

Shellfish Face Uncertain Future in High CO₂ World: Influence of Acidification on Oyster Larvae Calcification and Growth in Estuaries

A. Whitman Miller^{1*}, Amanda C. Reynolds¹, Cristina Sobrino², Gerhardt F. Riedel¹

¹ Smithsonian Environmental Research Center, Edgewater, Maryland, United States of America, ² Universidad de Vigo, Vigo, Pontevedra, Spain

Abstract

Background: Human activities have increased atmospheric concentrations of carbon dioxide by 36% during the past 200 years. One third of all anthropogenic CO₂ has been absorbed by the oceans, reducing pH by about 0.1 of a unit and significantly altering their carbonate chemistry. There is widespread concern that these changes are altering marine habitats severely, but little or no attention has been given to the biota of estuarine and coastal settings, ecosystems that are less pH buffered because of naturally reduced alkalinity.

Methodology/Principal Findings: To address CO₂-induced changes to estuarine calcification, veliger larvae of two oyster species, the Eastern oyster (*Crassostrea virginica*), and the Suminoe oyster (*Crassostrea ariakensis*) were grown in estuarine water under four pCO₂ regimes, 280, 380, 560 and 800 μatm, to simulate atmospheric conditions in the pre-industrial era, present, and projected future concentrations in 50 and 100 years respectively. CO₂ manipulations were made using an automated negative feedback control system that allowed continuous and precise control over the pCO₂ in experimental aquaria. Larval growth was measured using image analysis, and calcification was measured by chemical analysis of calcium in their shells. *C. virginica* experienced a 16% decrease in shell area and a 42% reduction in calcium content when pre-industrial and end of 21st century pCO₂ treatments were compared. *C. ariakensis* showed no change to either growth or calcification. Both species demonstrated net calcification and growth, even when aragonite was undersaturated, a result that runs counter to previous expectations for invertebrate larvae that produce aragonite shells.

Conclusions and Significance: Our results suggest that temperate estuarine and coastal ecosystems are vulnerable to the expected changes in water chemistry due to elevated atmospheric CO₂ and that biological responses to acidification, especially calcifying biota, will be species-specific and therefore much more variable and complex than reported previously.

Citation: Miller AW, Reynolds AC, Sobrino C, Riedel GF (2009) Shellfish Face Uncertain Future in High CO₂ World: Influence of Acidification on Oyster Larvae Calcification and Growth in Estuaries. PLoS ONE 4(5): e5661. doi:10.1371/journal.pone.0005661

Editor: Zoe Finkel, Mt. Alison University, Canada

Received: February 4, 2009; **Accepted:** April 27, 2009; **Published:** May 27, 2009

Copyright: © 2009 Miller et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Funding: Funding for this research was provided by the Seward Johnson Endowment provided through the Smithsonian Marine Science Network as well as federal appropriations made to the Smithsonian Institution.

Competing Interests: The authors have declared that no competing interests exist.

* E-mail: millerw@si.edu

Introduction

During the past 200 years the combustion of fossil fuels, deforestation, and land development have increased atmospheric concentrations of carbon dioxide by 36% and the rate of CO₂ emission is expected to increase during the coming century [1–3]. Approximately one third of all anthropogenic CO₂ has been absorbed by Earth's oceans [4–5]. Although the ocean has partially absorbed recently liberated anthropogenic atmospheric CO₂, this has come at the expense of significantly reduced pH (acidification) and altered carbonate chemistry in the ocean's surface waters. Since ca. 1760, the pH of the ocean's surface (the upper 200 m of the water column) has decreased by approximately 0.1 of a unit, and further reductions of 0.1 to 0.5 units are expected during the next 100 years [6–8]. There is widespread concern that these changes will produce irreversible ecological regime shifts in marine habitats, such as massive reductions in

coral reef habitats and their associated biodiversity as well as reduced availability of carbonate ions for calcifying biota [9–11].

To date most research has focused on CO₂-induced acidification in fully marine waters, while little attention has been devoted to lower salinity estuaries and temperate nearshore ecosystems. But coastal and estuarine biomes are among the most biologically productive and maintain some of the most extensive and measurable ecosystem services (e.g., commercial and recreational fisheries, fish and invertebrate nursery grounds, water purification, flood and storm surge protection, human recreation). Because they are shallower, less saline, and have lower alkalinity [12], estuaries and coastal marine habitats are more susceptible to changes in pH than the open ocean. Estuaries are also susceptible to substantial enrichment in CO₂, produced by the respiration of both natural and anthropogenic carbon. While many estuaries already have high and variable pCO₂ [13], atmospheric CO₂ enrichment will shift the baseline toward even higher values. For these reasons,

these ecosystems will likely experience more acute impacts from elevated CO₂ in coming decades.

A direct consequence of CO₂-induced acidification is the reduction of carbonate ion concentration [CO₃²⁻] in the water column. Of special concern in marine and estuarine systems is the effect of rising CO₂ on the saturation state of water with respect to calcium carbonate (Ω), where

$$\Omega = \frac{[Ca^{2+}][CO_3^{2-}]}{K'_{sp}}$$

and K'_{sp} is the apparent solubility product [14–15]:

$$K'_{sp} = [Ca_{(aq)}^{2+}][CO_{3(aq)}^{2-}]$$

Ω is therefore a proxy for the ease with which organisms can produce calcium carbonate (CaCO₃). The water's saturation states with respect to aragonite and calcite, the two most commonly biomineralized forms of CaCO₃, are diminished with increased partial pressures of CO₂ (pCO_2) [15].

In estuaries, Ω naturally decreases with decreasing salinity due to gradients in pH, Ca²⁺ and CO₃²⁻ produced by the dilution of seawater with river water (see Cai and Wang [13]). A recent study by Salisbury et al. [16] indicates that seasonal discharges of acidic riverine water will further exacerbate acidification in estuaries, suggesting the possibility of negative impacts to shell fisheries.

In Chesapeake Bay, Wong [12] showed that water from the James River and ocean mix conservatively and that salinity and

total alkalinity (TA) are linearly related from approximately 5 psu to 32 psu. At ~18 psu, TA in the Bay is ~1250 μ mol/kg-SW. If conservative mixing of water and equilibrium with current atmospheric CO₂ (380 μ atm) are assumed, the aragonite compensation point (i.e., $\Omega_{arag} = 1.0$) at summer temperatures should lie near the 18 psu isopleth. As atmospheric CO₂ increases, assuming Ca²⁺ and alkalinity remain at present levels, the aragonite compensation point in estuaries will shift seaward toward higher salinities. We contend that the aragonite compensation point has already shifted significantly toward the Bay's mouth since the beginning of the industrial era (Fig. 1). The compensation point for calcite, which is approximately 1.5 times less soluble than aragonite [14] occurs at lower salinities and will shift seaward in a similar way. In reality, estuaries are frequently not in equilibrium with the atmosphere, however, a change in atmospheric pCO_2 will shift the baseline equilibrium to which estuaries tend, and result in wider spread elevated pCO_2 .

We expect bivalve larvae to be more vulnerable than adults to increased CO₂ because larvae biomineralize aragonite, the more soluble form of CaCO₃, rather than calcite, the predominant material used in adult shell. Weiss et al. [17] further indicate that during biomineralization some bivalve larvae, (e.g., *Crassostrea gigas* and *Mercenaria mercenaria*) generate an even more soluble amorphous CaCO₃ as an ephemeral precursor to crystalline aragonite, and because larvae are generally less robust than adults to a variety of stresses. If larvae are indeed more susceptible to acidification, it may lead to their reduced performance, or even failure, ultimately leading to negative effects on oysters and other shellfish populations. Kurihara et al. observed at very high pCO_2

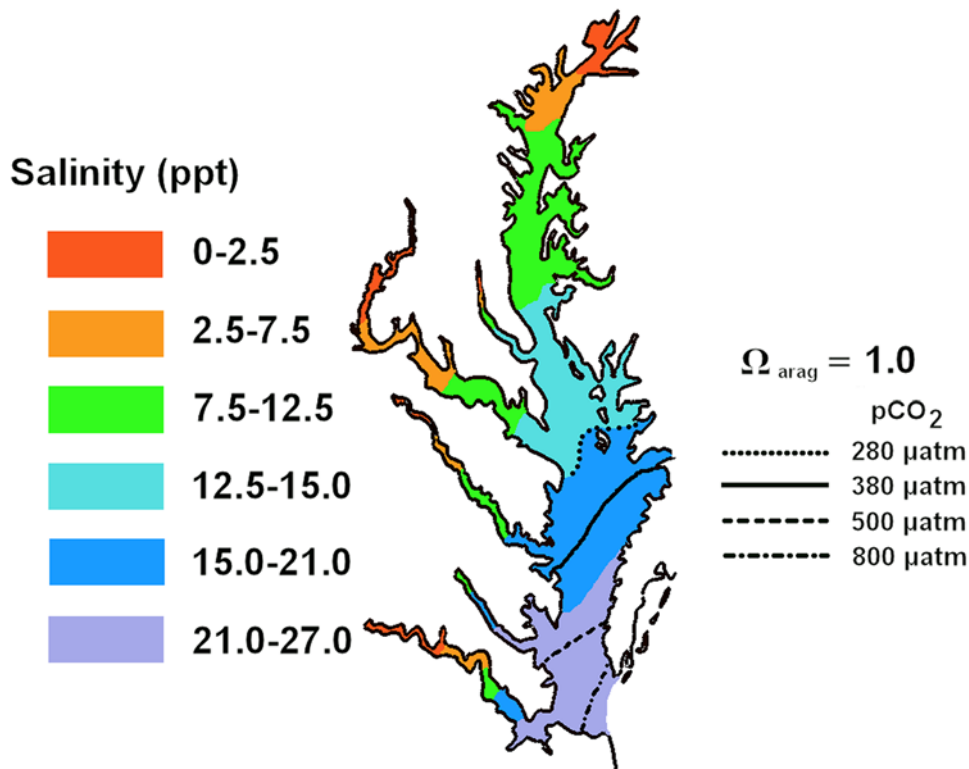


Figure 1. Projected mean summer positions of aragonite compensation points ($\Omega_{arag} = 1.0$) for Chesapeake Bay. Theoretical aragonite compensation positions were plotted under past, present and predicted atmospheric CO₂. Aragonite saturation was calculated using the program CO₂SYSL from alkalinity and atmospheric pCO_2 at 25°C and 0 mbar pressure, assuming conservative mixing of Atlantic and the Susquehanna River alkalinities, and mean summer salinities (1985–2006 – data from Chesapeake Bay Program [47]). Aragonite is supersaturated seaward of compensation lines.

doi:10.1371/journal.pone.0005661.g001

(2268 μatm) that embryonic development and shell formation in *Crassostrea gigas* was inhibited during the initial 48 h following fertilization [18]. How shifting carbonate chemistry will alter the ecological structure and function in benthic communities remains a critical gap in our knowledge [1–2].

We conducted experiments on the veliger larvae of two closely related euryhaline oyster species, the Eastern oyster (*Crassostrea virginica* [Gmelin 1791]) and the Suminoe oyster (*Crassostrea ariakensis* [Fujita 1913]), under conditions of continuously controlled pH/pCO₂ for up to 28 days. *C. virginica* is native to the Western Atlantic, but the recent landings are just 1% of their historical maximum in Chesapeake Bay, due in large measure to overfishing and disease [19–20]. The introduction of *C. ariakensis* to Chesapeake Bay has been considered in the hopes of restoring the oyster fishery and important ecosystem functions, such as water filtration and reduction of nutrients and sediment in the water column [21]. We hypothesize that (a) oyster larvae will exhibit reduced growth and calcification at elevated pCO₂ and (b) larval development will be adversely and measurably affected when $\Omega_{\text{arag}} < 1.0$. By extension, oyster fisheries, other shellfish, and calcifying benthic fauna are expected to be negatively influenced by acidification.

Materials and Methods

All oyster larvae tested were obtained from the Virginia Institute for Marine Science's Eastern Shore Laboratory, Wachapreague, VA, where *C. virginica* and *C. ariakensis* were spawned in

quarantined facilities. Experiment *Ca* 1 larvae were derived from *C. ariakensis* stocks originating in the Ariake Sea of Japan but accidentally transferred to the west coast of the US with shipments of *C. gigas* in the 1970s [22]. *Cv* 1 were derived from Wachapreague seaside *C. virginica* stocks. Table 1 summarizes all experimental parameters for experiments *Cv* 1 and *Ca* 1. At 72 hours post fertilization, D-stage larvae were transferred from Wachapreague to a quarantined laboratory at the Smithsonian Environmental Research Center in Edgewater, MD, and placed into culture. To test the effects of elevated pCO₂, reduced pH, and aragonite saturation state on larval growth and calcification, we cultured oyster larvae under controlled conditions that simulated a typical summer estuarine environment in upper mesohaline reaches of the Chesapeake Bay: salinity = 18 psu, temperature = 25°C, day/night cycle = 14:10 hrs. Experimental treatments were blocked across two incubators (Percival I-36 Controlled Environment Chambers with Philips 700 series 32 W fluorescent bulbs) and individual aquaria randomly positioned within each incubator. Incubator A contained 280 μatm and 380 μatm pCO₂ treatments and incubator B contained 560 μatm and 800 μatm for all experiments. Prior to experiments, the PAR irradiance was measured inside each incubator and shown to be similar (A = 167.7 ± 5.3 $\mu\text{E m}^{-2} \text{s}^{-1}$ compared with B = 169.2 ± 7.3 $\mu\text{E m}^{-2} \text{s}^{-1}$ (mean ± SEM).

Natural sea water was collected from Sinepuxent Bay, MD (~28 psu) and Delaware Bay, DE (~24 psu) and filtered through a 0.2 μm filter and diluted with deionized water to 18 psu. An open cell, two-point titration [23] determined the TA of the water

Table 1. Carbonate chemistry parameter values/sources and culture conditions for Eastern oyster (*Crassostrea virginica*) and Suminoe oyster (*C. ariakensis*) larvae experiments.

Parameter	<i>C. virginica</i> (<i>Cv</i> 1)				<i>C. ariakensis</i> (<i>Ca</i> 1)				Parameter Source
	Pre-IR	2008	2050	2100	Pre-IR	2008	2050	2100	
Simulation date	Pre-IR	2008	2050	2100	Pre-IR	2008	2050	2100	
Target pCO ₂ (μatm)	280	380	560	800	280	380	560	800	
Mean pCO ₂ (μatm)	284	389	572	840	291	386	581	823	CO ₂ SYS calc.
SEM	4.8	7.9	10.8	17.4	3.8	6.7	13.6	11.8	
Mean hourly pH	8.16	8.06	7.91	7.76	8.17	8.08	7.92	7.79	Direct Measure
SEM	0.002	0.005	0.005	0.006	0.004	0.006	0.008	0.006	
Mean TA ($\mu\text{mol/kg}$)	1229	1268	1283	1289	1297	1324	1357	1360	CO ₂ SYS calc.
SEM	15.7	18.4	17.2	15.7	16.6	15.5	23.9	16.4	
Mean TDIC ($\mu\text{mol/kg}$)	1126	1188	1232	1265	1187	1237	1301	1331	Direct Measure
SEM	15.1	17.8	16.9	15.7	15.3	14.6	23	15.8	
Mean [CO ₂] ($\mu\text{mol/kg}$)	8.8	12	17.7	25.9	9	11.9	18	25.4	CO ₂ SYS calc.
SEM	0.15	0.24	0.33	0.54	0.12	0.21	0.42	0.36	
Mean [HCO ₃ ⁻] ($\mu\text{mol/kg}$)	1045	1116	1169	1206	1100	1159	1234	1269	CO ₂ SYS calc.
SEM	14.1	16.8	16	14.9	13.9	13.6	21.7	15	
Mean [CO ₃ ⁻] ($\mu\text{mol/kg}$)	72.4	60.4	45.1	32.7	78.3	65.7	49.6	36.9	CO ₂ SYS calc.
SEM	0.8	1	0.7	0.6	1.4	1.3	1.4	0.8	
Mean Ω_{arag}	1.2	1	0.8	0.6	1.3	1.1	0.8	0.6	CO ₂ SYS calc.
SEM	0.01	0.02	0.01	0.01	0.02	0.02	0.02	0.01	
Salinity (psu)	18.2	18.2	18.2	18.2	18.2	18.2	18.2	18.2	Direct Measure
SEM	0.04	0.04	0.04	0.04	0.03	0.03	0.03	0.03	
Duration (d)	26	26	26	26	28	28	28	28	

Larvae were grown under four pCO₂ conditions (280, 380, 560, 800 μatm), simulating equilibration with atmospheric CO₂ in the preindustrial era, present (2008), 2050 and 2100 CE. All experiments were conducted at 25°C and under a 14 h:10 h light:dark cycle, simulating summer growing conditions in Chesapeake Bay. TA = Total Alkalinity, TDIC = Total Dissolved Inorganic Carbon.

doi:10.1371/journal.pone.0005661.t001

just prior to the onset of each experiment. Total alkalinity and $p\text{CO}_2$ targets for pre-industrial ($p\text{CO}_2 = 280 \mu\text{atm}$), present day ($p\text{CO}_2 = 380 \mu\text{atm}$), year 2050 ($p\text{CO}_2 = 560 \mu\text{atm}$) and year 2100 ($p\text{CO}_2 = 800 \mu\text{atm}$) settings, as projected by the IS92a “business as usual” scenario [24] were then entered into the computer program CO₂SYX.XLS [25]. The program was parameterized specifically with the two dissociation constants K_1 and K_2 for carbonic acid in estuarine waters of Cai and Wang [13] to determine corresponding experimental pH levels.

Larvae were grown in 4-L polycarbonate aquaria, under four $p\text{CO}_2$ treatments, and each treatment included three replicate aquaria that were independently controlled for $p\text{CO}_2/\text{pH}$ ($n = 12$). Filtered water samples ($0.2 \mu\text{m}$) were collected from each aquarium every few days throughout the experiments for total dissolved inorganic carbon (TDIC) analysis. (To maintain water quality, water was changed in all aquaria every two days. TDIC samples were taken 24–48 hours after a water change.) Water samples were capped without head space and analyzed immediately, or stored at 4°C and analyzed within 48 hrs. TDIC was measured using a total organic carbon analyzer, outfitted with a phosphoric acid inorganic carbon reagent reaction chamber and non-dispersive infrared detector (Schimadzu TOC-V with IC reactor kit). TDIC was then partitioned into $p\text{CO}_2$, bicarbonate, and carbonate and TA calculated with CO₂SYX.XLS [25] (Table 1.) Experiments were ended when larvae of the largest size classes achieved competence as indicated by the presence of eye spots.

Experiments *Cv* 1 (*Crassostrea virginica*) and *Ca* 1 (*C. ariakensis*) were inoculated with 15,000 four day-old D-stage larvae respectively, and were grown for up to 28 days. Aquaria received controlled quantities of the microalgae *Isochrysis galbana* (Prymnesiophyceae) as food. Microalgae were grown in semi-continuous cultures using f/2-Guillard medium. Equal quantities of microalgae were pipetted daily from cultures of known density into each aquarium, with daily doses increasing as larvae grew larger. Target inoculation densities in each aquarium increased from $1.5 \cdot 10^4 \text{ cells} \cdot \text{ml}^{-1}$ at the beginning of an experiment to $1.0 \cdot 10^5 \text{ cells} \cdot \text{ml}^{-1}$ during the final days, when larvae were larger and ingesting more food.

The $p\text{CO}_2$ in each aquarium was sustained by intermittent bubbling with CO₂-enriched air (commercially available, certified 1.0% CO₂) and continuous aeration with CO₂-stripped air. Soda-lime filters were used to strip CO₂. Continuous aeration with CO₂-stripped air oxygenated and mixed the water, while simultaneously driving CO₂ out of the water and pushing pH upward. Each aquarium was continuously monitored with a pH probe (NBS scale) and control system. Once a target pH set point was exceeded, an automated controller opened a solenoid and delivered the 1% CO₂ air mixture to an aquarium, thereby driving pH downward. The flow of CO₂ was automatically stopped when the pH set point was reached. Based on hourly pH measurements, the negative feedback control system was shown to maintain pH (and $p\text{CO}_2$) at near constant levels for up to 4 weeks. The four pH set points used corresponded to desired target $p\text{CO}_2$ treatments for water of known total alkalinity. Probes were calibrated prior to the experiment using NIST traceable buffers (pH = 4.00 and 7.00), recalibrated at least one time during the experiment, and again at the conclusion of the experiment to ensure proper operation.

At the end of each experiment, larvae from each aquarium were collected and fixed in 95% ethanol. Random samples were taken from each replicate and photographed at 20× magnification; ($n = 205 \pm 6.4$ shells per replicate (mean \pm SEM)). Larval shells were positioned on their sides and photographed in silhouette. Outer shell areas were measured using digital image analysis [26] (Scion

Image, ver. 4.0.3.2). All areas are reported as total outer surface areas (i.e., $2 \times$ photographed area = total surface area of both valves). The same samples were rinsed with deionized water to remove sea salts and ethanol, and the shells dissolved in trace metal grade HCl, and diluted to a known volume. Inductively coupled plasma/optical emission spectroscopy (ICP/OES) was used to measure the mean calcium content per shell from each replicate sample across treatments.

For each experimental treatment, the mean mass of CaCO₃ per unit area ($\mu\text{g}/\mu\text{m}^2$) was determined by dividing the mean CaCO₃ content of shells by the mean shell area. These values were then divided by the density of crystalline aragonite ($2.93 \text{ g}/\text{cm}^3$) [27] to estimate the mean apparent aragonite thickness of larval shells. However, this uniformly overestimates shell thickness slightly, because image analysis calculated shell area in silhouette, thereby underestimating the true three-dimensional shell surface. This bias should not affect comparisons with treatments for the same organism.

Results

Larval Development

Larval development proceeded from 96 h post fertilization to the eyed veliger and pediveliger stages (i.e., achieved competence) among the largest size classes in all treatments and across all experiments. Experiments *Cv* 1 and *Ca* 1 ran to larval ages of 30 d and 32 d, respectively.

Shell Area

Crassostrea virginica grew more slowly at elevated $p\text{CO}_2$ than at either 280 or 380 μatm treatments, as indicated by analysis of variance (*Cv* 1: $F_{3,8} = 6.605$, $n = 12$, $P = 0.015$, Fig. 2a). No significant differences in shell growth were observed across $p\text{CO}_2$ treatments for *C. ariakensis* (*Ca* 1: $F_{3,8} = 0.024$, $n = 12$, $P = 0.995$, Fig. 2b). Cumulative shell size frequencies for each species and treatment reveal: (1) that ninety percent of *C. virginica* shells exceeded the median size class of *C. ariakensis* and (2) clear treatment effects in *C. virginica* growth but none in *C. ariakensis* (Fig. 3).

Calcification

The amount of CaCO₃ biomineralized by *Crassostrea virginica* larvae decreased as $p\text{CO}_2$ increased (Fig. 2c). Analysis of variance confirmed significant differences among $p\text{CO}_2$ treatments (*Cv* 1: $F_{3,8} = 11.8026$, $n = 12$, $P = 0.0026$). By contrast, no clear trends or differences in biomineralization were apparent for *C. ariakensis* (*Ca* 1: $F_{3,7} = 0.6451$, $n = 11$, $P = 0.6103$, Fig. 2d).

Aragonite Saturation

In each experiment, aragonite conditions ranged from supersaturating ($\Omega_{\text{arag}} = 1.2–1.3$) to undersaturating ($\Omega_{\text{arag}} = 0.6$). *C. ariakensis* was unaffected by differences in aragonite saturation, but growth and calcification in *C. virginica* were significantly curtailed when $p\text{CO}_2$ was high and $\Omega_{\text{arag}} < 1.0$ (Fig. 2). Importantly, both species achieved net growth even when the saturation state of aragonite was well below 1.0.

Shell Thickness

Although cross sectional measurements of larval shells were not performed directly, we derived an estimate of average shell thickness that is theoretically attributable to aragonite volume. For each experimental treatment, the mean CaCO₃ content per shell was divided by the corresponding mean shell area ($\mu\text{g}/\mu\text{m}^2$). This value was then divided by the density of crystalline aragonite

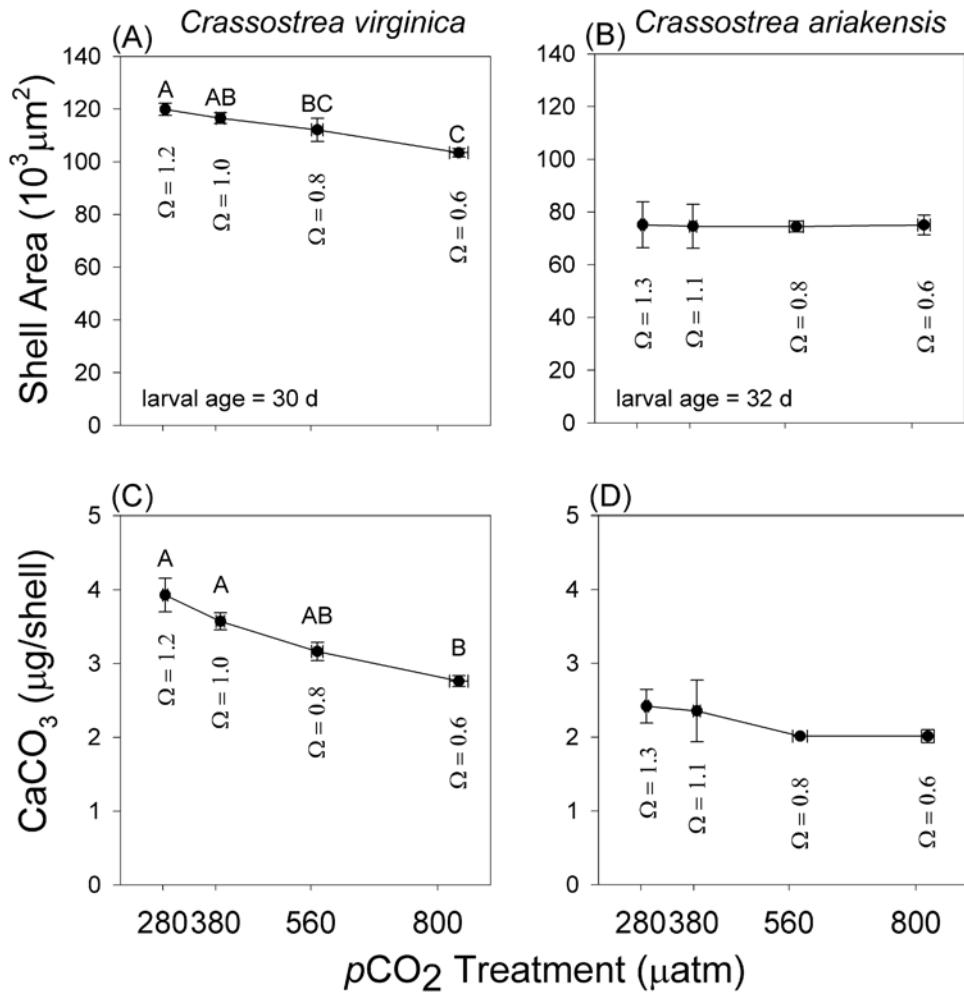


Figure 2. Effects of pCO₂ treatment on larval shell growth and calcification. Mean shell areas ± SEM (μm²) (panels A and B) and mean shell CaCO₃ content ± SEM (μg/shell) (panels C and D) reported by pCO₂ treatment ± SEM (μatm) for two oyster species. Corresponding aragonite saturation states (Ω_{arag}) are indicated for each treatment. Statistical differences determined by ANOVA and Tukey HSD tests. *C. virginica* grew more quickly than *C. ariakensis* under all treatments but experienced reduced growth at high pCO₂. The growth of *C. ariakensis* was not noticeably affected by elevated pCO₂ or aragonite saturation. *C. virginica* calcified less at elevated pCO₂ and Ω_{arag} < 1.0 (panel C), whereas calcification of *C. ariakensis* was not significantly influenced by elevated pCO₂ or aragonite saturation (panel D). doi:10.1371/journal.pone.0005661.g002

($2.93 \times 10^{-6} \mu\text{g}/\mu\text{m}^3$), leaving the mean apparent aragonite thickness in μm. The mean apparent aragonite thickness was strikingly similar across treatments and experiments (ranging from 9 to 11 μm) regardless of species or larval age (Fig. 4).

A second pair of similar experiments was conducted using a modified design (i.e., fed with a two algal species diet, use of a different strain of *C. ariakensis*, and a greater starting larval density). Because of ciliate contamination in one of the *C. virginica* treatments, the experiments had to be prematurely terminated. Nevertheless, at larval ages of 14 d, results were similar to those reported above, indicating significantly reduced shell area and calcium content for *C. virginica* at elevated pCO₂, but no significant pCO₂ effects for *C. ariakensis*.

Discussion

Most ocean acidification studies have focused on either warm water corals or pelagic biota [1–2], [28]. In general, marine fauna exhibit reductions in calcification at elevated pCO₂ (e.g., scleratinian corals, Gattuso et al. [29], Langdon [9]; pteropods,

Feely et al. [15], Orr et al. [8]; foraminifera, Bijima et al. [30]; see Fabry et al. [28] for review of marine faunal responses to elevated pCO₂). As a group, coccolithophores react more variably, appearing to show species-specific responses to elevated CO₂ [31], with Iglesias-Rodriguez et al. [32] reporting increased calcification in *Emiliana huxleyi* at pCO₂ at 750 μatm. Experiments to date have largely simulated elevated atmospheric CO₂ in fully marine settings (i.e., high salinity), meaning that water was supersaturated for CaCO₃ under all challenge treatments due the relatively high total alkalinity of the seawater.

In cases where marine mollusks have been subjected to aragonite undersaturation, the results have generally been deleterious. For example, Kurihara et al. [18] observed that calcification and shell formation was inhibited during early development of *Crassostrea gigas* larvae when seawater was undersaturated with respect to aragonite (Ω_{arag} = 0.68). When adult pteropods (*Clio pyramidata*) were exposed to seawater that was undersaturated for aragonite, their shells began to dissolve within 48 hours [8], [15], [28]. Green et al. [33] showed that newly set juvenile hard shell clams (*Mercenaria mercenaria*, <2.0 mm) grown in

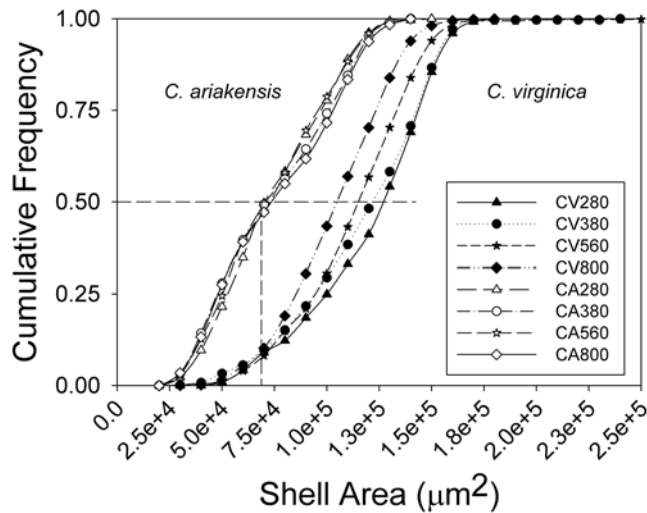


Figure 3. Effects of pCO₂ treatment on cumulative size frequency of larval shells (µm²/shell) for two oyster species. Black symbols and curves on right show results from *Crassostrea virginica*, experiment Cv 1, age=30 d. Open symbols and left curves plot *C. ariakensis* results (experiment Ca 1, age=32 d). pCO₂ treatments=280, 380, 560, 800 µatm. Number of shells per treatment=615±26.7 (mean±SEM). *C. virginica* grew to markedly larger sizes than *C. ariakensis*. Ninety percent of *C. virginica* shells were as large as or larger than the median *C. ariakensis* size class (dashed line). Growth of *C. virginica* was influenced by pCO₂ treatment and *C. ariakensis* was unaffected.
doi:10.1371/journal.pone.0005661.g003

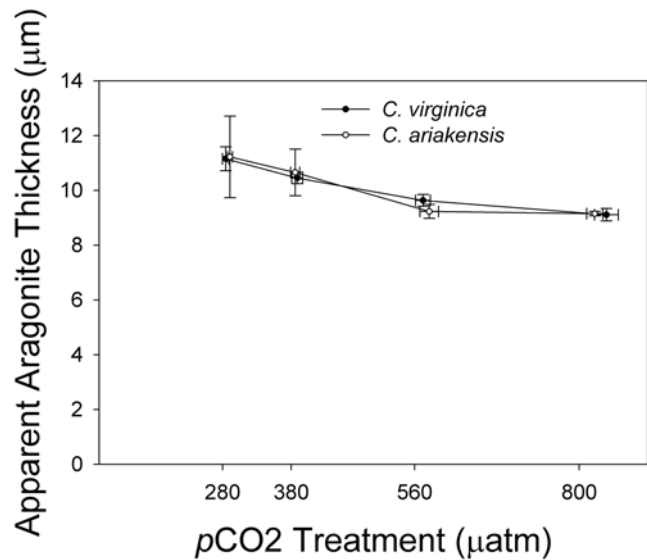


Figure 4. Effects of pCO₂ treatment on apparent aragonite shell thickness for two oyster species. Apparent aragonite thickness (µm) of larval shells = mean CaCO₃ content (µg)/mean shell area (µm²)/aragonite density (2.93 × 10⁻⁶ µg/µm³). Aragonite thickness was similar across species and varied little across pCO₂ treatments.
doi:10.1371/journal.pone.0005661.g004

benthic sediments with porewater undersaturated for CaCO₃ showed signs of corrosion and significantly increased mortality. Hall-Spencer et al. [34] have observed that the shells of adult gastropods living in ultra high pCO₂ habitats associated with natural underwater volcanic CO₂ vents in the Mediterranean also dissolve when Ω < 1.0.

In contrast, when *C. virginica* and *C. ariakensis* larvae were cultured continuously from 96 h post fertilization (D-stage) to ~30 d in typical estuarine conditions (salinity = 18 psu, TA ≈ 1225 µmol/kg) and exposed to elevated pCO₂ levels, both species appeared to grow, calcify and develop normally with no obvious morphological deformities, despite conditions of significant aragonite undersaturation (Ω_{arag} = 0.6–0.7). These findings demonstrate the physiological capacity of oyster larvae to withstand prolonged exposure (up to 28 days) to high pCO₂ and aragonite undersaturation, and run counter to expectations that aragonite shelled larvae should be especially prone to dissolution at high pCO₂ [28]. It should not be surprising that some mollusks can grow in undersaturated conditions; most fresh water mollusks are clearly well adapted to such conditions. Nevertheless, as atmospheric CO₂ continues to rise, all calcifying organisms will be encountering conditions that they have not experienced in recent geological history.

When Carrier and Palmer [35] studied the normal growth and development of *C. virginica* larval shells using scanning electron microscopy, they found uniform, extremely thin shells, 4–6 µm across a range of larvae 100–350 µm in length. The thinness of the shells for their area, ca. 2–4% of the total length, is likely an adaptation to planktonic living. In our study, both oyster species generated larval shells that were of similar mean thickness, regardless of pCO₂, Ω_{arag}, or shell area. We interpret the pattern of similar shell thickness as further evidence of normal larval shell development.

Regardless of their shared physiological tolerance to undersaturated aragonite, *C. virginica* and *C. ariakensis* have divergent responses to elevated pCO₂. *C. virginica* demonstrates consistently reduced growth and calcification at high pCO₂. Conversely, *C. ariakensis* larvae are not differentially affected by pCO₂, but do grow and calcify more slowly than *C. virginica* under all pCO₂ treatments. These differences suggest that CO₂-induced acidification is species-specific and will have unpredictable consequences in estuaries and nearshore ecosystems, even in closely related species.

From strictly thermodynamic considerations, the process of calcification in undersaturated environments (Ω_{arag} < 1.0) must require the input of exogenous energy to go forward. Weiner and Dove [36] describe two predominant processes of biomineralization widely used by marine and estuarine invertebrates. Extracellular mineralization is used by mollusks, scleratinian corals, bryozoans and some foraminifera and requires active pumping of ions or secretion and active transport of vesicles containing aqueous solutions of high ionic concentrations across cell membranes to produce conditions favorable for calcification. Coccolithophores, echinoderms, and other foraminifera use intracellular biomineralization, whereby nucleation of mineral structures occurs directly inside vesicles using similar active transport systems. The resulting carbonate structures are then secreted across the cell membrane (Weiner and Dove [36] and references therein). As aragonite becomes less saturated, the energetic costs for biomineralization should become progressively more expensive.

We do not perceive aragonite undersaturation as a “formal” barrier to calcification by marine or estuarine organisms in general. Aragonite saturation state is rather a convenient index to the ease with which biomineralization can be carried out. The higher the saturation state, the greater the relative availability of both Ca²⁺ and CO₃²⁻, and the lower the energy requirement for biochemical pumping to generate local supersaturation used when producing shell material. Just as species differ according to many environmental requirements, they likely differ in their ability or efficiency to supply energy for calcification. Marine taxa such as

corals and pteropods, which are well adapted to marine conditions where both Ca²⁺ and CO₃²⁻ have been abundant and relatively constant for millions of years, may have problems calcifying as CO₃²⁻ declines due to anthropogenic CO₂ enrichment, even though their water remains above saturation for both aragonite and calcite. In contrast, larvae of the euryhaline oysters *C. virginica* and *C. ariakensis*, cultured in mesohaline conditions with lower Ca²⁺ and CO₃²⁺ are able to generate and maintain aragonite shells when aragonite is somewhat below saturation. Although calcification is suppressed in lower saturation states for *C. virginica*, *C. ariakensis* is less affected by these conditions. Estuaries and open oceans differ substantially in their physical and chemical characteristics and dynamics, as do the evolutionary histories of their biota. For these reasons, expectations about the biological response to increasing atmospheric CO₂ and acidification ought to be different as well.

Because of extreme spatial and temporal heterogeneity in estuaries and coastal systems, uniform equilibrium with atmospheric CO₂ is neither expected nor achieved [37–38]. Estuarine habitats where shellfish settle and live often experience localized high pCO₂, up to as much as 6000 μatm, due to benthic respiration [39]. Our results suggest that for one key species, *C. virginica*, growth and calcification are diminished at relatively low enhancements of pCO₂, conditions that fall within the bounds of variation that currently exist in its native habitat. When combined with existing natural forcing, future increases to pCO₂ may influence significantly the success of larvae, and ultimately the success and distribution of these oysters in Chesapeake Bay and similar systems.

We predict that as atmospheric CO₂ concentrations increase, conditions in estuaries will be less favorable for calcification in all salinity zones and will pose new environmental stresses on their inhabitants. For some calcifying species, the added energetic burden of producing shell under less favorable conditions may restrict growth. Our results suggest that this will be the case for *C. virginica*, but not necessarily for *C. ariakensis*. As energy costs mount, calcifying biota may be faced with a trade-off, either to allocate more energy to calcification at the cost of growth and stored energy reserves, or to accept a lesser degree of calcification in exchange for greater growth and more stored energy.

When invertebrate larvae become physiologically stressed from chemical and physical factors encountered in their environment, they sometimes delay settlement and metamorphosis. Pechenik [40] lists temperature, salinity, nutrition, low dissolved oxygen, pollution, and ultraviolet radiation as possible stressors that may induce delayed metamorphosis, and further indicates that larvae are more sensitive than adults to such stressors (Pechenik [41] and references therein). High pCO₂ and reduced CaCO₃ saturation may produce similar effects. For planktotrophic larvae, even minor increases in energy expenditure for shell production may reduce energy reserves that are critical for larval growth and survival. Metamorphosis is energetically demanding and relies entirely on energy stores accumulated during the larval stage [41–42]. If larval growth and calcification are adversely affected by CO₂-induced acidification, larvae may well be energetically disadvantaged as they attempt metamorphosis, thereby suffering reduced survivorship and fitness. Furthermore, Pechenik [41] indicates that latent larval effects (i.e., impacts to post-metamorphic growth and mortality due to larval experience) may be common and species-specific among marine organisms.

Our findings suggest that as atmospheric CO₂ rises in coming decades, the larvae of *C. virginica*, and perhaps other estuarine species, will grow and calcify more slowly than today. Unless larvae can achieve settling/metamorphic competence more

efficiently in the future, slowed growth and calcification will lead to prolonged times in the water column, a situation that can result in higher pre-settlement larval mortality. Mortality from predation and disease increases the longer the larvae remain in the water column [43–44]. To illustrate the impact of relatively small changes in water column residence and mortality on recruitment, Kennedy [45] modeled the theoretical fate of *C. virginica* larval offspring from a single female, using natural daily mortality data from a variety of investigators. An 89% reduction in successful recruitment was postulated when metamorphosis was delayed five days (from 20 d to 25 d) and daily mortality rate increased from 20% to 25%. If higher water column mortality rates are coupled with lower larval quality at settlement (i.e., larvae settling with fewer energy reserves), then rates of recruitment could be expected to decrease in the future.

Whether or not prolonged time in the plankton and increased energy expenditures during larval growth and development from elevated CO₂ adversely affect later life stages and, ultimately, the population dynamics and ecology of *C. virginica* or other species is unknown. Clearly, the larvae of both *Crassostrea* species that we studied have the physiological capacity to grow and calcify in undersaturated conditions, but if and how increasing pCO₂ at all points in their habitat will adversely affect survivorship in the plankton as well as metamorphic success and recruitments dynamics remains to be tested.

On the Pacific coast of the United States, commercial oyster hatcheries are experiencing alarming and widespread difficulties keeping Pacific oyster (*C. gigas*) larvae alive in culture, with two of the largest hatcheries reporting production rates down by as much as 80%. Moreover, there has been little or no “natural” recruitment for several years in areas where *C. gigas* have previously established naturalized populations. (R. Downey, Pacific Coast Shellfish Growers Association, pers. comm.). In regions of upwelling along the continental shelf of western North America, Feely et al. [46] have determined that the pH and Ω_{arag} of surface waters are more acidic and have less aragonite saturation than expected. At water depths of 40–120 m in many locations along the coast, but at 0 m in the region near the California/Oregon border, pH was reported to be ~7.75 and Ω_{arag} < 1.0. Whether recent recruitment and aquaculture failures are linked to changes in carbonate chemistry are unknown, but should be investigated.

The natural spatial and temporal heterogeneity in salinity, alkalinity, pH, and pCO₂ in estuaries impose more complex environmental stresses on calcifying biota than in the open ocean where the environment is far less variable. As atmospheric CO₂ concentrations increase, the proportion of estuarine habitat undersaturated for aragonite will increase. In a high CO₂ world, we predict that the aragonite compensation point in estuaries (salinity isopleth where Ω_{arag} = 1.0) will shift seaward toward higher salinities. Because estuarine species have evolved in such variable environments, they may possess greater physiological capacity to respond to CO₂-induced acidification than fully marine taxa. Nevertheless, adaptive physiological tolerance of larvae may not be sufficient to sustain populations of calcifying benthic species, including widespread economically important fisheries, in the face of changing atmospheric CO₂.

Acknowledgments

We thank J.P. Megonigal, E. Jewett, P. Neale, and R. Osman for their consultation and technical advice. We thank S. Bonniwell and M. Luckenbach for supplying oyster larvae and culturing advice, as well as K. Erasmus, R. Lowenthal, and K. Stull, G. Riedel, D. Butera, S. Santagata, K. Klug, M. Hagan, and G.M. Ruiz for their contributions to these experiments and manuscript.

Author Contributions

Conceived and designed the experiments: AWM CS GFR. Performed the experiments: AWM ACR GFR. Analyzed the data: AWM ACR CS GFR.

References

- Royal Society (2005) Ocean acidification due to increasing carbon dioxide. Policy document 12/05 The Royal Society. 60 p.
- Kleypas JA, Feely RA, Fabry VJ, Langdon C, Sabine CL, et al. (2006) Impacts of Ocean Acidification on Coral Reefs and Other Marine Calcifiers: A guide for Future Research, report of a workshop held 18–20 April 2005, St. Petersburg, FL, sponsored by NSF, NOAA, and the U.S. Geological Survey. 88 p.
- Turley C, Blackford J, Widdicombe S, Lowe D, Nightingale PD, et al. (2006) Reviewing the impact of increased atmospheric CO₂ on oceanic pH and the marine ecosystem, In Schellnhuber HJ, Cramer W, Nakicenovic N, Wigley T, Yohe G, eds. Avoiding Dangerous Climate Change, Cambridge University Press, 8. pp 65–70.
- Sabine CL, Feely RA, Gruber N, Key RM, Lee K, et al. (2004) The Oceanic Sink for Anthropogenic CO₂. *Science* 305: 367–371.
- Canadell JG, Le Que re C, Raupach MR, Field CB, Buitenhuis ET, et al. (2007) Contributions to accelerating atmospheric CO₂ growth from economic activity, carbon intensity, and efficiency of natural sinks. *Proc Nat Acad Sci USA* 104(47): 18866–18870.
- Caldeira K, Wickett ME (2003) Anthropogenic carbon and ocean pH. *Nature* 425: 365.
- Caldeira K, Wickett ME (2005) Ocean model predictions of chemistry changes from carbon dioxide emissions to the atmosphere and ocean. *J Geophys Res Oceans* 110(C9), C09504: doi:10.1029/2004JC002671.
- Orr JC, Fabry VJ, Aumont O, Bopp L, Doney SC, et al. (2005) Anthropogenic ocean acidification over the twenty-first century and its impact on calcifying organisms. *Nature* 437(7059): 681–686.
- Langdon C (2002) Review of experimental evidence for effects of CO₂ on calcification of reef builders. *Proceedings of the 9th International Coral Reef Symposium 2*: 1091–1098.
- Gazeau F, Quiblier C, Jansen JM, Gattuso J-P, Middelburg JJ, et al. (2007) Impact of elevated CO₂ on shellfish calcification. *Geophys Res Lett* 34: L07603. doi:10.1029/2006GL028554.
- Carpenter KE, Abrar M, Aeby G, Aronson RB, Banks S, et al. (2008) One-third of reef-building corals face elevated extinction risk from climate change and local impacts. *Science* 321: 560–563.
- Wong GT (1979) Alkalinity and pH in the southern Chesapeake Bay and the James River estuary. *Limnol Oceanogr* 24: 970–977.
- Cai W-J, Wang Y (1998) The chemistry, fluxes, and sources of carbon dioxide in the estuarine waters of the Satilla and Althamaha Rivers, Georgia. *Limnol Oceanogr* 43: 657–668.
- Doney SC, Fabry VJ, Feely RA, Kleypas JA (2009) Ocean acidification: the other CO₂ problem. *Annu Rev Mar Sci* 1: 169–192.
- Feely RA, Sabine CL, Lee K, Berelson W, Kleypas J, et al. (2004) Impact of anthropogenic CO₂ on the CaCO₃ system in the oceans. *Science* 305: 362–366.
- Salisbury J, Green M, Hunt C, Campbell J (2008) Coastal acidification by rivers: A new threat to shellfish? *EOS Trans AGU* 89: 513.
- Weiss IM, Tuross N, Addadi L, Weiner S (2002) Mollusc larval shell formation: amorphous calcium carbonate is the precursor phase for aragonite. *J Exp Biol* 293: 478–491.
- Kurihara H, Kato S, Ishimatsu A (2007) Effect of increased seawater pCO₂ on early development of the oyster *Crassostrea gigas*. *Aquat Biol* 1: 91–98.
- Kirby MX (2004) Fishing down the coast: Historical expansion and collapse of oyster fisheries along continental margins. *Proc Acad Nat Sci U S A* 101: 13096–13099.
- Breitburg DL, Riedel GF (2005) Multiple Stressors in Marine Systems. In: EA Norse and LB Crowder editors. *Marine Conservation Biology: The Science of Maintaining the Sea's Biodiversity (MCB)*, Island Press, Washington, D.C. pp 167–182.
- OSB (2004) Nonnative Oysters in Chesapeake Bay. Ocean Studies Board, Division on Earth and Life Studies, National Research Council of the National Academies, The National Academies Press, Washington, D.C., 325 p.
- Breese WP, Malouf RE (1977) Hatchery rearing techniques for *Crassostrea rivularis*. *Aquaculture* 12: 123–126.
- Edmond JM (1970) High precision determination of titration alkalinity and total carbon dioxide content of sea water by potentiometric titration. *Deep Sea Res* 17: 737–750.
- Leggett J, Pepper WJ, Swart RJ (1992) Emissions Scenarios for the IPCC: An Update. In: J. T. Houghton, B. A. Callander, S. K. Varney editors. *Climate Change 1992: The Supplementary Report to the IPCC Scientific Assessment*, Cambridge University Press, Cambridge. pp 69–95.
- Pelletier G, Lewis E, Wallace D (2007) CO₂sys.xls: a calculator for the CO₂ system in seawater for Microsoft Excel/VBA, Washington State Department of Ecology/Brookhaven National Laboratory, Olympia, WA/Upton, NY, USA.
- Scion Image for Windows. Release alpha 4.0.3.2, © 2000–2001, Scion Corporation, www.scioncorp.com.
- Lide DR (2007) *CRC Handbook of Chemistry and Physics*, 88th Edition (Crc Handbook of Chemistry and Physics). CRC.
- Fabry VJ, Seibel BA, Feely RA, Orr JC (2008) Impacts of ocean acidification on marine fauna and ecosystem processes. *ICES J Mar Sci* 65: 414–432.
- Gattuso JP, Frankignoulle M, Bourge I, Romaine S, Buddemeier RW (1998) Effect of calcium carbonate saturation of seawater on coral calcification. *Global Planet Change* 18: 37–46.
- Bijima J, Honisch B, Zeebe RE (2002) Impact of the ocean carbonate chemistry on living foraminifera shell weight: comment on “Carbonate ion concentration in glacial-age deepwaters of the Caribbean Sea” by WS Broecker and E Clark. *Geochem. Geophys. Geosyst* 3: 1064. doi:10.1029/2002GC000388.
- Fabry VJ (2008) Marine calcifiers in a high-CO₂ ocean. *Science* 320: 120–122.
- Iglesias-Rodriguez MD, Halloran PR, Rickaby REM, Hall IR, Colmenero-Hidalgo E, et al. (2008) Phytoplankton calcification in a high-CO₂ world. *Science* 320: 336–340.
- Green MA, Jones ME, Boudreau CL, Moore RL, Westman BA (2004) Dissolution mortality of juvenile bivalves in coastal marine deposits. *Limnol Oceanogr* 49(3): 727–734.
- Hall-Spencer JM, Rodolfo-Metalpa M, Martin S, Ransome E, Fine M, et al. (2008) Volcanic carbon dioxide vents show ecosystem effects of ocean acidification. *Nature-online*; doi:10.1038/nature07051.
- Carriker MR, Palmer RE (1979) Ultrastructural morphogenesis of prodissoconch and early dissoconch valves of the oyster *Crassostrea virginica*. *Proc Natl Shellfish. Assoc* 69: 102–128.
- Weiner S, Dove PM (2003) An overview of biomineralization processes and the problem of the vital effect. In Dove PM, DeYoreo JJ, Weiner S, editors. *Biomaterialization, Reviews in Mineralogy & Geochemistry* 54. pp 1–29.
- Smith SV, Hollibaugh JT (1997) Annual cycle and interannual variability of ecosystem metabolism in a temperate climate embayment. *Ecol Monogr* 67: 509–533.
- Frankignoulle M, Abril G, Borges A, Bourge I, Canon C, et al. (1998) Carbon dioxide emission from European estuaries. *Science* 282: 434–436.
- Cai W-J, Pomeroy LR, Moran MA, Wang Y (1999) Oxygen and carbon dioxide mass balance for the estuarine-intertidal marsh complex of five rivers in the southeastern U.S. *Limnol. Oceanogr* 44: 639–649.
- Pechevik JA (1999) On the advantages and disadvantages of larval stages in benthic marine invertebrate life cycles. *Mar Ecol Prog Ser* 177: 269–297.
- Pechevik JA (2006) Larval experience and latent effects—metamorphosis is not a new beginning. *Integr Comp Biol* 46: 323–333.
- Burke K, Battler E, Miron G, Ouellette M, Tremblay R (2008) Larval quality of a nonnative bivalve species (European oyster, *Ostrea edulis*) off the east Canadian coast. *J Shellfish Res* 27(4): 701–710.
- Morgan SG (1996) Life and death in the plankton: larval mortality and adaptation. In: L. McEdward, editor. *Ecology of Marine Invertebrate Larvae*, CRC Press, New York, NY. pp 279–321.
- Underwood AJ, Fairweather PG (1989) Supply-side ecology and benthic marine assemblages. *Trends Ecol Evol* 4: 16–20.
- Kennedy VS (1996) Biology of larvae and spat. In: VS Kennedy, RIE Newell and AF Eble, editors. *The eastern oyster, Crassostrea virginica*, Maryland Sea Grant, College Park, MD. pp 371–441.
- Feely RA, Sabine CL, Hernandez-Ayon JM, Janson D, Hales B (2008) Evidence for upwelling of corrosive acidified water onto the continental shelf. *Science* 320(5882): 1490–1492.
- Chesapeake Bay Program. Chesapeake Bay Program Water Quality Database 1984–present. Accessed October 20, 2008, from <http://www.chesapeakebay.net/wquality.htm>.

Contributed reagents/materials/analysis tools: AWM ACR CS GFR. Wrote the paper: AWM ACR CS GFR.