



## Contribution to Special Issue: 'Towards a Broader Perspective on Ocean Acidification Research' Original Article

# Two intertidal, non-calcifying macroalgae (*Palmaria palmata* and *Saccharina latissima*) show complex and variable responses to short-term CO<sub>2</sub> acidification

Joana Nunes<sup>1\*</sup>, Sophie J. McCoy<sup>1</sup>, Helen S. Findlay<sup>1</sup>, Frances E. Hopkins<sup>1</sup>, Vassilis Kitidis<sup>1</sup>, Ana M. Queirós<sup>1</sup>, Lucy Rayner<sup>2</sup>, and Stephen Widdicombe<sup>1</sup>

<sup>1</sup>Plymouth Marine Laboratory, Prospect Place, West Hoe, Plymouth PL1 3DH, UK

<sup>2</sup>Marine Biology and Ecology Research Centre, Plymouth University, Drake Circus, Plymouth PL4 8AA, UK

\*Corresponding author: tel: +44 1752 633 407; e-mail: [jonu@pml.ac.uk](mailto:jonu@pml.ac.uk)

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Ocean acidification, the result of increased dissolution of carbon dioxide (CO<sub>2</sub>) in seawater, is a leading subject of current research. The effects of acidification on non-calcifying macroalgae are, however, still unclear. The current study reports two 1-month studies using two different macroalgae, the red alga *Palmaria palmata* (Rhodophyta) and the kelp *Saccharina latissima* (Phaeophyta), exposed to control (pH<sub>NBS</sub> = ~8.04) and increased (pH<sub>NBS</sub> = ~7.82) levels of CO<sub>2</sub>-induced seawater acidification. The impacts of both increased acidification and time of exposure on net primary production (NPP), respiration (R), dimethylsulphoniopropionate (DMSP) concentrations, and algal growth have been assessed. In *P. palmata*, although NPP significantly increased during the testing period, it significantly decreased with acidification, whereas R showed a significant decrease with acidification only. *S. latissima* significantly increased NPP with acidification but not with time, and significantly increased R with both acidification and time, suggesting a concomitant increase in gross primary production. The DMSP concentrations of both species remained unchanged by either acidification or through time during the experimental period. In contrast, algal growth differed markedly between the two experiments, in that *P. palmata* showed very little growth throughout the experiment, while *S. latissima* showed substantial growth during the course of the study, with the latter showing a significant difference between the acidified and control treatments. These two experiments suggest that the study species used here were resistant to a short-term exposure to ocean acidification, with some of the differences seen between species possibly linked to different nutrient concentrations between the experiments.

**Keywords:** DMSP, growth, macroalgae, net primary production, ocean acidification, *Palmaria palmata*, respiration, *Saccharina latissima*.

## Introduction

It has been > 10 years since the projections of Caldeira and Wickett (2003) that, by the end of the century, average surface ocean pH might decrease by 0.3–0.4 pH units from pre-industrial levels. In that time, the process of ocean acidification, which also affects seawater carbonate chemistry, has been an increasingly investigated subject (Riebesell and Gattuso, 2015). Many organismal responses to CO<sub>2</sub>-induced acidification have now been documented, with particular focus on calcifying algae and invertebrates, those expected to depend the most on seawater carbonate processes (Kroeker *et al.*,

2010, 2013). However, substantially less information is available on non-calcifying macroalgae.

In calcifying organisms, the lower carbonate saturation states which result from increased seawater CO<sub>2</sub> concentrations will significantly reduce an organism's ability to support this energetically expensive physiological process (Wood *et al.*, 2008). The energetic cost of maintaining calcification has a further impact on other key processes such as growth and reproduction (Findlay *et al.*, 2011; Burdett *et al.*, 2012; Ragazzola *et al.*, 2012; McCoy and Ragazzola, 2014). Ultimately, the impacts of ocean acidification on an

organism's health and performance can lead to significant changes in population viability and distribution, and consequently in species interactions and community structure (Findlay *et al.*, 2010; McCoy and Pfister, 2014; Queirós *et al.*, 2014).

More recently, there has been a growing interest in how ocean acidification might also affect non-calcifying species, especially organisms that contribute significantly to processes or activities that humans benefit from, i.e. ecosystem services (Beaumont *et al.*, 2008). One such group of organisms are non-calcifying macroalgae, which provide ecosystem services such as habitat structure and complexity, primary productivity, and biodiversity (Dayton, 1985), as well as commercial benefits such as the provision of industrial products, fertilizer, and food (Mabeau and Fleurence, 1993; Wei *et al.*, 2013). For example, kelp forests are one of the most productive habitats on Earth (Schaal *et al.*, 2010; Brodie *et al.*, 2014; Smale *et al.*, 2014) and their three-dimensional structure provides shelter, feeding, and nursery areas for many species (Steneck *et al.*, 2003; Christie *et al.*, 2009). Kelps are also an important carbon sink, which is becoming progressively more significant in light of rising levels of atmospheric CO<sub>2</sub> (Alonso *et al.*, 2012).

Many fleshy algae are expected to benefit from acidification, as an increase in CO<sub>2</sub> can potentially lead to increased productivity (Koch *et al.*, 2013) and growth (Hepburn *et al.*, 2011; Roleda *et al.*, 2012). Some macroalgal species can use both CO<sub>2</sub> and HCO<sub>3</sub><sup>−</sup> through the application of CO<sub>2</sub> carbon concentrating mechanisms (CCMs). These algae can then preferentially use HCO<sub>3</sub><sup>−</sup> over CO<sub>2</sub>, which obligate CO<sub>2</sub> users cannot do. The use of HCO<sub>3</sub><sup>−</sup> by CCM species is, however, more energetically costly than using CO<sub>2</sub> (Koch *et al.*, 2013). Changes in carbonate and CO<sub>2</sub> concentrations as a result of acidification will, therefore, affect the strategies used by CCM species and impact upon the competitive interactions between those species with and those without CCMs (Hepburn *et al.*, 2011; Cornwall *et al.*, 2012; Koch *et al.*, 2013).

If algae are indeed able to increase photosynthetic rates under higher CO<sub>2</sub> conditions, the energy available for somatic growth could consequently increase (Gutow *et al.*, 2014). At high CO<sub>2</sub>, CCM species may be able to switch to use CO<sub>2</sub> (less energetically demanding than HCO<sub>3</sub><sup>−</sup>) and allocate this additional energy to tissue growth (Zou and Gao, 2009). In addition, increased levels of CO<sub>2</sub> may also enhance the growth of turf-forming algae, which might prevent recruitment of other macroalgae, such as kelps (Connell and Russell, 2010). Differential responses between algal species to ocean acidification could thus critically alter the species composition of many marine habitats.

Environmental stressors, such as acidification, can impact more than just an alga's ability to photosynthesise and grow. Marine algae produce a variety of secondary metabolites that play a number of important cellular roles (Van Alstyne *et al.*, 2001; Krumhansl and Scheibling, 2012). The production of such compounds is influenced by environmental conditions and cellular fitness, such that stressors including desiccation (Renaud *et al.*, 1990), salinity (Pedersen, 1984), and elevated CO<sub>2</sub> (Swanson and Fox, 2007; Arnold *et al.*, 2012; Burdett *et al.*, 2012; Kerrison *et al.*, 2012) may alter the synthesis of these compounds. One such metabolite is dimethylsulphonio-propionate (DMSP) which is a tertiary sulphonium compound, with several roles, such as cryoprotectant, antioxidant, and precursor to anti-herbivore activated defences (Van Alstyne *et al.*, 2001; Van Alstyne, 2008). DMSP breaks down to produce dimethyl sulphide (DMS), a trace gas that constitutes the greatest biogenic source of reduced sulphur to the marine atmosphere. The atmospheric oxidation products of DMS participate in secondary

aerosol formation in the marine boundary layer, and thus play a key role in climate-related processes (Rap *et al.*, 2013). The world-wide distribution of dense macroalgal growth in coastal-zones may result in a significant flux of DMS to the atmosphere in these regions (Burdett *et al.*, 2012; Kerrison *et al.*, 2012). To date, there are very few studies on the effects of increased CO<sub>2</sub> on macroalgal DMSP production. DMSP production in the red coralline alga *Lithothamnion glaciale* has been shown to remain unmodified under continuous high CO<sub>2</sub> conditions; however, with an acute increase in CO<sub>2</sub>, both intracellular and water column DMSP concentrations were seen to increase, thought to be a result of damage to the algal cell walls (Burdett *et al.*, 2012). In non-calcifying macroalgae, the effects of CO<sub>2</sub> on DMSP concentration have been investigated in the green alga *Ulva*, where *U. lactuca* showed a significant increase in the production of extracellular total DMSP in high CO<sub>2</sub> treatments (Kerrison *et al.*, 2012).

The current study reports on two separate experiments which have both studied the physiological responses to CO<sub>2</sub>-induced seawater acidification in two ecologically important macroalgae from the Northeast Atlantic. The red alga *Palmaria palmata* and the kelp *Saccharina latissima*, which both possess CCMs, are common perennial macroalgae found primarily in the low intertidal zone of rocky shores. *Palmaria palmata* is an understory alga often found under the canopy-forming kelps, among other algae such as *Fucus serratus* and *Chondrus crispus* (Schaal *et al.*, 2010). The responses of *P. palmata* and *S. latissima* will inform our understanding of how ocean acidification will affect the function of these important nearshore ecosystems.

## Methods

### Sample collection and experimental set up

The two macroalgal species, *P. palmata* and *S. latissima*, were studied in separate experiments. Sixty *P. palmata* (Linnaeus) individuals (length of ~20 cm) were collected at low tide from Mount Batten (N 50° 21.371, W 4° 7.673), Plymouth, UK in October 2013 and the experimental exposures to CO<sub>2</sub>-induced seawater acidification ran from October to November. Thirty-six *S. latissima* (Linnaeus) individuals (length of ~40 cm) were collected at low tide from the same location in February 2013 and the experimental exposures ran from March to April.

Both experiments used the same exposure set-up. Algae were kept in clear acrylic tanks (*S. latissima*: 12 tanks – 60 cm long × 30 cm wide × 38 cm high, 3 individuals per tank; *P. palmata*: 30 tanks – 20 cm long × 20 cm wide × 40 cm high, two individuals per tank) supplied with recirculating seawater. The tanks were lit by four evenly spaced LED strip lights (Betta 120 cm, 15 K, 22 W) on 12-h light–dark cycles, positioned at a distance of 40 cm from the water surface. Irradiance (Photosynthetic Active Radiation: PAR) was ~40 μmol photons m<sup>−2</sup> s<sup>−1</sup>. During this initial settling phase, which lasted 1 week in the *P. palmata* experiment and four weeks in the *S. latissima* experiment, tanks were continuously bubbled with ambient air and algae were kept under ambient conditions (pH<sub>NBS</sub> = ~8.04).

In each experiment, half the experimental tanks were randomly allocated to the acidification treatment (OA: pH<sub>NBS</sub> = ~7.82), representative of an IPCC year 2100 OA scenario (IPCC, 2007), while the other half were allocated to ambient conditions (control: pH<sub>NBS</sub> = ~8.04). The pH in the acidification treatment is below the seasonal pH range (pH<sub>NBS</sub>: 8.00–8.44) reported for coastal waters near the sampling sites (Kitidis *et al.*, 2012). Acidification



was initiated once the settling phase was completed, and applied gradually by reducing the pH by  $\sim 0.02$  unit each day. For logistical reasons *S. latissima* had an acclimatization to acidification period of 2 weeks and *P. palmata* had an acclimatization to acidification period of 1 week. In both cases, the first set of measurements were taken after the acclimatization period, as soon as the target treatment level was reached. These measurements are referred to as  $T_0$  hereafter. For both experiments, the actual time of pH exposure (post acclimatization period) was the same and lasted 4 weeks. The second set of measurements were taken at the end of the pH exposure (4 weeks after  $T_0$ ), i.e. in April for *S. latissima* and in November for *P. palmata*, and will be collectively referred to as  $T_4$  hereafter. Acidification was achieved by continuously bubbling the experimental tanks with pre-mixed air and CO<sub>2</sub> following the method by Findlay *et al.* (2008). The pre-mixed high-[CO<sub>2</sub>] air passed through a Licor CO<sub>2</sub> analyser (LI-6262) and the atmospheric [CO<sub>2</sub>] was recorded daily. pH (NBS scale, Metrohm pH meter), temperature, and salinity (WTW LF197 combination temperature and salinity probe) were recorded three times a week. Total alkalinity (TA) samples were collected weekly from each tank in 250 ml amber glass bottles and poisoned with 100  $\mu$ l of a saturated mercuric chloride in deionised water solution. TA was measured using an open-cell potentiometric titration method with an automated titrator (Apollo SciTech Alkalinity Titrator Model AS-ALK2) following standard methods (Dickson *et al.*, 2007) and using certified reference materials (CO<sub>2</sub> CRM<sub>s</sub> (Batch 127), Dickson Laboratory, University of California). To determine dissolved inorganic nutrient concentrations, 50 ml of seawater was collected weekly from each tank. The water was filtered (Acrodisc syringe filter with 0.8/0.2  $\mu$ m Supor Membrane) into acid-cleaned, aged, 60 ml Nalgene bottles. The samples were frozen ( $-20^\circ\text{C}$ ) until ready for analysis. Analysis was carried out using a Bran and Luebbe AAIII segmented flow autoanalyser for the colorimetric determination of inorganic nutrients: combined nitrate + nitrite (Brewer and Riley, 1965), nitrite (Grasshoff, 1976), phosphate (Zhang and Chi, 2002), and silicate (Kirkwood, 1989). Nitrate concentrations were calculated by subtracting the nitrite from the combined nitrate + nitrite concentration. pCO<sub>2</sub>, bicarbonate and carbonate concentrations were calculated using CO2SYS (Lewis and Wallace, 1998; Pierrot *et al.*, 2006) with dissociation constants from Mehrbach *et al.* (1973) refit by Dickson and Millero (1987) and KSO<sub>4</sub> using Dickson (1990).

### Determination of net primary production and respiration

Net primary production (hereafter referred to as NPP) and respiration (hereafter referred to as  $R$ ) were assessed as changes in dissolved oxygen concentrations during light (mean PAR =  $34 \text{ mmol photons m}^{-2} \text{ s}^{-1}$ ) and dark incubation periods, respectively. One whole alga per tank was incubated in one light and in one dark incubation at  $T_0$  and again 4 weeks later ( $T_4$ ). Each incubation lasted for one and a half hours for *S. latissima* and for 2 h for *P. palmata*. Incubation times were determined by preliminary trials. Incubation chambers consisted of 5.3 l air tight acrylic tanks. A magnetic stirrer was placed at the bottom of each tank to homogenize oxygen concentration in the incubation water and chambers were placed in a water bath, on top of a large Variomag stirring plate. In addition to the algal incubations, blank incubations (seawater of each treatment only) were done in 0.9 l incubation chambers to correct for the contribution of planktonic and microbial activity. All individual algae were acclimatized to incubation conditions for 20–30 min, during which water flow into the

chambers was maintained. At the end of this period, each chamber was sealed and incubations initiated.

In the *S. latissima* experiment, dissolved oxygen concentrations were determined using the Winkler titration method (Carritt and Carpenter, 1966) on water samples taken at the beginning and at the end of each incubation. Acid washed, gravimetrically calibrated, 120 ml borosilicate glass bottles were carefully filled with incubation seawater using silicon tubing. For each incubated alga, triplicate samples were collected at: (a) Start (fixed immediately, see below), (b) Light (incubated under light before fixing), and (c) Dark (incubated in the dark before fixing). The concentration of oxygen (O<sub>2</sub>) in each bottle was determined by automated Winkler titration (Carritt and Carpenter, 1966) with photometric endpoint detection. Briefly, O<sub>2</sub> is precipitated with alkaline iodide (NaOH + NaI) and MnSO<sub>4</sub> and the samples stored in a water bath at room temperature until analysis (24–48 h). The precipitate is dissolved by addition of H<sub>2</sub>SO<sub>4</sub> and titrated against thiosulfate (transmission endpoint detection). The thiosulfate was calibrated every 3 days against 0.1 mol l<sup>-1</sup> KIO<sub>3</sub> standards (Sigma-Aldrich, product number: 34273). O<sub>2</sub> saturation with respect to atmospheric equilibrium was calculated from the solubility of O<sub>2</sub> at *in situ* temperature, salinity, and pressure (Benson and Krause, 1984; Garcia and Gordon, 1992).

In the *P. palmata* experiment, oxygen measurements were taken using a non-invasive optical O<sub>2</sub> analyser (5250i, OxySense, Dallas, USA) as detailed in Rastrick and Whiteley (2011) and Calosi *et al.* (2013). The OxySense fibre optic pen measures the fluorescence characteristics of an O<sub>2</sub> sensitive dot which was previously placed inside the incubation chambers. Before the first incubations, the oxygen optode was calibrated against the O<sub>2</sub> sensitive dots. Oxygen measurements were taken at the beginning and at 20 min intervals over a period of 2 h.

For both experiments, NPP was calculated as the O<sub>2</sub> concentration difference between 'Light' and 'Start' treated samples and  $R$  was calculated as the O<sub>2</sub> concentration difference between 'Dark' and 'Start'.

At the end of  $T_4$  incubations, the biomass of each alga was estimated as ash-free dry weight (AFDW = dry weight – ash weight). Algal dry weights were obtained after drying the algae at 60°C for 48 h and algal ash weights were obtained after combusting the dried algae at 350°C for 62 h. Each alga's  $R$  and NPP were corrected for biomass (AFDW) and the contribution of plankton and bacteria following the equation:

$$R_{\text{algal}} = \frac{R_{\text{incubation}} - R_{\text{blank}}}{\text{AFDW(g)}}$$

### DMSP sampling and analysis

Samples for DMSP analysis were collected at  $T_0$  and  $T_4$  from all individuals. Vegetative algal tissue samples were collected using a hole-borer size 10 (1.5 cm in diameter). To test for differences in DMSP production along the frond, half the algae were sampled at the base of the frond (just above the stipe) and the other half were sampled at the tip of the frond. The exact fresh weight of samples was recorded.

In the *S. latissima* experiment, algal samples were dried at 60°C for 48 h and dry weight recorded. Each sample was placed in a 20-ml serum vial containing 10 ml of Milli-Q water and one NaOH pellet, and the vial was quickly crimp sealed. In the *P. palmata* experiment, only fresh tissue samples were used (no drying) in smaller 8 mL serum vials containing 6 ml of 500  $\mu$ M sodium hydroxide. This was due to very low concentrations of DMSP in this species.

All samples were refrigerated in the dark until the time of analysis and analysed in the same way. Before analysis, samples were placed in a water bath overnight at 30°C to equilibrate DMS in the water and gas phases. One hundred to 250 µl of the supernatant was transferred to a glass purge tower using a Hamilton syringe for quantification of DMS. Samples were purged with helium at 60 ml min<sup>-1</sup> for 5 min while cryogenically trapping the DMS (Archer *et al.*, 2013). DMS concentrations were determined using a Varian 3800 gas chromatograph equipped with a pulsed flame photometric detector. DMS standards for calibration were prepared from DMSP (>98% purity; Centrum voor Analyse, Spectroscopie and Synthese, Rijksuniversiteit Groningen) following base hydrolysis in a 1.0 mol l<sup>-1</sup> NaOH solution. A five-point calibration was carried out at regular intervals throughout the sample analysis period, with an  $r^2$  for the resulting linear regression of nanogram sulphur vs. square root of the peak area of typically >0.995.

### Algal growth

Perforation areas at  $T_0$  were the 1.5 cm diameter circular perforations done for DMSP analysis. At  $T_4$  the perforations had become ellipses in some individuals. In *S. latissima* growth was assessed based on perforation area at  $T_4$  (perforation area =  $\Pi \times (1.5/2) \times (\text{perforation major axis}/2)$ ) and by perforation migration (calculated as the distance the perforation punched at  $T_0$  had migrated by the time of the  $T_4$  sampling point; Pfister, 1992).

Because *P. palmata* has smaller fronds than the kelp *S. latissima*, and because this experiment was run in autumn when algae were not actively growing, perforation area at  $T_4$  was considered a better method to estimate algal growth in *P. palmata*.

### Statistical analysis

The effects of acidification treatment (control cf. OA) and time ( $T_0$  cf.  $T_4$ ) on all measured parameters (NPP, R, DMSP concentration;

perforation area at  $T_4$  and perforation migration, i.e. algal growth, were only assessed for the effect of acidification) were individually investigated using generalized least-squares (GLS) analysis (Zuur *et al.*, 2009) given that data exhibited strong heteroskedasticity, therefore violating linear model assumptions. Linear model assumptions were verified by investigation of normal quantile–quantile (qq) plots and visual inspection of residual dispersion against treatments. Model structures investigated included acidification and time of exposure, and first-order interaction as fixed effects. To model heteroskedasticity various possible variance structures as a function of the predictor structure were compared. Models of increasingly higher complexity were then compared with simpler models hierarchically, using Akaike's information criterion (AIC) for each model, and estimation of likelihood-ratio statistics for evaluation of the improvement of fits. The best and most parsimonious fit for each response variable was selected. The effect of variance structures on model fit was investigated using restricted maximum likelihood estimates, while the effect of the predictor structure was investigated using maximum likelihood estimates (Pinheiro and Bates, 2000; Pinheiro *et al.*, 2007). The data from the two experiments were analysed separately.

## Results

### *Palmaria palmata*

Table 1 summarizes the seawater chemistry details during the  $T_0$  and  $T_4$  periods for the *P. palmata* experiment. The carbonate chemistry remained significantly different between control and acidified treatments (two-group  $t$ -test for pH,  $p < 0.01$ , two-group  $t$ -test for TA,  $p = 0.75$ ) throughout the duration of this experiment (two-group  $t$ -test for pH,  $p = 0.46$ , two-group  $t$ -test for TA,  $p = 0.81$ ).

Nitrate and phosphate concentrations varied throughout the experimental period (Table 3). In the *P. palmata* experiment, a

**Table 1.** Seawater chemistry (mean  $\pm$  SD) during the *Palmaria palmata* experiment.

Parameter	$T_0$		$T_4$	
	Control	OA	Control	OA
Temperature (°C)	15.8 $\pm$ 0.2	15.9 $\pm$ 0.1	15.8 $\pm$ 0.1	15.9 $\pm$ 0.1
Salinity	35.1 $\pm$ 2.4	35.0 $\pm$ 2.5	36.1 $\pm$ 2.0	36.0 $\pm$ 2.0
pH <sub>NBS</sub>	8.02 $\pm$ 0.02	7.90 $\pm$ 0.04	8.01 $\pm$ 0.01	7.85 $\pm$ 0.05
TA (µmol kg <sup>-1</sup> )	2718.7 $\pm$ 167.4	2738.7 $\pm$ 129.8	2735.0 $\pm$ 90.6	2745.5 $\pm$ 89.2
pCO <sub>2</sub> (µatm)	689.70 $\pm$ 43.73	960.27 $\pm$ 123.10	699.78 $\pm$ 34.55	1066.92 $\pm$ 157.28
Bicarbonate (µmol kg <sup>-1</sup> )	2362.98 $\pm$ 127.36	2461.22 $\pm$ 118.80	2376.07 $\pm$ 70.31	2484.87 $\pm$ 82.25
Carbonate (µmol kg <sup>-1</sup> )	147.60 $\pm$ 18.81	115.28 $\pm$ 10.98	149.00 $\pm$ 8.70	108.23 $\pm$ 12.12

Temperature, salinity, pH, and TA were measured. pCO<sub>2</sub>, bicarbonate, and carbonate concentrations were calculated using CO2SYS (Lewis and Wallace, 1998; Pierrot *et al.*, 2006) with dissociation constants from Mehrbach *et al.* (1973) refit by Dickson and Millero (1987) and KSO<sub>4</sub> using Dickson (1990).

**Table 2.** Seawater chemistry (mean  $\pm$  SD) during *S. latissima* experiment.

Parameter	$T_0$		$T_4$	
	Control	OA	Control	OA
Temperature (°C)	12.4 $\pm$ 0.7	12.2 $\pm$ 0.5	13.1 $\pm$ 2.5	13.7 $\pm$ 2.0
Salinity	36.6 $\pm$ 0.2	36.6 $\pm$ 0.3	35.7 $\pm$ 1.2	35.8 $\pm$ 1.2
pH <sub>NBS</sub>	8.09 $\pm$ 0.05	7.75 $\pm$ 0.04	8.06 $\pm$ 0.05	7.77 $\pm$ 0.04
TA (µmol kg <sup>-1</sup> )	2850.2 $\pm$ 91.3	2908.9 $\pm$ 200.7	2878.1 $\pm$ 165.4	2831.3 $\pm$ 114.3
pCO <sub>2</sub> (µatm)	580.91 $\pm$ 74.80	1392.12 $\pm$ 150.62	645.15 $\pm$ 78.22	1329.14 $\pm$ 145.68
Bicarbonate (µmol kg <sup>-1</sup> )	2448.21 $\pm$ 81.77	2708.09 $\pm$ 187.81	2495.17 $\pm$ 132.16	2622.84 $\pm$ 115.97
Carbonate (µmol kg <sup>-1</sup> )	167.27 $\pm$ 22.18	83.75 $\pm$ 9.91	160.21 $\pm$ 37.73	86.83 $\pm$ 6.69

Temperature, salinity, pH, and TA were measured. pCO<sub>2</sub>, bicarbonate, and carbonate concentrations were calculated using CO2SYS (Lewis and Wallace, 1998; Pierrot *et al.*, 2006) with dissociation constants from Mehrbach *et al.* (1973) refit by Dickson and Millero (1987) and KSO<sub>4</sub> using Dickson (1990).



significant decrease in both nutrients was observed over time in both acidified and control treatments (nitrate: nitrate–time, AIC = 28.2603, logLik = − 10.1301, d.f. = 4,  $p$ -value < 0.01; phosphate: phosphate–time, AIC = 35.8434, logLik = − 13.9217, d.f. = 4,  $p$ -value < 0.01).

In *P. palmata*, both acidification and time had a significant effect on NPP (NPP ~ OA + time; Table 4; Figure 1). In this experiment,  $R$  significantly decreased (i.e. higher O<sub>2</sub> concentration) in the acidified treatments, and this did not relate to time (R–OA; Table 4; Figure 2).

DMSP concentrations did not differ at different frond locations in *P. palmata* (mean base DMSP:  $0.67 \pm 0.80 \mu\text{g g}^{-1}$ , mean tip DMSP:  $0.61 \pm 0.79 \mu\text{g g}^{-1}$ ; AIC = 282.1255, logLik = − 137.0627, d.f. = 4, and  $p$ -value = 0.65; Figure 3). For this reason, in the *P. palmata* experiment, all samples were used in the DMSP analysis. DMSP concentrations did not differ significantly with either time or with acidification levels in this species (Table 5).

*Palmaria palmata* showed uniform growth along the frond, with no difference in growth between the base and the tip of the frond (AIC = − 72.5359, logLik = 39.2679, d.f. = 3,  $p$ -value = 0.32; Figure 4). For this reason, data from all individuals were used to test the effect of acidification on growth, which showed that exposure to acidified seawater had no significant impact on growth in *P. palmata* (Table 5). In fact, the *P. palmata* experiment did not show substantial algal growth from the initial perforation size of 1.77 cm<sup>2</sup>, with bottom perforations averaging at 2.16 cm<sup>2</sup> (Figure 4).

### *Saccharina latissima*

Table 2 summarizes the seawater chemistry details during the  $T_0$  and  $T_4$  periods for the *S. latissima* experiment. The carbonate chemistry

**Table 3.** Nitrate and phosphate concentrations (mean  $\pm$  SD) during oxygen incubations.

Species	Sampling time	Acidification treatment	Nitrate ( $\mu\text{mol l}^{-1}$ )	Phosphate ( $\mu\text{mol l}^{-1}$ )
<i>Palmaria palmata</i>	$T_0$	Control	$3.47 \pm 2.65$	$1.06 \pm 0.93$
		OA	$2.57 \pm 2.53$	$1.29 \pm 0.73$
	$T_4$	Control	$0.24 \pm 0.10$	$0.44 \pm 0.34$
		OA	$0.26 \pm 0.17$	$0.55 \pm 0.29$
<i>Saccharina latissima</i>	$T_0$	Control	$0.02 \pm 0.01$	$0.14 \pm 0.03$
		OA	$0.02 \pm 0.01$	$0.12 \pm 0.07$
	$T_4$	Control	$0.04 \pm 0.04$	$0.41 \pm 0.20$
		OA	$0.19 \pm 0.29$	$0.34 \pm 0.25$

**Table 4.** Results from GLS models for the effect of sampling time (time) and acidification treatment (OA) on NPP and  $R$  for *P. palmata* and *S. latissima*.

Species	Source of variation	NPP				R			
		AIC	logLik	d.f.	$p$	AIC	logLik	d.f.	$p$
<i>Palmaria palmata</i>	Time $\times$ OA	− 77.5536	44.7768	6	0.44	− 110.1263	61.0632	6	0.74
	Time + OA	<b>− 78.9664</b>	<b>44.4832</b>	<b>5</b>	<b>&lt;0.01, &lt;0.01</b>	− 112.0128	61.0064	5	<0.01, 0.08
	Time	− 71.8358	39.9179	4	<0.01	− 96.5592	52.2796	4	0.64
	OA	− 70.8763	39.4382	4	<0.01	<b>− 110.9779</b>	<b>59.4890</b>	<b>4</b>	<b>&lt;0.01</b>
	~1	− 63.0992	34.5496	3	na	− 98.3418	52.1709	3	na
<i>Saccharina latissima</i>	Time $\times$ OA	− 31.9217	21.9609	6	0.31	− 63.7202	37.8601	6	0.51
	Time + OA	− 32.8842	21.4421	5	<0.01, 0.07	<b>− 65.2886</b>	<b>37.6443</b>	<b>5</b>	<b>&lt;0.01, 0.02</b>
	Time	− 15.0206	11.5103	4	0.06	− 58.9119	33.4560	4	0.05
	OA	<b>− 31.5179</b>	<b>19.7589</b>	<b>4</b>	<b>&lt;0.01</b>	− 61.7799	34.8900	4	0.01
	~1	− 13.5983	9.7992	3	na	− 57.1323	31.5662	3	Na

AIC, Akaike information criterion; logLik, log-likelihood; d.f., degrees of freedom;  $p$ , level of confidence ( $\alpha = 0.05$ ). The values in bold represent the selected model.

remained significantly different between control and acidified treatments (two-group  $t$ -test for pH,  $p < 0.01$ , two-group  $t$ -test for TA,  $p = 0.89$ ) throughout the duration of this experiment (two-group  $t$ -test for pH,  $p = 0.88$ , two-group  $t$ -test for TA,  $p = 0.56$ ).

Nitrate and phosphate concentrations varied throughout the experimental period (Table 3). During the *S. latissima* experiment, both nitrate and phosphate significantly increased towards the end of the testing period (nitrate: nitrate–time, AIC = − 32.5912, logLik = 20.2956, d.f. = 4,  $p$ -value < 0.01; phosphate: phosphate–t, AIC = − 23.4771, logLik = 15.7385, d.f. = 4,  $p$ -value < 0.01).

In *S. latissima*, acidification had a significant effect on NPP, but time did not cause change (NPP–OA; Table 4; Figure 1). In *S. latissima*,  $R$  significantly increased in the acidified treatment and with time (R–OA + time; Table 4; Figure 2).

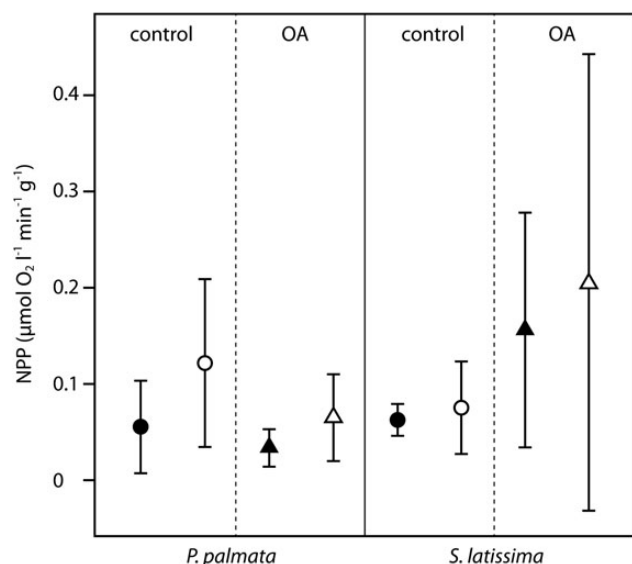
In *S. latissima*, DMSP concentrations varied between the base (mean base DMSP:  $12.40 \pm 7.18 \mu\text{g g}^{-1}$ ) and the tip of the frond (mean tip DMSP:  $1.83 \pm 0.96 \mu\text{g g}^{-1}$ ; Figure 3). This difference was highly significant (AIC = 349.0890, logLik = − 170.5445, d.f. = 4,  $p$ -value < 0.01). For this reason, in the *S. latissima* experiment DMSP analysis was focused on samples taken from the base of the frond only. DMSP concentrations did not differ significantly with either time of exposure or with acidification levels in *S. latissima* (Table 5).

Based on  $T_4$  perforation area, *S. latissima* growth was observed at the base of the frond, rather than at its tip (AIC = 51.5082, logLik = − 21.7541, d.f. = 4,  $p$ -value < 0.01; Figure 4). Therefore, subsequent analysis focused only on those samples taken at the base of the frond. During the 4-week exposure experiment there was no significant difference in growth between *S. latissima* grown in control conditions and those exposed to acidified seawater (Table 5). In *S. latissima* bottom perforations averaged at 5.43 cm<sup>2</sup> at  $T_4$  (Figure 4), showing substantial growth during the 4-week experiment.

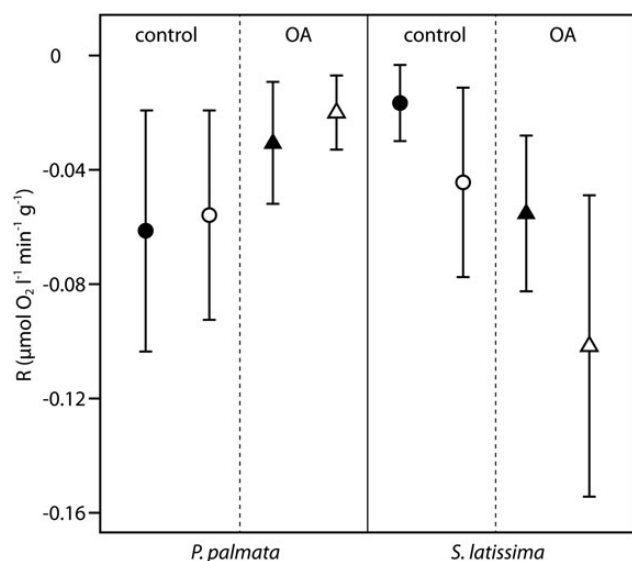
Based on perforation migration, and on bottom perforations only, acidification did have a significant effect on growth in *S. latissima* (growth by perforation migration–OA, AIC = 56.0671, logLik = − 24.0335, d.f. = 4,  $p$ -value = 0.02, Figure 5).

### Discussion

The current study describes two separate experiments which used the same experimental set-up to examine the impacts of CO<sub>2</sub>-induced acidification on key physiological functions in two species of



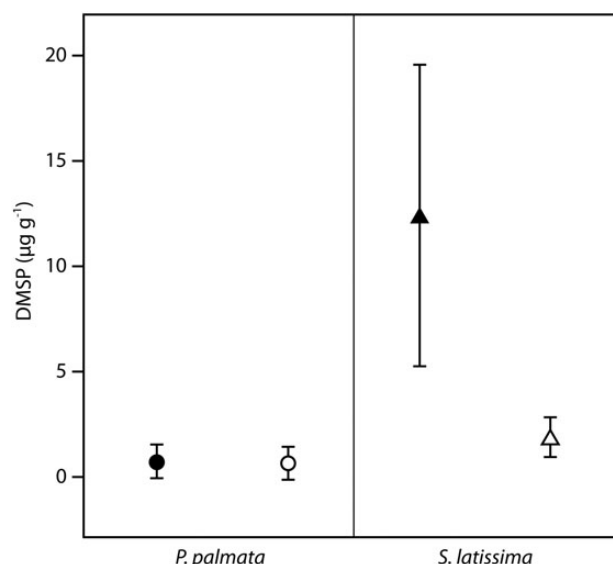
**Figure 1.** Mean  $\pm$  SD Net Primary Production, NPP, of *P. palmata* and *S. latissima* under control and acidified (OA) treatments. Black symbols represent  $T_0$ , white symbols represent  $T_4$ .



**Figure 2.** Mean  $\pm$  SD Respiration, R, of *P. palmata* and *S. latissima* under control and acidified (OA) treatments. Black symbols represent  $T_0$ , white symbols represent  $T_4$ .

intertidal fleshy algae. While the data from the two experiments cannot be directly compared in formal statistical analysis, the general responses and results do provide an insight into the variety of responses that can be seen across the macroalgal group, contributing towards an increased understanding of algal responses to ocean acidification.

A key factor in driving algal response over time could be nutrient supply. In the current studies, the statistical analysis showed that both nitrate and phosphate concentrations decreased significantly over the course of the *P. palmata* experiment, while the concentrations of these nutrients increased significantly over the course of



**Figure 3.** DMSP concentration (mean  $\pm$  SD) of *P. palmata* and *S. latissima* at the base (black symbols) and tip (white symbols) of the frond.

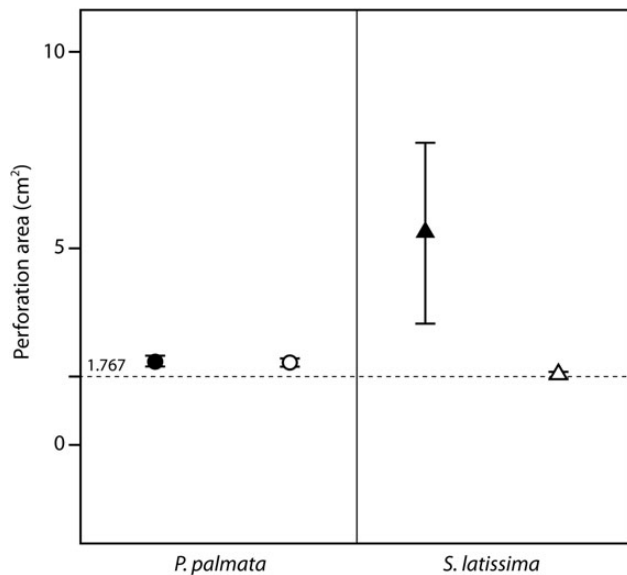
the *S. latissima* experiment. Despite the statistically significant increase in nutrient concentrations observed in the *S. latissima* experiment, which opposes that observed in the *P. palmata* experiment, the actual nutrient levels during the *S. latissima* experiment can be considered as extremely low and remained close to or even below the detection limit of the analytical techniques ( $0.02 \mu\text{mol l}^{-1}$  detection limit for nitrate). This, together with the fact, that *P. palmata* was tested in autumn, while *S. latissima* was tested in spring, might explain some of the contrasting responses reported here and the interspecies variability seen in some cases.

The observed responses of R to acidified conditions contrasted between the two species studied: *P. palmata* showed a significant decrease in R, whereas *S. latissima* exhibited a significant increase in R, in response to  $\text{CO}_2$ -induced acidification. For *P. palmata*, both NPP and R decreased significantly under higher acidification levels, and no increase in growth was observed. Given that  $\text{CO}_2$  is a resource for photosynthesis and might be expected to stimulate growth and function in non-calcifying algae, it is somewhat surprising that this alga appears down-regulating its photosynthesis and metabolism under high  $\text{CO}_2$ , and therefore limiting growth. However, this reduction in metabolic activity and lack of increased growth may not be caused by changes in acidification but may actually be a result of other important environmental factors. For example, daylength and net heat-flux, which would normally act as a cue to trigger growth, were held constant throughout the course of these experiments. The current results may, again, be confounded by the timing of the experiments. *P. palmata* specimens were collected in October when they may have already down-regulated their metabolism in response to environmental cues for the onset of winter. The suppressed growth of *P. palmata* in early autumn, a consequence of the low light and temperature typical of this time of year, has been previously reported (Martinez and Rico, 2002). Yet, despite the absence of growth, nutrients were depleted between  $T_0$  and  $T_4$  in the *P. palmata* experiment. Perhaps the algae took up the nutrients and stored these for future use.

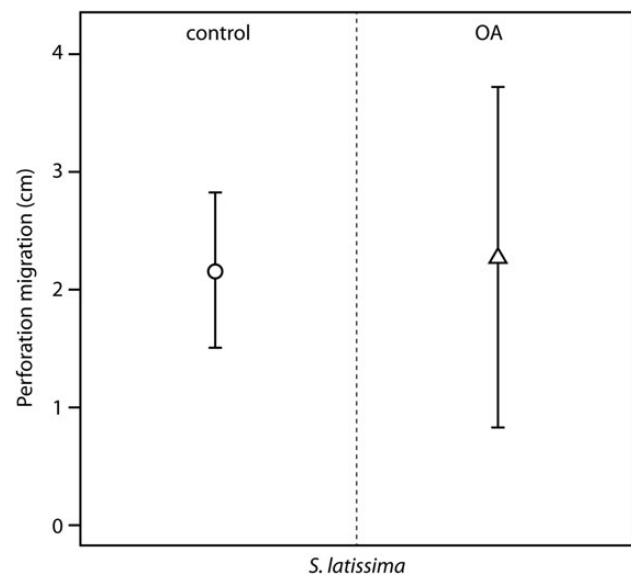
**Table 5.** Results from GLS models for the effect of sampling time (time) and acidification treatment (OA) on DMSP concentration and growth for *P. palmata* and *S. latissima*.

Species	Source of variation	DMSP				Growth (perforation area)			
		AIC	logLik	d.f.	p	AIC	logLik	d.f.	p
<i>Palmaria palmata</i>	Time + OA	280.7390	−135.3695	5	0.41, 0.20	na	na		na
	Time	279.4138	−135.7069	4	0.09	na	na		na
	OA	280.3574	−136.1787	4	0.16	−71.5484	38.7742	3	0.90
	~1	<b>280.3360</b>	<b>−137.1680</b>	<b>3</b>	<b>na</b>	<b>−73.5315</b>	<b>38.7658</b>	<b>2</b>	<b>na</b>
<i>Saccharina latissima</i>	Time + OA	250.5462	−121.2731	4	0.60, 0.58	na	na	na	na
	Time	248.8167	−121.4084	3	0.58	na	na	na	na
	OA	248.8581	−121.4291	3	0.60	84.7740	−39.3870	3	0.29
	~1	<b>247.1264</b>	<b>−121.5632</b>	<b>2</b>	<b>na</b>	<b>83.8798</b>	<b>−39.9399</b>	<b>2</b>	<b>na</b>

AIC, Akaike information criterion; logLik, log-likelihood; d.f., degrees of freedom; p, level of confidence ( $\alpha = 0.05$ ). The values in bold represent the selected model.

**Figure 4.** Mean  $\pm$  SD T<sub>4</sub> perforation area of *P. palmata* and *S. latissima* at the base (black symbols) and tip (white symbols) of the frond; the dotted line indicates the perforation area at T<sub>0</sub>.

*Saccharina latissima* showed an overall upregulation of both NPP and *R* when exposed to the acidified treatment, suggesting that these individuals are taking advantage of an increased availability of carbon. While the average level of NPP significantly increased in acidified seawater, it should be noted that the variability of this parameter also increased the longer the algae were under laboratory conditions (exposure time) as well as with increased acidification (Figure 1). Increased variability may suggest that algal “fitness” was more variable as time progressed and at high CO<sub>2</sub> levels, meaning that some individuals were able to withstand laboratory conditions and an acidified environment, while others were not. It is not clear if this increase in variability reflects differences in individual fitness at the genetic level, or differences in other factors, e.g. stored nutrients. It is unlikely that the small increase in nitrate and phosphate availability in the *S. latissima* experiment would have been sufficient to produce a significant algal response. These algae may therefore have experienced nutrient limitation from the beginning of the experiment, which could also partially explain the observed variability in responses. Perhaps nutrient limitation may have been exacerbated through

**Figure 5.** Mean  $\pm$  SD T<sub>4</sub> perforation migration of *S. latissima* at the base of the frond at control and acidified (OA) treatments.

the duration of the four week experiment and under acidification, manifesting itself as high variability in NPP and *R*. Though this interpretation is somewhat speculative, it further highlights the possibility of synergistic effects of different stressors (Russell *et al.*, 2009) and individual variability (Pistevos *et al.*, 2011). *Saccharina latissima* exhibited an increase in *R* at T<sub>4</sub> in both control and acidified treatments, possibly caused by poor acclimation to the experimental conditions, which would explain why these differences between T<sub>0</sub> and T<sub>4</sub> were also seen in the control treatment. Again, the variability in NPP and *R* response is especially evident at T<sub>4</sub> in acidified treatments, indicating that responses to acidification might have been exacerbated by the end of the experiment. Regardless of the mechanism, this variable response between individuals of the same species is a key element for future acclimation or potential adaption to changing environmental conditions.

Algal growth, in terms of perforation area, of both *P. palmata* and *S. latissima* remained unaffected by increased levels of CO<sub>2</sub> after 1 month of exposure. The fact that the effects of time and acidification on the NPP of *P. palmata* and *S. latissima*, respectively, were only

marginally significant, might suggest that not much extra energy was available to be allocated to somatic growth and support the perforation area results obtained here. In fact, *P. palmata* did not grow considerably, in either acidification or control treatments. However, in *S. latissima*, when estimating growth by comparing basal perforation migration between the control and the acidification treatment, it was apparent that perforations travelled (i.e. algae grew) more under acidified conditions than under control treatments. So in contrast to the perforation area results, it would appear that when growth is measured using the perforation migration method, there is some evidence that elevated CO<sub>2</sub> could have a stimulatory impact on *S. latissima* growth. The response of perforation migration to acidification is in accordance with the observed overall up-regulation of NPP and R, and suggests that a greater supply of CO<sub>2</sub> might indeed have slightly enhanced the performance of *S. latissima*. This highlights the need to consider CO<sub>2</sub> as both an important resource for non-calcifying macroalgae as well as a potential stressor. In addition, these growth results also demonstrate the importance of choosing the most appropriate method to assess particular biological responses.

In the kelp *Laminaria hyperborea*, an increase in growth rate has been previously linked to an increase in primary production in conjunction with stored nitrogen use (Abdullah and Fredriksen, 2004). Again, at least in the *S. latissima* experiment, nitrogen availability in the seawater was limited, which possibly further restricted the ability of the algae to grow. Furthermore, acidification did not affect algal growth during the stage of their life cycle studied here (mature sporophytes). It is worth considering that this may not be valid at other life-stages (e.g. juveniles or during reproduction). At the same time, it is likely that in these species algal growth is not prioritized when additional carbon is available. Macroalgae may instead preferably enhance reproduction or defences.

In the experiments here, there were no significant changes in DMSP concentration between acidified and control treatments in either *P. palmata* or *S. latissima*. This might be because both species produce relatively small quantities of this compound. Macroalgae reported to contain the highest concentrations of DMSP are Chlorophyta and Rhodophyta in the genus *Polysiphonia* (Van Alstyne et al., 2001; Van Alstyne, 2008). In green algae of the genus *Ulva*, concentrations of DMSP are typically 320–1600 mg g<sup>-1</sup> fresh weight (Van Alstyne and Puglisi, 2007). In contrast, and comparable to the current results, Lyons et al. (2007) found non-detectable levels of DMSP in *Laminaria digitata* and *Saccharina longicruris*, kelps from the Northwestern Atlantic coast, and also in *P. palmata*. It is thus possible that the species used in the current studies preferably invest in producing secondary metabolites other than DMSP when faced with a stressor, therefore maintaining their DMSP production unaltered between control and acidified treatments. Furthermore, the time of sampling of these experiments might have not been ideal, as seasonal variations in DMSP concentrations have been documented in *Codium fragile* ssp. *tomentosoides* (Chlorophyta) in Nova Scotia and shown to peak in winter then decrease through to autumn (Lyons et al., 2007). This seasonality might be related to an increased demand for the cryoprotectant properties of DMSP in winter. Additionally, DMSP occurs in high amounts in the genera *Enteromorpha*, *Ulva* and *Polysiphonia*, which are opportunistic macroalgae that can endure high salinity and desiccation (Van Alstyne et al., 2001). In the current experiments, the algae used were collected from the low intertidal and were typically aerially exposed for no longer than a couple of hours on an extreme

low tide, unlike *Enteromorpha*, *Ulva*, and *Polysiphonia* which are found at higher tidal elevations that become exposed during daily low tides for several hours.

The fact that *S. latissima* had significantly higher levels of DMSP at the base, rather than at the tip, of the frond, where tissues were also shown to be growing the most, suggests that this compound is mainly allocated to younger tissues. One hypothesis to explain the strategy of associating DMSP with younger tissues is that an alga should protect new growth, where resources have recently been allocated, in favour of old tissue at the frond tips that is most susceptible to physical damage. *Palmaria palmata* on the other hand, showed no differences in DMSP concentration or growth between base and tip of the frond. The lack of a difference in DMSP concentration between tissue locations might be supported by the fact that this species grows by dichotomous branching, as opposed to the elongated strap-shaped growth of a single frond in the kelp *S. latissima* (Bunker et al. 2010).

Finally, there are other compounds macroalgae produce to deal with changes in the environment. For instance, *S. latissima* in Canada has been found to invest in phlorotannin production when exposed to increased CO<sub>2</sub> levels (Swanson and Fox, 2007). However, this response was identified during a 55-day experiment. It is therefore also possible that the duration of the current experiments (4 weeks) was insufficient to identify detectable responses to CO<sub>2</sub> enrichment. Indeed, the distinct effects of short- and long-term exposures to climate stressors on marine organisms (Form and Riebesell, 2012; Godbold and Solan, 2013), have indicated that realistic climate change impact assessments may require far longer exposure times.

## Conclusions

North Atlantic macroalgal community structure and ecosystem functioning are expected to change in the future, as a consequence of the combined effects of acidification and warming (Brodie et al., 2014). However, in the present experiments few changes in the NPP, R, DMSP concentrations and algal growth of individual macroalgae exposed to CO<sub>2</sub>-induced seawater acidification were observed after 4 weeks. Specific elements of these experiments might have limited the study's ability to identify significant responses to ocean acidification, and it is recommended that future investigations of fleshy macroalgae consider seasonal variability in combination with longer exposure times. Nevertheless, the current studies have demonstrated that *P. palmata* and *S. latissima* possess a certain degree of resistance to ocean acidification. In light of these results, it is suggested that further experiments focus on the several parameters that might indicate macroalgal well-being, such as growth or grazing defences. Doing so in a multi-response approach, will help to disentangle possible trade-offs taking place in response to climate stressors. In addition, this study has highlighted the danger of extrapolating data from a single experiment to describe a generic acidification response for any particular species or group. Only by exploring the way in which underlying, seasonally variable, factors (such as organism condition, exposure history, reproductive/growth state, nutritional condition, or additional stressors or pressures) modify an organism's response to acidification, can a generic species-wide assessment be made. This will require multiple additional experiments to be conducted on species which may have already been studied. The effects of ocean acidification on habitat-forming non-calcifying macrophytes are likely to greatly modify present ecosystems, justifying the investment in such future research efforts for this group.



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