

Research



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Ocean acidification and rising temperatures may increase biofilm primary productivity but decrease grazer consumption

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Climate change may cause ecosystems to become trophically restructured as a result of primary producers and consumers responding differently to increasing CO₂ and temperature. This study used an integrative approach using a controlled microcosm experiment to investigate the combined effects of CO₂ and temperature on key components of the intertidal system in the UK, biofilms and their consumers (*Littorina littorea*). In addition, to identify whether pre-exposure to experimental conditions can alter experimental outcomes we explicitly tested for differential effects on *L. littorea* pre-exposed to experimental conditions for two weeks and five months. In contrast to predictions based on metabolic theory, the combination of elevated temperature and CO₂ over a five-week period caused a decrease in the amount of primary productivity consumed by grazers, while the abundance of biofilms increased. However, long-term pre-exposure to experimental conditions (five months) altered this effect, with grazing rates in these animals being greater than in animals exposed only for two weeks. We suggest that the structure of future ecosystems may not be predictable using short-term laboratory experiments alone owing to potentially confounding effects of exposure time and effects of being held in an artificial environment over prolonged time periods. A combination of laboratory (physiology responses) and large, long-term experiments (ecosystem responses) may therefore be necessary to adequately predict the complex and interactive effects of climate change as organisms may acclimate to conditions over the longer term.

1. Introduction

The structure and function of coastal marine ecosystems reflect the interaction between a range of anthropogenic and natural drivers that emanate from marine, terrestrial and atmospheric origins. Although there is broad recognition that changes to the physical environment can drive the biology of individual species (e.g. see review by [1]), there is less recognition of how multiple stressors may alter biological interactions [2]. For example, increasing temperatures are predicted to increase consumption of plant biomass by herbivores [3] but multiple stressors often alter this relationship [4]. Therefore, while the physico-chemical environment can be highly variable over short time-scales (e.g. tidal cycles and sudden weather events [1]), long-term alteration in conditions, such as with steadily increasing temperatures and ocean acidification, may exert strong influence on the interactions between coastal species and, subsequently, the structure and function of communities and ecosystems [5,6].

Global sea surface temperature (SST) has warmed by 0.4–0.8°C during the last century, although regional differences are evident [7,8]. The Northeast Atlantic is warming faster than the global average, by up to 1°C between the mid-1980s and 2000s with further warming of between 1.5°C and 4°C by 2100 predicted [9–11]. It is no surprise therefore that coastal marine species have shown some of the fastest responses to climate change in any system, with species-specific responses to thermal stress causing poleward shifts in biogeographic distributions towards cooler environments, as well as changes in phenology and regime shifts. Such alterations in the spatial and temporal distribution of marine organisms ultimately drive subsequent changes in ecosystem structure and functioning [1,12–15].

In addition to rising temperatures, another impact of rising anthropogenic CO₂ concentrations is ocean acidification. This phenomenon is caused by the rapid uptake of atmospheric CO₂ into the surface oceans, where the CO₂ causes a series of changes in seawater carbonate chemistry. These changes include a lowering of pH and carbonate saturation state as well as an increase in dissolved CO₂ and bicarbonate ions. These changes have already proved to be significant with a lowering of the average surface ocean pH by 0.1 unit observed since the onset of the Industrial Revolution [16]: a shift in pH that equates to the oceans having become 30% more acidic. Model forecasts suggest that average pH may decrease by as much as 0.5 pH units in total by 2100 [17]. Coastal benthic invertebrates and algae have shown a range of responses to elevated CO₂ in a number of short-term experiments and natural observational studies. These responses include impacts on calcification rates [18,19], immune function [20], reproduction and carryover effects in larval and juvenile stages of invertebrates [21], enhanced productivity in phytobenthos [22–25] but reduced calcification and growth in calcareous algae [26–28].

It has been suggested that species inhabiting these coastal ecosystems may be evolutionarily more robust with respect to changes in environmental pH [29]. However, extensive literature on the response of intertidal species to observed warming suggests that intertidal species may already exist close to their tolerance limits throughout large sections of their biogeographic distributions, and further warming may cause rapid range shifts [30]. Therefore, small changes in other environmental stressors, such as ocean acidification, may drive significant physiological and ecological responses [14,29,31,32]. The same may also be the case for tolerance to external pH conditions for coastal species, although intertidal species show varied and sometimes positive responses to reduced pH [33,34].

Evidence for the impacts of changes to single environmental conditions on individual species is rapidly growing [14,34], whereas data on the synergistic impacts of multiple stressors and how species interactions may moderate these effects across trophic levels are still lacking ([35,36], but see also [37]). It is acknowledged that these knowledge gaps are limiting the use of current ecosystem models that are otherwise well developed with respect to coastal biogeochemistry. In particular, current models lack sufficient biological detail at higher resolution than functional group level and do not incorporate trophic interactions other than via nutrient fluxes and pathways [38]. One major pathway to be addressed is the interaction between changing environmental temperature and concentration of CO₂ (and therefore reduced pH), the resultant composition and primary production within microbial communities and the implications

of altered food resources for grazing species. Simultaneously, changes in energy partitioning and energy budgets of invertebrates caused by chronic exposure to altered environmental conditions may affect rates of food consumption [39], and also the resultant ‘farming’ feedbacks of grazers on biofilms.

Benthic biofilms are composed of a community of photoautotrophic organisms, predominantly diatoms and cyanobacteria, together with heterotrophic microbes, bound together by an extracellular polymeric substance (EPS). Their formation starts with the development of a polysaccharide and protein conditioning layer, followed by bacterial adhesion, growth and expansion to form either a single or multispecies community [40]. Biofilm formation can occur on many natural and artificial surfaces and can form a major source of primary productivity in benthic systems [41,42]. Biofilms rapidly reorganize in response to environmental disturbance and are likely to be both indicators and drivers of community level change in response to ocean acidification. Photoautotrophic biofilms contribute significantly to intertidal primary productivity [43] and are the main food resource for many benthic grazers [44]. Biofilms also influence the structure and functioning of marine benthic communities by promoting or inhibiting the settlement of algal and invertebrate propagules [45–47].

This paper uses an integrative approach to examine the direct influence of elevated temperature and reduced pH on the biomass and composition of benthic biofilm, and the relative primary impacts of these drivers versus secondary effects via altered biofilm food supply on the grazing activity of *Littorina littorea* (Linnaeus 1758), a key grazing species of periwinkle present on rocky intertidal habitat across the northeast Atlantic. In addition, we test how different acclimation times (weeks versus months) before commencement of experiments may alter the experimental outcomes. These data provide new information on the relative roles of direct and indirect perturbations of warming and ocean acidification on primary productivity, trophic interactions and grazing activity in rocky intertidal systems.

2. Material and methods

(a) Experimental design and set-up

Adult *L. littorea* and biofilms were maintained under experimental conditions in 9 l (l × w × h: 30 × 20 × 15 cm) transparent plastic microcosms with lids. The microcosms were housed in the mesocosm facility located at the Plymouth Marine Laboratory, Plymouth, UK. The study was conducted over five weeks during July and August 2011. Experimental conditions were crossed combinations of temperature (ambient local August SST 14°C versus 18°C) and CO₂ (380 versus 750 versus 1000 ppm), with four replicate microcosms per treatment combination (total of 24 microcosms). Each microcosm was divided into three equal sections by mesh barriers, of which two sections contained a single *L. littorea* each (hereafter termed ‘grazed sections’) and one section which did not contain any *L. littorea* (hereafter termed ‘ungrazed sections’) (see below). The two *L. littorea* in each microcosm had experienced different conditions prior to the experiment: one *L. littorea* had been kept under the experimental conditions for five months (hereafter termed ‘5-month’), and one had been collected from the low intertidal at the same time as the settlement panels and exposed to laboratory conditions for two weeks prior to the start of the experiment (hereafter termed ‘2-week’).

Two weeks prior to the start of the experiment, six 5 × 5 cm PVC settlement panels not containing any biofilm were placed

into each microcosm (two panels per section). In addition, three settlement panels with natural biofilm communities were placed in the 'ungrazed' section of each microcosm, thus allowing a biofilm community to establish on the blank panels under the relevant experimental treatment. The panels with natural biofilm had previously been inoculated with biofilm in the low intertidal for six weeks from May to June 2011 and were only used to provide a seed for biofilm communities under experimental conditions and are not subsequently discussed here.

Water temperature was maintained to an accuracy of $\pm 0.5^\circ\text{C}$ using water baths surrounding the microcosms, with the bath water being maintained by bar heaters and pumps to circulate the water. CO_2 concentrations were manipulated by bubbling pre-mixed gas at the experimental concentrations at equal flow rates and pressures through all microcosms, which had lids to maintain a stable internal atmosphere. The pre-mixed gas was created following methods described by Findlay *et al.* [48]. Gas concentration was monitored using an infrared gas analyser (Licor LI-820, Nebraska, USA). Salinity, temperature (LF187, WTW, Weilheim, Germany) and pH (826 pH mobile with a glass electrode, Metrohm, Cheshire, UK) were monitored three times a week and total alkalinity (A_T) was measured weekly using standard methods proposed by Dickson & Millero [49] and then analysed by potentiometric titration using an Alkalinity Titrator (Model AS-ALK2, Apollo SciTech, Bogart, USA) and Dickson certified reference materials (Batch 100). Concentrations of bicarbonate (HCO_3^-), carbonate (CO_3^{2-}) and seawater $p\text{CO}_2$ were then calculated from measured A_T , pH, salinity and temperature using the CO2SYS program for Excel [50] with constants from Mehrbach *et al.* [51], as adjusted by Dickson & Millero [49] (table 1). A 4.5 l water change was carried out every other day in each microcosm with fresh seawater at close to experimental temperatures to ensure water quality, while maintaining a constant treatment temperature and pH.

(b) Consumption of biofilm by gastropods

The amount of biofilm consumed by grazers was quantified by subtracting the final percentage cover of biofilm on grazed plates from percentage cover of ungrazed plates in the same treatment (i.e. ungrazed – grazed = consumption). As none of the ungrazed plates attained a 100% cover of biofilm, this measure inherently takes growth of the biofilm into account, and so is a time-integrated measure of consumption. Percentage cover was quantified by placing a quadrat containing 25 random dots over a digital photograph of each panel and scoring as either presence or absence of biofilm with each dot equating to 4% cover. All panels were weighed before the start of the experiment. At the end of the experiment all settlement panels were dried in an oven at 60°C for 48 h and then re-weighed, with biofilm weight being end dry weight – plate weight.

(c) Gastropod resting metabolic rates

The resting metabolic rate of experimental gastropods was quantified at the end of the experiment during closed incubations during which changes in the oxygen saturation of seawater were measured using an oxygen meter (781, Strathkelvin Instruments, Glasgow, UK). The oxygen meter was calibrated using a two-point calibration prior to every incubation using aerated distilled water as 100% saturation and de-oxygenated distilled water prepared using sodium sulfate as 0% saturation. Individual snails were placed in small (69 ml) blacked-out glass jars which were sealed while submerged in the experimental microcosms to prevent air bubbles, and put into water baths set to representative treatment water temperature to maintain the experimental thermal regime during incubations. The *L. littorea* were allowed for a 15 min acclimation period in the jars with the lids on loosely before three replicate water samples were

taken with a syringe and run through the oxygen meter to determine the starting saturation. Bottles were sealed and left for 30 min along with a 'blank' bottle to control for any microbial respiration in the seawater. At completion of the incubation, the lid was partially removed and three 5 ml samples were immediately taken using syringes to measure the final oxygen concentration of the water.

Temperature, salinity and atmospheric pressure were also recorded for each incubation chamber (using the equipment detailed above), and Weiss Table [52] correction factors for oxygen saturation were used to calculate change in oxygen concentration per millilitre chamber volume per min. Following incubations, gastropods were euthanized and removed from their shells, and their soft tissue was dried on a paper towel and then weighed on an electronic balance so that final oxygen consumption could be calculated per gram of dry flesh weight ($\mu\text{mol O}_2 \text{ g}^{-1} \text{ dry weight (DW) h}^{-1} \text{ STP}$).

(d) Biofilm communities

(i) Clone library construction and sequence analysis

Biofilm material was scraped from the tiles with a sterile scalpel, placed into a 2 ml microtube and the DNA extracted using the PowerBiofilm DNA Isolation Kit (MO BIO) according to the manufacturer's instructions. Possible changes to the cyanobacterial and micro-algal community was first analysed using PCR–denaturing gradient gel electrophoresis (DGGE) using the PCR primer pair CYA-359F (5'-GGGGA ATYTCCG-CAATGGG-3') (containing a GC-clamp) and CYA-781R (5'-GACTACWGGGGTATCTAATCCW-3') following the methodology of Nubel *et al.* [46]. This primer pair amplifies 16S rRNA gene fragments which are specific for cyanobacteria and micro-algae chloroplast [53]. Each 25 μl PCR mixture contained 1 μl biofilm DNA, 5 μl PCR buffer (Promega), 2 mM MgCl_2 , 0.2 mM dNTPs, 2.5 U of GoTaq Flexi DNA polymerase (Promega) and 1 mM of each primer. PCR products were amplified using the following conditions: 95°C for 5 min, followed by 35 cycles of 95°C for 1 min, 58°C for 1 min and 72°C for 1 min with a final extension time of 5 min at 72°C . PCR products from each biofilm sample were run on polyacrylamide gradient gels with denaturing gradients of 20–60%. Gels were electrophoresed in $1 \times \text{TAE}$ at a constant temperature of 60°C for 16 h at 60 V. DGGE fingerprinting patterns were converted to presence/absence data using Gel Compar II software (Applied Maths, The Netherlands) and the data imported into PRIMER v. 6.1 multivariate analysis software [54] for statistical analysis. The four replicates from each treatment were also pooled and six clone libraries constructed. PCR products were cloned using the pGEM-T Easy Vector System I cloning kit (Promega) according to the manufacturer's instructions. Transformants were selected on Luria-Bertani agar plates containing ampicillin ($50 \mu\text{g ml}^{-1}$), X-gal ($40 \mu\text{g ml}^{-1}$) and 0.1 M isopropyl- β -D-thiogalactopyranoside. White colonies were screened by PCR using the vector primers M13F (5'-GTAAAACGACGGCCAG-3') and M13R (5'-CAGGAAACAGCTATGAC-3') and the resulting PCR products were sequenced using an ABI 3730 XL (LGC Genomics). Thirty two clones were sequenced from each clone library, providing 192 sequences in total. Sequences were processed using the QIIME pipeline (Quantitative Insights into Microbial Ecology v. 1.2) [55]. All 196 sequences were clustered into operational taxonomic units (OTUs) based on 97% sequence similarity using UCLUST. This 97% sequence similarity cut-off was chosen as it is typically considered to be representative of a species. To assign taxonomy to each OTU, a representative sequence from each OTU cluster was chosen, the representative sequences aligned using PyNAST, and taxonomy assigned by comparison with the Greengenes database (version February 2011) [56] which uses cyanodb taxonomy for classification of the Cyanobacteria [57].

Table 1. Seawater measurements from experimental aquaria: measured values of temperature (T), salinity (S), pH (NBS scale), total alkalinity (TA); and calculated values (using CO2SYS) of dissolved inorganic carbon (DIC), seawater $p\text{CO}_2$, saturation states for calcite (Ω_{calcite}) and aragonite ($\Omega_{\text{aragonite}}$), bicarbonate (HCO_3^-) and carbonate concentration (CO_3^{2-}).

target CO_2 (ppm)	380	750	1000	380	750	1000
target T ($^{\circ}\text{C}$)	14	14	14	18	18	18
T ($^{\circ}\text{C}$)	14.36 ± 1.05	14.45 ± 0.98	14.03 ± 0.93	19.09 ± 0.93	18.60 ± 1.24	18.19 ± 0.91
S	34.17 ± 0.33	34.09 ± 0.24	34.04 ± 0.21	34.41 ± 0.60	34.43 ± 0.57	34.52 ± 0.92
pH	8.25 ± 0.041	7.97 ± 0.076	7.79 ± 0.063	8.28 ± 0.064	7.98 ± 0.087	7.86 ± 0.065
TA ($\mu\text{mol kg}^{-1}$)	2440 ± 18	2452 ± 39	2437 ± 29	2451 ± 37	2441 ± 36	2442 ± 34
DIC ($\mu\text{mol kg}^{-1}$)	2168 ± 24	2300 ± 34	2351 ± 35	2115 ± 38	2248 ± 50	2314 ± 45
$p\text{CO}_2$ (μatm)	333 ± 32	663 ± 141	969 ± 82	296 ± 24	602 ± 104	867 ± 141
Ω_{calcite}	4.71 ± 0.26	2.94 ± 0.51	2.05 ± 0.13	5.82 ± 0.37	3.57 ± 0.51	2.64 ± 0.31
$\Omega_{\text{aragonite}}$	3.02 ± 0.17	1.89 ± 0.32	1.31 ± 0.08	3.77 ± 0.24	2.31 ± 0.33	1.71 ± 0.20
HCO_3^- ($\mu\text{mol kg}^{-1}$)	1959 ± 32	2153 ± 41	2228 ± 36	1863 ± 43	2079 ± 63	2175 ± 49
CO_3^{2-} ($\mu\text{mol kg}^{-1}$)	196 ± 11	123 ± 21	86 ± 5	242 ± 15	149 ± 21	110 ± 13

The Greengenes classification was also compared with the NCBI database. The QIIME pipeline and PRIMER v. 6.1 multivariate analyses software [54] were used to calculate alpha diversity for each clone library. Sequences obtained in this study have been deposited in GenBank under the following accession nos. KC895985–KC896007.

(e) Statistical analyses

Changes in the performance (percentage cover of biofilm consumed and metabolic rate) of the two sample groups of grazers (5-month and 2-week) in response to the different experimental treatments were first analysed using three-factor analysis of variances (ANOVAs). Grazer sample group, temperature and CO_2 were treated as fixed and orthogonal factors, with two levels in the factors of grazer sample group (5-month versus 2-week) and temperature (14°C versus 18°C) and three levels in the factor CO_2 (380 versus 750 versus 1000 ppm). Changes in percentage cover for the two settlement panels within each grazer treatment (5-month, 2-week or ungrazed) within each microcosm treatment were averaged and analysed with microcosms as replicates ($n = 4$). Following these analyses, the responses of grazers and biofilm to experimental treatments were analysed separately using two-factor ANOVAs, with the factors of temperature and CO_2 (treated as above). Data were checked for heterogeneity and $\ln(X + 1)$ transformed prior to analysis where necessary. Where significant treatment effects were detected, Student–Newman–Keuls (SNK) *post hoc* comparisons of means were run. All analyses were done with PERMANOVA for PRIMER v. 6.

3. Results

(a) Consumption of biofilm and gastropod metabolism

Overall, grazers that were exposed to altered CO_2 and temperature conditions for 5 months consumed more biofilm than the 2-week exposed grazers, with both CO_2 and temperature altering the consumption rate. The percentage cover of biofilm consumed differed significantly between groups of grazers, with consumption by 2-week grazers being reduced relative to the 5-month grazers (figure 1; $F_{1,36} = 20.76$, $p < 0.001$, electronic supplementary material, table S1).

When both sets of grazers are taken into account, the biggest driver of consumption was CO_2 concentration, with consumption of biofilm being less at 750 and 1000 ppm than that at 380 ppm (figure 1 and electronic supplementary material, table S1). For grazers exposed to experimental conditions for five months, both temperature and CO_2 had an effect on the amount of biofilm grazed but there was no significant interaction between these two factors (figure 1; $F_{2,18} = 1.01$, $p = 0.38$, electronic supplementary material, table S2a). Temperature increased grazing (figure 1; $F_{1,18} = 4.68$, $p = 0.04$, electronic supplementary material, table S2a) on average by 79%, 53% and 25%, at 380, 750 and 1000 ppm, respectively. In contrast, elevated concentrations of CO_2 reduced grazing (figure 1; $F_{1,18} = 5.92$, $p = 0.01$, electronic supplementary material, table S2a) by between 25 and 58%. Even though there appeared to be a similar trend in the effects of temperature and CO_2 on grazing by the 2-week grazers, no effect of temperature or CO_2 was statistically detected (figure 1; all $F_{1,18} < 1.34$, all $p > 0.2$, electronic supplementary material, table S2b). Therefore, the amount of biofilm consumed by *L. littorea* that were exposed to experimental conditions for 5 months was greater than that consumed by the 2-week individuals.

In contrast to grazing rates, resting metabolic rates did not differ between 5-month and 2-week grazers (figure 2; $F_{1,28} = 0.0034$, $p = 0.84$, electronic supplementary material, table S3). Temperature caused an overall increase in the metabolic rate of both groups of grazer (figure 2; $F_{1,28} = 4.25$, $p = 0.04$, electronic supplementary material, table S3), while CO_2 did not affect metabolism (figure 2; $F_{1,28} = 0.19$, $p = 0.82$, electronic supplementary material, table S3).

(b) Biofilm community

Both temperature and CO_2 affected the composition of the biofilms that grew on settlement panels. Elevated temperature but not CO_2 caused an increase in the percentage cover of biofilm present on ungrazed settlement panels (figure 3, electronic supplementary material, table S4). During the course of the experiment, visible differences in the colour of the biofilm communities that formed in different treatments became evident. The biofilms incubated at 14°C and 380

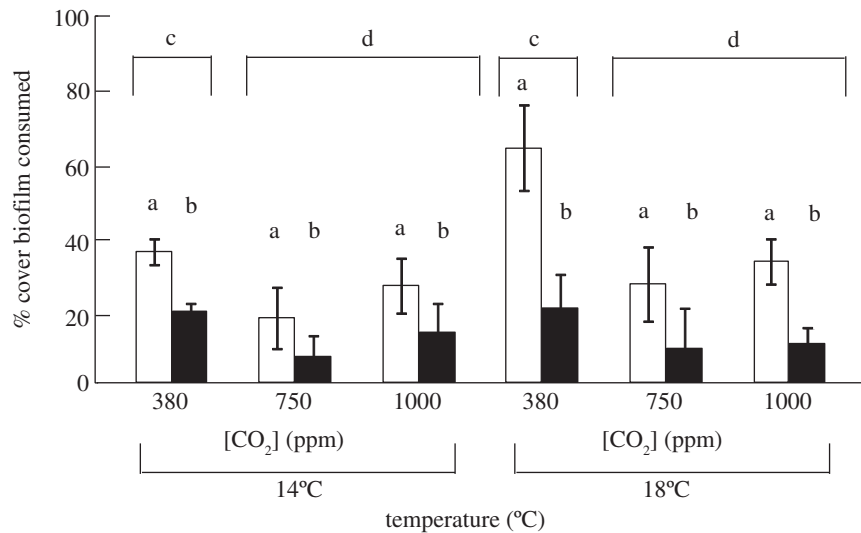


Figure 1. The effect of different combinations of temperature (14°C versus 18°C) and concentrations of CO₂ (380 versus 750 versus 1000 ppm) on the mean (\pm s.e.) percentage cover of biofilm consumed by gastropods pre-exposed to experimental conditions for five months (open bars) and two weeks (filled bars) prior to the experiment. SNK tests are presented on the figure, where different letters show significant differences. The amount of biofilm consumed differed between grazers (5-month > 2-week) and was affected by CO₂ (380 > 750 = 1000 ppm). There were no significant interactions between treatments (all interaction term $p > 0.1$).

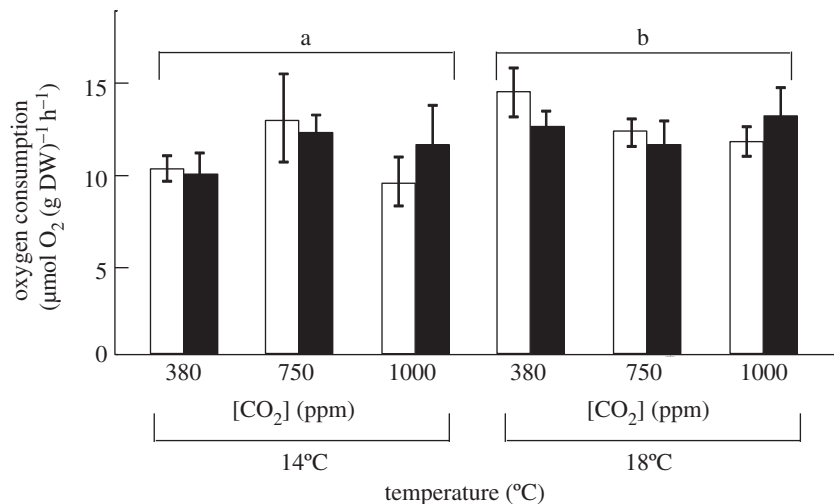


Figure 2. The effect of temperature (14°C versus 18°C) and CO₂ (380 versus 750 versus 1000 ppm) on the mean (\pm s.e.) metabolic rates ($\mu\text{mol O}_2 (\text{g DW})^{-1} \text{h}^{-1}$) of 5-month (open bars) and 2-week (filled bars) gastropods. SNK tests are presented on the figure, where different letters show significant differences.

and 750 ppm were mostly green in colour, whereas the majority of those within the other treatments contained patches of pink colouration (figure 4). This was an indication that the composition of the biofilm cyanobacterial community differed between treatments.

Possible changes to the community composition of the cyanobacterial community was first analysed using DGGE of cyanobacteria and micro-algae chloroplast 16S rRNA gene PCR products (figure 5). Although there was minor variability in DGGE banding pattern between replicates, particularly for the samples incubated at 1000 ppm CO₂, clear distinction in the microbial community between CO₂ ($F = 4.55$; $p = 0.003$) and temperature ($F = 4.68$; $p = 0.02$) was apparent, and so we analysed the community composition from each temperature/CO₂ treatment in detail using clone libraries. Analysis of sequence data obtained from clone libraries of PCR-amplified cyanobacteria and micro-algae chloroplast 16S rRNA gene sequences revealed that at 97% nucleotide identity 23 distinct OTUs were present. Species richness decreased at both elevated temperature

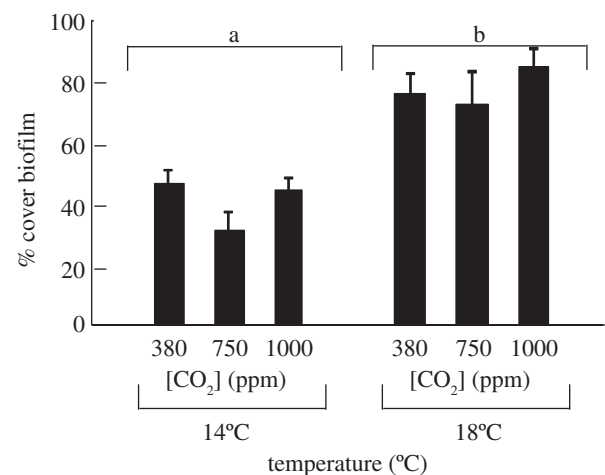


Figure 3. The effect of temperature (14 versus 18°C) and CO₂ (380 versus 750 versus 1000 ppm) on the percentage cover (\pm s.e.) of biofilm on ungrazed settlement panels. SNK tests are presented on the figure, where different letters show significant differences.

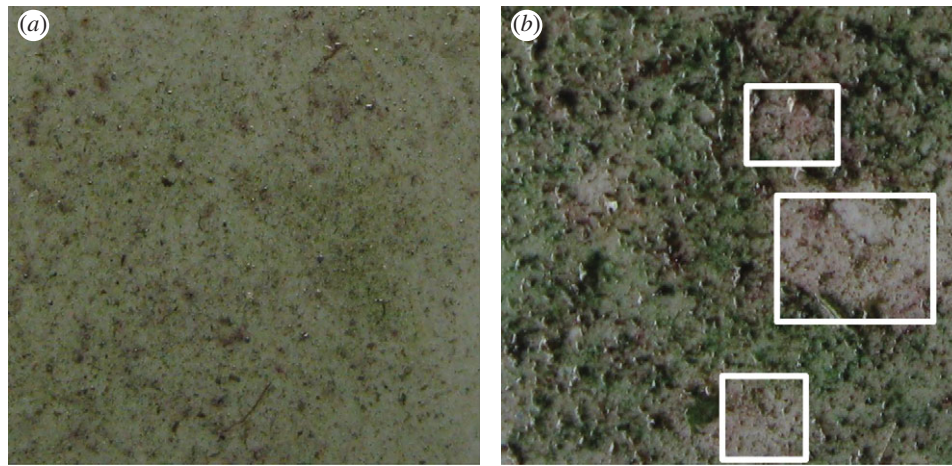


Figure 4. Photographs of the biofilms on settlement panels from different experimental treatments; (a) 14°C, 380 ppm; (b) 18°C, 1000 ppm. Note the relatively large patches of different biofilm in (b) (white boxes). (Online version in colour.)

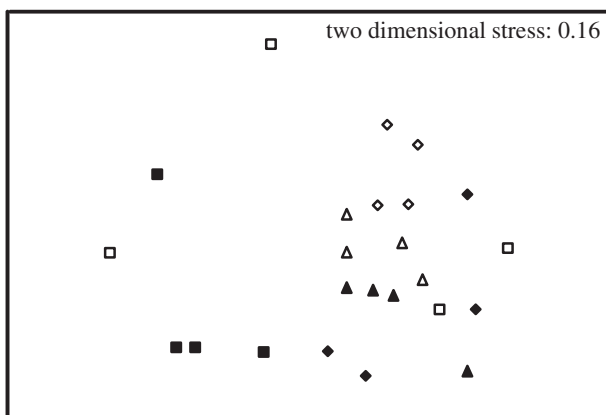


Figure 5. Effect of CO₂ and temperature on cyanobacterial community structure as assessed by PCR-DGGE. The plot shows a non-metric multi-dimensional scaling (MDS) ordination of a Bray–Curtis resemblance matrix, generated from presence/absence of DGGE bands. Open symbols represent samples incubated at 14°C and closed symbols are from 18°C incubations. Triangles, 380 ppm CO₂; diamonds, 750 ppm CO₂; squares, 1000 ppm CO₂.

and CO₂ (Margalef and ChaoI species richness, table 2), indicating a possible shift of the community composition within the biofilms exposed to elevated temperature and CO₂. To compare biofilm community composition within the different treatments, taxonomy was assigned by comparison of sequences to the Greengenes database [56]. The sequences were first grouped at class and order level taxonomies (table 2). This indicated that only three taxonomic orders were evident within the clone libraries: the Pseudanabaenales of the class Synechococcophycideae (60%), the Chroococcales of the class Oscillatoriophycideae (36%) and the eukaryote Stramenopiles (4%). Although the biofilms incubated at 14°C and 380 and 750 ppm were dominated by the order Pseudanabaenales, all other treatments contained equal numbers of Pseudanabaenales and Chroococcales. When examined in detail, this shift in community composition could be explained by the four most dominant OTUs, three Pseudanabaenales, two of which were most closely related to *Leptolyngbya* sp. (OTUs nos 10 and 20), and one which most closely related to *Halomicronema* sp. (OTU no. 4), and one Chroococcales which most closely related to *Dermocarpella* sp. (OTU no. 13) (table 2). The response of these OTUs

were opposite, with OTUs nos 10 and 20 decreasing and OTUs nos 4 and 13 increasing in abundance.

4. Discussion

Abiotic conditions in marine systems will continue to change substantially over the coming century, in particular temperature and CO₂ concentration, leading to warmer, lower pH oceans [8,17]. In an effort to predict the probable effects of these altered conditions on primary producers and primary consumers, and thereby on marine ecosystems, there has been a rapid increase in laboratory-based experimental studies [35]. Yet, for logistical reasons, a minimal acclimation period is used in many manipulative experiments and is assumed to be sufficient to provide results that could be indicative of real impacts in nature. Some recent studies have shown, however, that this assumption is not correct and that longer experimental periods may be necessary [58–60]. We show here that it is probable that responses to shifting abiotic conditions may be altered if a longer acclimation period is used as opposed to a shorter one (but see [61]). While the metabolic responses of *L. littorea* in our experiment did not differ according to time of exposure to treatments before the experiment, the amount of primary productivity that was consumed did differ; therefore, there was no difference from an individual perspective, but there was an ‘ecological difference’, with a greater rate of biofilm consumption observed for grazers exposed to experimental conditions for a shorter period of weeks compared to months.

Ectotherms are in general adapted to, and depend upon maintenance of the characteristic temperature window of their natural environment [1], the location and width of which determines the sensitivity of fauna to environmental temperature extremes, and can alter with lifestage or acclimation to differing environmental conditions [28,62]. Metabolic rate should increase as temperature increases above an optimum within this thermal tolerance range due to oxygen and capacity-limited thermal tolerance until the upper *pejus* (meaning getting worse) limit is reached, characterized by the onset of anaerobic metabolism [37]. At this juncture, the organism moves to a passive state characterized by the onset of a decrease in arterial *p*O₂ due to reduced ventilatory and

Table 2. Diversity and species richness indices estimates and taxonomic classification for biofilm communities that developed on settlement panels exposed to different combinations of temperature (14°C versus 18°C) and concentrations of CO₂ (380 versus 750 versus 1000 ppm). Numbers of operational taxonomic units (OTUs) per clone library and the Chao1 species richness indices were identified at 97% sequence similarity using the QIIME pipeline. Margalef species richness, Shannon diversity and Pielou's evenness were calculated from OTU abundance matrices using the Diverse test in PRIMER v. 6.1 [54]. Also shown are the number of sequences assigned to each taxonomic order within each treatment and the number of sequences within the four most dominant OTUs within the biofilm clone libraries.

temperature (°C)		14°C			18°C		
CO ₂ (ppm)		380	750	1000	380	750	1000
diversity metrics	no. OTUs (97%)	9	10	8	9	7	7
	Margalef species richness	2.31	2.60	2.02	2.31	1.73	1.73
	Chao1 species richness	14.08	14.3	10.87	11.83	7	7
	Shannon diversity	1.68	1.64	1.58	1.67	1.45	1.4
	Pielou evenness	0.77	0.71	0.76	0.76	0.75	0.72
taxonomic order	Chroococcales	6	7	15	14	13	16
	Pseudanabaenales	24	25	16	15	18	15
	Stramenopiles	2	0	1	3	1	1
dominant OTUs	no. 13 (<i>Dermocarpella</i> sp.)	4	4	15	14	13	15
	no. 10 (<i>Leptolyngbya</i> sp.)	15	17	6	7	10	9
	no. 20 (<i>Leptolyngbya</i> sp.)	5	3	4	1	0	0
	no. 4 (<i>Halomicronema</i> sp.)	0	0	0	4	5	4

cardiac performance [63], which can be characterized by alterations in feeding, growth and reproduction. The onset of this transition is the upper *pejus* temperature, T_p , defined as the temperature at which aerobic scope begins to decline [64]. Beyond upper *pejus* limits, oxygen supply capacity becomes limiting, maintenance demand rises, aerobic scope starts to decrease and hypoxaemia develops, resulting in a decline in whole organism functional capacity (e.g. exercise or growth performance). We found that increased metabolic rate in response to a 4°C warming (the 18°C treatment) was evident within individuals held in ambient CO₂ conditions, suggesting that these individuals shifted to be closer to the upper tolerance limits of the performance curve, but did not exceed the upper *pejus* limits for aerobic respiration. Recent investigations on numerous invertebrates have shown that organisms respond to a change in their environmental conditions by shifting their physiological and energetic states. Different responses appear between individuals and species as a result of their flexibility to make these alterations, i.e. their plasticity. A recent short-term (10 days acclimation + 30 days exposure) study specifically on *L. littorea* showed that elevated temperature and CO₂ concentration reduced metabolic rate and caused a shift in energetic status such that there was an increased reliance on anaerobic metabolism [65] with shifts in energetic partitioning contributing to decreases in shell growth (see discussion in [65,66]), which is a common response to elevated CO₂ in this species [67,68].

An alternative explanation to the different grazing activity among experimental treatments could be associated with the shift in community composition of the biofilm. In addition to greater primary productivity, shifts in biofilm community structure have been observed along pH gradients at natural CO₂ seeps [69]. *Littorina littorea* are Taenioglossan grazers that predominantly graze on micro-algae (diatoms and cyanobacteria) within the biofilm [70]. Biofilm

communities dominated by cyanobacteria have high nutritional quality, and this, together with their high turnover rates, can support high level of secondary production [71]. But, it is not known whether certain cyanobacteria are more nutritious than others. In the study of Witt *et al.* [72], biofilms incubated at 305 ppm contained diverse cyanobacterial species whereas only Chroococcales were present at 1140 ppm. This shift in biofilm community suggests that this group of predominantly unicellular cyanobacteria may have a selective advantage over other cyanobacteria at elevated CO₂, possibly owing to a higher growth rate. Not only did biofilm productivity in our experiment increase under elevated CO₂ (as seen in the percentage cover of biofilm on ungrazed plates; figure 2 [57]), but we observed a shift in biofilm community from one dominated more by cyanobacteria of the order Chroococcales to the order Pseudanabaenales at higher CO₂. If there was a corresponding shift in the nutritional value of the biofilms available to grazers (e.g. increases in algae [73] but cf. [74] in diatoms), then they would need to consume less to meet their nutritional needs (e.g. [75], but cf. [76]). Alternatively, the choice of food by gastropods, and the amount consumed, can be affected not only by the quality of the food but also by the feeding history of gastropods and their subsequent nutritional requirements [76,77]. Ultimately, it seems probable that the impact of multiple stressors on these organisms and their consumption of resources will be a combination of shifting energetics (i.e. a direct effect on the animal [78]) together with food availability and quality (i.e. an indirect effect through the food [73]).

In the short-to-medium term (present to 2050), temperature is predicted to exert a greater effect on performance and survival of temperate marine ectotherms than the decline in oceanic pH given the relative rates of change in these drivers compared to physiological performance and thermal tolerance windows [79]. In rocky intertidal systems, it is

probable that biofilm biomass will increase and undergo alterations in species diversity and potentially nutritional value under warming environmental conditions [69]. Temperature-driven increases in metabolic rate of boreal grazers, such as *L. littorea*, are likely to promote increased grazing rates to maintain 'normal' biological functions in the face of greater metabolic demands. Warming environmental conditions will also continue to directly negatively impact reproduction and recruitment of boreal species, with impacts at the population and species distributional range levels [13,80]. In the longer term, CO₂ emissions are predicted to rise in the range of 750–1000 ppm between 2050 and 2100 [17]. Our results add to the emerging body of evidence that CO₂-driven reductions in oceanic pH of up to 1 unit will exacerbate the effects of approximately 4°C warming ([16,23,81] but see also [82]). Metabolic depression driven by a shift of, and reduction in, the physiological performance window of *L. littorea* may reduce fitness of individuals within populations close to southern (higher latitude) range limits. Increased maintenance costs could decrease energy available for somatic and reproductive activity unless adaptation can keep pace with the shifting performance window [83,84]. Therefore, the direct effects of temperature and CO₂ on primary productivity within biofilms and primary consumption by grazers, coupled with altered interactions arising from changing quantity and/or quality of food resources available to grazers may thus have important implications for lower trophic levels and cascade up the food web.

Top-down control of primary productivity can be important in structuring marine ecosystems. The selective removal of filamentous micro- and macroalgae spores by benthic grazers provides habitat space that facilitates the settlement of habitat forming species (coral reefs: [85,86], temperate reefs: [87–89]) essential for the maintenance of a diverse benthic ecosystem. Thus, estimates of the amount of food consumed by grazers under experimentally manipulated environmental conditions can improve quantitative predictions of trophic interactions and increase understanding of the relative roles of species and processes in the maintenance of healthy, functioning ecosystems. In our experiment, grazers that were pre-exposed to the laboratory conditions only for two weeks consumed approximately 50% less cover of biofilm than grazers that had been pre-exposed to experimental conditions for five months. As the results from the control conditions also differed between grazers, this suggests that the individuals with short pre-exposure period were still acclimating to altered conditions within the experimental system and may have displayed an acute response to altered levels of temperature and pH. In contrast, individuals exposed to altered temperature and pH water conditions for prolonged periods showed a different response, likely to reflect chronic responses and, importantly, reflect different physiological mechanisms being employed to tackle short versus long-term exposure to deleterious environmental conditions. Indeed, it is being increasingly recognized that the combined effects of elevated temperature and CO₂ may not be of the magnitude suggested by early work involving short-term experiments using unrealistic pH levels, and that longer term experiments are required (fish: [90]; urchins: [58]; corals: [91]). The potential for confounding influences on biofilm composition and grazing rates of *L. littorea* from exposure to a stressor and from being held for long time periods in artificial tank environments cannot be resolved here. Ongoing studies recognize this and

incorporate sampling and measurement of the biotic parameters under study in the source field population at the same time as these are carried out for the mesocosm individuals. Comparisons of the control treatment animals with the source field population animals are able to show whether there has been an impact from individuals being held in artificial conditions during the experiment. It seems, therefore, that although short-term laboratory-based experiments provide value in identifying the potential mechanisms which underpin the response of physiological, behavioural and ecosystem-based processes to altered abiotic conditions, they are likely to overestimate the impact of novel conditions on individuals and underestimate the strength of grazers in structuring their environment as conditions change.

Longer term experiments are likely to be more representative of potential future changes to systems because they will not assess acclimation in individuals *per se*, which is only expressed in the system for the lifespan of the individual, but rather selection or other population level effects [92]. In our experiment, the 5-month gastropods that were used in the experiment were all individuals that had survived the 5-month exposure period and, as such, the different responses between 5-month and 2-week grazers could represent a combination of both acclimation and selection for stronger individuals. While we cannot distinguish between these two options in our experiment, if selection had occurred in the 5-month grazers it could be expected that the 2-week grazers would have greater variation in responses, which we did not see in our data. Experiments in other systems have shown that between five weeks (oysters [93]) and 16 months (sea urchins [58]) may be required for organisms to replenish energy stores and become fully acclimated to elevated CO₂ conditions. Increasingly, it seems that the acute effects of ocean acidification are not expressed in the second generation, possibly meaning that the effects of ocean acidification will be overestimated unless epigenetic expression is taken into account. For example, juvenile mortality may not be elevated under high CO₂, as previously thought, if parents are acclimated to high CO₂ conditions [58,93]. However, there may still be ecosystem-level effects as energetic resources may be reassigned to produce fewer offspring to maintain quality, meaning that population size will decrease [94]. In the case of keystone species such as sea urchins, this will have large ramifications for ecosystem structure and function. For example, long-term exposure to high CO₂ at natural CO₂ seeps may cause multi-generational declines in populations of herbivorous sea urchins, which could allow macroalgae to increase in abundance, though this relationship is not simple and may partly depend on other factors such as the tolerance of different species to high CO₂/low pH or abundance of preferred food [95]. While the use of small-scale laboratory experiments is informative with respect to the physiological responses of individuals to a range of environmental conditions, larger, long-term experiments will provide information on the ecosystem-level responses to these conditions.

5. Summary and conclusions

In summary, grazing rates in animals pre-exposed to experimental treatments for 5 months prior to the experiment were greater, and they were more sensitive to changes in temperature and CO₂, than grazing rates in 2-week animals.

Metabolic rate was affected only by temperature, and to maintain equivalent metabolic rates 5-month grazers needed more food than 2-week animals. Such experimental observations forecast a growing need to estimate energy budgets to identify whether such depressed consumption can be maintained in the longer term or whether animals are consuming metabolic reserves. Regardless, based on our experimental outcomes it appears that the metabolic rate of these grazers would need to be maintained at above present day levels to maintain energy budgets in response to forecast conditions of temperature and CO₂. In the absence of long-term acclimation of animals to the new environmental conditions, ecosystems are likely to become trophically restructured unless the community shift in biofilms increases nutritional quality and quantity of the primary consumers' food source, allowing more energy to be consumed to counter detrimental shifts in

the environment. In future, a combination of laboratory (physiology responses) and large, long-term experiments (ecosystem responses) may therefore be necessary to adequately predict the complex and interactive effects of climate change as organisms may acclimate to conditions over the longer term.

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