Ocean Acidification and the Loss of Phenolic Substances in Marine Plants

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Abstract

Rising atmospheric CO_2 often triggers the production of plant phenolics, including many that serve as herbivore deterrents, digestion reducers, antimicrobials, or ultraviolet sunscreens. Such responses are predicted by popular models of plant defense, especially resource availability models which link carbon availability to phenolic biosynthesis. CO_2 availability is also increasing in the oceans, where anthropogenic emissions cause ocean acidification, decreasing seawater pH and shifting the carbonate system towards further CO_2 enrichment. Such conditions tend to increase seagrass productivity but may also increase rates of grazing on these marine plants. Here we show that high CO_2 / low pH conditions of OA *decrease,* rather than increase, concentrations of phenolic protective substances in seagrasses and eurysaline marine plants. We observed a loss of simple and polymeric phenolics in the seagrass *Cymodocea nodosa* near a volcanic CO_2 vent on the Island of Vulcano, Italy, where pH values decreased from 8.1 to 7.3 and pCO_2 concentrations increased ten-fold. We observed similar responses in two estuarine species, *Ruppia maritima* and *Potamogeton perfoliatus*, in *in situ* Free-Ocean-Carbon-Enrichment experiments conducted in tributaries of the Chesapeake Bay, USA. These responses are strikingly different than those exhibited by terrestrial plants. The loss of phenolic substances may explain the higher-than-usual rates of grazing observed near undersea CO_2 vents and suggests that ocean acidification may alter coastal carbon fluxes by affecting rates of decomposition, grazing, and disease. Our observations temper recent predictions that seagrasses would necessarily be "winners" in a high CO_2 world.

Introduction

Increasing levels of atmospheric CO_2 can trigger accumulations of plant phenolic substances such as lignins, tannins, and phenolic acids and glycosides which serve as structural or chemical defenses against grazers and disease organisms [1–6]. In a meta-analysis of over one hundred separate studies Stiling and Cornelissen found that elevated CO_2 tends to increase plant C/N ratios and trigger the accumulation of tannins and other phenolics while also having significant effects on the abundance, consumption rates, development times, relative growth rates, conversion efficiencies, and pupal weights of a broad range of herbivores [7]. CO_2 enrichment can also alter the characteristics of leaf litter, inhibiting the activity of detritivores [1,8]. CO_2-stimulated accumulations of plant phenolics are often predicted by popular models of plant defense, especially resource availability models linking excess CO_2 and carbohydrates to an increased production of carbon-based defenses [9,10].

The availability of CO_2 is also increasing dramatically in oceans and estuaries. About a third of anthropogenic carbon emissions have been absorbed by the oceans, driving the process of ocean acidification wherein absorbed CO_2 generates carbonic acid,

increasing the concentrations of H^+, HCO_3^-, and dissolved CO_2, while lowering CO_3^{2-} concentrations and seawater pH [11]. In 150 years, the average ocean pH has dropped from 8.21 to 8.10 [12]. By the end of this century seawater pH is expected to fall another 0.3 to 0.4 units, leading to a 150% increase in H^+ and a corresponding increase in available CO_2 of ~300–400%. Acidification also occurs in estuaries where trends of decreasing pH have been detected amid the daily fluctuations driven by biological processes and tides [13,14]. Such high CO_2 / low pH conditions can stimulate the productivity of many marine photoautotrophs, including seagrasses which lack effective carbon-concentrating mechanisms [15–20]. For example, in mesocosm experiments CO_2 enrichment resulted in increases in photosynthesis, reproductive output, and carbohydrate levels in eelgrass, *Zostera marina* [21]. Similarly, studies of high CO_2 communities near submerged volcanic vents reveal luxurious seagrass beds with increased shoot densities and biomass, and leaves devoid of calcifying fouling organisms [22–24].

We have observed that seagrasses growing near undersea volcanic vents exhibit a greater-than-usual number of grazing scars and wondered if high CO_2 / low pH conditions may affect the

value of seagrass as a food item for herbivores. Such conditions may enhance the value of seagrasses as a food item by reducing the presence of calcareous epiphytes [22], altering tissue nutrients contents, or affecting the production of chemical and structural deterrents. The primary deterrent substances in seagrasses and most estuarine plants are phenolics, including simple and conjugated phenolic acids, condensed tannins, and lignins implicated as herbivore deterrents, digestion reducers, and antifoulants [25–34]. Many phenolics have antimicrobial properties; for example, phenolic acids from seagrasses inhibit the growth of the marine pathogen *Labyrinthula* which causes the seagrass wasting disease [35–37]. These compounds are synthesized via the shikimic acid and phenylpropenoid (SA/PP) pathway which is up-regulated by CO_2, visible and UV light, and photosynthesis, and sugars [27,38]. This suggests that the high CO_2 / low pH conditions of ocean acidification may trigger accumulations of these carbon-based chemical defenses in marine vascular plants, a prediction that is seemingly inconsistent with our previous observations of increased grazing near volcanic vents.

To test the assumption that ocean acidification would trigger accumulations of phenolic substances we examined seagrasses inhabiting the CO_2-enriched waters near an underwater volcanic seep on the Island of Vulcano, Italy and submerged aquatic vegetation in tributaries of the Chesapeake Bay, USA where ocean acidification was simulated using Free-Ocean-Carbon-Enrichment (F.O.C.E.).

Methods

Natural CO_2 vent site

Several submersed CO_2 vent systems are present in the Aeolian archipelago (Northeastern Sicily, Italy), generated by subduction processes in the Southern Tyrrhenian seafloor [39,40]. The southernmost volcanic island of Vulcano (38°25′08.52″N, 14°57′39.13″E) contains the most recently active center in the Gran Cratere at the top of the Fossa cone (last eruption 1888–1890) and several minor volcanic centers [41]. Most of the intense submersed seeps are located along southern and western shores of Baia di Levante (38°25′01.44″N, 14°57′36.29″E), where dispersed underwater leaks cover a 130×35 m shallow area (<1m depth) (Figure 1).

The Vulcano CO_2-seeps are particularly well-suited for studies of future ocean acidification. Gas composition at the seeps consists of >99% of carbon dioxide [42]. Dissolved hydrogen sulphide from the seeps, potentially toxic for cellular respiration, does not extend to the study sites. For example, while H_2S was found at a concentration of 273 and 166 μmol/Kg inside an intense bubbling site [43] it was undetectable (<2 ppm) at the sampling locations >20 m away (Parello et al. in prep.). At these distances sulphate (SO_4^-) levels are typical for oceanic waters (Parello et al. in prep.). This suggests that only a small proportion of the H_2S enters into the aquatic phase, where it oxidizes to non-toxic sulfate due to the high O_2 saturation (~100%) recorded across the bay.

A CO_2/pH gradient runs parallel to the northwestern coast of the Baia di Levante. The pH at the emission site ranges from 5.2 to 5.5 and the gradient reaches an ambient pH (~8.1 pH) at >350 m from an intense CO_2 leakage site. This CO_2 gradient encompasses a great variety of intertidal and subtidal communities at <2 m depth (Figure 1). *Cymodocea nodosa* occurs along the CO_2 gradient and has long been the dominant seagrass in the bay [44].

In May 2011 we collected leaf tissues from *C. nodosa* at 380, 300, and 260 m distances from the seep. Here, average pH values decrease from 8.1 to 7.3 and pCO$_2$ concentrations increased ten-fold from 422 to 4009 ppm, spanning both present-day conditions as well as those predicted for the next 150 years.

Free ocean carbon enrichment

Long-term manipulative experiments were conducted in tributaries of the Chesapeake Bay, USA, including the St. Mary's River (38°10′1.44″N, 76°26′33.50″W) in 2010 and the Severn River (39° 3′31.73″N, 76°32′38.17″W) in 2011. These experiments were conducted during the growing season (May-July) of each year. The St. Mary's River site contains perennial beds of Widgeon Grass *Ruppia maritima* (long form) at a depth of 1–2 m and salinities of 15–25 ppt (Figure 2). Here plants were acclimated to high CO_2/low pH conditions for four weeks prior to harvesting. The Severn River site includes a mixed grass bed of Widgeon Grass, *Ruppia maritima* (short form), and Redhead Grass, *Potamogeton perfoliatus* at similar depths and salinities of 4–7 ppt (Figure 3). At this site, high CO_2/low pH conditions were maintained for 1.5 mo with sampling occurring twice, at 4 and 6 wks.

The Free-Ocean-Carbon-Enrichment (F.O.C.E.) system was designed for *in situ* experiments requiring the manipulation of ocean pH under otherwise natural conditions. The process is analogous to free-air-carbon-enrichment (F.A.C.E.) commonly used to test the effects of atmospheric CO_2 increases on land. This portable F.O.C.E. system can be configured in several different ways to deliver either CO_2 gas or CO_2-enriched seawater on demand. Here, the system was configured to supply compressed CO_2 to five underwater diffusers, as determined by a computer-controlled 2.5 W solenoid. The electrical system can be located on site within a custom support buoy or on shore. Power is supplied by an internal 12V DC 78 amp-hour sealed AGM deep cycle battery mated to a Morning Star pure sine wave DC-to-AC inverter. Batteries can be charged by a 125 W Kyocera solar array with a 12V MPPT charge controller (Morning Star Corp., USA). For safety, the electronics and batteries are secured in a waterproof but vented enclosure; compressed gas cylinders must be protected from temperature extremes and gases produced by battery recharging must be exhausted. During these experiments, CO_2 flowed at 19 psi on a 20s/20s on/off cycle through 65–90 m of gas line to an underwater gang valve junction, and then to one of five replicate CO_2 diffusers secured within a 14×14 m experimental plot. Diffusers were anchored at similar depths and were separated by ≥5 m. Release of pure CO_2 creates a gentle stream of bubbles, similar to those found at the natural CO_2 vent sites. The system is capable of reducing seawater pH by >4.5 units; however, for these experiments the system was programmed to maintain a drop of ~0.5 pH units, roughly doubling pCO$_2$ levels, at a distance of 40 cm from each injector. For example, monitoring of multiple diffuser sites in July 2011 indicated average reductions in pH of 0.61 units with pCO$_2$ values 3.2 times ambient levels at these distances. In 2010, an average reduction of 0.41 pH units and a 3.0-fold increase in pCO$_2$ was recorded. This mimics the change in seawater chemistry and drop in surface ocean pH predicted for the next century under a business-as-usual scenario and span the range of pH used in previous mesocosm studies. Conditions were monitored at distances of 5, 40, 100, and 500 cm from each CO_2-injector, where plants were later harvested. At these distances we noted variations in carbonate chemistry that are the norm for estuarine systems, including those associated with tides, diurnal cycles, and a putative brown tide event on July 11, 2011 (note higher than typical ambient pH). The F.O.C.E system maintained a pH drop of ~0.5 units relative to this natural variation, day and night. We also verified that dissolved oxygen concentrations were not affected by the F.O.C.E. system. The carbonate system at these sites was determined by analyses of discrete water samples,
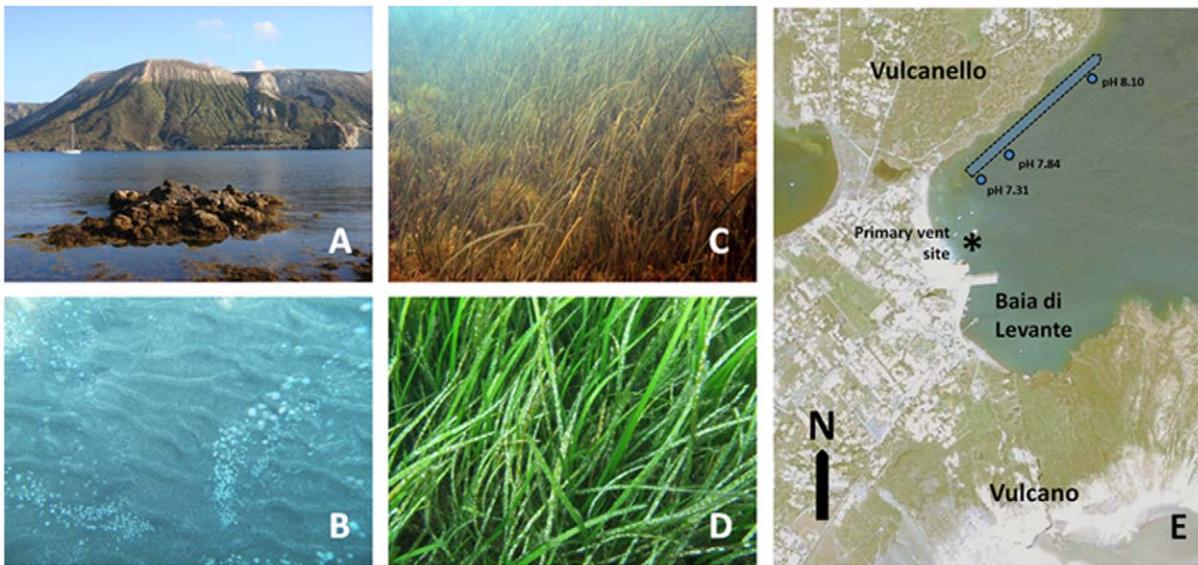
Figure 1. Location of the underwater CO_2 vent and seagrass meadows on the Island of Vulcano, Italy. (A) Location of near shore transect beneath the volcano, where dense populations of *Cymodocea nodosa* inhabit shallow grass beds (C,D). (E) The sampling sites spanned a range of high CO_2/low pH conditions at distances of 260, 300 and 380 m from the vent site (*) along the northern shoreline of the bay.
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real-time analyses of pCO_2 concentrations using an underway flow-through system, a solid-state pH probe, and – for comparison – a traditional glass electrode probe. Parameters not directly measured were calculated using the CO2SYS1.01 program (http://cdiac.ornl.gov.ftp.co2sys/.).

Permits

All necessary permits were obtained for the described field studies.

The harvesting of plant samples from the Chesapeake Bay sites were permitted by the Maryland Department of Natural Resources, and coordinated with local landowners including St. Mary's College of Maryland and Historical St. Mary's City the Round Bay Community Association in Round Bay, Maryland.

Seawater carbonate analyses

At each field site the seawater carbonate system was characterized multiple times, using a range of analytical methods.

Figure 2. Location of mid-salinity Free Ocean Carbon Enrichment (FOCE) experiments within the St. Mary's River, Maryland (USA) in May-July 2010. (A) Individual CO_2 diffusers (white) generated halos of high CO_2/low pH conditions (5 cm and 40 cm distances in grey; 100 cm locations in green; 500 cm ambient sites in blue.) (B) Dense meadow of *Ruppia maritima* from the near shore site. (C) Bubbles emitted from one of the CO_2 diffusers (compare to CO_2 natural vent site, Fig 1.B).
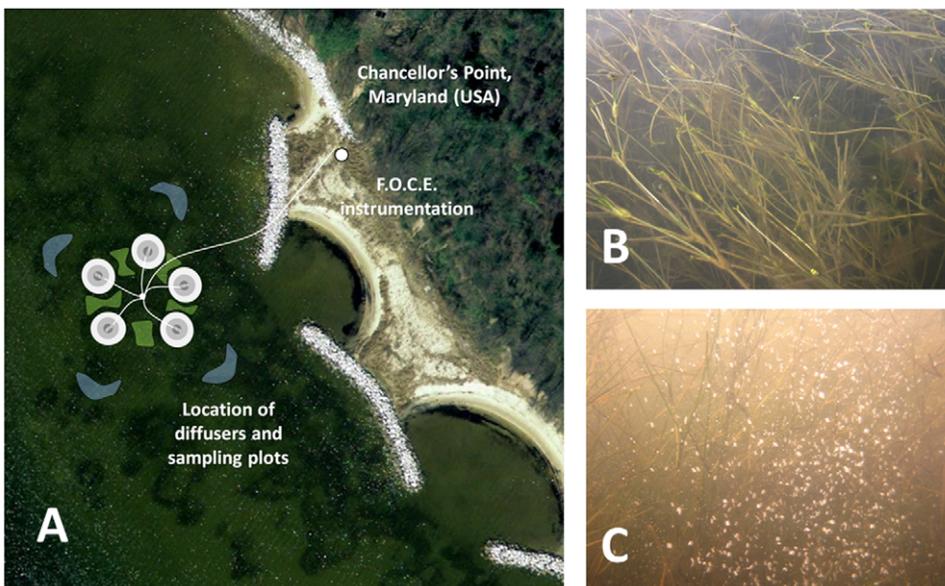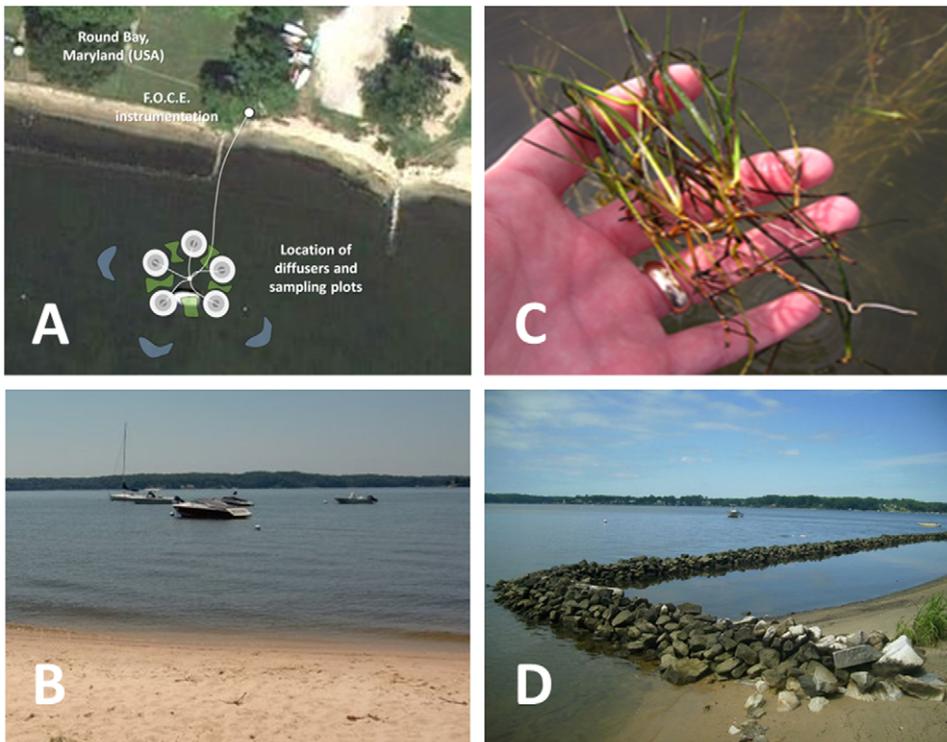doi:10.1371/journal.pone.0035107.g002

Figure 3. Location of low-salinity Free Ocean Carbon Enrichment (FOCE) experiments in the Severn River, Maryland (USA) in June-July 2011. (A) Individual CO_2 diffusers (white) generated halos of high CO_2/low pH conditions (5 cm and 40 cm distances in grey; 100 cm locations in green; 500 cm ambient sites in blue.) The FOCE system instrument package was located on shore (B,D) and maintained high CO_2/low pH conditions within mixed-species meadows of widgeon grass *(Ruppia maritima)* and redhead grass *(Potamogeton perfoliatus)* (C).
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Table 1. Concentrations of phenolic substances in *Cymodocea nodosa* shoots collected at various distances from the natural CO_2 vent site from the Island of Vulcano, Italy in May 2011.

Conditions	Seawater carbonate chemistry					
Distance from seep	**380m**	**300m**	**260m**			
Salinity	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			
pCO$_2$ (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			
Cymodocea nodosa	**Product concentration (mg g^{-1} WM)**			**General trend**	**Test**	**P**
Proanthocyanindins	13.62±1.66	10.17±0.73	10.92±0.87	*25% decrease**	**1**	**0.088**
Total phenolic acids	4.66±0.59 ^{a}	5.30±1.45 ^{ab}	1.89±0.19 ^{b}	*59% decrease***	**2**	**0.017**
Gallic acid	0.50±0.05	0.41±0.03	0.50±0.04	*no change*	1	0.252
Syr+4-HBA	0.07±0.01 ^{a}	0.05±0.01 ^{ab}	0.03±0.01 ^{b}	*58% decrease***	**1**	**0.006**
Vanillin	1.02±0.65	2.57±1.38	0.36±0.24	*65% decrease*	2	0.344
Acetovillinone	3.06±0.88	2.27±1.47	1.00±0.25	*67% decrease*	1	0.413
Coumaric acid	0.29±0.16	0.16±0.05	0.17±0.09	*41% decrease*	2	0.882
Ferulic acid	0.38±0.18	0.31±0.11	0.19±0.09	*50% decrease*	2	0.195
All phenolics†	109.30±4.39 ^{a}	104.51±4.48 ^{ab}	93.12±4.26 ^{b}	*15% decrease***	**3**	**0.026**

Values are means +/− SE.
Average (±SE) temperature, salinity and pH were collected on different visits from Sept 2009 to May 2011 (n = 60). Total Alkalinity (TA) was calculated from water samples collected at each site on April and November 2010 (n = 4). Statistical analyses: 1, one-factor ANOVA with Holm-Sidak multiple comparisons; 2, Kruskal-Wallis One Way Analysis of Variance on Ranks with Tukey or Dunns multiple comparisons; 3, Two-factor ANOVA on ranks with Holm-Sidak multiple comparisons. Letters indicate results of pairwise comparisons test $P<0.05$. †a combined analysis indicated significant variation in the overall concentrations of the various compounds (factor: compound type; $P<0.001$) but there was no significant difference in how the compound types responded to changes in pH (interaction term; $P = 0.900$).
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At the Baia di Levante location (Island of Vulcano, Italy), a 556 MPS YSI (Yellow Springs, USA) probe was used for rapid assessment of pH, salinity (ppt) and temperature (°C). Continuous measures were also collected in November 2010 and May 2011 at 15-min intervals for the at least 24 h. In common with other recent volcanic vents studies [23,45,46] we recorded pH fluctuations along a shallow CO_2 gradient in different weather conditions and at various hours of the day. On a regular basis (approximately every 45 days) from September 2009 to May 2011, the various seawater parameters were recorded in triplicates at 380 m (n = 60), 300 m (n = 60), and 260 m (n = 63) sites. The pH meter was accurate to 0.01 pH units and previously calibrated using TRIS/HCl and 2-aminopyridine/HCl buffer solutions [47]. For each site, pH means were calculated from hydrogen ion concentrations before re-converting back to pH values [45]. Water samples for Total Alkalinity (TA) were collected at each site along the pH gradient on April and November 2010 (n = 4) from a 100 ml water sample passed through 0.2 µm pore size filters, poisoned with 0.05 ml of 50% $HgCl_2$ to avoid biological alteration, and then stored in the dark at 4°C. Three replicate sub-samples were analyzed at 25°C using a titration system. The pH was measured at 0.02 ml increments of 0.1 N HCl. Total alkalinity was calculated from the Gran function applied to pH variations from 4.2 to 3.0, as mEq Kg^{-1} from the slope of the curve HCl volume versus pH. At the Chesapeake Bay sites the carbonate system at various distances from the diffusers was characterized periodically before and during the experiments, using several complimentary methods. During installation, pH was measured over periods of seconds to days using a YSI 556 MPS probe and a solid state probe (Honeywell Durafet II). The pH probes were calibrated with NIST traceable buffers (pH = 7.00 and 10.00) and measurements were made on the NBS scale). During experiments, more complete measurements were conducted. A portable flow-through underway pCO_2 system was used to measure real time pCO_2 values *in situ*, at the prescribed distances from CO_2 diffusers (in all four compass headings) and at representative control sites. The instrument design was based on the Palmer underway pCO_2 system produced by the Lamont-Doherty Earth Observatory with some modification to enable the system to be easily deployed in a small boat. The seawater equilibrator was based on the design for rapid equilibration under relatively low flow by W. McGillis (J. Salisbury, pers. comm.) and the infra-red gas analyzer was a dual (NDIR) model (CO_2meter.com) which logs CO_2 concentration, relative humidity, and temperature at 2-min intervals to a custom microprocessor designed specifically for this instrument. Total alkalinity (TA) samples were taken and processed via the spectrophotometric method of Yao and Byrne using an Ocean Optics (model USB2000) spectrophotometer [48]. TA and pCO_2 were used as master variables in CO2sys.xls to characterize the carbonate chemistry of the system. Dissociation constants K_1 and K_2 for carbonic acid in estuarine waters of Cai and Wang and NBS pH scale were selected to run the CO2sys model [49]. Here we present representative data for the St. Mary's River site collected at a range of distances from a single CO_2 diffuser in June 2010. For the Severn River site we present representative data collected from similar distances from multiple CO_2 diffusers in June 2010.

Biochemical analyses

Biochemical analyses of natural products were conducted for replicate plant tissues harvested from each site. Individual leaves (*C. nodosa*) or shoots with roots/rhizomes (*R. maritima, P. perfoliatus*) were harvested from each location, wiped clean of epiphytes, and analyzed separately. For *C. nodosa* the 2nd rank leaves were collected from separate shoots and identical 7 cm midsections were dissected. Leaf sections from three different shoots were pooled for each extraction. Separate extractions were analyzed for condensed tannins (n = 16 to 18/location) and phenolic acids (n = 8/location). For samples from the Chesapeake Bay, whole plants were collected from the specified distances from each CO_2 diffuser, and from control areas. At the St. Mary's River site 405 shoots were harvested at three distances from the five CO_2 diffusers, for a total of 15 locations. From each location, tissues from 27 shoots were pooled, 3 per extraction, to generate 9 replicate extractions. At the Severn River site plants were harvested at either three or four distances from the five CO_2 diffusers, for a total of 15–20 sampling locations sampled at each time interval. This resulted in the sampling of >900 *R. maritima* shoots in total. Fewer *P. perfoliatus* samples were obtained, as this species did not occur at every location. These tissues were pooled for extraction as described above, providing 9–15 extractions per location at each sampling interval. All samples were transported at −80°C, homogenized, and extracted in MeOH(aq) with 2% acetic acid for 24 h at 4°C in the dark. Concentrations of phenolic acids were determined by RP-HPLC using a method modified from previous studies [50,51]. One hundred µl of each extract was filtered to remove particulate matter and injected onto a semi-preparative RP-18 HPLC column (Supleco, Belfonte PA). We modified our previous methods to employ a gradient system, which more effectively resolved phenolic acid peaks from these species. Peaks were identified by comparison to commercial standards and concentrations (mg compound g^{-1} blade DM) determined using individual standard curves. Condensed tannins (proanthocyanindins), were quantified using a micro-titer plate assay derived from the acid-butanol method [52,53]. Total reactive phenolics were determined using a micro-Folin-Denis assay [54]. Standard curves were developed using quebrancho tannin obtained from A. Hagerman (Miami University of Ohio). Natural product concentrations were expressed as mg compound g^{-1} tissue wet mass. Our selection of these natural products for the analyses was based primarily upon their concentrations and previous reports of their putative roles as antimicrobials or antifeedants; however, we also sought to quantify concentrations of related compounds that are important precursors for phenolic biosynthesis, whether or not they have demonstrated bioactivity themselves.

Statistical analyses

Statistical analyses were conducted using SigmaStat. In experiments examining 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 Dunns multiple comparisons. For experiments comparing only two sites datasets were compared using Student's t-test or a Mann-Whitney Rank Sum Test. An α level of 0.05 was used to determine significance. P values between 0.05 and 0.10 are also noted in the tables.

Results

In these natural and manipulative experiments, we observed that high CO_2/low pH conditions were associated with a dramatic loss, rather than the predicted accumulations, of tannins and related phenolics in undersea vegetation. In experiments conducted at three sites, including high salinity and estuarine areas, we detected reduced levels of proanthocyanidins, hydrozybenzoates,

Table 2. Concentrations of phenolic substances in the "long" form of widgeon grass subjected to Free Ocean Carbon Enrichment within the St. Mary's River, Maryland (USA) in May-July 2010.

Conditions	Seawater carbonate chemistry					
Distance from injector	500cm	40cm	5cm			
Temperature (C)	25.0	25.0	25.0			
Salinity	17	17	17			
pH	8.4	8.0	6.9			
pCO_2 (µtm)	157.8	469.3	6792.0			
TA (µmol kg^{-1})	1467.0	1444.0	1455.0			
Ruppia maritima (long)	Product concentration (mg g^{-1} WM)			General trend	Test	P
Proanthocyanindins	5.92±0.99[a]	4.69±.068 [a]	2.41±0.31 [b]	*60% decrease***	2	0.002
Total reactive phenolics	3.43±.30 [a]	1.67±.26 [b]	1.88±.31 [b]	*45% decrease***	1	<0.001
Coumaric acid	0.31±.05 [a]	0.18±.01 [ab]	0.15±0.01 [b]	*53% decrease***	2	<0.001

Values are means +/− SE.
Statistical analyses: 1, one-factor ANOVA with Holm-Sidak multiple comparisons; 2, Kruskal-Wallis One Way Analysis of Variance on Ranks with Tukey or Dunns multiple comparisons. Letters indicate results of pairwise comparisons test $P<0.05$.
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hydroxycinnamates, and total reactive phenolics in three species of aquatic vascular plants.

In the Mediterranean, we found that levels of phenolic substances were significantly decreased in *Cymodocea nodosa* near CO_2 vents on the island of Vulcano, Italy (Table 1). Along a 120 m underwater transect levels of pCO_2 increased ten-fold from 422 to 4008 µatm, with a corresponding reduction of 0.8 pH

units. Over this distance concentrations of proanthocyaninidins and total phenolic acids decreased by 25% and 59%, respectively. We also detected decreased levels of specific hydroxycinnamic acid– and hydroxybenzoic acid-derivatives, including syringalde-hyde and 4-hyroxybenzoic acid. Some related compounds, e.g. gallic acid, vanillin, acetovinillone, and coumaric acid, were

Table 3. Concentrations of phenolic substances in low-salinity populations within the Severn River, Maryland (USA) after four weeks of Free Ocean Carbon Enrichment in June-July 2011.

Conditions		Seawater carbonate chemistry					
Distance from injector		500cm	100cm	40cm	5cm		
Temperature (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		
pCO_2 (µatm)		243±9	295±13	948±89	3465±527		
TA (µmol kg^{-1})		1122±0.5	1122±0.5	1122±0.5	1122±0.5		
Ruppia maritima (short)	Tissue	Product concentration (mg g^{-1} WM)		Trend	Test	P	
		100cm	40cm				
Proanthocyanindins	whole plants	25.00±7.19	6.33±0.55	*75% decrease***	1	0.018	
Syr+4-HBA	shoots	0.08±0.03	0.27±0.10	*330% increase**	1	0.081	
	roots	0.07±0.01	0.08±0.02	*no change*	1	0.647	
Vanillin	shoots	0.02±0.02	0.05±0.03	*no change*	2	0.415	
	roots	ND	ND	*no change*	ND	ND	
Acetovillinone	shoots	0.08±0.05	0.05±0.02	*no change*	2	0.917	
	roots	0.13±0.10	0.18±0.05	*no change*	2	0.210	
Coumaric acid	shoots	ND	<0.01	*no change*	2	0.424	
	roots	<0.01	<0.01	*no change*	2	0.797	
Ferulic acid	shoots	0.03±0.00	0.03±0.00	*no change*	1	0.451	
	roots	0.01±0.00	0.02±0.00	*no change*	1	0.185	

Values are means +/− SE.
ND, not detected. Statistical analyses: 1, Student's t-test; 2, Mann-Whitney Rank Sum Test; $P<0.05$.
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unaffected but, importantly, no compound or class of compounds increased in concentration under high CO_2/low pH conditions.

At estuarine sites we observed similar responses for *Ruppia maritima* and *Potamogeton perfoliatus*, two important submerged plants in Chesapeake Bay. In the polyhaline reach of the St. Mary's River (Maryland, USA) we detected a dramatic loss of phenolic substances in the "long form" of *Ruppia* after 63 days of CO_2 enrichment (Table 2). Plants located 40 and 5 cm from the F.O.C.E. CO_2-injectors (pH 8.0 and 6.9, respectively) possessed 45–60% lower levels of proanthocyanindins, *p*-coumaric acid, and total reactive phenolics than did control plants located 5 m from each injector (pH 8.4). We observed that daytime pCO_2 levels in these dense meadows site were ≤200 µatm due to uptake of CO_2 by rapid photosynthesis; the F.O.C.E. system successfully counteracted this, maintaining pCO_2 levels 2–3 times higher than ambient at a distance of 40 cm. During this two-month period plants grew >1m in height and became reproductive, bearing iridescent green flowers and semi-transparent seed pods. We observed no differences in the prevalence of reproductive structures (bud, flowers, or seed pods; data not shown) for CO_2 enriched plants compared to those outside the experimental areas. In the low salinity Severn River (Maryland, USA) we also detected CO_2-induced decreases in phenolics in the "short" form of *R. maritima* and in *P. perfoliatus*. After only 18 days, levels of proanthocyanindins dropped 75% in the leaves and rhizomes of *R. maritima* located 40 cm from CO_2 injectors, compared to those 150 cm away (Table 3). Phenolic acids in leaves or rhizomes were unchanged, except for a marginally significant increase in one phenolic acid present at trace amounts (<0.03% plant wet mass). By day 28, dramatically reduced phenolic levels of 61–85% were detected in Redhead Grass, *P. perfoliatus*, which exhibited lower levels of proanthocyanindins and total reactive phenolics near the CO_2 injectors (Table 4). Altogether, *R. maritima* and *P. perfoliatus* exhibited either a loss of phenolics at high CO_2 / low pH sites or no change at all.

In four populations of undersea vegetation from three different locations we observed eleven instances of CO_2-induced reductions in phenolics. This response is in stark contrast to the CO_2-induced accumulations of phenolics typically observed in terrestrial plants and predicted by popular models of plant defense.

Discussion

Our results demonstrate that ocean acidification can decrease levels of phenolic protective substances in marine and estuarine plants, the opposite effect to that typically observed for land plants exposed to atmospheric CO_2 enrichment. Dramatic reductions occurred in all four of the seagrass populations we tested, including those acclimated to naturally acidified conditions near volcanic vents and those exposed to free ocean carbon enrichment.

The phenolic contents of seagrasses, together with nitrogen contents and toughness, determine their palatability and grazing by isopods, urchins, fish, waterfowl, and turtles [55–57]. For example, Verges et al. demonstrated that chemical defenses from the seagrass *Posidonia oceanica* dramatically reduced the feeding of a wide range of consumers, including fishes and sea urchins [55]. More recently, Tomas et al. found that herbivorous isopods preferred tissues of eelgrass, *Zostera marina*, with low phenolic contents, and correspondingly high nutritional values [58]. Leaf toughness, which is determined in part by phenolic polymers such as lignin, is also an important determinant of herbivore feeding, especially for omnivorous fish [56]. Phenolics are important for mesohaline species such as *Ruppia spp.* and *Potamogeton spp.* which are noted for their high tannin levels and specialized brown

"tannin cells". Den Hartog and Kuo classified these plants as "eurysaline" species rather than true seagrasses, noting that many can tolerate a broad range of salinities but are usually not able to compete successfully with the seagrasses under oceanic conditions [59]. *R. maritima* inhabits temperate, tropical, and polar regions in part because of an impressive ability to tolerate different salinities, from brackish waters to salt ponds with salinities three times higher than the open oceans. Many of these "eurysaline" species are grazed heavily by water birds and invertebrates, which consume shoots, rhizomes, and seeds [60–63]. For example, at some locations in the Baltic Sea the exclusion of waterfowl resulted in up to an 80-fold increase in the density of *Potamogeton* sp. [61]. These eurysaline plants produce a variety of bioactive natural products, including simple and polymeric phenolics, which affect the feeding, digestion, and growth rates of these grazers. For example, Dorenbosch and Bakker conducted feeding experiments with five species of submerged macrophytes, including *Potamogeton pectinatus*, and noted that high phenolic species were the least grazed by omnivorous rudd and herbivorous grass carp [64]. Elkin et al. found that dietary tannins reduced the growth of waterducks and developing chicks by as much as a third [65]. Phenolics are not effective against all grazers however; for example, Cronin and Lodge reported that differences in the phenolic contents of *Potamogeton amplifolius* did not influence grazing by a freshwater crayfish [66].

Seagrasses also produce numerous antimicrobial substances, some of which are phenolics [67–69]. For example, caffeic acid and related phenolics inhibit the growth of *Labyrinthula*, a slime-mold like pathogen that causes periodic mass die-offs of certain seagrasses such as *Zostera* and *Thalassia* [37]. Decreased phenolic levels have been linked with outbreaks of the wasting disease in *Zostera marina* [70]. It has also been proposed that this pathogen spreads more quickly when shoot density (and, thus, blade-to-blade contact) is high, as they are at many CO_2-enriched sites [26]. Additional work is warranted to determine if high CO_2/low pH conditions may affect the phenolic substances of other seagrass genera, such as *Zostera* or *Thalassia,* in a way that could promote large scale die-offs associated with the seagrass wasting disease.

In general, the roles of phenolics in seagrasses and aquatic plants are analogous to those of terrestrial plants, where they act as antimicrobials and as deterrents and digestion reducers for many, but not all, invertebrate and vertebrate grazers [71–73]. Their relative importance compared to other factors is difficult to determine, especially since plant phenolic concentrations, nitrogen contents, and toughness are interrelated. However, in plant-animal interactions where phenolics are influential, their bioactivity is dosage-dependent; as a result, the observed reductions may help to explain our observations of increased rates of fish grazing near CO_2 vent sites. They may influence rates of herbivory and disease, important contributors to present-day seagrass declines [55,74–75].

The metabolic mechanism by which CO_2-enrichment caused dramatic reductions in seagrass phenolic contents remains unknown and deserves further study. The shikimic acid / phenylpropenoid pathway in plants leads to the deamination of the amino acid phenylalanine, providing the carbon skeletons required for phenolic biosynthesis [38,72]. Phenylalanine is a common precursor required both for the synthesis of phenolics and the proteins necessary for plant growth; as a result, these processes compete for resources and are often inversely correlated [76]. The notion that plants allocate finite resources to competing primary and secondary processes has been at the center of plant defense theory for the past half a century [77–80]. Many of these theories predict that excess carbon beyond that which can be used

Table 4. Concentrations of phenolic substances in low-salinity populations within the Severn River, Maryland (USA) after six weeks of Free Ocean Carbon Enrichment in June-July 2011.

Conditions		Seawater carbonate chemistry					
Distance from injector		**500cm**	**100cm**	**40cm**			
Temperature (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±0.02			
pCO_2 (µtm)		279±2	439±20	729±5			
TA (µmol kg^{-1})		1145±10	1145±10	1145±10			
Ruppia maritima (short)	Tissue	**Product concentration (mg g^{-1} WM)**			Trend	Test	P
		100cm	40cm	5cm			
Proanthocyanindins	shoots	1.76±0.39	1.24±0.28	2.44±1.29	*no change*	4	0.519
	roots	17.29±2.95 [a]	22.81±1.46 [b]	18.12±1.68 [a]	*intermediate****	**4**	**0.015**
Syr+4-HBA	shoots	ND	ND	ND	*no change*	ND	ND
Vanillin	shoots	ND	ND	ND	*no change*	ND	ND
Acetovillinone	shoots	0.07±0.02	0.07±0.02	0.07±0.02	*no change*	3	0.986
Coumaric acid	shoots	0.02±0.01	0.01±0.00	0.01±0.00	*no change*	3	0.960
Ferulic acid	shoots	0.02±0.00	0.02±0.00	0.02±0.00	*no change*	3	0.922
Potamogeton perfoliatus							
Proanthocyanindins	whole plants	0.65±0.44 [a]	ND [b]	0.10±0.10 [b]	*85% decrease****	**3**	**0.016**
Total reactive phenolics	whole plants	2.13±0.69	0.78±0.35	0.85±0.33	*61% decrease***	**4**	**0.100**

Values are means +/− SE.
ND, not detected. Statistical analyses: 3, Kruskal-Wallis One Way Analysis of Variance on Ranks with Tukey or Dunns multiple comparisons; 4, One-factor ANOVA with Holm-Sidak multiple comparisons. Letters indicate results of multiple pairwise comparisons tests, $P<0.05$.
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for plant growth is redirected to secondary metabolic processes, which in turn protect plant tissues when they may be most difficult to replace. Indeed, high levels of CO_2 and sugars as well as high irradiances – which result in elevated tissue carbon:nitrogen ratios – often trigger accumulations of plant phenolics. For instance, Cronin and Lodge reported that for *Potamogeton amplifolius* C:N ratios were increased 55% and leaf phenolics were increased 72% by high light [66]. However, when growth is not nutrient-limited these models instead predict the allocation of carbon to protein synthesis and growth [76]. In fact, in well-fertilized and rapidly growing plants, including seagrasses, phenolic contents have been found to be low [81], with few exceptions [66]. This could explain the response of the eurysaline plants from the Chesapeake Bay, which inhabit eutrophied waters where nutrient over-enrichment is a widespread problem, but perhaps not the response of seagrass from Vulcano, where waters are oligotrophic. This needs to be rigorously tested in future experiments combining analyses of natural product contents, plant nutrition, and measures of plant productivity.

Author Contributions

Conceived and designed the experiments: TMA AWM. Performed the experiments: TMA AWM MM JHS CM HL KM. Analyzed the data: TMA AWM MM. Contributed reagents/materials/analysis tools: TMA AWM MM JHS. Wrote the paper: TMA MM AWM JHS. Helped to organize the MedSeA training activity on the Island of Vulcano: MM JMH-S. Performed the statistical analysis: TMA.

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