culture buckets, respectively ( $361.9 \pm 9.90$  vs.  $368.6 \pm 9.10$  and  $979.3 \pm 5.71$  vs.  $1026.4 \pm 19.72$ , mean  $\pm$ SD, reservoir versus culture  $p\text{CO}_2$ , over 5 d). While this design does reduce the amount of treatment gas delivered to the gas-mixing reservoirs, this is more than compensated for by the resultant limited exchange between the larval culture seawater and the atmosphere.

In common with other standard larval-culturing techniques, our culturing system does not replicate interactions between

members of the larval community and the larvae do not experience natural disturbances in other parameters such as turbulence during their pelagic larval duration. Whereas our system, as currently configured, considers only one climate change variable at a time, it does lend itself to modification for work that addresses multiple climate change variables such as temperature by CO<sub>2</sub> interactions. While we acknowledge the need for larger-scale experiments that more closely reflect natural conditions (Schindler 1998), this small-scale culture system allows us to raise larval marine invertebrates in controlled conditions that support experimental work ranging from the analysis of larval form (e.g. O'Donnell et al. 2010) to the collection of large numbers of individuals for gene expression analysis (e.g. Todgham and Hofmann 2009). The ability to experimentally manipulate current and future environmental conditions of marine systems

is becoming increasingly relevant as projections for elevated atmospheric CO<sub>2</sub> and oceanic temperatures suggest that these perturbations to the natural system stand to impact the marine environment for centuries to come.

### Comments and recommendations

There are many factors to consider that affect the successful design of a CO<sub>2</sub> delivery system for a particular experimental condition. First, and most important, is the determination of the volume of treated seawater needed to meet the biological needs of the experimental organism. From this volume, it is possible to specify the equilibration apparatus required by altering the scale of the components of a design similar to that reported here, adding multiple equilibration units, or shifting to a different equilibration technology, such as membrane gas contactors. The size of the chosen equilibration apparatus, in combination with how efficiently the gas is mixed with seawater, will then dictate the volume of experimental treatment gas mixtures needed, setting the design criteria for the gas production components (e.g., MFCs, air compressor). We have provided our MFC specifics as well as our gas and water flow rates to serve as a rough starting point for these designs, although we would recommend scaling up the MFCs in the system we describe here to accommodate additional gas production for filling of the culture vessel headspaces. We recommend carefully evaluating your gas needs to ensure that your MFCs are of the proper scale. Undersized MFCs will limit the size of your experiments, while oversized MFCs may increase operational complexity and expense as well as reduce the precision of gas metering if not operated at full scale. In general, however, it is better to have more capacity than less.

We used a continually changing supply of seawater for the duration of our experiments. This flow-through design choice can present a challenge as the seawater source water may enter the system with variable water chemistry. This may be of particular concern in areas prone to upwelling or due to changes in the biological activity in the source pipes. Through continual monitoring of our seawater chemistry, we are able to ensure that our gas-mixing system overwhelmed fluctuations in the pCO<sub>2</sub> of the incoming seawater. In fact, on day 3 of our experiment, the incoming pCO<sub>2</sub> of our FSW dropped by approximately 150 ppm, but the effect of this change was largely smoothed out by our CO<sub>2</sub> equilibration methods (see Figs. 4 and 5). It would also be possible to modify this CO<sub>2</sub> delivery system to draw water from a single seawater sample stored at the start of an experiment if resources were available to handle these potentially large volumes of seawater.

The strength of the system that we describe is that our gas-mixing and CO<sub>2</sub> delivery system is matched with a methodology for performing seawater chemistry on site, as larval development progresses. This latter point is particularly important for our experiments because rapid return of seawater chemical parameters provides confidence about the state

and stability of the CO<sub>2</sub> concentrations throughout larval development. The issue of performing 'in-house' chemistry is also significant because the costs and time required for service laboratories to perform analysis of the multiple water samples produced from well-designed experiments quickly becomes cost prohibitive, particularly in studies that feature a significant time-course component. In addition, the gas-mixing, CO<sub>2</sub> delivery, and water chemistry analysis systems that we describe are portable and can be easily transported: a feature that allows ocean acidification experiments to be performed at marine field stations, remote field locations, or at numerous points across a species' biogeographic range. We emphasize, however, that performing 'in-house' seawater chemistry also requires a stringent 'in-house' quality control system to ensure the accuracy of our measurements. Finally, this methodology supports the movement in the ocean acidification research community to converge on similar techniques (OCB Subcommittee on Ocean Acidification 2009; NRC Report 2010; Riebesell et al. 2010), especially with regard to the seawater chemistry, to facilitate the collection of comparative data sets across research groups.

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