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Effects of Reduced pH on Health Biomarkers of the Seagrass *Cymodocea Nodosa*

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ABSTRACT Ocean acidification is a growing problem that may affect many marine organisms in the future. Within 100 years the pH of the ocean is predicted to decrease to 7.8, from the current ocean pH of around 8.1. Using phenolic acid levels as a stress indicator as well as respiration and chlorophyll content as a measure of health, the effect of lowering pH was tested on the seagrass, *Cymodocea nodosa*, in a controlled environment. Plant samples, water, and soil were taken from the Bay of Cádiz, Spain, and placed in aquaria in a temperature-controlled room. One control group was left untreated with a pH of approximately 8.1, while experimental groups maintained pH levels of 7.8 and 7.5. Using High Performance Liquid Chromatography (HPLC), the concentration of the phenol rosmarinic acid was quantified in the plants. Average concentration for the control group was 1.7 μg g^{-1}, while it was 2.9 μg g^{-1} for pH group 7.8, and 10.1g g^{-1} for pH group 7.5. To evaluate the overall health of *C. nodosa* within the three groups, chlorophyll concentration and photosynthesis/respiration rates were determined. A one-tailed ANOVA test was conducted using the chlorophyll concentrations of the three groups. With an F-value of 1.360 and a p-value of 0.287, the differences between the groups were not statistically significant. Although the raw data shows a slight decrease in chlorophyll content between the control group and the pH group 7.5, these discrepancies might have been larger or smaller due to sampling or experimental error. Additionally, the average values with their respective standard deviations were calculated for the respiration rates and oxygen production of each group. A one-tailed ANOVA was also used to determine the relationship between rosmarinic acid content and pH levels between the groups, with an F-value of 5.1423 and a p-value of 0.050.

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Research completed in 2018

INTRODUCTION

Ocean acidification is a rising long-term anthropogenic problem in the marine eco-system. Research studies have been conducted to understand how human activity leads to acidification and many have found that domestic and industrial wastewater as well as pollution of solid waste have large contributions in addition to increasing atmospheric CO_2 (Maranho, André, Delvalls, Gagné, Martín-Díaz, 2015). Such human activities contaminate marine ecosystems with chemicals known as polycyclic aromatic hydrocarbons (PAHs), which are produced by the combustion of organic matter and deposited into bodies of water (Maranho et al., 2015). Ocean acidification can be defined as the decline of the ocean's pH level as a result of CO_2 absorption by the ocean. It has been predicted that if CO_2 levels continue increasing, then in the year 2100 the ocean's pH will be around 7.80-7.85 (Arnold et al., 2012). Previous studies have examined the impact of a higher CO_2 would have on the pH of the ocean. Current pH levels of the ocean are around 8.1, which would mean in the year 2100 the pH will drop by 0.3 units (Arnold et al., 2012). This can be a significant problem for many marine organisms as they may be sensitive to a change in pH. With a decrease in pH also comes a decline of carbonate ion concentration and decline in saturation states of calcium carbonate minerals (Cox et al., 2015). Calcium carbonate minerals are used by marine organisms such as coral to build their skeletons (Azevedo, De Schryver, Hendriks, Jhuijbregts, 2015). A decrease in available calcium carbonate minerals can leave marine organisms more vulnerable to predators or disease and also can cause the degradation of coral reefs (Cox et al., 2015). According to previous research, the decrease in pH and increase in CO_2 levels can be mitigated by seagrass meadows, which act as a natural buffer to these changes (Hendriks et al., 2014). Due to the high metabolic activity of seagrass meadows, the CO_2 levels can be reduced while O_2 levels increase by the process of photosynthesis (see Equation 1). However, after a certain point, a decrease in pH should negatively affect the health of the seagrass and decrease their overall species diversity due to the fact that some species may respond more efficiently to these changes than others. Although seagrasses are fairly resilient to changes in pH, salinity, and temperature, they are still delicate organisms that require specific nutrients and amounts of light in order to thrive (Drew, Edward A., 1978).

$$6CO_2 + 6H_2O \rightarrow C_6H_{12}O_6 + 6O_2$$

Equation 1. The balanced equation uses carbon dioxide (CO_2) and water (H_2O) to produce glucose ($C_6H_{12}O_6$) and oxygen (O_2) through light energy, indicated as an arrow.

Nearly all species of seagrass require a high amount of light in order to photosynthesize. Additionally, seagrasses require mixing of the surrounding water, which is often provided by the motion of the tide, in order to gain necessary nutrients (Duarte, Carlos M., 1990). These nutrients include carbon, primarily from the abundant CO_2 (made even more abundant due to ocean acidification), nitrogen, and phosphorus. These are the three primary nutrients that are necessary for the seagrass to grow and remain healthy (Duarte, Carlos M., 1990).

The pH level of the ambient marine environment also affects the health of the seagrass. If the ambient pH level decreased dramatically, then this stress may cause seagrasses to produce more metabolites, which are organic compounds that are involved in the growth, development, and defense of the plant (Grignon-Dubois et al., 2012). One form of metabolite that provides a variety of deterrent and ecological functions for plants are phenolic acids, which are chemicals found in both terrestrial and aquatic plants. These compounds can be identified by their aromatic carbon rings with hydroxyl groups attached to the ring. Their major roles are to serve as disease resistance, herbivore deterrents, and to provide storage of nutrients for the plant (Grignon-Dubois et al., 2012). Previous literature has shown that an increase in phenolic acid content in the seagrass *Zostera marina* has been shown when a pathogenic infection, such as wasting disease, is present (Vergeer & Develi, 1997). An increase

in environmental CO_2 and reduced pH level should induce a stress response and cause an increase in the phenolic content of the plant in order to protect itself. However, one study found that at a pH level of 7.3, phenolic content in *C. nodosa* decreased (Arnold et al., 2012). Although these results were found with the same species that was observed in this study, the experiment was done *in situ* comparing individuals from different locations (which varied in their temperature, water chemistry etc.) rather than in a controlled, lab-based experiment in which only pH was manipulated. This previous study also did not look at the specific phenolic, rosmarinic acid, which is another major phenolic compound found in many species of seagrass. Rosmarinic acid (see Figure 1) was chosen as the phenolic compound analyzed in this study due to its natural abundance in *C. nodosa*, which means that it would be easier to quantify if there were changes in its concentration before and after experimental treatment (Arnold et al., 2012). In a controlled lab environment in which pH levels are decreasing, the rosmarinic acid content would likely increase to protect the plant while in a changing environment.

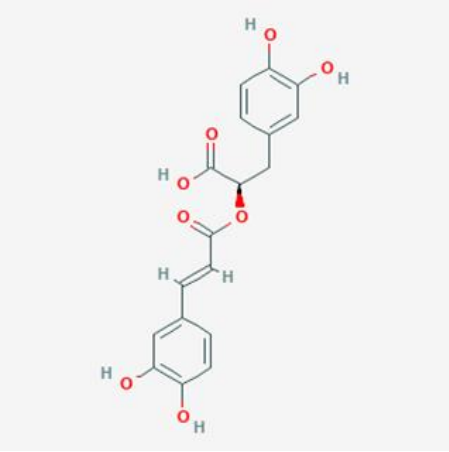

Figure 1. Chemical structure of the phenolic compound, rosmarinic acid (accessed from PubChem, 2019).

The focus of this study was to induce pH stress on the seagrass, C. nodosa, in a controlled lab setup, which previous studies have not done before. The temperature, soil and water quality, and nutrient availability were all controlled and the pH levels were manipulated in order to determine if this had an effect on the phenolic acid content of the seagrass, while also monitoring other health biomarkers such as photosynthetic rates and chlorophyll concentration. Based on the background information regarding seagrass health, a pH decrease in the seagrasses' environment will influence the phenolic acid levels, respiration rate, and chlorophyll concentration. With a pH decrease in the seagrasses' environment, there will be an increase in the phenolic acid content, decreased rate of respiration, and decreased chlorophyll level concentration.

METHODS

C. *nodosa* Collection and Determination of Sediment PAH Levels

Initial observations of the study site called Clean Point (Andalusia, Spain 36.490624°, -6.265793°) were that it had a low density population of seagrass and low diversity overall. This was because the seagrass was mainly seen in patches and not many other plants were observed around the area. Also, the study site was in very close proximity to a waste treatment facility. Sediment samples were collected from the study site and compared to another site (Cádiz, Spain 36.466341°, -6.249099°) across the Bay of Cadiz that had less human activity. Gas Chromatography-Mass Spectrometry (GC-MS) was used to analyze soil extracts from both sites to compare the contents in terms of PAH, since they were expected to be highly present pollutants in the region. More specifically the PAH of benzo(a)pyrene (252.31 g/mol), benzo(a)anthracene (228.28g/mol), pyrene (202.25 g/mol), and anthracene (178.23 g/mol) were searched for since they were highly present according to a previous study (Maranho et al 2015).

A plastic container was filled with sediment sample taken from the study site. The collected sediment sample was sieved of any large objects within it with a woven wire mesh (2.52 mm x 1.6 mm). A 10 g moist sample of the filtered sediment sample was massed using a scale. Then 10 g of anhydrous sulfate was massed in a weigh boat. The sample along with the anhydrous sulfate was transferred to a mortar

and pestle and was mortared by hand for 5 minutes. Then 10 mL of acetone was added to the mixture and the sample was transferred to a 50 ml beaker. The sample was taken from the beaker and distributed equally into centrifuge tubes, which were vigorously mixed 5 minutes. The tubes were centrifuged at 12,000x g for 10 minutes to separate the liquid from the solid material. Then liquid sample was removed from the centrifuge tubes and filtered through a 0.2 mm syringe filter. Finally, the liquid was transferred into vials for injection into GC-MS. GC-MS was carried out by the University of Cadiz central services coordinator Maria J. Ortega using standard methods.

C. nodosa samples were collected from Clean Point (Andalusia, Spain 36.490624°, -6.265793°) in the Bay of Cadiz in July 2018. The collection site was a shallow area in the south end of the city of Cadiz. This site was observed to have a high human impact from trash and pollution. Approximately 70 shoots were collected from the site at low tide. Samples were then transported on ice to the laboratory within 30 minutes. Once in the lab, samples were measured and planted into aquaria. Aquaria were prepared by filling with approximately 12.6 liters of seawater and 3 inches of sand from the collection site and allowing them to settle before seagrass samples were added. Seagrass rhizomes and roots were gently inserted into the sand. Air stones and pumps were placed in each tank in order to ensure access to oxygen and proper water mixing for the seagrass. Seagrass was allowed to acclimate overnight at 18.1 °C with a salinity of 36 ppt. There were 9 total aquaria, with 7 plants per tank. Control tanks were maintained at a pH of 8.1 to mimic the natural environment. The experimental groups maintained pH values of 7.8 and 7.5 approximately. The pH levels were maintained for 12 hours, with each pH condition having 3 replicate tanks. To measure pH levels, a pH meter was inserted to the tanks every 30 minutes. For the experimental tanks, 5% HCl was used to maintain pH levels when needed.

Oxygen Production and Respiration Measurement

After two days of experimental treatment, three seagrass plants were removed from each tank for a total of six plants per group. This meant that there would be two replicates for each experimental run of the respirometry equipment and that each pH group would have three plants per run. The dimensions of each plant were recorded using a ruler and used to calculate the total surface area of the leaf. Then, they were placed in cups of seawater, collected from the Bay of Cádiz, in order to keep them from drying out. The production of oxygen, during photosynthesis, and its use, during respiration, was measured using a closed respirometry setup with two intervals of 30 minutes, one providing light conditions and the other providing dark. Two cylindrical Plexiglas chambers, each containing three plants from the control group (pH 8.1), were completely submerged in a water bath, which allowed two respirometer trials to run simultaneously for each experimental group. Each chamber was connected to an Eheim pump with a flow rate of 5.0 l/min (Deizisau, Germany) using polyvinyl chloride (PVC) tubing, which circulated oxygenated/deoxygenated water through the system to an oxygen probe without letting oxygen escape into the water bath (see Figure 2).

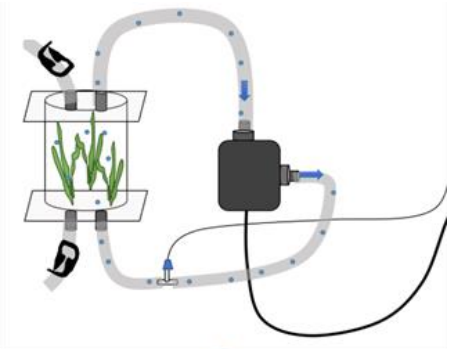

Figure 2. Respirometer set-up for one trial run where blue circles indicate oxygen and blue arrows indicate the flow through the apparatus.

The concentration of oxygen produced or used by the seagrass was measured using a multi-channel oxygen meter (Witrox 4, Loligo Systems, Viborg, Denmark). Measurements

were taken for two trials of each pH group for a total of 60 minutes with values recorded by the oxygen meter every second.

Chlorophyll Extraction

The method used for extracting chlorophyll pigments was modified from a previous study that used freshwater algae instead of seagrass (Arar, E. J., 1997). Plants were directly removed from the respirometer set-up and sorted for chlorophyll extraction. The total wet mass of each plant was recorded to ±0.0000 grams for 6 plant units per group for a total of 18 plants. Then, a razor blade was used to remove any dead leaves and epiphytes from the primary shoot of the plant as well as the rhizome segment. Each plant was then cut into four equal sections, from which, the upper middle section was used for chlorophyll sample (see Figure 3.). The wet mass and approximate length was recorded for all 18 plants. The lengths of the plants were between approximately 3 and 5 centimeters.

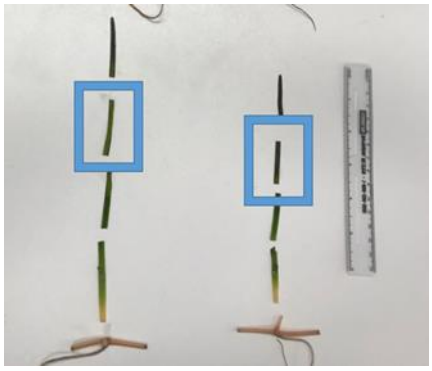

Figure 3. Leaf sections that were cut from the seagrass shoots with the upper middle sections that were used highlighted with a blue box.

Forceps were used to place the first plant sample from the control group (pH 8.1) into the bottom of a clean, dry dounce homogenizer. Then, the homogenizer was filled with 2 mL of a 90% acetone solution The sample was crushed in solution until the chlorophyll pigment was fully extracted (only translucent cell particulates were left at the bottom and none of the solution remained on the sides of the homogenizer). Next, 2 mL of the extracted chlorophyll solution was transferred to a 2 mL centrifuge tube. This process was repeated for all 18 plant samples, using a clean, dry homogenizer each time and placing each labelled tube into a test tube rack for storage. The sample tubes were centrifuged at 12,000x g for 15 minutes and approximately 2 mL of the supernatant liquid was then transferred to clean, labelled test tubes using a 1 mL micropipette. The first sample of the control group (pH 8.1) was transferred to a 2 mL cuvette and prepared to be inserted into the spectrophotometer. The spectrophotometer was blanked using 2 mL of the 90% acetone solution in a 2 mL cuvette. Absorbance readings were then recorded for the first control sample at wavelengths of 750, 664, 647, and 630 nm. This process was repeated for all other 17 plant samples using clean, dry cuvettes and pipette tips each time. These values were inserted into Jeffrey and Humphrey's Trichromatic Equation (see Equation 2.) in order to calculate the concentration of chlorophyll a in each solution.

$$C_{E,a} = 11.85\ (Abs\ 664) - 1.54\ (Abs\ 647) - 0.08\ (Abs\ 630)$$

Equation 2. Calculation of $C_{E,a}$, the concentration (mg/L) of chlorophyll a in the extraction solution, based on absorbance valuess at various wavelengths.

Analysis of Rosmarinic Acid

The method used for analyzing the rosmarinic acid content with HPLC was modified from a previous study that focused on the phenolic acid content of Z. marina (Achamlale et al., 2009). For the extraction of rosmarinic acid, 3 plants from each tank were collected together and cut 7.62 centimeters from the rhizomes. Top leaves of the plants were kept, while rhizomes and roots were discarded. Leaves from each tank were pat dry and wet weights were collected. Samples were then dried in an oven overnight at 80 °C. Dried leaves were then homogenized with a mortar and pestle, and 0.11 grams of each were weighed out. Samples were then added to a 5.5 mL of ethanol: water solution (60:40), and stirred on a stir plate at room temperature for an hour. After mixing, solutions were centrifuged at 12,000 x g for 10 minutes. Using a syringe, 1 mL of the liquid solutions was filtered and inserted into a 2 mL HPLC vials. Samples were then entered into an RP-HPLC PDA detector with a wavelength range of 210 to 400 nm. The HPLC model used was XEVO-G2S and the

samples were analyzed by central services at UCA and a specific column model was not provided. The solvents were water with 0.1 formic acid and HPLC grade methanol. Flow rate was 0.6 (ml/min).

RESULTS

Sediment PAH Levels

The site of collection, which exhibited a lot of human activity, and a less populated site were compared in terms of PAHs. The specific PAH's that were searched for in both sediment samples were benzo(a)pyrene (252.31 g/mol), benzo(a)anthracene (228.28g/mol), pyrene (202.25 g/mol), and anthracene (178.23 g/mol) as stated earlier. In terms of qualitative data the site of data collection appeared to have a higher abundance of all four PAHs when compared to the less populated site. Due to insufficient separation of peaks in the GC method, exact differences could not be quantified between individual PAHs.

Oxygen Production/Usage

Oxygen data was collected for a period of 60 minutes, including a 30 minute light period and a 30 min dark period. A representative graph is shown in Figure 4.

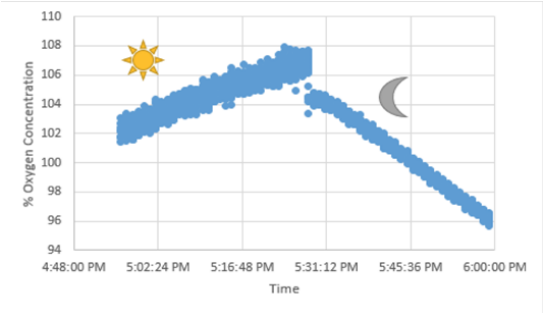

Figure 4. A representative graph of the oxygen production/use of *C. nodosa* where the upward slope indicates light-saturated conditions (respiration and photosynthesis) and the downward slope indicates a period of darkness (respiration only).

The data points taken every second were used to calculate a rate of photosynthesis and respiration for each group in percent oxygen per hour per gram of plant. These average rates are shown in Table 1.

	pH 8.1 (control)	pH 7.8	pH 7.5
Photo.	7.024 +/- 0.65	6.407 +/- 0.82	7.635+/- 1.2
Resp.	5.086 +/- 0.012	2.487+/- 0.23	4.635+/- 1.4

Table 1. The average rate of photosynthesis and respiration of two replicates in % ⋅ hr^{-1} ⋅ g^{-1} for pH values 8.1 (control), 7.8 , 7.5 Chlorophyll Concentration

After preparing the extracted chlorophyll solution, absorbance readings were taken at wavelengths of 750, 664, 647, and 630 nm producing four different values for each sample as shown in Table 2. These values were then used as inputs for the trichromatic equation.

	750 nm	664 nm	647 nm	630 nm
pH 8.1	0.01	1.51	0.79	0.38

Table 2. Example absorbance values for a sample from pH 8.1.

$$C_{E,a} = 11.85\,(1.51) - 1.54\,(0.79) - 0.08\,(0.38)$$
$$C_{E,a} = 16.65\ mg/L$$

The calculation shown above used absorbance values from the first of six samples in the pH 8.1 group. The concentration of chlorophyll *a* in mg/L of extracted solution was calculated for each sample from all three pH groups (8.1, 7.8, and 7.5). The average values with standard deviations were calculated for each pH group and compiled into Table 3**,** shown below.

pH 8.1 (control)	pH 7.8	pH 7.5
12.4 +/-3.7	13.3 +/- 4.6	10 +/- 1.5

Table 3. The average values of chlorophyll *a* concentration in mg/L of solution for the Control, pH 7.8, and pH 7.5 groups with their standard deviation values.

A one-way analysis of variance test (ANOVA) was conducted to determine the effect of decreasing the ambient pH on the chlorophyll pigment concentration of the seagrass with 3 different conditions (pH of 8.1, 7.8, and 7.5). The ANOVA showed that the effect of

decreasing pH on chlorophyll pigment concentration was not significant, $F(2,15) = 1.3595$, $p = 0.287$. Although the average chlorophyll concentration of the Control group (pH 8.1) was higher than that of the Experimental group, which had the lowest pH of 7.5, the difference is not significant but shows that further studies should be conducted to corroborate these findings.

Rosmarinic Acid Concentration

To find the total concentration of rosmarinic acid in each plant sample, a standard curve using concentrations of 0.01, 0.1, and 0.5 ppm and the equation of the line was used (see Equation 3).

$$y = 385.76x + 66.89$$

Equation 3. Line of best fit for the area of under the peak in the HPLC chromatograms (y) and the concentration of rosmarinic acid (x) in ppm.

Average concentrations were found in micrograms per gram of dry weight ($\mu g\ g^{-1}$). Highest average was found in experimental pH group 7.5 (10.1 $\mu g\ g^{-1}$), with lowest average found in the control group (1.7 $\mu g\ g^{-1}$) (see Table 4).

pH 8.1 (control)	pH 7.8	pH 7.5
1.7 +/- 1.2	2.9 +/- 1.6	10.1 +/- 0.8

Table 4. Average rosmarinic concentration found in **µ** g g-1 for pH values 8.1, 7.8, and 7.5 with standard deviation values.

A one-way ANOVA test was done to determine if there was a relationship between the concentration of rosmarinic acid at the 3 pH values of (8.1, 7.8, 7.5). The ANOVA test showed that there was a significant relationship between rosmarinic acid content and changing pH, $F(2,6) = 5.1423$, $p = 0.050$. Therefore, the data met the assumptions of the ANOVA since there was a significant difference among the three groups.

DISCUSSION

An overall trend was seen in the seagrass that as the level of pH decreased in the seagrass' surroundings the rosmarinic concentration increased. The same did not hold for the respiration/oxygen production or the chlorophyll concentration of the seagrass. The values of the rate of respiration between the seagrass at a normal pH of 8.1, pH of 7.8, and a pH of 7.5 were relatively similar. Similarly, the chlorophyll concentration in the seagrass also was not significantly different between groups. Therefore, a clear trend could not be concluded for the chlorophyll and respiration in the seagrass when comparing all three pH groups. The increase in phenolic acid (rosmarinic acid) results disagreed with the *in situ* experiment that was done on this specific seagrass species (Arnold et al 2012). Both results show a change in phenolic acid concentration as the seagrass was stressed, but the directions of the changes were different.

Although phenolic acids play a major role in plant protection from microbial grazing and from changes in the environment, further analysis needs to be done to better understand the relationships between phenolic acids and changing pH levels. Additionally, the roles of each type phenolic acid in seagrass species must be better studied as there is limited knowledge of this. It has been observed that in the species *Z. marina*, caffeic acid plays a major role in disease resistance, but it is unknown the roles that other phenolics play in that species and in *C. nodosa* (Buchsbaum, Short, & Cheney, 1990). Rosmarinic acid has many different antimicrobial and antioxidant properties in plants, but it may not play a major role in this species of seagrass.

Future replications of this experiment could benefit from more extensive methods of standardization in terms of the seagrass and derived samples. Specifically for the chlorophyll analyses, the samples should be based off of dry mass only so that any extra water does not interfere with the standardization of the samples and their masses. Additionally, when cutting sections of the seagrass for the

samples, it should be noted whether or not one plant is particularly unhealthy, which would cause it to have less green pigment and more of a brown color. This could lead to statistical outliers in the dataset. In terms of the experiment as a whole, the results might be more informative if more time was allocated to the control and experimental groups in order to see clear differences based on the treatment. In this study, the groups were only given a maximum of 2 days in their respective conditions. Although there were differences between them in terms of chlorophyll content and respiration, there would likely be a more significant difference between the control and experimental groups for phenolics, chlorophyll, and respiration if they had more time to adjust to their conditions. A longer experimental treatment could lead to less respiration and chlorophyll content on the seagrass with lower pH while having an increased rosmarinic acid concentration. Another variable to consider for future replications of this experiment is the ability of the water, soil, and seagrass to act as buffers and naturally resist changes in pH. This meant that the experimental tanks needed to be checked more frequently and additional HCl needed to be added in order to keep the tanks at the appropriate experimental pH values. For the most accurate results, the pH of the tanks should be monitored more frequently, perhaps every 10-15 minutes, and more acid may need to be added for each increment or the ambient pH may need to be lower than was used for this experiment.

CONCLUSION

Overall, it can be concluded that ocean acidification had a significant effect on the concentration of rosmarinic acid the seagrass between a pH of 8.1 to 7.5 but not on the other health biomarkers examined. When analyzing the results statistically, we can conclude that ocean acidification has a significant effect on the concentration of rosmarinic acid in *C. nodosa.* The statistics calculated from the ANOVA test were found to be $F(2,6) = 5.1423$, $p = 0.050$. This showed that the difference in the data collected between the three groups is significant at different pH levels of 8.1 ,7.8, and 7.5. Therefore, a decrease in pH does show change in the rosmarinic acid content.

Further studies may be conducted to focus on the relationship between phenolic compounds and their role in seagrass health in controlled lab environments rather than *in situ*. This may yield information on how to effectively maintain seagrass populations around the world by mitigating the anthropogenic stressors, which is fundamental to the health and diversity of the aquatic-ecosystem.

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