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The Effects of Ocean Acidification on the Development and Calcification of the Larval Shells of the Red Abalone *Haliotis rufescens* Swainson, 1822

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Summary

This study focuses on the effects of increasing atmospheric carbon dioxide and decreasing pH on the development and calcification of larval red abalone shells. Experimental bowls were inoculated with 2 mL of a homogenous mixture of red abalone embryos. Six replicate bowls were placed inside each of four plexiglass gas chambers: a control chamber injected with 200 cc of air, a chamber injected with 200 cc of 10% CO_2, another injected with 400 cc of 10% CO_2, and a fourth chamber injected with 600 cc of 10% CO_2. After 48 hours, the chambers were opened, and the abalone larvae were transferred into culture flasks, fixed with formalin, and examined under an inverted compound microscope. Statistically significant differences between the treatments (increasing levels of CO_2 and decreasing pH) on red abalone shell development and calcification were observed even at the lowest volume of CO_2 injected (i.e., 200 cc of CO_2).

These results strongly suggest that increasing levels of CO_2 and decreasing levels of pH result in abnormal abalone larval development. The results also support the use of CO_2 injection into gas chambers to mimic the real-life processes of ocean acidification and the use of the EPA-approved red abalone chronic toxicity test protocol, which is typically used to assess toxicity of sewage and treated sewage (effluent), as an appropriate method for investigating the effects of ocean acidification on the larval development of the abalone.

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Introduction

Since the Industrial Revolution, the burning of fossil fuels has led to a sharp increase in atmospheric CO_2 levels. The ocean absorbs approximately a quarter of this CO_2, resulting in the production of carbonic acid (1). As the carbonic acid dissociates, hydrogen ions are formed, causing the ocean to become more acidic, a phenomenon known as ocean acidification. By the end of this century, the acidity of the ocean surface is predicted to decrease by 0.3 to 0.5 pH units (2,3). This drop in pH will result in increased availability of hydrogen ions to combine with carbonate ions, thereby decreasing the availability of carbonate ions for calcifying organisms.

Ocean acidification has already impacted many species of marine organisms that require calcium carbonate to calcify their shells. Coccolithophores, foraminiferans, pteropods, echinoderms, corals, coralline algae, and mollusks are all examples of organisms that have been adversely affected by ocean acidification (4). Larval sea urchins, when put in more acidic solutions (decrease of 0.3 to 0.5 pH units), have been found to have considerably less calcification of their spines (5). The growth of coral reefs, which are extremely important ecosystems that support a diverse community of marine organisms, has also been impacted as a result of ocean acidification. Since 1990, coral polyps have been forming thinner and more fragile $CaCO_3$ skeletons (6). For a comprehensive review of the impacts of ocean warming and acidification on various marine invertebrates, see Byrne, 2011 (7).

The red abalone *Haliotis rufescens* Swainson, 1822 is an edible mollusk, the largest abalone species in the world, and commonly found in northern California (8). It typically inhabits subtidal rocky areas to approximately 40 m depth and its biogeographic range is from Oregon south to Baja California (9).

Unfortunately, the red abalone populations have declined significantly as a result of overharvesting, predation by sea otters, and disease, i.e., chronic wasting disease known as withering syndrome (10). Since much of the wild populations of abalone have been decimated, culturing abalone has become a successful mariculture. Abalone culturing began in California in the 1960s and has now become a multimillion dollar business (11). In 2008, California abalone culture was valued at about $9 million (12) and worldwide, the value is estimated between $450 to $900 million per year (13).

Due to the ecologic and economic importance of abalone species, as well as their potential vulnerability to ocean acidification, several studies have documented negative effects of ocean acidification on abalone development. When raised at a pH of 7.6-7.8, *Haliotis coccoradiata* Reeve, 1846 larval shells developed abnormally and were under calcified (14). In the study on northern abalone (*Haliotis kamtschatkana*) by Crim et al. (15), 60% of the larvae kept at 800 ppm of CO_2 developed normal shells, many of which were

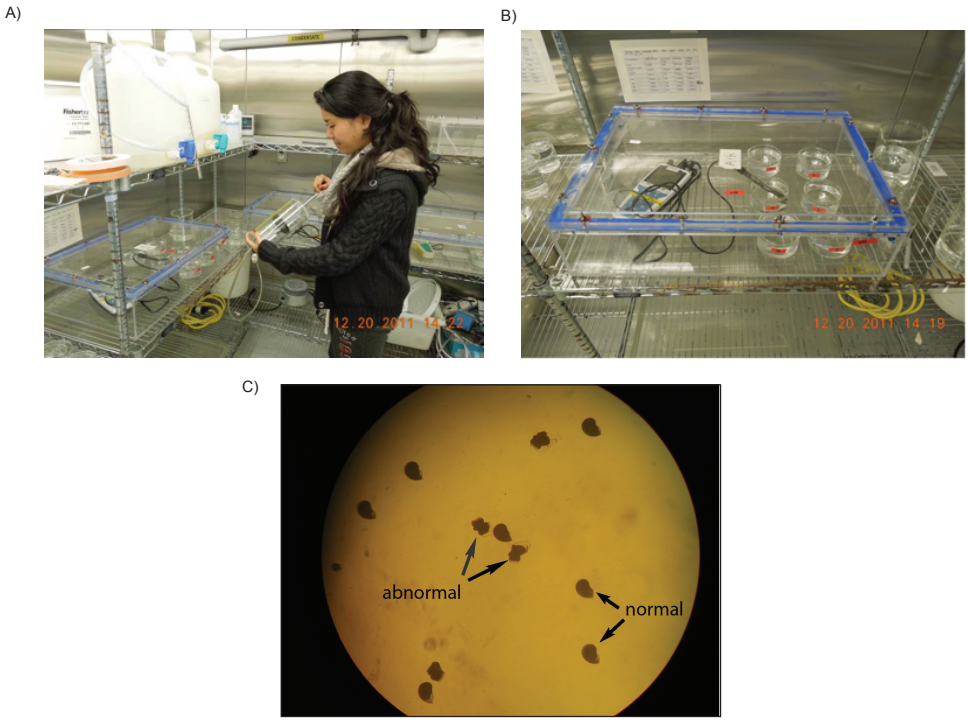

Figure 1: A) Injection of 10% CO_2 into experimental plexiglass gas chambers. B) Dissolved oxygen (DO), pH, and temperature meter inside plexiglass gas chamber with probe inside water quality bowl. C) Red abalone *Haliotis rufescens* larvae with normal and abnormal shells. Note: unusual shape and lack of calcification in abnormal shells. Photos courtesy of EMD, City of Los Angeles.

smaller than the controls. At 1800 ppm, the abalone larvae either developed an extremely abnormal shell or lacked a shell. In another study on red abalone by Zippay and Hoffman (4), lower pH was also shown to have a negative effect on the abalone's thermal tolerance and late-veliger survivorship. These combined results clearly indicate that ocean acidification will have a detrimental impact on abalone growth and survival.

Other studies on mollusks such as California mussels (*Mytilus californianus*) have shown that more acidic waters led to the development of smaller, thinner shells and a third less body mass (16). Studies on other marine calcifying organisms such as sea urchins (17) and corals also have shown that ocean acidification negatively impacts calcification (7, 14) .

This study investigated the effects of increasing atmospheric CO_2 and decreasing pH on the development of the embryos and calcification of the larval shells of red abalone *Haliotis rufescens*. Furthermore, the use of the EPA-approved chronic red abalone toxicity test (EPA/600/R-95/163), which is a standard test method used to assess the toxicity of sewage treatment plant effluent, as an appropriate method for studying the effects of ocean acidification on red abalone larvae, as well as using air-tight plexiglass gas chambers injected with different amounts of CO_2, was evaluated.

Results

In order to replicate environmental conditions and evaluate the effects of increasing CO_2 concentration and decreasing pH levels on the development of red abalone shells, four plexiglass chambers were used. The control chamber was injected with 200 cc of air, and the other three chambers were injected with increasing

Treatments	pH prior to CO_2 Injection	pH 24 hrs After CO_2 Injection	pH 48 hrs After CO_2 Injection	Average pH after CO_2 Injection
Blank (Control)	8.18	7.91	8.00	8.03[a]
200 cc CO_2	8.18	7.74	7.65	7.70[b]
400 cc CO_2	8.17	7.50	7.41	7.46[b]
600 cc CO_2	8.16	7.43	7.30	7.36[b]

[a] Mean of pH prior to and after CO_2 injection.
[b] Mean of pH after CO_2 injection.

Table 1: Initial pH prior to CO_2 injection into plexiglass chambers and after 24 and 48 hours following injection of 200, 400, and 600 cc of 10% CO_2.

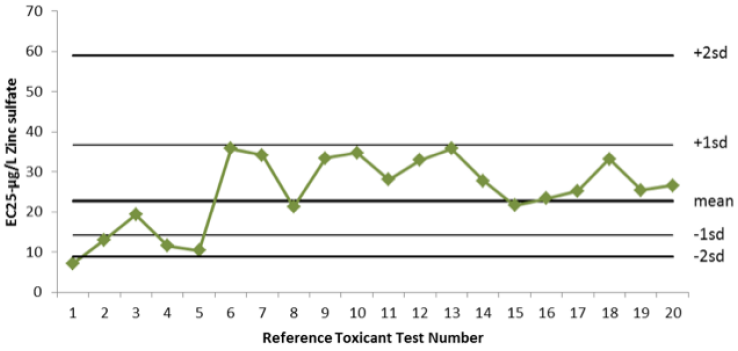

Figure 2: Control chart of reference toxicant tests with n1-n19 conducted by EMD (City of Los Angeles) staff and n20 representing the present test initiated on December 20, 2011. Of note, the present reference toxicant test falls within the ± 1 SD and is extremely close to the mean of 22.92 µg/L, indicating that the abalone embryos tested in this experiment were healthy and fell within the normal range of sensitivity to the reference toxicant ($ZnSO_4$). Abbreviations: EC25 = effective concentration 25, which represents the concentration of a toxicant that induces a response in one fourth of the total number of test organisms at that concentration; sd= standard deviation.

volumes of CO_2 (100, 400, and 600 cc of 10% CO_2). Each chamber contained six replicate bowls, and each replicate bowl contained 2 mL of a homogenous mixture of red abalone embryos in 200 mL of seawater (**Figure 1A and 1B**). Water quality measurements were taken prior to injection, after the addition of embryos, and after 48 hrs of incubation in the plexiglass chambers. Embryo development was monitored microscopically after 48 hrs of incubation in the plexiglass chambers. **Figure 1C** illustrates the differences between normal and abnormal abalone shells.

The water quality bowls in all four plexiglass gas chambers had similar pH levels (8.16-8.18) (**Table 1**) prior to injection of CO_2. After injection of 200 cc of air into the control chamber, the pH was 8.00 at the close of the test (48 hours after test initiation). The pH of the gas chambers injected with CO_2 ranged from 7.65 to 7.30 (**Table 1**). Salinity remained constant at 34‰, while the temperature of the water and DO varied slightly, ranging from 14.5 °C to 15.5 °C and from 7.36 to 8.03 mg/L, respectively.

Larval shell development in the controls was 99.67% normal, which passes the test acceptability criterion of at least 80% normal embryo shell development in the controls for this EPA-approved toxicity test. In the reference toxicant test, the abalone embryo shell development response (LOEL=32 µg/L) was significantly different from the controls at 56 µg/L. This meets the test acceptability criteria of a valid reference toxicant test. The designated point estimate, EC25, which is the concentration of a toxicant that induces a response in one-fourth of the total number of test organisms, fell within the upper and lower limits of the control chart, coming extremely close to the mean of 22.92 µg/L (n=19) (**Figure 2**).

Increasing levels of CO_2 and decreasing levels of pH caused more abalone to exhibit abnormal shell development (**Figure 3**). In the control chamber, 99.67% of the abalone shells developed normally, compared with 91.17% in the chamber injected with 200 cc CO_2, 29.83% in the 400 cc CO_2 chamber, and only 1.33% in the 600 mL CO_2 chamber (**Table 2**). Equal variance (p = 0.03) was determined by Bartlett Equality of Variance, and a non-normal distribution by Shapiro-Wilk Test for Normality (p = 0.003). Both a one-way analysis of variance (ANOVA) and a Steel's Many-One Rank Test indicate that a significant difference exists between the percent normal larval shell development in the treatment bowls and in the controls [$F(3,15) = 1256$; $p < 0.0001$ for ANOVA, p = 0.006 for all three treatments for Steel's Many-One Rank Test).

A similar trend was observed for percent normal calcification of the larval red abalone shell cultured under increasing serial levels of 10% CO_2. The controls exhibited 99.33% normal calcification (i.e., opaque larval shells), with the 200 cc, 400 cc, and 600 cc treatment bowls displaying decreasing percentages of normal shell calcification, 91.54%, 67.17%, and 29.11%, respectively (**Table 3, Figure 4**). Homogeneity of variance as determined by the Bartlett Equality of Variance indicates unequal variance (p = 0.006), and the Shapiro-Wilk Test for Normality (p = 0.0005) reflects a non-normal distribution. A one-way analysis of variance (ANOVA) revealed that a significant difference exists between the percent normal larval shell calcification results in the treatment bowls and the controls [$F(3,15) = 141.6$; p = <0.0001) as does a Steel's Many-One Rank Test (p = 0.009, 0.006, and 0.006, for the 200 cc, 400 cc, and 600 cc treatments, respectively) (**Table 4**). Although statistically significant differences exist between all three treatments (i.e., 200, 400, and 600 cc CO_2) and the controls for both shell development and shell calcification, the 91.17% and 91.54% normal development and calcification in the

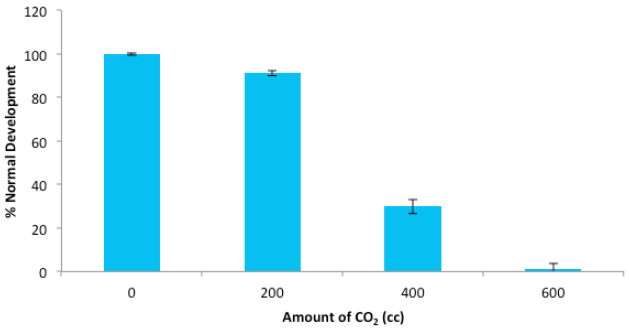

Figure 3: Mean percent normal red abalone larval shell development after exposure to elevated CO_2 levels (200, 400, 600 cc of 10% CO_2). Error bars represent 1 SD.

Treatments	Number of bowls	% Normal Calcification	Std. Dev.	95% LCL	95% UCL
Blank (Control)	6	99.67	0.52	98.64	100.70
200 cc CO_2	6	91.17	1.17	88.83	93.50
400 cc CO_2	6	29.83	3.19	23.46	36.21
600 cc CO_2	6	1.33	2.34	-3.34	6.01

Table 2: Percent normal shell development of larval red abalone with increasing levels of CO_2.

200 cc treatment, respectively, may not be considered biologically different from the controls as defined by EPA.

Discussion

These results strongly suggest that increasing levels of CO_2 and decreasing levels of pH cause the red abalone shells to develop abnormally. Additionally, conducting this test by injecting CO_2 into airtight gas chambers closely mimics the real-life processes of ocean acidification. Finally, the EPA-approved abalone chronic toxicity test is a valid method for investigating the effects of ocean acidification on larval abalone.

The method of injecting CO_2 into the chambers proved effective in lowering the pH of the seawater significantly. It also successfully simulated what happens in the ocean: CO_2 is absorbed, rather than bubbled into the water. Therefore, the injected CO_2 into gas-tight plexiglass gas chambers closely mimics the actual environmental conditions. In the chamber injected with 400 cc CO_2, the pH decreased by 0.76 units. In the rocky intertidal waters, where red abalone inhabits, the pH is expected to decrease by 0.3-0.5 units by the year 2100 (18, 2). This level of pH reduction is predicted to cause larval red abalone shells to develop the deformities exhibited by the abalone in the 200 cc CO_2 chamber, where pH decreased by a similar amount 0.3. By the year 2250, the pH of the ocean surface is estimated to drop by 0.6-0.7 units, which will likely cause deformities in shells similar to those observed in the 400 cc CO_2 chamber.

The results of this research project suggest that the EPA-approved abalone chronic toxicity test may be used as a valid method to study the effects of ocean acidification on the larval development of abalone and that the strict test acceptability criteria help to ensure a valid test. In addition, the acid-washing protocol, water quality measurements, reference toxicant test, and other requirements of this EPA method ensure standardization of the testing method and may eliminate or greatly reduce confounding factors that increase the variability of the test.

The adverse impacts of decreasing pH on larval red abalone shell development are significant both economically and ecologically. Calcification and shell development were severely affected for the abalone in both the 400 cc and 600 cc CO_2 chambers. More than half of the animals developed non-calcified, deformed, or broken shells. Studies on *Haliotis kamtschatkana* by Crim et al., *H. coccoradiata* by Byrne et al., and *H. rufescens* by Zippy and Hofmann clearly have shown that ocean acidification will impair abalone growth and survival (15, 14, 4). Both the northern and the red abalone are endangered; therefore, ocean acidification will only contribute to their population decline.

Further investigations on the effects of ocean acidification on abalone survival in later stages of life should be conducted in order to fully understand how populations are being affected. Effects on fertilization must also be studied to understand if the abalone can still reproduce effectively and at a normal rate.

Materials and Methods

Obtaining Abalone Embryos

Four gravid female and four gravid male abalones were ordered from American Abalone Farms (Davenport, CA). Prior to inducing spawning, the abalones were kept in a marine aquarium and fed fresh giant kelp *Macrocystis pyrifera* (Linnaeus). Female and male abalones were placed in two separate buckets and maintained in aerated seawater at 15 °C. One male was removed from the spawning process because it was crawling up the sides of the bucket. The females were induced to spawn by adding 30 mL Tris solution (12.1 g of Tris buffer mixed with 50 mL of deionized water) and 25 mL H_2O_2 solution (10 mL of 30% H_2O_2 mixed with 40 mL of deionized water). After five minutes, 30 mL of the Tris solution and 25 mL of the H_2O_2 solution were added to the male abalone bucket and mixed. The abalones were maintained at 15 °C and the water aerated. After 2.5 hours, the female abalones and the

Treatments	Number of bowls	% Normal Calcification	Std. Dev.	95% LCL	95% UCL
Blank (Control)	6	99.33	1.21	96.91	101.75
200 cc CO_2	6	91.54	9.29	72.95	110.13
400 cc CO_2	6	69.17	2.48	64.20	74.14
600 cc CO_2	6	29.11	5.70	17.72	40.50

Table 3: Percent normal calcification of larval red abalone shells with increasing levels of CO_2.

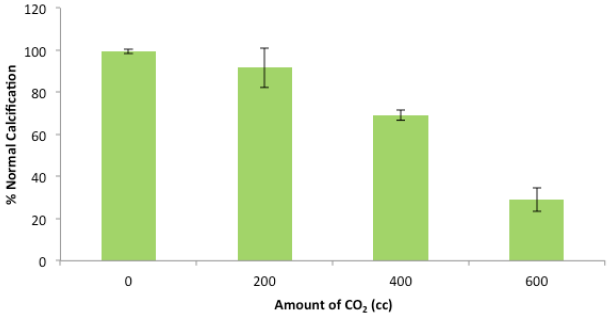

Figure 4: Mean percent normal calcification of red abalone larval shell after exposure to elevated CO_2 levels (200, 400, 600 cc of 10% CO_2). Error bars represent 1 SD.

eggs were rinsed four times before transferring the eggs to a third, fertilization bucket.

Upon release from the males, the sperm were siphoned into a beaker every 15 minutes. Then, 200 mL of water containing fresh sperm was poured into the fertilization bucket with the eggs. 15 °C seawater was added at a low-flow rate, mixing the eggs and sperm, until the fertilization bucket was three-quarters full. The eggs were then allowed to settle. The water in the fertilization bucket was siphoned off, taking care not to include any eggs, and seawater was added back again at a low-flow rate. The eggs were then allowed to settle once again. After 15 minutes, fertilization was deemed to be complete, and the fertilized eggs were siphoned into a 1000 mL beaker. Approximately 300 embryos were pipetted into each experimental bowl.

Reference Toxicant Test

A reference toxicant test was conducted alongside the controls and the treatment bowls to determine the sensitivity of the red abalone embryos to a known concentration of toxicant. The abalone embryos were inoculated into different concentrations of $ZnSO_4$ (0, 10, 18, 32, 56, and 100 µg/L). After 48 hours in the environmental chamber maintained at 15 °C, the embryos were screened, preserved, and examined under an inverted compound microscope to determine the LOEL (lowest observable effect level), i.e., the lowest toxicant level at which normal shell development was significantly different than the controls. This level was then compared to 19 previous Environmental Monitoring Division (EMD), Bureau of Sanitation, City of Los Angeles reference toxicant tests to determine if the test abalone embryos fell near the mean and within the upper and lower control limits (± 1 or 2 standard deviations).

CO_2 Injection and Introduction of Embryos

To investigate the effect of increasing CO_2 and decreasing pH, four plexiglass gas chambers (61 cm x 41.9 cm x 11.3 cm) were used: a control injected with 200 cc of air, one injected with 100 cc of 10% CO_2, another injected with 400 cc of 10% CO_2, and the fourth chamber injected with 600 cc of 10% CO_2. Each chamber contained six replicate bowls, which were detergent-washed, deionized water-rinsed, acetone-rinsed, 2N HCl acid-rinsed, and finally deionized water-rinsed [City of Los Angeles, Environmental Monitoring Division (EMD) acid-washing SOP]. The bowls were then filled with 200 mL of seawater. Initial dissolved oxygen (DO), pH, salinity, and water temperature were all measured with Hach HQ30d portable dissolved oxygen meter with LDI101 rugged optical probe, Thermo Scientific Orion 3-Star plus pH portable meter, Fisher Scientific handheld refractometer (catalog # 13-946-27), and Thermo Scientific Orion Star A222 conductivity portable meter, respectively, calibrated using manufacturers' specifications.

Two mL of a homogenous mixture of fertilized embryos were transferred into the bowls. After the embryos were pipetted into each bowl, water quality measurements were recorded. The lids of the chambers were bolted down with wing nuts, and the appropriate amount of air or CO_2 was injected into the chambers (**Figure 1A**). Each chamber contained a portable pH meter that provided a continuous readout of pH and temperature for one bowl in each experimental chamber (**Figure 1B**). All the bowls were incubated for 48 hours at 15 °C ± 1 °C within a walk-in environmental chamber with a 16:8 light to dark photoperiod.

Closing the Experiment

After 48 hours, final water quality measurements (DO, pH, salinity, and temperature) were recorded. The contents of the bowls were carefully poured into a 37µ-mesh screen. The abalone larvae were collected using seawater to rinse the edge of the screen. A funnel was placed over an open 50 mL culture flask pre-labeled with gas-chamber identification and bowl number. The screen was inverted over the funnel and the abalone

Control vs Treatment	Test Stat	Critical	DF	Ties	P-Value	Decision (α: 5%)
Blank vs 200 cc CO_2	22	26	10	1	<0.009	Significant Effect
Blank vs 400 cc CO_2	21	26	10	0	<0.006	Significant Effect
Blank vs 600 cc CO_2	21	26	10	0	<0.006	Significant Effect

Table 4: Results of Steel's Many-One Rank Test of normal larval shell calcification of the red abalone with increasing serial concentrations of CO_2.

larvae were rinsed into the flask with seawater. 10% buffered formalin was added to the culture flasks to preserve the larvae. The volume of the seawater in the culture flasks was doubled to create a 5% buffered formalin solution. The culture flasks were sealed tightly and gently shaken to mix the solution. One hundred larvae from each were examined at 100x magnification using an inverted microscope. A hand-counter was used to count normal and abnormal larval shell developments, as well as normal, partial, or completely absent calcification of the larval shell. Normal shell development produces smooth, curved shells striated with calcareous deposits, while abnormal development included severe deformations such as shells separated from the animal, as shown in **Figure 1C**. Calcification of the shell was indicated by the shell's opacity. Opaque shells indicate normal calcification, while partially or completely clear shells reflect partial or absent calcification.

Data Analysis

Data were entered into the Comprehensive Environmental Toxicity Information System™ (CETIS), a toxicity data analysis and database software application written and published by Tidepool Scientific Software, to perform tests for equal variances, normality, and to conduct statistical analyses to determine any significant differences between any (or all) the treatments and the controls.

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References

1. Sabine, C. L., R. A. Feely, N. Gruber, R.M. Key, K. Lee, J. L. Bullister, R. Wanninkhof, C. S. Wong, D. W. R. Wallace, B. Tilbrook, F. J. Millero, T.-H. Peng, A. Kozyr, T. Ono, and A. F. Rios. 2004. The ocean sink for anthropogenic CO_2. *Science* 305: 367-371.
2. Feely, R. A., S. C. Doney, and S. R. Cooley. 2009. Ocean acidification: Present conditions and future changes in a high-CO_2 world. *Oceanography* 22 (4): 36-47.
3. Doney, S. C., V. J. Fabry, R. A. Feely, and J. A. Kleypas. 2009. Ocean acidification: the other CO_2 problem. *Annual Review of Marine Science* 1: 169-192.
4. Zippay, M. L. and G.E. Hofmann. 2010. Effect of pH on gene expression and thermal tolerance of early life history stages of red abalone (*Haliotis rufescens*). *Journal of Shellfish Research* 29(2): 429-439.
5. Brennand, H.S., N. Soars, S. A. Dworjanyn, A. R. Davis, M. Byrne. 2010. Impact of ocean warming and ocean acidification on larval development and calcification in the sea urchin *Tripneustes gratilla*. *PLoS ONE* 5(6): e11372.
6. Kleypas, J. A. and K. K. Yates. 2009. Coral Reefs and Ocean Acidification. *Oceanography* 22 (4): 108-117.
7. Byrne, M. 2011. Impact of ocean warming and ocean acidification on marine invertebrate life history stages: vulnerabilities and potential for persistence in a changing ocean. *Oceanography and Marine Biology: An Annual Review* 49: 1-42.
8. Hines, A.H. and J.S Pearse. 1982. Abalones, shells, and sea otters: dynamics of prey populations in central California. *Ecology*, 63: 1547-1560.
9. California Fish and Game. 2005. Abalone recovery and management plan. California Department of Fish and Game Marine Region, Monterey, California, USA.
10. Moore, J.D., T.T. Robbins, and C.S. Friedman. 2000. Withering syndrome in farmed red abalone *Haliotis rufescens*: thermal induction and association with a gastrointestinal *Rickettsiales*-like prokaryote. *Journal of Aquatic Animal Health*, 12(1); 26-34.
11. Leighton, D.L. 1989. Abalone (genus *Haliotis*) mariculture on the North American Pacific Coast. U.S. Department of Commerce, *Fishery Bulletin*, 87: 689-702.
12. Moore, T.O. and J.D. Moore. 2008. Culture of abalone, *Haliotis* spp. Status of the Fisheries Report. 18-1 to 18-6.
13. Cook, P.A. and H.R. Gordon. 2010. World abalone, supply, market, and pricing. *Journal of Shellfish Research*, 29(3); 569-571.
14. Byrne, M., M. Ho, E. Wong, N.A. Soars, P. Selvakumaraswamy, H. Shepard-Brennand, S.A. Dworjanyn, and A.R. Davis. 2010. Unshelled abalone and corrupted urchins: development of marine calcifiers in a changing ocean. *Proceedings of the Royal Society B* 276: 1883-1935.
15. Crim, R. N., J. M. Sunday, and C. D.G. Harley. 2011. Elevated seawater CO_2 concentrations impair larval development and reduce larval survival in endangered northern abalone (*Haliotis kamtschatkana*). *Journal of Experimental Marine Biology and Ecology* 400: 272-277.
16. Gaylord, B., T.M. Hill, E. Sanford, E.A. Lenz, L.A. Jacobs, K.N. Sato, A.D. Russell, and A. Hettinger. 2011. Functional impacts of ocean acidification in an ecologically critical foundation species. *Journal of Experimental Biology*, 214: 2586-2594.
17. O'Donnell, M.J., A.E. Todgham, M.A. Sewell, L.M. Hammond, K. Ruggiero, N.A. Fangue, M.L. Zippay, and G.E. Hofmann. 2010. Ocean acidification alters skeletogenesis and gene expression in larval sea urchins. *Marine Ecology Progress Series*, 398: 157-171.
18. Sharp, J.H. 2007. Ocean acidification. CO2.cms.udel.edu/ocean_acidification.htm.