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The ecological impacts of multiple environmental stressors on coastal biofilm bacteria

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Abstract

Ecological communities are increasingly exposed to multiple interacting stressors. For example, warming directly affects the physiology of organisms, eutrophication stimulates the base of the food web, and harvesting larger organisms for human consumption dampens top-down control. These stressors often combine in the natural environment with unpredictable results. Bacterial communities in coastal ecosystems underpin marine food webs and provide many important ecosystem services (e.g. nutrient cycling and carbon fixation). Yet, how microbial communities will respond to a changing climate remains uncertain. Thus, we used marine mesocosms to examine the impacts of warming, nutrient enrichment, and altered top-predator population size structure (common shore crab) on coastal microbial biofilm communities in a crossed experimental design. Warming increased bacterial α-diversity (18% increase in species richness and 67% increase in evenness), but this was countered by a decrease in α-diversity with nutrient enrichment (14% and 21% decrease for species richness and evenness, respectively). Thus, we show some effects of these stressors could cancel each other out under climate change scenarios. Warming and top-predator population size structure both affected bacterial biofilm community composition, with warming increasing the abundance of bacteria capable of increased mineralization of dissolved and particulate organic matter, such as Flavobacteriia, Sphingobacteriia, and Cytophagia. However, the community shifts observed with warming depended on top-predator population size structure, with Sphingobacteriia increasing with smaller crabs and Cytophagia increasing with larger crabs. These changes could alter the balance between mineralization and carbon sequestration in coastal ecosystems, leading to a positive feedback loop between warming and CO_2 production. Our results highlight the potential for warming to disrupt microbial communities and biogeochemical cycling in coastal ecosystems, and the importance of studying these effects in combination with other environmental stressors.

KEYWORDS
bacteria, Bacteroidetes, biofilms, body size, food webs, metabarcoding, multiple stressors, nutrient enrichment, warming, α-diversity

1 | INTRODUCTION

Coastal marine ecosystems are among the most important providers of ecosystem services, but are also some of the most heavily exploited (Barbier et al., 2011). Despite only making up 4% of the Earth's total land area, more than a third of the World's population live in coastal environments and draw on the services they provide (Barbier, 2017). Due to this close synergy with the human population, coastal environments are disproportionately impacted by human activity (e.g. compared to the open ocean) and have already changed considerably from pre-industrial states (Jonkers et al., 2019). Marine food webs are underpinned by microbes, which despite their small size, drive global nutrient cycles due to sheer volume of numbers and unremitting activity (Hutchins & Fu, 2017). Yet, how microbial communities have, and will continue to respond to multiple anthropogenic stressors remains uncertain (Cavicchioli et al., 2019).

The balance between autotrophic and heterotrophic microbial activity plays a key role in balancing CO_2 budgets (Sarmento et al., 2010). Any perturbation to this balance could result in a positive feedback loop, escalating climate change. Of particular significance in the oceans are the rates of microbial degradation of high-molecular-weight biopolymers; which determine whether carbon fixed by autotrophs is mineralized as CO_2, or sinks into the deep ocean where it can be sequestered for hundreds of years (Eppley & Peterson, 1979). Coastal sediments are an important contributor to carbon sequestration, accounting for 13–28% of marine carbon storage at a rate many times the global ocean average (Bauer et al., 2013; Cai, 2011). It is therefore of paramount importance to understand the effects of multiple anthropogenic stressors, such as warming, nutrient enrichment, and altered top-predator (common shore crab) population size structure, on coastal marine microbial communities.

Under business-as-usual scenarios, the global mean sea surface temperature is expected to increase to 1.5°C above pre-1950 levels by 2050 and 3.5°C by 2100 (IPCC, 2019). Warming is generally predicted to lead to a decline in biodiversity for marine macro-flora and fauna (Gruner et al., 2017), but increases in species richness with warming have also been observed for microbial communities (Pold et al., 2015; H. Wang et al., 2019; Yvon-Durocher et al., 2015). Microbial respiration, turnover, and growth rates increase with warming, potentially driving these increases in microbial species richness (Rivkin & Legendre, 2001; Vázquez-Domínguez et al., 2007; White et al., 1991). This could be further exacerbated in biofilms under warming, since autotrophs extrude more organic matter as their activity increases, stimulating heterotrophic bacteria (Watanabe, 1980; Zlotnik & Dubinsky, 1989). This increased organic matter from autotrophs may stimulate the abundance, diversity, and activity of bacterial heterotrophs, particularly degraders of complex biomolecules (Saha et al., 2020; von Scheibner et al., 2014; Webster et al., 2011). Depending on how bacterial growth efficiency scales with respiration, this could result in a greater proportion of marine carbon being mineralized

as CO_2, rather than being exported to deep sediments via the 'biological carbon pump' (López-Urrutia & Morán, 2007; Rivkin & Legendre, 2001; Vázquez-Domínguez et al., 2007). Indeed, it has been shown that the carbon cycle will shift towards heterotrophic bacterial processes as the climate warms (Kvale et al., 2015; Vaqué et al., 2019).

Nutrient levels in coastal waters have risen over the last 200 years due to increasing industrialization of these areas (Smith et al., 1999). This will be further exacerbated in the coming century by increased runoff resulting from more frequent extreme precipitation events (Christensen & Christensen, 2003). With more nitrogen input into the oceans coming from anthropogenic sources, natural denitrification is overwhelmed, destabilizing coastal food webs, biogeochemical cycles, and associated ecosystem services (Gruber & Galloway, 2008). This can have a dramatic effect on microbial communities in coastal environments, including decreased diversity, increased abundance of denitrifiers, and coastal eutrophication (Graves et al., 2016; Wang et al., 2019). The relationship between increased productivity (caused by increased nutrients) and species richness depends on spatial scale, with flat, humped, or negative relationships often observed at local scales, and positive relationships at regional scales (Chase & Leibold, 2002; Harrison et al., 2006). At the local level, we expect diversity to decline as increased energy input to the base of food webs typically results in dominance of a few species via competitive exclusion due to reduced competition for basal resources (Huston, 1979; Rosenzweig, 1971). The associated decline in bacterial diversity may, in turn, reduce ecosystem functioning and increase the vulnerability of microbial communities to other stressors, even if diversity is increased at the regional level (Delgado-Baquerizo et al., 2016).

Size-selective harvesting of the oldest and largest top predators may result in selective pressure for rapid growth and early reproduction, causing changes in the size structure of marine communities (Enberg et al., 2012). Changes in community size structure can also interact with other stressors as they propagate through the food web. For example, differential top-down and bottom-up control in coastal food webs can alter the effects of nutrients and warming (Binzer et al., 2016). Loss of large fauna, or even just changes in their body size, can cause trophic cascades that alter the biomass of lower trophic level consumers and subsequently their resources at the base of the food web (Jochum et al., 2012; Myers et al., 2007; O'Connor & Bruno, 2007; O'Connor et al., 2013). These effects may propagate down to bacterial biofilm communities. For example, larger crabs should exert greater predation pressure on grazing arthropods, whose lower abundance may support more algal biofilms and associated bacteria, although the lower arthropod biomass may support fewer chitin degraders. It is therefore important to account for changes in top-predator populations as they might alter the interactions between bacteria and other stressors in unpredictable ways.

Environmental stressors such as warming, nutrient enrichment, and harvesting of top predators are not present in isolation and
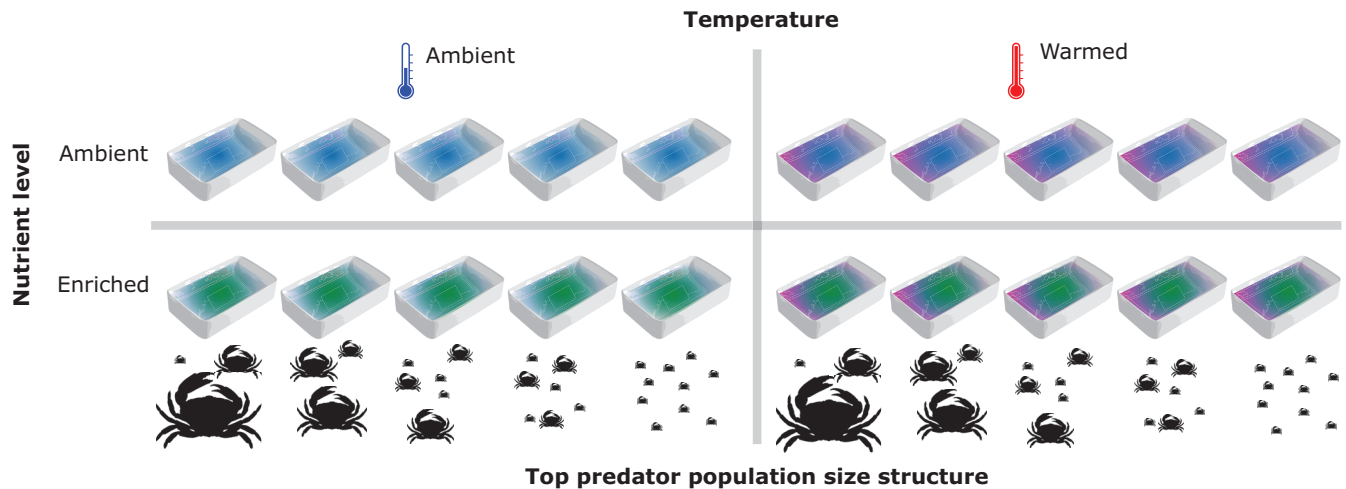
FIGURE 1 Schematic of the experimental design

often combine in surprising ways that cannot be predicted from their individual impacts (Crain et al., 2008; Vye et al., 2015, 2017). For example, the effects of warming on diversity may be reversed depending on nutrient status, or can be moderated by changes in community size structure (Binzer et al., 2016). Warming and nutrients can have synergistic effects on productivity (Davidson et al., 2018), but antagonistic effects on invertebrate interaction strengths have also been observed (Mcelroy et al., 2015). Changes in higher trophic-level taxa may even alter top-down control on bacterial biofilms, whose fates are inextricably linked with their hosts (Saha et al., 2020), but these food-web effects can also be modulated by other combined stressors, such as nutrient enrichment (O'Connor et al., 2015). It is therefore critical to assess the effects of climate change stressors in combination, rather than isolation.

Surfaces in marine environments are ubiquitously enveloped in complex agglomerations of microorganisms called biofilms (Flemming & Wuertz, 2019), which consist of bacteria, algae, protozoa, fungi, archaea, viruses, and metazoa bound in a matrix of extracellular polymeric substances. Biofilms are complex ecosystems in their own right, but they also interact with the wider environment, playing important roles in nutrient cycling, degradation of pollutants, and photosynthesis (Flemming et al., 2016). As the interface between their constituent taxa and the wider environment, biofilms are a crucial aspect of these organisms' ecology (Besemer, 2015). This makes bacterial biofilms foundational to understanding how the effects of anthropogenic stressors will propagate through complex marine food webs.

Using marine tidal mesocosms that mimic naturally occurring temperate rock pools, we investigate the effect of multiple stressors (warming, nutrient enrichment, and top-predator population size structure) on bacterial biofilm communities to test the following hypotheses: (H1) Warming will lead to more diverse bacterial biofilms, with more degraders of complex biomolecules; (H2) Nutrient enrichment will lead to less diverse bacterial biofilms with a less even distribution of diversity; and (H3) Decreasing top-predator population

size structure will lead to a reduction in bacteria associated with arthropods (e.g. chitin degraders) and an increase in the relative abundance of bacteria associated with algae.

2 | MATERIALS AND METHODS

2.1 | Experimental design

We used a subset of treatments from an outdoor marine mesocosm experiment conducted at Queen's University Marine Laboratory in Portaferry, Northern Ireland from April to June 2013 (see Mcelroy et al., (2015) for full experimental details). Briefly, 100 × 45 L tanks were constantly supplied with gravel-filtered seawater from the adjacent Strangford Lough, delivered *via* overhead dump buckets that simulated the turbulence of the natural rocky shore. The coarse filter allowed the passage of microbes, meiofauna, and algal spores, ensuring that each mesocosm experienced a semi-natural degree of connectivity to the Lough. Living algal specimens consisting of *Cladophora* spp. (4 g), *Corallina* spp. (16 g), *Fucus serratus* (21 g), *Mastocarpus stellatus* (3 g), and *Ulva lactuca* (1 g) were introduced to each mesocosm at the outset of the experiment. These were collected from natural assemblages on the shoreline of Strangford Lough (which is adjacent to the laboratory) to provide a simplified community representative of the dominant algae in the Lough (in addition to the micro-algae supplied through the natural seawater). The algae and mesh where washed with a pyrethrum-based pesticide (10 g L^{-1} Vitax Py Spray Insect Killer Concentrate) to remove epifauna before they were introduced to the mesocosms. Pyrethroids work by disrupting nerve function in insects and are reported to have low toxicity to non-target taxa (including macroalgae), they are also rapidly degraded in the environment so we expect no side effects of the pesticide wash on the algae (Downes et al., 2000). A random mix of 125 amphipods (consisting of *Chaetogammarus* spp., *Gammarus* spp., *Orchestria* spp., and *Talitrus* spp.) was also added to each tank on six occasions over the course of the experiment. *Chaetogammarus*

spp. and *Gammarus* spp. were by far the dominant species in these additions, so any species-specific effects should have been small.

The experimental treatments involved temperature (two levels: ambient and warmed) crossed with nutrient enrichment (two levels: ambient and enriched), and top-predator population size structure (a continuous variable; Figure 1). Temperature was elevated by $3.5 \pm 0.4°C$ (mean ± standard error) using Elite 300 W aquarium heaters. Nutrient enrichment was achieved by addition of 140 g of Osmocote Pro 3–4 Months slow-release fertilizer pellets (consisting of 5 N: 1 P: 2 K), contained in four perforated 50 ml tubes. Identical tubes containing gravel were included as procedural controls in the unenriched mesocosms. The population size structure of the common shore crab (*Carcinus maenas*) was manipulated in an allometric design (Schneider et al., 2012), where the number of individuals increased as the average body size decreased to mimic the size structure of natural populations (Mcelroy et al., 2015). Note that while the shore crab is commercially fished in Western Europe and the east coast of North America (Klassen & Locke, 2007), we are using it here as a model organism for studying the community-level impacts of changes in top-predator size structure, rather than looking at the species-level response of shore crabs to fishing pressure.

Sampling took place immediately after the end of the 6-week experiment. Biofilms from five replicates of the four temperature × nutrient combinations were sampled, ensuring the full range of top-predator body size treatments was captured across the replicates for each combination. This involved scraping a sterilized razor blade (43 mm wide) in a straight line from the bottom to the top of each mesocosm (approximately 235 mm tall) for a scraped area of approximately 100 cm^2. The scraped biofilm was immediately transferred to a sterilized 1.5 ml tube, topped up with 96% ethanol, and frozen at −20°C until processing. Note that we chose a central starting location for each scrape on a side with the same orientation for each mesocosm.

2.2 | Bacterial biofilm community characterization

The bacterial biofilm community structure was characterized by Illumina MiSeq amplicon sequencing of the V3-4 region of the bacterial 16S rRNA gene, which has been widely used in marker gene studies to target bacterial diversity (Clark et al., 2018; Ferguson et al., 2016, 2017). DNA was extracted from a 250 mg (wet weight) subsample of each biofilm scrape using MoBio PowerSoil DNA isolation kits (MoBio Laboratories, Inc.) following the manufacturer's instructions.

Amplicon libraries were prepared using a two-step PCR. First the V3-4 region of the bacterial 16S rRNA gene was PCR amplified using the primers 341F 5'-CCTACGGGNGGCWGCAG-3' and 805R 5'-GACTACHVGGGTATCTAATCC-3' modified with Illumina-specific overhang adapter sequences, the reverse primer was further modified with a 12 base pair (bp) Golay barcode (ATCACCAGGTGT) and linker sequence (CC; Caporaso et al., 2012). PCR mixtures (25 µl) consisted of 1 µl DNA template, 12.5 µl of 2x KAPA HiFi HotStart

ReadyMix (Roche), and 0.2 µM of each primer. Thermocycling consisted of 95°C for 30 s, 25 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 30 s, with a final elongation of 72°C for 10 min, and was performed in a Gene Amp® PCR system 9700 Thermocycler (Applied Biosystems). The PCR products were cleaned using AMPure XP beads (Beckman Coulter) according to the manufacturer's instructions. Unique pairs of eight-base indexes (Nextera XT v1, Ilumina) were then attached to PCR products using a secondary eight-cycle PCR (Illumina, 2013). The indexed PCR products were then cleaned using AMPure XP beads (Beckman Coulter) and quantified with a Quant-iT dsDNA assay kit (Thermo Fisher Scientific) with a NanoDrop 3300 fluorospectrometer (Thermo Fisher Scientific) according to the manufacturer's instructions. The samples were then mixed in equimolar amounts and sequenced on a MiSeq System (Illumina) with a MiSeq Reagent Kit v2 (2 × 300 bp sequences) at The Earlham Institute (formerly The Genome Analysis Centre, Norwich Research Park, Norwich, NR4 7UH, UK). Raw sequence data have been submitted to The National Center for Biotechnology Information (NCBI) Sequence Read Archive under the Accession no. PRJNA714953.

2.3 | Bioinformatics

MiSeq reads were analysed following the guidelines for paired-read Illumina amplicon libraries in Dumbrell et al., (2017). Briefly, quality filtering was carried out with SICKLE (Joshi & Fass, 2011), and reads were trimmed from the 3′ end when the PHRED score over a 30 bp window dropped to <30. Error correction was carried out on the trimmed reads with BayesHammer (Nikolenko et al., 2013) implemented with the default settings in SPAdes v3.7.1 (Bankevich et al., 2012). The paired forward and reverse reads were then paired-end aligned and primers removed using the PEAR algorithm (Zhang et al., 2014) implemented in PANDAseq (Masella et al., 2012). Further quality filtering was carried out in Mothur v1.35.1 (Schloss et al., 2009) to remove reads with ambiguous bases (N's), homopolymer inserts longer than eight, or that were overly short or long in comparison with the target amplicon length (<400 bp or >450 bp). The quality filtered paired aligned reads were then clustered into de-novo operational taxonomic units (OTUs) at a 97% similarity threshold using VSEARCH v2.1.2 (Rognes et al., 2016). Chimeric sequences were removed using de-novo chimera checking in UCHIME (Edgar et al., 2011) retaining 1906 OTUs. The representative reads from each OTU were then assigned a taxonomic group using the RDP classifier algorithm (Wang et al., 2007). OTUs assigned to chloroplast or cyanobacteria were removed from the dataset as these may originate from plastid genomes and not bacteria. At this point, one sample (a replicate of warming and enrichment with average crab body size of 1.7 g) was removed from further analysis due to only containing a few poor-quality reads (<5% of the average library size in the other samples). As normalization to account for uneven sequencing depth between samples is required for metabarcoding data, read depth in each sample was normalized by rarefaction to the same sampling depth of 9146 reads across all

the libraries following recommendations in Weiss et al., (2017) and McKnight et al., (2019).

To determine whether the functional potential of the bacterial biofilm community was changing with the shifts in microbial community structure, a metagenome was inferred for each sample with Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt2) v2.2.0-b (Douglas et al., 2020). In short, the OTUs were aligned and placed into a reference tree of 16S rRNA sequences (Barbera et al., 2018; Czech et al., 2020) with known genomes from the Integrated Microbial Genomes Database (Markowitz et al., 2012). Gene family abundances (EC numbers) were then predicted with Castor (Louca & Doebeli, 2018) with a Nearest-sequenced taxon index cut-off of two to remove poor-quality matches. Metagenomes for each sample were constructed and pathway-level abundances (MetaCyc; Caspi et al., 2013) were then predicted based on EC number with MinPath (Ye & Doak, 2009).

2.4 | Statistical analysis

For α-diversity, we calculated species richness (the total number of OTUs) and the reciprocal of Simpson's index so that higher values represent a more even community (1/D). Simpson's index was calculated based on OTU read abundances after normalization by rarefaction as previously described. As our analyses are based on OTU read abundances generated from amplicon samples with the same potential biases inherent to all PCR-based metabarcoding, we followed all current guidelines to minimize biases Zinger et al., (2019), and have no evidence to suggest that if any biases were present that they would be quantitatively different across samples. Generalized linear models were used to test the main effects of temperature, nutrient enrichment, and crab body size, and the interaction between temperature and nutrient enrichment, on α-diversity (Bates et al., 2014). The interactions between crab body size and the other predictors were not fitted as there was insufficient replication for the comparisons. We used a negative binomial error term for species richness (Venables & Ripley, 2002) and a Poisson error term for community evenness. The generalized linear models were simplified by stepwise deletion from the maximal model until the minimum adequate model was found. Whenever crab body size was removed as a main effect, it was inserted as a random effect in a generalized linear mixed model to control for variation due to crab body size in the temperature and nutrient treatments. We ensured that the final models conformed to the assumptions of normality, homogeneity of variance, and independence.

We used multivariate generalized linear models (with a negative binomial error term to account for overdispersion) to explore changes in community composition (Wang et al., 2012). The normalized OTU abundances were fitted to the predictors and simplified to the minimum adequate model as described for α-diversity. Multivariate and univariate p values were obtained by log-likelihood ratio tests and model-free bootstrapping with 10,000 permutations of probability integral transform residuals. All statistical analyses were carried out in R 3.6.3 (R Development Core Team, 2019), the figures were prepared with the R package 'ggplot2' (Wickham, 2016), the sequence data were processed with the R package 'Phyloseq' (McMurdie & Holmes, 2013), generalized linear models and generalized linear mixed models were fitted with the R package 'lme4' and 'MASS' (Bates et al., 2014; Venables & Ripley, 2002), and multivariate generalized linear models were fitted with the R package 'Mvabund' (Wang et al., 2012).

3 | RESULTS

3.1 | Effects of multiple stressors on α-diversity

Warming and nutrient enrichment had significant, yet opposite effects on species richness and diversity (Table 1; Figure 2). Warming increased species richness and resulted in a more even distribution of diversity (Figure 2a,c; Hypothesis one), whereas nutrient enrichment resulted in a less diverse community dominated by only a few OTUs (Figure 2a,c; Hypothesis two). There was no significant interaction between warming and nutrient enrichment, that is, the effects combined additively to cancel each out such that the positive effect of warming on diversity was counteracted by the negative effect of nutrient enrichment. There was also no evidence for a

TABLE 1 Generalized linear models describing changes in α-diversity (species richness and community evenness), community composition, and the relative abundance of functional pathways from metagenomes predicted by PICRUSt2 with respect to warming (Warm), nutrient enrichment (Nut), top-predator population size structure (Size), and the warming × nutrient interaction (Warm:Nut). Average crab body size was fitted as a random factor following model simplification for α-diversity. Subscript p values are for the terms that were removed during model selection. The coefficients have been back-transformed for ease of interpretation

	Species richness			Community evenness			Community composition		Predicted functional capacity	
	$\chi^2_{(df)}$	β^a	p	$\chi^2_{(df)}$	β	p	$\chi^2_{(df)}$	p	$\chi^2_{(df)}$	p
Warm	$13.7_{(1,17)}$	85	**<.001**	$111_{(1,17)}$	22.9	**.002**	$3492_{(1,17)}$	**.001**	$2659_{(1,17)}$	**.016**
Nut	$18.4_{(1,17)}$	−65	**<.001**	$90_{(1,17)}$	−9.3	.051	$2371_{(1,17)}$	.086		.486
Size	*Added to random effects*		0.102	*Added to random effects*		.763	$2852_{(1,17)}$	**.013**		.19
Warm:Nut			.609			.9031		.104		.378

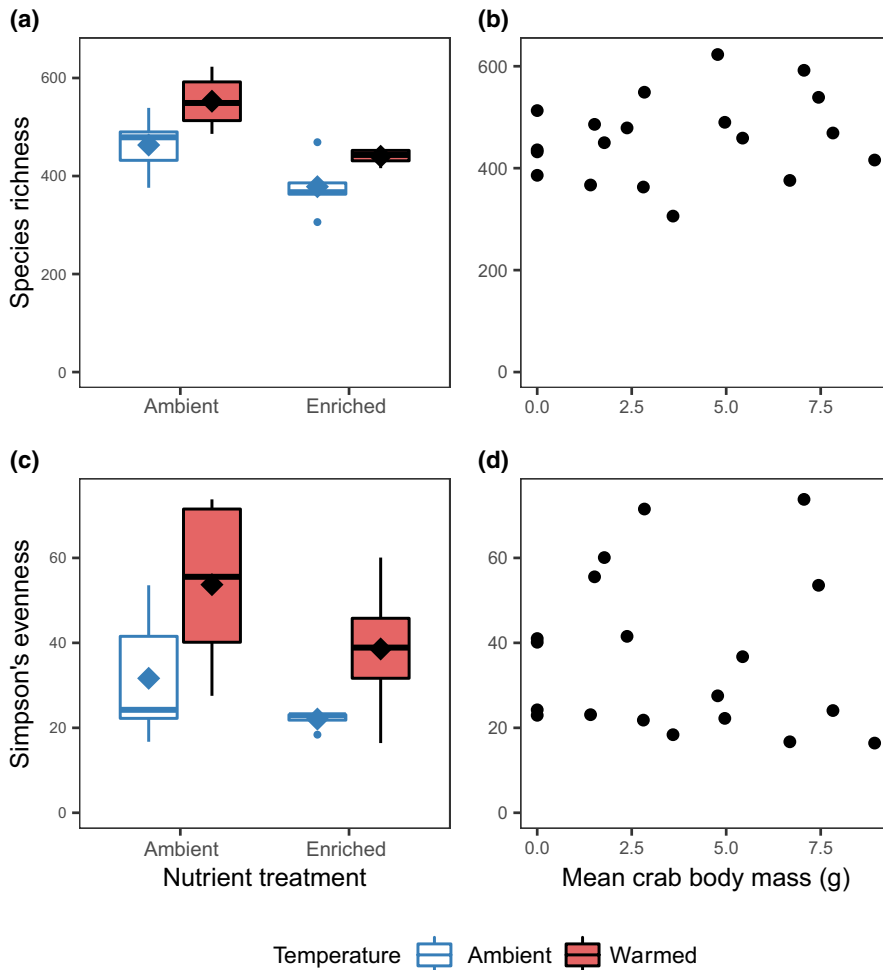^{a}β denotes back-transformed model coefficients.

FIGURE 2 Effects of warming and nutrient enrichment (a, c), and top-predator body size (b, d) on the α-diversity measures, species richness, and community evenness. For boxplots, the diamonds denote the mean, the boxes show the 25th, 50th (median), and 75th percentiles, and the whiskers extend to the most extreme datapoint no more than 1.5 times the interquartile range

top-down effect of top-predator population size structure on either measure of α-diversity (Figure 2b,d).

## 3.2 | Effects of multiple stressors on bacterial community composition

The Classes Alphaproteobacteria (38%), Flavobacteriia (13%), Verrucomicrobiae (10%), and Gammaproteobacteria (8%) dominated the bacterial biofilm community. Both warming and crab body size had significant effects on bacterial community composition (Table 1; Hypothesis one and three). Warming had a greater influence over bacterial community structure than top-predator population size structure, with the abundance of 911 of the 1468 OTUs best explained by warming (ΔAIC <0; Figure S1a). Nutrient enrichment did not have a significant effect on the bacterial biofilm community and there was no significant interaction between warming and nutrient enrichment (Table 1).

Warming had a significant effect on the relative abundance of 203 OTUs (Figure S1b), most of which were from the phyla Proteobacteria and Bacteroidetes (122 and 52 OTUs, respectively). Among the dominant taxa, the most notable changes with warming were increases in the relative abundance of Bacteroidetes and Gammaproteobacteria, and a decrease in the relative abundance of

Alphaproteobacteria (Figure 3). OTUs from Bacteroidetes almost exclusively increased with warming (Figure S1b); with Sphingobacteriia, Flavobacteriia, and Cytophagia showing 2-, 6-, and 6.5-fold increases in relative abundance with warming, respectively (Figure 3; Hypothesis one). Proteobacteria OTUs showed a mixture of negative and positive changes in abundance (Figure S1b); but overall Alpha- and Deltaproteobacteria decreased with warming (2- and 1.6-fold decreases respectively), and Gamma- and Epsilonproteobacteria increased with warming (3.5- and 11-fold increases, respectively; Figure 3). There were also significant increases in relative abundance with warming for Planctomycetes (5.2- and 8.6-fold increases for classes Phycisphaerae and Planctonmycetia, respectively) and the Classes Opitutae, Deinococci, and Chitinivibrionia (which were absent at ambient temperature). There was a significant decrease with warming in the relative abundance of Verrucomicrobiae (5.9-fold decrease) and Clostridia (not present with warming).

Although nutrient enrichment had no overall effect on the bacterial assemblage (Table 1), there were 81 OTUs for which relative abundance or presence was individually correlated with nutrient enrichment (Figure S1b). In general, nutrient enrichment decreased the relative abundance of OTUs (64 OTUs negative $\log_2$-fold change; Figure S1b) with 20 of these OTUs entirely absent from nutrient-enriched mesocosms. A core of 17 OTUs significantly increased in relative abundance with nutrient enrichment (Table S1), the most

FIGURE 3 Mean changes in relative abundance of bacterial classes with warming and crab body size. Points show the average log$_2$-fold change in relative abundance for the OTUs significantly correlated with warming or crab body size (multivariate generalized linear models). Colour denotes phylum and size denotes the number of significant OTUs. See Figure S1b for the log$_2$-fold change for the individual OTUs for all factors
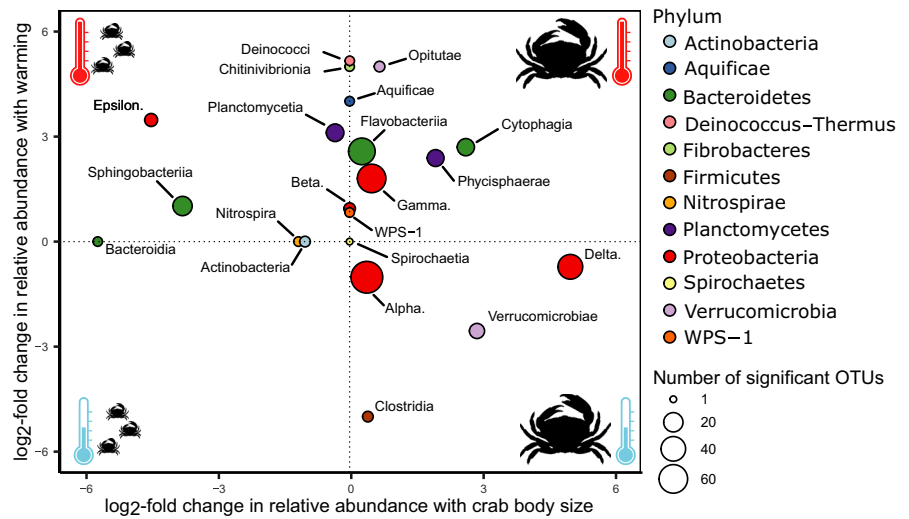
notable (in terms of total relative abundance) was two OTUs assigned to the Alphaproteobacteria genera *Sphingorhabdus* and *Sulfitobacter*, which made up 2.1% and 5% of the total community in nutrient-enriched mesocosms respectively (being the highest for any single OTU). Although there were no significant interactions at the community level, 12 OTUs were significantly correlated with nutrient enrichment and warming (Table S2). In all cases, the effects cancelled each other out, that is, abundance increased with warming and decreased with nutrient enrichment.

Top-predator population size structure had a significant effect on the relative abundance of 118 OTUs, mostly from the classes Alphaproteobacteria, Flavobacteriia, and Gammaproteobacteria (33, 23, and 25 OTUs, respectively; Figure S1b). However, the average change in abundance for the aforementioned groups was small (<1.5-fold change). There were large increases in abundance with crab body size for Deltaproteobacteria, Cytophagia, Verrucomicrobiae, and Phycisphaerae; and decreases for Sphingobacteriia, Epsilon-proteobacteria, and Bacteroidia (Figure 3). However, crab body size also modulated the overall effects of warming. For example, the phylum Bacteroidetes had a positive correlation with warming, but the increase was due to Cytophagia when crabs were large and Sphingobacteriia when crabs were small (Figure 3). The relative abundance of 27 OTUs were correlated with both crab body size and warming (Table S2). In all cases, the effect was in the same direction for both predictors, that is, increasing (or decreasing) crab body size resulted in more positive (or negative) effects of warming on relative abundance.

3.3 | Effects of multiple stressors on predicted functions of bacterial communities

Warming was the only factor to have a significant effect on the predicted functional potential of the bacterial communities (Table 1), indicating that the aforementioned shifts in community composition due to warming could result in functionally distinct communities. There was an increase in predicted abundance of pathways for the processing of complex carbohydrates (e.g. starch degradation III, chitin derivatives degradation, and Bifidobacterium shunt; Figure S2) and subsequent processing of degradation products (e.g. fucose and rhamnose degradation; Figure S2), supporting our first hypothesis. Warming also increased the predicted abundance of genes related to cycling of C1 compounds to CO_2, and genes involved with nitrogen and sulphur cycling (e.g. nitrate reduction VI, sulphate reduction I; Figure S2). Genes predicted from pathways involved in arginine degradation (a key chemical in biofilm regulation) also increased with warming. Predicted genes related to bacterial autotrophy, specifically the synthesis of photosynthetic pigments in Proteobacteria, decreased with warming.

4 | DISCUSSION

Our results support the growing number of studies demonstrating an increase in bacterial diversity with warming (Pold et al., 2015; Wang et al., 2019; Yvon-Durocher et al., 2015). This may indicate a greater resilience of coastal biofilm communities to climate change compared with higher trophic-level organisms (Delgado-Baquerizo et al., 2016; Gruner et al., 2017). More diverse bacterial communities tend to cycle a wider range of carbon substrates, which may reduce the amount of carbon sequestered by coastal ecosystems (Zhang et al., 2018). Changes in bacterial community composition associated with diversity are thus key to understanding the consequences of warming. Here, warming resulted in a bacterial community capable of degrading complex biomolecules (e.g. complex polysaccharides such as chitin), supporting our first hypothesis. This was evidenced by increases in the Bacteroidetes classes Sphingobacteriia, Flavobacteriia, and Cytophagia; Proteobacteria class Gammaproteobacteria; PVC group classes Phycisphaerae, Planctonmycetia, and Opitutae; and chitin-degrading group Chitinivibrionia. These groups are common in biofilms, and are all known to be degraders of complex biological polymers (Cottrell & Kirchman, 2000; Fukunaga et al., 2009; Reichenbach, 2006). These observed shifts may have been

driven by increased autotrophic activity and resulting exudation of organic matter, stimulating heterotrophic biofilm bacteria (Watanabe, 1980; Zlotnik & Dubinsky, 1989). Similar shifts have been observed in Phyto-Bacterioplankton, including tighter coupling between autotrophs and heterotrophs (von Scheibner et al., 2014; Vázquez-Domínguez et al., 2007). Warming favours a higher contribution to marine primary production from picophytoplankton (Morán et al., 2010; Sarmento et al., 2010), which will elevate the importance of the 'microbial loop' under warming scenarios across all marine habitats. Consequently, the balance between marine microbial autotrophy and heterotrophy will be of key importance in global carbon budgets under warming scenarios (Sarmento et al., 2010).

The observed increase in predicted functional pathways for degradation of complex biomolecules with warming provides further evidence to support our first hypothesis. Sinking of particulate organic matter (marine snow, consisting of complex biological polymers) transports carbon into the deep ocean, where it can be buried in sediment for thousands of years (Eppley & Peterson, 1979). This process, known as the biological carbon pump, removes about a third of anthropogenic carbon (Sabine et al., 2004). But the efficiency of this mechanism is determined by the rate of microbial degradation of marine snow, which determines how much marine snow is mineralized as CO_2 before it sinks into the deep ocean (Kwon et al., 2009). The aforementioned bacterial groups that we found to be enriched by warming are commonly attached to marine snow (DeLong et al., 1993; Vojvoda et al., 2014), where they are key degraders of complex biomolecules (Fontanez et al., 2015; Lyons & Dobbs, 2012). Therefore, increases in their abundance or activity could divert marine carbon from long-term sequestration back into food webs, ultimately resulting in a positive feedback loop between CO_2 production and warming. This hypothesis requires further testing, the approach used in this study can only infer the function of the bacterial community based on matching OTUs with known genomes (Douglas et al., 2020). Metagenomics (or metatranscriptomics) could be used to confirm these genes are present in the community or direct measurement of carbon cycling processes (such as Biolog microarrays) could be used to quantify changes in rates and diversity of carbon molecules processed by bacteria.

Nutrient enrichment led to a less diverse microbial community, dominated by fewer taxa, supporting our second hypothesis. This has been observed elsewhere for bacterial communities in coastal lagoon and wetland sediments (Kearns et al., 2016), but not universally (Bowen et al., 2011). This decrease in diversity was driven by decreases in relative abundance, as well as local (and sometimes total) extinction of taxa in the nutrient-enriched conditions, contrasted with increase in relative abundance of a few dominant taxa (especially Alphaproteobacteria), thus lowering the overall diversity of the community through non-targeted, competitive exclusion (Huston, 1979; Rosenzweig, 1971). The specific Alphaproteobacteria OTUs with the highest relative abundance after nutrient enrichment (*Sphingorhabdus* and *Sulfitobacter*) are both degraders of xenobiotics

and in the case of *Sulfitobacter* associated with organic sulphur cycling (Jiao et al., 2011; Silva et al., 2018). Although we saw no associated changes in the predicted functional potential of the bacterial community, lower diversity tends to erode resilience and leave ecosystems more vulnerable to other stressors (Delgado-Baquerizo et al., 2016).

The effects of nutrient enrichment and warming on bacterial richness and diversity were in direct opposition. A similar cancelling effect of these two stressors was shown for interaction strengths between crabs and the amphipod and algal assemblages in the same experiment (Mcelroy et al., 2015). These opposing effects may enable less competitive bacteria to persist in nutrient-rich coastal ecosystems exposed to warming, maintaining microbial biodiversity. The effect of nutrient enrichment is to flood the system with readily accessible resources, whereas warming increased the processing of more recalcitrant substrates (as indicated by the associated taxonomic shifts). Therefore, the expected increase in bacterial diversity with warming was undermined by the greater availability of resources in the presence of nutrient enrichment. This highlights the importance of studying multiple stressors in the context of climate change, given the potential for some stressors to counteract the effects of others (Crain et al., 2008). For example, modelling studies have shown that warming can have a positive effect on diversity in eutrophic conditions and a negative effect in oligotrophic systems (Binzer et al., 2016). Although we did not observe an interactive effect such as this, the combination of stressors was nevertheless important, as they negated each other's overall effects.

The impacts of warming and nutrients on food-web structure are also predicted to depend on population size structure (Binzer et al., 2016). Our results showed that decreasing top-predator body size altered bacterial community composition, but not α-diversity, partially supporting our third hypothesis. One potential mechanism for this could be a cascading effect of reduced predation by smaller crabs on grazing arthropods, whose greater abundance could thus suppress algal biofilm growth (Jochum et al., 2012), with subsequent changes in the bacterial communities associated with both groups. In support of this, the abundance of Bacteroidia, Sphingobacteriia, Actinobacteria, and Epsilonproteobacteria increased with decreasing top-predator body size, which could be associated with increasing arthropod abundance. These bacterial groups are commonly found in the gastrointestinal tracts of animals (Binda et al., 2018; Xu & Gordon, 2003), and Actinobacteria are a particularly common constituent of the marine arthropod microbiome (Cornejo-Granados et al., 2018). Other typical members of the marine arthropod microbiome did not vary with top-predator population size structure, however (e.g. Alphaproteobacteria, Gammaproteobacteria, and Verrucomicrobiae). Therefore, changes in the relative abundance of bacterial groups cannot be explained entirely by effects of top-predator population size structure on arthropod abundance. We also did not detect any evidence for cascading effects on algal biofilm bacteria (e.g. Alpha- and Gammaproteobacteria).

Crabs may directly affect bacterial biofilms, in addition to their indirect effects through the food web. For example, smaller crabs are more active, which may disturb bacterial biofilms, and they are more likely to feed on biofilms (as observed during the experiment). In support of this, treatments with smaller crabs had a greater abundance of bacterial groups considered to be early colonizers of marine biofilms (Sphingobacteriia and Actinobacteria; Battin et al., 2016; Lee et al., 2008) and those associated with bioturbation (Epsilonproteobacteria; Ashforth et al., 2011). They also contained fewer bacterial predators, which implies that the biofilms associated with those treatments were less mature (Williams et al., 2016). In contrast, treatments with larger crabs had a greater abundance of slow-growing, late biofilm colonizers (such as Verrucomicrobiae and Planctomycetia; Abed et al., 2019; Pollet et al., 2018).

Overall, the pathways of indirect effects from top predators to microbial communities are much more complex than anticipated, requiring quantification of ecological network structure to disentangle. It is possible that species-specific effects due to variation in the amphipod assemblage contributed to this complexity. Importantly, the effects of warming depended on top-predator body size, for example, warming impacts on the bacterial community were driven by Cytophagia when crabs were large and Sphingobacteriia when crabs were small. This demonstrates that changes in top-predator population size structure can alter the effects of other stressors. Top-down control has also been shown to influence the effects of other anthropogenic stressors on food-web structure in mesocosm experiments (Greig et al., 2012; Jochum et al., 2012). These results highlight the importance of considering trophic interactions in future research on environmental stressors, to help disentangle the direct and indirect effects of climate change.

5 | CONCLUSIONS

We have shown that warming, nutrient enrichment, and declining body size of top predators can act alone and in tandem to alter the community composition of coastal bacterial biofilms. Predicted functional consequences of these structural changes with warming included alterations to key biogeochemical processes. Warming had by far the biggest impact on the bacterial biofilm community, increasing α-diversity and eliciting the dominance of bacteria capable of degrading complex biomolecules. As this could divert marine carbon from long-term sequestration back into food webs (ultimately resulting in a positive feedback loop between CO_2 production and warming), it is vital to formally test this hypothesis in future research. Nutrient enrichment cancelled out the effects of warming on α-diversity, however, and top-predator body size modified the taxonomic shifts observed with warming, but not the predicted function of the bacterial biofilms. This highlights the need for more experimental research into the impacts of multiple stressors on microbial communities to help disentangle the surprising ways in which they can combine to alter the structure and functioning of natural systems.

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SUPPORTING INFORMATION

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