An automated monitoring and control system for flow-through co-cycling hypoxia and pH experiments

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Abstract
Acidification research has exploded in recent years, however, experiments testing effects of co-cycling hypoxia and pH on ecological and physiological processes are rare, despite the pervasiveness and potential importance of co-varying fluctuations in these parameters. Co-cycling dissolved oxygen (DO) and pH are difficult to precisely control, as gases used for manipulation influence both parameters. We successfully developed a LabVIEW™-based system capable of monitoring and controlling co-varying DO and pH in raw seawater flow-through aquaria. Using feedback from Oxyguard DO probes and Honeywell ion sensitive field effect transistor Durafet pH sensors, our system controls ratios of nitrogen, oxygen, carbon dioxide, atmospheric air, and CO2-stripped air within a total gas flow rate through mass flow controllers, to achieve target co-cycling DO and pH values in five treatments. Our system performed well in two long-term experiments investigating effects of diel-cycling hypoxia and pH on eastern oyster (Crassostrea virginica) feeding, growth, fecundity, Perkinsus sp. (Dermo) infection dynamics and immune response. In our 2013 adult oyster experiment, the severe low DO treatment averaged only 0.04 mg L\(^{-1}\) higher than the 0.50 mg L\(^{-1}\) target, and the moderate hypoxia averaged only 0.05 mg L\(^{-1}\) higher than the 1.30 mg L\(^{-1}\) target over 48 d of cycles. Mean pH for the hypercapnia plateau was within 0.02 above the 7.00 target. In our 2013 spat experiment, daily minimum DO in the severe and moderate hypoxia treatments were both within 0.06 mg L\(^{-1}\) of the 0.50 and 1.3 mg L\(^{-1}\) targets, respectively; hypercapnia plateau pH values were within 0.01 of our 7.00 target.

There is increasing interest in the combined effects of hypoxia (low dissolved oxygen [DO] concentrations) and acidification of estuaries and coastal waters where anthropogenic CO2 emissions and nutrient loads potentially exacerbate natural diel, tidal, and seasonal cycles of DO and pH (Doney et al. 2009a,b; Duarte et al. 2013; Melzner et al. 2013). In shallow estuarine systems, the magnitude of diel fluctuations can be very large; DO can range from anoxia to supersaturation, and pH can vary by a full pH unit or more (Fig. 1), as a result of diel variation in the balance between photosynthesis and respiration (Nixon and Oviatt 1973; Hackney et al. 1976; D’Avanzo and Kremer 1994; Tyler et al. 2009; Wallace et al. 2014). Wind, current, and tidally-driven upwelling, incursions, and advections can also cause rapidly changing but relatively short-term variation in DO and pH (Feely et al. 2010; Booth et al. 2012). When coupled with diel variation, even simple tidal mixing can modulate DO and pH conditions or cause more extreme cycles, depending on such factors as season, adjacent local conditions, and timing, stage, and scale of the tidal signal (Baumann et al. 2015). Although research on ocean acidification (OA) has exploded in recent years (Wernberg et al. 2012), and various forms of hypoxia have been studied for decades (Diaz 2001; Gray and Ying 2002), experimental examinations of co-cycling hypoxia and pH and the effects these co-varying environmental factors may have on ecological and physiological processes are rare (e.g., Bogue 2013; Frieder et al. 2014; Gobler et al. 2014). In contrast, there is a rich literature database examining the effects of constant hypoxia—and more recently, constant hypercapnia—on biological, physiological, and resultant ecological processes (e.g., Breitburg et al. 2009; Levin et al. 2009; Melzner et al. 2013).

Co-cycling DO and pH are difficult to precisely control simultaneously in experimental systems because these parameters are tightly coupled and the gases used to alter in-water DO and pH influence each other during manipulations. For example, bubbling with nitrogen to sparge oxygen from the water to lower DO also displaces CO2, causing pH to rise. To examine effects of cycling OA, several systems have been developed to perform controlled experiments, including a

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gas-seawater equilibration system named MSEAS: the Multiple Stressor Experimental Aquarium at Scripps (Bockmon et al. 2013), the Northwest Fisheries Science Center (NWFSC) OA facility (NOAA 2010), a closed-recirculating system at University of Delaware (Bogue 2013) adapted from an earlier DO-controlling apparatus (Grecay and Stierhoff 2002) and a gas-controlled aquarium system (Barry et al. 2008). Many of the previously developed experimental systems have been aimed at experimental control in small-volume containers (especially larval and zooplankton studies, e.g., McGraw et al. 2010), constant hypoxia and/or hypercapnia (e.g., Fangue et al. 2010), or under a recirculating system with little or no flow-through seawater (Grecay and Stierhoff 2002). Such systems are not suitable for experiments involving high numbers of large filter-feeding organisms, such as oysters, that require either a high flow of raw seawater or a substantial quantity of supplemental food stock (e.g., algae).

Our laboratory at Smithsonian Environmental Research Center (SERC) in Edgewater, Maryland, U.S.A. was interested in how diel-cycling hypoxia and hypercapnia affect eastern oyster mortality, feeding, growth and Perkinsus sp. (a protistan parasite causing Dermo disease) acquisition and progression (Clark 2014; Keppel 2014; Keppel et al. 2015). We successfully developed a custom LabVIEW™-based system capable of monitoring and controlling five separate treatments of diel-cycling fluctuations in 30 high through-put flow-through 75 L aquaria. The system maintains a constant total gas flow rate (TFR), and varies ratios of nitrogen (N₂), oxygen (O₂), carbon dioxide (CO₂), atmospheric air, and CO₂-stripped air through mass flow controllers (MFCs) to achieve target DO and pH values on a diel cycle. The gas bubbling approach that we employed is one of the preferred methods of pH manipulation; it has been reported to very closely mimic ongoing and future changes in the seawater carbonate chemistry, and offers precise control over extended time periods (Gattuso and Lavigne 2009). Our system was named “DOOM” Treatment Control; DOOM stands for Dissolved Oxygen Oyster Mortality, and is a legacy acronym referring to the original experiments and endpoint we measured in our earlier experiments (Breitburg et al. 2015). In this article, we provide the LabVIEW program (Supporting Information) as well as a description of the system and its performance.

Materials and procedures

Software and hardware

We developed a custom National Instruments (NI) LabVIEW™ (National Instruments Corp., Austin, Texas, U.S.A.) software-based system to monitor and control DO and pH (Supporting Information). We wrote the logic and controlling equations, and consulted a programmer (J. Jariwala, Applied Control Engineering, Owings Mills, Maryland, U.S.A.) to code the written logic for LabVIEW™ (a powerful graphical programming platform) and create a user-interface based, in part, on his work on Targett’s cycling control system (Bogue 2013). The advantage of LabVIEW™ software, besides the ability to integrate with many kinds of equipment, is that all treatments can be monitored and manipulated concurrently instead of in sequence. The LabVIEW™ software interfaces with an NI cDAQ-9178 CompactDAQ chassis (eight slot USB) fitted with seven NI boards: NI9215 (n = 1), NI9265 (n = 4), and NI9472 (n = 2). The NI boards

![Fig. 1. Examples of two different types of cycling DO and pH conditions, shading indicates nighttime, (a) diel cycling DO and pH in the Honga River, MD during 1 week in 2008. Data from Maryland Department of Natural Resources (MD-DNR) Shallow Water Monitoring Program, eyesonthebay.net, and (b) tidally-influenced cycling DO and pH recorded outside the Kirkpatrick marsh of SERC’s Global Change Research Wetland on the Rhode River, MD during 1 week in 2014 (Breitburg, unpubl.).](image)
interface with three Honeywell UDA 2182 analyzer units that send pH data to the computer from six Honeywell Ion Sensitive Field Effect Transistor (ISFET) Duraflow III pH probes (Honeywell International, Morristown, New Jersey, U.S.A.). A Campbell Scientific CR1000 datalogger interprets signals from six Oxyguard Standard DO model III probes (Oxyguard International A/S, Birkeroed, Denmark) and makes data available for query by the system. The NI boards also interface with one Global Water WE100 barometer (Global Water, College Station, Texas, U.S.A.), one Instrumentation Northwest Aquistar CT2X Conductivity Smart Sensor (Instrumentation Northwest, Kirkland, Washington, U.S.A.), five two-way solenoids for emergency gas shutoff, five two-way solenoids for emergency backup air, five three-way solenoids for switching between atmospheric air and CO2-stripped air (W.W. Grainger, Lake Forest, Illinois, U.S.A.), and 15 Dakota MFCs (Dakota Instruments, Orangeburg, New York, U.S.A.). For the purposes of our experiments, we used MFCs with the following flow rate ranges for each gas type: atmospheric air/CO2-stripped air 0–40 LPM (n = 5), CO2 0–0.5 LPM (n = 4), O2 0–10 LPM (n = 3), and N2 0–40 LPM (n = 3). We only purchased MFCs to control the minimum number of gases required for each treatment in our experiments, hence the unbalanced numbers of MFCs for each gas type. Although our experiment room has an emergency backup generator, there is a short delay between a power outage and the generator startup. For this reason, our computer and apparatus hardware were fitted with backup uninterruptible power supply (UPS) units to hold equipment on while the power switches over to the emergency generator.

DO and pH manipulation

DOOM Treatment Control receives DO and pH data from probes located in one of each of the six replicate aquaria per treatment, and compares the in-tank measurements to a user-changeable spreadsheet of target DO and pH values for the current day and time. The duration of time steps for each day can be input at any scale; in the experiments presented in this article we used 30 min increments. The frequency of checking actual vs. target DO and pH is user-controllable on the Setup/Control screen (Fig. 2), as is the frequency of DO and pH adjustments. While performing instantaneous readings with the aquaria, we have not seen any lag in DO or pH readings with our sensors at the gas and water flow rates that were used. The minimum flow rates we tested were 0.1 LPM (in each aquarium) for water and a gas flow rate of 1.5 LPM; the maximum rates we tested were 1 LPM of water and gas flow rate 6 LPM per aquarium. It is possible to alter the amount of time the system waits before checking DO and pH and making any adjustments, for example, if a lag is detected and a longer wait time between adjustments is desired. As always, care must be taken that water flow and mixing in aquaria are above the minimum flow requirements for the sensors to ensure correct readings and minimum lag.

After checking actual vs. target values, DOOM controls ratios of each gas necessary to achieve the target values within a user-defined “total gas flow rate” or “TFR” (e.g., 30 LPM) using the coded logic calculations. For manipulation of DO, the system constantly calculates the current water DO saturation using the measured barometric pressure, temperature, and salinity. Spreadsheet target values for DO can be entered either as numerical values in mg L⁻¹ or as percent saturation relative to the current system-calculated temperature- and salinity-based 100% oxygen saturation value (e.g., “80% saturation” or “0.99 × saturation”). Nitrogen gas from liquid N2 dewars fitted for gas removal is used in calculated ratios with air to lower DO to hypoxic levels via displacement of oxygen. Atmospheric air is used to maintain and/or to achieve oxygen concentrations approximating normoxia (input as 99% of system calculated current 100% DO saturation), and in ratios with N2 to reach target DO concentrations below 100% saturation. Oxygen (mixed with atmospheric or CO2-stripped air) is added to attain supersaturated DO conditions. CO2 is also added during hypoxic phases to compensate for N2 displacement of CO2. For treatments requiring pH manipulation, gaseous CO2 is added to the TFR gas mix ratio to lower pH to target hypercapnic levels. A three-way solenoid valve is used to allow the system to switch between atmospheric air and CO2-stripped air. Atmospheric air is used to maintain and/or achieve normocapnia, while CO2-stripped air is used to lower pCO2, thereby raising pH.

Logic

The control logic of DOOM follows a sequential flowchart (Fig. 3): reading values, checking thresholds, and making adjustments. The instruments are read on a continual basis, and adjustments are made periodically. During the “Startup” procedure, DOOM Treatment Control initializes all user-changeable fields with the correct set of options, reads, and checks values from all setup and daily files, updates all front panel options and controls based on an input setup file, and puts the system into “safe state”—all MFCs off except air MFCs (which are automatically set to TFR), and sets all solenoid valves to their fail-safe setting (except emergency air solenoids). When the system is in “Idle” and the treatment is set to “Auto,” all alarms are reset and DOOM will not allow a “Start” until all errors are handled.

After the system is set to “Start,” it will go through the “Find Step” procedure by loading the daily steps and calculating targets from the user-changeable spreadsheets which were automatically loaded at Startup. The next task is to check whether actual DO and pH values of each treatment fall outside “Critical” alarm limits as set in the input file. If so, the critical alarm and message are sent and the system moves to “Critical Action” (see section “Alarms and Critical Actions”). The system then performs a similar check to
determine system excursions outside alarm values, and if necessary, sounds an auditory alarm and sends alarm messages. Once successfully past the “Alarm and Critical” checks, the system then determines whether enough time has passed since the last pH adjustment (this pH check time setting is user-controllable on the Setup/Control screen), and if so, it moves on to “Adjust pH”, manipulating the CO₂ MFC and Air valve for each treatment, as needed. To adjust pH, the system checks if the actual pH falls within an acceptable (user defined) range around the target pH. If it does, gas flows are not altered. If actual pH is outside the acceptable range, the system changes the gas flow rates proportionally to the difference between actual and target pH values. The system then follows with a similar protocol for DO, with “DO Check Time” determining time since last DO adjustment (again, user-changeable on the Setup/Control screen), and, if necessary, moving into “DO Adjust” and manipulating air, N₂, and O₂ MFCs as needed. The system then moves into a “Wait Interval” until the check interval time has elapsed (again, this setting can be altered on the Setup/Control screen) and then the logic begins looping through the steps until a user chooses to terminate the sequence by choosing to “Exit,” which sets all valves and MFCs to their safe states and exits the application. Adjusting the check intervals for DO and pH (e.g., 10 s and 20 s, respectively) and the wait interval (10 s) to be short is one way we achieve tight control.

Cycle phases

We designated names for each section of a simplified DO/pH cycle to assign differing logic for each phase (Fig. 4). Each treatment can be given a different cycle determined by both the desired DO and pH levels input in the target values spreadsheet, and by the cycle phase names. If desired, each daily input file can have a different cycle for each treatment, but each daily input file must have the same number of time steps. Cycles do not necessarily have to be constrained to one 24 h period, such that a cycle may take several days, or
multiple cycles may be run in 1 d. It is important to stress that the system can be set up to control a wide range of DO and pH level at cycling or constant conditions by inputting the desired targets in the spreadsheet and designating the appropriate cycle phase name. The rate of change between cycle phase plateaus (i.e., phases 2, 4, 5, and 7 in Fig. 4) can be manipulated by adjusting the target values used at each time step and number of time steps assigned to each cycle phase. Between each time step where the target DO and pH values are inputted, the system automatically calculates six “micro-targets” based on a linear regression between the previous and next targets, and updates these targets on the user interface. For example, if the input spreadsheet is set up with time steps and target values in 30 min increments, the target value changes every 5 min. As such, rate of change can be manipulated using shorter main time steps (i.e., 1 min increments vs. 30 min increments).

Target DO and pH and their durations are flexible and can be changed between or during experiments to meet research goals, or, for example, to match target pH to desired $\Omega_{\text{aragonite}}$ or $\Omega_{\text{calcite}}$ as background environmental conditions change (which may require monitoring additional parameters). The description below provides an example based on our use of the system and matches terminology in the DOOM Treatment Control program we provide (Supporting Information).

The control treatment, which maintains a constant normoxia (which we input as $0.99 \times 100\%$ saturation) and constant normocapnia (the $pCO_2$ achieved with dissolved $CO_2$ in equilibrium with the atmosphere under existing temperature, salinity, and alkalinity, but used here for pH targets set for control treatments), has all cycle phases coded as “normoxic plateau” (see Figs. 4, 5a). For the “Cycling severe hypoxia/Cycling pH” treatment (see Figs. 4, 5e), in which both DO and pH are manipulated on a diel cycle, the treatment begins at a “normoxic plateau,” after which DO and pH are decreased over 3 h to low target values (for this treatment 0.5 mg L$^{-1}$ and pH 7.0) and held at a “low plateau” for 4 h. Following the low plateau DO and pH are returned to normoxia and normocapnia over 3 h and held for a 2-h “normoxic/normocapnic” plateau, after which DO and pH levels are raised to supersaturated and a high pH value (e.g., 8.10), respectively, and held for 3 h at a “supersaturated plateau.” At the end of this phase, the DO and pH are returned to, and held at, a normoxic plateau and the cycle can then repeat. Figure 5 shows the target DO and pH cycles for each treatment used in both experiments. The plateaus were included in the cycles to compensate for potential variation among replicate tanks in the rate of decline or increase in DO and pH, although we saw very little variation among
tanks at the beginning or end of plateaus, which would seem to indicate that replicates tracked well during periods of change.

**Laboratory setup**

Our 2013 water handling and delivery system was designed to address several problems identified in pilot tests: (1) the need to deliver adequate food and water for up to 90 one-yr-old (35–70 mm) oysters per aquaria at a rate of 0.5 LPM, and (2) the fact that the $pCO_2$ of our incoming estuarine water was variable and frequently substantially higher than desired in our experimental treatments (for example, $pCO_2$ range was 2100–3134 μatm in incoming water during our 2013 adult oyster experiment). Raw Rhode River estuarine water enters the SERC wet lab experimental room from a head tank via gravity feed. The water is piped into Holding Tank 1 (HT1) located at the far end of five 567 L holding tanks (HT1–HT5) plumbed together in sequence (Fig. 6). To lower $pCO_2$, the river water is strongly bubbled with CO$_2$-stripped air using soda lime filled CO$_2$-stripping columns as it passively moves from its entry in HT1 to the final HT5; additionally, the majority of the mud and other solids settle out before reaching HT5. As the incoming water has substantially lower algal content than the Rhode River, HT5 receives

![Fig. 5. Target DO and pH cycles for each of the five treatments (a–e) we used for 2013 experiments. The solid black line represents the dissolved oxygen cycle and the dashed line represents the pH cycle, which were set up over a 24 h timescale.](image-url)
Fig. 6. Schematic diagram of the overall setup of the control system. The top portion illustrates the air and gas system, where dotted lines represent data communication between the computer program and instruments, and solid black arrowed lines indicate gas flow. The lower panel diagrams the water and algal supplement systems. Additional sensors for barometric pressure, water temperature and salinity are not pictured.
a constant input of DTs Premium Reef Blend Phytoplankton, a mix of *Nannochloropsis oculata*, *Phaeodactylum tricornutum* and *Chlorella* (Innovative Marine Aquaculture, Palmetto, Florida, U.S.A.), to supplement algae lost during the water transport and pCO$_2$ stripping processes. Water delivery to the aquaria originates from HT5 and is pumped via Cole-Parmer Masterflex L/S peristaltic pumps (Cole-Parmer, Vernon Hills, Illinois, U.S.A.) to the center bottom of each aquaria (*n* = 30) at 0.5 LPM; water passively leaves aquaria by overflow. Aquarium water flow rates and in-tank and water in-flow chlorophyll $a$ levels were checked at least weekly.

Air for the experiment is provided via five Thomas air compressors (Model 688CE44), one for each treatment. Moisture removal prior to each air MFC was handled by coiling 30 m of tubing (one 30 m coil per treatment) in a chest freezer to induce water condensation, which was subsequently collected in custom-made clear polyvinyl chloride (PVC) drip legs with tubing attached to solenoids at the bottom. Drip legs emptied water every 2 h by automated control via a repeat-cycle timer (Omron Corp., Kyoto, Japan; Fig. 6) when solenoids were energized. After the initial water removal, a three-way solenoid controlled by DOOM Treatment Control directed whether the air went directly to a final drying column of indicating silica (recharged thrice weekly) or through a soda lime filled CO$_2$-stripping column prior to the final drying column. Lastly, before entering the MFCs air was sent through 5 $\mu$m particle filters. Gas delivery pressures for N$_2$, CO$_2$, and O$_2$ were set as necessary for the pressure requirements of each MFC.

DOOM Treatment Control manipulates ratios of each gas required to achieve targets within a user-defined TFR (e.g., 30 LPM) using the coded logic calculations. For each treatment, the separate gases exiting the MFCs are plumbed together for mixing and connected to a separate gas manifold for each treatment, Check valves were used at each tubing junction to eliminate gas back-flow (Pentair Aquatic Eco-Systems, part no. 228225). The gas manifolds split the gas mix to each replicate aquarium fitted with a tight acrylic lid (with holes drilled for gas/water inputs and for discrete water quality measurements), where the delivered gas (5 LPM/tank) is bubbled through two Sweetwater® glass-bonded diffusers (3.81 cm L x 1.27 cm W, Pentair AquaticEco, part no. AS1) located at the bottom center of each aquaria, to facilitate mixing.

“Alarms” and “Critical” actions

At each time increment (as input on DOOM Setup screen), DOOM Treatment Control compares reported DO and pH values to a user-adjustable spreadsheet of allowable “Alarm” and “Critical” values. If a reported value enters the range of alarm values, but does not breach the critical values, an auditory alarm sounds in the experiment room and the system sends an automated email and/or text message to any designated recipients with the report of the alarm and the treatment(s) affected. If a reported value triggers a critical response, an auditory signal sounds, emails/text messages are sent, and the solenoid of the affected treatment(s) is/are closed to stop all gases except air from reaching the aquaria; a separate “emergency” solenoid is also opened to allow additional “emergency air” from a separate compressor to enter the affected critical treatment. We assumed that extra time at normoxic/normocapnic conditions, which could happen when a sudden squall rapidly mixes shallow water, was less problematic to the integrity and interpretation of our long-term experiments than pH or oxygen concentrations sufficiently low to trigger acute responses. All solenoids used in the setup were strategically used as either fail-open or fail-closed so as to be the safest for assuring experiment viability in the event of a power failure.

System flexibility

We designed this system to be flexible for immediate and future use; we can modify many conditions easily while the system is underway, and also alter the way the system functions for a wide range of possible future experiments. Although the results shown are from diel-cycling experiments, it is important to note that any cycle can be mimicked, or DO and pH can be held constant. As values for DO and pH targets and alarms are pulled from user-changeable spreadsheets (one for each day of the week), we can effectively establish any experimental conditions we desire for DO, pH and alarm settings separately for up to five treatments and have differing conditions on a daily basis. We can manipulate values in the spreadsheets and reload them to DOOM Treatment Control at any time without shutting down the system.

The majority of our system settings are user-changeable on the “Setup/Control” tab of DOOM Treatment Control (Fig. 2). We can manipulate the TFR to change the total amount of gas delivered to each treatment. There are also user changeable time intervals for the frequency of checking current DO and pH values against alarm and critical values, frequency of data recording, and frequency of gas ratio alterations to achieve DO and pH targets. We also included multiplication factors that act to increase or decrease the magnitude of our calculated gas additions or subtractions to reach target DO and pH values; these help assure we reach targets even under different environmental conditions, biological load, container sizes, or under differing water or gas flow rates.

Within DOOM Treatment Control, each of the five treatments can be turned “On” or “Off” (so that we can run an experiment with less treatments, if desired) and can also be set for one of four different system manipulation conditions—“pH only,” “DO only,” “DO and pH” adjustments, or “control” where DOOM uses only air and CO$_2$-stripped air to maintain normoxia and normocapnia (Fig. 2). Treatments can also be put into “Automatic” or “Manual” settings. In
“Automatic,” DOOM controls the treatment manipulations and the gas ratios, and under the “Manual” setting a user can manually input desired gas ratios. The “Manual” setting is very useful in circumstances where overriding the program is desirable, for example, in the case of an N₂ dewar emptying. When an N₂ dewar empties the treatment will no longer receive nitrogen and the measured in-aquarium DO value will begin to climb above the target value. As this occurs, the system will attempt to add increasingly more nitrogen (by increasing the N₂ MFC flow value) to drive DO back to the lower DO target. However, with an empty N₂ dewar no change in DO can actually occur, but with no feedback from the MFC the system will still attempt to sequentially increase the N₂ on the MFC up to the maximum TFR setting. We do not want the system to send a large volume of gas to the treatment and effect such a rapid DO change when the N₂ dewar is replaced. To prevent this, we can switch to “Manual” and adjust gas ratios manually to return the treatment to the target DO before resetting the treatment to “Automatic.”

Another important feature is the ability to change the status and treatment location of each DO and pH probe. A drop-down menu allows us to assign probes to each treatment and change this assignment whenever necessary, and also allows us to denote a probe as “normal,” “calibrate,” or “problem.” The probe locations and status codes are recorded alongside the data, which results in easy automatic coding of our data for periods of probe repair, calibration, and treatment reassignments.

Although we have continuous monitoring of aquarium water temperature and salinity, and in-room barometric pressure for system calculation of DO saturation, we also included the option for manual settings for these parameters. In the event of a probe calibration or malfunction, or to run the system in a way that considers other nonmeasured parameters, we can switch to a manual input instead of using real-time measured data. The system stores the most recent manually inputted value even when set to the in-tank measured value. The measured parameters are linked to a table of acceptable values, and if the measured value falls outside the allowable bounds, the system will revert to the manually inputted value for the temperature, salinity and/or barometric pressure. This ensures that potential erroneous data from probe malfunctions, for example, are not used in treatment manipulations.

Discrete measurements

As continuous measurements of parameters are recorded for only one replicate aquarium for each treatment, we also took discrete measurements in every aquarium ($n = 30$) to assure consistency in DO and pH values among replicates within treatments, and to help identify problems in water or gas delivery. DO, temperature, and salinity were measured in every aquarium 1–3 times daily using a handheld YSI ProPlus (Yellow Springs Instruments, Yellow Springs, Ohio, U.S.A.); pH was measured on the same schedule using a handheld Oakton Acorn 5+ pH meter fitted with a glass probe (Oakton Instruments, Vernon Hills, Illinois, U.S.A.). The discrete pH measurements were performed with special attention given to calibration and salinity stabilization. Recorded values were cross-checked with Durafet pH values as well as with pH values calculated using CO2SYS (Pelletier et al. 2007) with measured salinity, temperature, $p\text{CO}_2$, and alkalinity. We used the constants of Cai and Wang (1998) which are the best option available in CO2SYS for our salinity range as the majority of their riverine samples used to develop the constants ranged in salinity from 0 to 15. Total alkalinity ($A_t$) was determined thrice weekly via two-stage, open-cell, potentiometric titration using a Tazo Schott-Gerate piston burette titrator and a Corning pH Analyzer 350 in 2012 according to Standard Methods 2320 (APHA 1992) and in 2013 via two-stage, open-cell, potentiometric titrations following Dickson’s Guide to Best Practices for Ocean CO₂ Measurements (2007) and use of Scripps Institution of Oceanography Certified Reference Material (CRM) to validate our measurements. Necessary frequency of alkalinity sample measurement will depend on the variability of the particular system studied, the level of precision needed, and logistical constraints; this issue is not unique to this control system.

$p\text{CO}_2$ of each treatment and cycle phase was measured periodically throughout the experiments via aquaria water equilibration with air through custom constructed $p\text{CO}_2$ equilibrators and measured using Li-Cor 840A CO₂ Gas Analyzers (Li-Cor, Lincoln, Nebraska, U.S.A.). The discrete pH values calculated using CO2SYS (Pelletier et al. 2007) and use of Scripps Institution of Oceanography Certified Reference Material (CRM) to validate our measurements. Necessary frequency of alkalinity sample measurement will depend on the variability of the particular system studied, the level of precision needed, and logistical constraints; this issue is not unique to this control system.

Calibrations and carbonate chemistry

All Oxyguard DO probes were calibrated biweekly in water-saturated air using oxygen saturation tables and cross-checked daily against the YSI ProPlus. The YSI ProPlus was calibrated for DO in the YSI-provided calibration sleeve before each use, and crosschecked with DO saturation tables; conductivity was calibrated biweekly.

The Oakton handheld pH probe was calibrated before each use at the same temperature as the aquaria. After calibration the probe was left in an aquarium to stabilize to the salinity conditions prior to making discrete measurements. Durafet pH sensors were calibrated weekly in a water bath at the same temperature as the aquaria. As the salinity of our seawater typically ranges from 5 to 15 we chose to use National Bureau of Standards, now the National Institute of Standards and Technology (NBS) buffers for pH probe calibrations. Sea water scale buffers are not appropriately scaled for measurement of estuarine water pH, yet the use of NBS buffers when measuring seawater can also cause pH probe drift and measurement errors due to the difference in ionic
Table 1. Experimental conditions in 2013 oyster adult and spat experiments performed using DOOM Treatment Control.

<table>
<thead>
<tr>
<th>Experiment conditions</th>
<th>2013 adult oyster</th>
<th>2013 spat</th>
</tr>
</thead>
<tbody>
<tr>
<td>Expt length/# days cycling</td>
<td>10 week/48 d</td>
<td>5 week/23 d</td>
</tr>
<tr>
<td># Treatments/# replicates</td>
<td>5/6</td>
<td>5/6</td>
</tr>
<tr>
<td>Aquaria size/# aquaria</td>
<td>75 L/30</td>
<td>75 L/30</td>
</tr>
<tr>
<td>Water inflow rate (LPM)</td>
<td>0.5</td>
<td>0.3</td>
</tr>
<tr>
<td># Eastern oysters/tank</td>
<td>90 1yo oysters</td>
<td>20 oyster spat</td>
</tr>
<tr>
<td>Mean temp (°C)±SE</td>
<td>26.25 ± 0.03</td>
<td>24.35 ± 0.02</td>
</tr>
<tr>
<td>Mean salinity±SE</td>
<td>8.21 ± 0.01</td>
<td>11.90 ± 0.01</td>
</tr>
<tr>
<td>mean alkalinity</td>
<td>1400.92 ± 28.76</td>
<td>1678.87 ± 4.97</td>
</tr>
</tbody>
</table>

(μmol kg⁻¹·SW)±SE

strength (Millero 1986; Dickson et al. 2007). For estuarine water pH measurements, the NBS scale are the most commonly used set of buffers (Frankignoulle and Borges 2001). To assure that our Durafet pH readings were not adversely affected by estuarine salinity, we ran a test of our probes following the acid titration method described by Covington and Whitfield (1988). We created a set of seven water samples ranging in salinity from 5.48 to 29.17 using Corallife Scientific Grade Marine Salt and RO water, and also included an SIO CRM (Batch 132) with a listed salinity value as 33.44. Using the calculation defined in Covington and Whitfield (1988), a regression relating hydrogen ion concentration as measured by Durafet pH probe to actual H⁺ ions added resulted in a calculated correction factor for the effect of salinity on the pH measurements of the Durafet probes, $y = -0.0032x + 0.9722$ where $y$ is the correction factor and $x$ is salinity (see also Hanson 1973). All pH data collected by Durafet sensors was corrected accordingly. The Licor840A was calibrated using pure dry nitrogen gas (0 ppm CO₂) and a custom span gas mixture with a CO₂ amount of 8227 ppm in 2012 and 8015 ppm in 2013 (Roberts Oxygen, Maryland, U.S.A.).

**Assessment**

We present data from two experiments as illustrations of the ability of DOOM Treatment Control to effectively manipulate co-varying DO and pH on a diel cycle under flow-through water conditions (Table 1). Experiments ranged in length from 5 week to 12 week, and had water flow rates to each aquarium between 0.3 LPM and 0.5 LPM. To examine the performance of the system under differing biological loads, we include one experiment with a high biological load (90 one-year-old eastern oysters per 75 L aquaria, starting length range 35–70 mm) and one experiment with low biological load (20 eastern oyster spat of three postsettlement age classes in each 75 L aquaria, mean starting shell areas were <1 mm², 3.7 mm², and 18.3 mm²). A comparison of the continuous data with experiment targets is used to indicate the effectiveness of DOOM Treatment Control to achieve and maintain targets, whereas variability among tanks based on discrete measurements indicates the ability of our overall system setup to translate the one “master” aquarium’s measured values and subsequent gas ratio changes to the remaining five replicate aquaria for each treatment. All errors reported in this article are standard errors (SE).

**Assessment—continuous monitoring**

Our 2013 experiment using adult oysters tested effects of diel-cycling DO and pH on *Perkinsus* sp. (Dermo) acquisition and progression, hemocyte function, growth, and reproduction (Keppel 2014; Keppel et al. 2015). It is important to iterate that the data recorded by continuously monitoring sensors is output as 1 min and 10 min means, so all means and SEs reported from continuous monitors are calculated from very large sample sizes. The system maintained an overall normoxia/normocapnia plateau DO saturation range of 93.8–98.1% with an overall mean normoxic DO concentration of 7.34 mg L⁻¹ ± 0.02 (n = 32,410) and pH of 7.90 ± 0.001 (n = 31,765).

Figure 7 shows an example week of DO and pH cycles (panels a and b, respectively) achieved in the 2013 adult oyster experiment, as well as the difference between achieved and target values (offset; panels c and d). During low plateau, the DO offsets in cycling treatments are close to zero (Fig. 7c). Offsets tend to be highest during cycle phases when targets are rapidly changing. Offsets were also sometimes higher during normoxic phases than low plateau because we chose to only allow the program to use air or CO₂-stripped air for normoxia targets even though the high biological load in aquaria often resulted in oxygen concentrations a bit below 100% saturation. We considered this level of precision during high oxygen phases adequate for our experiments, but more precise control could be achieved by setting the system to fully control DO at normoxia using all gases, including oxygen.

The pH offset at low plateau depended on the oxygen as well as the pH target (Fig. 7d). Nitrogen used to displace oxygen also displaced CO₂, resulting in a slightly positive pH offset in treatments with cycling low oxygen. As with DO, the pH offset during periods with a high rate of change tended to be higher than during plateau phases, and variability in the normoxia/normocapnia treatment reflected the fact that CO₂ was not used to lower pH. The box and whisker plots in Fig. 8 are provided to illustrate both the system accuracy (median lines within the boxes align with target values) and the precision (note that outlier values follow the same pattern among treatments).

In late summer/early fall 2013, we performed a 5-week long experiment on eastern oyster spat growth under co-varying diel-cycling DO and pH (Keppel 2014). Although performed in the same year as the experiment described above, the later season start of this experiment resulted in cooler and more saline conditions than those of the summer 2013 adult oyster experiment (Table 1). Cooler temperatures resulted in a higher
overall mean normoxia plateau DO of $7.65 \text{ mg L}^{-1} \pm 0.002$ (94.4–101.3% DO saturation range, $n = 13,156$), and along with higher alkalinity contributed to our higher overall mean pH at normocapnia plateau of $8.08 \pm 0.000$ ($n = 11,423$). We did not include example weeks of DO and pH, offsets, or box and whisker plots for the spat experiment, as the data are very similar to those from the adult oyster experiment.

**Assessment—discrete measurements**

As the discrete DO and pH measurements were taken in all aquaria, comparing these values at each cycle phase for each treatment tests how well the replicate aquaria mimic the conditions in the “master controlled” aquaria (Figs. 9, 10, and Table 2). Variation among replicates of each treatment was very low (Figs. 9, 10; note SEs in Table 2). For example, the coefficient of variation during the low plateau phase in the severe cycling DO/cycling pH treatments were 5.4%, and 0.1%, and 6.6%, and 0.4% for DO and pH in the adult oyster and spat experiments, respectively (Table 2). In general, spikes in DO and pH were rare (i.e., open circles in Figs. 9, 10). Spikes in data can be from erroneous probe spikes (e.g., due to an amphipod or bubble on a sensor) where the actual value has
not strayed from the target value, or can be true in-water excursions from target values (e.g., due to gases running empty or air line ruptures). As it would be very difficult to determine variation among aquaria during periods of rate change, measurements were not taken during these time periods. Target levels would change during the hour or more it takes a technician to manually measure DO and pH in all aquaria. However, we routinely recorded full sets of discrete measurements at the very start of low plateau to make sure all aquaria had reached target. During measurements at this time point, all tanks were either at target, or very close, so we assume that there is little variation during periods of rate change. Discrete measurements were not taken during the supersaturated phase of the spat growth experiment.

**Discussion**

The DOOM Treatment Control automated system successfully monitored and controlled co-varying diel-cycling fluctuations of DO and pH in high-throughput, raw-water, flow-through aquaria, simulating cycles found in the field. The ability of the LabVIEW™ software to monitor and manipulate all treatments and parameters concurrently results in very tight control. More importantly, the capacity of DOOM Treatment Control to manipulate DO and pH parameters either separately, or in concert, provides the capability to tease apart the relative importance of each of these variables separately, as well as their effects as co-occurring stressors. As the system can be set to control any environmentally relevant DO and pH cycles or constant levels, at any time scale, DOOM Treatment Control can effectively mimic conditions ranging from diel-cycles to upwelling conditions to tidal mixing situations. In addition, the current system can run 12 replicates per treatment, offering an adequate level of replication for flow-through experiments in fairly large containers. These capabilities make DOOM Treatment Control a useful tool for investigating the co-varying effects of DO and pH. Although our experiments utilized standard daily cycles, it would be simple to input target values at short time intervals to mimic the more irregular fluctuations seen in some shallow estuarine and marine waters. It is also possible to set up daily spreadsheets with different cycle levels per day, or to input slower changes in DO and pH, or multiple days at steady levels. The LabVIEW™ program we provide will enable other researchers to view our code, build on our work, and modify the system to meet other research goals.

**Shortcomings and potential improvements**

The most serious shortcomings of DOOM Treatment Control are not associated with the system logic, but are instead largely a product of (1) the problems that arise when gas cylinders/dewars empty, (2) pH probe deficiencies, (3) lack of water temperature control, and (4) the labor intensive nature of the experiments using large volumes (up to 21,600 L d⁻¹) of raw estuarine water.

As mentioned in the “System Flexibility” section, the system is unaware when gas cylinders empty. Subsequently, when DO and/or pH values begin to fall outside the target range, the system will attempt to adjust the MFCs to correct the in-tank DO or pH until the MFC is either at 0 or at maximum TFR. If the gas is empty no change will actually occur and the system will eventually sound an alarm, and if not corrected, will eventually trigger the “Critical” alarm response. The problem of empty cylinders could be alleviated by integrating feedback from the MFCs to alert users when gas levels are running low before DO and pH values fall outside of alarm and/or critical values, or by setting up
automatic-switching valves hooked to multiple gas cylinders or dewars, or by using gas generators.

The pH probe deficiency problem is a bit more difficult to resolve with currently available commercial sensors. Although all parameters of the carbonate chemistry system can be sampled and measured, we wanted DOOM Treatment Control to use pH as the measurement and control variable as it can be continuously monitored with commercially available sensors, and there is a plethora of pH data to which we can compare our results. ISFET pH probe technology, including Honeywell Durafet ISFET probes, has been touted as the industry standard for their insensitivity to pressure changes, validity under changing salinities, low sensor impedance, precision, longevity and long-term stability (Le Bris and Birot 1997; Sandifer and Voycheck 1999; Shitashima et al. 2002; Martz et al. 2010). However, we did
not find that these probes were as reliable under eutrophic or fluctuating conditions characteristic of an estuarine environment (in particular, salinity fluctuations), as in field tests that were performed under more oceanic conditions. Discussions with colleagues indicated that our experience is not unique. We intended to record $pCO_2$ as another continual measurement of the carbonate chemistry system, but because our raw water clogged the equilibrator spray, and our equilibrator had high water flow requirements matching or exceeding the maximum water flow rate into our

**Fig. 10.** Box and whisker plots of pH values for each cycle phase for each treatment for the 2013 adult oyster experiment (Standard percentile method, SigmaPlot). Plots were very similar for the spat experiment (data not shown).
Table 2. Means (± standard error [SE]) and percent coefficients of variation for DO, pH, temperature, and salinity discrete measurements at each cycle phase of each treatment for both 2013 oyster experiments. Means, standard deviations, and standard errors were calculated by replicate, then among replicates.

<table>
<thead>
<tr>
<th></th>
<th>Mean DO mg L⁻¹±SE (CV%)</th>
<th>Mean pH±SE (CV%)</th>
<th>Mean temp °C±SE</th>
<th>Mean salinity±SE</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Normoxia</td>
<td>Low plateau</td>
<td>Supersat</td>
<td>Normoxia</td>
</tr>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normoxia/</td>
<td>7.53 ± 0.017</td>
<td>7.65 ± 0.013</td>
<td>7.57 ± 0.016</td>
<td>7.92 ± 0.006</td>
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<tr>
<td>normocapnia</td>
<td>(0.6)</td>
<td>(0.4)</td>
<td>(0.5)</td>
<td>(0.2)</td>
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<td>7.59 ± 0.015</td>
<td>7.54 ± 0.012</td>
<td>7.90 ± 0.005</td>
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<td>(0.5)</td>
<td>(0.4)</td>
<td>(0.2)</td>
</tr>
<tr>
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<td>9.92 ± 0.009</td>
<td>7.91 ± 0.007</td>
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<td>(0.2)</td>
<td>(0.2)</td>
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<td>0.51 ± 0.010</td>
<td>9.92 ± 0.022</td>
<td>7.90 ± 0.007</td>
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<td>(4.6)</td>
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<td>7.90 ± 0.007</td>
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<td>(0.5)</td>
<td>(0.2)</td>
</tr>
<tr>
<td><strong>2013 spat</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Normoxia/</td>
<td>7.80 ± 0.019</td>
<td>7.94 ± 0.082</td>
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<td>8.09 ± 0.007</td>
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<td>(2.3)</td>
<td></td>
<td>(0.2)</td>
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<td>7.96 ± 0.019</td>
<td></td>
<td>8.03 ± 0.004</td>
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<td></td>
<td>(0.2)</td>
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<td>0.55 ± 0.006</td>
<td></td>
<td>8.03 ± 0.005</td>
</tr>
<tr>
<td>DO/normocapnia</td>
<td>(0.6)</td>
<td>(2.5)</td>
<td></td>
<td>(0.1)</td>
</tr>
<tr>
<td>Cycling severe</td>
<td>7.76 ± 0.026</td>
<td>0.56 ± 0.017</td>
<td></td>
<td>8.03 ± 0.012</td>
</tr>
<tr>
<td>DO/cycling pH</td>
<td>(0.7)</td>
<td>(6.6)</td>
<td></td>
<td>(0.3)</td>
</tr>
</tbody>
</table>
aquaria (0.5 LPM), we were unable to run this measurement system continuously. Under different conditions, it would be possible to integrate LICOR measurements of pCO₂ to improve the description of the carbonate chemistry within an experiment.

Another limitation of our system is the lack of integrated water temperature control. Adding temperature control to the existing system would stabilize the treatment conditions within the experimental containers. Doing so in a high-volume raw-water flow-through system would be both expensive and logistically difficult. Moderating fluctuations in temperature of the incoming water during the summer by air conditioning the experiment room, and, in early autumn, via a recirculating water heater unit and submersible aquarium heaters in the holding tanks was sufficient to meet the goals of our experiments and approximated seasonal fluctuations in the adjacent Rhode River. However, other experimental uses might require more precise control.

Perhaps the most onerous of the system shortcomings is its labor intensive nature resulting from our high raw-water turnover, and results in rapid use of expendable materials such as gases, soda lime, silica drying agent, peristalsis tubing, water and gas delivery tubing, airstones, and filtration materials.

**Comments and recommendations**

The accuracy of the reproducible DO and pH values of DOOM Treatment Control is critically linked to the accuracy of the sensors used and the calibration procedures for the probes. Although DOOM Treatment Control has been proven to reliably reach intended target values, the system is reliant on the user certifying that the sensors are working and calibrated correctly so that correct values are reported and used for system calculations and gas manipulations.

Because of the flexibility of the LabVIEW program and the ability to adjust or replace other individual components, the system as a whole can be modified for use for a wide range of experimental organisms and container sizes. Although the experiments reported here used a direct-bubble approach, we see no reason why our system could not also be modified, for example, to instead manipulate header tanks, without any programming changes. For 2014 and 2015 experiments in our laboratory performed with *Menidia beryllina* and *Menidia menidia*. (inland and Atlantic silversides, respectively), we modified the program provided here to control additional treatments and ran experiments with substantially lower bubbling rates (i.e., lower TFR), lower water flow rates, and with headspace in the aquaria to allow for aquatic surface respiration. All experiments resulted in equally successful manipulation and control. Most importantly, the ability of the DOOM Treatment Control system to concurrently run multiple treatments with DO and/or pH cycles of biologically realistic magnitude, and at any timescale, provides a powerful tool to evaluate both the separate and synergistic effects of these environmental factors.

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