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# Ocean acidification and seagrasses: evidence for reduction in polyphenolic-based chemical defenses and an increase in herbivory

Christopher James Mealey
*Dickinson College*

# Ocean acidification and seagrasses: evidence for reduction in polyphenolic-based chemical defenses and an increase in herbivory

By

Christopher J. Mealey

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_____ Date_____

Prof. Thomas Arnold, Supervisor

_____Date_____

Prof. Scott Boback, Reader

_____ Date_____

Prof. John Henson, Reader

**Abstract**

Atmospheric carbon dioxide ($CO_2$) has increased by about 40% since the Industrial Revolution, with current levels residing around 395ppm. A portion of this excess $CO_2$ is absorbed by the oceans resulting in the increase of $H^+$ and carbonic acid concentrations, as well as a corresponding reduction in mean pH. This phenomenon is termed 'ocean acidification' (OA). Multiple studies demonstrate a decline in calcification of many marine organisms as a result of OA, but greater photosynthetic productivity in algae and seagrasses has also been reported. However, little is known regarding the effects of OA on the chemical defenses produced by these marine angiosperms. Three forms of $CO_2$ enrichment were utilized in this study to observe the effects OA may have on secondary metabolite accumulation in four species of seagrass. These include a Free Ocean Carbon Enrichment (F.O.C.E.) system – Severn River, MD (USA), a natural volcanic vent – Vulcano (Italy), and the naturally acidified Myora Springs – North Stradbroke Island (AUS). Additionally, herbivory tests examined preferences of juvenile black rabbitfish on eelgrass grown in low and normal pH regions near the naturally acidified Myora Spring (AUS). Phenolic acids, the main chemical defenses of these species, were identified and measured via HPLC, whereas more complex tannin concentrations were measured by colorimetry. The results of this experiment observed a significant decrease, about 60% in some instances, in the production of these secondary metabolites corresponding to a decrease in average oceanic pH and an increase in $pCO_2$ concentrations. The reduction in the accumulation of these chemical defenses within the observed seagrasses implies a greater susceptibility to herbivory and harmful pathogens, which reveals location dependent impacts of OA on marine plants.

## Introduction

Anthropogenic carbon dioxide ($CO_2$) has increased an alarming 40% from pre-industrial revolution levels of 280ppm to a current level of 395ppm [1]. Approximately 80% of the emitted $CO_2$ has been absorbed by the oceans where it is sequestered and cycled through various marine ecosystems [2]. However, this uptake of $CO_2$ by surface water results in the reduction of global oceanic pH levels, termed 'ocean acidification' (OA) [1-3]. This process involves the dissolution of excess $H^+$ and bicarbonate in the water column [4]. The influx of $H^+$ and $HCO_3^-$ in seawater has produced a drop of 0.1 units in global pH since the industrial revolution, with further reductions of ~0.3-0.4 pH units expected by 2100 [1, 3]. Soloman et al. (2009) predict an irreversible change to global temperature, sea level rise, and $pCO_2$ levels if a 'business as usual' approach to carbon emissions persists.

OA disrupts calcification processes due to a reduction in aragonite, calcite, and magnesium saturation levels [1, 5]. As a result, OA affects calcifying organisms, such as cnidarians, molluscs, echinoderms, crustaceans, and calcareous algae [6, 7]. Select mesocosm experiments revealed negative responses in calcification and growth in acidified environments by a majority of coral species, as well as in bay scallops and oysters; the latter being important target species for commercial fisheries [7-9]. Implications for the decrease in calcification rates on coral reefs are addressed by Hoegh-Guldberg et al. (2007) and include a dramatic decline in global coral reef ecosystems, with a resulting take-over by macroalgae. Additionally, many coral reef fauna and fishes dependent upon the shelter and food provided by coral colonies will suffer population declines due to the effects of OA on coral reef ecosystems [5].

Not all organisms seem to respond negatively to ocean acidification, however. Marine primary producers, e.g. seagrasses and non-calcifying, fleshy macroalgae, appear to respond

positively to a greater availability of $pCO_2$ for utilization in photosynthesis and growth [10, 11]. Jiang et al. (2010) found increases in photosynthesis, growth, and C:N ratios (in below ground tissue) of *Thalassia hemprichii* from a pH of 8.10, corresponding to current conditions, to levels as low as 7.75 and 7.50; expected for years 2100 and 2200, respectively. Additionally, *Zostera marina* experienced an increase in rhizome biomass between these same pH ranges [11]. Certain coccolithophore species have also exhibited increased photosynthesis and calcification as a result of greater $pCO_2$ [12]. Another benefit for seagrass health is the reduction in calcification exhibited by epiphytes that frequently reside on seagrass blades and limit light availability to the plant [13].

Despite the overwhelming evidence that marine primary producers can benefit from excess $pCO_2$, it may be too early to declare them as 'winners' because we know very little about how OA will affect trophic processes, such as herbivory. For example, the effects of OA on protective chemical defenses produced by plants and fleshy macroalgae has only been minimally acknowledged and addressed. Marine plants, similar to their terrestrial counterparts, produce a broad range of natural products with anti-grazer and anti-microbial properties [14]. It is possible that the production of these chemical defenses, like calcium carbonate derived structures, could be compromised by high $CO_2$- levels and the corresponding reduction in oceanic pH. The production of chemical defenses depends upon the availability of resources, the growth strategies of specific species, and selective pressure from attackers [14]. Most models of plant defense presume that available resources must be allocated among competing metabolic processes so that defenses are "costly". It is assumed that rapidly growing plants can tolerate or "outgrow" grazers and, thus, do not invest heavily in chemical defenses. On the other hand, slow growing species are expected to be chemically-rich. For example, Herms and Mattson (1992) famously

addressed the dilemma of plants ("to grow or defend") by acknowledging that plants must direct carbon and nitrogen towards growth *or* the production of chemical defenses. These models are most reliable when considering polyphenolic chemical defenses produced by the shikimic acid and phenylpropenoid pathways, in which there are specific metabolic mechanisms forcing a growth vs. defense tradeoff [15]. For polyphenolics, it has been observed that in nitrogen poor environments that limit growth the plant will focus its carbon resources towards the production of carbon-based defenses [14, 15]. Therefore, it is hypothesized that excess $pCO_2$ in the water column may increase the C:N ratio of marine plants and algae, in turn, increasing the production of carbon based chemical defenses; a trend that has been observed in many studies regarding terrestrial plants [16, 17].

Chemical defenses in marine angiosperms are common and important to study due to their antimicrobial and herbivore deterrent properties [15, 18-22]. In general, these chemical defenses exhibit similar properties and functions to their terrestrial counterparts, but have yet to be analyzed to a great extent in marine environments. A study by Bushmann and Ailstock (2006) observed antimicrobial defenses against bacterial epiphytes residing on seagrass blades, but it was uncertain as to which compounds were specifically inhibiting their growth. Additionally, carbon based phenolic acids and tannins have been shown to inhibit the growth of the wasting disease pathogen, *Labyrinthula* sp., and feeding by various waterfowl species, e.g. the Canada goose [24]. The roles of the specific phenolic metabolites have yet to be completely understood, but compounds such as free and conjugated phenolic acids, condensed tannins, lignins and terpenoids have been found in various concentrations in such seagrass species as *Ruppia maritima*, *Cymodocea nodosa*, *Posidonia oceanica*, *Syringodium filiforme*, *Zostera marina* and *Potamogeton perfoliatus* [25-27].

This study aims to 1) identify the presence of commonly found phenolic acids in four seagrass species, *Ruppia maritima*, *Cymodocea nodosa*, *Potamogeton perfoliatus,* and *Zostera capricorni* and 2) to observe the effects of ocean acidification on the production of phenolic acids, condensed tannins and lignins within their tissues. The results of some of these studies were recently published [28]. Subsequent research involved herbivory experiments with *Siganus fuscescens,* black rabbitfish, feeding on *Z. capricorni* seagrass grown within a naturally-acidified environment within Moreton Bay, Queensland, Australia. This research was conducted as a part of Dickinson's Global Scholars program (2012). Phenolic acid analysis for the *Z. capricorni* samples will continue in 2013. It is expected that ocean acidification will preference *S. fuscescens* feeding upon seagrass grown within a higher pH environment where less secondary metabolites are proposed to be produced.

## Methods

*Chesapeake Bay Study Site*

One study site existed in the Severn River, MD (USA), during the growing season of May to June 2011, where *Ruppia maritima* (widgeon grass – short form) and *Potamogeton perfoliatus* (redhead grass) were exposed, *in situ*, to acidified conditions *via* a Free Ocean Carbon Enrichment (F.O.C.E.) system (Figure 1; 28). This mixed bed of seagrasses resided in about 1-2 meters of water with salinity around 4 (Table 1). The F.O.C.E. system was designed by Dr. Tom Arnold and serves to incorporate controlled pH fluctuations into a focused area to observe variation between natural and acidified environments [28]. The system bubbles $CO_2$ freely from a series of injectors to simulate pH levels corresponding to present conditions and levels expected 50 and 100 years from now. The pH of the water was measured using a solid state probe (Honeywell Durafet II) and taken at distances of 5, 40, 100 and 500 cm from each injector. In addition, a Mar$CO_2$m system (Marine $CO_2$ meter - Whitman Miller, Smithsonian Environmental Research Center) was utilized to record *in situ* $pCO_2$ concentrations, temperature and salinity surrounding the study site [29]. Total alkalinity (TA) was determined according to the Yao and Byrne spectrophotoscopic method using an Ocean Optics spectrometer. Seagrass samples were collected four and six weeks after initial placement of the F.O.C.E. system. Permission for set up of the system was provided by the Sullivan's Cove community association and collecting permits were received from the Maryland Department of Natural Resources.

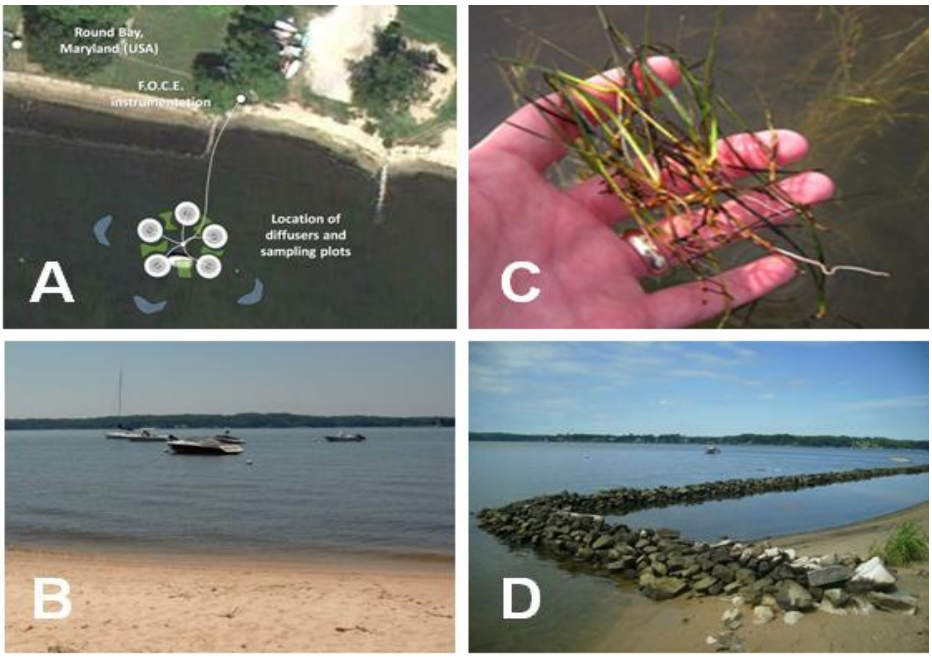


Figure 1. F.O.C.E. system placement in a seagrass bed in the Severn River, MD. Seagrasses include *R. maritima* and *P. perfoliatus*. (A) Five diffusers created a pH gradient corresponding to normal and future levels. Blue arches indicate control samples unaffected by the $CO_2$ injectors. Gray circles represent the lowest pH, white represents the middle concentrations, and green represents pH levels corresponding with current conditions. (C) *Ruppia maritima* was the primary seagrass collected, but patches of *P. perfoliatus* were found during the 6 week collection period. (B and D) show the environment surrounding the seagrass bed in relation to shore.

### *Vulcano, Italy Study Site*

Natural volcanic vents provide suitable regions for studying the effects of climate change because they have been known to reduce the pH in areas surrounding these $CO_2$ seeps [13]. Seagrass grown in these regions are susceptible to the consistently acidified conditions and therefore provide sufficient samples for use in studying climate change. *Cymodocea nodosa* growing in proximity to a naturally occurring volcanic vent was collected from varying pH concentrations surrounding Vulcano Island, Italy, with those farthest from the bubbling vents residing in ambient pH concentrations (Figure 2). A 556 YSI probe was used to measure the pH,

salinity, and temperature of the region. The chemistry of the water has been well characterized over the years to indicate a strong influence of $CO_2$, making these volcanic vents a suitable region for studying naturally occurring OA [30, 31]. Samples of *C. nodosa* were collected in May 2011 along a pH gradient from 8.1 to 7.3. Collected samples were transported back to Dickinson College in liquid nitrogen and stored at -80ºC.

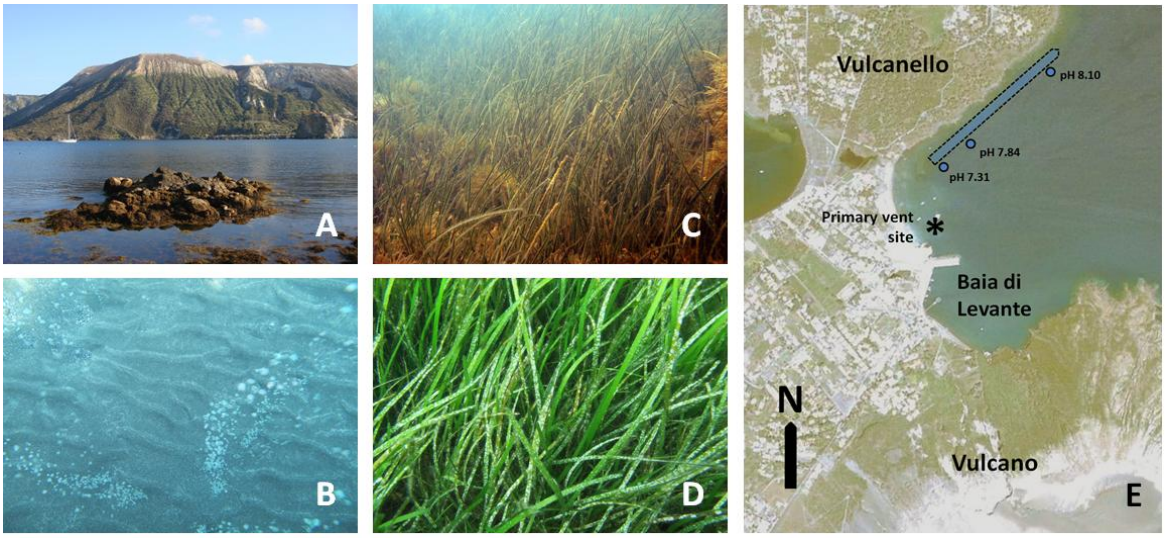


Figure 2. Study location in Vulcano, Italy. A) View of Baia di Levante from a distance. B) Image of the bubbles seeping from the volcanic vents. C & D) Images of *C. nodosa* growing in the region. E) Distant view of the study site. The asterisk represents the location of the vent. The blue arrow shows the direction of water flow, with the blue dots indicating seagrass collection sites, as well as the corresponding pH.

*Queensland, Australia Study Site*

*Zostera capricorni* was collected on March 16, 2012 from Moreton Bay adjacent to the mouth of Myora Springs. This region is naturally acidified, with a pH around 4.9 at the start of the spring, where it is then diluted by the fresh water flow into the nearby harbor. *Z. capricorni* samples were collected during low tide from various locations: control areas distant from the mouth of the spring, corresponding to current conditions (pH=8.78), and experimental areas near

the mouth corresponding to conditions expected by the year 2100 (pH=8.3). Separate water samples were extracted from each location and analyzed for pH, total alkalinity, temperature, salinity, total nitrogen, and sulfate by the National Association of Testing Authorities (NATA). $pCO_2$ was calculated *via* the program $CO_2$sys by using the measured pH and TA.

In addition to *Z. capricorni*, collection of juvenile black rabbitfish, *Siganus fuscescens*, were gathered during low tide during the evening of March 15, 2012. A seine net was used to capture *S. fuscescens* from One Mile Landing. These specimens were then transported back to the Moreton Bay Research Station wet lab where they were sorted by size and utilized for further feeding experiments. Juvenile *S. fuscescens* were used because they are common to the region and are known to feed on *Z. capricorni*.

*Feeding Experiment*

Feeding experiments were conducted in the Moreton Bay Research Station wet lab. Eleven tanks were prepared for feeding tests and contained two small and one large juvenile rabbitfish (N=3), determined prior to the experiment. Seawater used in the tanks was collected from the same location as *S. fuscescens*. In addition to the 11 experimental tanks, one empty tank served as a control to ensure no outside variables were affecting the mass of the seagrass.

Shoots of *Z. capricorni* were cut to a uniform length of 10cm. Samples were cleaned of epibionts and mass was measured. Five shoots were placed into a cut 15 mL polypropylene test tube, leaving the leaves exposed for consumption. Sand was placed into the bottom of the tubes to anchor the seagrass to the bottom of the tank. This process was identical for both the control (pH 8.78) and experimental (pH 8.3) seagrass specimens.

One control and one experimental tube were placed into each tank to determine feeding preference for *S. fuscescens*. Feeding tests took place approximately 24 hours after initial collection of *S.* fuscescens to establish equal starvation among the rabbitfish. Completion of the feeding experiment was based on the rate of consumption by each individual tank. When approximately 50% of one feeding tube was consumed, the experiment was concluded. At the conclusion of each experiment, leaves were removed from the tanks and again dried, weighed, and measured for length.

*Seagrass Chemical Analysis*

Seagrass samples collected *in situ* were transported back to Dickinson College and stored at –80°C. Samples were then homogenized and extracted in methanol (aq) plus 2% acetic acid for 24 hr in the dark at 4°C. Simple phenolic acid concentration was measured *via* reverse phase – high performance liquid chromatography (RP-HPLC). The extracts were then filtered and 100µL of solution was injected onto a semi-preparative RP-18 HPLC column. Prepared standards of common phenolic acids, e.g. *p*-coumaric, caffeic, ferulic, acetovillinone, syr+4-HBA, vanillic and gallic acid, were used prior to the extracted samples to establish peaks for compound identification and estimated concentration [26]. Total reactive phenolic and condensed tannin concentrations were determined using a micro-Folin-Denis assay, which were standardized from a quebrancho tannin sample, and measured by colorimetry. These analyses were performed for samples from *R. maritima, P. perfoliatus*, and *C. nodosa*. Further analysis still has to be performed for samples from *Z. capricorni*. In addition, C/N rations and lignin concentrations have yet to be measured for all seagrass species. SigmaStat was used to determine any significant variation in compound concentrations among all analyzed samples.

## Results

*Chesapeake Bay Study Site*

The FOCE system altered $CO_2$/pH levels in the Severn River, MD while maintaining consistent levels of salinity, total alkalinity (TA) and temperature no matter the distance from the injector. This isolates the $pCO_2$ and pH as contributing factors to potential changes in seagrass chemistry. An inverse relationship exists between the $pCO_2$ levels and pH: a pH of 8.34 and $pCO_2$ concentration of 243 µtm was found a distance of 500 cm from the injector, a pH of 7.82 and $pCO_2$ concentration of 948 µtm at a distance of 40 cm from the injector, and a pH of 7.32 and $pCO_2$ concentration of 3465 µtm only 5 cm away from the injector. The salinity of 4.3 indicates a nearly freshwater environment in which *R. maritima* and *P. perfoliatus* thrive (Table 1).

Table 1. Seawater chemistry 4 weeks after introducing the F.O.C.E. system in the Severn River, MD. TA = total alkalinity. Values are means with ± SD.

| **Conditions** | **Seawater Chemistry** | | | |
|---|---|---|---|---|
| **Distance from injector** | **500cm** | **100cm** | **40cm** | **5cm** |
| **Temp ◦C** | 28.3 | 28.3 | 28.3 | 28.3 |
| **Salinity** | 4.3 | 4.3 | 4.3 | 4.3 |
| **pH** | 8.34±0.01 | 8.26±0.02 | 7.82±0.04 | 7.32±0.06 |
| **pCO2 (µtm)** | 243±9 | 295±13 | 948±89 | 3465±527 |
| **TA (µmol kg-1)** | 1122±0.5 | 1122±0.5 | 1122±0.5 | 1122±0.5 |

The phenolic compounds found in *R. maritima* after the four week study period include proanthocyanindins (condensed tannins), syr+4-HBA, vanillin, acetovillinone, coumaric acid, and ferulic acid. A significant decrease was found among proanthocyanindins with an average

concentration of 25 mg $g^{-1}$ WM in a pH of 8.26 and an average concentration of 6.33 mg $g^{-1}$ WM at a pH of 7.82. All other compounds remained at relatively even mean concentrations within the pH gradient: with around 0.08 mg $g^{-1}$ WM of syr+4-HBA, between 0.02 and 0.05 mg $g^{-1}$ WM of vanillin, between 0.05 and 0.08 mg $g^{-1}$ WM of acetovillinone, and 0.03 mg $g^{-1}$ of ferulic acid. Only trace amounts of coumaric acid were found with concentrations less than 0.01 mg $g^{-1}$ WM (Figure 3).

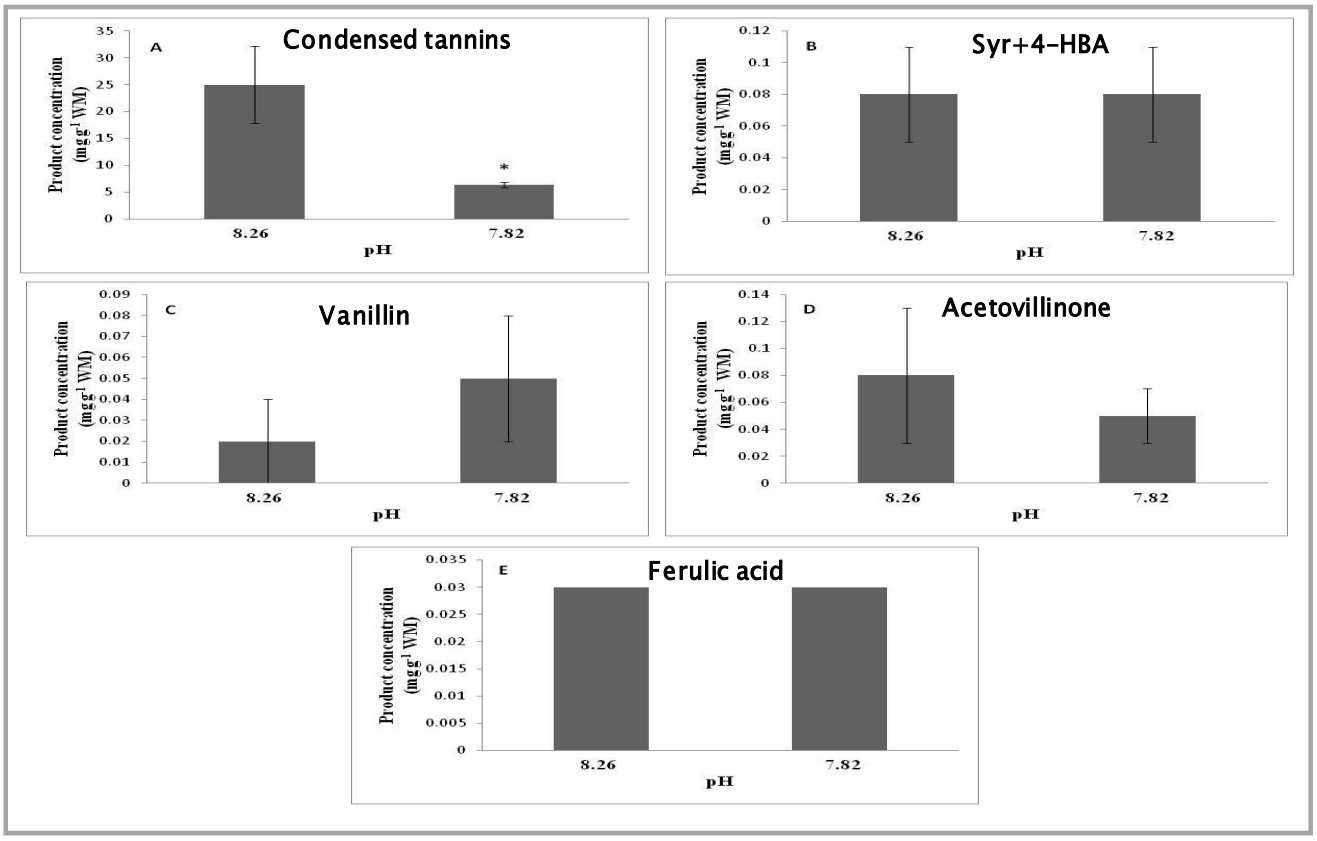


Figure 3. Secondary metabolite concentrations in *R. maritima* shoot tissue after 4 weeks of F.O.C.E. manipulation in the Severn River, MD. **A**: Proanthocyanindins (Student's t-test: $p<0.05$); **B:** Syr+4-HBA; **C:** Vanillin; **D:** Acetovillinone; **E:** Ferulic Acid. There were only trace amounts of coumaric acid so it was excluded from presentation. Values are means ± SD. WM = wet mass.

The chemistry of the water remained fairly consistent between the four and six week collection times. Temperature, salinity, and TA remained constant between the varying distances from the injector and exhibit similar levels to the four week collection data. The correlation between a decreasing pH, ranging from 8.29 to 7.94, with increasing concentrations of $pCO_2$ continued to be evident and further supported the use of these factors as determining potential changes in seagrass chemistry (Table 2).

Table 2. Seawater chemistry taken 6 weeks after introducing the F.O.C.E. system in the Severn River, MD. TA = total alkalinity. Values are means ± SD.

| **Conditions** | **Seawater Chemistry** | | |
|---|---|---|---|
| **Distance from injector** | **500cm** | **100cm** | **40cm** |
| **Temp ◦C** | 29.5 | 29.5 | 29.5 |
| **Salinity** | 4.9 | 4.9 | 4.9 |
| **pH** | 8.29±0.01 | 8.11±0.02 | 7.94±0.03 |
| **pCO2 (µtm)** | 279±2 | 439±20 | 729±5 |
| **TA (µmol kg-1)** | 1145±10 | 1145±10 | 1145±10 |

The phenolic compounds found in *R. maritima* upon completion of the six week study period include proanthocyanindins, acetovillinone, coumaric acid, and ferulic acid (Figure 4). Additionally, proanthocyanindins and total reactive phenolics were measured within whole plant tissue of *P. perfoliatus* (Figure 5). A significant decrease in proanthocyanindins, located in *R. maritima* roots, was found between pH levels of 8.11 and 7.94, with average concentrations of 22.81 mg $g^{-1}$ and 18.12 mg $g^{-1}$ respectively (Figure 4). The remaining phenolic compounds found within *R. maritima* saw no significant change in concentrations among the pH gradient (Figure

4). In *P. perfoliatus*, a significant decrease was also found in levels of proanthocyanindins with 0.65 mg $g^{-1}$ WM at a pH of 8.29 and 0.10 mg $g^{-1}$ WM at a pH of 7.94; no trace of proanthocyanindins was detected at a middle pH of 8.11 (Figure 5). There was no significant change in concentrations of total reactive phenolics found within *P. perfoliatus* (Figure 5).

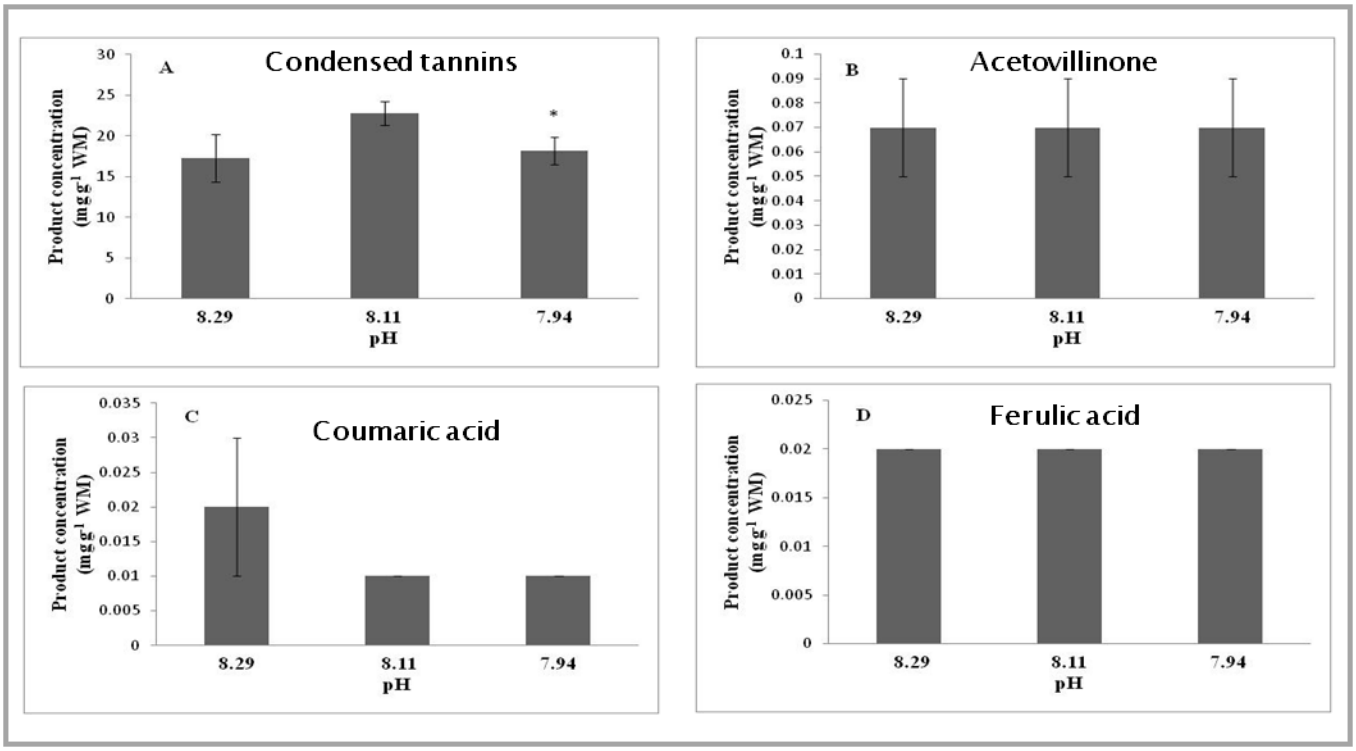


Figure 4. Secondary metabolite concentrations in *R. maritima* tissue after 6 weeks of F.O.C.E. manipulation in the Severn River, MD. **A**: Proanthocyanindins (roots: one-factor ANOVA w/ Holm-Sidak multiple comparisons, $p<0.05$); **B:** Acetovillinone (shoot); **C:** Coumaric Acid (shoot); **D:** Ferulic Acid (shoot). There were only trace amounts of syr+4-HBA and vanillin so they were excluded from presentation. Values are means ± SD. WM = Wet mass.

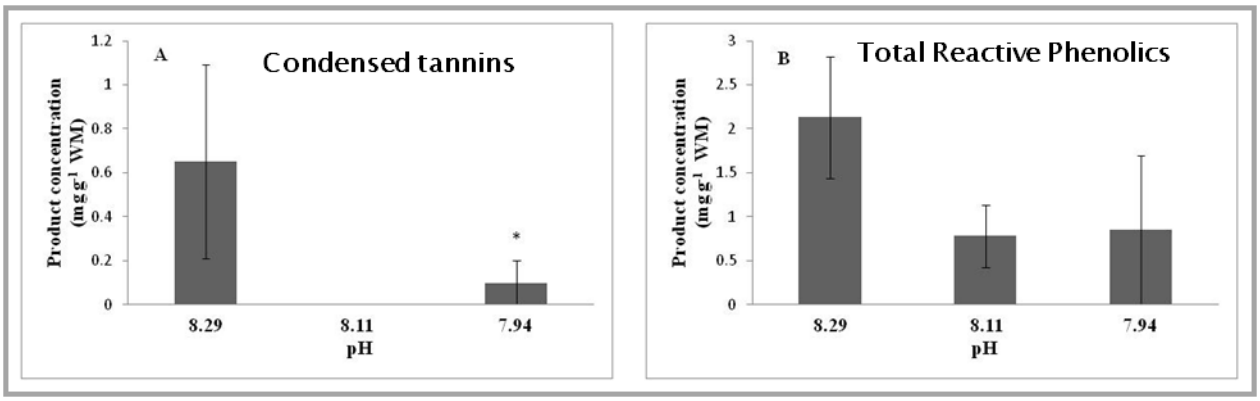


Figure 5. Secondary metabolite concentrations in whole plant *P. perfoliatus* tissue after 6 weeks of F.O.C.E. manipulation in the Severn River, MD. **A:** Proanthocyanindins (Kruskal-Wallis One Way Analysis of Variance on Ranks with Tukey or Dunns multiple comparisons, $p<0.05$) **B:** Total Reactive Phenolics. Values are means ± SD. WM = Wet mass.

*Vulcano, Italy Study Site*

An increase in $pCO_2$ near the vent site corresponded with a decrease in pH, with levels ranging from 8.11 to 7.32 as the distance from the volcanic vent decreased (Table 3). The salinity and total alkalinity of the water surrounding the volcanic vents in Vulcano, Italy remained constant; salinity levels were measured around 37 indicating a very saline environment in which *C. nodosa* grow (Table 3).

Table 3. Seawater chemistry in Vulcano, Italy in proximity to the naturally occurring volcanic vents. Values are means ± SD. TA= total alkalinity.

| **Conditions** | **Seawater Chemistry** | | |
|---|---|---|---|
| **Distance from vent** | **380m** | **300m** | **260m** |
| **Salinity** | 37.16±0.07 | 37.12±0.06 | 37.05±0.1 |
| **pH** | 8.11±0.01 | 7.84±0.04 | 7.32±0.05 |
| **pCO2 (µtm)** | 422±43 | 976±269.5 | 4009±1442.7 |
| **TA (µmol kg-1)** | 2549.6±29.6 | 2555.9±28.9 | 2592.5±48.3 |

The defensive compounds found in *C. nodosa* include proanthocyanindins, total phenolic acids, gallic acid, syr+4-HBA, vanillin, acetovillinone, coumaric acid, ferulic acid, and all combined phenolics. Significant decreases were found in levels of proanthocyanindins, between a pH of 8.11 and 7.84, total phenolic acids, pH between 7.84 and 7.32, syr+4-HBA, between all three pH levels, and all combined phenolics, among all three pH levels. Levels of these compounds dropped from 13.62 to 10.17 mg $g^{-1}$ WM, 5.30 to 1.89 mg $g^{-1}$ WM, 0.07 to 0.05 to 0.03 mg $g^{-1}$ WM, and 109.30 to 104.51 to 93.12 mg $g^{-1}$ WM, respectively. All other compounds saw no significant change in concentrations between pH levels (Figure 6).

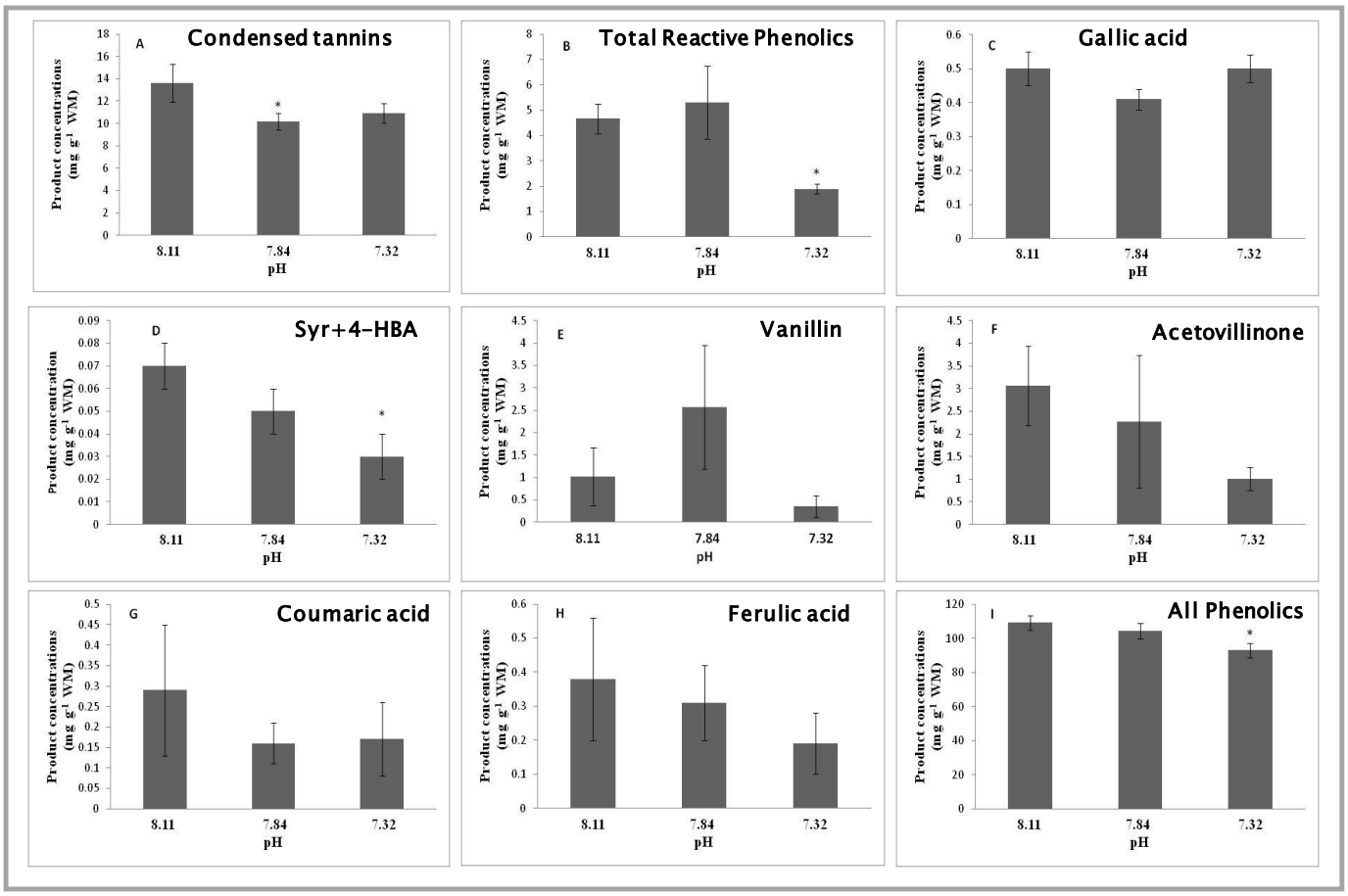


Figure 6. Secondary metabolite concentration in *C. nodosa* whole plant tissue located near the volcanic vent in Vulcano, Italy. **A**: Proanthocyanindins (Student's t-test, p=0.088); **B:** Total Phenolic Acids (Mann-Whitney Rank Sum Test, p<0.05); **C:** Gallic Acid; **D:** Syr+4-HBA (Student's t-test, p<0.05); **E:** Vanillin; **F:** Acetovillinone; **G:** Coumaric Acid; **H:** Ferulic Acid; **I:** All Phenolics (Kruskal-Wallis One Way Analysis of Variance on Ranks with Tukey or Dunns multiple comparisons, p<0.05). Values are means ± SD. WM = Wet mass.

*Queensland, Australia Study Site*

The water chemistry at the Myora Springs site in Moreton Bay, Australia saw consistent temperatures (around 25-26◦C), salinities (35), and levels of total nitrogen, total alkalinity and sulfate between the study sites, i.e. experimental and control. The only significant variation in water chemistry was $CO_2$/pH, with experimental locations showing a pH of 8.3, control locations a pH of 8.78, and a mean pH within the spring itself around 5.2. Some of these pH levels appear

higher than normal and may be a result of a combination of collection during low tide and photosynthesizing periods. The difference in pH (~0.4 units) simulates the variability between current and future expected levels. The $pCO_2$ levels were calculated from the measured pH and TA, using the program $CO_2$sys, and observed levels around 390 ppm (control pH 8.78) and around 2000 ppm (experimental pH 8.3). This suggests that any preference in feeding may be an indirect result of variation in $pCO_2$/pH levels, corresponding to any potential impacts pH variation may have on the seagrass chemistry (Table 4).

There was an obvious preference of juvenile rabbitfish feeding upon high $CO_2$/low pH seagrass leaves. Upon conclusion of the feeding tests, a statistically significant preference was found for *S. fuscescens* feeding on *Z. capricorni* grown within the low pH, experimental study site. Specifically, a 55.2% loss of mass was observed in the experimental seagrass groups, with only a 25.4% loss in mass of the seagrass grown in the high pH control group (Figure 7). Additionally, although not a statistically significant result, there was a 34.5% change in mean length of *Z. capricorni* blades in the low pH, experimental groups, whereas only a 15.0% change in mean length was found in the high pH, control groups (Figure 8).

Table 4. Water Chemistry tested from three study sites within Myora Springs and nearby delta on North Stradbroke Island (AUS). The means of pH, temperature, and salinity were calculated from five samples taken at each site. The means for alkalinity, total N, and sulfate were calculated and measured from three samples sent to NATA. The samples for Myora springs included those taken from the start of the spring near the road. Values are means +/- SE. TA= total alkalinity.

| **Conditions** | **Seawater Chemistry** | | |
|---|---|---|---|
| **Site** | **Experimental** | **Control** | **Myora Spring** |
| **Temperature (◦C)** | 24.9±0.18 | 26.6±1.09 | 22±0.21 |
| **Salinity** | 35.3±0.22 | 35.4±0.33 | 1.4±0.33 |
| **pH** | 8.3±0.07 | 8.78±0.03 | 5.2±0.19 |
| **Total N (mg/L)** | 0.27±0.02 | 0.31±0.03 | 0.24±0.004 |
| **TA (mg/L)** | 120±0.00 | 120±0.00 | <2±0.00 |
| **$SO_4$ (mg/L)** | 2467±81.6 | 2567±147.2 | 2.57±0.36 |

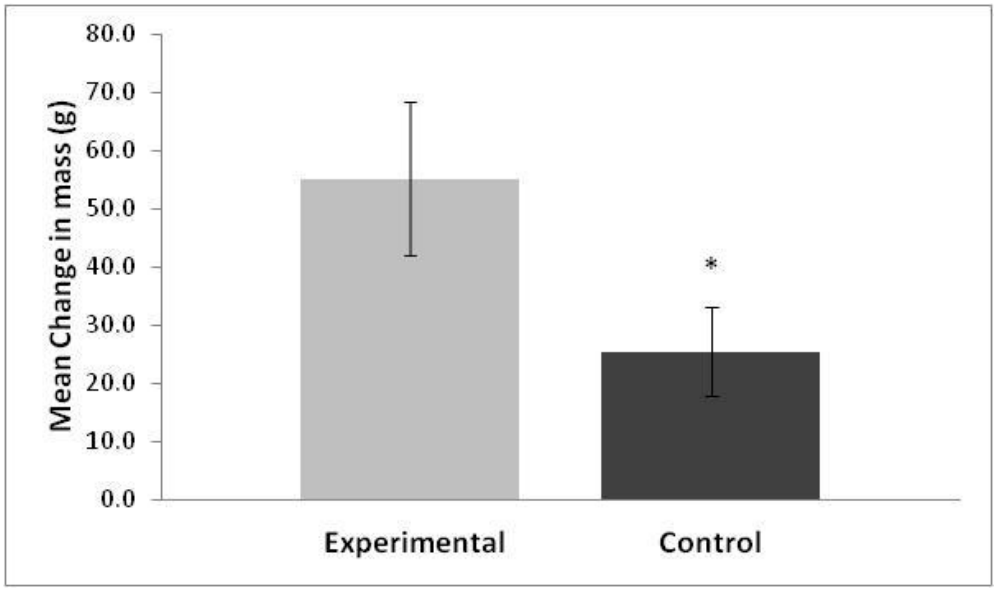


Figure 7. Mean change in mass (g) of *Zostera capricorni*, collected at Myora Springs, from start to end of the feeding experiment. The control group represents seagrass collected from water with a pH of 8.78 and the experimental seagrass was collected from water with a pH 8.3. Values are means ± SE. Student's t-test: p=0.05, N=22, df=20.

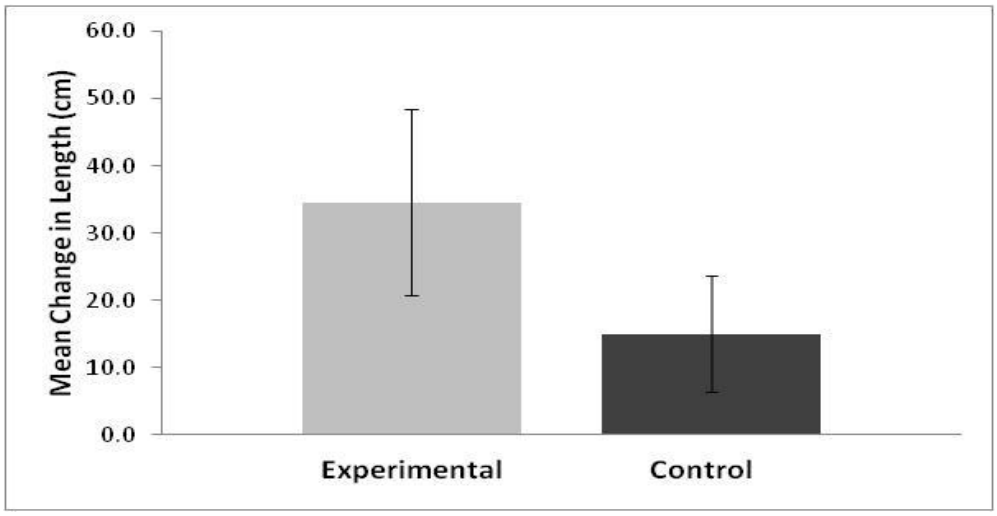


Figure 8. Mean change in length (cm) of *Zostera capricorni*, collected at Myora Springs from start to end of the feeding experiment. The control group represents seagrass collected from water with a pH of 8.78 and the experimental seagrass was collected from water with a pH of pH 8.3. Values are means ± SE. Student's t-test: p=0.357, N=16, df=14. *N=5 for the control site due to miscommunication in the experimental process.

**Discussion**

The results of these experiments demonstrated that elevated $pCO_2$ caused either no change or a significant decrease in secondary metabolite concentrations of seagrasses, refuting the original hypothesis. The observed decreases in these chemical compounds directly oppose the typical observations that excess $CO_2$ increases secondary metabolite concentrations of terrestrial plants [16, 17]. This response may indicate that OA boosts seagrass growth and the allocation of resources away from secondary metabolism or that pH induced stress is a key factor leading to the decreased levels observed in defensive compounds produced by the seagrass.

The study sites in the Severn River, Maryland and Vulcano, Italy possess different environmental conditions, varying dramatically in their water chemistry and especially, their salinities. However, these environmental differences did not alter the observed results. *R. maritima, P. perfoliatus* and *C. nodosa* all experienced a significant decrease in at least one observed phenolic compound within their tissues, with the remaining compounds exhibiting no significant change in secondary metabolite concentrations. This finding is consistent with the results of similar experiments conducted in the St. Mary's River in 2009 [28]. Together these results are opposite to the response of many terrestrial plants which have shown $CO_2$-induced increases in phenolics by 16% and in terpenes by 8% for example [17]. The increases seen in terrestrial angiosperms occurred due to exposure to both elevated $CO_2$ levels and $O_3$ concentrations in the atmosphere; however, the main factor was identified statistically as the elevated $CO_2$ [17].

The feeding experiments performed in Australia in which high $CO_2$ acclimated and "normal" *Z. capricorni* were offered to the herbivorous fish, *S. fuscescens,* also suggested

differences between marine and terrestrial systems. Here, a significant preference was shown for seagrass grown within a high $CO_2$/low pH environment. This data may be consistent with our previous findings since some marine herbivores are known to prefer seagrass with decreased levels of secondary metabolites [24, 32]. We have not yet been able to analyze the tissue chemistry for phenolic concentrations due to time constraints. However, it is expected that the trends will remain consistent by expressing a decrease in secondary metabolites within *Z. capricorni*. This expression would explain the elevated levels of herbivory on the seagrass residing in a lower pH environment.

Due to the antimicrobial and herbivore deterrent properties of these phenolic compounds and terpenoids, it can be expected that many of these seagrass species may grow increasingly susceptible to pathogens, or herbivory, as a result of more acidified oceanic environments. Verges *et al*. (2007) examined the deterrent properties of phenolic compounds against sea urchins, gastropods and fish, which provide relief from over-grazing by these herbivores. Without these defensive substances the seagrass become more vulnerable to grazing pressure, which can result in a significant decline in seagrass meadows [32]; especially since these secondary metabolites have been shown to deter feeding more efficiently than the presence of a tough structural defense, i.e. $CaCO_3$ or lignin [22].

The reduction in marine plant chemical defenses may be counteracted by the dramatic improvements in growth and photosynthesis as a result of elevated $CO_2$ levels in the oceans, even under low light conditions [6, 10-11]. It is possible that $CO_2$-fueled seagrasses will tolerate, rather than resist, grazing and pathogen infection. This has important implications for coastal marine ecosystems. For example, a study by Manzello *et* al. (2012) observed the potential for seagrass meadows to counteract OA by capturing $CO_2$ during photosynthesis, thus serving as a

refuge for nearby coral reefs. Ultimately, the seagrasses in this study provided a temporary refuge to the reef from the stress caused by OA, but the effectiveness was hindered by their inefficient carbon concentrating mechanisms [33]. In order to achieve a high enough photosynthesis level to raise the pH and relieve sufficient stress on the corals, seagrass meadows would have to encompass areas significantly larger than the reef itself to make any sort of significant contribution to sequestering excess $CO_2$ in the water.

Ocean acidification has yielded a variety of responses by marine ecosystems, with some organisms benefitting from excess $CO_2$ while others exhibit adverse affects to the lower pH of the surrounding environment. Seagrass species are no different and, in fact, may exhibit both of these trends within a single organism. The results from this study suggest that the potential for seagrass to act as a refuge for coral reefs is at risk because the decrease in defensive compounds leaves them more susceptible to herbivory and disease. Therefore, the enhanced growth and photosynthesis exhibited by seagrass due to elevated levels of $CO_2$ may be counterbalanced by their increased susceptibility to herbivory or destructive pathogens. However, this relationship requires much more examination before determining whether seagrass not only benefit from elevated $CO_2$ in the oceans, but also whether these angiosperms can truly act as a 'savior' for marine ecosystems in response to the seemingly irreversible effects of climate change.

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