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# Responses of marine benthic microalgae to elevated $CO_2$

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**Abstract** Increasing anthropogenic $CO_2$ emissions to the atmosphere are causing a rise in $pCO_2$ concentrations in the ocean surface and lowering pH. To predict the effects of these changes, we need to improve our understanding of the responses of marine primary producers since these drive biogeochemical cycles and profoundly affect the structure and function of benthic habitats. The effects of increasing $CO_2$ levels on the colonisation of artificial substrata by microalgal assemblages (periphyton) were examined across a $CO_2$ gradient off the volcanic island of Vulcano (NE Sicily). We show that periphyton communities altered significantly as $CO_2$ concentrations increased. $CO_2$ enrichment caused significant increases in chlorophyll *a* concentrations and in diatom abundance although we did not detect any changes in cyanobacteria. SEM analysis revealed major shifts in diatom assemblage composition as $CO_2$ levels increased. The responses of benthic microalgae to rising anthropogenic $CO_2$ emissions are likely to have significant ecological ramifications for coastal systems.

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## Introduction

Increasing atmospheric $CO_2$ is causing unprecedented changes in seawater chemistry (National Research Council 2010) that are expected to have profound and widespread consequences for marine ecosystems since some organisms may benefit whilst many others are likely to be disadvantaged (Hall-Spencer et al. 2008; Doney et al. 2009; Kleypas and Yates 2009; Kroeker et al. 2010; Diaz-Pulido et al. 2011; Hepburn et al. 2011). The saturation state of calcium carbonate falls as ocean pH is lowered (Caldeira and Wickett 2003; Orr et al. 2005; IPCC 2007), so research into the effects that these chemical changes may have on primary producers has predominately focused on calcifiers (Engel et al. 2005; Langer et al. 2009; Martin and Gattuso 2009), although work is also now emerging on the effects of elevated $CO_2$ on non-calcareous groups of algae.

The predicted changes in $CO_2$ and bicarbonate ($HCO_3^-$) availability have the potential to stimulate photosynthesis in marine photoautotrophs. In response to low ambient $CO_2$ concentrations, most marine microalgae have evolved a carbon concentrating mechanism (CCM) to elevate concentrations at the site of carbon fixation (Beardall and Giordano 2002; Raven and Beardall 2003; Raven et al. 2011). Increases in dissolved $CO_2$ are predicted to cause down-regulation of microalgal CCM capacity (Giordano et al. 2005; Hopkinson et al. 2011) and, given the energetic costs of CCMs (Raven 1991), will potentially allow

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more energy for other growth processes. As the carbon acquisition mechanisms and efficiencies of CCMs differ between algae, it is thought that rising $CO_2$ will benefit different species to varying degrees (Hein and Sand-Jensen 1997; Tortell et al. 2000; Rost et al. 2003; Beardall and Raven 2004; Riebesell 2004; Fu et al. 2008b) and may result in dramatic community shifts with profound consequences for marine biogeochemistry (Hutchins et al. 2009).

Periphyton are microflora living attached to the surfaces of submerged objects (Azim et al. 2005). Periphytic communities (predominantly benthic diatoms and cyanobacteria) are major constituents of marine biofilms forming an important functional component of marine benthic ecosystems (Underwood 1984; Hill and Hawkins 1991; Thompson et al. 2004). Marine photoautotrophic biofilms contribute significantly to primary productivity (Hawkins et al. 1992; Bustamante et al. 1995), providing a main food source for a variety of grazers (Hawkins et al. 1989; Hill and Hawkins 1991; Jenkins et al. 2001). They also play important roles in determining the structure and dynamics of the overlying benthic communities by enhancing and/or inhibiting the settlement of invertebrates and macroalgal propagules (Meadows and Williams 1963; Huang and Boney 1984; Thompson et al. 1998).

The potential effects of ocean acidification on photoautotrophic biofilms and benthic microalgae have received relatively little attention (Torstensson et al. 2011; Witt et al. 2011). Research into the effects of ocean acidification on microalgae has mainly focused on planktonic species in laboratory or mesocosm experiments (Kim et al. 2006; Fu et al. 2007; Levitan et al. 2007). Several diatom species are relatively insensitive to changes in pH (Hinga 2002), and some oceanic diatoms show little response over a large $CO_2$ range (Tortell et al. 1997; Burkhardt et al. 2001), although other experiments have shown positive responses of diatoms to $CO_2$ enrichment (Riebesell et al. 1993; Burkhardt and Riebesell 1997; Burkhardt et al. 1999). Hopkinson et al. (2011) calculated that a doubling of ambient $CO_2$ could reduce CCM expenditure and lead to an increase in diatom productivity as $CO_2$ levels rise over the course of this century. Shifts in the dominance and composition of planktonic diatom assemblages with $CO_2$ enrichment have also been revealed and attributed to taxon-specific differences in $CO_2$ sensitivity (Tortell et al. 2002, 2008; Kim et al. 2006; Trimborn et al. 2009). There is also evidence that cyanobacterial responses to ocean acidification are species specific. Several oceanic, bloom-forming genera (*Trichodesmium*, *Synechococcus* and *Crocosphaera* spp.) improve resource allocation and increase their rates of photosynthesis, nitrogen fixation and growth in response to $CO_2$ enrichment (Lu et al. 2006; Barcelos e Ramos et al. 2007; Hutchins et al. 2007; Levitan et al. 2007; Fu et al. 2007, 2008a; Kranz et al. 2009); *Prochlorococcus*, on the other hand, has showed little response (Fu et al. 2007).

Our current understanding of the impacts of ocean acidification on microalgal assemblages is very limited as no research has been carried out in situ and there is likely much to learn from observations in areas that are naturally enriched with $CO_2$ (Liu et al. 2010). Short-term culture work has yielded contradictory results attributed to differences in the experimental design and control of carbonate chemistry (Iglesias-Rodriguez et al. 2008; Hurd et al. 2009). Short-term perturbation experiments can provoke stress responses (Wood et al. 2008), which may overestimate the impacts of acidification on marine organisms (Hendriks et al. 2010), do not reflect natural conditions and are unable to account for the adaptive capability of organisms. However, ocean acidification research is starting to be augmented with in situ experiments utilising areas that are naturally enriched with $CO_2$ such as cold-water $CO_2$ vents (Hall-Spencer et al. 2008; Fabricius et al. 2011; Rodolfo-Metalpa et al. 2011), hydrothermal vents (Couto et al. 2010; Vizzini et al. 2010; Bianchi et al. 2011) and upwelling areas (Thomsen et al. 2010). Such sites allow investigations of the ecosystem-level responses to ocean acidification although this approach has not yet considered effects on periphyton.

Here we present the results of an investigation that compared periphyton assemblages on artificial substrata installed along a coastal $CO_2$ gradient at a shallow water cold vent system off the island of Vulcano, NE Sicily. Our aim was to test the hypothesis that periphyton assemblages respond to $CO_2$ gradients and to characterise any changes in diatom and cyanobacteria populations to better understand the ecological effects of ocean acidification.

## Materials and methods

### Study site

This study was conducted between 17th September and 8th October 2010 along a stretch of rocky coast off the island of Vulcano (38°25′ N, 14°57′ E), part of the Aeolian Island chain, NE Sicily (Fig. 1). This is a microtidal region where volcanic $CO_2$ vent activity acidifies the seawater producing a pH gradient ranging from ~8.2 to ~6.8, running parallel to the coast. Three sites along this gradient were selected; Station 1 (S1) was located outside the vent and had a normal, relatively stable mean pH (8.18) representing ambient $CO_2$ levels (Table 1) whereas S2 and S3 had widely varying but intermediate and low mean pH (mean pH 8.05 and 7.49, respectively) due to their proximity to $CO_2$ vents. Median $CO_2$ levels at S2 represent conditions forecasted for around the middle of this century (Nakićenović and Swart 2000), whilst at S3 they go beyond those expected due to ocean acidification, yet remain useful

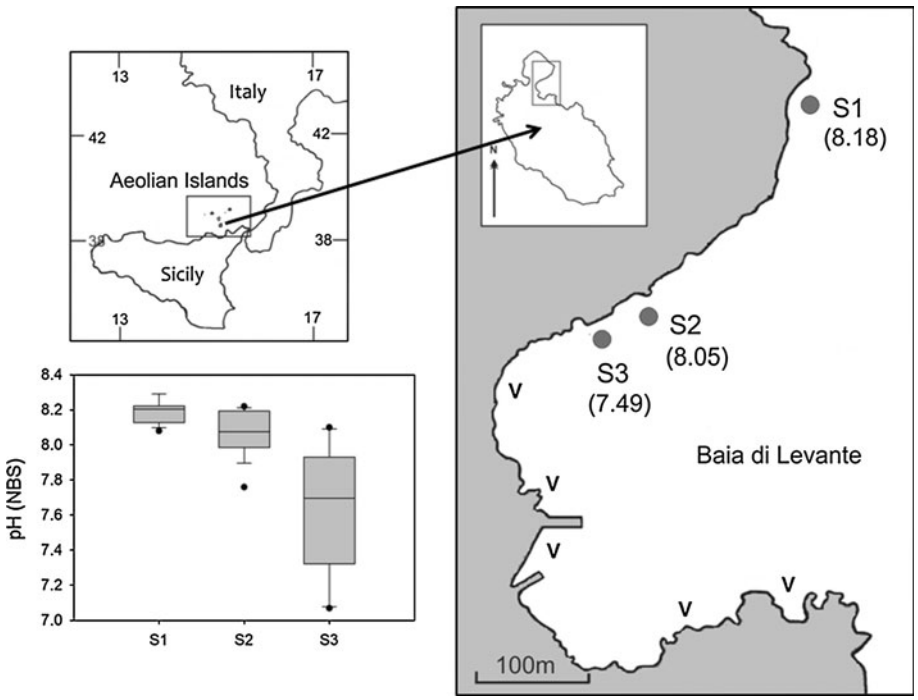


**Fig. 1** Map of the study area, Baia di Levante (Vulcano Island), showing sampling stations *S1*, *S2* and *S3*, v = gas vents. Data represent the mean pH of each station ($n = 18$ per station). The graph shows pH range along the $CO_2$ gradient measured at various intervals between September 2009 and October 2010; median = *horizontal line*, 25th and 75th percentiles = *vertical boxes*, 10th and 90th percentiles = *whiskers* and *dots* = min/max values

**Table 1** Seawater carbonate chemistry measurements along a $CO_2$ gradient on the island of Vulcano

| Station | | pH range (NBS scale) | $pCO_2$ ($\mu$tm) | TA (mmol kg$^{-1}$) | DIC (mmol kg$^{-1}$) | $CO_3^{2-}$ (mmol kg$^{-1}$) | $HCO_3^-$ (mmol kg$^{-1}$) | $\Omega_{calcite}$ | $\Omega_{aragonite}$ |
|---|---|---|---|---|---|---|---|---|---|
| S1 | Max | 8.29 | 331 | 2.625 | 2.197 | 0.32 | 1.871 | 7.54 | 7.97 |
| | Median | 8.21 | 419 | | 2.233 | 0.29 | 1.929 | 7 | 4.65 |
| | Min | 8.08 | 603 | | 2.339 | 0.22 | 2.101 | 5.26 | 3.77 |
| S2 | Max | 8.22 | 410 | 2.642 | 2.23 | 0.31 | 1.912 | 7.38 | 4.91 |
| | Median | 8.08 | 592 | | 2.401 | 0.19 | 2.193 | 4.45 | 2.89 |
| | Min | 7.76 | 1,429 | | 2.512 | 0.12 | 2.349 | 2.96 | 1.96 |
| S3 | Max | 8.1 | 599 | 2.736 | 2.409 | 0.25 | 2.14 | 6.05 | 4.02 |
| | Median | 7.71 | 1,611 | | 2.656 | 0.1 | 2.508 | 2.28 | 1.49 |
| | Min | 7.07 | 7,454 | | 2.95 | 0.02 | 2.682 | 0.54 | 0.35 |

Temperature (range 18.6–27.7°C), pH (NBS scale) and salinity (=38) were measured on several occasions between September 2009 and October 2010 ($n = 18$). Total alkalinity (TA) is point measurement taken on 02/10/10. The remaining parameters were calculated using $CO_2$ SYS programme (using the constants of Roy et al. 1993 and Dickson for $KSO_4$)

for examining response boundaries (Barry et al. 2010). All stations were at ambient temperature, and although sulphur was present at the $CO_2$ vent systems on the south of Baia di Levante, it was localised and undetectable at stations S1–S3.

## Carbonate chemistry and physical measurements

At all three stations, a calibrated YSI (556 MPS) pH (NBS scale) meter was used to measure temperature, pH and salinity at a depth of ~0.5 m. We recorded rapid pH fluctuations along this coastal gradient (over 1 unit in under ~4 h at S3), so the lack of precision in using the NBS scale for seawater measurements (approximately 0.05 pH, Dickson et al. 2007) was considered acceptable for this study. On the recommendations of Hoppe et al. (2010), total alkalinity (TA) alongside pH was measured to calculate carbonate chemistry. TA was measured at each station from a water sample that had been passed through a 0.2-µm pore size filter (stored in the dark at 4°C), and an AS-Alk 2 Total Alkalinity Titrator (Apollo SciTech Inc, Georgia, USA) was used to determine TA. The remaining parameters of the carbonate system were then calculated using $CO_2$ SYS software (Lewis and Wallace 1998). Light intensity was measured at 1–2 m depth in May 2011 at S1 and S3 using Hobo light loggers (Onset).

Means and interquartile ranges (IQ), more suitable for use over standard errors with highly variable pH data (Kerrison et al. 2011), were calculated from hydrogen ion concentrations before re-converting back to pH values. Means were calculated for each station using pH data collected from several visits to the vents between 2009 and 2010 (September–October 2009, April 2010, July 2010, September–October 2010).

### Sample collection

At each station, perspex slides (75 × 25 mm) were horizontally attached to four anchored floats (6 slides per float placed at 25-mm intervals), suspended in the shallow subtidal zone (<1 m depth) ($n = 24$ slides per station). Perspex (hydrophobic surface) was selected over glass (hydrophilic) to maximise the amount of microalgal attachment (Sekar et al. 2004). The slides were removed after 21 days by which time they had established biofilms that were visible to the naked eye. Half the slides ($n = 12$ per station) were chosen randomly for chlorophyll extraction. They were immediately preserved at −20°C (for <48 h) and stored at −80°C upon return to the laboratory (for <2–3 weeks) to prevent chlorophyll degradation (Thompson et al. 1999). The remaining half were fixed in glutaraldehyde (2.5% with filtered seawater) for 1 h in the dark, rinsed and then frozen as above until epifluorescence analysis (< 1 month) and viewing under the scanning electron microscope (SEM).

### Sample analysis

The photosynthetic standing stock of each slide ($n = 12$ per station) was measured from chlorophyll extracted using 100% ethanol (placed in boiling ethanol, ~70°C, for 2 min). Ethanol was chosen solvent for this experiment as it is an efficient extractant of chlorophyll from resistant material and provides the most reliable estimates for use with natural assemblages of mixed microalgae (Ritchie 2008). The absorbance of each sample at 632, 649, 665, 696 and 750 nm was measured using a Cecil CE2011 spectrophotometer, and the concentration of the total chlorophyll *a* (chl *a*) in the sample (μg $cm^{-2}$) was calculated using the quadrichroic equation of Ritchie (2008).

The relative proportions of cyanobacteria and diatoms within the periphyton on each slide ($n = 12$ per station) was determined through quantification of their epifluorescence using confocal laser scanning microscopy (CLSM). Epifluorescence microscopy can quantify the coverage of individual components of the periphyton and is more useful than light microscopy, which only allows a limited number of cyanobacterial types to be clearly distinguished (Nagarkar and Williams 1997). In addition, diatom cells were difficult to count directly with light microscopy (thick films on some slides prevented adequate passage of light) so areal coverage of their epifluorescence was used as proxy for abundance. Slides were viewed using a Radiance 2000 CLSM (Bio-Rad, UK), excitation 488 nm; emission 570–590/70, 650 nm DCLPXR and 620–660 nm. Thirty images at a fixed, low-power magnification (×10) were taken at random locations across each slide, and the average percentage cover of cyanobacteria and diatom fluorescence was digitally quantified using Image J software (v 1.43, National Institutes of Health, Bethesda, MD, USA).

The composition of the attached diatom assemblage from five replicate slides at each station was analysed by SEM. Slides were cut into ~1 $cm^2$ pieces, air-dried (Hill and Hawkins 1990) and coated with gold prior to observation with a JEOL JSM 5600 LV SEM. Each slide was examined at fixed magnification (×500), and the abundance of different diatom genera (identified according to Round et al. 1990) was recorded from counts in ten randomly positioned photographs on the colonised areas of the slide. Diatoms that could not be accurately identified were assigned to numbered groups (i.e. unidentified pennate 1, 2, unidentified naviculoid 1).

### Statistical analysis

Differences in periphyton assemblages among stations were tested using one-way ANOVA (percentage data arc sin transformed). A paired *t* test was used to compare the differences in light intensities between S1 and S3. Data that failed tests for normality (Shapiro–Wilk) and equal variances (Levene Median test) were analysed by Kruskal–Wallis one-way analysis of variance on ranks. Pairwise multiple comparisons were performed using Holm-Sidak method or SNK. These statistical analyses were performed using SigmaPlot 11.0.

The abundances of diatom genera were used to calculate Shannon diversity (Shannon and Weaver 1949) and Simpsons index of dominance (Simpson 1949) for each slide. The similarity of community assemblages across the different slides (total $n = 15$) was examined by hierarchical cluster analysis using IBM SPSS Statistics 18. Only genera representing over 1% abundance were included in this analysis including any of the numbered unidentified diatoms groups. Assemblages were clustered using a dissimilarity coefficient (squared Euclidian distance) and Ward's method (minimum variance clustering).

## Results

Between September 2009 and October 2010, mean surface seawater pH decreased with increasing proximity to $CO_2$ vents (S1 = 8.18, S2 = 8.05, S3 = 7.49, $n = 18$) and was

significantly different between all stations (Kruskal–Wallis test, $H_2 = 34.499$, $P < 0.001$). The pH at S1 fell within the normal range of coastal waters (IQ: 8.13–8.22) whilst stations exposed to high $CO_2$ had a greater range in pH that increased with proximity to the main venting area (S2 IQ: 8.00–8.19, S3 IQ: 7.36–7.89, $n = 18$). Table 1 shows the carbonate chemistry profile of each sampling station. Temperature (range 18.6–27.7°C, April–October), salinity (38) and TA (range 2.6–2.7 mmol $kg^{-1}$) remained relatively constant among stations. The highest median values for $pCO_2$ and DIC were found at S3 (1611 µatm and 2.7 mmol $kg^{-1}$, respectively), which had the lowest calcite and aragonite saturation levels (2.28 and 1.49 Ω, respectively). Periods of calcium carbonate under-saturation occurred during the lowest range of pH at S3 (Ω calcite 0.54 and Ω aragonite 0.35). We found no significant difference in midday (noon–13:00) light intensities between S1 (mean lux = 36,935 ± 3,641, $n = 13$) and S3 (mean lux = 38,895 ± 4,234, $n = 13$) ($t_{24} = -0.351$, $P = 0.729$). During periphyton experiments in September–October 2010, the pH at S1, S2 and S3 was found to fall within the established range of the long-term data (8.26, 8.01 and 7.36, respectively), and temperature (~23–24°C), salinity (38) and TA (2.6–2.7 mmol $kg^{-1}$) remained constant between stations.

Mean chl *a* concentrations were significantly different between stations (ANOVA, $F_{(2,33)} = 69.682$, $P < 0.001$), and the highest values were measured on slides from S2 (0.99 ± 0.05 µg $cm^{-2}$), which were almost fivefold higher than those at S1 (0.19 ± 0.03 µg $cm^{-2}$) (Fig. 2). Pairwise comparisons (SNK) revealed that slides from S2 and S3 had significantly greater chl *a* concentrations than S1, but no significant difference could be detected between S2 and S3.

A significant difference in diatom abundance (mean % cover on slides) was detected between stations (ANOVA, $F_{(2,33)} = 610.212$, $P < 0.001$); greater values were recorded with increasing $CO_2$ levels (Holm-Sidak pairwise comparisons, S3 > S2 > S1, $P < 0.001$) (Fig. 3). The highest abundances were found on slides at S3 (60 ± 1.11%), a sevenfold increase from S1 (8.5 ± 0.60%). There was no significant difference in the percentage cover of cyanobacteria in the periphtyic communities between stations (ANOVA, $F_{(2,33)} = 3.041$, $P = 0.061$) that remained relatively low (<2%) across the gradient (Fig. 3).

The mean diversity (H′) of periphytic diatom communities was significantly different among stations (Kruskal–Wallis test, $H_2 = 7.969$, $P = 0.019$). S3 had a significantly lower diversity (1.59 ± 0.14) than the other stations whilst the diversity at S1 and S2 (2.3 ± 0.11, 2.2 ± 0.28 respectively) did not differ significantly (SNK pairwise comparisons, S2 vs. S3 $P < 0.05$, S1 vs. S3 $P < 0.05$, S2 vs. S1 $P > 0.05$) (Fig. 4). The dominance index also varied significantly between stations (ANOVA, $F_{(2,14)} = 5.147$, $P = 0.024$) with significantly higher values at S3 than S1 and S2 (SNK pairwise comparisons, 0.28 ± 0.03, S3 vs. S2 $P < 0.05$, S3 vs. S1 $P < 0.05$). Mean values for S1 and S2 (0.15 ± 0.02 and 0.18 ± 0.08 respectively), however, did not differ significantly (SNK pairwise comparisons, S2 vs. S1 $P < 0.05$).

Cluster analysis of relative abundances of periphytic diatom genera for each slide yielded four separate groups (Fig. 5). The slides from S1 formed widely separated, distinct groups whilst slides from S2 and S3 were more closely linked, indicating greater similarities in assemblage compositions. Figures 6 and 7 highlight the marked differences in community composition between the $CO_2$-enriched stations (S2 and S3) and the reference station (S1). A dramatic shift in community composition occurred as the relative abundance of *Toxarium* and *Grammatophora* increased from 2.1 and 0.5%, respectively, in the S1 assemblages to 41 and 24% in S3. Figure 8 displays mean cell counts of the most numerous taxa from the SEM images but cannot be accurately scaled-up for whole slide totals as there was a higher incidence of uncolonised spaces at S1. It shows that the changes in carbonate chemistry between S1 and S2 correlate with the growth of some genera (*viz.*; *Toxarium*, *Grammatophora*, *Bacillaria*, *Navicula*, *Cocconeis*, *Amphora*) whilst reducing the abundance of others (*viz.*; *Cyclophora*, *Neosynedra*, *Rhabdonema*, *Nitzschia*). Some

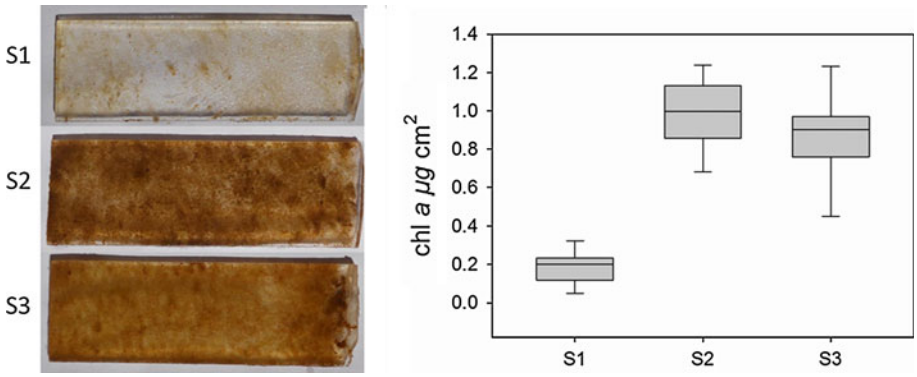


**Fig. 2** Images of biofilms that colonised the slides after 3 weeks at *S1*, *S2* and *S3*. Chart shows chlorophyll *a* concentration (µg $cm^{-2}$) of colonised slides at *S1*, *S2* and *S3* ($n = 12$ per station). Median = *horizontal line*; 25th and 75th percentiles = *vertical boxes* and 10th and 90th percentiles = *whiskers*

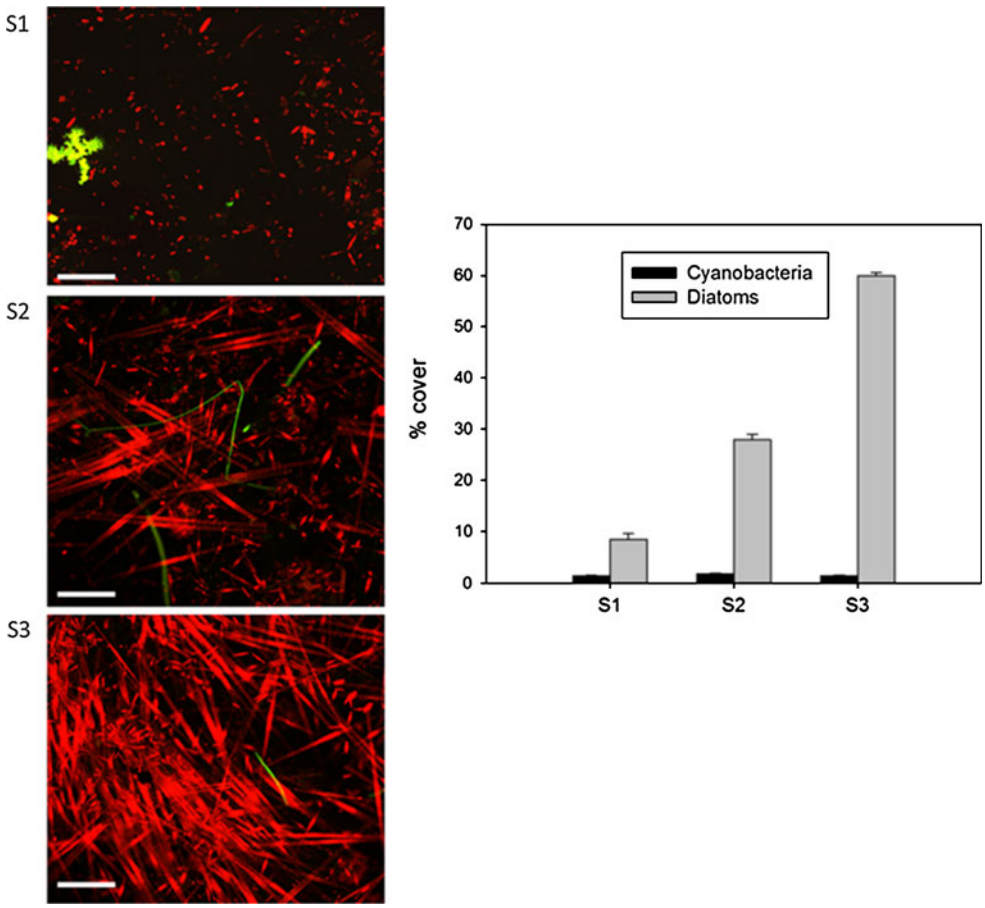


**Fig. 3** Epifluorescence microscope photographs of slides colonised in *S1–S3*. Images show an increase in diatoms (pennates) from *S1* to *S3* whilst cyanobacteria (filamentous and coccoid) cover remains relatively low at each station (*scale bars* = 50 μm) (*Red* = diatoms, *green/yellow* = cyanobacteria). *Graph* shows relative percentage cover ± SE (based on chlorophyll fluorescence) of cyanobacteria and diatoms along the $CO_2$ gradient ($n = 12$ per station) (colour figure online)

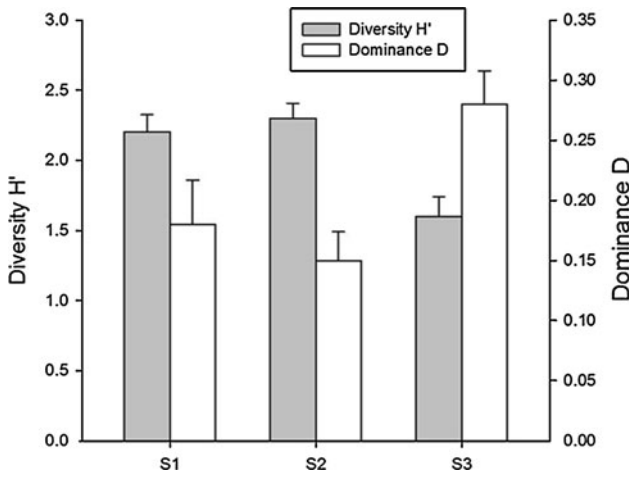


**Fig. 4** Mean ± SE values ($n = 5$ per station) for diversity (Shannon H′) and dominance (Simpsons index D) of periphytic diatom assemblages along the $CO_2$ gradient

genera, on the other hand, did not appear to be affected by the changes in pH/$CO_2$ (*viz.*; *Licmophora*, *Striatella*). The subsequent changes that occurred with further elevations in $CO_2$ at S3 correspond to the increased dominance of *Toxarium* and *Grammatophora* and further reductions in other genera (*viz.*; *Striatella*, *Navicula*, *Amphora*).

## Discussion

There have been relatively few studies exploring the impact of $CO_2$ enrichment on temperate benthic ecosystems, in particular the microphytobenthos. This paper presents the first assessment of the responses of periphytic assemblages to elevated $CO_2$ in situ. In order to advance our understanding of how ocean acidification may impact coastal benthic ecosystems, it is essential to determine what changes will occur at the level of the primary producers. This field study adds to a growing body of evidence from $CO_2$ vent sites that reveal the important biological and ecological changes that are likely to occur with increasing $CO_2$ emissions (Martin et al. 2008; Cigliano et al. 2010; Dias et al. 2010; Rodolfo-Metalpa et al. 2010; Porzio et al. 2011; Lombardi et al. 2010).

The large pH variability found within the Vulcano vent zone has also been observed at other vent sites (Hall-Spencer et al. 2008; Kerrison et al. 2011) and is likely the result of changes in wave action and currents. This can be considered a drawback to in situ studies because accurate dose–response relationships become difficult to determine and most surface waters will not be characterised by such rapid variability as the oceans acidify (Riebesell 2008).

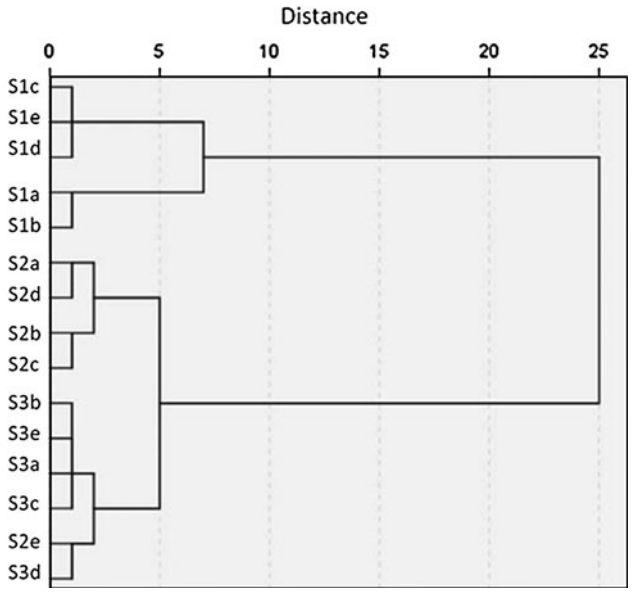


**Fig. 5** Cluster analysis of the similarity of periphytic diatom assemblage composition based on Ward's method with squared Euclidian distance for all the slides sampled along the $CO_2$ gradient ($n = 5$ per station). Analysis consists of all genera present over 1%, including any of the unidentified groups

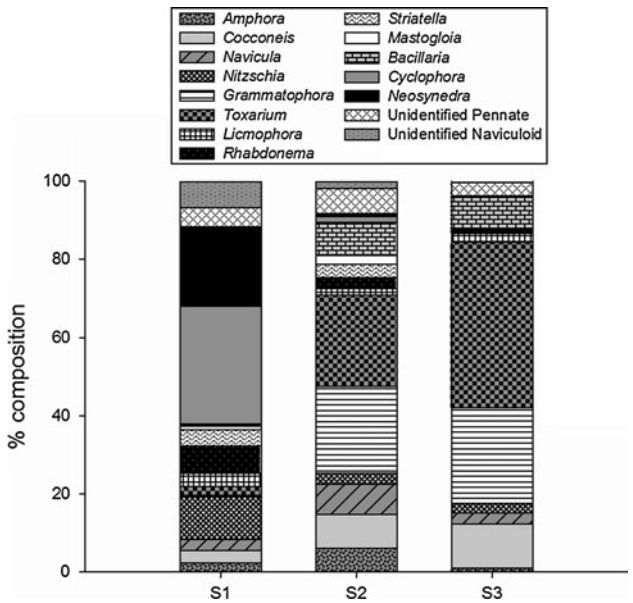


**Fig. 6** Relative composition of the periphytic diatom assemblages along the $CO_2$ gradient, including all genera present over 1% and with all unidentified diatoms grouped as unidentified pennate or naviculoid

Despite this, mean pH differs significantly between sampling stations so the relative changes on spatial scales yield important information about the effects of $CO_2$ enrichment on benthic communities. Potential confounding variables such as light, temperature, total alkalinity, and salinity remained relatively constant between stations. Whilst nutrient levels have not been measured on this occasion, the relatively small size of the study area (length ~300 m) and lack of river outflows in the bay imply that they also should not have been a significant source of variation in the microalgal assemblages we sampled. The biomass of phototrophic biofilm biomass is known to be regulated by grazing gastropods (Hill and Hawkins 1991; Mak and Williams 1999; Stafford and Davies 2005), populations of which have been found to decrease at low pH at other $CO_2$ vent systems (Hall-Spencer et al. 2008). The design of the experimental slides in this study, however, eliminates this as a potential confounding factor as the floats to which they were attached were suspended above the benthos (via thin nylon wire), out of the influence of macroinvertebrate grazing.

By using chl *a* as an index of the photosynthetic standing crop (Underwood 1984), periphyton biomass was found to increase substantially (fivefold) at the $CO_2$-enriched stations. This indicates that elevations in $CO_2$ stimulate primary productivity in these benthic assemblages. Diatom epifluorescence increased significantly across all stations with increasing $CO_2$ concentrations, with a sevenfold difference between S1 and S3 station. This appears to be attributed to the increase in abundance of large pennate types. A potential methodological shortcoming, however, must be addressed here; epifluorescence measurements, despite providing useful percentage surface cover data, may not yield accurate information concerning the vertical density of the biofilm. The self-shading effects that can occur within thicker biofilms (McNamara and Hill 2000) may be source of variance in chl *a* measurements, and the reduced light intensity has the potential to induce higher concentrations without the corresponding increase in biomass. In addition, as pH may vary in different layers of a biofilm, future studies should incorporate the use of pH microelectrodes to measure the potential gradient through the microalgal layers.

Laboratory studies have revealed that pH can affect the adhesion of diatoms to hard surfaces; however, attachment is reportedly favoured in more alkaline conditions greater than pH 7 (Sekar et al. 2004). The greater abundance of diatoms at S2 and S3 is therefore not likely to be the result of pH-induced preferential attachment. Our data contradict the results of some previous experiments on benthic and planktonic diatom species that suggest their responses to ocean pH changes will be negative (Torstensson et al. 2011) or small (Tortell et al. 1997, 2000; Burkhardt et al. 1999). Our data support the notion that some diatoms will benefit from increasing $CO_2$ through a reduction in the energetic costs of their CCMs, optimising resource allocation (Beardall and Giordano 2002; Rost et al. 2008; Trimborn et al. 2009; Hopkinson et al. 2011). These results are also in agreement with field incubations that showed $CO_2$ stimulation of phytoplanktonic communities (Tortell

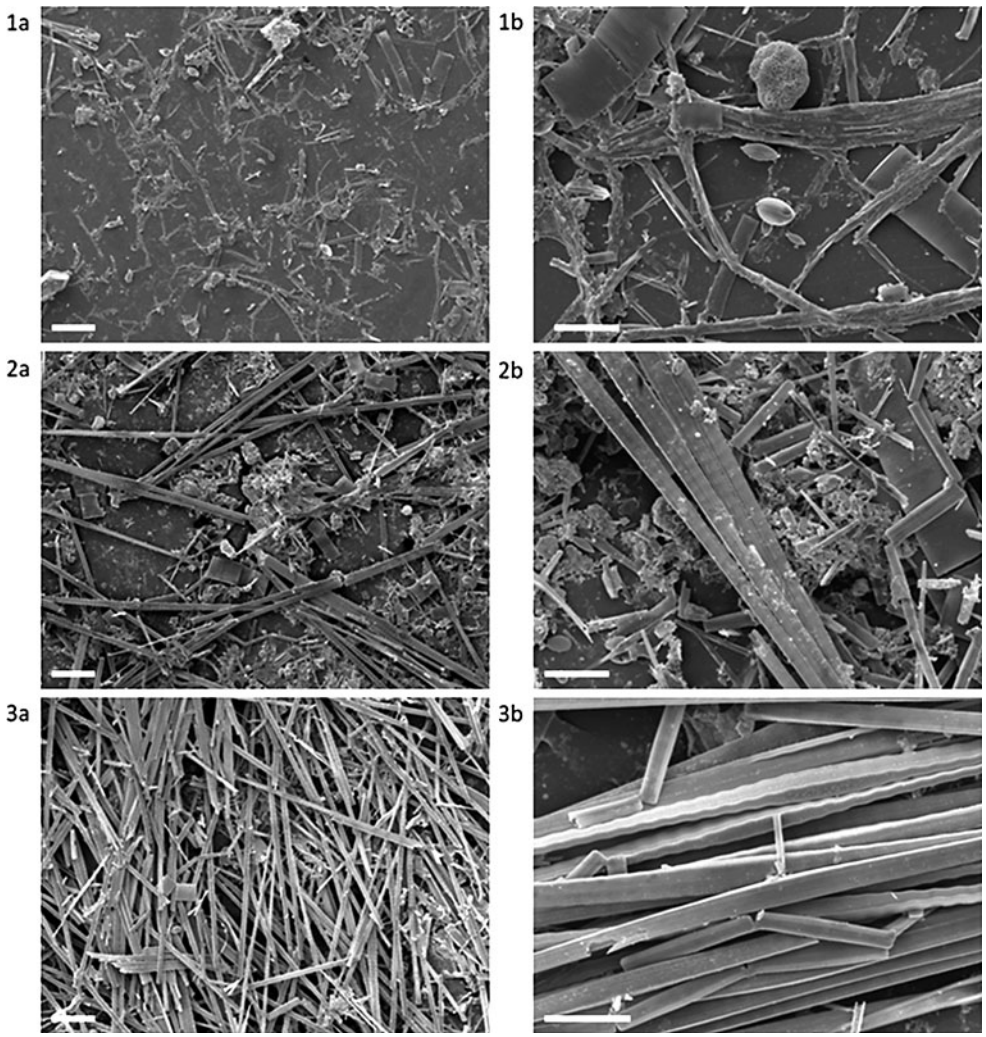


**Fig. 7** SEM images of slides colonised by periphytic diatoms at the three stations; S1 (**1a**, **1b**), S2 (**2a**, **2b**) and S3 (**3a**, **3b**). Diatom colonisation increases with rising $pCO_2$ concentrations along the gradient (**1a**, **2a**, **3a**). Changes in the community composition also occur with colonies of *Grammatophora* spp. and *Toxarium undulatum* dominating in S2 and S3 (**2b**, **3b**) (*Scale bars*: **1a**, **2a**, **2b** = 100 µm, **1b**, **2b**, **3b** = 50 µm)

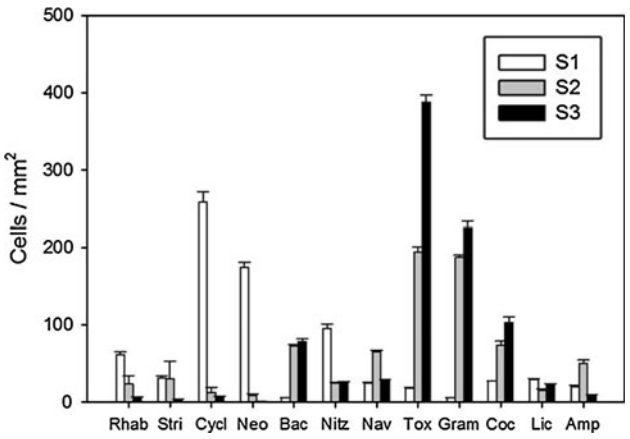


**Fig. 8** Changes in the mean diatom abundances between *S1*, *S2* and *S3*; *error bars* represent standard error ($n = 5$ per station)

et al. 2002, 2008) and support the findings from a recent biofilm laboratory experiment which observed a promotion of diatom dominated biofilms in high $CO_2$ treatments (Witt et al. 2011). Our epifluorescence findings indicate that periphytic diatoms, rather than cyanobacteria, were responsible for the differences measured in chl *a* concentrations and strongly indicate that $CO_2$ enrichment may stimulate the growth of temperate benthic diatoms.

Experimental studies of bloom-forming cyanobacteria usually show a positive response to $CO_2$ enrichment (Barcelos e Ramos et al. 2007; Hutchins et al. 2007; Levitan et al. 2007; Fu et al. 2008a; Kranz et al. 2009). We did not record this response as cyanobacteria coverage on the slides remained low (<2%) at each station. This is consistent with reports of highly efficient CCMs in cyanobacteria (Badger and Price 2002). A similar neutral

response to elevated $CO_2$ has also been observed in endolithic cyanobacteria (Tribollet et al. 2006). Primary production saturation under ambient $pCO_2$, attributed to CCM activity or oligotrophic conditions, limiting production was thought to underlie this response and may also apply to the Mediterranean cyanobacteria assemblages of the present study. It can be assumed that the reduction in pH at the $CO_2$-enriched stations does not limit cell attachment as several laboratory studies have shown that ~pH 7 creates favourable conditions for cyanobacteria adhesion (Stanley 1983; Vanhaecke et al. 1990; Matsumoto et al. 2000).

The majority of the diatom genera that colonised the slides were the attached forms, araphid and monoraphid groups (e.g. *Cocconeis*, *Amphora*, *Toxarium*, *Grammatophora, Cyclophora*); however, free-living motile forms, pennate biraphids, were also present within the biofilms (e.g. *Nitzschia*, *Navicula*). SEM analysis of the composition of diatom populations revealed two contrasting assemblages between S1 and the $CO_2$-enriched stations (S2 & S3). The changes in carbonate chemistry caused some populations to increase whilst others decreased. Whilst this indicates a shift in competitive outcomes and assemblage structure, it only applies for the genera identified; species-specific changes in these populations need to be investigated further. Diversity was significantly reduced at S3. As $CO_2$ concentrations increased, large and chain-forming pennates (*Toxarium* and *Grammatophora*) began to dominate the periphyton assemblages. Similar results have also been found in Southern Ocean phytoplankton assemblages where elevated $CO_2$ conditions promoted a shift to larger chain-forming *Chaetoceros* spp. (Tortell et al. 2008). The authors attributed this to differences in surface area to volume ratios between genera that would influence competitive outcomes under increasing $CO_2$ concentrations. Diffusion limitation becomes increasingly important as cell size increases (Chisholm 1992; Kiørboe 1993), under present $CO_2$ conditions carbon uptake in smaller species is maximised by high surface area to volume ratio whilst larger species (which have smaller surface area to volume ratios and longer diffusion paths) are at a competitive disadvantage. Elevations in $CO_2$ levels may therefore change the competitive abilities among different size classes of diatoms.

Our findings indicate that periphytic diatoms exhibit a non-uniform response to $CO_2$ enrichment; this is most likely due to taxon-specific differences in their sensitivity to $CO_2$ concentrations and presumably due to their kinetics of carbon use. $CO_2$-induced community shifts have also been observed in many other photoautotrophic assemblages (Tortell et al. 2002, 2008; Fu et al. 2007; Russell et al. 2009; Trimborn et al. 2009; Connell and Russell 2010; Porzio et al. 2011) adding to evidence that increasing $CO_2$ emissions are likely to lead to structural and functional changes in a wide variety of marine and coastal systems. The establishment of microalgal assemblages on artificial substrata is a complex process. Differences in seasonal recolonisation and succession events (Anderson 1995; Munda 2005) and artificial substratum type (Tuchman and Blinn 1979; Edyvean et al. 1985; Sekar et al. 2004) play an important part in determining the final periphyton assemblage. The diatom composition data presented in this study therefore provides an indication of elevated $CO_2$ effects on biofilm assemblages rather than a precise analogue for the future effects of $CO_2$ enrichment. Similar studies should be repeated seasonally, using a variety of substrata and based across different $CO_2$ gradients to better constrain the large-scale changes that we can expect in response to increases in $CO_2$ emissions.

The periphyton assemblages analysed here showed significant changes resulting from $CO_2$ enrichment. We confirm that increasing $CO_2$ can stimulate the growth of some benthic diatom species, particularly large, chain-forming genera, promoting the primary productivity in shallow water coastal habitats. This is likely to have wide-ranging consequences from local-scale influences on the structure of overlying benthic communities to effects on food web structure and larger-scale biogeochemical cycles.

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