Climate Change Stressors Destabilize the Microbiome of the Caribbean Barrel Sponge, *Xestospongia muta*

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Running title

Climate change effects on a sponge microbiome

Abstract

The effect of climate change, both thermal stress and ocean acidification, on coral reefs is of increasing concernwith the effects on calcification at the organismal level, and changes in the ratio of accretion to erosion on larger spatialscales of particular interest. But far fewer studies have been done on non-calcifying organisms, such as sponges, that haveimportant ecological roleson coral reefs. Here we report the results of a combined thermal stress and ocean acidification experiment on the ecologically dominant barrel sponge, *Xestospongia muta*, found on coral reefs throughout the Caribbean basin. The results show that ocean acidification alone, as well as its interaction with elevated seawater temperature,has significant effects on the sponge microbiome. Specifically, thesignificant interactive effects of thermal stress and ocean acidification led to a decline in the productivity potential of the symbiotic cyanobacteria in these sponges with a subsequent impact on nutrient transfer, as carbohydrate, between symbiont and host. Additionally, while neither environmental stressor predictably changed sponge microbiome community composition, ocean acidification alone reduced the stability of sponge microbiomes and their predicted functions. Future changesin ocean acidification and thermal stresspredicted by current climate models could negatively impact the microbiomes of coral reef organisms and therefore also affect theirorganismal performance and fitness in the future.

Keywords

Sponges, microbiome, coral reefs, ocean acidification, climate change, Caribbean

1. Introduction

The worldwide decline of coral reefs has been attributed to multipleenvironmental stressors but the consequences of climate change on coral reefs arewidespread, long-term and potentially irreversible (Hoegh-Guldberg et al., 2007; Hough-Guldberg and Bruno, 2010). Both ocean acidification (OA) and elevated seawater temperatures (SWT)are known threats to coral reefs (Hoegh-Guldberg et al., 2007),with the interactive effects of increasing concern (Boyd et al., 2014; Pandolphi et al., 2011). Climate models estimate that 90% of the increased heat content of the atmosphere,as a result of the release of greenhouse gases (e.g., CO_2), issequestered in the oceans resulting in increasingseawater temperature (Donner et al.,2005) with multiple ecological effects including: species range extensions, increased incidence of opportunistic disease, enhanced storm effects, and changes in food-web structure (Doney et al., 2012). Simultaneously with increases in SWT,the increase in atmospheric CO_2 has caused a reduction of 0.1 pH units in the ocean, equivalent to a 30% decreaseover pre-industrial era levels. Climate models predict additional declines of as much as 0.3-0.4 pH units by the year 2100 (Doney et al., 2012). However, these predictions are for the open ocean and there is now evidence that coastal marine environments, including coral reef ecosystems,currently exceed these predictions withsignificant temporal and spatial variability (Hofmann et al., 2011). A major ecological and economic concern is that lowering the pH and the aragonite saturation state [Ω_{arag}] will inhibit calcification in marine organisms (Ries et al., 2009).

Elevated SWT can causecoral bleaching,a stress responsewith significant impacts to coral reefs worldwide (Hoegh-Guldberg et al., 2007; Lesser, 2004). But fewer studies have considered thecombined effects of elevated SWT and OA in combination (Anthony et al.,2011;Boyd et al., 2014), despite the fact that interacting stressors can be more damaging than the independent

68 effect of any single stressor (Byrne et al.,2013). Additionally, there have been far fewer studies
69 of the effects of climate change stressors on non-calcifying taxa on coral reefs. For example,
70 sponges are now recognized as a dominant taxon on many Caribbean coral reefs (Colvard et
71 al.,2011), and it has been predicted that many coral reefs could become sponge dominated in the
72 future (Bell et al.,2013). The ecological importance of sponges to coral reef communities is
73 unequivocal (Bell et al.,2008)with sponges involved in a number of important roles on coral
74 reefs including: benthic-pelagic coupling via filtration of large quantities of dissolved and
75 particulate organic matter (de Goeij et al.,2013; Lesser and Slattery, 2013), nutrient cycling on
76 coral reefs (Fiore et al., 2013 a, Southwell et al., 2008)and primary productivity and synthesis of
77 secondary metabolites (Taylor et al.,2007). Many of the functional roles of sponges
78 aredependenton diverse assemblages of symbiotic microorganisms(Erwin and Thacker, 2008;
79 Fiore et al.,2010; Taylor et al.,2007).The barrel sponge, *Xestospongia muta,* is a long-lived and
80 dominant component of the Caribbean benthic coral reef fauna (McMurray et al., 2010) whose
81 multiple contributions to coral reef ecology and biogeochemistryhas been shown to be mediated
82 by a diverse symbiotic microbiome (e.g., Fiore et al., 2013 a, b).Here, the results of an
83 experiment examining the independent and interactive effects of SWT and OA on*X. muta*, and its
84 symbiotic microbes, are presented.
85
86 **2. Materials and Methods**
87 *2.1. Study sites, experimental design and statistical analysis*
88 Barrel sponges(*Xestopongia muta*) were collected from South Perry Reef (17 m depth) on Lee
89 Stocking Island in the Bahamas. All samples were collected whole by cutting the sponges from
90 the substrate without compromising the spongocoel integrity and maintaining the pumping

activity of the sponges. Sponges ranged in size from 73-401 g wet weight. Sponges were randomly picked and acclimatized (N=5 for each treatment group) in ambient pCO_2 and temperature reef seawater for 3 dwithin individual aquaria in a raceway system. Sponges were then acclimatized from the ambient conditions to experimental conditions over 2 d in a fully orthogonal matrix design of CO_2 concentrations that reflected pCO_2 (~390 ppm) conditions at the time of the experiments and predicted CO_2 concentrations in the year 2100 under an A2 climate model (pCO_2 of ~800 ppm) scenario, as well as current and predicted summer SWT (IPCC, 2007). The seawater pH values for these conditions were held constant using the pH-stat approach with WTW 3310 pH meters (accuracy = ±0.005 pH capability). The mean treatment temperatures were maintained using JBJ titanium heaters (± 0.5 °C capability) and measured using HOBO Water Temperature Pro v2 data loggers (Onset Corp). Temperatures fluctuated on a diel basis in theflow through system as a result of daily heating and cooling, but still were within the 2-5.4°C predicted by the IPCC 2007 A2 model for the open ocean. The treatment groups were: Control (present day temperature and pH), Reduced pH (present day temperature and A2 pH), Elevated Temperature (A2 temperatures and present day pH) and Elevated Temperature and Reduced pH (A2 temperatures and pH). Sponges in all treatment groups were held in individual aquaria (8 L) with flowing seawater (1.0 L h^{-1}), and exposed to natural solar radiation under neutral density cloth for 12 d. The maximum irradiance of photosynthetically active radiation (PAR; 400-700 nm), measured with a LI-COR LI-192 cosine corrected underwater quantum sensor, was 450-500 µmol quanta m^{-2} s^{-1} and replicated the total irradiance of the site and depth of collection (e.g., Lesser and Gorbunov 2001). All treatment and time effects, and their interaction, were tested using ANOVA with *post hoc* multiple comparison testing (i.e., Tukey's HSD) as required.

114 *2.2. Carbonate chemistry*

115 Independent seawater samples (N=3) were collected from the experimental aquaria at the end of
116 the experiment and analyzed for total alkalinity (TA; µmol kg seawater^{-1}), total CO_2 (µmol kg
117 seawater^{-1}) and the partial pressure of carbon dioxide ($pCO2$; µatm)at the University of New
118 Hampshire Ocean Process Analysis Laboratory (OPAL). TA was analyzed using an Apollo Sci-
119 Tech AS-A2 automated analyzer, which employs the Gran titration procedure with a precision of
120 0.1% and pCO_2 (± 4 µatm) using a LiCor 840A gas analyzer. The initial pH for the titration was
121 measured on the sample using a Thermo Orion combination electrode (precision ±0.027 pH
122 units). Certified reference materials were used to ensure the precision of the measurements
123 (Dickson et al., 2007). pH_T and Ω Ca were then calculated with CO2calc software (Robbins et al.
124 2010)using the temperature of each treatment, a salinity of 37.0 ppt, and the inorganic carbon
125 dissociation constants from Mehrbach et al. (1973) as refitted by Dickson and Millero (2007).
126
127 *2.3. Active fluorescence*

128 The quantum yield of photosystem II (PSII) fluorescence was measured using a pulse amplitude
129 modulated (PAM) diving fluorometer (Walz Inc.) at noon and midnight daily, equivalent to the
130 maximum and minimum irradiances for each day of sampling, during the entire experiment. The
131 *in vivo* fluorescence of chlorophyll varies between a minimum yield, F_o, and maximum yield, F_m.
132 The difference between $F_o$and $F_m$fluorescent yields (F_m-F_o) is the variable fluorescence, F_v and
133 the ratio F_v/F_m or $\Delta F/F_m'$ is the maximum (i.e., dark adapted) and steady state (i.e., in the light),
134 respectively, quantum yield of PSII fluorescence(Warner et al., 2010). The PAM fluorometer
135 utilized was a blue LED version (470 nm excitation, emission >630 nm) with the fiber-optic
136 probe held 1 cm from the sponge in a perpendicular orientation. A saturation pulse of 0.8 s at

3000 µmol quanta m^{-2} s^{-1} was used to record maximal fluorescence (F_m) at a gain setting of 6. While studies on cyanobacteria are generally conducted using red excitation, the cyanobacteria, *Synechococcus* sp., in *Xestospongia muta* contain phycoerythrin and as a result they absorb more efficiently in the blueportion of the spectrum. Additionally, the blue LED detects a wider emission signal (>630 nm versus >700 nm for the red LED PAM) making it more sensitive then the red LED version of the PAM. Lastly, as irradiance and pigment content has the greatest influence on cyanobacterial quantum yields (Campbell et al., 1998), and all sponge treatment irradiances and treatment pigment concentrations were not significantly different from each other (Lesser, unpublished data), these quantum yield measurements are a very good approximation of treatment effects on the quantum yield of cyanobacterial PSII fluorescence. All sponge measurements (N=3) were taken at the same instrument settings. In cyanobacteria the quantum yields of PSII fluorescence, under constant pigment concentration and irradiances,also correlate very well with rates of photosynthesis (i.e., oxygen evolution) especially when the same sample is measured repeatedly as was done here (Campbell et al., 1998). All quantum yield measurements are ratios and therefore *a priori* not normally distributed. These measurements were transformed (Log +1) prior to analysis and back transformed for presentation. Only the measurements at the end of the experiment were analyzed and reported herewith ocean acidification, SWT and time of day (i.e., midday or midnight; for each sampling time: N=20 from four treatment groups and five sponges)each treated as an independent factorusing a three-factor ANOVA with interaction for the analysis of the data.

2.4. Metagenetic analysis of 16S rRNA genes

For the metagenetic analysis of 16S rRNA genes, DNA extractions were performed on the experimental sponge samples (N=3 for each treatment) at the end of the experiment as described byFiore et al. (2013 a). A section of each sponge,includingboth the pinacoderm and outer mesohyl of the sponge, was cut into smaller pieces for processing. The 16S rRNA genes of each sample was amplified and barcoded for multiplexed pyrosequencing using Titanium adapter sequences A (forward primer) and B (reverse primer), and a 10 bp barcode sequence added to the PCR primers. Primers designed to amplify both Bacteria and Archaea (hypervariable V6 region) were used, consisting of the forward primer U789F (5'-TAGATACCCSSGTAGTCC-3') and the reverse primer U1068R ('-CTGACGRCRGCCATGC-3'). Samples were pyrosequenced on a ROCHE/454 GS FLX+ platform (Roche, Branford, CT, USA) at the University of Illinois W.M. Keck Center for Comparative and Functional Genomics (Urbana-Champaign, IL, USA). The sequence analysis is similar to that described in Fiore *et al.* (2013) and uses the Quantitative Insights Into Microbial Ecology (QIIME) pipeline (Caporaso et al., 2010) on the Amazon Elastic Compute Cloud (EC2), except where noted. Raw sequence reads were filtered for quality by discarding short reads (<200 bp), or reads with more than two mismatches with the primer sequence, or with ambiguous nucleotides, or with an average quality score less than 25. A custom Perl script based on the QIIME script "split_libraries.py", was used to trim primers from the sequences, assign reads to their sample of origin (based on MID tags), and reverse complement the reads originating from the B adapter (reverse reads). Trie clustering (QIIME team, unpublished, http://qiime.org) was used to collapse reads that are prefixes of each other into clusters and discard singleton reads as described in Fiore et al. (2013). Chimeric sequences were identified and removed using USEARCH 6.1 (Edgar 2010) in QIIME. Reads were then

clustered into OTUs using UCLUST de novo clustering (97% similarity) (Edgar 2010). Taxonomy was assigned to representative sequences for each OTU using the Ribosomal Database Project (RDP) classifier with a minimum cutoff of 0.8 (Wang et al.,2007) in QIIME. The OTU table generated in QIIME was rarefied (min=4331), and any OTUs with less than 4,331 sequences were removed prior to downstream analysis.ANOSIM was utilized to examine treatment effects on the community composition of experimental sponges. Analysis of microbial or functional β-diversity was quantified using weighted UniFrac distances for microbial communities (a quantitative, phylogenetic β-diversity metric) or Bray-Curtis divergences for functional profiles (a quantitative, non-phylogenetic measure) in each individual sponge. These metrics are then used to assess the stability of the individual sponge samples as it relates to any treatment effects. The significance of differences between all pH treatments, all A2 treatments, or all treatment categories was assessed using permutational *t*-tests of distances, witha Bonferroni correction applied to comparisons between treatments to account for multiple comparisons. Sequences resulting from pyrosequencing were deposited in the iMicrobe data repository under project accession number CAM_P_0000957 (ftp://ftp.imicrobe.us/projects/103/samples/).

2.5. Functional analysis of sponge microbiomes using PICRUSt

Functional profiles for 16S rRNA gene sequence data were predicted using the program Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt) version 1.0 (Langille et al.,2013). PICRUSt uses an algorithm that estimates the functional gene content of Bacteria or Archaea for which no genome is available. PICRUSt does this through evolutionary modeling of the copy number of each gene family, based on the strain's

206 phylogenetic relationship with all Bacteria and Archaea for which sequenced genomes are
207 available. The phylogenetic predictability of sponge samples was calculated using Nearest
208 Sequenced Taxon Index (NSTI) scores, which measure the average branch length on the
209 reference phylogeny (Greengenes version 13.8) between the representative sequence for each
210 OTU in a sample compared against a sequenced genome, weighted by the abundance of that
211 OTU.
212
213 *2.6. Proximate biochemical composition*
214 The proximate biochemical composition of *Xestospongia muta* was determined from lyophilized
215 tissue (including host and microbial symbionts) samples of replicate sponges (N=5) from each
216 treatment. For each sponge, freeze-dried "tissue" was ground into a fine powder using a Wiley
217 Mill. Levels of soluble protein and soluble carbohydrate were determined colorimetrically using
218 the Bradford(1976) and Dubois et al. (1953)techniques, respectively as described by Slattery and
219 McClintock (1995). Lipid was measured gravimetrically using the technique of Freeman et
220 al.(1957),and ash was determined by placing tissues in a muffle furnace for 4 h at 500°C(Paine,
221 1971). The remaining refractory material was calculated by subtraction and considered to be
222 insoluble protein(Lawrence, 1973).
223
224 **3. Results**
225 *3.1. Carbonate chemistry*
226 Spongeswere acclimatized to either current levels of OA (pCO_2 ~390ppm; resulting pH: 8.07 ±
227 0.05 [SD]) or predicted pH levels for the year 2100 (pCO_2 = 800ppm; resulting pH: 7.81 ± 0.12
228 [SD]) under an A2 climate model(IPCC 2007). Within each pCO_2 treatment, sponges were also

229 acclimatized to current temperatures (mean 29.2 ± 0.9 [SD]°C; Range 27.3-30.1°C), or elevated
230 temperatures (mean 31.4 ± 1.07 [SD]°C; Range 29.3-33.4°C) representative of predicted
231 summertime means for the year 2100(IPCC 2007) along with the daily variability that
232 characterizes coastal environments.The mean differences in treatment conditions were significant
233 for both OA (*t*-test; two-tailed, P<0001) and temperature (*t*-test; two-tailed, P<0001). All
234 measured and predicted carbonate chemistry values for each treatment group arepresented in
235 Table 1. These predictions were based on the IPCC 2007 Coupled Model Intercomparison
236 Projectthathave now been replaced with new climate model scenarios, the Representative
237 Concentration Pathways (RCP) from the IPCC 2014(Rogelj et al., 2012) report.The A2 climate
238 model conditions used in these experiments were closest to the RCP6.0climate model when you
239 include the CO_2 equivalents for CH_4 and N_2O (Rogelj et al., 2012).
240
241 *3.2. Active fluorescence*
242 The effects of SWT and OA on the photosynthetic symbionts were assessed usingboth the
243 maximum (F_v/F_m) and steady state ($\Delta F/F_m'$) quantum yields of photosystem II (PSII)
244 fluorescence measured at noon and midnightwitha pulse amplitude modulated (PAM)
245 fluorometry (Fig. 1). No visible signs of "bleaching" were observed in experimental sponges.
246 Because the interaction of temperature, pH and time on the quantum yields of PSII fluorescence
247 was significant (ANOVA: F=9.6, P=0.004), no further analysisofany independent effects was
248 considered. A Tukey's HSD multiple comparison test on the complete matrix of treatment
249 groups reveals that significant decreases in the quantum yields of PSII fluorescence were
250 observed (Fig. 1) in thereduced pH and elevated temperature treatments,with no differences
251 between noon and midnight measurements, when compared to control values. However,

252 thecombined elevated temperature and reduced pH treatments(0.426 ± 0.014 [SE]) had the
253 lowest quantum yieldsobserved compared to the controltreatment group (0.621 ± 0.018 [SE]).
254
255 *3.3. Metagenetic analysis of 16S rRNA*
256 Pyrosequencing of the 16S rRNA genefor *Xestospongia muta* symbionts yielded 282,298 reads
257 (average read length 299 nucleotides). Following quality filter steps and removal of singleton
258 reads there were 206,995 reads that were then clustered into OTUs at 97% similarity. A total of
259 967 OTUs remained following removal of chimeric sequences, singletons and contaminants from
260 the sponge samples, and these included 19known bacterial and two archaeal phyla (Fig. 2). Two
261 groups of unclassified prokaryotes were clustered as "Unclassified Bacteria" and "Unclassified
262 Archaea" (Fig. 2). These results are similar to those for *X. muta* from other locations around Lee
263 Stocking Island, as well as from populations in the Cayman Islands and Florida Keys (Fiore et
264 al.,2013 a).An analysis of similarity (ANOSIM) revealed no significant differences between
265 treatments for the sponge microbial communities(Global R=0.005, P=0.31).
266
267 *3.4. Functional analysis of sponge microbiomes using PICRUSt*
268 To assess whether functional changes in the symbiotic communities occurredin*Xestospongia*
269 *muta* from each treatment group, functional profiles were calculated using the PICRUSt software
270 package. This approach predicts the gene families present in microbial metagenomes by
271 modeling the gene families present in each OTU using available fully sequenced genomesas a
272 reference (Langille et al.,2013). No significant differences were found between predicted
273 functional categories by PICRUSt. Testing of whether any functional categories were enriched
274 was done by summarizing the PICRUSt-predicted gene family counts (i.e.,KEGG orthology or

KO counts), and no significant differences were found by Kruskal-Wallis tests of KEGG Pathway abundance, summarized at the second or third levels of the KEGG functional hierarchy ($p > 0.05$, FDR $q > 0.05$). Sponge samples were also tested for differences in the abundance of specific functional categories (N=264) across all treatments by g-test of predicted results, rarified to 20,000 counts per sample. The top-three categories were all genes involved in photosynthesis: 'photosynthesis proteins', 'photosynthesis', and 'photosynthesis – antennae proteins'. Using a false discovery rate control for multiple comparisons, only the first of these categories wassignificant (FDR $q = 0.14$, $p = 0.0005$). The mean relative abundance for photosynthesis proteins,after rarefaction,was lowest in the elevated temperature and reduced pH treatment group.

In order to testthe potential accuracy of phylogenetic predictionsof functional profiles in these samples, the average phylogenetic distance between microbial OTUs in sampled sponges versus the nearest sequenced microbial genome was calculated. These distances were weighted by the abundance of each OTU in the sample using the Nearest Sequenced Taxon Index, or NSTI (Langille et al.,2013). The mean NSTI score was 0.26,with values ranging from 0.11 to 0.31 (Fig. 3), and were lowest when reference genome coverage was bestrepresented in the characterized microbiome (Fig. 2). A two-factor ANOVA of the independent and interactive treatment effects on the Log +1 transformed NSTI values shows no treatment effects were detected (ANOVA: $P > 0.05$).

Finally, a test of whether OA or elevated SWT altered the extent of inter-individual variation in the sponge microbiome or its predicted function was conducted. Within each treatment, changes in sample-to-sample turnover in microbial community composition, as a measure of community stability (i.e., β-diversity), were assessed using Weighted UniFrac

298 distances, and changes in predicted function were assessed using Bray-Curtis divergences. The
299 resulting β-diversity values were compared for either all reduced pH treatments (Fig.4a,b), all
300 elevated temperature treatments (Fig.4c,d), or each treatment individually (Fig.4e,f). Despite the
301 absence of significant changes in community composition (see Fig. 1), reduced pH alone
302 measurably increased the inter-individual variation in sponge microbiomes, while the elevated
303 temperature alone treatment reduced this variation (Fig. 4).
304
305 *3.5. Changes in the proximate biochemical composition of sponges*
306 Quantifying the proximate biochemical composition (i.e., protein, lipid and carbohydrate)of the
307 holobiont at the end of the experiment revealed no significant effects of OA, SWT or their
308 interaction on the concentration of protein or lipids (Fig. 5). The concentration of carbohydrates,
309 however, showed a significant interactive effect of reduced pH and elevated
310 temperatures(ANOVA: $F=18.8$, $P=0.0005$). Post-hoc multiple comparison tests (i.e., Tukey's
311 HSD) shows that the A2predicted temperature and pH treatment group (i.e., Elevated
312 temperature and reduced pH) had significantly ($P<0.05$) lower concentrations of carbohydrate
313 when compared to all other treatment groups (Fig. 5).
314
315 **4. Discussion**
316 The interactive effects of elevated SWT and OA are of increasing concern in marine ecosystems
317 generally (Boyd et al.,2014), and on coral reefs specifically (Hoegh-Guldberg and Bruno, 2010).
318 In addition to scleractinian corals, spongescan also be negatively affected by the impacts of
319 environmental stressors, including elevated SWT (Fan et al.,2013*;* Webster et al.,2013)
320 orOA(Goodwin et al.,2013). Alternatively, sponges can also benefit from the effects of

increasing CO_2; studies done at natural CO_2seeps that simulate future OA conditions,in the absence of elevated SWT, show that sponges with photoautotrophic symbionts (e.g., *Synechococcus* sp.) increase in abundance compared to adjacent sites with contemporary carbonate chemistry (Morrow et al.,2014).This is consistent with studies on free-living *Synechococcus* that showed an increase in their rates of photosynthesis and cell division (Fu et al.,2007)when exposed to similar conditions to those described here for the A2 predicted temperature and pH treatment group.While the symbiotic cyanobacteria of sponges can supply a significant amount of carbon to their host via autotrophy (Wilkinson, 1983), cyanobacteria can also be negatively affected by OA conditions under nutrient limitation (e.g., Shi et al.,2012), a situation unlikely to occur *in hospite*for sponges (Fiore et al., 2010).

Cyanobacteria in the genus *Synechococcus* are well-described symbionts of *Xestospongia muta*(Fiore et al., 2013 a) and a previous study has shown that *X. muta* harbors different symbiotic phylotypes of *Synechoccoccus spongiarum* (Erwin and Thacker, 2008 a). Observations on other sponge species(Erwin and Thacker, 2008 b) suggest that different phylotypes of cyanobacteria in *X. muta*may confer varying abilities to translocate photoautotrophically derived organic products to the host. One study on *X. muta*, using isotopic tracers (e.g., $NaH^{13}CO_3$), shows that the bacterial community does readily fix carbon and translocate labeled products to the host (Fiore et al., 2013 b).

Whenthe symbiotic cyanobacteria of sponges are exposed to elevated pCO_2alone their abundance increasessignificantly with the most important predicted functional role of these symbionts being photosynthesis (Morrow et al., 2014), while elevated SWT alone has been shown to significantly decrease steady state quantum yields of PSII fluorescence in sponges with cyanobacteria (Cebrian et al., 2011). The results presented here show that the interactive effects

of OA and elevated SWT affect the photosynthetic apparatus of the symbiotic cyanobacteria in *Xestospongia muta* by decreasing the number of functional PSII units. Additionally, the sponges in this experiment not only experienced the mean predicted future values for the A2 climate model, but also the diel variability naturally experienced on shallow coral reefs (e.g., Hoffmann et al., 2011) where the extreme values can actually exceed the predicted A2 predicted climatology. Under these experimental conditions,*X. muta* showed a decrease in both the steady state and maximum quantum yields of PSII fluorescence. This is the result of either non-photochemical quenching with state transition changes as the underlying mechanism (Campbell et al., 1998), or chronic photoinhibition with damage to PSII that would result in a decrease in productivity (Gorbunov et al., 2001). While state transitions could potentially be affecting these measurements, both dark-adapted and effective quantum yields of PSII fluorescence show the same patterns. Since state transitions largely occur during darkacclimation (Campbell et al., 1998), if one compares maximum and effective yields state transitions appear to have had very little effect on the treatment specific patterns which are the same for both fluorescent measurements.These decreases in PSII quantum yields would then be mechanistically linked to lower rates of photosynthesis in cyanobacteria (Campbell et al., 1998), and the observed decrease in carbohydrate concentration in the holobiont.

Other studies have shown the effects of both elevated SWT and OA on sponges includingdecreases in the biosynthesis of secondary metabolites known to be involved in chemical defense (Duckworth et al.,2012). Additionally, studies on the excavating sponge *Cliona orientalis* have shown that the interactive effects of OA and elevated SWT can negatively affect energetic budgets, but sponge growth and reef bioerosion would still increase(Fang et al.,2013, 2014). Experiments using a multiplexed qPCR approach withsponge explants exposed

367 to elevated SWT have shown significant effects on host function as sponges became necrotic
368 during the experiment (Fanet al., 2013). In the same experiment the microbiome community of
369 necrotic sponge explants was significantly different from other treatment groups as were the
370 metagenomic and metaproteomic profiles (Fanet al., 2013).
371 The experiment presented here, using intact whole sponges, provides a more ecologically
372 realistic scenario on the effects of multiple stressors occurring simultaneously on this
373 sponge.While thesephysiological changes were not accompanied by significantcommunity
374 changes in the microbiome of *Xestospongia muta*, predictedOA, but not elevated SWT,
375 significantly increased the inter-individual variation in the composition (Fig.4a,c,e) and predicted
376 function (Fig.4b,d,f) of the *X. muta* microbiome. This may reflect a decreased ability of the host
377 to regulate its microbiome under stressful conditions. Along with this destabilization of sponge
378 microbiomes, significant changes in the predicted abundance of microbial 'photosynthesis
379 proteins'were observed using PICRUSt. This decrease in photosynthesis proteinsis consistent
380 with the significant decreasein the quantum yields of PSII fluorescence for the cyanobacterial
381 symbionts of *X. muta* in the A2predicted temperature and pH treatment group. PICRUSt is
382 dependent on the availability of reference genomes to translate microbial taxonomy into
383 predicted function, andthe NSTI values observed here are 2-3 times greater (~0.24 to 0.29 across
384 treatments, Fig. 3) than the gut microbiome of diverse terrestrial mammals but comparable with
385 other underexplored environments such as the Guerrero Negro microbial mats (NSTI =0.23)for
386 which insufficient genomic resources are available (Langille et al.,2013). Thus current function
387 predictions in sponges shouldserve to highlight the need for targeted cultivation and sequencing
388 of underexplored lineages in the sponge microbiome in order to improve the accuracy of future
389 predictions.

While it is true that genomic information for the symbionts of sponges and many other marine invertebrates is generally lacking,genome databases are populated with much genomic data for two key photosymbionts detected in these samples: the genus *Synecococcus* sp., including *CandidatusSynecococcus spongiarum*, a well described cyanobacterial symbiont of sponges (Burgsdorf et al., 2015) and Chloroflexi, a group ofphotoheterotrophic green non-sulfur bacteria that is tolerant of both elevated temperatures and lower pH.The latter increased by ~20% in both treatments exposed to elevated temperatures (Fig. 2). Choloroflexi are often found in syntrophic relationships with cyanobacteria, and the extensive literature on their roles in microbial mat physiology should inform future studies on their functional roles in sponges (Klatt et al., 2013).

5. Conclusions

Experiments simulating coastal environments, including coral reefs, are important sinceincreasing SWT and OA predicted by climate modelsfor open ocean ecosystems in the year 2100 already impacts these nearshore ecosystems. The datapresented here highlight how much remains to be discovered about the physiology of sponges and theirmicrobiomes under present or predictedclimate change conditions. The microbiome of *Xestospongia muta*is clearly less represented in the available genomes thanmany other organisms with microbial symbiontsor unexplored environments (Langille et al.,2013)which currently limits the predictive power to quantify their function, and changes in function, under predicted climate change scenarios using programs like PICRUSt. PICRUSt, however, has been used successfully here to show our best current estimate of the functional consequences of changes in sponge microbiomes based on available genomic data. A similar approach produced ecologically plausible predictions for a

413 con-specific sponge, *Xestospongia testudinaria*, with similar NSTI scores (de Voogd et al.,
414 2015). As discussed in previous works (de Voogd et al., 2015) comparisons of PICRUSt
415 accuracy using paired sponge samples deeply sequenced using both 16S rRNA amplicons and
416 shotgun metagenomics is needed in order to quantify whether PICRUSt's prediction accuracy in
417 sponges is more like soils (which achieved high accuracy despite NSTIs ~0.17), or more like the
418 Guerrero Negro hypersaline microbial mats.
419 Regardless, the results presented here indicate that *X. muta*and its microbiome
420 respondsnegatively to the independent effects of OA, and the interactive effects of elevated SWT
421 and OA at the physiological and organismal levels. The ecological consequences (e.g., decrease
422 in fitness), if any, for the effects of climate change related stressors are not currently known for
423 this ecologically important member of coral reef communities throughout the Caribbean basin
424 (McMurray et al., 2010). Recent studieshave clearly shown the importance of metabolic
425 interchange between the host and symbionts of *X. muta*(Fiore et al., 2015) such that
426 understanding the effects of predicted climate change on the physiology of *X. muta* and their
427 symbionts is important, and how those effects translate to the level of reef communities is
428 essential.
429
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597
598

599 **Figure Legends**

600 Figure 1. Treatment effects on the maximum ($F_v/F_{m,,}$ grey bars [midnight]) and steady state
601 ($\Delta F/F_m'$, white bars [midday]) quantum yields of photosystem II (PSII) fluorescence (mean ±
602 SE)for*Xestospongia muta*. Superscripts denote groups not statistically different from one
603 another using multiple comparison testing (Tukey's HSD).

604

605 Figure 2. Average relative abundance of OTUs (97% similarity) at the class level for each
606 treatment group for *Xestospongia muta*included unclassified Bacteria, unclassified Archaea,
607 Crenarchaeota, Euryarchaeota, Acidobacteria, Actinobacteria, AncK6, Bacteroidetes,
608 Chloroflexi, Cyanobacteria, Fermicutes, Gemmatimonadetes, H178, Nitrospirae, OD1,
609 PAUC34f, Poribacteria, Proteobacteria, SRB1093, Spirochaetes, TM7, Thermi and
610 Verrucomicrobia.

611

612 Figure 3. The Nearest Sequenced Taxon Index (NSTI, mean ± SD) for all treatment groups.
613 Samples with more organisms closely related to those with sequenced genomes have lower NSTI
614 scores.

615

616 Figure 4. Effects of pHand thermal stress on thestructure and function of *Xestospongia muta*
617 microbiomes. Box plots show the effects of predicted (IPCC 2007 A2 scenario) pH and
618 temperatures on sponge microbiomes. Phylogenetic β-diversity was measured using the
619 weighted UniFrac metric, while predicted functional β-diversity was assessed using the Bray-
620 Curtis distance between PICRUSt predicted functional profiles for each sample. Significance
621 between conditions was assessed by Bonferroni-corrected permutational *t*-tests. All pairwise

622 differences were tested, only those with Bonferroni-corrected $p < 0.10$ are shown. (A, B) Effects
623 of pH across all temperature regimes (i.e. Control andElevated Temperature treatments*vs.*
624 ReducedpH andReduced pH andElevated Temperature treatments). (C, D) Effects of
625 temperature across all pH regimes.(E, F) separate and combined effects of pH and temperature.
626
627 Figure. 5. Proximate biochemical composition (mean ± SE) of *Xestospongia muta* (μg mg^{-1}
628 tissue) for each treatment group. Superscripts denote groups not statistically different ($P>0.05$)
629 from one another using multiple comparison testing (Tukey's HSD).
630

Table

Table 1. Values for directly measured and calculated carbonate chemistry parameters for the experimental treatments. Parameters of carbonate seawater chemistry were calculated from TA, TCO_2, pCO_2, temperature and salinity using the free-access CO2Calc package.

Treatment	**TA (± SD)*** (µmol kg^{-1} SW)	**TCO_2 (± SD)*** (µmol kg^{-1} SW)	**pCO_2 (± SD)*** (µatm)	**pH_T#**	**Ω_{arg}#**
Control	2376 ± 26	2019 ± 32	408	8.035	4.02
Reduced pH	2403 ± 48	2162 ± 44	717	7.841	2.86
Elevated Temperature	2376 ± 19	2019 ± 27	444	8.005	4.15
Elevated temperature and Reduced pH	2403 ± 52	2162 ± 53	861	7.772	2.97

* -directly measured
# -calculated
SW -seawater
pH_T -total scale

FIGURE 1

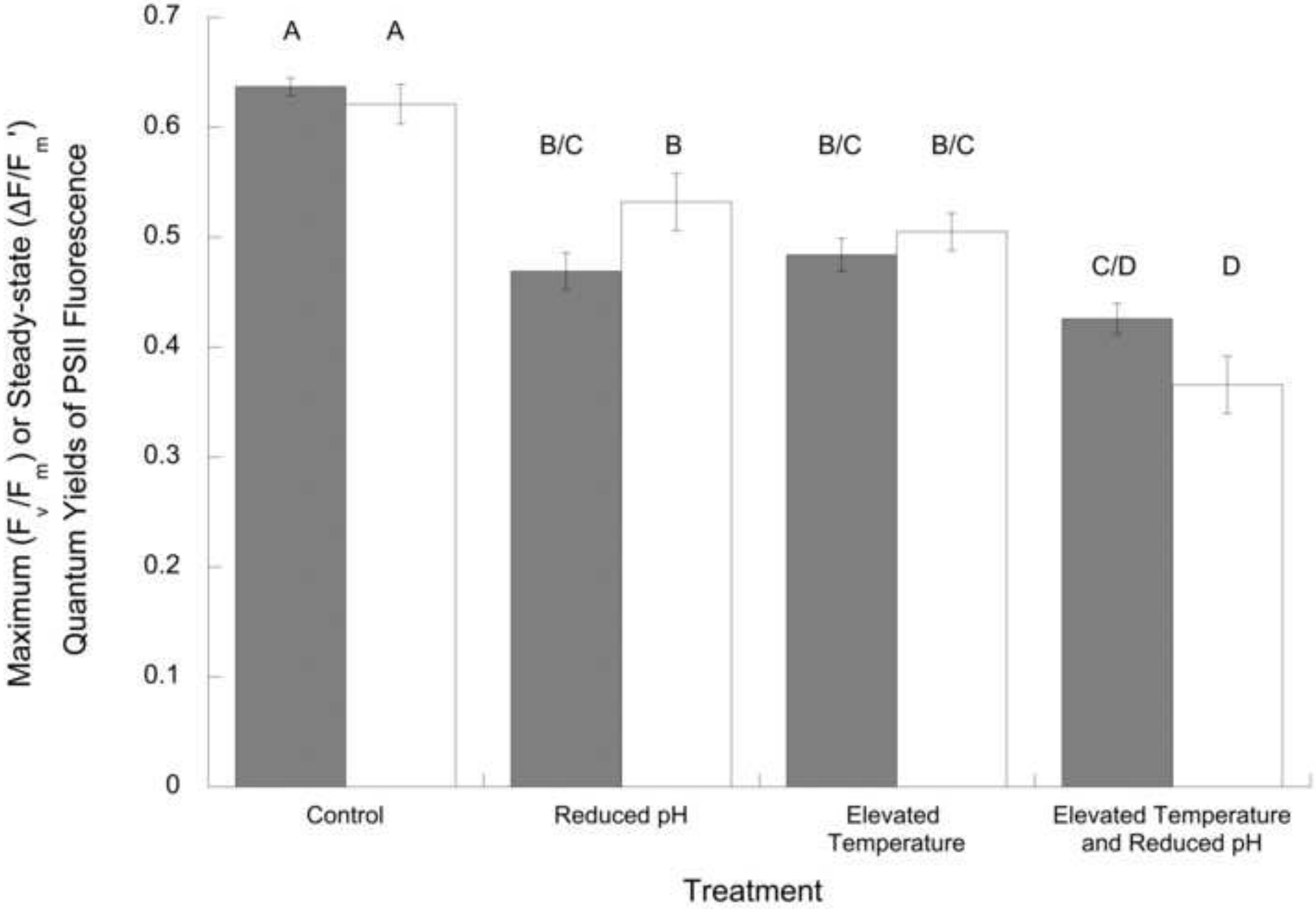

Maximum (F_v/F_m) or Steady-state ($\Delta F/F_m'$) Quantum Yields of PSII Fluorescence
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
A
A
B/C
B
B/C
B/C
C/D
D
Control
Reduced pH
Elevated Temperature
Elevated Temperature and Reduced pH
Treatment

FIGURE 2

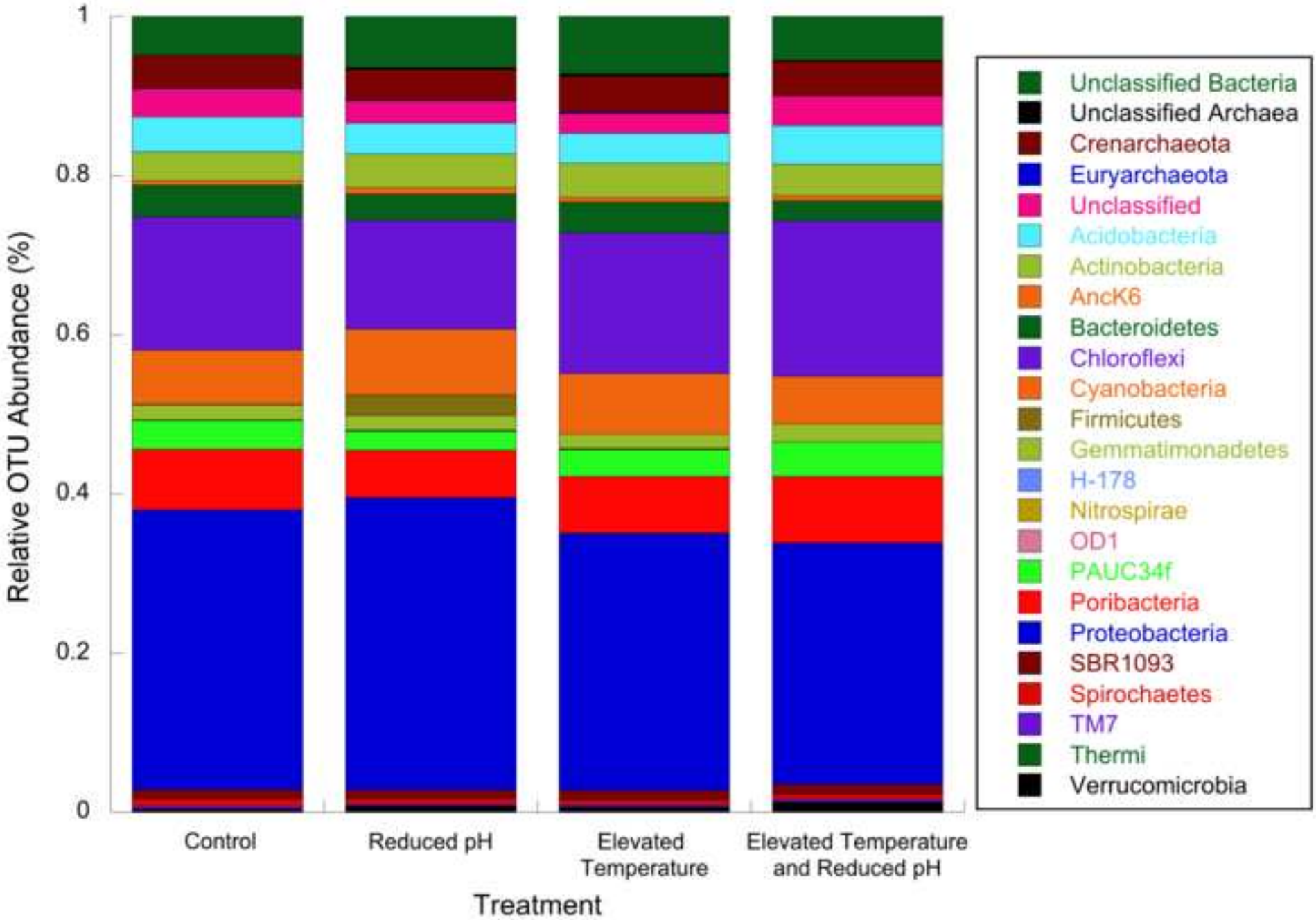

Relative OTU Abundance (%)
1
0.8
0.6
0.4
0.2
0
Control
Reduced pH
Elevated Temperature
Elevated Temperature and Reduced pH
Treatment
Unclassified Bacteria
Unclassified Archaea
Crenarchaeota
Euryarchaeota
Unclassified
Acidobacteria
Actinobacteria
AncK6
Bacteroidetes
Chloroflexi
Cyanobacteria
Firmicutes
Gemmatimonadetes
H-178
Nitrospirae
OD1
PAUC34f
Poribacteria
Proteobacteria
SBR1093
Spirochaetes
TM7
Thermi
Verrucomicrobia

FIGURE 3

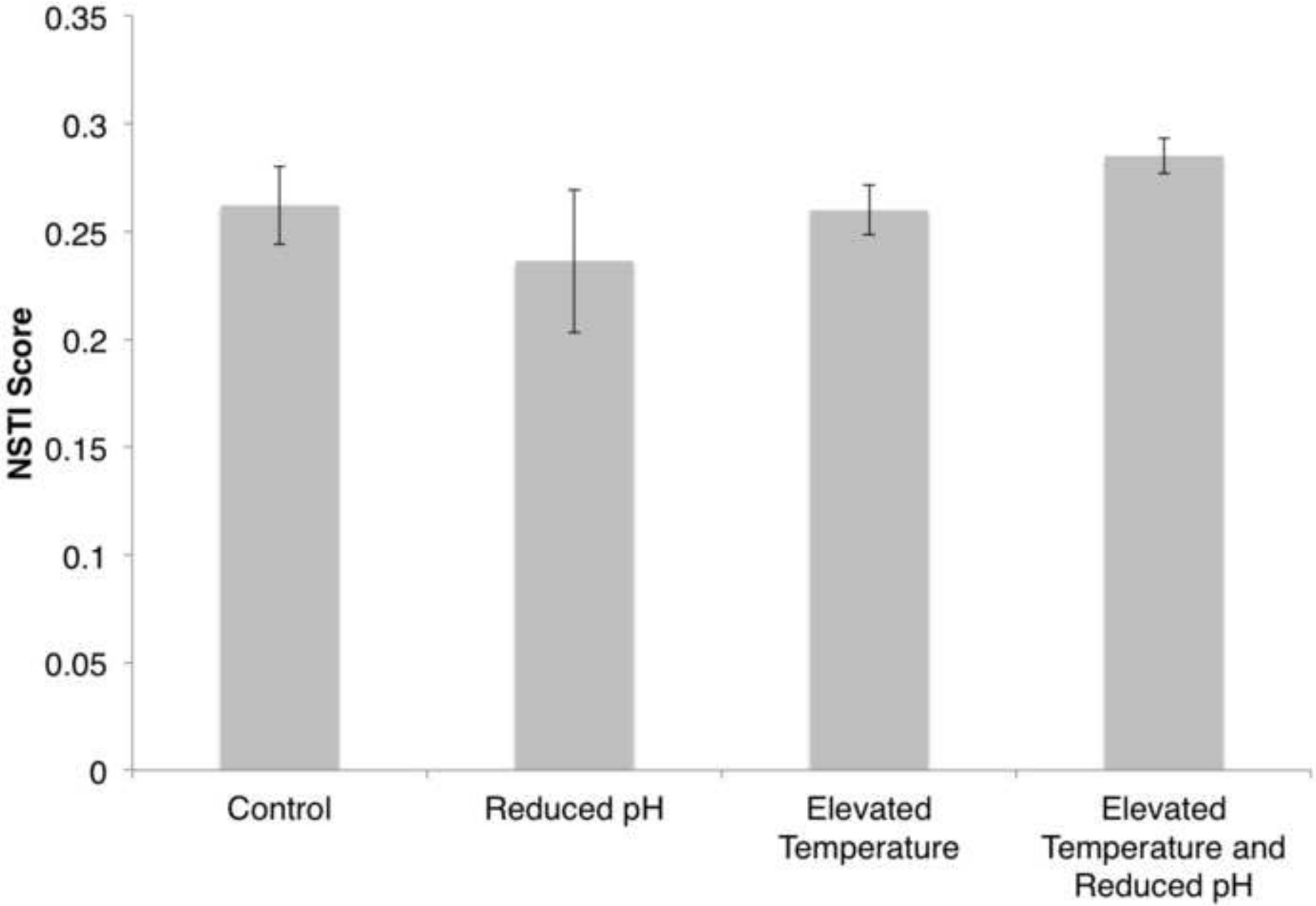

NSTI Score
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
Control
Reduced pH
Elevated Temperature
Elevated Temperature and Reduced pH

FIGURE 4

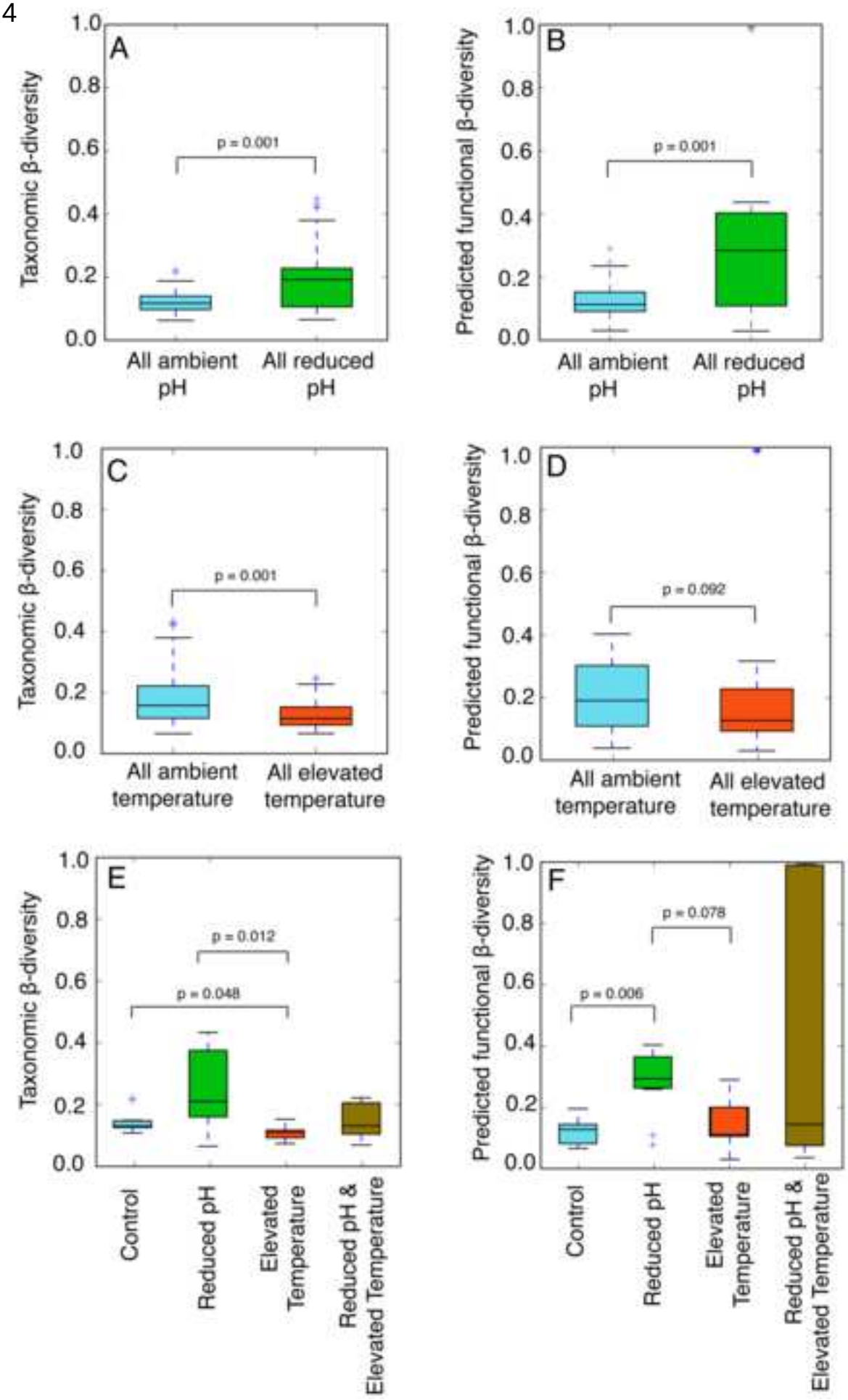
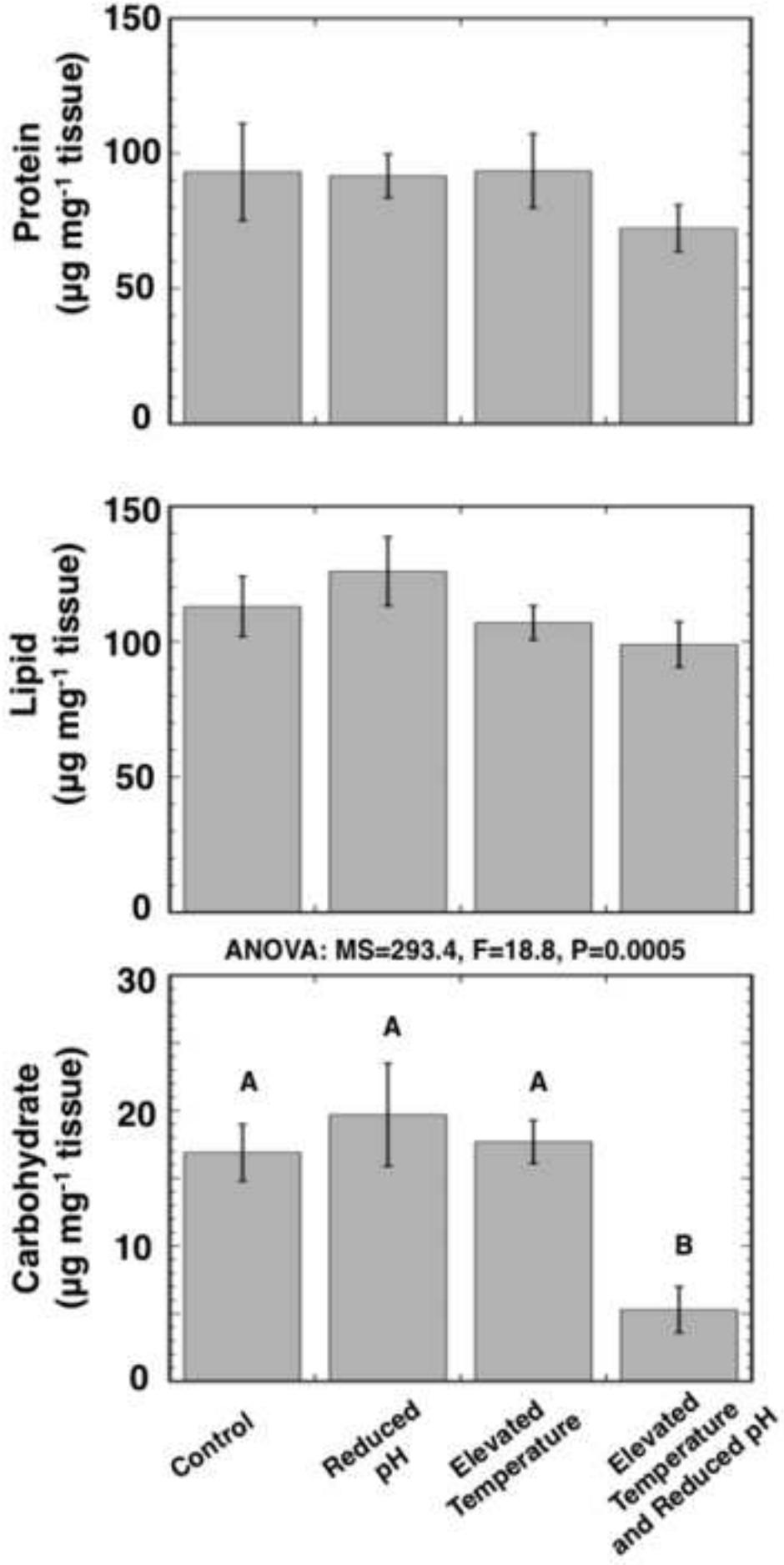

A
Taxonomic β-diversity
1.0
0.8
0.6
0.4
0.2
0.0
p = 0.001
All ambient pH
All reduced pH
B
Predicted functional β-diversity
p = 0.001
All ambient pH
All reduced pH
C
Taxonomic β-diversity
p = 0.001
All ambient temperature
All elevated temperature
D
Predicted functional β-diversity
p = 0.092
All ambient temperature
All elevated temperature
E
Taxonomic β-diversity
p = 0.012
p = 0.048
Control
Reduced pH
Elevated Temperature
Reduced pH & Elevated Temperature
F
Predicted functional β-diversity
p = 0.078
p = 0.006
Control
Reduced pH
Elevated Temperature
Reduced pH & Elevated Temperature

FIGURE 5


Protein (µg mg-1 tissue)
150
100
50
0
Lipid (µg mg-1 tissue)
150
100
50
0
ANOVA: MS=293.4, F=18.8, P=0.0005
Carbohydrate (µg mg-1 tissue)
30
20
10
0
A
A
A
B
Control
Reduced pH
Elevated Temperature
Elevated Temperature and Reduced pH