8-1-2018

Future warming and acidification result in multiple ecological impacts to a temperate coralline alga

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10.1111/1462-2920.14113
Huggett, M. J., McMahon, K., & Bernasconi, R. (2018). Future warming and acidification result in multiple ecological impacts to a temperate coralline alga. *Environmental microbiology*, 20 (8), p. 2769-2782. Available here

Future warming and acidification result in multiple ecological impacts to a temperate coralline alga

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Running title: Coralline algal microbiomes and climate change

Key words: coralline algae, reef biofilms, larval settlement, TRFLP, 16S tag sequencing, climate change

Originality-Significance Statement

This study examines, for the first time, the impact of predicted future climate change on the coralline alga *Amphiroa anceps* and associated biofilms as well as subsequent larval settlement by the abundant Australian sea urchin *Heliocidaris erythrogramma.* In particular, both ocean warming, and acidification are considered separately as well as in a combined warming and acidification treatment. This is one of the first attempts to investigate the effects of predicted future environmental change on key organisms from temperate reefs as most of the existing data is focussed on tropical or subtropical species.

This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process which may lead to differences between this version and the Version of Record. Please cite this article as an 'Accepted Article', doi: 10.1111/1462-2920.14113

Abstract

Coralline algae are a crucial component of reef systems, stabilising reef substrate, providing habitat and contributing to accretion. Coralline algae and their surface microbial biofilms are also important as settlement cues for marine invertebrates, yet few studies address the impact of future environmental conditions on interactions between coralline algae, reef microbes and settlement by larvae of marine invertebrates. We exposed the temperate coralline algal species *Amphiroa gracilis* to warming and/or acidification scenarios for 21 days. Algae became bleached but photosystem II (PSII) function was not measurably impacted. Settlement by larvae of the sea urchin *Heliocidaris erythrogramma* was reduced and the structure of the prokaryotic community associated with *A. gracilis* was altered. Coralline algae in ambient conditions were dominated by Alphaproteobacteria from the *Rhodobacteraceae* including *Loktonella*; those under warming were dominated by Bacteroidetes and Verrucomicrobia; acidification resulted in less *Loktonella* and more Planctomycetes; and a combination of warming and acidification caused increases in Bacteroidetes, Verrucomicrobia and the Alphaproteobacteria family *Hyphomonadaceae*. These experiments indicate that predicted future environmental change may reduce the ability of some temperate reef coralline algae and associated reef microbes to facilitate settlement of invertebrate larvae as well as having a direct impact to algae via bleaching.

Introduction

During the past one hundred years global levels of atmospheric carbon dioxide have increased rapidly (Fabry *et al.*, 2005; Hartmann *et al.*, 2013) causing subsequent ocean acidification (Sabine, 2004; Doney *et al.*, 2009) and warming (Rhein *et al.*, 2013). Impacts of ocean warming include unprecedented amounts of coral bleaching (Hoegh-Guldberg, 1999; Heron *et al.*, 2016), species retreating pole wards (reviewed by Wernberg *et al.*, 2011), changes to key biological processes (Hoegh-Guldberg and Bruno, 2010) and a reduction in biological habitat complexity (Hoegh-Guldberg and Bruno, 2010; Wernberg, Russell, Moore, *et al.*, 2011). Acidification impacts skeletal formation

and physiology (Fabry *et al.*, 2008), survival, development and growth (Kroeker *et al.*, 2013) and is predicted to result in substantial shifts in near shore species assemblages through direct impacts to calcifying species and indirect effects via species interactions (Wootton *et al.*, 2008). Given that reefs will face the cumulative impacts of these stressors within the next century there is an urgent need for studies addressing the combined effects of warming and acidification on significant marine ecosystem functions.

Coralline algae are important in global carbon cycles (Lee and Carpenter, 2001), abundant in benthic marine systems worldwide (Steneck, 1986) and provide reef structural strength and stability (Aedy, 1986; Davies *et al.*, 2004). Articulate coralline algae can be considered 'autogenic ecosystem engineers' (Jones *et al.*, 1994) that modify the structure of benthic reefs and contribute to higher diversity and abundance of fish and invertebrates (Nelson, 2009). Demonstrated impacts of temperature and acidification to coralline algae include bleaching (Webster *et al.*, 2011), structural weakening (Ragazzola *et al.*, 2012), reduced recruitment (Fabricius *et al.*, 2015), increased vulnerability to herbivory (Johnson and Carpenter, 2012), as well as photosystem damage, necrosis and death (Martin and Gattuso, 2009). Temperate species are predicted to be particularly vulnerable to the impacts of ocean acidification due to the higher saturation of carbon dioxide in cold water (Fabry *et al.*, 2005; Kamenos *et al.*, 2013). Yet much of the effort to date on impacts of climate change to coralline algae has been directed toward tropical and subtropical species (McCoy and Kamenos, 2015), highlighting the necessity for investigations in temperate systems.

Reef microbiomes are pivotal to ecosystem health and play multiple roles in marine systems such as animal nutrition and development (McFall-Ngai *et al.*, 2013), global biogeochemical cycling (MacIntyre *et al.*, 1996), protection from disease (Ritchie, 2006), facilitation of recruitment by invertebrates (Keough and Raimondi, 1995) and algae (Joint *et al.*, 2002), antifouling (Holmström *et al.*, 2002; Dobretsov *et al.*, 2006) and pathogenicity (Egan *et al.*, 2014). Given their short generation

times and proximity to the surrounding environment, microbes respond rapidly to changes in the environment (Ottesen *et al.*, 2013; Yeo *et al.*, 2013). Microbial community structure, measured by species richness or alpha-diversity, can be strongly impacted resulting in a dysbiosis of the assemblage when environmental conditions change (Lidbury *et al.*, 2012; Raulf *et al.*, 2015; Roder *et al.*, 2015). Current data indicate that dysbiosis of host-associated assemblages is species-specific and when it does occur can result in either increased or decreased alpha diversity (Morrow *et al.*, 2015), showcasing the complexity and unpredictability of microbial response to environmental change. Given their essential role in ecosystem function, more data are thus urgently needed to understand how reef biofilms respond to future predicted climate change.

A vital determinant of marine benthic community structure is the ability of larvae to successfully settle and recruit. For many species of marine invertebrates either coralline algae, microbial biofilms or a combination of both have been shown to facilitate this significant process (Keough and Raimondi, 1995; Webster *et al.*, 2004; Huggett *et al.*, 2006). Coralline algae from the genera *Corallina* and *Amphiroa,* and their associated microbial communities, induce high rates of settlement by larvae of the temperate Australian sea urchin *Heliocidaris erythrogramma* (Huggett *et al.*, 2006; Nielsen *et al.*, 2015). Despite the key functional role that coralline algae and their associated microbial communities have been demonstrated to play in larval settlement of marine invertebrates there are few studies (Webster *et al.*, 2011; Webster *et al.*, 2013; Whalan and Webster, 2014) that address the impact of future climate change on this interaction, and less from temperate ecosystems (O'Leary *et al.*, 2017). Given the importance of coralline algae and their associated microbiomes to reef function, and the current lack of data from temperate systems, we aimed to (1) determine the response of the articulated coralline alga *Amphiroa gracilis* to either increased temperature or acidification or a combination of both, (2) measure any changes in associated microbiome structure and (3) determine if larval settlement by *Heliocidaris erythrogramma* on *A. gracilis* is impacted by predicted warming and/or acidification.

Materials and Methods

Experimental setup

Eighty individual *Amphiroa anceps* algae were collected from Mindarie Keys, Western Australia (31°41'8.16"S, 115°41'54.02"E) on November 25th 2013, held in aerated seawater and transported to Edith Cowan University, approximately 30 minutes' drive away. Each individual was fragmented into five pieces, strung up using nylon fishing wire, then all fragments from the one individual were placed into a single 2 L tank and allowed to heal for 1 week at ambient conditions (pH 8.1, temperature 20.5°C, salinity 36 ppt), giving a total of 80 x 2 L tanks. Individuals were fragmented so that they could be easily harvested over time for the different measures described below.

On December 3rd 2013 conditions were altered into four treatment groups at a rate of 0.06°C h^{-1} and 0.01 pH units h^{-1} across a 48 hour period to mimic future warming and/or acidification scenarios. Current conditions were considered 20.5°C and pH 8.1. The future conditions approach the upper threshold for near future climate predictions of local conditions during summer, 24°C and pH 7.6 (2070-2100) (Hartmann *et al.,* 2013). Seawater was collected from the nearby coast and held in a 20,000 L aerated holding tank. Experimental tanks were randomly allocated. There were four 100 L reservoir tanks, each feeding 20 individual algal containers following Russell *et al.* (2009). The four treatments included (1) the control: 20.5°C, pH 8.1, designated ambient 'A', (2) decreased pH: 20.5°C, pH 7.6, designated future (F) pH 'FPH' (3) increased temperature: 24°C, pH 8.1, designated F temperature (T) 'FT' and (4) combined increased temperature and decreased pH: 24°C, 7.6, designated 'FTPH'. Temperature was controlled using aquarium heaters (200W; Aqua One) and pH with a self-regulating CO_2 controller (Aquatronica). Treated seawater entered the bottom of each 2 L treatment tank and left via overflow vents in the top at ~40 L /hr, returning via filtration to the reservoir tank. A minimum of 60 L seawater was replaced every 2-3 days within each reservoir tank. Tank pH, temperature and salinity were monitored daily and total alkalinity (A_T) was measured

weekly with a Metrohm 716 Titrino (method adapted from Williams *et al.*, 2009). pCO_2 bicarbonate, carbonate and aragonite saturation state (Ω) were subsequently calculated from pH and A_T measurements using CO2SYS (Pierrot *et al.*, 2006). Fluorescent lights (Sylvania 36 W) providing ca 40 mmol m^{-2} s^{-1} (water surface) were placed above treatment tanks on a 14:10 h light:dark cycle to mimic natural conditions. The experiment was run for 21 days, with various measurements (larval settlement assays, microbial community analysis, PAM fluorometry, bleaching assessment) done after 7, 14 and 21 days, as described below.

Algal photosystem II (PSII) function

Photosystem II (PSII) function of the alga were measured with Rapid Light Curves (RLC) using a pulse-amplitude modulated fluorometer (Diving-PAM fluorometer - Walz GmbH, Effeltrich, Germany) following Ralph & Gademann (2005). The value of a RLC as opposed to just a measure of Effective Quantum Yield (EQY) of photosystem II, is that it provides insights into photosynthetic function under fluctuating light conditions, which is more representative of a field situation (Ralph and Gademann, 2005). Photosynthetic yield was measured with a fibre optic cable placed directly on the least faded terminal algal segment through nine pre-determined steps of increasing light intensity (0, 4, 12, 25, 45, 65, 100, 140 and 180 µmols PAR m^{-2} s^{-1} on average, 10 seconds each). This range of light intensity was selected as it produced the required response to calculate PSII function. This was replicated on a randomly selected fragment from five of the tanks from each treatment on day 7, 14 and 21 of the experiment. Only five of the twenty tanks from each treatment were assessed due to time constraints of the measuring process. These were selected randomly over the course of the experiment, and no fragment in a tank was measured twice. In addition, 5 individuals were measured on the day of collection. Electron Transport Rates (ETR) were calculated following the standard RLC protocol (Beer *et al.*, 2001). The ETR-Irradiances were fitted to the equation described by Jassby and Platt (1976) to estimate ETR_{max}, (the maximum ETR rate) and the half-saturating irradiance (E_k) using SigmaPlot (version 7). The absorbance factor (AF) was set to 0.84 in the ETR

calculations so ETR_{max} should be considered a relative rate $rETR_{max}$. In addition, EQY of PSII was extracted from the first reading of the RLC.

To assess whether the experimental conditions affected algal photosystem II (PSII) function as measured by $rMax_{rETR}$, E_k and EQY of PSII a 2-way interacting PERMANOVA was used with the factors Time ('7', '14', '21' d) and Treatment ('A', 'FT', 'FPH', 'FTPH') using the software package PERMANOVA. A PERMANOVA was selected as the data could not be transformed to meet the assumptions of an analysis of variance (ANOVA). PERMDISP was used to test the dispersion of the data within treatments, and all variables had equal dispersion. Although 5 replicates per treatment were measured, only 4 were used in the statistical analysis as some data had to be discarded due to measurements errors. Where significant interactions of main affects were detected pairwise tests were performed in PERMANOVA.

Algal bleaching

Bleaching was characterised as visible whitening of the blade tissue, following Marzinelli *et al.* (2015). At the end of the experiment (Day 21) ten individual tanks per treatment type were randomly selected. One fragment per tank (40 in total) was placed against a white background and photographed. Images were imported into Adobe Photoshop, a grid was overlaid so that approximately 100 grid intercept points occurred across the individual alga, and each grid intercept point was recorded as either healthy, faded (partial loss of colour) or bleached (complete loss of colour). Numbers for each category were converted to percent of total cover per individual. To assess whether the experimental conditions effected algal bleaching, at the end of the experiment, 1-way PERMANOVAs were used with the factor Treatment ('A', 'FT', 'FPH', 'FTPH'). PERMANOVA was used as the data could not be transformed to meet the assumptions of an ANOVA. Prior to PERMANOVA analyses data was tested to ensure equal dispersion. The two PERMANOVAs were performed on the variables % faded tissue and % bleached tissue.

Heliocidaris erythrogramma *larval culture and settlement assays*

Heliocidaris erythrogramma is a dominant sea urchin found throughout temperate Australia (Keesing, 2001). For larval rearing, adult urchins were collected from the same site as algae in Mindarie Keys and injected with 0.5M KCl to induce spawning. Fertilisations were done with eggs and sperm from a minimum of two males and two females and larvae were reared in 0.2 µM filtered seawater (FSW) in aerated beakers at 19°C. After approximately 3 days larvae were competent (Williams and Anderson, 1975) and a haphazard selection of larvae was used in assays. On Day 14 and Day 21 of the experiment, a fragment of algae from each tank was harvested and approximately 50 mg (wet weight) of algal tissue was haphazardly selected, removed from the fragments, rinsed gently in FSW and placed in sterile 5 mL petri dishes with 3.5 mL of FSW. To test for spontaneous settlement, ten 0.2 µM filtered seawater controls were added to the experimental design. Five competent *H. erythrogramma* larvae were added to each dish and the number of larvae that had settled and metamorphosed was recorded after 48 hours. Separate larval cultures were reared to accommodate larval settlement assays done at the two time points. To assess whether the experimental conditions affected *H. erythrogramma* larval settlement (20 per treatment) a 1-way ANOVA was used with the factor Treatment ('A', 'FT', 'FPH', 'FTPH') in SigmaPlot version 13.0 (Systat Software Inc). Larval settlement between time points could not be compared given that each batch of larvae has distinctive levels of larval competency.

Microbial community characterisation: T-RFLP and MiSeq Illumina analysis of SSU rRNA genes

At Day 14 and Day 21 a fragment from each tank that had not been handled for settlement assays or PAM fluorometry was gently rinsed with FSW and frozen at -80°C. Genomic DNA was extracted using 24:1 chloroform:isoamylalcohol, bead beating of the sample, precipitation with isopropanol and cleaning with ice cold ethanol (see Petersen *et al.*, 2004 for full method) then used for terminal restriction fragment length polymorphism (TRFLP) and 16S rRNA MiSeq Illumina sequencing. For

TRFLP analysis (Liu *et al.*, 1997), the general bacterial primers 27F-B-FAM (5'- FAM-AGRGTTYGATYMTGGCTCAG-3') and 519R (5'GWATTACCGCGGCKGCTG-3') (Weisburg *et al.*, 1991) were used. PCR included: 12.5 µl Gotaq colourless mastermix, 10 µM each of primer, 12.5 µg BSA, approximately 1-2 ng of mixed environmental genomic DNA and water to 25 µL (94°C for 2 min then 30 cycles of 94°C for 1 min, 55°C for 1 min, and 72°C for 2 min, then 72°C for 10 min). Amplicons were purified and digested with *HaeIII* (Invitrogen, Life Technologies). Restricted amplicons were purified and separated via capillary electrophoresis on an automated ABI 3730 Genetic Analyser (Applied Biosystems, Foster City, CA). PeakScanner software (Applied Biosystems) was used to estimate the size and relative abundance of the resulting terminal restriction fragments. Fragment lengths were rounded to the nearest integer value, aligned, and manually corrected. Peaks were excluded using the variable percentage threshold method (Osborne *et al.*, 2006). Only 10 to 12 samples per treatment successfully amplified.

Five replicates from each treatment from Day 21 of the experiment were sequenced using 28F (5'GAGTTTGATCNTGGCTCAG3') and 519R (5'GTNTTACNGCGGCKGCTG3') primers for the 16S rRNA gene V1-V3 region. These five were randomly selected from the subset of replicates that successfully amplified in the TRFLP analysis. PCR was done with HotStarTaq Plus Master Mix Kit (Qiagen, USA): 94°C for 3 minutes, then 28 cycles of 94°C for 30 seconds, 53°C for 40 seconds and 72°C for 1 minute, then 72°C for 5 minutes. Libraries were sequenced using Illumina 600 cycle MiSeq® v3 Reagent Kits. The resulting data was denoised and processed using the MOTHUR (Schloss *et al.*, 2009) MiSeq standard operating procedure (Kozich *et al.*, 2013). Sequences <450bp and sequences containing ambiguous base calls or homopolymer runs above 8 bp were removed. Sequences were aligned to the SILVA 16S rRNA alignment (Yilmaz *et al.*, 2014), then unique sequences were identified and operational taxonomic units (OTUs) (>97% similarity) were defined. Chimeras were removed with UCHIME (Edgar *et al.*, 2011) and sequences classified as mitochondria, chloroplast or eukaryotic or of unknown origin were removed. In order to present an unbiased dataset all samples

were subsampled to 17,144 sequences. OTUs were taxonomically classified using BLASTn against a curated database derived from SILVA v123, greengenes (gg_13_8_99), RDP II (v14) and NCBI (www.ncbi.nlm.nih.gov; DeSantis *et al.*, 2006; Wang *et al.*, 2007; Quast *et al.*, 2013). The MiSeq dataset was deposited in the NCBI Sequence Read Archive (SRA) database with the accession number (PRJNA385209).

Sorenson (Bray-Curtis) distance was used on normalised percentage abundance TRFLP and MiSeq sequencing data and non-metric multidimensional scaling (NMDS) was used to visualize patterns in microbial community structure among treatments. PERMANOVA was used to examine the differences in microbial assemblages between treatments. Prior to PERMANOVA analyses data was tested to ensure equal dispersion. For both the TRFLP and MiSeq data, a 1-way PERMANOVA, with the factor Treatment ('A', 'FT', 'FPH', 'FTPH') was done at each time point (14 days and 21 days for TRFLP data, 21 days for MiSeq data). SIMPER analysis was used to identify OTUs that were driving patterns of separation between groups. NMDS, PERMANOVA and SIMPER analyses were done using the software packages PRIMER 6 (PRIMER Ltd., Plymouth, UK; Clarke, 1993).

To assess for any differences in alpha-diversity of microbial communities at the end of the experiment, Shannon's H', (TRFLP data, 10 to 12 samples, MiSeq data, 4 or 5 samples), Pielou's eveness and Chao Indices (MiSeq data, 4 or 5 samples) were calculated based on data from Day 21. One way PERMANOVAs with the factor Treatment ('A', 'FT', 'FPH', 'FTPH') were done using the software package PERMANOVA. A PERMANOVA was selected as the data could not be transformed to meet the assumptions of an ANOVA. Prior to PERMANOVA analyses data was tested to ensure equal dispersion.

Phylogenetic analysis

Representative sequences for each OTU and 16S sequence data from comparable studies of coralline algal and reef microbial communities under future climate change (Webster *et al.*, 2011, 2013a 2013b, accession numbers HM177481-HM178920; JQ179496-JQ178383; JX177366-JX177430) were aligned using the online SINA aligner (Pruesse *et al.*, 2012) and imported into ARB (Ludwig *et al.*, 2004). Alignments were checked within ARB and manually corrected where necessary. Phylogenetic trees were calculated using full length 16S rRNA (>1200 bp) sequences for close relatives of target sequences using the maximum likelihood, neighbour-joining and maximum parsimony methods in ARB. OTU sequences (~450bp length) were subsequently added to trees without altering tree topology using the 'quick add mark' tool in ARB. The robustness of the maximum likelihood tree was checked by 1000 bootstrap resamplings of the data using the CIPRES online tool (version 3.3) (Miller, M.A., Pfeiffer, W., and Schwartz *et al.*, 2010).

Results

Tank conditions

The treatment conditions of pH (7.8-7.9 vs. 8.1-8.2) and temperature (19°C vs 23°C) were maintained throughout the 21 day experiment. Conditions within each of the treatments for temperature, pH and salinity remained stable across the 21 days of the experiment (Table 1). However, in the increased temperature treatments, pCO_2 levels and total alkalinity rose between day 7 and day 14, resulting in a subsequent increase in both aragonite and calcite saturation states (Table 1). Both the ambient and the decreased pH treatments had steady values for all measured parameters across the duration of the experiment (Table 1). Given the interaction between temperature and pH resulting in a drift in alkalinity and pCO_2, a two-factor interacting analysis of temperature and pH was not considered appropriate, and each treatment was considered separately, rather than as two levels within the two factors 'temperature' and 'pH'.

Amphiroa gracilaris *photosystem II (PSII) function*

There was no significant interaction between time and treatment for the $rETR_{max}$ values, but both individual factors were significant ($p<0.05$, Supplementary Table 1). At day 21 $rETR_{max}$ values were higher than at day 7 across all treatments (Fig. 1A) and with lower pH (FPH) *A. gracilis* had higher $rETR_{max}$ values, but only under ambient temperature conditions, not under future warming (FTPH) (Fig. 1B). There were no significant interactions between time and treatment for the E_k values of *A. gracilis* over the experiment, nor for the treatment, but time was significant ($p<0.05$, Supplementary Table 1). E_k increased over time, with day 21 readings higher than those at day 7 (Fig. 1C). There was a significant interaction between time and treatment for EQY of PSII ($p<0.05$, Supplementary Table 1, Supplementary Figure 1). After 1 week EQY was higher in the future temperature treatment (FT) relative to current (A) and future temperature and pH conditions (FTPH), but the EQY of FT declined over time. By week 3 it was significantly lower than the future pH treatment (FPH), but not compared to ambient or future temperature and pH conditions (Supplementary Table 1, Supplementary Figure 1).

Bleaching

After 21 days only algae in the ambient control tanks were healthy, all other treatments had significantly high amounts of impacted tissues (either fading, or both fading and bleaching ($p<0.05$, Figs. 1D. and 2, Supplementary Table 1). Reduced pH (FPH) alone did not result in more bleaching compared to the ambient treatments (A), however there was significantly more bleaching in the future temperature treatments (FT) under ambient pH and when combined with future pH (FTPH), although this was borderline (*p=0.042*). The greatest amount of faded tissue was observed in the future pH treatments, 38%, but when combined with future temperate the amount of faded tissue reduced to approximately 30% of their surface area, similar to the future temperature (FT). To minimise disturbance to the algae during the experiment, bleaching levels were not recorded daily via removal from the tanks and imaging of each individual. However, regular observations indicated

that bleaching progressed over the course of the 21 day period and reached maximum levels by the end of the experiment.

Larval settlement

After algae had been at experimental conditions for 14 days, there was a statistically significant effect of treatment for the amount of settlement by larvae of the sea urchin *Heliocidaris erythrogramma* ($p<0.05$, Supplementary Table 1). Larval settlement declined when larvae were placed with algae that had been treated with increased temperature (FT ~25% and FTPH ~32%) in comparison to those under ambient temperature (A ~56%) but not under decreased pH (FPH ~40%) (Fig. 3.). This pattern remained the same after 21 days ($p<0.05$, Supplementary Table 1) with lower settlement on both warming treatments (FT ~17% and FTPH ~20%) in comparison to controls (A ~41%) but not in decreased pH (FPH ~32%) treatments (Fig. 3).

Microbial community analysis

The MiSeq dataset contained 478,512 high quality sequences, ranging from 17,144 to 44,917 sequences per sample. Sequences from each sample were subsampled so they contained equal numbers of sequences (17,144) resulting in 1722 OTUs (clustered at 97% similarity) which were used in all analyses. For both time points, the microbial communities associated with each treatment were significantly different to all other treatments. This is shown by both the TRFLP data (at Day 14 and Day 21) and MiSeq data (at Day 21 only) with all treatments significantly different from each other (1 way PERMANOVA, $p<0.05$. Figure 4 and Figure 5). Furthermore, after 21 days the nMDS plots indicate that these differences were more defined, with clusters becoming more discrete, indicating that samples from the same treatment become more similar to one another after longer exposure to treatment conditions.

At neither time point did alpha diversity of the TRFLP data, as measured by Shannon's *H'*, vary between treatments (Table 2, 1 way PERMANOVA, p>0.05). The total number of TRFs detected at day 14 was 234 (mean 48 ± 2 SE) and at day 21 was 291 (mean 43 ± 6 SE). Similarly, there were no differences in alpha diversity, measured by Pielou's evenness *J* and Shannon's *H*; or in Chao1 richness, between the four treatment types when examined with 16S rRNA MiSeq sequencing (1 way PERMANOVA, p>0.05, Table 2).

Impacts to microbial community structure by climate change

SIMPER analysis (Supp. Table 1) showed that algae at ambient temperature and pH (A) were characterised by Alphaproteobacteria (OTU1, OTU2, OTU6, OTU23 and OTU35) from the Rhodbacteraceae genera *Loktonella, Tateyamaria* and *Thalassobacter* and the OCS116 clade (OTU13) and OTU3 from the Bacteroidetes family Flammeovirgaceae (genus *Reichenbachiella*). When algae were exposed to high temperature (FT), the two most abundant Alphaproteobacteria OTUs (OTU1 and OTU2) persisted but other dominant OTUs declined and algae were instead characterised by other members of the Rhodbacteraceae (genus *Roseovarius,* OTU10) and the OCS116 clade (OTU15) as well as a Verrucomicrobiaceae (OTU8), Hyphomonadaceae (OTU4) and OTU15 from the Alphaproteobacteria order *Rhizobiales.* Of these, OTU10 and OTU4 were both closely matched to clone sequences from diseased corals (Accession numbers FJ203288 and EF123355). Under increased acidification (FPH), algae maintained similarly high abundances of several OTUs to controls but were also characterised by the Alphaproteobacteria OTU22 (Rhodobacteraceae genus *Tateyamaria*) and Planctomycetes OTU46 (Phycisphaerae). OTU2 (Rhodobacteraceae genus *Loktonella)* decreased under acidification, contributing to differences between these treatments and controls. Under both increased temperature and acidification (FTPH) microbial communities were characterised by OTU5 (Planctomycetaceae), OTU7 and OTU9 (both Flavobacteriaceae).

Phylogenetic analysis and heatmapping of abundant OTUs revealed differences between various treatments at several taxonomic levels (Figure 5). In general, most differences in dominant OTUs were not related to high level (e.g. Phylum level) taxonomic differences, but instead occurred at lower taxonomic levels (family, genus or OTU). For example, the Alphaproteobacteria family Rhodobacteraceae was dominant in all samples, but at the genus level *Loktonella* decreased in low pH treatments while *Tateyamaria* persisted. At the OTU level, within the genus *Tateyamaria*, OTU22 increased under increasing acidification while OTU1 and OTU6 did not. Similarly, within the Bacteroidetes family Flammeovirgaceae the genus *Reichenbachiella* was characteristic of controls, but under warming this genus decreased in abundance and was replaced with the genus *Marinoscillum*. Notably, each Phyla was present in all treatments, and high level taxonomic differences were not clearly evident. Finally, all OTUs that were abundant in controls (present on average above 1% of all sequences) were maintained under all of the treatment conditions. In contrast the rare OTUs (present on average below 0.1% of all sequences) from controls were mostly lost in one or more treatment with only 31% maintained across all treatments and 21% of rare OTUs lost from all algae treated with either increased temperature, acidification or a combination of both (Supplementary data).

Discussion

Both *Amphiroa gracilis* and its associated microbial biofilm were impacted by future warming and acidification scenarios. Warming treatments caused algal tissue to fade and bleach and there were low levels of sea urchin larval settlement. Warming also resulted in substantial differences in the microbial community composition including increased abundances of OTUs from the genera *Aquimarina* (Flavobacterales), *Roseovarius* (Alphaproteobacteria) and *Marinoscillum* (Bacteroidetes) and reduced abundances of specific Proteobacteria, Verrucomicrobia and Planctomycetes OTUs. Acidification triggered high levels of faded algal tissue and differences in the microbial assemblages including a reduced abundance of OTUs from the genera *Loktanella* and *Tateyamaria*

(Alphaproteobacteria) and the KI89A clade (Gammaproteobacteria) and increases in OTUs from *Reichenbachiella* (Bacteroidetes) and the genus *Tateyamaria*, but did not impact larval settlement. The cumulative impact of both warming and acidification resulted in low levels of larval settlement, fading of algal pigments and a difference in the microbial community assemblage but the inclusion of low pH reduced the complete progression to bleaching. This is the first experiment that has tested the resilience of temperate reef biofilms associated with coralline algae to the likely impacts of either future warming or acidification, and one of the first studies to test the cumulative impact of both warming and acidification on subsequent settlement by invertebrate larvae.

Future warming and/or acidification treatments all resulted in strong differences in the microbial community composition, and these differences persisted after three weeks exposure. Notably, there was an increase in Bacteroidetes including OTU9 (*Aquimarina*, Flavobacterales) which was most strongly associated with differences between the control and warming treatment. Interestingly, despite the different sequencing technologies used in our study and Webster *et al.*, (2011) both studies found closely related *Aquimarina* sequences became important members of the biofilm community after warming. *Aquimarina* have been associated with aging of the canopy forming brown algal species *Cystoseira compressa* (Mancuso *et al.*, 2016) and with leaching in the red alga *Delisea pulchra* (Kumar *et al.*, 2016). *Aquimarina* strains can also be agarlytic and are found ubiquitously associated with lobster lesions (Quinn *et al.*, 2012), suggesting a possible opportunistic role for this species when the host associated microbial assemblage breaks down. A further two OTUs, OTU10 (*Roseovarius)* and OTU28 (unclassified Bacteroidetes) were among those driving the compositional differences under increased temperature and matched most closely to putative disease organisms of white syndrome in the corals *Montastraea* (Sunagawa *et al.*, 2009) and *Porites* (Séré *et al.*, 2013). In contrast, OTUs that became abundant with acidification were most closely matched to organisms previously recorded in healthy host tissues from sponges and corals. The putative disease-associated OTU10 also contributed to differences among acidification treatments

and controls, but it decreased under acidification, suggesting a moderating effect of acidification even under high temperature. These differences advocate that temperature, a key threat to temperate marine ecosystems via heat waves and gradual warming (Wernberg, *et al.*, 2012; Smale and Wernberg, 2013; Hyndes *et al.*, 2016), may result in increased abundances of putative pathogens on coralline algae and that while acidification was not linked to disease-associated organisms the microbial community composition displayed substantial differences which may have unknown impacts to algae and their interactions within temperate systems.

While the overall microbial community composition was different, none of the abundant OTUs (>1% abundance) were lost from treated algae. In contrast, many rare OTUs were lost when algae were treated with either increased temperature, acidification or a combination of both. Loss of the rare 'seed bank' (Lennon and Jones, 2011) microbiome associated with coralline algae under climate change scenarios may be a consistent threat as major loss of rare OTUs was also observed when the tropical encrusting coralline alga *Hydrolithon onkodes* was exposed to increased acidification (Webster *et al.*, 2013). Rare bacteria are important members of the microbial community as they can become abundant in response to changes in environmental conditions, and there is mounting evidence that they play an important role in ecosystem functioning (Caporaso *et al.*, 2012; Aanderud *et al.*, 2015). In particular, when rare organisms share only distant phylogenetic similarity with abundant taxa, they are likely to provide different functional capabilities, and thus it may be of key importance that they are maintained (Coveley *et al.*, 2015). Given that predicted future climate change scenarios are above the threshold for maintenance of the majority of the rare microbiome it is pivotal that more research is aimed at understanding the precise functional capacity of these organisms and the implications of their loss.

Different species of coralline algae host distinct microbial community assemblages (Nielsen *et al.*, 2015), suggesting that properties of the host species control assembly of surface biofilms. When

microbial community composition is tightly linked to the host, the stability of the composition is thought to be reflective of the status of the host environment. For example, Roder *et al.*, (2015) recorded a marked difference in alpha diversity within microbial communities associated with corals from the preferred habitat over marginal environments, with those in marginal habitats having high alpha diversity, indicative of dysbiosis. Such loss in structure within the host associated microbial assemblage was not observed in our study, or in previous studies on either warming (Webster *et al.*, 2011) or acidification (Webster *et al.*, 2013) impacts to coralline algal biofilms. Stable alpha diversity across treatments may indicate that coralline algal hosts do not exert control over microbial community structure as tightly as other hosts, such as corals and sponges. Understanding the putative differences in alpha diversity that may be expected in different hosts has important implications for field studies that examine environmental perturbations, as some eukaryotic hosts may be more resilient in terms of maintenance of community structure, and thereby may represent more robust tipping points for environmental management.

Bleaching can be defined as whitening of localized, nonuniform parts of the thallus (Campbell *et al.*, 2011; Case *et al.*, 2011) and microbial pathogens are thought to facilitate bleaching (Fernandes *et al.*, 2011; Marzinelli *et al.*, 2015) which can be exacerbated by thermally stressed conditions (Case *et al.*, 2011). *Amphiroa gracilis* displayed high levels of bleaching in the temperature treatment, which was moderated by the addition of acidification. Coralline algae display high sensitivity to photodamage under elevated temperature (Webster *et al.*, 2011; Vásquez-Elizondo and Enríquez, 2016) but there is less of a consensus regarding the impacts of acidification given that lower pH can enhance photosynthesis, but reduce calcification (Semesi *et al.*, 2009; Webster *et al.*, 2013; Nash *et al.*, 2015). Under combined warming and acidification there was no evidence that photosystem II (PSII) function of *A*. *gracilis* will be negatively impacted. Significant bleaching did occur, although, unlike the cumulative stress of temperature and acidification leading to high levels of bleaching reported by (Martin and Gattuso, 2009) ocean acidification reduced the amount of bleaching due to

temperature stress, similar to other studies (Scherner *et al.*, 2016; Schoenrock *et al.*, 2016; Vásquez-Elizondo and Enríquez, 2016). Based on these responses *A. gracilis* is sensitive to warming and acidification, and based on the higher sensitivity to warming are likely to be at risk from heat wave events.

Settlement by larvae of the sea urchin *Heliocidaris erythrogramma* was substantially decreased when *A. gracilis* was treated with warming and acidification. Despite intense investigations (Williamson *et al.*, 2000; Swanson *et al.*, 2006; Tebben *et al.*, 2011, 2015) the exact identity of the morphogens that result in successful larval settlement remain unclear. What is clear however, is that effects of increasing atmospheric carbon dioxide, particularly changes in temperature, causes a substantial difference in the microbial community composition of coralline algae, results in bleaching and stress to algae, and also results in a significant loss of efficacy in inducing larval settlement by both corals and sea urchins, as shown here and in studies of coralline algae elsewhere (Webster *et al.*, 2011, 2013b, 2016). Previous studies on the impact of climate change to the efficacy of coralline algae and biofilms to induce settlement by larvae of marine invertebrates have focused on a variety of coral species (Webster *et al.*, 2011; Doropoulos *et al.*, 2012; Doropoulos and Diaz-Pulido, 2013; Uthicke *et al.*, 2013) and have shown a substantial reduction of settlement by coral larvae to both warming and acidification. In temperate ecosystems corals are absent or in very low abundances (Miller and Hay, 1996) and other organisms including sea urchins are among the dominant organisms that define the structure of kelp forest ecosystems through processes such as bioerosion and herbivory (Steneck *et al.*, 2002; Wright *et al.*, 2005). Loss of sea urchin recruitment will result in multiple ecosystem level impacts and under a warming ocean may even facilitate the recovery of kelp forests in the short term (Gagnon *et al.*, 2004; Fagerli *et al.*, 2013). However, long term impacts are likely to include substantial changes in algal community species composition (Miller and Hay, 1996), and biodiversity (Wright *et al.*, 2005) which will impact the entire ecosystem and thus there is

an urgent need to understand the likelihood that urchin recruitment will be reduced under future climate conditions.

While heat waves can develop in a matter of weeks (Smale and Wernberg, 2013) *in situ* increases in temperature and acidification are unlikely to occur as quickly as were done in this experiment and thus the results need to be taken with caution. For example, Kamenos *et al.*, (2013) found that coralline algae that were acclimatised to increased temperature over a longer period (8 days) were less impacted by acidification than those that were acclimated quickly (2 days). Furthermore, while temperature can be relatively stable in marine ecosystems, pH is now known to fluctuate across a 24 hour period, and static conditions therefore do not mimic realistic field conditions (Albright *et al.*, 2015). However there is good evidence, at least for temperate coralline algae, that static acidification conditions result in the same trends in impacts to growth as those under fluctuating pH (Cornwall *et al.*, 2013). Static laboratory based experiments also provide highly useful information regarding limits of environmental change (Webster *et al.*, 2016) and such experiments, together with field based observations and manipulations, are an important component of understanding impacts of future climate change (Wernberg, *et al.*, 2012).

Climate change will result in substantial changes to marine ecosystems, and here we have shown that temperate microbial biofilms associated with coralline algae are no exception. Both temperature and acidification caused substantial changes in the microbiome including a loss of the rare seed bank microbiome. Disease-associated organisms were more abundant in microbiomes exposed to high temperature, algae were more stressed in the temperature treatments, and settlement by sea urchin larvae was greatly reduced. In contrast, after three weeks' exposure to ocean acidification larval settlement remained high, and no disease-associated sequences were detected. Our data indicate that future environmental conditions will result in a reduction in the health of temperate coralline algae combined with low sea urchin recruitment. Given the

importance of sea urchins and geniculate coralline algal species to temperate reefs, these changes are likely to have ecosystem wide consequences, highlighting the critical need for studies focused on understanding multi-species interactions under changing climate.

Acknowledgements

The authors thank Ms Audrey Cartraud, Ms Deborah van Gÿen and Ms Alba Esteban for assistance in the field, Mrs Natasha Dunham for data collation and Mr Chris Bording from the Pawsey Supercomputing Centre for computing assistance. This research was supported by use of the Nectar Research Cloud, a collaborative Australian research platform supported by the National Collaborative Research Infrastructure Strategy (NCRIS).

Conflict of Interest Statement

This work was funded by Edith Cowan University (ECU) internal funding to Professor Pierre Horwitz and an ECU Faculty of Health, Engineering and Science grant to MJH.

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Figure Legends

Figure 1: Mean $rETR_{max}$ (+SE); after 7, 14 and 21 days of the experiment (A) and across all treatments (B); (C) mean Ek (+SE) after 7, 14 and 21 days of the experiment; and (D) Mean % of tissue bleached and faded (+ SE) after 21 days. A: ambient, FPH: future (decreased) pH, FT: future (increased) temperature and FTPH: combined future pH (decreased) and temperature (increased). Treatments that share a letter are not significantly different from one another.

Figure 2: Examples of algae from each treatment, as indicated, taken after 21 days of the experiment. Arrows indicate tissue that was considered healthy (pink), faded (green) and bleached (white).

Figure 3: Settlement by larvae of *Heliocidaris erythogramma* on *Amphiroa gracilis* after the alga had 14 (light bars) and 21 (dark bars) days of exposure to treatments. A: ambient, FPH: future (decreased) pH, FT: future (increased) temperature and FTPH: combined future pH (decreased) and temperature (increased). Treatments that share a letter are not significantly different from one another. Lowercase letters: 14 days; uppercase letters: 21 days.

Figure 4: Multidimensional scaling plots of square root transformed Bray-Curtis similarity data based on TRFLP after (A) 14 and (B) 21 days of the experiment. Ambient: black triangles; future (decreased) pH: grey squares; future (increased) temperature: inverted grey triangles; and combined future pH (decreased) and temperature (increased): open circles. Microbial community structure in all treatments were significantly different from all others at both time points.

Figure 5: Heatmap clustering visualisation (x axis) and maximum likelihood phylogenetic tree of abundant OTUs (those whose mean is >1% of sequences in at least one treatment) (y axis). Top: Cluster dendogram of Bray-Curtis similarity between each sample based on square root transformed 16S sequencing data. A: ambient, FTPH: future temperature and pH, FT: future temperature, FPH: future pH. Scale bar represents 0.10 substitutions per nucleotide position, closed circles indicate nodes with >90% bootstrap support, open circles represent nodes with 80-90% support.

Supplementary Figure 1: Mean Effective Quantum Yield (EQY) of PSII (+SE); after 7, 14 and 21 days of the experiment, and across all treatments. A: ambient, FT: future temperature, FPH: future pH, FTPH: future temperature and pH. Lines above bars represent statistical comparisons of treatment within each time point and letters represent statistical comparisons of time points within each treatment. Bars that share a line or a letter are not statistically different from one another. Uppercase letters: 7 days; lowercase letters: 14 days, Greek letters: 21 days.

Table 1: Water chemistry parameters across the duration of the experiment. Numbers are means (5 per treatment), with standard error in parentheses. A_T = total alkalinity, Ω_{Ca} and Ω_{Ar}: saturation state of calcium and aragonite. A: ambient, target 19°C and pH 8.1; FT: future temperature, target 23° and pH 8.1; FPH: future pH, target 19°C, pH 7.6; FTPH: future temperature and pH, target 23°C and pH 7.6.

	Temperature (°C)	pH	pCO_2 in (µatm)	A_T µM/kg SW	Salinity (ppt)	Ω_{Ca}	Ω_{Ar}
A							
7 days	19.0 (0.03)	8.1	379 (10)	2327 (6)	37.4 (0.02)	4.6 (0.09)	3.0 (0.06)
14 days	19.3 (0.04)	8.1	325 (3)	2393 (20)	37.3 (0.04)	5.3 (0.10)	3.5 (0.06)
21 days	19.3 (0.03)	8.1	342 (3)	2420 (2)	36.8 (0.11)	5.3 (0.02)	3.4 (0.01)
FT							
7 days	22.9 (0.04)	8.2	325 (3)	2762 (5)	38.3 (0.04)	7.5 (0.04)	4.9 (0.03)
14 days	23.5 (0.03)	8.2	280 (2)	2930 (4)	37.4 (0.08)	9.1 (0.05)	6.0 (0.03)
21 days	22.6 (0.03)	8.2	289 (3)	2967 (6)	37.4 (0.06)	8.9 (0.03)	5.9 (0.02)
FPH							
7 days	19.1 (0.04)	7.8	746 (18)	2319 (4)	37.5 (0.05)	2.8 (0.05)	1.8 (0.03)
14 days	19.5 (0.04)	7.8	816 (10)	2398 (3)	37.1 (0.07)	2.8 (0.03)	1.8 (0.02)
21 days	19.3 (0.03)	7.9	650 (24)	2438 (3)	36.9 (0.05)	3.4 (0.09)	2.2 (0.06)
FTPH							
7 days	22.9 (0.04)	7.8	889 (29)	2574 (5)	36.5 (0.11)	3.4 (0.08)	2.2 (0.05)
14 days	23.4 (0.04)	7.8	1013 (20)	2850 (2)	37.5 (0.2)	3.8 (0.06)	2.5 (0.04)
21 days	22.6 (0.03)	7.8	1059 (10)	2904 (8)	37.4 (0.10)	3.6 (0.02)	2.4 (0.01)

Table 2: Richness and diversity of microbial communities in treatments based on 16S tag sequencing data after 21 days exposure to treatments. **Shannon's H measurements in italics (below) for TRFLP data.* A: ambient, FT: future temperature, FPH: future pH, FTPH: future temperature and pH.

	Samples (n)	Mean no. of OTUs	Chao1 Richness	Pielou's evenness *J*	Shannon's *H*
A	4	436.7 (± 24.3)	479.15 (± 54.92)	0.65 (± 0.01)	3.96 (± 0.06)
	*12**				*3.64 (± 0.07)**
FT	4	400 (± 27.0)	444.18 (± 61.03)	0.65 (± 0.01)	3.91 (± 0.06)
	*12**				*3.76 (± 0.11)**
FPH	5	456 (± 42.6)	519.80 (± 112.84)	0.63 (± 0.03)	3.82 (± 0.22)
	*12**				*3.46 (± 0.07)**
FTPH	4	439 (± 27.7)	496.21 (± 55.07)	0.66 (± 0.02)	4.00 (±0.15)
	*12**				*3.91 (± 0.09)**

Figure 1

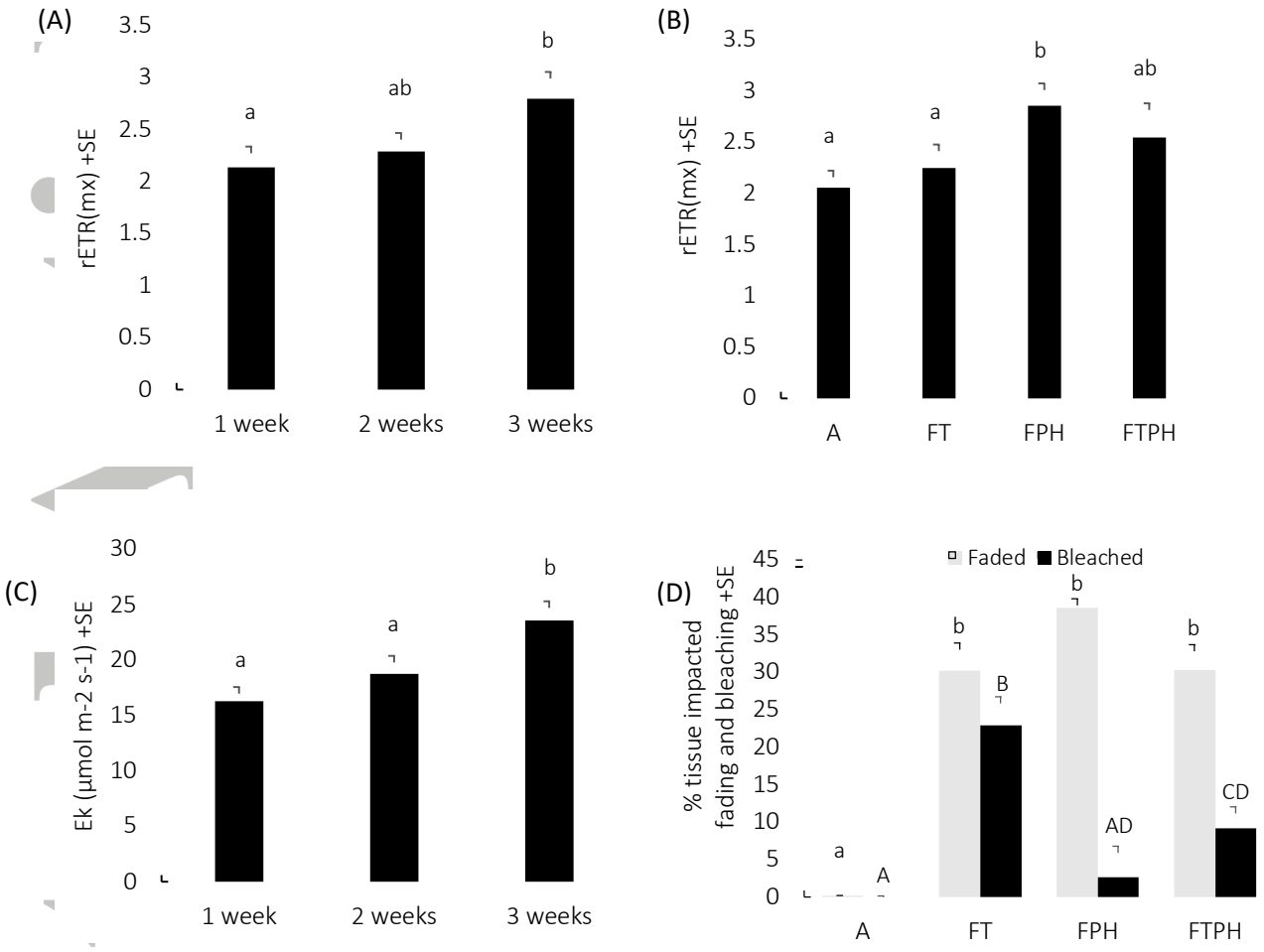

Figure 2:

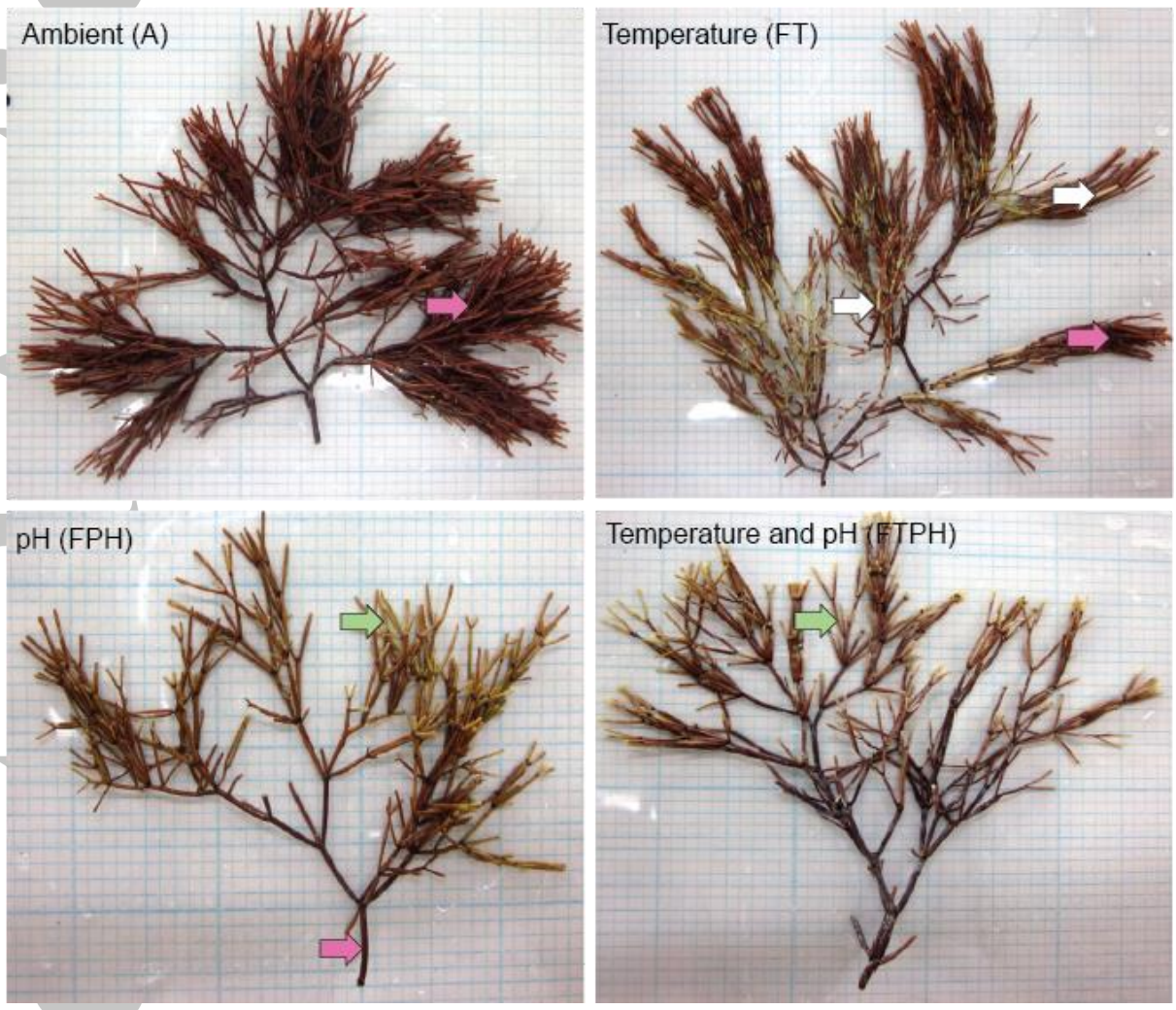

Figure 3:

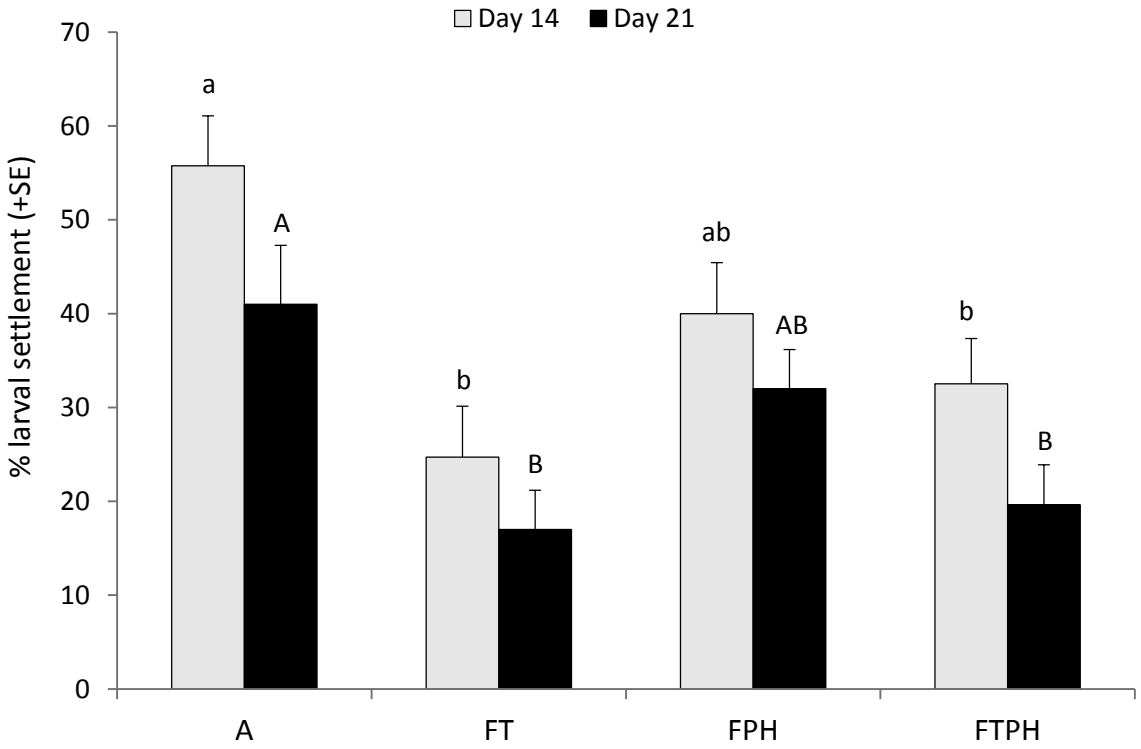

Figure 4: