Impacts of Groundwater Discharge at Myora Springs (North Stradbroke Island, Australia) on the Phenolic Metabolism of Eelgrass, Zostera muelleri, and Grazing by the Juvenile Rabbitfish, Siganus fuscescens

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Abstract

Myora Springs is one of many groundwater discharge sites on North Stradbroke Island (Queensland, Australia). Here spring waters emerge from wetland forests to join Moreton Bay, mixing with seawater over seagrass meadows dominated by eelgrass, Zostera muelleri. We sought to determine how low pH / high CO2 conditions near the spring affect these plants and their interactions with the black rabbitfish (Siganus fuscescens), a co-occurring grazer. In paired-choice feeding trials S. fuscescens preferentially consumed Z. muelleri shoots collected nearest to Myora Springs. Proximity to the spring did not significantly alter the carbon and nitrogen contents of seagrass tissues but did result in the extraordinary loss of soluble phenolics, including Folin-reactive phenolics, condensed tannins, and phenolic acids by ≥87%. Conversely, seagrass lignin contents were, in this and related experiments, unaffected or increased, suggesting a shift in secondary metabolism away from the production of soluble, but not insoluble, (poly)phenolics. We suggest that groundwater discharge sites such as Myora Springs, and other sites characterized by low pH, are likely to be popular feeding grounds for seagrass grazers seeking to reduce their exposure to soluble phenolics.

Introduction

North Stradbroke Island (NSI) is one of the world’s largest sand islands, covering 285 km² and framing the east side of Moreton Bay in southeast Queensland, Australia. The island, also known by its aboriginal name of Minjerribah, is surrounded by extensive seagrass meadows [1,2] which are grazed by fishes, turtles, and dugongs and serve as nursery grounds for other native species (e.g., [3–6]).

The unusual geomorphology of NSI allows rainwater to recharge a massive aquifer of groundwater, forming a “central mound” or lens of freshwater under the island [7]. Pressure within this aquifer prevents saltwater intrusion and drives groundwater out beyond the coastline and into numerous creeks, swamps, window lakes, and springs on the island. One such release point is Myora Springs, located on the west coastline of NSI. Myora springs discharges approximately 2.4 million liters day⁻¹ of groundwater through a mangrove forest and paperbark (Melaleuca quinquenervia) swamp and into Moreton Bay [8–10]. Here spring waters mix with seawater over shallow seagrass meadows dominated by eelgrass, Zostera muelleri. The degree of mixing is dependent upon the tides; for at least several hours per tidal cycle, at low tide, the effluent extends over 2500 m² of exposed seagrass meadow. Several observations suggested that spring effluent might be impacting the shallow water marine community at this site; for example, anecdotal reports from local fisherman describe pitted and eroded shells in this area and we observed a lack of calcareous epiphytes on seagrasses near the shoreline, both of which suggest possible low pH conditions [11]. Indeed, a preliminary study in January 2012 revealed that spring effluent, pH 5, extended over exposed seagrass meadows during low tides, depressing seawater pH by ~0.5 units at a distance of 10–20 m from the edge of the mangrove forest, without detectable changes in salinity at this distance.

Previously we found that low pH conditions and the corresponding increases in seawater CO₂ levels were associated with a dramatic loss of protective phenolic substances, including phenolic acids and condensed tannins, in four different populations of seagrass ([12], also see [13]). These results were surprising since terrestrial plants exposed to elevated CO₂ conditions often exhibit increased levels of many ‘carbon-based’ secondary metabolites, including (poly)phenolics [14–21]. Indeed, light and...
carbon availability stimulate the shikimic acid and phenylprope- noid (SA/PP) pathways that synthesize most plant phenolics [22– 24]. Whatever the cause, such a loss of seagrass phenolics may have ecological consequences; they serve as herbivore deterrents, digestion reducers, and antifoulants and some possess antimicro- brial properties, inhibiting the growth of the marine pathogen Labyrinthula which causes the seagrass wasting disease [25–37].

In the earlier set of experiments [12] the potential impacts on herbivores were not investigated because there were few important seagrass grazers at those study sites. In contrast, there are numerous large herbivores consuming significant quantities of seagrass in Moreton Bay. Here we sought to determine how the spring affects the dominant macrophyte Z. muelleri and interactions with one co-occurring grazer, the black rabbitfish (Siganus fuscescens).

Methods

Study Site

Myora Springs is located on the west coast of North Stradbroke Island, Queensland, Australia (7°30′54.7″S 153°27′43.5″E). It is one of many island habitats, including creeks, gullies, swamps, and perched lakes, receiving freshwater from a pressurized lens of groundwater [8,9]. In this case spring water flows through several types of forested wetlands, including a paperbark swamp, eucalypt woodland, and mangrove forest, before reaching Moreton Bay [10]. Preliminary surveys, conducted at low tide, suggested that spring waters flowing westward onto exposed seagrass meadows, mixed with seawater from Moreton Bay to generate a pH gradient. We examined seagrass patches along this gradient, focusing on areas located 5–10 m (near spring) and 30–50 m (background) from the spring outflow which differed in pH but not salinity during low tides (Fig 1).

Water analyses

Preliminary analyses of the site were conducted in March and April 2012 using handheld instruments [12]. In April 2012 a series of discrete water samples from the spring itself and additional “near spring” and “background” sites were collected. Samples were immediately transported on ice to SGS Environmental in Rocklea, Queensland (NATA accreditation #2562, site 2070) for analysis of pH, alkalinity, total nitrogen, total Kjeldahl nitrogen, nitrates, nitrites, and sulfates. Corresponding pCO 2 values were calculated using the CO2SYS 1.05 program [30,39] with constants of Cai and Wang [40].

Specimen collections

The collection of juvenile black rabbitfish, Siganus fuscescens, was conducted by nighttime seineing in One Mile Harbor, Dunwich under the auspices of permit SBS/182/12/URG/ GOODMAN FOUNDATION to I.R. Tibbetts. Fish were held overnight following the animal care policies of Queensland Animal Care and Protection Act 2001 (Animal Care and Protection Regulation 2012) in shaded tanks supplied with fresh seawater from the seawater system at the Moreton Bay Research Station (MBRS), University of Queensland as approved by the institution’s Institutional Animal Care and Use Committee (IACUC). Zostera muelleri was collected from Myora Springs site under the auspices of permit #Q52011/MAN151 from the Queensland Department of Environment and Resource Management awarded to T. Arnold. Collections were made at ten “background” sites, located 30–50 m from the spring outflow, and ten “spring” sites located 5–10 m from the mouth of the spring. Plants were immediately transported in cool dark insulated containers, between layers of seawater moistened towels, to MBRS.

Feeding experiments

For feeding trials, blades from shoots of Z. muelleri were gently wiped clean of fouling organisms and clipped to consistent 10 cm lengths. Three to five shoots, each possessing 3–4 blades were fastened together, weighed, and affixed at one end to plastic tubes filled with sand, to mimic the morphology and orientation of intact eelgrass shoots (Fig 1c). The average mass for these clusters of eelgrass shoots was 0.85 g wet mass (WM). Similar sections, and bulk plants, were stored at ~80°C and subsequently transported to Dickinson College, Carlisle, PA (USA) excised in dry ice. Paired choice feeding trials were conducted in a series of 12 shaded 50 liter tanks containing four juvenile rabbitfish each (Fig 1d). Fish had been acclimated to tanks and had not had access to food for 24 hours prior to the trials. Weighted groupings of seagrass shoots, one from “background” sites and one from “near spring” sites, were placed into randomly selected ends of each tank at the start of the trials. Trials concluded when one food item was ~50% consumed; clumps were removed, blotted dry, weighed and measured. Each fish participated in only 1 trial. To control for potential non-grazing weight loss a set of seagrass food items (n = 3 from both sites) were placed into similar tanks without fish for corresponding time periods [41]. We observed no autogenic loss of tissue for these controls.

Tissue analyses

Analyses of seagrass metabolites were conducted for leaf tissues (without roots and rhizomes) harvested from each location. Leaves were gently wiped clean of epiphytes. For elemental analyses second and third rank leaves from four Z. muelleri plants per location were combined, dried, and homogenized. Samples (3–5 mg each) were analyzed for carbon and nitrogen contents as well as 13C and 15N signatures on a Europa isotope ratio mass spectrometer at the Stable Isotope Laboratory at the University of California Davis. Biochemical analyses were conducted on tissues samples stored at ~80°C. Leaf tissues for 3–5 shoots per location were homogenized using a HT high throughput grinder and extracted in MeOHaq with 2% acetic acid (except in the case of phenolic acid analyses) for 24 h at 4°C in the dark. Concentrations of phenolic acids were determined by RP-HPLC using a gradient method modified described previously [12,42]. Fifteen μl of each extract were injected onto an Agilent Eclipse Plus RP-18 HPLC column. Phenolic acids previously attributed to Z. muelleri [42] were identified by comparison to commercial standards and concentrations (mg compound g−1 blade DM) determined using individual standard curves. Condensed tannins (proanthocyanidins) and Folin-reactive phenolics were quantified using microplate assays derived from the acid-butanol and micro-Folin methods [43–46] with standard curves developed using quebracho tannin obtained from A. Hagerman (Miami University of Ohio) and gallic acid (Sigma), respectively. Natural product concentrations were expressed as mg compound g−1 tissue wet mass (WM) or %WM. Lignin contents were determined for Z. muelleri shoots from 10 locations. Five to eight shoots per location were cleaned, pooled, air-dried, and homogenized. Well-mixed 75 mg aliquots were analyzed using the methods of Foster et al [47]. Acetyl bromide soluble lignin (%ABSL) was calculated using the coefficient 17.75 and expressed as mg g−1 cell wall unit.

Statistical analyses

Statistical analyses were conducted using SigmaStat. Datasets comparing two groups were analyzed using two-tailed Student’s t-
tests or Mann-Whitney Rank Sum Test with an \( \alpha \) level of 0.05. Water chemistry data and datasets resulting from the analyses of tissues from previous experiments [12] which examined three sampling sites groups were compared using ANOVAs with Holm-Sidak multiple comparisons or, when transforming data did not satisfy test assumptions, with Kruskal-Wallis One Way Analysis of Variance on Ranks with Tukey or Dunn’s multiple comparisons.

**Results**

We observed that for the common seagrass *Z. muelleri* proximity to Myora Springs was associated with increased grazing by juvenile black rabbitfish and a dramatic and nearly complete loss of soluble phenolics, such as phenolic acids and condensed tannins, but not insoluble lignins.

**Site characterization**

As expected, the spring effluent was cool, fresh, and relatively acidic. The spring was not a significant source of alkalinity, sulfates, total Kjeldahl nitrogen and nitrite but did contain elevated levels of nitrate (Table 1). During low tides the effluent extended over the exposed seagrass meadows, over an area of \( \sim 2500 \) m\(^2\), mixing with Moreton Bay seawaters. The plume of spring water influenced seawater chemistry 5–10 m from the source; here, pH was depressed an average of 0.5–0.8 units and corresponding pCO\(_2\) values were increased \( \sim 400\% \), reaching an average level of 1749 ppm (Table 1). Spring water dilution of seawater resulted in slightly lower levels of sulfates, total Kjeldahl nitrogen, and total N, compared to unaffected sites located 30–50 m away (Table 1). At this distance, we observed no significant reductions in salinity or water temperatures, during low tides. *Zostera muelleri* was the dominant seagrass, with *Halophilla ovalis* interspersed. The presence of shallow channels of spring water.

**Figure 1.** Spring water from Myora Springs emerges from a mangrove forest (A) and discharges over shallow seagrass beds in Moreton Bay, Australia, during low tides (B). Shoots of *Zostera muelleri*, collected from “near spring” sites 5–10 m from the mouth of the spring and “background” sites at distances of 30–50 m were used in pair-choice feeding trials. Clusters of shoots from each location (C) were offered as food items to juvenile black rabbitfish, *Siganus fuscencens* (D).

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near the shore and occasional ~1 m diameter depressions in the sediment (stingray feeding sites) generated some patchiness within an otherwise dense seagrass meadow. Except for the aforementioned differences in epiphytic cover seagrasses from the various sites were indistinguishable. We did not observe signs of grazing (e.g. bite marks) on Z. muelleri leaves. However, this is not surprising since most co-occurring herbivores, including rabbitfish, tend to consume whole blades or shoots, without leaving tell-tale grazing scars.

Feeding experiments
Rabbitfish began to consume Z. muelleri immediately and most feeding trials were completed within 30 minutes, when ~50% of the “near spring” tissue mass had been consumed. S. fuscescens fed preferentially upon seagrass blades collected near the spring (Figure 1; Mann-Whitney Rank Sum Test, P = 0.012). A total of 48 fish, from twelve replicate aquaria, consumed significantly more “near spring” tissue (55% or an average of 400 mg tissue trial⁻¹), compared to tissue from plants collected at “background” sites (25% or an average of 200 mg tissue trial⁻¹).

Elemental analyses
Neither the carbon and nitrogen contents nor the C:N ratio of seagrass foliage were significantly altered by proximity to the spring (Table 1). The δ¹³C and δ¹⁵N signatures of plant tissues were both significantly increased near the spring (Student’s t-test; P = 0.001 and P = 0.003, respectively). These values and the observed increases are similar to previously reported isotopic signatures for Z. muelleri on NSI [48].

Biochemical analyses
We found that Z. muelleri from sites >30 m from the spring outflow accumulated significant levels of proanthocyanidins (condensed tannins) and possessed many, but not all, of the expected phenolic acids previously reported for this species (see [41]). The concentrations of all soluble phenolics decreased significantly, often becoming undetectable in plants sampled near the shore and occasional ~1 m diameter depressions in the sediment (stingray feeding sites) generated some patchiness within an otherwise dense seagrass meadow. Except for the aforementioned differences in epiphytic cover seagrasses from the various sites were indistinguishable. We did not observe signs of grazing (e.g. bite marks) on Z. muelleri leaves. However, this is not surprising since most co-occurring herbivores, including rabbitfish, tend to consume whole blades or shoots, without leaving tell-tale grazing scars.

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Discussion
Myora Springs surface water flowing through paperbark and mangrove forests was low in salinity, alkalinity, pH, total and Kjeldahl N, and sulfates but higher in nitrates, compared to Moreton Bay seawater (Table 1). At the coast, spring water mixed with seawater, generating a gradient of abnormally low pH conditions within a ca. 2500 m² area during low tides. The pH of seawater over emergent seagrasses was reduced by ~0.7 units; for example, in early April we observed pH 7.8 waters at sites within 10 m of the effluent, compared to an average of pH 8.5 for seawater collected 30–50 m away. Similar conditions were recorded throughout March and April 2012. Most other conditions were unaffected, or affected to a minimal degree. Eelgrass shoots collected near the outflow of the spring were apparently healthy and, except for a lack of calcareous epiphytes, indistinguishable from plants collected 30–50 meters away.

Seagrass proximity to the spring altered the feeding behavior of a native grazer, the black rabbitfish, Siganus fuscescens. In paired-choice feeding trials juvenile rabbitfish immediately identified and

### Table 1. Chemical characteristics of water near Myora Springs, North Stradbroke Island, Australia in April 2012.

| Conditions | Seawater chemistry |
|------------|--------------------|
| Distance from seep | Spring | 5–10 m | 30–40 m | Test | P |
| Temperature (°C) | 23.15±0.66<sup>a</sup> | 24.92±0.16<sup>ab</sup> | 26.56±0.97<sup>b</sup> | 1 | 0.019 |
| Salinity | 2.7±0.8<sup>a</sup> | 35.3±0.2<sup>b</sup> | 35.4±0.3<sup>b</sup> | 2 | 0.009 |
| Total Kjel. Nitrogen (calc) (mg/L) | 0.117±0.003<sup>a</sup> | 0.192±0.039<sup>ab</sup> | 0.287±0.024<sup>b</sup> | 1 | 0.021 |
| Alkalinity - Total as CaCO₃ (mg/L) | 2<sup>c</sup> | 120.0±0.0 | 120.0±0.0 | 2 | 0.071 |
| Nitrate as N (calc) (mg/L) | 0.117±0.009<sup>a</sup> | 0.049±0.014<sup>b</sup> | 0.001±0.001<sup>c</sup> | 1 | 0.001 |
| Nitrite + Nitrate as N (mg/L) | 0.120±0.006<sup>a</sup> | 0.054±0.014<sup>b</sup> | 0.002±0.001<sup>c</sup> | 1 | 0.001 |
| Nitrite as N (mg/L) | 0.004±0.000 | 0.005±0.001 | 0.005±0.000 | 1 | 0.706 |
| Total Nitrogen as N (mg/L) | 0.237±0.003 | 0.248±0.026 | 0.307±0.024 | 1 | 0.136 |
| Sulphate as SO₄ (mg/L) | 2.6±0.3<sup>a</sup> | 1937.5±531.3<sup>ab</sup> | 2566.7±120.2<sup>b</sup> | 2 | 0.023 |
| pH Value @25°C | 5.5±0.1<sup>a</sup> | 7.8±0.3<sup>b</sup> | 8.5±0.0<sup>b</sup> | 2 | 0.001 |
| pCO₂ (µatm) | 174±15<sup>a</sup> | 572±4<sup>b</sup> | 3 | 0.002 |

Statistical analyses: 1, one-factor ANOVA with Holm-Sidak multiple comparisons; 2, Kruskal-Wallis One Way Analysis of Variance on Ranks with Tukey or Dunn multiple comparisons; Student’s t-test. * below detection limit, did not test. Values are means ±/− SE. doi:10.1371/journal.pone.0104738.t001
preferentially consumed Zostera muelleri leaves collected nearest to the spring. They removed approximately twice the tissue from these plants, in terms of total mass and percent mass lost, compared to those collected from more distant sites (Fig 2). In fact, they often ignored seagrasses collected from more distant sites until much of the other, more preferred leaves were consumed.

The feeding preferences of marine grazers, including fishes, can often be influenced by seagrass carbon and nitrogen contents which, in turn affect leaf toughness and digestibility [49–52] but this did not seem to be a factor in our feeding trials simply because these characteristics did not differ significantly in the seagrass leaves from the two locations. For example, nitrogen input from Myora Springs was relatively low, even though nitrates were elevated, and we did not detect a significant change in Z. muelleri tissue %N near the spring (Table 1). The %N contents and 15N signatures we observed for Z. muelleri tissues were comparable to values reported for relatively other un-impacted sites in Moreton Bay [48]. The small change in tissue 15N signatures near the spring may indicate some limited input of anthropogenic nitrogen [48,53] but this did not alter the nitrogen content of the seagrasses (Fig. 3).

Similarly, we found that carbon contents of Z. muelleri tissues were not altered significantly by proximity to spring effluent. This was surprising since Z. muelleri the estimated pCO2 concentrations were elevated ~400% near the spring, at least during low tides. Leaf δ13C signatures suggested that carbon assimilation had been enhanced in the recent past, as would be expected for plants possessing poor carbon concentrating mechanisms (e.g., [54–56]) at these high CO2 locations (Fig. 3). As the unaltered %C of %N contents would suggest, seagrass tissue C:N ratios were unchanged. They were similar to those reported for this species in nearby locations [53].

To determine if it is common for seagrass C and N contents to be unaffected by high CO2 / low pH conditions we analyzed tissues from a previous study examining the response of other seagrass species at Mediterranean CO2 vents sites and in free ocean carbon enrichment experiments [12]. These results were indeed similar: low pH / high CO2 conditions did not alter carbon and nitrogen contents, carbohydrate levels, or protein contents in these earlier studies (Tables 2,3; see [12] for site descriptions). Similarly, C:N ratios from these earlier studies did not change, or change in any particular direction. We detected similar decreases in the δ13C values of Cymodocea nodosa growing near this high CO2 vent near the island of Vulcano, Italy which is consistent with the previous reports of Vizzini et al [56] who detected a similar decrease in δ13C values of Posidonia oceanica exposed to high CO2 vents near Panarea, Italy.

In contrast the secondary chemistry of Z. muelleri near Myora Springs was dramatically different (Fig. 3). Levels of Folin-reactive phenolics were reduced 87% in leaf tissues of these plants, compared to those located 30-50 m away. Condensed tannins, normally present at relatively high concentrations (15.3% of seagrass wet mass), were undetectable in leaf tissues collected near Myora Springs. Similarly, concentrations of phenolic acids decreased ≥93% with some compounds normally present in this species reduced to undetectable levels. In general, the phenolic metabolites of Z. muelleri responded to low pH conditions near Myora Springs in much the same way that other seagrass species respond to low pH and high CO2 conditions elsewhere. Previous studies revealed comparable losses of seagrass phenolics in Cymodocea nodosa and Posidonia sp. near high CO2 vents in the Mediterranean Sea and Ruppia maritima and Potamogeton perfoliatus exposed to free ocean carbon enrichment in the Chesapeake Bay [USA] [12,13].

We quantified leaf lignin contents because these polyphenols are also products of the SA/PP pathway [57] and can influence herbivore feeding behaviors and digestion efficiencies [38]. We hypothesized that concentrations of lignin, like those of the other SA/PP phenolics, would be reduced; however, surprisingly, we found that lignin concentrations of Z. muelleri were not affected by proximity to the spring (Fig 3). To determine if this was a common occurrence we again analyzed stored tissues of Cymodocea nodosa and Ruppia maritima from our previous studies [12]. In these cases we observed significant increases in lignin in seagrasses exposed to low pH / high CO2 waters (Tables 2,3). The lignin contents of seagrasses collected near a natural CO2 vent site and exposed to high CO2/low pH conditions in FOCE experiments in the Chesapeake Bay were 150% and 124% higher, respectively, than lignin contents of nearby seagrasses. The results of all three studies indicate that while levels of soluble phenolics were consistently reduced at the various low pH / high CO2 sites, the concentrations of lignin did not decrease. These observations would be familiar to those studying the response of terrestrial plants to high CO2: for example, in land plants exposed to elevated atmospheric CO2 concentrations in free air carbon enrichment studies lignin levels are often increased or unaffected, but are rarely reduced (e.g., [59–62]). For studies of aquatic plants, such as seagrasses, we must also consider the potential impact of seawater pH, irrespective of elevated CO2 availability. Low pH can trigger ‘acid growth’ which softens plant cell walls by stimulating the activity of pH-sensitive expansin enzymes and the expression of related genes [63,64]. Expanded cell wall structures are subsequently stabilized [64], in part by acid-induced lignification [65]. Instances of enhanced lignin production, where they occur [12], could help to explain the depletion of phenolic acids pools, as some of these compounds are required precursors in lignin biosynthesis. This suggests a growth vs. defense tradeoff that would be difficult to untangle, given that many of these SA/PP compounds have multiple roles in plants [66].

Many marine grazers, including fish, turtles and dugongs, tend to prefer species with lower levels of neutral digestible fiber (a combination of lignins and cellulosic substances) [67–70]. However, since we did not observe altered lignin levels in the leaves of Z. muelleri here this would not explain the feeding preferences exhibited by the rabbitfish in our feeding trials. Rather the ‘per
The nutritional quality of Z. muelleri, and the feeding behavior of grazing fish, is most likely to have been determined by soluble phenolics. These compounds are the only characteristics of the food items shown to have changed significantly in response to spring effluent. This may explain the near-instantaneous food choices of Siganus fuscescens during feeding trials. We observed that fish seemed to select preferred food items using either visual or olfactory cues, perceiving differences we could not. They generally did not browse or taste multiple food items during feeding trials. We hypothesize that the changing phenolic chemistry of Z. muelleri was dramatic enough to be readily sensed by juvenile rabbitfish. Many fish and sea turtles have UV sensitive vision capable of detecting wavelengths of light absorbed strongly by plant phenolics, at least when young [71–73]. As fish mature UV sensitive cones are often modified and detect only blue light [71–72]. Interestingly, this is when rabbitfish lose their proclivity for consuming seagrasses.

Overall, our observations demonstrate the groundwater discharge at Myora Springs alters the phenolic qualities of nearby seagrasses and the feeding behavior of certain herbivores. Similar...
results were observed in previous studies examining seagrasses exposed to high CO₂ / low pH conditions near natural vent sites and generated by a series of simplified FOCE experiments [12]. Those studies sought to simulate, as closely as possible, future conditions of ocean acidification. In contrast, the situation at the Myora Springs site, where the exposure to low pH conditions is intermittent, is probably not an accurate simulation of anthropogenic ocean acidification. Nevertheless, these are naturally-

### Table 2. Composition of *Cymodocea nodosa* shoots collected at various distances from the natural CO₂ vent site from the Island of Vulcano, Italy in 2011 and 2013.

| Conditions | Seawater carbonate chemistry¹ |
|------------|-------------------------------|
| Distance from seep | 380 m | 300 m | 260 m |
| Salinity (ppt) | 37.16 ± 0.07 | 37.12 ± 0.06 | 37.05 ± 0.1 |
| pH (units) | 8.11 ± 0.01 | 7.84 ± 0.04 | 7.32 ± 0.05 |
| pCO₂ (µatm) | 422 ± 43 | 976 ± 269.5 | 4009 ± 1442.7 |
| TA (µmol kg⁻¹) | 2549.6 ± 29.6 | 2555.9 ± 28.9 | 2592.5 ± 48.3 |

| Cymodocea nodosa | Value | Trend | Test | P |
|------------------|-------|-------|------|---|
| Carbon (%) | 23.78 ± 6.89 | 31.89 ± 4.08 | 38.98 ± 3.58 | increase | 1 | 0.161 |
| Nitrogen (%) | 1.22 ± 0.35 | 1.31 ± 0.17 | 1.75 ± 0.17 | none | 1 | 0.302 |
| C:N ratio | 19.02 ± 0.69 | 24.25 ± 0.06b | 22.25 ± 0.20ab | intermediate | 2 | <0.001 |
| δ¹³C | -11.42 ± 0.03a | -11.68 ± 0.05ab | -11.90 ± 0.19b | decrease | 2 | 0.013 |
| δ¹⁵N | 1.69 ± 0.18a | 0.13 ± 0.04b | 0.15 ± 0.06ab | decrease | 2 | 0.013 |

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### Table 3. Composition of widgeon grass subjected to Free Ocean Carbon Enrichment within the St. Mary’s River, Maryland (USA) in May-July 2010.

| Conditions | Seawater carbonate chemistry¹ |
|------------|-------------------------------|
| Distance from injector | 500 cm | 40 cm | 5 cm |
| Temperature (°C) | 25.0 | 25.0 | 25.0 |
| Salinity (ppt) | 17 | 17 | 17 |
| pH | 8.4 | 8.0 | 6.9 |
| pCO₂ (µatm) | 157.8 | 469.3 | 6792.0 |
| TA (µmol kg⁻¹) | 1467.0 | 1444.0 | 1455.0 |

| Ruppia maritima | Value | Trend | Test | P |
|-----------------|-------|-------|------|---|
| Carbon (%) | 23.79 ± 2.40 | 29.10 ± 2.06 | 23.98 ± 2.20 | none | 1 | 0.190 |
| Nitrogen (%) | 2.34 ± 0.20 | 2.41 ± 0.11 | 2.08 ± 0.18 | none | 1 | 0.345 |
| C:N ratio | 10.24 ± 0.93 | 12.21 ± 1.16 | 11.57 ± 0.77 | none | 1 | 0.406 |
| δ¹³C | -13.20 ± 0.26 | -12.67 ± 0.25 | -12.82 ± 0.20 | none | 1 | 0.296 |
| δ¹⁵N | 11.06 ± 0.05 | 11.12 ± 0.08 | 10.93 ± 0.06 | none | 1 | 0.129 |
| Crude protein (%DM) | 15.34 ± 0.39 | 13.82 ± 0.56 | 13.92 ± 0.41 | none | 1 | 0.096 |
| Adj. protein (%DM) | 13.56 ± 0.37 | 12.36 ± 0.66 | 12.26 ± 0.42 | none | 1 | 0.167 |
| Starch (%DM) | 3.08 ± 0.77 | 4.00 ± 0.82 | 3.76 ± 0.70 | none | 1 | 0.684 |
| Sugar (%DM) | 9.14 ± 2.12 | 10.40 ± 0.90 | 8.70 ± 1.62 | none | 1 | 0.750 |
| Lignin (%DM)² | 12.60 ± 0.62a | 15.67 ± 0.57ab | 15.25 ± 1.11ab | increase | 1 | 0.027 |

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¹Seawater carbonate chemistry previously published in Arnold et al 2012.
²Lignin analyses conducted by the Cumberland Valley Forage lab in Cumberland Co, Maryland using the methods of Goering and Van Soest (1970). Values are means ± SE.

Statistical analyses: 1, one-factor ANOVA with Holm-Sidak multiple comparisons. Letters indicate results of pairwise comparisons test P<0.05.

Values are means ± SE.

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occurring low pH conditions, a type of "coastal acidification", which may have existed at Myora Springs for at least the last 105,000 years [10] and is common at other coastal groundwater discharge sites. Based on these findings we would predict that such sites would be popular feeding grounds for seagrass grazers seeking to reduce their exposure to soluble phenolics.

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Author Contributions

Conceived and designed the experiments: TA IT. Performed the experiments: TA IT GF TW AV. Analyzed the data: TA. Contributed reagents/materials/analysis tools: TA IT. Contributed to the writing of the manuscript: TA IT GF.

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