Cascading Effects of Ocean Acidification in a Rocky Subtidal Community

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Abstract

Temperate marine rocky habitats may be alternatively characterized by well vegetated macroalgal assemblages or barren grounds, as a consequence of direct and indirect human impacts (e.g. overfishing) and grazing pressure by herbivorous organisms. In future scenarios of ocean acidification, calcifying organisms are expected to be less competitive: among these two key elements of the rocky subtidal food web, coralline algae and sea urchins. In order to highlight how the effects of increased pCO_2 on individual calcifying species will be exacerbated by interactions with other trophic levels, we performed an experiment simultaneously testing ocean acidification effects on primary producers (calcifying and non-calcifying algae) and their grazers (sea urchins). Artificial communities, composed by juveniles of the sea urchin *Paracentrotus lividus* and calcifying (*Corallina elongata*) and non-calcifying (*Cystoseira amentacea* var *stricta*, *Dictyota dichotoma*) macroalgae, were subjected to pCO_2 levels of 390, 550, 750 and 1000 µatm in the laboratory. Our study highlighted a direct pCO_2 effect on coralline algae and on sea urchin defense from predation (test robustness). There was no direct effect on the non-calcifying macroalgae. More interestingly, we highlighted diet-mediated effects on test robustness and on the Aristotle's lantern size. In a future scenario of ocean acidification a decrease of sea urchins' density is expected, due to lower defense from predation, as a direct consequence of pH decrease, and to a reduced availability of calcifying macroalgae, important component of urchins' diet. The effects of ocean acidification may therefore be contrasting on well vegetated macroalgal assemblages and barren grounds: in the absence of other human impacts, a decrease of biodiversity can be predicted in vegetated macroalgal assemblages, whereas a lower density of sea urchin could help the recovery of shallow subtidal rocky areas affected by overfishing from barren grounds to assemblages dominated by fleshy macroalgae.

Introduction

The partial pressure of CO_2 (pCO_2) in the atmosphere has increased by about 40% (267 to 384 ppm) since the beginning of the industrial revolution, leading to changes in the Earth's climate and in terrestrial ecosystems functioning [1]. This increase in atmospheric CO_2, mainly due to anthropogenic emissions, led to a worldwide modification of the seawater carbonate system, causing a decrease in ocean pH. Mean surface ocean pH has decreased by approximately 0.1 unit between pre-industrial time and the 1990s [2–3], and a further decrease of approximately 0.4 units is predicted to occur by the end of the century [3]. This "ocean acidification" process may have profound impacts on marine biota, mostly through the direct effects of pH on inter-cellular transport mechanisms that control the physiology and metabolism of marine organisms [4] and the decreased availability of CO_3^{2-}, used by many species to build calcareous shells and skeletons. Temperate rocky reef communities, as coral reefs, are particularly threatened by ocean acidification, since most of their organisms use carbonates to build calcareous structures. These organisms are

well known to be particularly sensitive to acidified conditions (e.g. [5–7]; see [8] for a recent review). The two main benthic calcifiers, corals and algae, secrete the most soluble forms of $CaCO_3$. Corals secrete aragonite, while calcifying algae secrete either aragonite (green algae, such as *Halimeda* spp.) or high-magnesium calcite (coralline red algae) [9]. Experiments investigating the effects of elevated pCO_2 on photosynthesis and/or calcification of calcifying algae show complex and species-specific responses, with variable results depending on the pathway of carbonate deposition and on its relative amount [9].

Along temperate Mediterranean coasts, shallow subtidal areas are dominated by macroalgae that provide a range of ecosystem services such as food source, shelter from predation and disturbance, proper settlement substrate and nursery for marine vertebrates and invertebrates. Macroalgal communities are particularly sensitive to anthropogenic disturbances and are, accordingly, used for ecological status assessment under the European Water Framework Directive 2000/60/EC [10–12]. Shifts between alternative states have already been reported [13–14], such as a loss of canopy-forming species (i.e. fucoids) in favour

of turfs [15–16] or barrens of coralline algae [13,17] in response to human pressures, *e.g.* urbanization of the coastline and overfishing (that favour proliferation of sea urchins).

Macroalgae also exhibit different sensitivities to increased sea temperature and ocean acidification [9,18–20]. Non-calcifying algae generally show increased production and growth in response to elevated CO_2 [21–23], while fucoid canopy algae, even though particularly sensitive to human impacts, are considered to be less sensitive to pH variations and their primary production might be even expected to increase in acidified waters [24]. Corallinales species are the dominant calcifying algal group and appear to be the most sensitive to ocean acidification: elevated pCO_2 negatively affects their recruitment [25], growth [26] and calcification [7,27–29]. They exhibit calcium carbonate dissolution and decreased surface percent cover at lower pH and may be less competitive for space, driving a shift from dominance of calcifying to non-calcifying algae [25,30–32].

Sea urchins are generally the most effective benthic herbivores in shallow subtidal areas [33]. Although their grazing activity can facilitate the settlement of new species by providing patches of bare substrate, with an increase in biodiversity [34], when they are particularly abundant as a direct or indirect consequence of human disturbance (*e.g.* overfishing, [13]; date-mussel fishery, [17]), they can dramatically deplete non-calcifying algae, changing the seascape with the creation of extensive barren grounds dominated by coralline algae.

Sea urchins also show sensitivity to decreased pH: their carbonate structures (skeleton and grazing apparatus) are made up of the very soluble high-magnesium calcite, both in adult, juvenile and larval stages [35–37]. Adult sea urchin sensitivity to ocean acidification has been reported particularly by *in situ* records in naturally acidified areas [18], while laboratory experiments have mainly tested effects of low pH on larval stages or gene expression in the developmental process [36,38–41].

Macroalgae and sea urchins therefore strongly interact to shape the ecological state of rocky ecosystems. Studies on their response to ocean acidification have been so far performed separately through laboratory experiments on isolated species [19,21–22,35,42] and through *in situ* observations in naturally acidified areas [18,30,43], but it is crucial to assess the cascading effects of their individual responses [44].

The effects of elevate pCO_2 on species interactions are still poorly known and the extent of consequences at the ecosystem level hard to assess. Most of the laboratory studies on ocean acidification have been focused on fitness and physiological processes of individual species [45]. Only a few studies, so far, have dealt with species interactions in naturally acidified locations [18,43–44], that mimic future acidified scenarios but whose ecology is potentially affected by surrounding areas (see [44] for in depth discussion). Individual species fitness can affect communities and/or ecosystem functioning and biodiversity by cascade processes [8,46] and pCO_2 effects on upper trophic levels may be modulated (ameliorated or exacerbated) by indirect effects intrinsic to interactions with other trophic levels [47]. These effects cannot be ascertained without simultaneously testing response to ocean acidification on interacting species.

In this context, we performed one of the first, to our knowledge, laboratory experiments on ocean acidification taking into account species interactions. We used calcifying (*Corallina elongata*), and non-calcifying (the fucoid *Cystoseira amentacea* var. *stricta*, hereafter *Cystoseira amentacea*, and *Dictyota dichotoma*) macroalgae, and sea urchins (*Paracentrotus lividus*), under 4 different pCO_2 conditions, relevant to present conditions and future scenarios [48].

The experimental design enabled testing the macroalgal responses to increasing pCO_2 as a function of their carbonate content, in presence or absence of grazers, and the sensitivity of sea urchins to ocean acidification, through direct and diet-mediated effects, in order to assess whether low pH conditions may lead to different grazing capabilities, as a function of their requirement for carbonate ions to build-up their carbonate structures, and to different levels of defence to predation. Another novelty of the present study is the focus on the juvenile urchin stage, whose morphology and feeding behaviour are the same of adults, but whose growth rate (skeletons and jaws) is faster and purportedly more sensitive to pH decrease [49]. Given their faster growth rate, their responses (in terms *e.g.* of jaw and test growth) under short-term experimental conditions may better be assessed.

Materials and Methods

Experimental set-up

Juveniles *of Paracentrotus lividus*, about 4 months old, were provided by a sea urchin hatchery in Camogli (NW Mediterranean Sea, Italy), where they had been reared after *in vitro* fertilization. A total of 144 juveniles, with a test diameter of 5 to 6 mm, had been randomly selected and moved to the Laboratoire d'Océanographie de Villefranche, where the experiment was performed, one month before the start of the experiment. Algal specimens were collected in the Bay of Villefranche (NW Mediterranean Sea, France) at a depth of less than 5 m, and transported to Villefranche laboratory. No specific permits were required for collecting specimens in the present location: the Bay of Villefranche is not subject to particular protection restrictions. The location is not privately-owned or protected in any way. For each of the 3 selected algal species, *Corallina elongata*, *Cystoseira amentacea* and *Dictyota dichotoma*, 32 samples of around 5 g fresh weight (FW) were collected, cleaned of epiphytes, fastened with a rubber band and placed in experimental aquaria. The field collection did not involve endangered or protected species. All algal specimens were acclimated in a thermostated room at 22°C, for at least one week before the start of the experiment: the relatively short acclimation period was due to the decision of not adding nutrients for the macroalgal culture. We decided not to add any culture medium to the tanks in order to avoid possible biases due to fact that nutrient enrichment and pH interplay in different directions on growth of non-calcifying and coralline algae [23] and could possibly exert negative effects on juvenile urchins.

Four pCO_2 levels, chosen according to best practices [50] and IPCC projections [48], were used: (1) present day, $pCO_2 = 390$ µatm (control), (2) optimistic scenario, $pCO_2 = 550$ µatm, (3) realistic scenario (close to what expected for 2100), $pCO_2 = 750$ µatm and (4) pessimistic scenario, $pCO_2 = 1000$ µatm.

Unfiltered seawater, pumped from a depth of 10 m in the Bay of Villefranche, was continuously supplied to four 200 l header tanks. The chosen pCO_2 levels in the experimental tanks were obtained by bubbling pure-CO_2 using a continuous pH-stat system (IKS, Karlsbad, Aquastar). In the control tank, pCO_2 was maintained at 390 µatm by bubbling CO_2-free air produced by stripping CO_2 from ambient using soda lime, and adjusting to the appropriate level through the IKS system. pH values corresponding to each pCO_2 level were estimated based on desired pCO_2, total alkalinity (A_T), temperature and salinity using the R package seacarb [51]. pH electrodes from the pH-stat system were inter-calibrated every 2 days using a glass combination electrode (Metrohm, electrode plus) calibrated on the total scale using TRIS buffer solutions with a salinity of 35 [52].

The whole system comprised 48 experimental units allocated in 16 aquaria (20 l), 4 aquaria for each pCO$_2$ level (Fig. 1). Three smaller containers, each representing one experimental unit, were placed in each aquarium. Manipulated seawater from the four header tanks was delivered to experimental units at a rate of about 6 l h^{-1}. Each experimental unit was directly provided inflow water from the respective reservoir at any given pH with an individual pipe. The water filled experimental units and flowed out of the smaller containers, filling the aquarium, then was discharged through an overflow system. For each pCO$_2$ level, two aquaria (6 experimental units) with only algae and two aquaria (6 experimental units) with algae and sea urchins were set up. The design enabled us to tease apart weight loss due to sea urchin grazing and direct pCO$_2$ effects on algal species. One of the three different algal species was allocated to each of the three experimental units in each aquarium (Fig. 1); two 5 g samples of each species were placed in their respective units. In the dedicated aquaria, six juvenile urchins were placed in each experimental unit. The experimental units within each aquarium were covered with a net in order to prevent urchin movement from one section to another, and to force them to graze on a single algal species, but with mesh large enough not to reduce light and water flow.

Irradiance values in the aquarium were maintained at about 215 µmol photons m^{-2}s^{-1}, corresponding to the irradiance at ca. 5 m depth in the Bay of Villefranche in June-July [19]. Light was supplied by two 39 W fluorescent tubes (JBL Solar Ultra Marin Day), with reflectors, above each aquarium (at a distance of ca. 10 cm from the water surface), with a 12:12 h L:D photoperiod.

Carbonate chemistry and specimens measurements

Seawater samples for total alkalinity (A_T) measurements were collected twice a week, filtered on GF/F membranes and immediately analyzed. A_T was determined potentiometrically using a Metrohm titrator (Titrando 80) and a glass electrode (Metrohm, electrode plus) calibrated using first NBS buffers (pH 4.0 and pH 7.0, to check that the slope was Nernstian) and then using TRIS buffer solutions. Triplicate titrations were performed on 50 ml sub-samples at 25°C and A_T was calculated as described by Dickson et al. (2007). Titrations of standard seawater provided by A.G. Dickson (batch 106) yielded A_T values within 2.4 µmol kg^{-1} of the nominal value (standard deviation =

4.6 µmol kg^{-1}). All parameters of the carbonate chemistry were determined from pH$_T$, A_T, temperature and salinity using the R package seacarb [51].

Algae and urchins wet weights were measured at the beginning and at the end of the experiment (precision: 0.001 g). At the end of the experiment, all specimens were air dried and stored for following analyses. Macroalgal weight loss under experimental conditions was calculated as the difference between algal wet weight at the beginning and at the end of the experiment.

A subset of 24 sea urchins (one for each experimental unit) was dissected and immersed in 1% (w:v) sodium hypochlorite for 30 min in order to remove organic matter and facilitate the separation of structural elements. The length of all portions of Aristotle's lantern and the diameter of the test were measured under a stereomicroscope and the ratio between the length of the jaw pyramids of the Aristotle's lantern and the diameter of the test was calculated (hereafter referred as jaw/test ratio).

Test robustness was measured on another sub-set of 24 sea urchin specimens using a custom-made device designed to measure the static force required to crush sea urchin tests (adapted from [53]). Sea urchins were positioned upside down (in order to mimic fish predator attack) in a glass column. Then, a hollow piston, built to fit and run within the column, was inserted inside the column and progressively filled with lead pellets in order to increase the pressure, until the crushing of the urchin test. The static force required to crush sea urchin tests was measured as the weight (g) of piston and lead added: data were normalized by the diameter of the test.

Finally, scanning electron microscopy (SEM) images of different parts of sea urchins (apical disc, spines and jaw pyramids) were taken using an Environmental Scanning Electron Microscope (E-SEM VEGA3, TESCAN) at the University of Genoa.

Statistical analyses

The effect of pCO$_2$ on calcifying (C. elongata) and non-calcifying (C. amentacea, D. dichotoma) algae in presence or absence of P. lividus on the percent algal weight loss (arcsin transformed) was tested using a 3-way crossed ANOVA, after test for normality and homogeneity of variance: factor 1 "algal species", 3 levels (C. elongata, C. amentacea, D. dichotoma); factor 2 "pCO$_2$", 4 levels (pCO$_2$ = 390, 550, 750, 1000 µatm); factor 3 "urchins", 2 levels (absent/

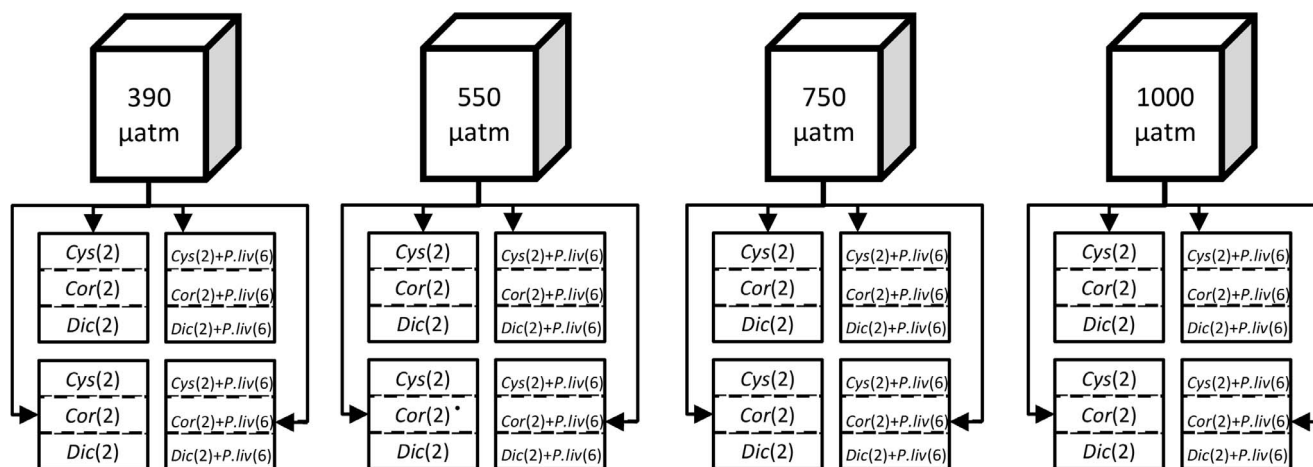**Figure 1. Scheme of the experimental set-up.** The top boxes represent the four reservoirs in which the pCO$_2$ was regulated. Squares represent the 16 experimental aquaria, divided into sub-sections as described in the Methods, to yield a total of 48 independent experimental units. The numbers of samples of each macroalgal item and of P. lividus are reported in brackets.
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present). Replicate values (n = 2) were the mean weight losses of the two samples of algae in each experimental unit. Student–Newman–Keuls (SNK) tests were performed for the *a posteriori* comparisons of means to check for differences among factor levels when the ANOVA detected significant effects.

Then, 2-way crossed ANOVA and SNK tests was performed on untransformed data to assess potential changes in the jaw/test ratio of the sea urchins fed with the three macroalgae and exposed to the four different pH levels. Considered factor were: factor 1 "algal species", 3 levels (*C. elongata*, *C. amentacea*, *D. dichotoma*); factor 2 "pCO_2", 4 levels (pCO_2 = 390, 550, 750, 1000 µatm). Replicate values (n = 2) were the mean values derived from urchins investigated in each independent experimental unit. A 2-way ANOVA, testing the same factors, and SNK tests were performed also on untransformed data of the normalised weight required to crush the test, in order to detect potential differences in test robustness among urchins fed with different algae and kept at different pCO_2 levels.

Results

Parameters of the carbonate chemistry are reported in Table 1. pH_T was maintained at an average (±SD) of (1) 8.09 ± 0.04, (2) 7.98 ± 0.06, (3) 7.84 ± 0.04 and (4) 7.70 ± 0.03, in the four treatments, respectively. A_T levels remained stable across treatments and during the whole experiment.

Although irradiance and temperature in the experimental aquaria were chosen in order to mimic natural conditions, no growth of algal thalli was observed during the experiment in any of the treatments. Accordingly, all algal specimens showed a general decrease in weight in all treatments, including in the experimental units without sea urchins. As expected, weight loss was much larger in the units where urchins were present and represented between 65 and 100% of the amount of algae placed in the aquaria at the beginning of the experiment. In general, *C. amentacea* and *D. dichotoma* showed larger weight loss than *C. elongata*, irrespective of the pCO_2 level: across all pH treatments *C. amentacea* lost around 90–100% of its weight when grazed by urchins and 55–65% when not grazed (Fig. 2a). *D. dichotoma* lost around 85–100% in presence of urchins and 45–55% when urchins were absent (Fig. 2b). *C. elongata*, probably more tolerant to artificial conditions, lost noticeably less weight than the other two algae in the treatments without urchins: around 40% in the control, till 60% at the highest pCO_2 treatment (Fig 2c). Yet, weight loss of *C. elongata* increased with increasing pCO_2, both in the presence (from 75 to 95%) and absence of sea urchins (Fig. 2c). The statistical significance of these results is shown in Table 2 which highlighted a significant effect of the "algal spp." and "pCO_2" interaction

($p<0.05$) and of the factor "urchins" ($p<0.001$) on all algal species. The SNK test on the factor "urchins" confirmed the expected larger weight loss when urchins were present at all pCO_2 levels (presence > absence; $p<0.01$). For the interaction between factors "algal spp." and "pCO_2", SNK test revealed a significantly larger weight loss of *C. elongata* at elevated pCO_2 treatments than at the control conditions (pCO_2 (550 = 750 = 1000) > 390 µatm; $p<0.01$; Table 2), while no differences were observed for the two non calcifying species.

Some mortality of sea urchins, unrelated to the pCO_2 level, occurred during the first week (19 out of 144 specimens, 15 of them were fed with *D. dichotoma*). The remaining ones were in very good conditions after one month, and showed positive increase in size. The jaw/test ratio showed a weak significant difference only between control pCO_2 and 750 µatm (390 > 750 µatm; p<0.05) and no significant interaction between factor "algal species" and "pCO_2" was observed (ANOVA; Table 3). Significant differences were found for the factor "algal species" ($p<0.01$) and the SNK test highlighted significant higher values of the ratio in urchins fed with *C. elongata*, compared to *C. amentacea* and *D. dichotoma* (*C. elongata* > (*C. amentacea* = *D. dichotoma*); $p<0.01$). In Figure 3, box plots show jaw/test ratio values for urchins fed with the three different algae, kept at the four different pH conditions.

The effects of pCO_2 and algal species on test robustness (Fig. 4) were both significant (ANOVA, $p<0.05$; Table 4). Test robustness was significantly lower in urchins kept at the highest pCO_2 level (pCO_2 (390 = 550 = 750) > 1000 µatm; $p<0.05$) and changed according to the diet, across all pH treatments: tests of urchins fed with *C. elongata* were significantly stronger compared to urchins fed with the two non-calcifying species (*C. elongata* > (*C. amentacea* = *D. dichotoma*)). Scanning Electron Microscope images performed on the apical disc, spines, and Aristotle's lantern of sea urchins showed that only the Aristotle's lantern was affected by the experimental treatments, while the other portions investigated did not reveal clearly detectable differences. The surface of latero-radial sides of the Aristotle's lantern, usually characterized by fine and dense tridimensional mesh of calcite trabeculae [54], examined with SEM at a magnification of 8500x, showed a porous structure with larger and more irregularly shaped holes, increasing signs of corrosion and structural breaks as a function of increasing pCO_2 (Fig. 5). The porous structure of Aristotle's lantern of urchins fed with *C. elongata* at 390 µatm (Fig. 5a) looked denser and Aristotle's lantern surface was smoother than those of urchins fed with the same diet but maintained at elevated pCO_2 (*e.g.* 1000 µatm, Fig. 5b) and also to those of urchins fed with non-calcifying macroalgae (*e.g.* urchins fed with *C. amentacea*, Fig. 5c) at the same pCO_2 (390 µatm). The density and preservation of the

Table 1. Parameters of the carbonate system and temperature in each treatment (mean ± SD).

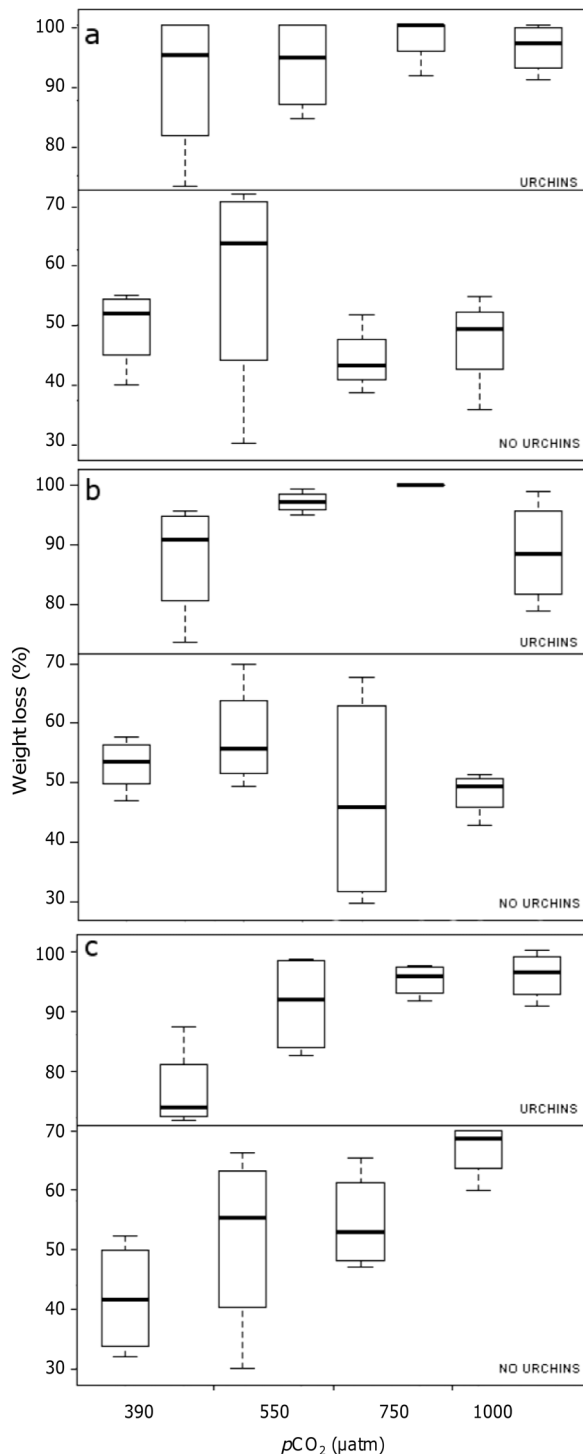| Treat	pH_T	pCO_2 (µatm)	A_T (mmol kg^{-1})	CO_3^{2-} (mmol kg^{-1})	HCO_3^- (mmol kg-1)	C_T (mmol kg-1)	$\Omega_{calcite}$	$\Omega_{aragonite}$	T(°C)
T1	8.09 ± 0.04	382 ± 41	2.531 ± 0.005	0.260 ± 0.023	1.891 ± 0.059	2.162 ± 0.037	6.10 ± 0.55	4.023 ± 0.378	24.18 ±1.12
T2	7.98 ± 0.06	528 ± 87	2.530 ± 0.005	0.215 ± 0.022	2.002 ± 0.055	2.232 ± 0.036	5.03 ± 0.52	3.322 ± 0.344	24.40 ±1.30
T3	7.84 ± 0.04	755 ± 87	2.530 ± 0.006	0.167 ± 0.017	2.121 ± 0.039	2.309 ± 0.025	3.91 ± 0.40	2.581 ± 0.268	24.32 ±1.08
T4	7.70 ± 0.03	1093 ± 72	2.530 ± 0.005	0.126 ± 0.010	2.222 ± 0.019	2.378 ± 0.012	2.95 ± 0.23	1.947 ± 0.154	24.33 ±0.97

Figure 2. Box plot on percent weight loss as a function of *p*CO₂ (µatm), for each macroalga: (a) *Cystoseira amentacea*, **(b)** *Dictyota dichotoma* **and (c)** *Corallina elongata.* Medians are highlighted in bold; bars represent the 25% and 75% quartiles; whiskers represent the lowest and highest data points.
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trabecular structure of the Aristotle's lantern of urchins that grazed on non-calcifying macroalgae exhibited a larger damage at elevated *p*CO₂ (Fig. 5d).

Table 2. Three-way ANOVA on algal weight loss.

Source	SS	DF	MS	F	P
algal species	1493.56	2	746.78	17.65	0.0000
*p*CO₂	655.71	3	218.57	5.16	0.0068
Urchins	12076.74	1	12076.74	285.38	**0.0000**
algal spp. X *p*CO₂	726.15	6	121.02	2.86	**0.0302**
algal spp. X urchins	63.24	2	31.62	0.75	0.4844
*p*CO₂ X urchins	337.50	3	112.50	2.66	0.0711
algal spp. X *p*CO₂ X urchins	167.34	6	27.89	0.66	0.6829
Residuals	1015.62	24	42.32		
Total	16535.87	47			

SNK tests:
Algal species (pCO₂): at 390 µatm C. amentacea = D. dichotoma > C. elongata (p<0.01); at 550 µatm, C. amentacea = D. dichotoma > C. elongata (p<0.05); at 750 µatm: D. dichotoma > C. elongata (p<0.05); at 1000 µatm: C. amentacea = D. dichotoma = C. elongata;
pCO₂ (Algal species): for C. amentacea and D. dichotoma 390 = 550 = 750 = 1000 µatm; for C. elongata pCO₂ 550 = 750 = 1000 > 390 µatm (p<0.01);
Urchins: presence > absence (p<0.01).
All factors are orthogonal and fixed. Number of replicates = 2. Cochran's test is not significant (C = 0.19). The significant effects are highlighted in bold. F values were calculated *versus* MS of residuals.
doi:10.1371/journal.pone.0061978.t002

Discussion

Effects of ocean acidification and grazing on calcifying and non-calcifying macroalgae

The role that ocean acidification could play on macroalgal assemblages, potentially affecting different processes, such as photosynthesis, growth, calcification rate and competitive ability of macroalgae, depending on their carbonate content and deposition pathway, is difficult to unravel. Macroalgae rely for
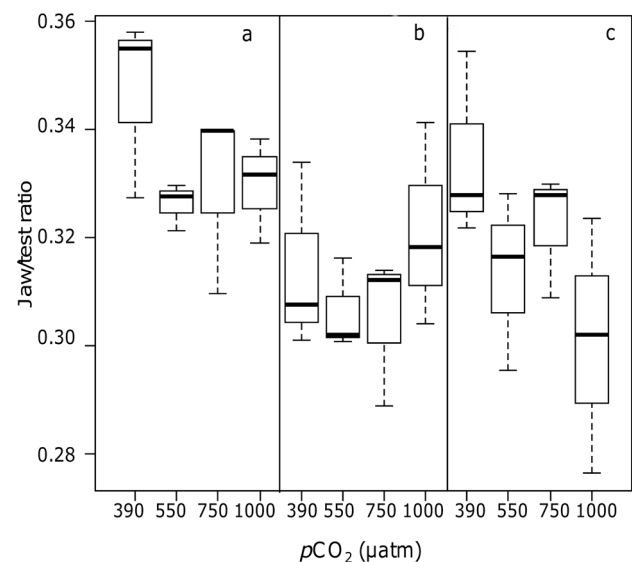

Figure 3. Box plot on the jaw/test ratio as a function of *p*CO₂ and algal diet: sea urchins fed with (a) *Corallina elongata,* **(b)** *Cystoseira amentacea* **and (c)** *Dictyota dichotoma.* Medians are highlighted in bold; bars represent the 25% and 75% quartiles; whiskers represent the lowest and highest data points.
doi:10.1371/journal.pone.0061978.g003

Table 3. Two-way ANOVA on the jaw/test ratio.

Source	SS	DF	MS	F	P
algal species	0.0036	2	0.0018	10.13	**0.0027**
pCO_2	0.0019	3	0.0006	3.61	**0.0458**
algal spp. X pCO_2	0.0024	6	0.0004	2.20	0.1157
Residuals	0.0021	12	0.0002		
Total	0.0100	23			

SNK tests:
Algal species: *C. elongata* > (*C. amentacea* = *D. dichotoma*) (p<0.01);
pCO_2: 390 > 750 µatm (p<0.05).
All factors are orthogonal and fixed. Number of replicates = 2. Cochran's Test is not significant (C = 0.22). The significant effects are highlighted in bold. F values were calculated *versus* MS of residuals.
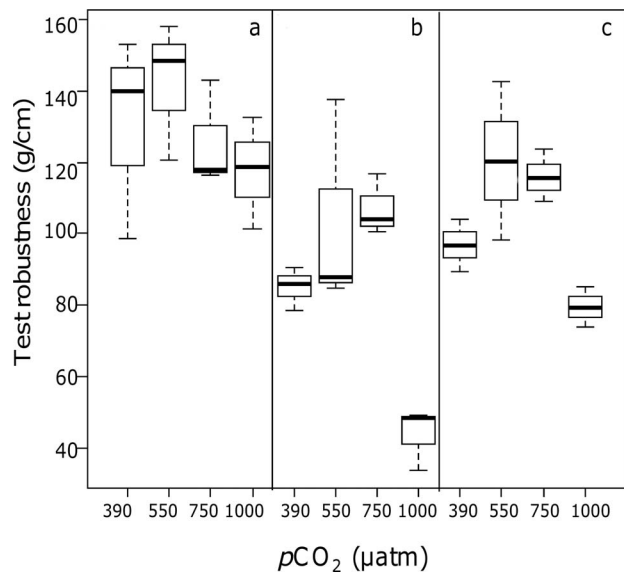doi:10.1371/journal.pone.0061978.t003

Figure 4. Box plot on test robustness, expressed as the weight (g) needed to crush the urchin test normalized by the test diameter, as a function of pCO_2 and algal diet: sea urchins fed with (a) *Corallina elongata*, **(b)** *Cystoseira amentacea* **and (c)** *Dictyota dichotoma.* Medians are highlighted in bold; bars represent the 25% and 75% quartiles; whiskers represent the lowest and highest data points.
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photosynthesis on CO_2 and/or HCO_3^- [55] which will increase in the ocean acidification scenario, potentially stimulating primary production [56]. Yet, the decrease in pH reduces CO_3^{2-}, which is used by calcifying macroalgae for the production and maintenance of carbonate structures [57–59], reducing growth [23,25] and calcification [28–29]. Additionally, calcifying species may also be negatively affected by increased CO_2 indirectly, as a consequence of the increased competitive ability of non-calcifying macroalgae [60].

The present study confirms previous findings, both based on *in situ* observations [18,30] and laboratory experiments [19–20,23,60], which have shown different responses of calcifying and non-calcifying macroalgae to ocean acidification.

The weight loss of the non-calcifying species, *Cystoseira amentacea* and *Dictyota dichotoma* was unaffected by pCO_2. In contrast, the calcifying seaweed *Corallina elongata* showed a significantly larger weight loss in the three elevated pCO_2 treatments than in the control condition. The sensitivity of coralline algae to elevated pCO_2 has already been reported by previous studies, some of which actually contrasted the response to nowadays pCO_2 conditions compared to pCO_2 levels higher than those projected in the incoming decades (ranging between 700 and 1500 µatm; *e.g.* [7,19,25,29,61]). The present study, testing pCO_2 levels in the range projected in the coming century, demonstrates that *Corallina elongata* exhibits a negative response even at a relatively low pCO_2 level (550 µatm), suggesting a significant impact in a few decades.

In the presence of urchins, all macroalgal species lost significantly more weight at any pCO_2 compared to experimental units where urchins were absent. More interestingly, the presence of urchins increased algal weight loss at any elevate pCO_2 compared to controls. The apparently smaller weight loss of *C. elongata* in the grazed condition (Fig. 2), compared to the non-calcifying species, was actually due to the lower weight loss of the calcified species also under the un-grazed condition. These findings suggest that urchin grazing pressure will be unaffected in an acidified scenario, and, as a consequence, grazing activity is expected to exacerbate pCO_2 effects on macroalgae, particularly calcifying ones.

All algal species showed a weight loss during the experiment. It did not prevent testing the effects of the main factors (pCO_2 and presence/absence of urchins). An increase in growth of non-calcifying species at elevated pCO_2 was anticipated based on previous studies [21–22,23,62–63], which would suggest that photosynthesis of certain benthic autotrophs will increase in a high-CO_2 world, except when other constituents such as nitrogen, phosphorus, and iron are limiting [8,64]. The weight loss observed

in the present study may be explained by nutrient limitation and/ or artificial conditions of the experiment (*e.g.* light). Additionally, in the Mediterranean Sea, the macroalgal biomass is highest in the spring; growth is much lower in June-July, the time at which the experiment was performed.
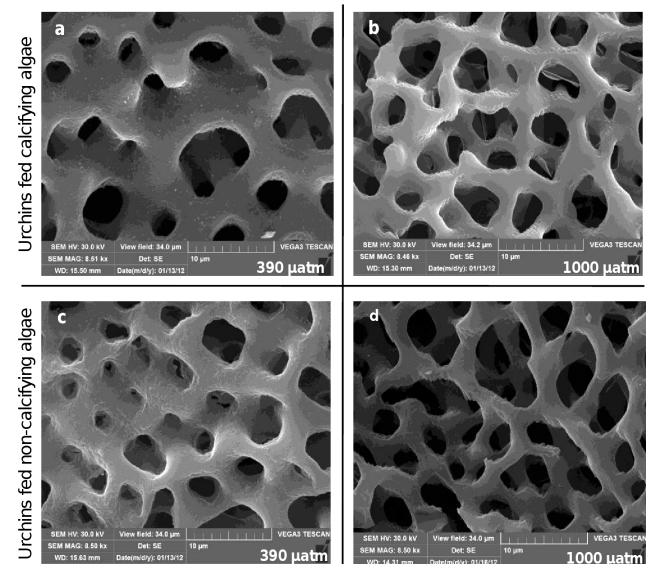

Figure 5. SEM images (8500x) of portions of the Aristotle's lantern of urchins fed calcifying (*C. elongata***; a, b) and non-calcifying (***C. amentacea***; c, d) macroalgae and maintained at pCO_2 levels of 390 (a, c) and 1000 µatm (b, d) for 1 month.**
doi:10.1371/journal.pone.0061978.g005

Table 4. Two-way ANOVA on test robustness.

Source	SS	DF	MS	F	P
algal species	6164.02	2	3082.01	6.55	**0.0119**
pCO_2	6253.78	3	2084.59	4.43	**0.0257**
algal spp. X pCO_2	1367.76	6	227.96	0.48	0.8078
Residuals	5645.56	12	470.46		
Total	19431.13	23			

SNK tests:
Algal species: *C. elongata* > (*C. amentacea* = *D. dichotoma*) ($p < 0.05$);
pCO_2: (390 = 550 = 750) > 1000 μatm ($p < 0.05$).
All factors are orthogonal and fixed. Number of replicates = 2. Cochran's Test is not significant (C = 0.26). The significant effects are highlighted in bold. F values were calculated *versus* MS of residuals.
doi:10.1371/journal.pone.0061978.t004

Combined effect of ocean acidification and diet on sea urchins

Sea urchins are the major grazers on rocky subtidal habitats [33] and their carbonate structures are particularly susceptible to pH reductions across their different developmental stages (larval skeletal rods, adult test, teeth and spines; [35,65–66]).

Few studies have focused on the juvenile stage, which is potentially one of the most susceptible to acidified conditions, given their higher growth rate compared to adults. In fact, even a small change in pCO_2 (200 μatm) is reported to increase mortality, decrease growth and cause a thinner and more easily breakable test in one year old urchins [49].

The present study shows significant pCO_2 effects on test robustness: sea urchins maintained at the lower pH (pH$_T$ 7.7) exhibited a less robust test than the ones maintained at higher pH. Yet, lower test robustness, *per se*, did not affect mortality during the short experimental time. In addition to direct pCO_2 effects, our study points out that the effect of ocean acidification on juveniles of *Paracentrotus lividus* is mediated by their diet, both concerning test robustness and jaw size, potentially affecting grazing capacity. The food source caused differences in the robustness of the test: urchins fed with *C. elongata* displayed a significantly stronger test than the ones fed with the two non-calcifying species.

These findings (the threshold of pH$_T$ 7.7 in sea urchin sensitivity and the role of coralline in their diet for strengthening the test) are in agreement with the findings of Hall-Spencer *et al.* (2008) in naturally acidified areas: a threshold value of mean pH$_T$ 7.8 is reported below which sea urchins are absent; accordingly, in these pH$_T$ conditions coralline algae are missing. Combining the results of these two studies suggests that a pH$_T$ value of 7.7–7.8 and the lack of corallines in urchin diet does not increase directly the sea urchin mortality but, rather, makes them more susceptible to predation, because of the less robust tests.

Additional diet related differences were observed in the jaw/test ratio, a parameter notoriously variable according to the trophic condition of the individual [67–69]. Higher values of this index, that means larger lantern compared to the test size, were found in urchins fed with *C. elongata* than in urchins fed with the other species, irrespectively of the pCO_2 level. In Figure 3 appear that pCO_2 modulates the diet effects as the ratio in sea urchins fed with *C. elongata* was higher at 390 μatm than at the three more elevated pCO_2 levels. Sea urchins fed with *D. dichotoma* only exhibited a clear difference between the control and the highest pCO_2 treatment (1000 μatm) (Fig. 3c). No difference among pCO_2 treatments was observed in specimens fed with *C. amentacea* (Fig. 3b). The different response to the three algal species,

additionally modulated by pCO_2 in the calcifying species (ratio higher in urchins fed with *C. elongata* in the control condition, and lower with increasing pCO_2 and in the urchins fed with the non-calcifying algae), may be due to the different toughness of the algal tissues. It is hypothesized that sea urchins need a larger grazing apparatus (relative to the body size) when fed with more calcified algal structures: in *Corallina*-fed urchins the ratio decreases sharply from the controls to the first intermediate pCO_2 level, showing a fast response to *C. elongata* decalcification, that makes the alga more easily grazable [27].

Scanning Electron Microscope images on latero-radial sides of Aristotle's lantern highlighted, for all sea urchins exposed to experimental conditions, a progressive weakening of the calcite trabecular structure as a function of increasing pCO_2. While the Aristotle's lantern of urchins fed with *C. amentacea* and *D. dichotoma* looked generally less tough in all treatments, *Corallina*-fed urchins displayed denser and smoother structures in the controls compared to controls fed with the other algae, with a clear reduction in thickness and increase in corrosion as a function of increasing pCO_2.

Taken together, these results suggest that the uptake of carbonate from the diet is fundamental in modulating sea urchin response to ocean acidification. The carbonate content of *C. elongata*, even at the higher pCO_2, makes the test of the juveniles much stronger and their jaws larger compared with specimens fed with non-calcifying algae. These findings, that show a direct pCO_2 effect on sea urchin defense from predation (test robustness) but also strong indirect effects mediated by the diet on test robustness and on jaw size, were achieved thanks to the experimental setup of an artificial community, designed for assessing interactions among primary producers and their grazers.

Cascading effects of ocean acidification on prey-predator dynamics

Ocean acidification, whether causing the loss of keystone/critical species or the reduction in their activity (*e.g.* predation, grazing, bioturbation), could have consequences at the ecosystem level [70], for example reducing habitat complexity and interfering with biological controls, such as the top-down control of sea urchins on non-calcifying macroalgae [44]. Our results highlight that in the different combinations of pCO_2 and diet, sea urchins showed different morpho-functional features, more as a consequence of diet than of pCO_2. Notwithstanding these differences, the grazing pressure of the urchins was similar across all the treatments. The observed combined effects of ocean acidification and macroalgal diet on test robustness and on jaw/test ratio may have severe consequences at the ecosystem level. The decrease in test robustness due to the diet and pCO_2 is anticipated to make juveniles of *P. lividus* more susceptible to predation by fish, *e.g. Diplodus* spp. [71–72], because the decreased robustness of sea urchin skeletons makes them less resistant to static loads whenever they are attacked by crushing predators [53].

The consequent reduced sea urchin density is expected to cause a reduction of their grazing pressure on macroalgal assemblages, with different effects according to the state of the habitat. In barren ground habitats, the reduced abundance of sea urchins would favor the recolonisation of non-calcifying macroalgae [73], potentially causing an increase in macrobenthic biomass and biodiversity. Conversely, in well vegetated, undisturbed environments, biodiversity may be negatively affected by i) the loss of coralline species, ii) the reduced number of colonizable patches produced by sea urchins grazing [34], iii) the lack of the succession promoting role of coralline algae which positively affects recovery and complexity in rocky reef communities after disturbance ([74–

77], authors unpublished data). Consequently, the predicted effects of ocean acidification will lead to a decrease in biodiversity in well vegetated, undisturbed environments, while, considering the interaction with cumulated human impacts [78–79], particularly overfishing (favoring barren grounds formation), non-additive antagonistic effects on macroalgal biomass are expected. The proliferation of sea-urchins due to overfishing will be mitigated in a ocean acidification scenario, also due to higher vulnerability to predation, as shown in this study, favoring the recovery from barren grounds to well vegetated assemblages. The barren recovery trajectory is expected to lead to an increase of macroalgal biomass, but this may be locally unpredictable [80], depending on a multitude of biotic and abiotic factors (*e.g.* abundance of other grazers, extent of barren area and distance from well vegetated areas, availability of propagules, presence of other human impacts).

The recovery of foundation species (*e.g. Cystoseira*), in absence of coralline macroalgae, will be potentially prevented by the installation of turfs, able to inhibit their recruitment and known to be promoted in the framework of cumulated human impacts (see [81]).

These findings stress the need to move from experiments on individual species to species interactions in order to better understand the both direct and indirect effects driven by top-down and bottom-up processes, in order to build more reliable predictions of future scenarios under the interaction of high pCO$_2$ conditions and cumulated human impacts.

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Author Contributions

Conceived and designed the experiments: VA MC LM FG J-PG. Performed the experiments: VA FG SA. Analyzed the data: VA MC LM. Contributed reagents/materials/analysis tools: FG SA J-PG MC. Wrote the paper: VA MC LM FG PF J-PG.

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