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Seagrass can mitigate negative ocean acidification effects on calcifying algae

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The ultimate effect that ocean acidification (OA) and warming will have on the physiology of calcifying algae is still largely uncertain. Responses depend on the complex interactions between seawater chemistry, global/local stressors and species-specific physiologies. There is a significant gap regarding the effect that metabolic interactions between coexisting species may have on local seawater chemistry and the concurrent effect of OA. Here, we manipulated CO₂ and temperature to evaluate the physiological responses of two common photoautotrophs from shallow tropical marine coastal ecosystems in Brazil: the calcifying alga *Halimeda cuneata*, and the seagrass *Halodule wrightii*. We tested whether or not seagrass presence can influence the calcification rate of a widespread and abundant species of *Halimeda* under OA and warming. Our results demonstrate that under elevated CO₂, the high photosynthetic rates of *H. wrightii* contribute to raise *H. cuneata* calcification more than two-fold and thus we suggest that *H. cuneata* populations coexisting with *H. wrightii* may have a higher resilience to OA conditions. This conclusion supports the more general hypothesis that, in coastal and shallow reef environments, the metabolic interactions between calcifying and non-calcifying organisms are instrumental in providing refuge against OA effects and increasing the resilience of the more OA-susceptible species.

Seagrass meadows and calcifying algae beds are benthic communities that play unique roles in the removal, storage and release of carbon from seawater, via photosynthesis and/or calcification¹. Coastal communities are metabolically responsible for 85% of the organic carbon and 45% of the inorganic carbon (C_i) buried in coastal sediments^{2–4}. CO₂ is essential to photosynthesis, yet its increase in seawater reduces pH and carbonate ions, threatening the calcification process⁵. However, these ecosystems naturally experience large vertical and horizontal variations in abiotic parameters, namely pCO₂ and temperature⁶, that can vary from 400 to 10,000 μatm² and 15 to 30 °C⁷, respectively. Research has suggested that exposure to natural fluctuations alongside possession of phenotypic plasticity may help organisms and populations to resist or acclimate to novel anthropogenic conditions^{6,8}.

Little is known about the existing interactions between calcifying and non-calcifying primary producers under OA and temperature rise. Whether it is via alteration of seawater chemistry, allelopathy or other molecular signaling, neighboring marine plants interact by influencing each other's metabolisms⁹. Changes in benthic macrophyte communities are projected for the future¹⁰ where altered competition dynamics between fleshy and calcifying algae already have been shown to drive ecosystem shifts under elevated CO₂ conditions¹¹. The current incomplete understanding of these interactions and the consequent mechanisms that drive ecosystem changes limit our ability to make realistic predictions for the effects of OA and warming on future community structure.

Seagrasses can act as buffers to OA by absorbing large quantities of CO₂ and increasing the pH of seawater^{12–14}. Diel pH fluctuations of 0.7–1 pH due to the photosynthesis and respiration of seagrass beds, have been reported in different locations^{13,15}. Increased ambient pH levels during the day can become locally significant to the point where they have a positive effect on the calcification of co-occurring calcifying algae^{12,13}. However, since oceanic

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Source of variation		Calcification			GPP		
<i>H. cuneata</i>	df	MS	F	p	MS	F	p
CO ₂ treatment (CO ₂)	1	0.61	7.06	0.017	0.99	0.85	0.370
Seagrass presence (S.P.)	1	0.44	5.10	0.038	31.24	26.69	0.000
S.P.*CO ₂	1	0.11	1.25	0.281	1.83	1.57	0.229
Temperature	1	0.03	0.15	0.706	0.03	0.01	0.918
<i>H. wrightii</i>							
CO ₂	1	—	—	—	40.64	1.09	0.321
Temperature	1	—	—	—	40.17	0.98	0.351

Table 1. ANOVA results showing (1) the effect of elevated CO₂ and seagrass presence on the calcification and gross primary production (GPP) for *H. cuneata*, (2) the effect of elevated CO₂ on GPP for *H. wrightii* and (3) the effect of temperature on the calcification and GPP of both primary producers (highlighted in gray). Significance was considered when $p < 0.05$.

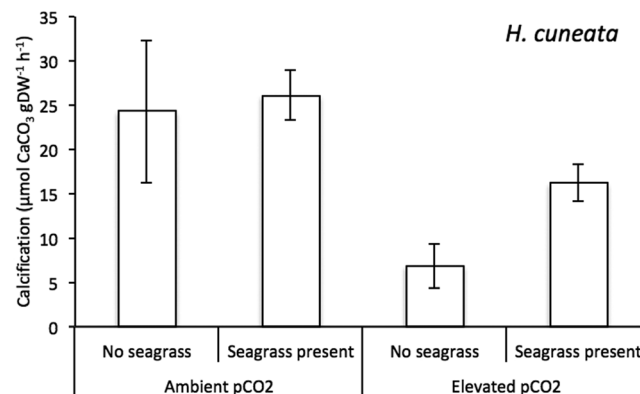


Figure 1. Mean calcification responses of *H. cuneata* ($n = 5$) \pm SEM in the presence & absence of seagrass, under ambient (380 μ atm) and elevated (822 μ atm) pCO₂ levels. Values were normalized to the dry, decalcified weight (DW) of only *H. cuneata*.

conditions are rapidly changing, information is needed about how the presence of seagrasses will affect calcifying algae responses under OA and temperature rise.

Most of the studies regarding the impact of global stressors evaluate the isolated responses of primary producers, using unifactorial models or eventually considering the combined role of OA and temperature rise in the fitness of a specific and isolated biological indicator¹⁶. Thus far, the expected trend for seagrasses is neutral to positive physiological responses to OA¹, yet the magnitude of change and affinity for DIC species varies^{17,18}. The isolated effects of temperature and CO₂ on the seagrass genus *Halodule* Endlicher^{17,19,20} and their isolated and combined effects on the calcifying green algae genus *Halimeda* J.V. Lamouroux have been widely addressed^{21–28}. The general consensus of OA studies on *Halimeda* indicates negative to neutral calcification responses and neutral to positive photosynthetic responses to CO₂-enriched seawater, due to species specificity^{22,24,26–33}.

To date, two studies have considered the effects of seagrass-calcifying algae interactions under ambient conditions^{12,13}, but none have addressed how OA and temperature rise influence these ecophysiological interactions. The species-specific nature of the isolated responses emphasizes the necessity to conduct studies that address OA and temperature rise together in order to better understand the mechanisms behind the presence/absence of interactions between these drivers. Short-term mesocosm experiments that simulate rapid heat waves and acidification, as observed in different regions, are fundamental tools to predict complex ecosystem interactions^{34,35}. It is also necessary to introduce realism in these simulations by representing the high-frequency semidiurnal or diurnal variability that dominates coastal or shallow environments³⁶. Recent studies reveal that under OA, net photosynthesis of the kelp *Ecklonia radiata* was almost 50% lower when pH fluctuated than when it was static³⁷. This natural variability imposes particularities that can limit or stimulate primary production and must be reproduced in order to properly simulate the predictable future scenarios.

Here we investigate the effects of OA on the photosynthesis and calcification of the seagrass *Halodule wrightii* and the green alga *Halimeda cuneata* via a full factorial mesocosm design. We simulate OA and warming by exposing the calcifying alga and the seagrass to the following four combinations of ambient and elevated pCO₂ and temperature: 28 °C & 320 μ atm, 28 °C & 822 μ atm, 30 °C & 320 μ atm and 30 °C & 822 μ atm. Most importantly, we examine the degree to which the photosynthetic carbon uptake of *H. wrightii* influences seawater chemistry under OA through short-term incubations. We aim to determine whether this can act as a metabolic feedback on the photosynthesis and calcification of *H. cuneata*, considering that these two species of macrophytes coexist in the shallow tropical waters off the Brazilian coast³⁸. We hypothesize that *H. wrightii* is capable of using the excess DIC resulting from OA to increase its photosynthetic activity. In mitigating the effects of OA on seawater

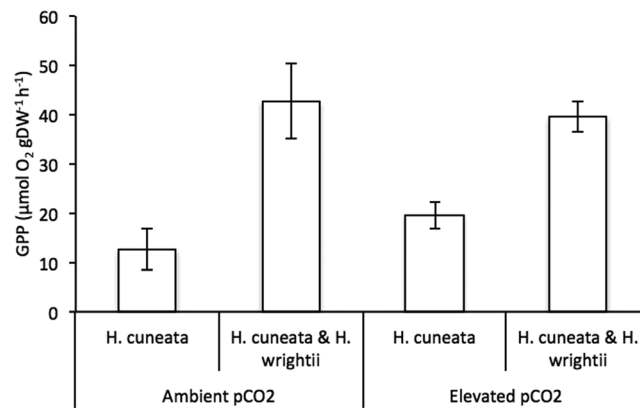


Figure 2. Mean gross primary production (GPP) responses of *H. cuneata* ($n = 5$) \pm SEM and *H. cuneata* & *H. wrightii* together ($n = 5$) \pm SEM, under ambient (380 μ atm) and elevated (822 μ atm) pCO₂ levels. Values were normalized to the dry, decalcified weight (DW) of the species present.

Species	CO ₂	Seagrass presence	Calcification (μ mol CaCO ₃ g ⁻¹ h ⁻¹)	GPP (μ mol O ₂ g ⁻¹ h ⁻¹)	Δ HCO ₃ ⁻ (μ mol g ⁻¹ h ⁻¹)	Δ CO ₂ (μ mol g ⁻¹ h ⁻¹)	Δ CO ₃ ²⁻ (μ mol g ⁻¹ h ⁻¹)	Δ DIC (μ mol g ⁻¹ h ⁻¹)	$\Delta\Omega_{Ar}$ (g ⁻¹ h ⁻¹)	Δ pH
<i>H. cuneata</i>	Ambient	—	24.29 \pm 8.07	12.61 \pm 4.10	-68.28 \pm 20.08	-0.74 \pm 0.16	5.43 \pm 2.11	-63.58 \pm 19.44	0.08 \pm 0.03	0.08 \pm 0.04
	Elevated	—	6.85 \pm 2.49	19.45 \pm 2.65	-29.87 \pm 10.65	-1.46 \pm 0.71	6.12 \pm 2.98	-25.20 \pm 8.76	0.09 \pm 0.05	0.07 \pm 0.02
	Ambient	+	26.09 \pm 2.84	42.72 \pm 7.58	-131.25 \pm 18.09	-1.63 \pm 0.48	31.47 \pm 4.66	-101.42 \pm 13.98	0.49 \pm 0.07	0.21 \pm 0.05
	Elevated	+	16.15 \pm 2.09	39.46 \pm 3.13	-99.13 \pm 14.62	-2.53 \pm 0.64	27.23 \pm 4.73	-74.43 \pm 10.13	0.42 \pm 0.07	0.25 \pm 0.06
<i>H. wrightii</i>	Ambient	n/a	—	677.48 \pm 169.87	-1097.94 \pm 237.31	-20.06 \pm 7.7	313.53 \pm 39.11	-804.47 \pm 206.7	4.85 \pm 0.6	0.16 \pm 0.03
	Elevated	n/a	—	464.11 \pm 60.23	-1484.45 \pm 163.84	-33.0 \pm 9.21	490.06 \pm 71.52	-1027.38 \pm 117.81	7.57 \pm 1.11	0.27 \pm 0.05

Table 2. Mean values of calcification, gross primary production (GPP) and the changes in bicarbonate (Δ HCO₃⁻), carbon dioxide (Δ CO₂), carbonate (Δ CO₃²⁻), total DIC (Δ DIC), aragonite saturation state ($\Delta\Omega_{Ar}$) and pH (Δ pH) \pm SEM for *H. cuneata* and *H. wrightii*. These result from ambient and elevated pCO₂ levels of 380 and 822 μ atm, respectively and the absence/presence of seagrass (for *H. cuneata* only).

chemistry, we hypothesize that the negative effects of OA on the calcification rate of *H. cuneata* may consequently be ameliorated.

Results

After exposure to treatments for 10 d, short-term incubations (illustrated in Supplementary Fig. S1) of *H. cuneata* and *H. wrightii* separately, as well as together, were conducted in order to understand their physiological responses to OA. We also sought to obtain the magnitude of the effect that the primary production of these macrophytes, particularly the seagrass, has on surrounding seawater chemistry under the stress of OA. The ultimate objective was to determine whether biologically altered seawater might be sufficient enough to mitigate the effects of OA on *H. cuneata* calcification.

H. cuneata and *H. wrightii* fared differently under OA conditions. While the calcifying alga experienced negative physiological consequences (Fig. 1), the seagrass showed a neutral response (Fig. 2, Table 1). We report a significant effect of CO₂ enrichment on *H. cuneata* calcification ($p = 0.017$, Table 1), causing it to suffer a 72% decrease under elevated pCO₂. Simultaneously, we observed a shift in carbonate chemistry when *H. cuneata* was incubated in the elevated pCO₂ treatment, where HCO₃⁻ decreased by 56% and total DIC, by 60%, but carbonate and CO₂ remained the same (Fig. 3, Table 2).

We did not detect a significant effect of CO₂ on the gross primary production (GPP) of either *H. cuneata* ($p = 0.370$) or *H. wrightii* ($p = 0.321$) when incubated separately (Table 1), yet for *H. cuneata* there was an increasing trend (Fig. 2, Table 2). When *H. cuneata* and *H. wrightii* were incubated together, the resulting GPP also did not differ from ambient to elevated pCO₂ (Table 2), but the overall values were clearly driven by *H. wrightii* production. The GPP of *H. wrightii* was about 53 times higher than that of *H. cuneata* at ambient conditions and 23 times higher under elevated CO₂ (Table 2).

We observed significant changes in seawater chemistry (Table 3) resulting from the incubation of *H. cuneata* and *H. wrightii* separately and together. It's worthy to note, however, that observed shifts that we attribute to an organism's metabolism also reflect the natural chemical equilibrium change that occurs following biological inorganic carbon uptake. The challenge of teasing apart the biological effect and the equilibrium change warrants relative interpretation of DIC uptake. Surprisingly, *H. wrightii* did not increase DIC uptake under OA, showing no significant differences in total DIC consumption between ambient and elevated CO₂ ($p = 0.356$, Table 3). However, due to its comparably higher GPP, *H. wrightii* still took up 40 times more total DIC than *H. cuneata*

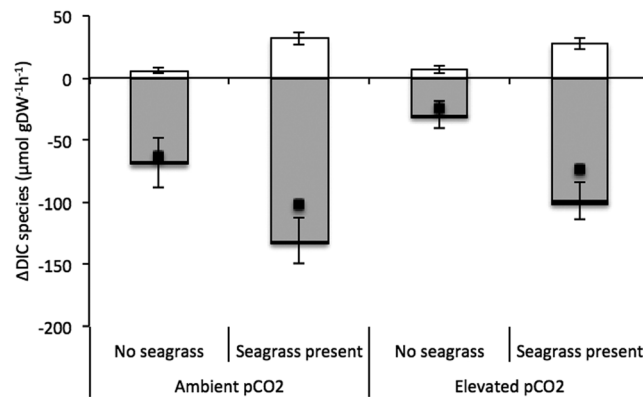


Figure 3. Changes in DIC species (HCO_3^- , CO_2 & CO_3^{2-}) \pm SEM during the incubation of *H. cuneata* with and without seagrass present, at ambient (380 μatm) and elevated (822 μatm) pCO_2 levels. Negative changes indicate consumption of the DIC species. White bars refer to ΔCO_3^{2-} , grey bars refer to ΔHCO_3^- and black bars refer to ΔCO_2 . Total DIC (ΔDIC) is the sum of the change in all DIC species and is represented by the black scattered points. When no seagrass was present, values were normalized to the dry, decalcified weight (DW) of *H. cuneata* ($n = 5$). When seagrass was present, values were normalized to the sum of the dry, decalcified weight of *H. cuneata* + *H. wrightii*.

Source of variation		ΔHCO_3^-			ΔCO_2			ΔCO_3^{2-}			ΔDIC			$\Delta\Omega_{\text{Ar}}$			ΔpH		
<i>H. cuneata</i>	df	MS	F	p	MS	F	p	MS	F	p	MS	F	p	MS	F	p	MS	F	p
CO_2 treatment (CO_2)	1	9140	7.28	0.015	4.59	2.83	0.111	82	1.37	0.258	7126	7.65	0.013	0.02	1.38	0.256	0	0.25	0.621
Seagrass presence (S.P.)	1	18490	14.72	0.001	6.43	3.97	0.063	2386	40.0	0.000	8040	8.63	0.009	0.57	39.0	0.000	0.09	13.46	0.002
S.P.* CO_2	1	62	0.05	0.827	0.24	0.15	0.704	113	1.89	0.187	11	0	0.917	0.03	1.86	0.191	0	0.06	0.805
<i>H. wrightii</i>																			
CO_2	1	331964	1.92	0.209	372.19	1.08	0.333	69252	4.02	0.085	110422	1.0	0.356	16.44	3.95	0.087	0	2.81	0.138

Table 3. Results from the ANOVA used to test the effect of elevated CO_2 and seagrass presence on DIC species evolution for *H. cuneata* incubations and the ANOVA used to test the effect of elevated CO_2 on DIC species evolution for *H. wrightii* incubations. Significance was considered when $p < 0.05$.

under elevated CO_2 (Table 2). Consequently, when incubated alone under elevated CO_2 , *H. wrightii* was able to metabolically increase seawater pH by 0.27 ± 0.05 units and aragonite saturation state (Ω_{Ar}) by 7.57 ± 1.11 units (Table 2). In contrast, under the same conditions, *H. cuneata* only increased seawater pH by 0.08 ± 0.04 and Ω_{Ar} by 0.08 ± 0.03 (Fig. 4, Table 2). When incubated with *H. cuneata*, seagrass presence was a significant factor in determining the evolution of HCO_3^- , CO_3^{2-} and total DIC as well as $\Delta\Omega_{\text{Ar}}$ and ΔpH in seawater (Table 3). We were not able to quantitatively separate the photosynthetic rates of the alga and the seagrass when they were incubated together. So in order to estimate the effect of the seagrass, we relied on the magnitude of metabolic change for which the seagrass was solely responsible when incubated alone, as mentioned previously.

The seagrass was able to mitigate OA and significantly impact the alga's physiology. We report significant effects of seagrass presence on *H. cuneata* calcification ($p = 0.038$), but no interactive effect was found between CO_2 treatment and the latter ($p = 0.281$, Table 1). When *H. wrightii* and *H. cuneata* were incubated together at ambient pCO_2 , there was interestingly no observed change in calcification. However, at elevated pCO_2 , the metabolic interaction between the two mitigated the negative impact of OA and the calcification rate of *H. cuneata* was reduced by only 34% (as opposed to 72% when alone; Fig. 1, Table 2).

During the experimental period, the ambient CO_2 treatment had a mean pCO_2 of $380 \mu\text{atm} \pm 7$ SEM and a mean pH of 8.197 ± 0.006 SEM and for the elevated CO_2 treatment, a mean pCO_2 of $822 \mu\text{atm} \pm 16$ SEM and a mean pH of 7.923 ± 0.007 SEM (Table 4). pH fluctuated throughout the experiment due to natural variation from the adjacent reef, as shown in Supplementary Fig. S2. Mean A_{T} was 2278 ± 2 (ambient) and 2280 ± 7 (elevated; Table 4). There was no effect of CO_2 treatments on mean seawater TA ($p = 0.723$). The ranges of these parameters as well as the remaining physicochemical characterisation of seawater are found in Table 4. Temperature showed no significant effects or interactions on all descriptors (Table 1). Supplementary Fig. S3 shows the average PAR values observed throughout the day during the experimental period.

Discussion

The presence of the seagrass *H. wrightii* mitigated the negative effect of OA on the calcification of the alga *H. cuneata*. This is the first study to confirm that under elevated CO_2 concentrations, seagrass is still capable of maintaining comparatively high photosynthetic rates, and in turn, creating seawater conditions that are conducive to the calcification of sympatric, and otherwise ill-fated, calcifying algae. On their own, *H. cuneata* and *H. wrightii* responded differently to OA. The alga suffered decreased calcification and both the alga and the seagrass showed

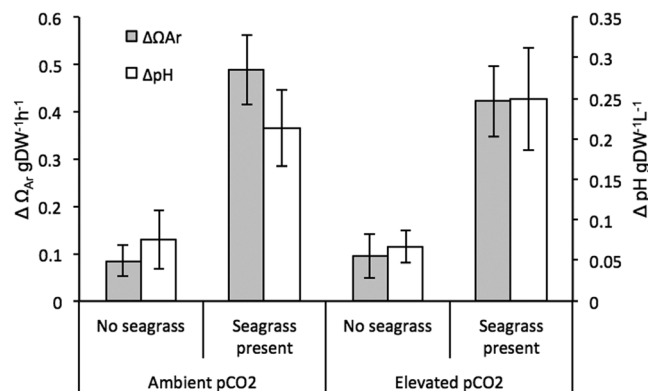


Figure 4. Changes in aragonite saturation state ($\Delta\Omega_{Ar}$; grey bars) and pH (ΔpH ; white bars) of seawater \pm SEM resulting from the incubation of *H. cuneata* in two CO_2 treatments (380 μatm & 822 μatm) and in the absence/presence of seagrass. When no seagrass was present, values were normalized to the dry, decalcified weight (DW) of *H. cuneata* ($n = 5$). When seagrass was present, values were normalized to the sum of the dry, decalcified weight of *H. cuneata* + *H. wrightii*.

	Ambient CO_2 treatment	Elevated CO_2 treatment RCP 6.0
S	37.7 ± 0.3	37.6 ± 0.4
T ($^{\circ}C$)	28.99 ± 0.09	29.04 ± 0.09
pH (NBS)	8.197 ± 0.006 (7.990–8.395)	7.924 ± 0.007 (7.685–8.145)
TA ($\mu mol kgSW^{-1}$)	2278 ± 2	2280 ± 7
pCO_2 (μatm)	380 ± 7 (203–666)	822 ± 16 (431–1486)
Phosphate (μM)	0.17 ± 0.06	
Nitrate & Nitrite (μM)	1.22 ± 0.07	
Ammonium (μM)	1.64 ± 0.14	

Table 4. Seawater characterisation of the two CO_2 treatments. Shown are the means \pm SEM (and ranges for pH and pCO_2) for each parameter.

no significant photosynthetic response. It has widely been shown that calcifying organisms respond negatively to OA, whereas fleshy plants respond neutrally or positively^{1,39}. The difference in the magnitude of the metabolic effect that each organism had on the surrounding seawater was substantial, where the dominant effect of *H. wrightii* played to the calcifying alga's advantage under OA. It is worthy to note that the range of temperature initially tested (28–30 $^{\circ}C$) to simulate ocean warming need not be considered a frontrunner threat to *H. cuneata* and *H. wrightii* photosynthesis and calcification due to the absence of an observable effect of temperature on these processes.

One of the more interesting findings of our study is the apparent asymmetry between photosynthetic and calcification responses to OA in *H. cuneata*. The calcified macroalgae showed signs of physiological stress, seeing as we observed a substantial decrease (72%) in its calcification. On the other hand, the photosynthetic response was not significant due to variation within treatment, however the fact that there was a 54% increase is worthy of consideration. These two processes occur side by side at the cellular level. Calcification occurs in intercellular spaces (inter-utricles spaces, or IUSs), which are separated from bulk seawater by a layer of utricles where photosynthesis is concentrated⁴⁰. DIC uptake during photosynthesis is found to increase the pH of IUSs and calcium carbonate precipitation is favoured. Conversely, calcification produces H^+ and CO_2 , which balance the change in pH and CO_2 concentration produced by photosynthesis⁴⁰. Due to the close proximity of these processes, under ambient conditions, calcification is reported to be closely coupled to photosynthesis in the genus *Halimeda*^{41,42}. Thus, when photosynthesis increases, calcification is expected to increase, and vice versa. However, when we elevated CO_2 in this study, calcification was compromised despite an increasing trend in GPP (Table 2), which suggests that there may be a certain degree of independence between these processes.

The understanding of this uncoupling lies in the carbonate chemistry dynamics of the location where photosynthesis and calcification intersect, the IUSs. Ultrastructure data suggests that the structure and size of utricles and IUSs in *Halimeda* may help to explain the carbonate chemistry of the IUSs and thus, OA responses^{24,41}. Peach *et al.* 2017 found an inverse relationship between diffusive pathway type and mineral content, where species with longer utricles and thinner pathways contained more aragonite than those with shorter utricles and wider pathways. Morphological parameters were not one of our response variables, but based on our results and previously established calcifying mechanisms for *Halimeda*^{40,43} and aquatic plants⁴⁴, we suggest that the diffusive pathway of *H. cuneata* permits corrosive bulk seawater to replenish the IUSs at a much faster rate than it can be biologically-regulated, thus partially inhibiting calcification⁴⁵. We also suggest that dissolution may be

contributing to the low pH and DIC-rich environments in the IUSs. The degree to which the alga may be experiencing dissolution is also not evident, due to the difficulty of disentangling the effects of dissolution and decreased calcification in OA studies^{42,46}. The alga may be using DIC directly from dissolution as substrate for photosynthesis, thus explaining why DIC from bulk seawater was in lesser demand, shown by the 60% decrease in DIC consumption. However, the GPP of the alga was not capable of ameliorating the imbalance in carbonate chemistry of IUSs enough to stimulate calcification, thus our data supports the hypothesis that photosynthesis and calcification become uncoupled under OA¹. Nonetheless, we cannot be certain of the source of DIC for this increasing trend in photosynthesis, nor the reason that the alga was unable to further increase GPP. Further research on this species using microsensors would be essential to ascertain these unknown thresholds that explain the apparent disparity between photosynthetic and calcification responses to OA⁴⁶.

We initially expected an increase in the GPP of *H. wrightii*, since additional CO₂ substrate is expected to stimulate primary production in fleshy marine plants. However, our results show that there was no significant change in photosynthesis at elevated CO₂. Recent meta-analyses report neutral to positive photosynthetic responses of seagrasses to elevated CO₂^{1,47}, which maintains our results within the range of expected responses. In addition, a study that exposed tropical *H. wrightii* to reduced pH observed an increase of only 20% in its photosynthetic rate, followed by a prominent plateau that was attributed to a preference for HCO₃⁻ use¹⁷. In the same study, the absence of a change in photosynthesis in *H. wrightii* after the exposure to acetazolamide (AZ), an inhibitor of carbonate anhydrase (CA), a common enzyme that aids in conversion of HCO₃⁻ to CO₂, indicated that this species has an alternate and more efficient mechanism for HCO₃⁻ use when compared to other seagrasses¹⁷. Our observations likely indicate that *H. wrightii*'s neutral response to OA is due to the efficiency of its mechanism of HCO₃⁻ use. Due to its much higher photosynthetic rate (464 ± 60 (SEM) $\mu\text{mol O}_2 \text{ gDW}^{-1} \text{ h}^{-1}$), when compared to *H. cuneata* (19.5 ± 2.7 (SEM) $\mu\text{mol O}_2 \text{ gDW}^{-1} \text{ h}^{-1}$) at elevated pCO₂, *H. wrightii* removes 40 times more DIC from seawater, thus increasing the pH⁴⁸, CO₃²⁻ availability and aragonite/calcite saturation states⁴⁹. Although the seagrass did not increase its GPP, its capacity to biologically alter its surrounding seawater chemistry was enough to influence the metabolism of coexisting *H. cuneata*. We did not quantify the density of the studied *Halodule* bed, which is a factor that is shown to affect the magnitude of *Halimeda*-seagrass interactions^{12,50}. Our results show that *Halodule* populations are likely to withstand intermediate OA scenarios, yet local irradiance, temperature and nutrient conditions may very well play a determinant role in the magnitude of the metabolic interactions between seagrasses and sympatric calcifying macroalgae. Interspecific variations in seagrass photosynthesis due to diverse DIC assimilation mechanisms will also put some species at an advantage over others^{17,51}. This was observed at volcanic CO₂ vent sites, where seagrass community composition shifted according to seawater pH⁵². The extent to which populations are acclimated to elevated conditions may determine their long-term resilience.

Acidified seawater is ultimately unfavourable for *H. cuneata* calcification, however we demonstrate that high-performing primary producers such as *H. wrightii* are capable of providing significant refuge for these calcifying algae via biologically altering seawater chemistry. Previously, a 1.6-fold increase in the calcification rate of *Halimeda renchii* was observed in seagrass beds at ambient CO₂ levels¹³. In our study, it was unexpected that seagrass presence did not also increase *H. cuneata* calcification at ambient CO₂. Based on the high GPP of *H. wrightii* observed at ambient CO₂, one would expect the consequent IUS carbonate chemistry to be exceptionally favourable and to stimulate calcification. Regardless, the issue is that future oceans will possess a much higher pCO₂ than that of today's oceans. Our results show that under OA, the presence of seagrass will likely foster calcification rates during the day that are comparable to those at current pCO₂. Recent findings anticipate, however, that other factors of different functional scales will cause variation in this buffering capacity. There are often other marine macrophytes coexisting with *Halimeda* and seagrass, namely macroalgae. Benthic community composition is known to alter seawater chemistry at different magnitudes^{53,54}, greatly due to species-specific irradiance optima and CCM mechanisms, therefore influencing the community's OA buffering capacity. Modeled projections incorporating effects of OA and net community metabolism (NCM) on carbonate chemistry in seagrass meadows predict long-term offsets of CO₂, but also NCM-driven extremes in carbonate chemistry under OA⁵⁵. In particular, future pH levels at night are expected to be extremely low due to the intensified effect of OA on respiration⁵⁵. This has implications for the net calcification of *Halimeda* that we weren't capable of addressing and would need to be analysed in future studies. Additionally, Cyronak *et al.* (2018) reveal that the spatial and short temporal variation of carbonate chemistry in seagrass beds can be even greater than diel variability, thus potentially impacting the buffering capability of seagrasses across even smaller scales⁵⁶. The fate of calcifying algae under OA may very well lie in the composition of the accompanying photoautotroph community as well as their associated NCM dynamics⁵⁴.

Solid predictions of whether and which calcifying algae will adapt to OA & temperature rise are generally still insufficient⁵⁷, partially due to the lack of incorporation of species interactions effects and natural seawater variability. Most of the available information produced until now has been based on stable values of pH and temperature³⁷ and few global studies exist that address marine plant interactions alongside OA and temperature rise⁵⁸. Despite the academic value of these efforts, their utilization in depicting future scenarios should be considered with caution, since natural variability of physical/chemical conditions is a selective pressure and a major driver of marine ecosystem functioning. Likewise, although there are known limitations to not manipulating CO₂ directly into each experimental tank⁵⁹, our design was chosen as the most feasible, which gave priority to the incorporation of diel pH and CO₂ variability. Each tank was an isolated experimental unit with a certain degree of intrinsic variability and our results do not suggest that our design has biased the experimental outcome.

The degree of physiological tolerance or increased performance to changes in CO₂ and temperature in the marine environment can be due to trans-generational plasticity, phenotypic buffering, or plasticity within generations (or 'classical' plasticity) from which 'true' evolutionary adaptation may arise⁶. Data supports that genetic variation in traits important for OA and temperature rise is prevalent in near-shore plants⁶. Based on our results, we suggest that the large natural variability of temperature and CO₂ in shallow coastal environments has selected

for phenotypic plasticity and co-evolutionary tools involving the metabolic interaction between *H. cuneata* and *H. wrightii*, thus potentially providing resilience and adaptability to OA. Plasticity in response to OA and temperature rise will help maintain population resilience under changing environments⁶⁰. If *Halimeda* species have adequate genetic variability to generate phenotypes with different CO₂ tolerances and optima, then it is likely that inter or intraspecific variability in fitness will be observed, where OA winners are likely to be those coexisting with seagrasses. The responses we observed are a contribution to the understanding of possible shifts in composition of relevant communities⁶¹, but they also highlight the relevance of coastal plant metabolic interactions as a dynamic biological factor that should be considered in the management of natural habitats, namely marine protected areas, in view of future climate scenarios.

Methods

Study area and experimental design. The experiment was performed in a large-scale, flow-through mesocosm designed by Projeto Coral Vivo, located at its research station on Araçápe beach, Bahia, Brazil. The mesocosm was designed to test the effects of ocean acidification and warming (among other factors) on reef organisms⁶², while closely mimicking the adjacent reef conditions. Araçápe beach's (16° 29' 28.6" S 39° 3' 58.4" W) fringing reef develops 100 m off the coast of the Marine Mesocosm, where the seagrass *H. wrightii* and the upright calcifying green alga *H. cuneata* coexist. The open flow and proximity of the mesocosm to the fringing reef make this system highly realistic since it is able to maintain experimental conditions (seawater composition, temperature, diel pH and CO₂ variability, turbidity, salinity, plankton density, photoperiod, rainfall, irradiance... etc) that are very similar to those in the adjacent reef.

With the interest of simulating moderate predictions of ocean acidification and warming, levels of pCO₂ and temperature were chosen based on the IPCC RCP 6.0 scenario⁶³ and modeling of future atmospheric emissions^{64,65}. We initially established a full factorial design of ambient temperature and pCO₂ and elevated temperature and pCO₂, totaling four treatments. The ambient levels were the unaltered present temperature (28 °C) and pCO₂ (380 µatm) of seawater from the adjacent reef, and the elevated CO₂ and temperature treatments were achieved by manipulating seawater to target +2 °C (30 °C) and +0.25 pH, or +442 µatm (822 µatm). The pCO₂ was calculated for each treatment using the TA and mean pH values via CO2SYS⁶⁶.

Seawater from 500 m offshore was continuously pumped into four 5,000-L underground sumps where the four CO₂ and temperature treatments were applied. pCO₂ was manipulated in two sumps using a custom reactor system that introduced fine bubbles of CO₂ into constantly mixed seawater. Similarly, a 1.9 m 15,000 W heater was placed in each of the two underground sumps where seawater temperature was to be elevated. Seawater pCO₂ and temperature were not fixed. We used a custom-made Reef Angel Open-Source Controller, which elevated and regulated pCO₂ and temperature levels with respect to ambient fluctuations. Treatments were applied to header sumps and not directly to experimental tanks based on feasibility and limitation of resources. Mixed treatment water was fed to four 310-L reservoir tanks, from which flow was regulated to 16, fully randomized 130-L raceway experimental tanks (n = 4 per treatment). Experimental tanks were continuously supplied with seawater at a flow rate of ~10 L·min⁻¹, achieving a renewal rate of 5 x per hour. The experimental tank area was covered in shade cloth to uniformly reduce the intensity of natural sunlight by 70%, simulating the amount of incident solar radiation measured at approximately 2 m where organisms were collected on the reef. Duarte *et al.* (2015) reported a complete description of the mesocosm design and functioning.

Sampling. Approximately 160 specimens of *H. cuneata* and 1,500 shoots of *H. wrightii* were collected at a depth of 2 m using SCUBA by carefully removing the entire holdfast and rhizome, respectively, and were brought to the holding aquariums of the Marine Mesocosm for sorting and removal of epibionts. Sediment from the first 10 cm of the sampling area was also collected and used as substrate for subsequent planting in the mesocosm. Ten *H. cuneata* thalli were placed upright in a plastic tray (40 × 17 × 4 cm), with the holdfasts anchored in 3 cm of sand. One tray was placed in each of the 16 experimental tanks. Approximately 50 seagrass shoots were replanted in each of 2 plastic trays with 3 cm of sediment in each experimental tank. Each of the 16 experimental tanks thus possessed 3 trays, 1 with *H. cuneata* and 2 with *H. wrightii*. Organisms were acclimated at ambient temperature and pCO₂ for 15 days. Treatments commenced upon completion of the acclimation period, reaching target levels within 24 hours and were applied for a total of 10 days.

Abiotic parameters. Salinity (Refractometer: Instrutherm RTS-101ATC), dissolved oxygen & temperature (Portable dissolved oxygen meter: Instrutherm MO-900), incident irradiance (Quantometer: apogee MQ-200) and pH (pHmeter: Gehaka ISO 9001) were measured daily in each experimental tank. Handheld pH meter and pH sensors were calibrated to NBS buffers daily and sensor drift was checked weekly using a bench top Gehaka pH meter. The remaining abiotic parameter meters were calibrated as per recommended in their factory manuals, using appropriate calibration solutions. The daily average photosynthetically active radiation (PAR) was monitored with light loggers (HOBO), which were positioned underwater at the level of the organisms in the experimental tanks. Nutrient concentrations were monitored every 3 days in each tank⁶⁷. For the monitoring of seawater carbonate chemistry, water samples were retrieved from each experimental raceway tank (n = 3) and were immediately refrigerated. Total alkalinity measurements, were performed using a custom USB4000 spectrophotometer (Ocean Optics, Dunedin, USA) and compared to certified reference material (Scripps Institute of Oceanography, USA).

Primary production and calcification. In order to isolate and assess the potential metabolic interactions between *H. cuneata* and *H. wrightii*, short-term (2.5 h) incubations were administered at the beginning and end of the experiment. Incubations were conducted on each species separately, as well as with the two species together

($n = 3$ per species/combination). Oxygen evolution and change in total alkalinity were measured; the former was used to calculate gross primary production (GPP) and the latter, for calcification rates and change in dissolved inorganic carbon (Δ DIC). GPP and Δ DIC were calculated for each species, whereas calcification was only calculated for the alga. An irradiance of $750 \mu\text{mol quanta m}^{-2} \text{s}^{-1}$ was used during the incubations since this was the average midday irradiance during the time period when the incubations were conducted.

Incubations did not take place in the experimental raceway tanks. An incubation setup was constructed outside of the experimental tanks using 28-L boxes, impermeable plastic bags as chambers and an illuminator. The illuminator structure was equipped with four metallic vapor lamps (220 V, REV426A4, Serwal) and positioned over four independent dark 28L boxes ($22.5 \times 35 \times 50$ cm, Marfinita). Each box was connected to an individual raceway experimental tank by a 12 mm (diam.) hose so that seawater was constantly renewed in the box. Thus, each box corresponded to a treatment (Supplementary Material Fig. S1). In each box, four transparent 29×39 cm incubation bags impermeable to dissolved oxygen served as incubation chambers and were filled with approximately 500 ml of seawater. Three of the bags received the following biological material: (1) only 1–2 *H. cuneata* thalli, (2) 1–2 *H. cuneata* thalli and 10 shoots of *H. wrightii* or (3) only 10 shoots of *H. wrightii* (illustrated in Supplementary Material Fig. S1). The fourth bag contained only seawater in order to monitor any background changes in oxygen concentration and total alkalinity (A_T) due to microorganisms. Water samples were taken directly from treatment boxes for initial measurements of dissolved oxygen concentration (DO) and A_T immediately prior to commencement of incubations. All visible air bubbles were removed from the bags before their sealing. At the end of the incubation period, water samples were taken from each bag for the final measurements of DO and A_T . Water volume was measured and *H. cuneata* and *H. wrightii* were removed, dried at 60°C and weighed. Basal segments were removed from each sample, decalcified with nitric acid (0.6M HNO_3) and weighed to determine the dry decalcified weight.

Oxygen evolution. Five initial water samples (12 ml) were collected from each treatment box ($n = 3$) at the beginning of the incubation. At the end of the incubations, five water samples were taken from each bag ($n = 3$) with a 60 ml syringe fitted with a small tube. Samples were immediately treated with manganese chloride and alkaline-iodide reagents upon removal and refrigerated for 72 hours. They were then treated with a sulfuric acid reagent and analysed spectrophotometrically according to the Winkler method adapted by Labasque⁶⁸ in order to calculate the dissolved oxygen concentration, or O_2 production.

GPP values were calculated by normalizing O_2 production to incubation time, volume of water and the decalcified dry weight of the incubated tissue ($\mu\text{mol O}_2 \text{gDW}^{-1} \text{h}^{-1}$), after removing background O_2 fluctuations due to microbial activity.

Calcification. One initial water sample (180 ml) was collected from each treatment box pre-incubation and one final water sample was taken from each bag post-incubation ($n = 3$). Water samples were immediately refrigerated until analysis. Alkalinity anomaly measurements were performed using the aforementioned Ocean Optics equipment. The CO2SYS program⁶⁶ was used to calculate all DIC species and components of seawater carbonate chemistry. Changes in each DIC species (ΔHCO_3^- , ΔCO_2 and ΔCO_3^{2-}) and total DIC (Δ DIC) were calculated by subtracting the pre-incubation value from the post-incubation value.

Calcification rates were calculated for *H. cuneata* using the following equation⁶⁹:

$$g = -0.5 \frac{\Delta A_T V}{DW \Delta t} \quad (1)$$

where $g = \mu\text{mol CaCO}_3 \text{g}^{-1} \text{h}^{-1}$, ΔA_T = change in total alkalinity, V = volume of incubated seawater, DW = dry, decalcified weight of *H. cuneata* and Δt = incubation time (h).

Statistical analysis. A three-way analysis of variance (ANOVA), was performed on the *H. cuneata* calcification and GPP data ($\log(x + 1)$ transformed), with the factors CO_2 (two levels), temperature (two levels), and seagrass presence (two levels). A two-way ANOVA was used to test *H. wrightii* GPP data ($\log(x + 1)$ transformed), with the factors CO_2 and temperature (two levels each). Significance level was set at $p = 0.05$. Due to the absence of any temperature effect and a strong trend in the data with respect to CO_2 , we proceeded to pool the samples from the same CO_2 treatment for a more robust analysis and in order to preserve important ecological implications. All data passed assumptions of normality of residuals and homogeneity of variances. Subsequently, two-way ANOVAs were applied to *H. cuneata* calcification, GPP data ($\log(x + 1)$ transformed), ΔHCO_3^- , ΔCO_2 , ΔCO_3^{2-} , $\Delta\Omega_A$, ΔpH and Δ total DIC data ($\log(x + 1)$ transformed), with the factors CO_2 (two levels) and seagrass presence (two levels). One-way ANOVAs were applied to *H. wrightii* GPP data ($\log(x + 1)$ transformed), ΔHCO_3^- , ΔCO_2 , ΔCO_3^{2-} and Δ total DIC data ($\log(x + 1)$ transformed), with CO_2 (two levels) as a factor. Fisher's LSD post hoc tests were used for pairwise comparisons of significant effects. All statistical analyses were performed using IBM SPSS Statistics 24.

Data Availability

The datasets generated during and/or analysed during the current study are available from the corresponding author upon reasonable request.

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Author Contributions

E.B. contributed to all steps of the experimental design, experiment, data analysis and manuscript preparation. J.S. contributed to the experimental design, data analysis and manuscript preparation. C.M. contributed to the experimental design and experiment. P.H. contributed to experimental design, experiment, data analysis and manuscript preparation.

Additional Information

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