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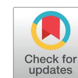
No bacterial-mediated alleviation of thermal stress in a brown seaweed suggests the absence of ecological bacterial rescue effects



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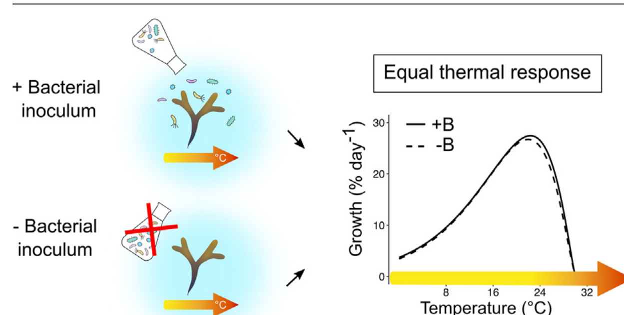
HIGHLIGHTS

- Addition of bacteria does not affect the thermal response of a common brown seaweed.
- Seaweed thermal response curves are highly genotype-specific.
- Lower temperatures had the most pronounced effect on the seaweed microbiomes.

GRAPHICAL ABSTRACT



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ABSTRACT

While microbiome alterations are increasingly proposed as a rapid mechanism to buffer organisms under changing environmental conditions, studies of these processes in the marine realm are lagging far behind their terrestrial counterparts. Here, we used a controlled laboratory experiment to examine whether the thermal tolerance of the brown seaweed *Dictyota dichotoma,* a common species in European coastal ecosystems, could be enhanced by the repeated addition of bacteria from its natural environment. Juvenile algae from three genotypes were subjected for two weeks to a temperature gradient, spanning almost the entire thermal range that can be tolerated by the species (11–30 °C). At the start of the experiment and again in the middle of the experiment, the algae were inoculated with bacteria from their natural environment or left untouched as a control. Relative growth rate was measured over the two-week period, and we assessed bacterial community composition prior to and at the end of the experiment. Since the growth of *D. dichotoma* over the full thermal gradient was not affected by supplementing bacteria, our results indicate no scope for bacterial-mediated stress alleviation. The minimal changes in the bacterial communities linked to bacterial addition, particularly at temperatures above the thermal optimum (22–23 °C), suggest the existence of a barrier to bacterial recruitment. These findings indicate that ecological bacterial rescue is unlikely to play a role in mitigating the effects of ocean warming on this brown seaweed.

1. Introduction

The ongoing effects of global climate change, particularly ocean warming, severely impact marine ecosystems worldwide (Hoegh-Guldberg and Bruno, 2010; Hughes et al., 2017; Bryndum-Buchholz et al., 2019). Whether and how species will tolerate these environmental changes largely depends on their potential to adapt and/or acclimatize to rapidly

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changing conditions (Munday et al., 2013). Over the past two decades, it has been increasingly acknowledged that associated microorganisms may influence the resilience of their hosts to environmental stressors, including temperature (e.g., Moran and Yun, 2015; Ziegler et al., 2017; Voolstra and Ziegler, 2020). In particular, the diversity of the microbial reservoir, their versatility, and their short generation time, allow for a much faster response towards anthropogenic changes than can be expected from their host (Bang et al., 2018; Stock et al., 2021). Yet, studies directly assessing the effects of microorganisms on the stress tolerance of their host remain scarce in marine systems, with the exception of coral research (e.g., Ziegler et al., 2017; Rosado et al., 2019; Peixoto et al., 2021; Santoro et al., 2021). However, such information is crucial to understand the full potential of marine organisms to cope with forecasted environmental changes and to disentangle the complex ecological interactions that play a role herein.

In plants, the role of associated microbial communities in conferring host resilience to stress is well documented (Yang et al., 2009; Zia et al., 2021). Especially rhizosphere bacteria have been shown to alleviate the influences of abiotic stresses through a number of different mechanisms, including alterations in phytohormone levels, metabolic adjustments, antioxidant defences, and the stimulation of root growth (Khan et al., 2021). As such, research on stress induced rhizosphere-plant feedbacks has identified numerous bacteria that can improve plant fitness under environmentally extreme conditions (e.g., Numan et al., 2018). This highlights the need to further explore the importance of associated microbial communities in marine systems, which would help to better predict the response of marine primary producers to global climate change and to anticipate the role of microorganisms therein.

Seaweeds are key components of coastal ecosystems, where they constitute habitat, shelter, and a source of food for a wide diversity of marine organisms (Christie et al., 2009; Marzinelli et al., 2016; Teagle et al., 2017). In addition, they have been shown to provide important ecosystem services, such as nutrient cycling, coastal protection, and carbon sequestration (Costanza et al., 1997; Bennett et al., 2016). Like plants, seaweeds harbour diverse bacterial communities that assist them with a variety of functions, including growth (Marshall et al., 2006; Spoerner et al., 2012), morphological development (Matsuo et al., 2005; Marshall et al., 2006), protection against biofouling (Dobretsov and Qian, 2002; Wahl et al., 2012), and resource acquisition (Chisholm et al., 1996; Helliwell et al., 2011; Weigel et al., 2022). Given the vital importance of seaweeds in the functioning of coastal ecosystems and the increasing awareness of the relevance of microorganisms to overall host health, seaweed-bacteria interactions have received a large increase in interest during the past decade (e.g., Egan et al., 2013; van der Loos et al., 2019 and references therein). In particular, significant advances have been made in understanding the response of algal-associated bacterial communities to environmental (Mensch et al., 2016; Saha et al., 2020; Dobretsov et al., 2021) and seasonal changes (Bengtsson et al., 2010; Mancuso et al., 2016; Serebryakova et al., 2018). However, when it comes to the role of associated bacteria in conferring host resilience to stress, studies in seaweeds are lagging far behind those in plants. To date, only a single study has directly investigated the link between bacterial communities and the ability of a seaweed host to cope with environmental stress in a controlled environment (Dittami et al., 2016). Here, the authors showed that the brown seaweed *Ectocarpus* could only withstand reduced salinity levels in the presence of its associated bacteria. While promising, it remains unclear whether such beneficial effects also hold true under different stressors and for other species.

Among climate change effects, ocean warming is one of the major stressors adversely affecting seaweed ecosystems (Wernberg et al., 2011; Araújo et al., 2016; Smale, 2020). Since temperature largely determines geographical ranges in many seaweed species (Lüning, 1990), even small changes in thermal regimes can have profound effects on seaweed distributions. At the microbiome level, thermal stress has been shown to affect the composition and structure of algal-associated bacterial communities in a wide range of seaweeds (Webster et al., 2011; Stratil et al., 2013; Minich et al., 2018; Mensch et al., 2020; Morrissey et al., 2021; Paix et al., 2021). However, the effects of these changes on the fitness of the algal host have yet to be validated. In addition, while most studies focus on the effects of ambient vs. one or multiple warming scenarios, a more holistic approach that evaluates the effects of temperature on algal fitness across the full tolerance range might be much more informative (Harley et al., 2012; Arnold et al., 2019).

Here, we explore whether the addition of bacteria can enhance the thermal tolerance of the brown seaweed *Dictyota dichotoma*. This species is a common annual macroalga in European coastal ecosystems, where its distribution ranges from southern Norway, along the coastlines of the northeast Atlantic Ocean, to the Mediterranean Sea and the Canary Islands (Tronholm et al., 2010). *D. dichotoma* is an ideal study species due to its amenability to laboratory experiments, existing information regarding its thermal tolerance, and its easy propagation in a lab context (Bogaert et al., 2016). In this study, we grew juvenile algae under a full gradient of optimal and suboptimal temperatures, while manipulating the availability of bacteria present in their environment. We anticipated that the addition of novel bacteria would enhance the thermal tolerance of *D. dichotoma*, resulting in lower growth reduction at both the upper and lower parts of the temperature range, which has repeatedly been suggested for seaweeds (e.g., Ghaderiardakani et al., 2020) and has been observed in plants (Hussain et al., 2018 and references therein).

2. Material and methods

2.1. Culture conditions and algal genotypes

To evaluate whether bacteria can enhance the thermal performance of *Dictyota dichotoma*, we created genetically identical juvenile algae (F2 generation) using laboratory-reared male and female gametophyte cultures as a parental generation (F1 generation). These parental algae were derived from wild, fertile sporophytes (F0 generation) collected in 2019 from a population located in Goes, the Netherlands (51.541° N, 3.930° E). Temperatures at this location range from below 5 °C during the coldest month to over 22 °C during the hottest month and occasionally exceed 24 °C on warm days (Fig. A.1). Following collection and spore release, the obtained spores were isolated in 48-well culture plates (one spore per well) filled with 1 mL of autoclaved natural seawater enriched with 10 mL L^{-1} modified Provasoli medium (West and McBride, 1999), hereafter referred to as enriched seawater medium. We supplemented the medium with GeO_2 (1.8 mg L^{-1}; Sigma-Aldrich) to prevent diatom contamination and we added the broad-spectrum antibiotic streptomycin (18.2 mg L^{-1}; Sigma-Aldrich) to suppress bacterial growth. The spores were then left to germinate and grow during at least 14 days in thermal incubators under a 12 h:12 h light:dark cycle, controlled by mechanical timers, and a temperature of 20 °C. This temperature was chosen based on a study conducted by Bogaert et al. (2016), in which 20 °C was identified as the optimal temperature for growth in *D. dichotoma*. From then on, all cultures were kept under these conditions of daylength and temperature unless stated otherwise. After the initial 14-day period, we transferred all clean juveniles to 24-well culture plates filled with 2 mL of enriched seawater medium to develop for at least two more weeks. Following the development period, we selected one or more clean gametophyte individuals per original sporophyte. These F1 individuals were further maintained in 30 mL crystalizing dishes filled with enriched seawater medium under low light levels (3–4 μmol photons m^{-2} s^{-1}) provided by cool white LEDs (Ohmeron, Belgium), until crosses were performed. To produce offspring, fertile male and female algae were placed together in a large crystallizing dish (150 mL) to cross during three consecutive days. During this time, the gametophyte parents produced haploid gametes through mitosis, which created genetically identical, diploid offspring following gametic fusion. After three days, the parental individuals were removed, and the medium of the successful crosses was completely refreshed. The resulting sporophytes were grown together in their respective dishes for another 10–12 days under a light intensity of 18–23 μmol photons m^{-2} s^{-1} provided by cool-white LED lights, until the start of the experiment. In total, three crosses, using distinct parental individuals, were performed. Each

cross yielded offspring with a unique genotype, resulting in three genotypes, hereafter referred to as Goes B, Goes C, and Goes D (F2 generation) (Fig. 1).

2.2. Preparation of the bacterial inoculum

We prepared the bacterial inoculum with seawater collected in between *D. dichotoma* specimens at the original sampling location of the F0 sporophytes (Goes, the Netherlands) on November 3rd and 10th 2021 (surface water temperature of 11.1 and 10.3 °C respectively; buoy Plaat van Oude Tonge, Rijkswaterstaat, the Netherlands). The seawater was filtered twice over a 5 μm GLA-5000 PVC PALL (USA) filter to remove larger organisms, after which bacteria were collected by filtering 120 mL of seawater over a 0.1 μm supor-100 PALL (USA) filter. A tenth of the filter was frozen immediately at −20 °C and stored for further bacterial DNA analyses (see Section 2.6). The remainder of the filter was then transferred to 25 mL of enriched seawater medium, and the bacteria were resuspended by vigorous shaking and vortexing. Each time, the inoculum was made on the same day of its use, while the seawater was collected the day before the inoculum was prepared. Over 800 colony forming units per mL (BD Difco Marine agar) were present in the inoculum at each time point.

2.3. Experimental design

To assess the effect of bacterial recruitment on the thermal response of *D. dichotoma*, we used a fully crossed design, manipulating algal genotype (Goes B, Goes C, Goes D), temperature (11, 16, 20, 22, 24, 27, and 30 °C ± 1 °C), and bacterial inoculum (supplemented/not supplemented) (Fig. 1). For each combination of genotype, experimental temperature, and bacterial inoculum treatment, we used three replicate juvenile algae (n = 3), comprising 126 algae in total. Note that these algal individuals still harbour associated bacteria (see Section 3.2 for details), as it was not possible to create axenic *D. dichotoma* individuals. The experiment was conducted in temperature-controlled incubators (Lovibond, Germany) and the aforementioned seven temperature treatments were chosen to cover nearly the entire thermal range that can be tolerated by *D. dichotoma,* as indicated by pilot studies previously conducted in our lab. Throughout the experiment, individual algae were grown in separate crystallizing dishes (30 mL) at a light intensity of 18–26 μmol m^{-2} s^{-1} provided by cool white LEDs. Daylength conditions and culture medium were identical to those mentioned for the parental cultures. At the start of the experiment, the replicates of the bacterial inoculum treatment received 250 μL of bacterial suspension while the other group of replicates was left untouched. Following this step, all algal individuals were brought to their respective temperatures using a thermal ramping of 2 °C per half hour. For the temperature treatments of 11, 20, and 27 °C, we used sterile galvanized half metal rings (31.2 mm^2) as controls to assess bacterial settlement on non-living surfaces. These temperatures represent a sublethal cold stress treatment (11 °C), a near-optimum temperature (20 °C), and a sublethal heat stress treatment (27 °C). The experiment ran for 14 days and the medium was refreshed once after seven days, together with the bacterial inoculum when present. To mimic algal growth, an additional half metal ring was added to the controls during the refreshment. For comparison purposes, the experiment was conducted simultaneously on all three algal genotypes, ensuring that each genotype received the same bacterial inoculum when appropriate.

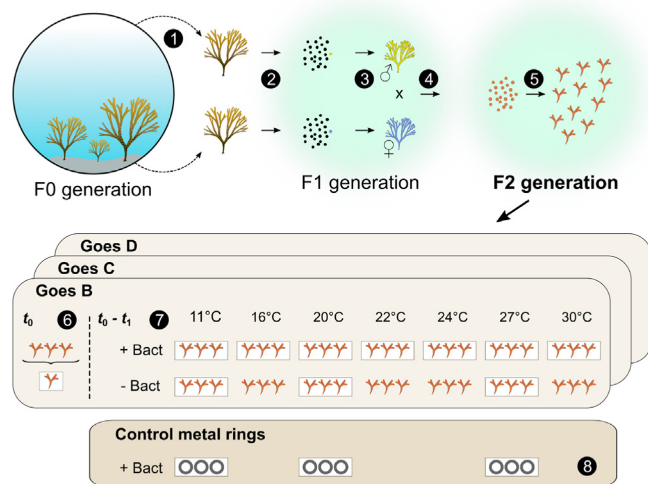

Fig. 1. Graphical overview of the experimental design. (1) Fertile sporophytes are sampled in Goes, the Netherlands (F0 generation). (2) Spores are released in the lab. (3) A number of spores are isolated and grown for a few weeks. One or more clean juveniles are selected to further develop into adult gametophytes (F1 generation). (4) Unrelated male and female gametophytes are crossed, producing genetically identical zygotes. (5) Zygotes develop into juvenile sporophytes for 10–12 days (F2 generation). (6) At the start of the experiment (t_0), three juvenile sporophytes per cross are pooled and their bacterial communities are sequenced. (7) Next, 42 juveniles per cross are selected: 21 juveniles receive bacteria from their natural environment (+ Bact), the other 21 juveniles are left untouched (− Bact). Juveniles are equally divided over a gradient of seven temperatures. Growth rate is measured over two weeks, and the bacterial community composition of each juvenile in a white box is sequenced at the end of the experiment (t_1). This is done simultaneously on juveniles from three crosses, representing three distinct genotypes (Goes B, Goes C, and Goes D). (8) Bacterial inocula are added to sterile metal rings, placed at 11, 20, and 27 °C, to assess bacterial settlement on non-living surfaces.

2.4. Growth measurements

We photographed the juvenile algae at the beginning and at the end of the experiment and quantified their surface area using the image analysis software ImageJ (Schindelin et al., 2015). The relative growth rate (RGR) of each juvenile was calculated by applying the following equation:

$$\mathrm{RGR}\left(\%\mathrm{day}^{-1}\right) = \frac{\ln(S_{t1}) - \ln(S_{t0})}{t_1 - t_0} * 100 \qquad (1)$$

where S_{t0} and S_{t1} are the surface areas at timepoints t_0 and t_1 in days, representing the beginning and the end of the experiment. Negative growth rates were converted to zero. Prior to conducting further analyses, we removed four algal individuals from our dataset, either because they got fertile during the experiment or because they showed stress due to handling, leaving 122 data points for further analysis.

2.5. Collection of bacterial samples

The bacterial communities associated with *in situ D. dichotoma* specimens were characterised using ten individuals sampled on the 29th of November 2022 at the location where the inocula and the F0 generation were previously collected (8.6 °C surface water temperature; buoy Plaat van Oude Tonge, Rijkswaterstaat, the Netherlands). A tip from each individual was washed in autoclaved seawater and stored at −20 °C.

To assess the initial bacterial community present on each algal genotype, we haphazardly selected and pooled three individuals per genotype (n = 1 replicate per genotype) at the start of the experiment (t_0). These algal individuals were selected from the remaining offspring that were not used in any treatment. They were then washed in enriched seawater medium and stored at −20 °C for further analyses. The specimens at the end of the experiment (t_1) were harvested to assess the effect of the different treatments on the bacterial community of each algal individual. Specifically, following the surface area measurements, we washed and stored all surviving individuals that received a bacterial inoculum across the full temperature gradient as well as those that did not receive an inoculum at 11, 20, and 27 °C (Fig. 1). The metal rings, which were used as controls to assess bacterial settlement on non-living surfaces, were processed in the same way as the algal samples.

2.6. DNA extraction, Nanopore sequencing, and bioinformatics analysis

DNA of the frozen samples was extracted with the QIAamp DNA Mini Kit (QIAGEN, Hilden, Germany) with an additional bead-beat step after the addition of the first buffer to enhance cell lysis. The extracted DNA was processed and sequenced as in van der Loos et al. (2021), using Oxford Nanopore Technology (ONT). Base-calling was done with Guppy (v. 5.0.14, ONT) using the super high accuracy mode and reads shorter than 1200 or longer than 1700 bp were discarded using NanoFilt (v. 2.8.0; De Coster et al., 2018). The sequences from the inocula and experimental samples were processed using a modified version of the NanoCLUST pipeline (Rodríguez-Pérez et al., 2021). Briefly, reverse reads were reoriented using VSEARCH (v. 2.21; Rognes et al., 2016), and operational taxonomic units (OTUs) were delineated for each barcode separately by clustering with UMAP (v. 0.4.6; McInnes et al., 2018) based on their 5-mer frequencies. A consensus sequence was drafted from the reads assigned to each cluster using CANU (v. 2.0; Koren et al., 2017), which was further polished using Racon (v. 1.4.13; Vaser et al., 2017) and medaka (v. 1.0.3; https://github.com/nanoporetech/medaka). Chimeric consensus sequences were detected and removed across all samples using DADA2's removeBimeraDenovo (v. 1.22.0; Callahan et al., 2016) in R (v. 4.1.2). We aligned the remaining consensus sequences using ssu-align (v. 0.1.1) also masking ambiguous positions. A phylogenetic tree was constructed from this alignment using FastTree (v. 2.1.11). Taxonomy was assigned to the consensus sequences using the SILVA database (v. 123) with VSEARCH, and all samples were rarefied to 2372 reads. We agglomerated OTUs from different samples based on the tree using *tip_glom* from the *phyloseq* package with h = 0.05 (v. 3.14; McMurdie and Holmes, 2013). Due to the high abundance of chloroplast-derived sequences in the *in situ* collected samples and our interest in potentially rare taxa, the *in situ* collected samples were analysed as in van der Loos et al. (2021), using Kraken2 (v. 2.1.2; Wood et al., 2019), which classified each sequence instead of creating consensus sequences first. The same reference sequence database as the other samples was used to ensure taxonomic assignment was comparable between samples. Kraken-biom (v. 1.0.1; Dabdoub, 2016) was used to reformat Kraken2 output prior to importing into R. Reads/OTUs that could not be assigned to a bacterial taxon or that were assigned to chloroplasts were removed prior to the statistical analyses.

2.7. Statistical analyses

All statistical analyses were performed using R v. 4.1.2 (R Core Team, 2020), unless stated otherwise. Graphics were made using the R package *ggplot2* (Wickham, 2016). To obtain thermal response curves, we modelled the relationship between RGR and temperature according to the equation from Blanchard et al. (1996):

$$\mathrm{RGR}\ (T) = G_{\max}\left(\frac{T_{\max}-T}{T_{\max}-T_{\mathrm{opt}}}\right)^{\beta}\exp\left[-\beta\left(\frac{T_{\max}-T}{T_{\max}-T_{\mathrm{opt}}}-1\right)\right] \quad (2)$$

where T is the temperature (in °C), $G_{\max}$ is the maximum growth rate at the optimum temperature T_{opt}, $T_{\max}$ is the maximum temperature for growth, and β is a dimensionless scaling parameter. To obtain parameter estimates, model fitting was performed for each combination of genotype and bacterial treatment by using non-linear least squares regression, employing the *nlsLM* function implemented in the R package *minpack.lm* (Elzhov et al., 2013). Next, we examined whether the thermal response curves differed between genotypes (Goes B, Goes C, Goes D) and bacterial inoculum (supplemented/not supplemented), by using an information-theoretic approach. Specifically, we tested whether the observed growth rates were better explained by one general thermal response curve (i.e., a reduced model) or by multiple response curves (either fitted to each genotype, to each bacterial inoculum treatment, or to both the different genotypes and bacterial inoculum treatments, with the latter representing the full model). Models were fitted with the NLIN procedure implemented in SAS v. 9.4 (SAS Institute inc., Cary, North Carolina, USA) and the best model was selected based on the Akaike Information Criterion corrected for small sample size ($\mathrm{AIC_c}$; Anderson, 2008). While this approach immediately assesses the thermal response over the full gradient of temperatures, it may miss differences in growth linked to the inoculum treatment at certain specific temperatures. Therefore, we compared growth rates among temperature, genotype, and bacterial treatment using a three-way analysis of variance (ANOVA) with Type III sum of squares and all variables and their interactions included as fixed factors R package *car,* (Fox et al., 2012). All data were tested for homogeneity of variances using Levene's test and the residuals of the model were checked for normality with a Shapiro test. In addition, we visually checked the distribution of the residuals using a normal QQ plot. When appropriate, post-hoc pairwise comparisons were conducted using the functions *pairs* and *emmeans* from the R package *emmeans* (Lenth, 2018). To account for multiple testing, p-values were adjusted using the Benjamini-Hochberg procedure (Benjamini and Hochberg, 1995).

We visualized differences between experimental bacterial communities using a Bray-Curtis based non-metric multidimensional scaling (nMDS) in the R package *phyloseq* (McMurdie and Holmes, 2013). The effect of bacterial inoculum addition was examined for the temperatures at which both bacterial treatment groups (inoculum supplemented/not supplemented) were sampled (i.e., 11, 20, and 27 °C). To this end, we performed a three-way permutational analysis of variance (PERMANOVA), including temperature, genotype, and bacterial inoculum as well as their interaction as *a priori* factors. This analysis was based on a Bray-Curtis dissimilarity matrix using the *adonis2* function of the R package *vegan* (Oksanen et al., 2018). The effect of temperature on bacterial community composition was assessed on a subset of the data that included all algal samples that received an inoculum, as these were sampled across the full temperature gradient. A two-way PERMANOVA was run in the same way as before, including temperature, genotype, and their interaction as *a priori* factors. Pairwise differences were tested using the *pairwise.adonis2* function implemented in the *pairwiseAdonis* package (Martinez Arbizu, 2020) when appropriate. As there was no multivariate homogeneity between groups, the resulting significance values should be interpreted with care (Anderson and Walsh, 2013). The diversity for each community was calculated as the number of species observed (n_{obs}) and the Shannon diversity index (H'). We compared the bacterial diversity between communities using Kruskal-Wallis Rank Sum Tests followed by Wilcoxon tests when appropriate. To identify OTUs that covary with temperature, we calculated the Kendall rank correlation coefficient between the relative abundance of the OTUs and the experienced temperature for samples that were taken across the full gradient (i.e., only those that received a bacterial inoculum).

3. Results

3.1. Thermal response curves

The relative growth rate (RGR) of *Dictyota dichotoma* was strongly influenced by temperature. By fitting Eq. (2) to the growth data, we found that the optimum temperature for growth, T_{opt} (± SE), ranged from 21.96 (± 0.28) to 22.96 (± 0.37) °C while the maximum growth rate, $G_{\max}$, (± SE), ranged from 21.50 (± 0.83) to 27.45 (± 0.68) % day^{-1} (Figs. 2, A.2, Table A.1). In contrast, the 30 °C treatment was lethal for all algal individuals, with a corresponding growth rate of 0 % day^{-1} (Figs. 2, A.2). The growth data were best described by a model allowing only the different genotypes to have separate thermal response curves, with different values for the T_{opt}, $G_{\max}$, and β parameters (i.e., this model had the lowest $\mathrm{AIC_c}$; see Tables A.2, A.3, and Fig. A.3 in supplementary information). This indicates that the thermal response curves of juvenile *D. dichotoma* individuals differed notably among genotypes, but not among bacterial inoculum treatments (supplemented/not supplemented) (i.e., the $\mathrm{AIC_c}$ of the genotype-specific model was lower than that of the full model and the bacteria-specific model, Tables A.2, A.3, and Fig. A.3). These results were congruent with the outcome of the three-way ANOVA, which indicated a significant effect of temperature ($F_{6,80}$ = 698.2;

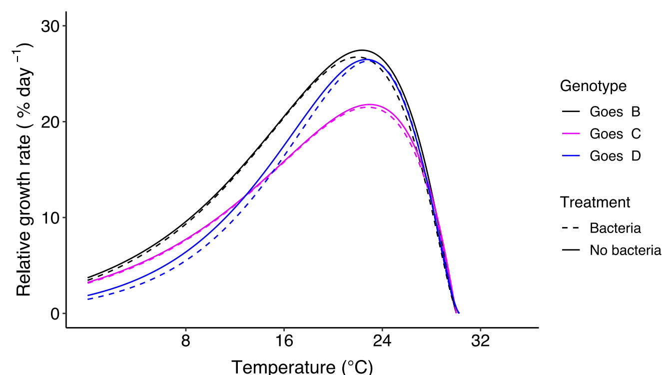

Fig. 2. Thermal response curves differ according to algal genotype but not bacterial inoculum treatment. Fitted lines show the thermal response of relative growth rate of juvenile *D. dichotoma* individuals, predicted according to Eq. (2). Response curves were fitted for each algal genotype, depicted by colour (black = Goes B, pink = Goes C, blue = Goes D), fully crossed with two bacterial inoculum treatments, depicted by line type (dashed line = bacterial inoculum supplemented, solid line = no bacterial inoculum supplemented).

$p < 0.0001$), algal genotype ($F_{2,80} = 51.9$; $p < 0.0001$) and their interaction ($F_{12,80} = 6.6$; $p < 0.0001$), but not of bacterial inoculum treatment ($F_{1,80} = 2.4$; $p = 0.1235$) on algal growth rate (see Table A.4 in supplementary information). Pairwise comparisons, testing for differences in RGR between genotypes at each temperature, revealed that Goes B significantly outperformed Goes C and Goes D at 11 and 16 °C, while both Goes B and Goes D significantly outperformed Goes C at 20, 22, and 24 °C (see Table A.5 in supplementary information). No significant differences between genotypes were observed at 27 and 30 °C (Table A.5).

3.2. Bacterial communities associated with D. dichotoma specimens in situ

The bacterial communities varied substantially between the *in situ* collected *D. dichotoma* specimens, with different genera dominating the communities. About half of the community consisted of Pseudomonadota (Proteobacteria), mostly Rhizobiales, Rhodobacterales, Chromatiales, Thiotrichales and Alteromonadales (Fig. 3). The gammaproteobacterial genus *Marinomonas* was present in eight out of the ten individuals with a mean abundance of 1.1 %. *Marinobacter* (Gammaproteobacteria) and *Labrenzia* (Alphaproteobacteria) were present in some cases but always below 1 %. *Arcobacter*, a genus within the Campylobacterota (Epsilonproteobacteria), was detected in four out of the ten individuals with a mean abundance of 0.058 %. Bacteroidota (Bacteroidetes), mostly Sphingobacteriales and to a lesser extent Flavobacteriales, was the second most abundant phylum and represented on average about one eight of the bacteria present in the community. No *Fabibacter* was detected. Actinomycetota (Actinobacteria), Deinococcota (Deinococcus-Thermus), Bacillota (Firmicutes) made up about a tenth of some communities while being absent in others.

3.3. Effects of temperature, bacterial inoculum, and algal genotype on experimental bacterial communities

Alphaproteobacteria (Rhodobacteraceae, Sphingobacteriales, Rhizobiales), Gammaproteobacteria (Alteromonadaceae), and Cryomorphaceae (Flavobacteriia) were prominent in the initial communities associated with the three different algal genotypes used in the laboratory experiment (Fig. 3). The genotypes had distinct bacterial

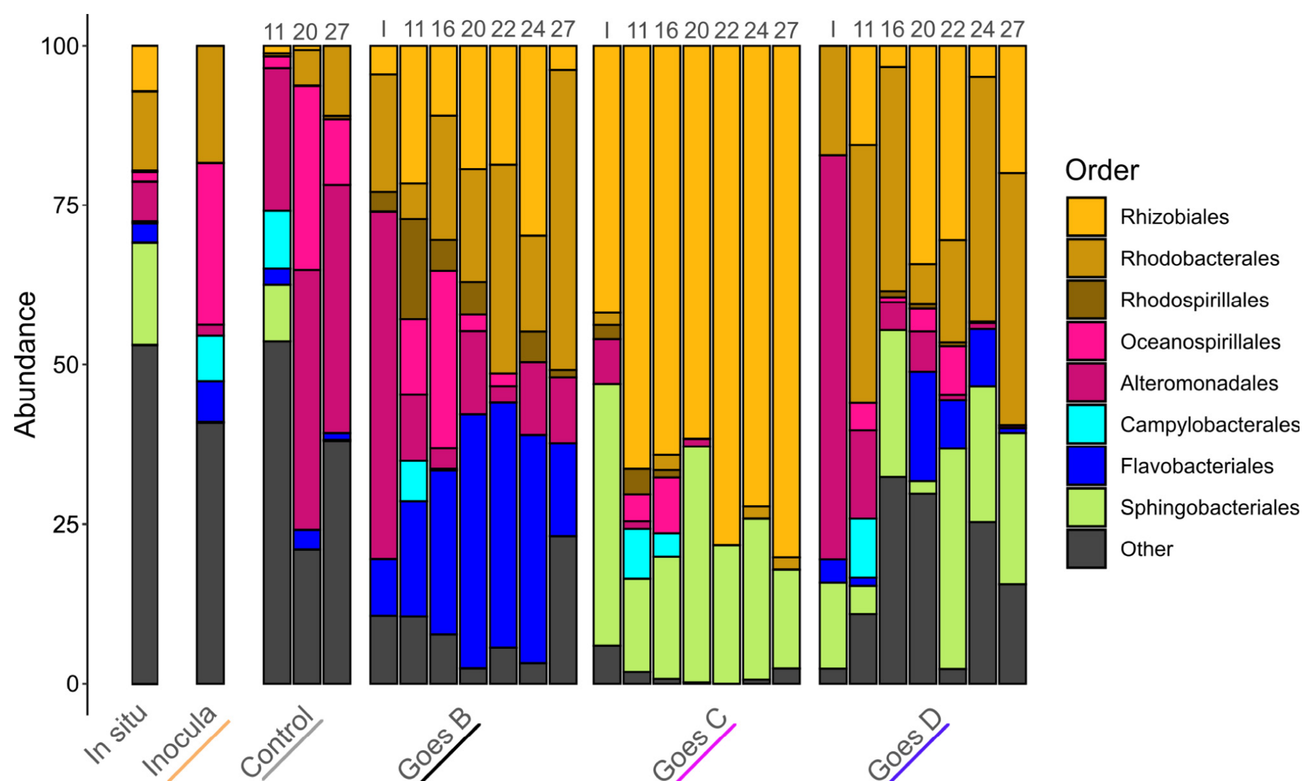

Fig. 3. Barplots show variation in bacterial community composition across algal genotypes and temperature treatments. Numbers on top of the plot indicate temperature treatments, while 'I' represents the initial community prior to the thermal experiment. For comparison purposes, only samples that received a bacterial inoculum are shown for each temperature treatment. Barplots are grouped according to sample type, underlined in colour at the bottom of the plot (from left to right: transparent = *in situ* specimens, orange = bacterial inocula, grey = control metal rings, black = Goes B, pink = Goes C, blue = Goes D).

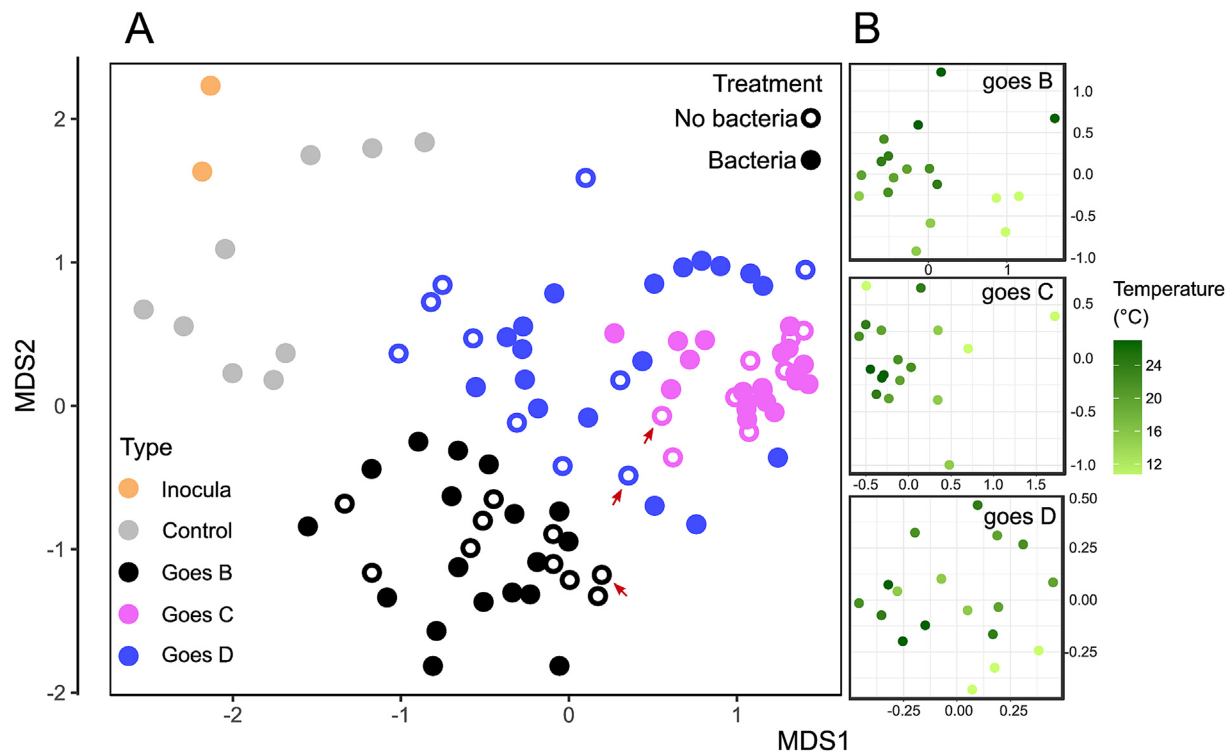

Fig. 4. Non-metric multidimensional scaling (nMDS) plots show variation in bacterial community composition between genotypes, and to a lesser extent, temperatures. (A) nMDS plot of the full dataset; individual samples are coloured based on the sample type (orange = bacterial inocula, grey = control metal rings, black = Goes B, pink = Goes C, blue = Goes D), while the bacterial inoculum treatments are depicted by symbol fill (filled = bacterial inoculum supplemented, not filled = no bacterial inoculum supplemented). Red arrows indicate algal samples taken prior to the experiment, illustrating the initial bacterial community of each genotype. (B) nMDS plots for algal samples that received a bacterial inoculum, taken at the end of the experiment. Colours indicate temperature treatments and samples are grouped per algal genotype (Goes B, Goes C, and Goes D).

communities although they shared a *Marinobacter* (Alteromonadales), an unidentified Alteromonadaceae strain (Alteromonadales), a *Labrenzia* (Rhodobacterales), and a *Fabibacter* (Cytophagales). Goes B started with the most diverse bacterial community ($n_{obs} = 27$, $H' = 2.55$; compared to $n_{obs} = 11$ and 14, and $H' = 1.79$ and 1.94 for Goes C and D respectively).

The communities found in the bacterial inocula were dominated by Pseudomonadota (Proteobacteria), mostly from the SAR11-clade, but also other Alphaproteobacteria (Rhodobacterales), Gammaproteobacteria (Oceanospirillales, Alteromonadales, Vibrionales), and Deltaproteobacteria (Bdellovibrionales; Fig. 3). Campylobacterota (Epsilonproteobacteria; Campylobacterales) were detected, as were Bacteroidota (Bacteroidetes; Flavobacteriales) and Actinomycetota (Actinobacteria; Acidimicrobiales; Fig. 3).

The addition of bacterial inocula to the algal individuals did not strongly impact the associated communities. Apart from the *Arcobacter* and *Marinomonas*, detected at lower temperatures, several Rhodobacteraceae (e.g., *Stappia* and *Sulfitobacter*) were detected in a few of the algal microbiomes without any clear pattern across replicates nor temperature. As a result, there was no significant impact of the bacterial addition on the structure (PERMANOVA: $p > 0.05$; supplementary table A.6) or diversity (p_{nobs} & $p_{H'} > 0.05$) of the bacterial communities associated with the algae.

The exposure of the algae to different temperatures resulted in a minor, but significant effect across the temperature gradient (PERMANOVA: $p = 0.003$, Figs. 3, 4; supplementary table A.6). In the algal samples where an inoculum was added, four OTUs were consistently negatively influenced by increasing temperatures (Kendall correlation ≤ -0.3): Two *Arcobacter OTUs* and a *Marinomonas* that were most likely not present in the initial communities (Fig. 5) and a member of the OM27_clade (Bdellovibrionaceae). The latter OTU was not detected in the initial communities but was found in some of the algal-associated communities that did not receive a bacterial inoculum, suggesting it was present in some of the original communities but at very low abundances. When considering the samples at the three temperatures for which we have the controls, again only negative correlations were found. These included the same *Arcobacter*, member of the OM27_clade and *Marinomonas*, but also a *Thalassospira* and Hyphomicrobiaceae OTU, which were already present in at least two of the initial communities. No OTUs consistently increased with increasing temperature. In general, the bacterial diversity was negatively affected by temperature (i.e., was lower at higher temperatures), especially for the algal samples that received a bacterial inoculum (Linear

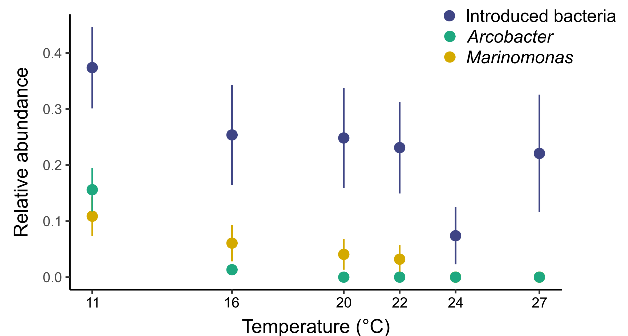

Fig. 5. Two bacterial genera, *Arcobacter* and *Marinomonas,* had a higher chance of establishing in the *Dictyota*-associated bacterial communities at lower temperature. The average relative abundance ± SE of bacteria that were able to settle in the *Dictyota*-associated bacterial communities after being provided by the inocula is shown (n = 54). The total relative abundance of introduced bacteria is indicated in blue, while all OTUs identified as *Arcobacter* or *Marinomonas* are indicated in green and yellow, respectively.

regression, for the algal samples where an inoculum was added: p_{nobs} = 0.0126 & $p_{H'}$ = 0.0029; for the samples at the three temperatures for which we have the controls p_{nobs} > 0.05 & $p_{H'}$ = 0.0421).

The composition and structure of the microbiomes differed significantly between the three *Dictyota* genotypes at the end of the experiment in both datasets (PERMANOVA: $p \leq 0.001$; pairwise adonis $p \leq 0.001$, Figs. 3, 4, supplementary Table A.6). The differences predominantly reflected the differences that were already present between the initial communities, with Goes B still having the most diverse community (12 ± 6 OTUs; H' = 1.66 ± 0.44) of the three genotypes (Goes C: 7 ± 3 OTUs, H' = 1.20 ± 0.28; Goes D: 8 ± 5 OTUs, H' = 1.28 ± 0.47). The diversity was significantly different between genotypes (Kruskal-Wallis rank sum test p_{nobs} = 0.0014; $p_{H'}$ = 0.0003; Wilcoxon tests n_{obs} $p_{B\text{-}C}$ = 0.0007; $p_{B\text{-}D}$ = 0.0044; Wilcoxon tests H' $p_{B\text{-}C}$ < 0.0001; $p_{B\text{-}D}$ = 0.0053). The latter values were calculated on the whole dataset at the end of the experiment, in order to get a full overview of the bacterial diversity found on each genotype. In addition, the metal rings had a markedly different community compared to the algae (pairwise adonis $p \leq 0.001$), with for instance *Maricaulis* (Alphaproteobacteria), *Neptuniibacter* (Gammaproteobacteria) being typically abundant in these communities. The communities associated with these rings had a significantly (Wilcoxon tests p_{nobs} < 0.0001; Wilcoxon tests $p_{H'}$ = 0.0004) higher bacterial diversity associated with them (25 ± 11 OTUs, H' = 2.27 ± 0.58) compared to the algae. However, there might be a bias as rings lack chloroplast 16S rRNA genes which might impact amplification and sequence processing.

4. Discussion

In this study, we explored whether supplementing bacteria enhanced the thermal tolerance of the brown seaweed *Dictyota dichotoma* along a broad temperature gradient. Contrary to expectations, the addition of bacterial inocula did not alter the growth of the seaweed at any temperature tested.

At the microbiome level, the addition of bacteria had little effect on the diversity and composition of the seaweed-associated communities. As the inocula were made from bacteria collected in between a natural population of *D. dichotoma*, and sequencing showed that they contain many taxa typically found on those seaweeds (e.g., Rhodobacterales, Flavobacteriales, and Alteromonadales), it is highly unlikely that they would not contain bacteria that could associate with the seaweed. Our results therefore suggest the existence of a barrier to bacterial recruitment, which is also evidenced by the more diverse communities found on the control metal rings. If novel bacteria fail to establish into the algal microbiome, the host cannot benefit from the diversity of the environmental microbial reservoir to rapidly respond to changing environmental conditions. On the other hand, this can also protect the host from potential pathogens that could otherwise establish on an already stressed organism. For instance, *Vibrio*, which was detected in the inocula, is known to be an important seaweed pathogen (Ward et al., 2020; Yang et al., 2020).

Failure to establish into the seaweed associated community might be mediated by the algal host, its associated microbial communities, or the interaction between both. As we are not able to obtain axenic *D. dichotoma*, we cannot differentiate between the effects of host and bacteria. It is known that seaweeds have evolved a number of mechanisms to control and shape their associated microbial communities, including the release of reactive oxygen species (see Weinberger, 2007 and references therein), shedding of their outermost cell layers (Nylund and Pavia, 2005), and the production of specific chemical metabolites (Lachnit et al., 2013; Saha and Weinberger, 2019). The genus *Dictyota* is known for producing a wide array of secondary metabolites, which provide an effective defense mechanism against competitors and herbivores (see Chen et al., 2018 and references therein) and largely inhibit the settlement of invertebrate and coral larvae on or near the seaweed surface (Schmitt et al., 1995; Walters et al., 1996). These effects may be extended to the microbiome level as several studies demonstrated the influence of *Dictyota* species on the microbiome of adjacent organisms (Barott et al., 2012; Morrow et al., 2013; Zaneveld et al., 2016). Next to the host, seaweed-associated bacteria have been shown to produce complex compounds (Singh et al., 2015) that can control the establishment of novel bacterial species (Rao et al., 2005). Such microbe-microbe interactions can have positive consequences for the algal host, for example, by providing protection against microbial pathogens (Saha and Weinberger, 2019; Li et al., 2022a, 2022b), and they may increase in importance when host defense mechanisms are impaired under stress (Longford et al., 2019). Bacteria might also indirectly influence the outcome of the microbial community on the fitness of the seaweed by mediating the invasion success of others (Nappi et al., 2022). While it is not clear which of these mechanisms is at play here, our results indicate that the low level of novel bacterial recruitment is consistent across *D. dichotoma* genotypes and is not dependent on the composition or diversity of the initial bacterial communities, as these differ among genotypes.

In line with previous studies on brown seaweeds (e.g., Mensch et al., 2016; Minich et al., 2018), we did find an effect of temperature on the algal-associated bacterial communities. In particular, low temperature communities were enriched in certain bacteria that were absent or less abundant at higher temperatures and, hence, correlated negatively with temperature. It is worth noting that the bacterial inocula were sourced from seawater which had approximately the same temperature as the lower experimental temperatures. Since temperature is known to drive bacterial seawater communities (Sunagawa et al., 2015), it is possible that bacteria in the inocula had a higher relative fitness at those lower temperatures. Three bacterial species stand out, namely two *Arcobacter* strains and a *Marinomonas* strain, as they were the only bacteria that were consistently capable of establishing at lower temperatures after being supplemented by the inocula. Both genera have often been detected on seaweeds (Hollants et al., 2011; Ojha et al., 2017; Selvarajan et al., 2019), but their ecology is currently not well understood (Satomi and Fujii, 2014; Salas-Massó et al., 2016). Their presence on the control metal rings and absence on the *in situ* collected seaweeds suggests that they might not be specifically associated with the seaweed but rather proliferate on any available surface at those lower temperatures in our experimental setting. While these bacteria seem to be superior competitors under cold conditions, their establishment did not affect the growth rate of the algal individuals in any way. This highlights the need to link microbiome analyses to host physiology, as the gain (or loss) of bacterial symbionts may not necessarily have implications for host fitness under stressful conditions.

By fitting thermal response curves, we found that the optimal temperature for growth ranged between 21.96 and 22.96 °C. These results are largely congruent with a previous study on *D. dichotoma*, which identified 20 °C as the optimal growth temperature among 8, 12, 16, 20, and 24 °C (Bogaert et al., 2016). Importantly, our results indicated that the thermal response of *D. dichotoma* differed substantially between genotypes. As there is a difference in host genetics and in initial bacterial community composition between these genotypes, we hereby approached host and associated bacterial community to be an ecological unit. It seems most plausible that the observed differences in thermal response result from the host itself. Further research is needed to elucidate the existence of intraspecific, and even intra-population, variation in thermal response, which represents another important mechanism that can buffer organisms under global warming (e.g., Herrando-Pérez et al., 2019; Bishop et al., 2022).

As another avenue for future studies, it may be interesting to explore the effects of exchanging bacteria between seaweed populations that are naturally growing at different temperatures. Such studies could elucidate whether bacteria associated to warm-origin populations have a higher ability to confer thermal stress resilience, which is particularly relevant given that many seaweed species, including *D. dichotoma* (Tronholm et al., 2010), experience a wide range of thermal regimes throughout their geographical distribution.

5. Conclusion

To conclude, this study has shown that the addition of bacteria does not affect the growth of *Dictyota dichotoma* across its thermal range, which is

likely due to a failure of growth promoting bacteria to establish in the seaweed-associated bacterial community. While the role of bacteria on host performance under different environmental conditions is typically done by using axenic material as reference, our results suggest that such an approach might overestimate the role of the bacterial addition in conferring host resilience to stress. The differences in the bacterial communities observed under experimental conditions compared to those associated with *D. dichotoma in situ* illustrate that we need to further increase complexity of our experimental setup in order to better understand the effect of environmental changes on naturally growing populations of this seaweed. In light of global warming, most research is focused on evaluating the effects of elevated temperatures, but we noticed the most pronounced effect of temperature on the bacterial communities at lower temperatures. Future research focusing on the lower thermal limit of the host might expose interesting bacterial-host dynamics that would otherwise remain unnoticed.

CRediT authorship contribution statement

Soria Delva: Conceptualization, Methodology, Formal analysis, Investigation, Visualization, Writing - original draft, Writing - review & editing. **Bernard De Baets:** Funding acquisition, Writing - review & editing. **Jan M. Baetens:** Funding acquisition, Writing - review & editing. **Olivier De Clerck:** Writing – review & editing, Supervision, Funding acquisition. **Willem Stock:** Conceptualization, Methodology, Formal analysis, Investigation, Writing – original draft, Writing – review & editing, Visualization, Supervision, Funding acquisition.

Data availability

The high throughput sequence data is available in the NCBI BioSample database under BioProject ID PRJNA931960.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2023.162532.

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