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Controlled experimental aquarium system for multi-stressor investigation: carbonate chemistry, oxygen saturation, and temperature

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Abstract

As the field of ocean acidification has grown, researchers have increasingly turned to laboratory experiments to understand the impacts of increased CO_2 on marine organisms. However, other changes such as ocean warming and deoxygenation are occurring concurrently with the increasing CO_2 concentrations, complicating the anthropogenic impact on organisms. This experimental aquarium design allows for independent regulation of CO_2 concentration, O_2 levels, and temperature in a controlled environment to study the impacts of multiple stressors. The system has the flexibility for a wide range of treatment chemistry, seawater volumes, and study organisms. Control of the seawater chemistry is achieved by equilibration of a chosen gas mixture with seawater using a Liqui-Cel® membrane contactor. Included as examples, two experiments performed using the system have shown control of CO_2 between approximately 500–1400 µatm and O_2 from 80–240 µmol kg^{-1}. Temperature has been maintained to 0.5 °C or better in the range of 10–17 °C. On a weeklong timescale, control results in variability in pH of less than 0.007 pH units and in oxygen concentration less than 3.5 µmol kg^{-1}. Longer experiments, over a month, have been completed with reasonable but lessened control, still better than 0.08 pH units and 13 µmol kg^{-1} O_2. The ability to study the impacts of multiple stressors in the laboratory simultaneously, as well as independently, will be an important part of understanding the response of marine organisms to a high-CO_2 world.

1 Introduction

Due to increasing anthropogenic carbon dioxide in the atmosphere, the amount of inorganic carbon in the oceans is increasing and the pH decreasing, a process commonly known as ocean acidification (Caldeira and Wickett, 2003). In the past decade, ocean acidification has received increasing attention from the scientific community, particularly the impact of the expected changes in carbonate chemistry on organisms (Doney et al., 2009a). Experimental studies in the laboratory are an important part of this research (Doney et al., 2009b). While significant insight has been gained from these laboratory experiments, it has also become clear that the questions are not simple to answer, and that reasonable control of the experimental conditions can be challenging. In 2010 EPOCA published a *Guide to best practices for ocean acidification research and data reporting* (Riebesell et al., 2010) which includes several relevant chapters for setting up experimental aquaria with the intent to control carbonate chemistry. Various options for modifying carbonate chemistry are suggested, including bubbling or mixing high-CO_2 water, which changes dissolved inorganic carbon (DIC) at constant total alkalinity (A_T), or adding acids and bases, or carbonate and bicarbonate, potentially changing both A_T and DIC. Some discussion has surrounded these various methods, and the differences in carbonate chemistry have been evaluated and found to be small (Schulz et al., 2009). Despite this, bubbling is recommended as the "first choice" because it "exactly mimics carbonate chemistry changes occurring in the years to come" (Gattuso et al., 2010). However, bubbling can lead to difficulties in sustaining phytoplankton cultures (Shi et al., 2009), so often header tanks are used for equilibration to eliminate the impact of bubbling on the experimental organism.

A variety of experimental systems have been designed and used, with varying degrees of success, by researchers interested in control over carbonate chemistry in the laboratory. Several experiments have been performed by bubbling seawater directly with gas mixtures created by combining pure CO_2 with ambient or CO_2-stripped air to create an elevated partial pressure of CO_2 ($p(CO_2)$) (Miller et al., 2009; Talmage and Gobler, 2009). One published system bubbles a custom gas mixture while monitoring $p(CO_2)$ (Fangue et al., 2010), and another uses acid additions to create constant pH seawater as determined by spectrophotometric measurements (McGraw et al., 2010). Although the field has come a long way in a short time, gaps in knowledge and in execution of some studies still exist (Gattuso and Lavigne, 2009). Most published methods manipulate small volumes of water, limiting experimental organism biomass. They also generally modify header tanks that then supply seawater to the culture vessels, rather than direct modification. Use of a more precise, reliable, and flexible experimental aquarium design, such as that presented here, should help to elucidate and confirm some of the biological responses to ocean acidification.

In addition to changes in the carbon parameters, there are other expected impacts of anthropogenic climate change on the ocean. Temperatures in the upper 300 m of the oceans are rising (Lyman et al., 2010), and changes in stratification have occurred (Palacios et al., 2004). Oxygen saturation is expected to decrease as the oceans warm, stratify, and absorb carbon (Keeling et al., 2010; Shaffer et al., 2009). Predictions of the consequences of ocean acidification must consider synergistic effects between changing inorganic carbon parameters and changes to these other variables. Multi-stressor, or multi-variable, interactions have gained a lot of attention recently as researchers have begun to examine the many simultaneous impacts that climate change will have on organisms (Boyd, 2011; Pörtner et al., 2005). However, a review of marine climate change papers found that most were single-factor experiments, most often focusing on acidification alone (Wernberg et al., 2012). In particular, the need for experiments focusing on the combination of CO_2 and O_2 has been identified (Melzner et al., 2012; Pörtner et al., 2005). Oxygen and pH are strongly, positively correlated in systems dominated by photosynthesis and respiration, as has been documented recently in coastal upwelling systems (Frieder et al., 2012; Paulmier et al., 2011). The implications for organisms simultaneously experiencing low pH and low oxygen seawater are only just starting to be investigated. The ability to modify CO_2 and O_2 levels independently in an experimental laboratory, in addition to temperature, will be critical to understanding the response of organisms that live in natural environments with these multiple stressors.

Presented here is the Multiple Stressor Experimental Aquarium at Scripps (MSEAS) designed to address some of the challenges facing the scientific community regarding ocean acidification research and to facilitate the study of organisms under future ocean scenarios. The aim of this study was to develop a system capable of independent manipulation and control of the inorganic carbon chemistry, oxygen levels, and temperature of the seawater in each tank. Additionally, the system is designed with flexibility so that it may be adapted for a variety of marine organisms and life stages. Finally, this study demonstrates the stability and accuracy of the system by presenting data from two separate experiments.

2 Methods

2.1 Carbon parameter control

Achieving good control in an experimental laboratory setting is difficult, in part because the carbonate chemistry is complicated. Factors affecting control of the carbonate chemistry include gas exchange, temperature influences, and direct modification by the study organisms through processes such as photosynthesis, respiration, and calcification. Another obstacle is that measurement of the carbon parameters is neither simple nor inexpensive (Dickson et al., 2007; Dickson, 2010). The acid-base chemistry in clean seawater can be described using known equilibrium constants given salinity, temperature, pressure and the total boron/salinity ratio, together with two other measured parameters (typically A_T DIC, $p(CO_2)$, and/or pH). As only two parameters are needed to characterize the system in an aquarium setting, controlling two of these parameters will result in complete control over the carbonate chemistry.

Controlling two parameters at once is not accomplished easily. However, by assuming a constant A_T only a single parameter needs to be controlled continually (Dickson, 2010). Constant A_T is a reasonable assumption for the seawater system that supplies MSEAS, thus achieving control of one carbon parameter (Fig. 1). Over a three-year period, the observed range of A_T for the seawater system was 50 µmol kg^{-1} This would change pH calculated from A_T and constant $p(CO_2)$ by less than 0.01 pH units. More often A_T is similar from one day to the next, showing large changes over months, rather than days. Consequently the assumption of constant A_T would only lead to small errors in the understanding of the carbonate chemistry when paired with controlled $p(CO_2)$, and thus serves as a reasonable assumption for this experimental system.

However, some care must be taken to ensure that the assumption of constant A_T remains true in an aquarium setting. Organisms continually modify the chemistry of their environment. For A_T this usually means calcification or the assimilation and remineralization of other nutrients and ions (for further discussion see Wolf-Gladrow et al., 2007). To maintain A_T levels throughout the duration of the experiment, some action must be taken to counteract these modifications. The most straightforward solution is the addition of new seawater to the tanks, which replenishes A_T. Sufficient overturning, depending on the organism and biomass in the aquaria, will guarantee that the A_T in each tank reflects the assumed constant A_T of the seawater system.

Alkalinity is particularly useful as a control variable for carbonate chemistry in an ocean acidification experimental laboratory because it is not affected by changes in temperature and is conservative with respect to mixing. Most importantly, the addition or removal of CO_2 (and O_2) gas from seawater does not change A_T. This allows for modification of the total amount of CO_2 in the seawater to control a second parameter, without invalidating the assumption of constant A_T. In this setup, the second parameter is controlled by reacting a gas of a particular CO_2 and O_2 content with seawater in a way that allows a desired $p(CO_2)$ and oxygen percent saturation to be achieved. This equilibration of a known gas with seawater has the same resulting carbonate chemistry as if bubbling had been used to modify the seawater sample.

2.2 Apparatus

To achieve the desired chemistry in MSEAS, a gas mixture is equilibrated with seawater using a Membrana Liqui-Cel® 2.5 × 8 Extra-Flow membrane contactor with × 50 fiber for each aquarium (Fig. 2). The desired gas composition (N_2, O_2, CO_2) is mixed from individual gas cylinders using Omega® mass flow controllers (FMA 5418 0–5 SLM, FMA 5411 0–2 SLM, and FMA 5402 0–10 sccm, respectively). The mass flow controllers are operated by a laptop running NI LabVIEW™ software with communication using a voltage generating NI 9265 4-Channel Analog Output Module™ combined with an NI USB-9162 Single Module Carrier™. Mass flow controller function is monitored using a NI USB-6210 Multifunction DAQ™ (Fig. 2). Mixing individual gases gives the user complete control over both the CO_2 and O_2 concentrations. After the three gases mix in the desired proportions, the line is split, providing an identical gas mixture to two or more replicates. Currently the system is designed with two sets of three mass flow controllers, allowing for two treatment levels. A submerged MARINELAND® Maxi-Jet 400 Power Head pumps seawater from each treatment tank through a 5 µm filter and then through the tank's associated Liqui-Cel membrane contactor. The gas mixture is introduced to the Liqui-Cel in the opposite direction, maximizing equilibration. The newly equilibrated seawater is returned to the corresponding treatment tank and the gas flows to waste (Fig. 2). It is the continuous recirculation and equilibration of the treatment seawater that enables such precise control of the CO_2 and O_2 levels. Temperature of each tank is maintained with a titanium coil through which temperature-controlled water flows from a Thermo Scientific NESLAB™ RTE 7 Refrigerated Bath.

The system design allows the seawater in each aquarium to be exchanged in a flow-through mode, where raw seawater is continuously added at a slow rate, replenishing nutrients, and maintaining A_T levels. The resultant increased volume of seawater overflows, removing organism waste. This rate of overturning must be optimized, and will be organism and biomass dependent. The system can also be run as a closed system, with no input of seawater, although changes in A_T quickly become a concern.

Although the system was originally designed with a large, 50 L tank in mind, the size of the treatment tank is easily exchangeable. Several of the experiments performed have used much smaller volumes, to fit the experimental organism better, and to maximize control over the chosen parameters. The size of the tank, and therefore the volume of water needing to be equilibrated, must be chosen for each experimental organism. Smaller volumes of water will recirculate through the membrane and equilibrate with the gas more often, leading to better control.

Equilibration between the gas and seawater using the Liqui-Cel is done separately for each replicate, so there is no mixing of treated seawater: each tank is modified directly and independently. The only shared part of any replicate is the gas composition and the original source water, eliminating many of the pseudoreplication problems that continue to plague other ocean acidification aquarium experimental designs (Wernberg et al., 2012; Havenhand et al., 2010). In addition, because of the independent equilibration for each replicate, there is no concern over inference of the carbonate chemistry based on measurements made in a header tank.

One of the main advantages of MSEAS, is that it allows the user to choose any desired CO_2 and O_2 composition, within a large range. The CO_2 level of the gas mixture can be chosen by the user to be any value between 0 and 5000 ppm, although the maximum $p(CO_2)$ of any experiment performed to date is approximately 1500 µatm. Barry et al. (2010) includes suggestions of CO_2 levels for ocean acidification laboratory experiments; the recommendation for a two treatment system is for one treatment near a “present-day” atmospheric value of 385 ppm and the other at a “future” value of 750 ppm. MSEAS is well suited to perform experiments at these specified values, but also has the advantage of flexibility in terms of its target CO_2. This is especially valuable, in that it easily allows investigation of environments that are not at equilibrium with the atmosphere. For example, the coastal region of western North America experiences upwelling events, in which seawater already eleveated in CO_2 flows onto parts of the continental shelf (Feely et al., 2008). For studying impacts of anthropogenic ocean acidification on organsims living in elevated CO_2 environments such as this, an incremental increase to the known elevated value is recommended, using increases similar to those between the original recommended treatment values (385 and 750 ppm) (Barry et al., 2010). This is easily accomplished using the described aquarium system.

3 Assessment and discussion

The usefulness and capability of this system is exhibited by the experiments performed to date. Two of these experiments are described below as examples of the stability that can be maintained – one a week long, the other lasting longer than a month. Both experiments modified the CO_2 and O_2 of the seawater for each treatment and were performed at different temperatures. Discrete samples for A_T, pH, and O_2 were taken daily during the experiments and analyzed at SIO. A_T samples were poisoned with saturated mercuric chloride and stored for later analysis which was done by open-cell titration. Discrete pH samples were analyzed spectrophotometrically on the same day as sampling. Values are reported at the in situ temperature and on the total pH scale. Discrete oxygen samples were pickled immediately and analyzed a few days later by Winkler titration. Temperature was monitored every five minutes in all tanks by HOBO Pendant® Temperature/Light Data Loggers.

3.1 Experiment M7

A week-long experiment was performed on mussel larvae (*Mytilus gallorprovincialis*) with the described system – one treatment of "ambient" pH and oxygen levels, typical of a California coastal upwelling environment, and the other with low pH and oxygen levels. For this experiment, the treatment tanks were round 7.5 L buckets with lids. The larvae were protected from the flowing seawater recirculation by containment in a smaller nested bucket that freely exchanged seawater with the main tank. All treatments were held at an average temperature of 17.2 °C. Alkalinity varied only slightly over the week, and consequent control of pH and oxygen levels were very good (Table 1 & Fig. 3).

In addition to the discrete sampling, a Honeywell Durafet® pH sensor was used continuously throughout the experiment to monitor the carbonate chemistry on short timescales, switching between tanks daily. The Durafet sensor data in Fig. 4 shows day-long variability in pH beginning each day when sensor's location was changed. Four consecutive examples are given, one from each tank. Some fluctuation is seen, possibly a respiration signal. This fluctuation is possible due to the somewhat passive approach to controlling the carbonate chemistry in the current system design. The constant composition gas mixture is supplied to each Liqui-Cel, but there is no way to measure and counteract the influence of outside factors on the carbonate chemistry of the seawater. Fluctuations such as those seen in Fig. 4 could be dampened if a more active approach to pH control was taken, using information gathered by chemical sensors in the tanks as feedback signals to the gas mixing system. Durafet pH sensors are well suited to monitor the carbonate chemistry, and for oxygen, an optode that measures in real time would be appropriate. Based on the information from these sensors, the gas composition supplied to the Liqui-Cels could be continually adjusted to compensate for any divergences from the desired seawater pH and oxygen levels. The adjustments to the gas mixture would help dampen or eliminate the small diurnal signal seen, but would also eliminate any large changes in seawater chemistry that might happen over the course of the experiment.

3.2 Experiment S32

The system can be used on much longer time scales than a week, demonstrated by a 32-day experiment investigating the impacts of varied pH and oxygen levels on squid embryos (*Doryteuthis opalescens*). The experiment was performed in square 50 L insulated tanks, with the squid egg capsules attached to the bottom. Some turbulence was caused by the recirculation of the seawater for equilibration. Target pH and oxygen levels were chosen based on values recorded at a location near Scripps (Nam et al., 2011). For this experiment, low pH and high oxygen levels were paired in one treatment, and high pH and low oxygen levels in the other. This is in contrast to experiment M7 which paired low oxygen and low pH, and demonstrates the system flexibility and independent control of chosen seawater chemistry. The longer duration of this experiment reflects a growing need in the scientific community to understand the effects of chronic exposure to low pH on organisms. Results from discrete samples indicate adequate control for a successful biological experiment, even over this extended period (Table 2 & Fig. 5). However, there are clear discrepancies from target values and both gradual and abrupt changes during the experiment, some of which are easily explained.

The control of the seawater chemistry in this system is based on the mole fraction of CO_2 and O_2 in the gas that is supplied to the Liqui-Cel for equilibration. Any changes in that mole fraction will be apparent in the resulting seawater chemistry. Throughout experiment S32 several deliberate changes were made to the control parameters: on 11 March 2012, the amount of oxygen in the gas mixture was increased from 5.4 % to 6.9 % of the total gas flow in Treatment A, and from 19.7 % to 20.1 % in Treatment B. The subsequent increase in the dissolved oxygen content of the seawater on that day is apparent in Fig. 5. Similarly, the CO_2 fraction in the gas of Treatment B was increased from 1500 ppm to 1600 ppm during the experiment, likely causing the decrease in pH seen. The abrupt increase in seawater temperature of all tanks beginning 23 March 2012 results from an increase in the temperature setting of the thermostat baths. These changes are reflected in Table 2 by the much larger standard deviations for pH, oxygen level, and temperature than were observed during experiment M7. The reason for the significant decrease in pH in only Treatment A Replicate 2 on 24 March 2012, which is then maintained the rest of the experiment, is unknown.

Likely the easiest way to improve the consistency of pH and oxygen levels in this system would be to improve the seawater temperature control. In both example experiments, the temperature of the treatment seawater in the tanks was influenced by the room air temperature, which was strongly influenced by the San Diego weather. This effect was much stronger in S32, with a daily seawater temperature cycle of approximately 0.5 °C occurring throughout most of the experiment. This likely contributed to the lesser control over the carbonate chemistry compared to experiment M7. These temperature changes strongly influence the carbonate parameters: a 1 °C change in temperature causes about a 0.015 change in pH and a 20–50 µ atm change in $p(CO_2)$. Between experiments S32 and M7 the location of MSEAS was moved from Birch Aquarium's OAR Building, which is completely un-insulated, to the Experimental Aquarium in Kaplan Laboratory at SIO, which is more protected. This change in location likely accounted for the difference in seawater temperature control, as the variability in the room temperature is much less in Kaplan compared to OAR.

4 Conclusions

MSEAS will be useful to help elucidate responses of organisms to expected future ocean scenarios, which involve changes to multiple physical and chemical parameters. The system design allows for manipulation of any one or multiple of the three control parameters: CO_2 concentration, O_2 levels, and temperature. This independent control is a potentially useful experimental approach to investigating mechanisms underlying organismal responses. The automated prototype presented here is easily scalable to larger numbers of replicates by splitting the gas line (ensuring adequate gas flow) and adding Liqui-Cels for each tank. Control of additional treatments requires more mass flow controllers for creation of a separate gas composition, in addition to Liqui-Cels and tanks.

The use of Liqui-Cel membrane contactors in the system design allows for rapid equilibration between the gas and seawater. Equilibration by bubbling can be quite slow depending on the volume of seawater needed (Schulz et al., 2009). Membrane contactors eliminate any concern over the direct impacts of bubbling on the experimental organism, and the active equilibration allows for longer well-controlled experiments. Liqui-Cels make the system well suited to convert into a setup with feedback from chemical sensors in the aquaria, achieving a more active control over the carbonate chemistry. They also allow for the transition of the system to one with intentional variability in the three controlled parameters, whether weekly, daily, or tidal time scales of variability. The fast equilibration of the Liqui-Cels, as well as the direct modification of the treatment seawater, makes this system well suited for this task. There is a growing need to understand the responses of organisms that live in variable environments and how those responses may change in the future (Andersson and Mackenzie, 2012; Dufault et al., 2012).

MSEAS has been used successfully to study several organisms, life stages, and parameters. The flexibility of the system design has allowed for experimental organisms ranging from mussel larvae to juvenile abalone to adult oysters. Experiments on moon jellies have been completed modifying only the oxygen levels in the seawater. Inversely, in another experiment O_2 levels were held steady between treatments while CO_2 concentration and temperature were modified. Combined with experiments M7 and S32, these examples show the large range of possible biological questions that can be examined using MSEAS, to expose a variety of species to future ocean conditions.

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Table 1. Average ± standard deviation for chemical parameters during experiment M7. The $p(CO_2)$, $\Omega_{calcite}$, and $\Omega_{aragonite}$ reported here were calculated using CO2calc (Robbins et al., 2010) with dissociation constants from Mehrbach et al. (1973) as refit by Dickson and Millero (1987). For most values $n = 8$, except for oxygen for which some samples from each tank were lost (see Fig. 3).

	Temp (°C)	Salinity	Alkalinity ($\mu mol\,kg^{-1}$)	$pH_{(in\text{-}situ)}$ Total Scale	Oxygen ($\mu mol\,kg^{-1}$)	Calc $p(CO_2)$ (μatm)	Calculated $\Omega_{Calcite}$	Calculated $\Omega_{Aragonite}$
Treatment A								
Replicate 1	17.2 ± 0.3	33.65 ± 0.01	2249.3 ± 6.1	7.924 ± 0.004	230.9 ± 2.2	546.1 ± 5.8	3.09 ± 0.03	1.99 ± 0.02
Replicate 2	17.2 ± 0.2	33.64 ± 0.01	2250.4 ± 5.9	7.905 ± 0.007	227.7 ± 1.3	574.6 ± 9.5	2.96 ± 0.05	1.91 ± 0.03
Treatment B								
Replicate 1	17.0 ± 0.4	33.66 ± 0.01	2254.1 ± 7.0	7.619 ± 0.007	86.2 ± 3.4	1188.4 ± 17.7	1.64 ± 0.03	1.05 ± 0.02
Replicate 2	17.2 ± 0.3	33.65 ± 0.01	2250.5 ± 4.0	7.612 ± 0.005	83.9 ± 2.7	1209.8 ± 15.8	1.62 ± 0.02	1.04 ± 0.01

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Table 2. Average ± standard deviation for chemical parameters during experiment S32. The $p(CO_2)$, $\Omega_{Calcite}$, and $\Omega_{Aragonite}$ reported here were calculated using CO2calc (Robbins et al., 2010) with dissociation constants from Merbach et al. (1973) as refit by Dickson and Millero (1987). For most values $n = 29–32$, except for oxygen for which some samples from each tank were lost (see Fig. 5).

	Temp (°C)	Salinity	Alkalinity (μmol kg^{-1})	$pH_{(in\text{-}situ)}$ Total Scale	Oxygen (μmol kg^{-1})	Calc $p(CO_2)$ (μatm)	Calculated $\Omega_{Calcite}$	Calculated $\Omega_{Aragonite}$
Treatment A								
Replicate 1	11.2 ± 0.5	33.50 ± 0.07	2239.1 ± 5.5	7.923 ± 0.035	86.4 ± 8.3	540.7 ± 48.7	2.49 ± 0.15	1.58 ± 0.10
Replicate 2	11.6 ± 0.5	33.51 ± 0.05	2241.8 ± 4.5	7.908 ± 0.072	83.0 ± 12.9	570.4 ± 107.9	2.46 ± 0.32	1.57 ± 0.21
Treatment B								
Replicate 1	11.3 ± 0.5	33.49 ± 0.07	2241.1 ± 5.8	7.559 ± 0.029	241.1 ± 9.1	1337.3 ± 97.4	1.15 ± 0.06	0.73 ± 0.04
Replicate 2	11.6 ± 0.6	33.51 ± 0.06	2244.2 ± 7.1	7.552 ± 0.026	241.7 ± 7.6	1364.2 ± 88.1	1.15 ± 0.06	0.73 ± 0.04

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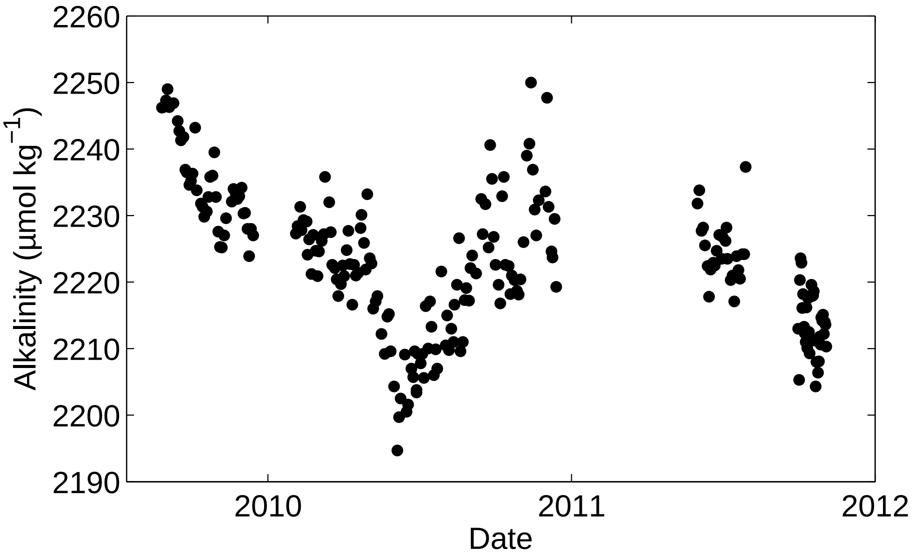

Fig. 1. Measured total alkalinity (µmol kg^{-1}) in the Scripps Institution of Oceanography seawater system over several years. Discrete samples were poisoned for later analysis. The mean is 2223 and the standard deviation is 11 µmol kg^{-1}

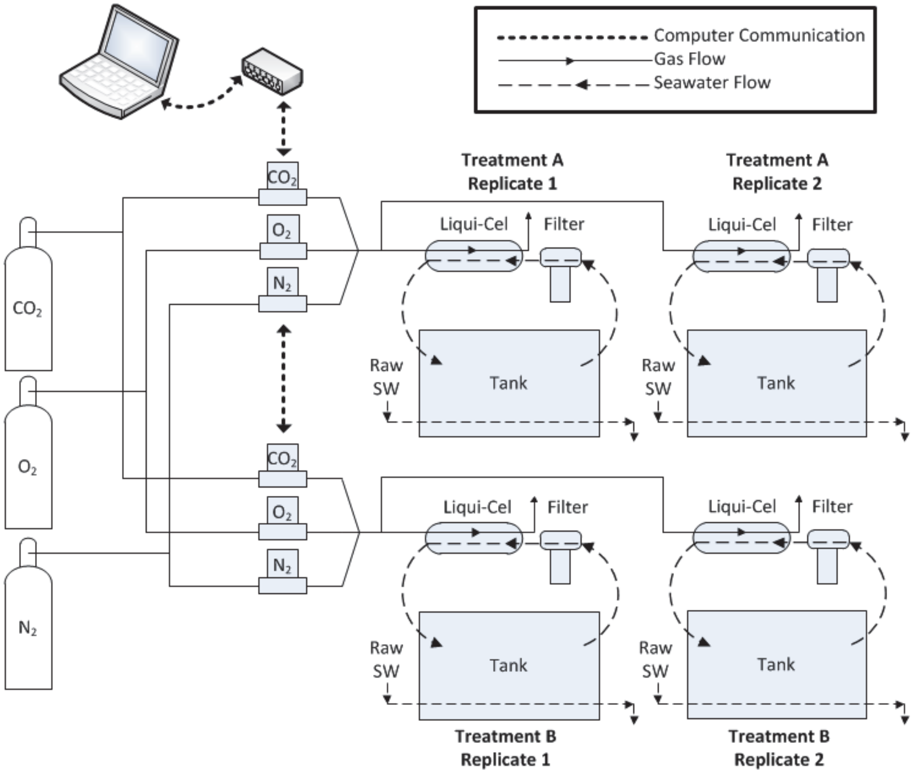

Fig. 2. Schematic of aquarium control system. Solid lines indicate gas tubing and flow, and thin dashed lines indicate seawater tubing and flow. Thick dashed lines represent data communication between instruments.

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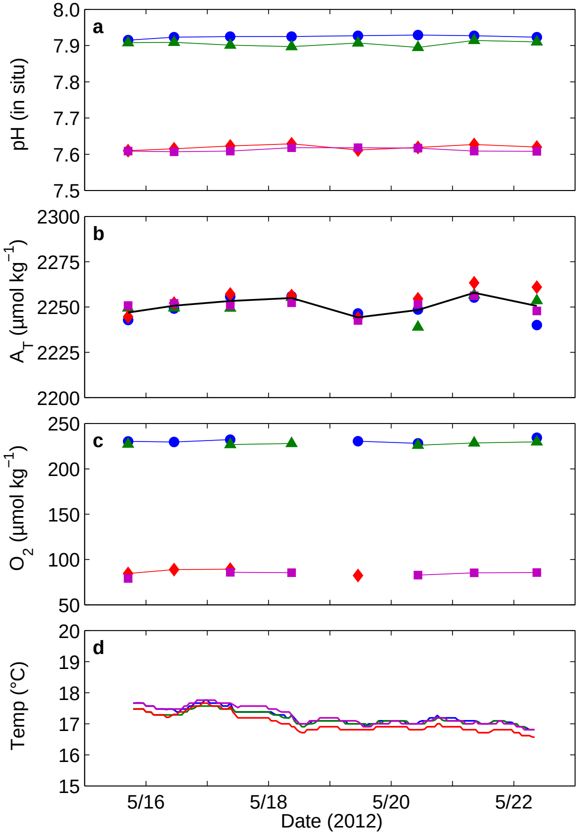

Fig. 3. Measured data from experiment M7. **(a)** pH; total pH scale, **(b)** A_T; the solid black line connects the daily average for all four tanks, **(c)** oxygen, and **(d)** temperature. Symbols indicate discrete samples: Treatment A Replicate 1 (blue circles), Treatment A Replicate 2 (green triangles), Treatment B Replicate 1 (red diamonds), and Treatment B Replicate 2 (purple squares).

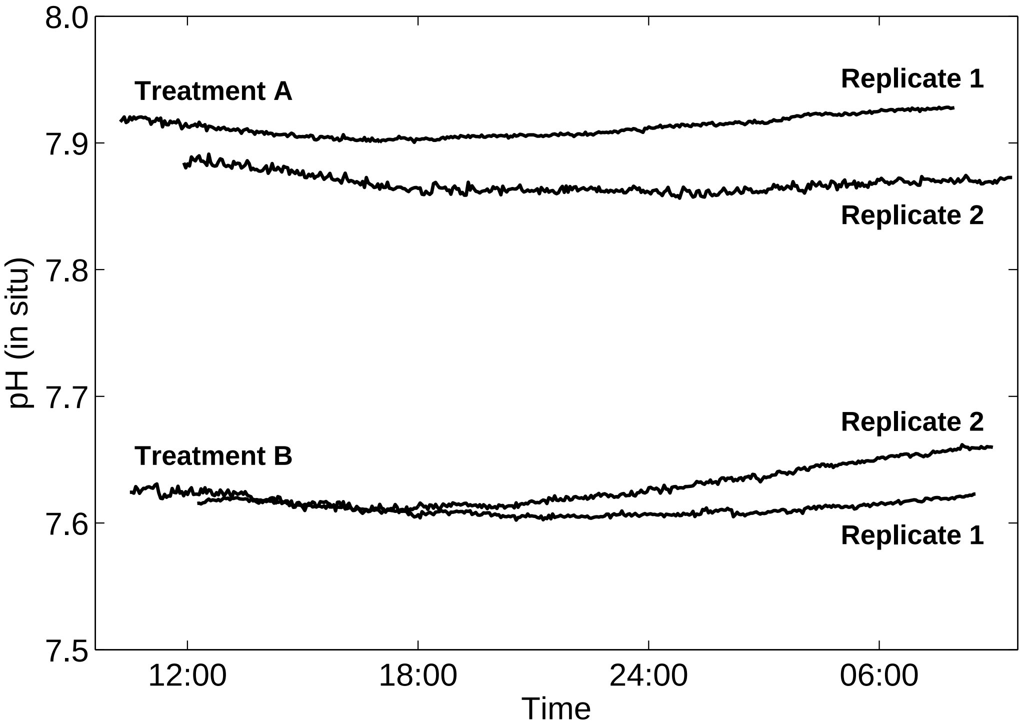

Fig. 4. pH recorded by a single Durafet sensor. The sensor was used to monitor each of the four tanks on four consecutive days. Data begins and ends at the time the sensor was moved each day, allowing for sensor equilibration once placed in the tank.

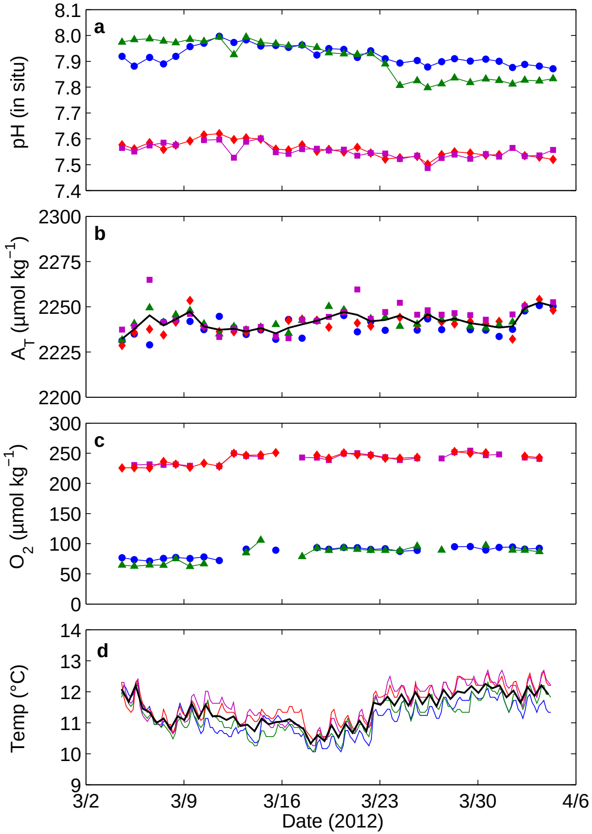

Fig. 5. Measured data from experiment S32. **(a)** pH; total pH scale, **(b)** A_T; the solid black line connects the daily average for all four tanks, **(c)** oxygen and **(d)** temperature; the thick black line is the average of all tanks calculated at 12-h intervals. Symbols indicate discrete samples: Treatment A Replicate 1 (blue circles), Treatment A Replicate 2 (green triangles), Treatment B Replicate 1 (red diamonds), and Treatment B Replicate 2 (purple squares).