



# Simulated Heatwaves Lead to Upregulated Chemical Defense of a Marine Foundation Macrophyte Against Microbial Colonizers

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Climate change is characterized not only by an increase in mean temperature, but also an increase in the variability around the means causing extreme events like marine heatwaves. These events are expected to have strong influence on the ecology of marine foundation species such as the eelgrass *Zostera marina*. Bacterial and macroscopic foulers are ubiquitous in the marine environment; they can have detrimental impacts on macrophytes and warming is known to enhance bacterial fouling. Thus, to investigate the consequence of heatwaves on the chemical defense of eelgrass against microbial colonizers, we incubated *Z. marina* plants in the Kiel Outdoor Benthocosm system under ambient control conditions and two different heatwave treatments: a treatment experiencing two spring heatwaves followed by a summer heatwave, and a treatment only experiencing just the summer heatwave. The capacity to deter microbial colonizers was found to be significantly up-regulated in *Z. marina* from both heatwave treatments in comparison to *Z. marina* under control conditions, suggesting defense regulation of *Z. marina* in response to marine heatwaves. We conclude climate extremes such as heatwaves can trigger a regulation in the defense capacity, which could be necessary for resilience against climate change scenarios. Such dynamics in rapid regulation of defense capacity as found in this study could also apply to other host plant – microbe interactions under scenarios of ongoing climate change or extreme climate events like heatwaves.

**Keywords:** chemical defense, *Zostera marina*, heatwaves, fouling, climate extremes, plant–climate interactions, climate change

## INTRODUCTION

Climate change is predicted to affect life on earth. Many species have already shifted their geographical range in response to global warming and a poleward redistribution of species has been documented in both terrestrial and marine environments (Sunday et al., 2012). Global and regional climate models predict not only an increase of mean temperatures but also increased duration and frequency of severe heatwave events by 17 and 34%, respectively (Oliver et al., 2018). Marine species are often less adapted to fluctuating temperatures than terrestrial organisms, as the general

buffering capacity of the ocean often prevents abrupt temperature change (Hoegh-Guldberg and Bruno, 2010), thus making them more vulnerable toward abnormally high temperature.

Macrophyte based coastal ecosystems – and in particular seagrass meadows – often lack species redundancy, which can make them particularly vulnerable to warming stress (Ehlers et al., 2008). Temperature stress effects on such habitat forming species may therefore result in changes of whole coastal environments (Holling, 1973; Hughes et al., 2003). In many parts of the world seagrasses are of great importance for the near shore marine ecosystems, and *Zostera marina*, also known as eelgrass, is one of the most dominant seagrass species in temperate areas (Den Hartog and Kuo, 2006). The eelgrass ecosystem is very productive and supports a diverse assemblage of organisms (Hemminga and Duarte, 2000; Den Hartog and Kuo, 2006). Within this system, eelgrass together with seaweeds provides a supportive nursery habitat for several fish and shellfish species that are of economic importance (Orth et al., 2006; Plummer et al., 2013) and the basis of a complex food web that supports all other organisms. Eelgrass meadows have numerous ecological functions that are important for the stability of the ecosystem and at the same time for fisheries (Duarte, 2002). Hence, loss of eelgrass on a larger scale can lead to serious consequences and devastation of the seagrass ecosystem (Hemminga and Duarte, 2000; Waycott et al., 2009). The currently observed decline of eelgrass in most coastal environments (Waycott et al., 2009) often appears accelerated by warming. For example, a heatwave of 3 weeks during the summer of 2003 caused major losses and die-offs of eelgrass in Europe (Greve et al., 2003; Reusch et al., 2005). High temperature (30°C) was also found to have severe negative impacts on eelgrass in the lower Chesapeake Bay, affecting growth, tissue integrity, nitrogen metabolism, and protein/enzyme synthesis (Hammer et al., 2018). Also in the future such heatwaves are likely to cause further losses of eelgrass through direct physiological stress due to damage to photosystems or negative carbon balance (Winters et al., 2011). At the same time incidences of infectious diseases often increase with high temperatures, including wasting disease of *Z. marina* (Sullivan et al., 2018; Brakel et al., 2019), which might increase the sensitivity of eelgrass further.

The optimum water temperature for growth of *Z. marina* is between 10 and 20°C. Temperatures of 25°C or more can largely decrease photosynthetic rates and survival of *Z. marina* (Nejrup and Pedersen, 2008). While current maximum summer temperatures (around 24°C) in the southern Baltic Sea are still tolerated by eelgrass, additional temperature increase in the coming decades may threaten the fitness of eelgrass plants in this brackish inland sea (Salo and Pedersen, 2014). In the Kiel Fjord, summer water temperature has been predicted to increase between 3 and 5°C and winter temperature could increase even by 5–10°C before the end of the century (BACC 2006). Communities and ecosystems in the western Baltic Sea have already suffered from climate change and knowledge of the vulnerability of key coastal habitat forming species such as *Z. marina* to temperature change is important for the development of management concepts for this environment (Ehlers et al., 2008).

Being a significant source of dissolved organic carbon for heterotrophic bacteria (Asmala et al., 2019), eelgrass – like other aquatic macrophytes – is continuously subject to colonization by epibacteria (Fahimipour et al., 2017). Many of the consequences of this microbial settlement are detrimental, for example microbial settlers often attract macrofoulers (Wahl et al., 2012), and seagrass is not an exception (Hughes et al., 2009; Waycott et al., 2009). The microbiota of eelgrass roots and leaves comprise specific symbiotic components that provide important metabolic functions (Cucio et al., 2016; Ettinger et al., 2017). Instead of a specific core microbiome eelgrass leaves bear a fraction of the bacterial community that is present in the surrounding water (Bengtsson et al., 2017; Fahimipour et al., 2017). This strongly suggests that microbes settle on eelgrass leaves more or less randomly and they then undergo a filtering community assembly mechanism (Fahimipour et al., 2017). The later was shown to be driven at least in part by chemical defense compounds that are present on the host surface (Guan et al., 2017). Seagrasses defend themselves against excessive colonization; one of the strategies is the production (Fusetani, 2011; Marhaeni et al., 2011; Zidorn, 2016) and release of functional compounds that repel bacteria (Guan et al., 2017). In particular, phenolic secondary metabolites from seagrasses have repeatedly been identified as bioactive compounds that may affect bacterial settlers (Iyapparaj et al., 2014) and macrofoulers (Davis et al., 1989). Recently Rosmarinic acid was identified as a main defense compound against bacterial settlers that colonize the surface of *Z. marina* (Guan et al., 2017).

Although temperature has been reported to be an important factor influencing the production of phenolic compounds in terrestrial plants (Løvdal et al., 2010), equivalent research with marine plants has rarely been conducted, except by Vergeer et al. (1995), who suggested that a negative relationship between temperature and the content of phenolic defense compounds (that are active against the wasting disease pathogen *Labyrinthula zosterae*) may exist in *Z. marina*. Further, the concentrations of phenolics in eelgrass can be subject to seasonal variation. Ravn et al. (1994) detected the highest tissue concentrations of Rosmarinic acid in eelgrass during the warm season. The same was also observed with surface concentrations of this compound, which were repeatedly observed to be high in summer (Guan et al., 2017, 2019) and low during the cold season (Guan et al., 2019; Papazian et al., 2019).

The environmental stress hypothesis (ESH) predicts unfavorable environmental conditions could generally limit resources or the physiological capacity for chemical defenses in host macrophytes (Menge et al., 2002; Weinberger et al., 2011). For example, in the red seaweed *Laurencia dendroidea* severe conditions of temperature and salinity promoted a decrease in concentrations of the surface associated anti-fouling compound elatol, as predicted by the ESH (Sudatti et al., 2011). In other cases, chemical defense of marine macrophytes has been found to contradict the ESH. For example, surface associated defense is unaffected in the brown seaweed *Fucus vesiculosus* at a relatively high temperature of 25°C (Saha et al., 2014).

The first goal of the present study was to investigate whether *Z. marina*, under heatwave treatments, undergoes upregulation or downregulation of its chemical defense against

microsettlers when compared to *Z. marina* exposed to control conditions. A second goal was to investigate whether repeated heatwaves would result in a weaker or stronger defense than one single heatwave.

To test this eelgrass was incubated in outdoor benthocosms under the same conditions as in natural assemblages, except for heatwave treatments. Control treatments (tanks under control conditions without heatwaves; 0HW) were compared to treatments with one simulated summer heatwave (1HW) and treatments which experienced two simulated spring heatwaves along with the simulated summer heatwave (3HWs). Response variables studied were (a) anti-settlement activity of surface extract of *Zostera* against bacterial colonizers, (b) abundance of bacterial colonizers on eelgrass and (c) concentrations of the defense compound Rosmarinic acid on the surface of eelgrass leaves.

## MATERIALS AND METHODS

### *Z. marina* and Sediment Collection

*Z. marina* was collected by scuba diving at a water depth of 3–4 m from Falkenstein, Kiel, Germany (54°38' N/10°17' E) in May 2015. Sediment was collected from the same site and on the same day, using a Van Veen grab sampler that collected ~15–20 cm of the top sediment. The sampling site is located in a distance of 7 km from the experimental site in the Kiel Fjord (southwest Baltic Sea), a brackish water environment, where the average surface water salinity is around 16, with a possible variation between 10 and 24. *Z. marina* was transported in cooler boxes to the lab and maintained for one night in 300 L tanks containing Baltic Sea water (waterflow at 1800 L/d) in a climate room at 15°C, under a light intensity of 20–30  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ , before they were stocked into the outdoor mesocosms as described below.

### Experimental Setup

#### Benthocosm System and Temperature Treatment

The experiment was conducted in the Kiel outdoor benthocosms facility (KOB). The KOB is a mesocosm system located on aluminum floats in the inner Kiel Fjord, at ~7.5 km from the collection site. The KOB allows for the control and manipulation of various environmental parameters to obtain target treatments (**Supplementary Figure S1**; for details about and images of the KOB see Wahl et al., 2015). Six benthocosm tanks – each with a volume of 3,000 L (inner size: 2 m  $\times$  2 m  $\times$  0.9 m) – were used in this experiment. Each tank was evenly divided into two independent subunits by implementing a PVC wall. The water temperature in each subunit was adjusted with an accuracy of close to 100% (see below) by aquarium heaters and coolers. These were controlled by designed software (GHL Profilux Control or GHL Control Center) that allows for the realization of complex temperature treatments. In total 12 controllers were applied to permanently control and record the temperature in each of the subunits and an additional sensor was used to measure the temperature in the Kiel Fjord. Seawater was constantly pumped from the Fjord (water depth: 1–1.2 meter) into the tanks at

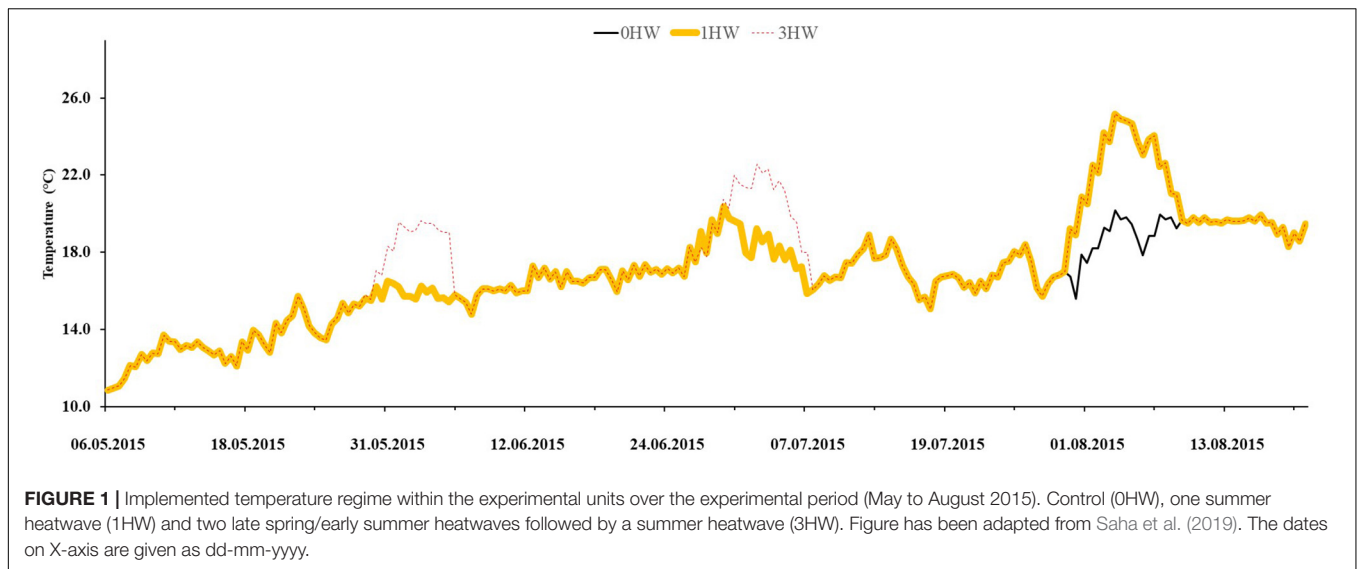
an exchange rate of 1,800 L per day, while wave generators prevented stratification within the tanks and simulated near-natural conditions. As a consequence, salinity in the benthocosms experienced the same fluctuations as salinity in Kiel Fjord surface water (Wahl et al., 2015). During the experiment described here, salinity varied between 13.9 and 16.4 with a mean of 15.4 (Pansch et al., 2018).

To set up the temperature regimes in the tanks we analyzed temperature fluctuations in the Kiel Fjord from May to September in 15 preceding years (2000–2014) and in time intervals of 5 min (Pansch et al., 2018). The temperature regime of 2009 was identified as the least divergent from the mean, i.e., as the regime exhibiting the least extremes. This temperature fluctuation regime was therefore realized in two tanks (four subunits) as the control treatment. The remaining four tanks received the same temperature treatment, but with an intermittent simulation of heatwaves. Three heatwaves (3HWs) were applied in two tanks (four subunits) in May, July and August, each lasting 7 day, while only the last of these heatwaves, i.e., 1HW, was applied in two other tanks (four subunits) in August only, when the seasonal temperature increase had reached its annual maximum.

To identify realistic heatwave treatments, we analyzed temperature fluctuations in the Kiel Fjord between 2000 and 2014. We considered heat waves as events during which the daily temperature increases over two or more consecutive days exceed a given threshold; and we defined this threshold as the highest daily increase that allowed finding, at least, one heat wave in each year of the time series. In the period 2000–2014, this threshold corresponded to 0.7°C/day. This threshold was then used to identify heat waves and to further analyze them. Increases up to 1.2°C d<sup>-1</sup> and a maximum temperature anomaly of +5.2°C above average occurred twice in these years (for further details see Pansch et al., 2018). Hence, the 1st (May) and 2nd (July) heatwave of the 3HW treatment followed a temperature increase of 1.2°C d<sup>-1</sup> over 3 days, remained at the elevated temperature (+3.6°C) for 4 days and dropped to control conditions over a period of 2 days. The heatwave in August in both treatments (3HWs and 1HW) increased by 1.7°C day<sup>-1</sup> over 3 days, remained at the elevated temperature (+5.2°C) for 4 days and dropped to control conditions within 2 days. These temperature increases applied during the heatwave treatments represent the maximal increases observed within two consecutive days during the years 2000–2014. Maximum temperatures obtained were 20.4°C for the control treatments and 25.2°C for 1HW and 3HW treatments (see **Figure 1** for the treatment applied). This maximum temperature of 25.2°C exceeds maximum temperatures that occurred 2000–2014 in shallow, open waters of the Kiel Fjord by about 1°C (Pansch et al., 2018). The achieved temperature treatments were largely identical with programmed daily and seasonal fluctuations. Deviations from programmed values were only recorded during some exceptionally warm days, mostly during late afternoon.

#### Set-Up With Eelgrass

Eelgrass was planted into individual 5 L aquaria made from polystyrene (26 cm  $\times$  16 cm  $\times$  15 cm) that contained



sediment to a depth of  $10 \pm 1$  cm. Six individual plants were planted in each aquarium to obtain an initial density of 144 shoots  $m^{-2}$ , corresponding with eelgrass meadows of low density (Holmer and Nielsen, 1997). Eleven aquaria – together containing 66 plants – were placed at the bottom (70 cm water depth) of each benthocosm subunit. In addition, various benthic invertebrates and macroalgae that are common in the Kiel Fjord (e.g., *Idotea baltica*, *Littorina littorea*, *Gammarus spp.*, *Mytilus edulis*, *Balanus improvisus*, *Fucus vesiculosus*, *Agarophyton vermiculophyllum*) were inoculated into all benthocosm subunits at identical densities and natural abundances (Werner et al., 2016), in order to create a diverse benthic ecosystem. The performance of benthic and free living animals and macrophytes has been presented in Pansch et al. (2018) and Saha et al. (2019). Briefly, the 3HW treatments resulted in a reduction of longitudinal growth by 40% and increased formation of side branches in *Z. marina*, whereas photosynthesis and respiration rates were not significantly affected by both treatments. Symptoms of wasting disease throughout the experiment were generally low and not affected by the treatments and also the defense capacity of *Z. marina* against the wasting disease pathogen *Labyrinthula zosterae* was not measurably compromised (Saha et al., 2019).

### Sampling for *Z. marina* Surface Extract Generation, Rosmarinic Acid, and Epibacteria Quantification

Samples for *Z. marina* surface extract generation, Rosmarinic acid quantification and quantification of epibacteria on the surface of *Z. marina* were taken immediately prior to the start of the experiment in May, after application of two spring heatwaves in July and after the end of the experiment, i.e., after the summer heatwave in August. At each of these occasions, two plants were sampled from each subunit and processed as described below. The replication

level was four in each case, with each subunit acting as a true replicate.

### Surface Extract Generation

The “dipping method” (de Nys et al., 1998) was modified to obtain compounds from the surface of sampled eelgrass leaves as described in Guan et al. (2017). Briefly, spin-dried leaves (entire leaves) were dipped into 2-propanol for 5 s, which allowed to extract surface compounds without disruption of epidermal cells (see Guan et al., 2017 for details). This method is benign and does not extract intracellular compounds. The concentration of compounds was related to the extracted surface volume, which was determined by a product of the leaf surface area and the mean thickness of 30  $\mu m$  of the leaf surface boundary layer of marine plants (Wahl et al., 2010). The leaf surface was calculated from the leaf wet weight, using the conversion factor of  $78.9903 \text{ cm}^2 \text{ g}^{-1}$  (Guan et al., 2017). The obtained surface extracts were filtered through glass fiber-filters with a mesh size of 0.2  $\mu m$  (Carl Roth, Karlsruhe, Germany), evaporated *in vacuo* and stored at  $-20^\circ\text{C}$ . For all subsequent steps the extracts were redissolved in methanol to yield stock solutions at the same 3.33-fold natural concentration (for example, extract originating from a surface volume of 1,000  $\mu l$  dissolved in 300  $\mu l$  of methanol).

### Defense Capacity Test

Inhibition or reduction in bacterial settlement and attachment represents the first line of defense against microbial challenge (Lane et al., 2009). Thus, an anti-*settlement assay* was employed as the most relevant criterion for determining anti-microfouling defense, as it quantifies both repellent and toxic effects (Wahl et al., 1994). Anti-settlement assays were done to quantify the defense capacity of *Z. marina* surface extracts against fouling bacterial strains isolated from various substrates as described previously (Saha et al., 2011, 2012, Guan et al., 2017). Bioassays were conducted with five

bacterial isolates originating from non-living substrates on North Sea and Baltic Sea coasts (Saha et al., 2016) that had been cryopreserved at  $-80^{\circ}\text{C}$ . The isolates were *Alteromonas stellipolaris*, *Pseudoalteromonas carrageenivora*, *Marivita litorea*, *Polaribacter dokdonensis*, and *Vibrio cyclitrophicus*. All bacteria were grown in sterile liquid nutrient medium [5 g peptone (Carl Roth, Karlsruhe, Germany), 3 g yeast extract (Carl Roth, Karlsruhe, Germany), 16 g artificial sea salt and 1 L deionized water] at  $25^{\circ}\text{C}$  for 1 or 2 days before they were tested individually in the assays.

To test 1-fold natural concentrations of extracted compounds, 30  $\mu\text{l}$  of each extract dissolved in methanol at 3.33-fold-natural concentration were added into eight wells (to account for the variability in the bacterial settlement rates) on 96 well microtiter plates and diluted with 70  $\mu\text{l}$  of pure methanol. Control wells received 100  $\mu\text{l}$  of pure methanol instead. The solvent was then evaporated in a freeze-dryer and 100  $\mu\text{l}$  of bacterial suspension (with O.D. between 0.5 and 0.8) were added into four of each eight wells, while sterile seawater was added into the remaining four wells. After 2 h of settlement at  $25^{\circ}\text{C}$  in darkness, the wells were emptied and immediately rinsed with 100  $\mu\text{l}$  of sterile seawater. Syto 9 (Invitrogen, Waltham, MA, United States) dissolved in sterile sea water was then applied at a final concentration of 5  $\mu\text{M}$  to stain the DNA of attached bacterial cells (as described in Saha et al., 2011, 2012; Guan et al., 2017). After the staining procedure excess dye was rinsed away with sterile sea water and the fluorescence intensity was measured with a Hidex Chameleon IV plate reader (excitation: 477–491 nm, emission: 540 nm). Extract replication was four times for each of the treatment tanks and control tanks.

## Quantification of Rosmarinic Acid

Rosmarinic acid was quantified by HPLC on a semi-preparative LichroCart 250/10 Purospher STAR-rp 18 ec column (VWR, Radnor, United States) using a Varian 940-LC with diode array detection (Varian 940-LC, Agilent Technologies, Palo Alto, CA, United States). The solvent system ranged from 0.1% formic acid dissolved in Acetonitrile to 0.1% formic acid dissolved in water, as described previously (Guan et al., 2017). The detection of Rosmarinic acid was based on its light absorption at 350 nm and concentrations were derived from peak areas after calibration of the system with purchased Rosmarinic acid.

## Epibacterial Abundance on *Z. marina*

Biofilms from 1  $\text{cm}^2$  of *Z. marina* surface (1 cm from the tips) were collected by swabbing with a sterile cotton tip, followed by vortexing the cotton tip for 30 s in a sterile Eppendorf vial containing 1 ml of sterile-filtered seawater (SSW, 16 ‰). The relative abundance of diatoms (and any other possible photoautotrophs) in 100  $\mu\text{L}$  of subsample was determined by measuring the fluorescence of chlorophyll *a* at 477–491 nm (excitation) and 677 nm (emission), using a plate reader (Hidex Chameleon IV, Turku, Finland) and 96-well microtiter well plates (Greiner). Subsequently, the relative density of all microfoulers (including bacteria and diatoms) was determined by staining all the particles in the same 100  $\mu\text{L}$  subsample with the fluorescent DNA-binding dye Syto 9 at 5  $\mu\text{M}$  (Invitrogen

GmbH). Following an incubation time of 10 min in darkness, fluorescence was subsequently measured (excitation 477–491 nm, emission 540 nm), using the same plate reader. Replication was four for each treatment. The first measurements provided data on treatment effects on the relative microalgal density at the algal surface, while the second measurement provided similar information on all epibiotic cells. Thereafter, the bacterial abundance was determined in relative units (RU) by subtracting the first measurement from the second one.

## Statistical Analysis

A log effect ratio was used to express the relative activity strength of extracts (i.e., defense capacity) from each plant in RU. It is calculated as the logarithm of the ratio of fluorescence intensity of target microbes settled in presence of extract and fluorescence intensity of target microbes settled on solvent controls. Thus, negative values indicate an inhibitory effect of an extract against a target bacterial strain, while “0” would indicate the absence of such an effect. Mean log effect ratios of the activity strength against all tested bacterial strains are presented here.

Subreplicates – data obtained within the same tank at the same time point – were averaged and tanks were treated as replicated experimental units. Data obtained within the same unit at different time points were treated as repeated measures. Two-way-repeated-measures-ANOVA – with the factor sampling month as within-measures factor and the factor heatwave treatment as between-measures factor – was used to investigate the interactive and single effects of both factors on the activity strength against bacteria, the production of Rosmarinic acid in eelgrass, and the log-transformed densities of bacteria colonizing eelgrass plants. Shapiro-Wilk’s test was used to test whether data within groups were normally distributed ( $p < 0.05$ ) and Levene’s test was used to test for homogeneity of variances ( $p < 0.05$ ). Tukey’s HSD test was used to compare the means of single groups. The relationships between the abundance of bacteria settled on *Z. marina*, activity strength of surface extracts and concentrations of the defense compound Rosmarinic acid were analyzed with simple linear regression ( $p < 0.05$ ). All statistical analyses were conducted with the Statistica 8.0 software package (StatSoft, Tulsa, OK, United States).

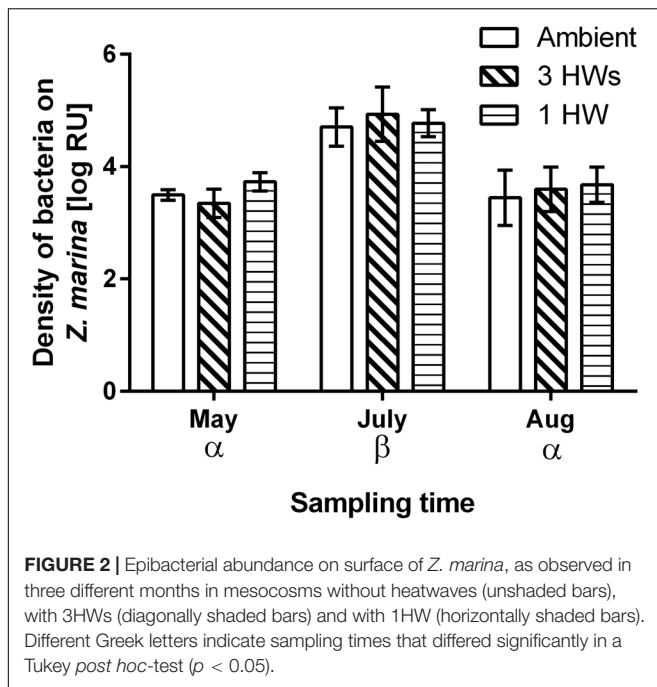
## RESULTS

### Variation of Bacterial Density on *Z. marina* Surface

Bacteria that settled on eelgrass differed significantly between sampling times (Figure 2 and Table 1). They peaked in July (0HW: 4.71 RU; 1HW: 4.93 RU, 3HWs 4.77 RU) and had lower and similar densities in May and August, but were unaffected by the heatwave treatments ( $p = 0.2714$ , Figure 2 and Table 1).

### Defense Capacity and Concentration of Rosmarinic Acid

All five tested bacterial isolates were deterred by *Z. marina* surface extracts (Supplementary Figure S2). However, sensitivities of



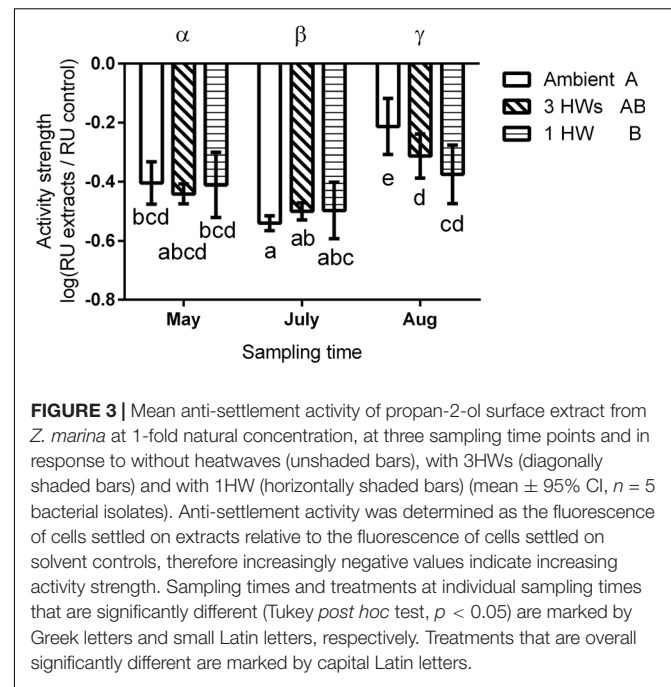
**TABLE 1 |** Two-way-repeated measures ANOVA of bacterial density on *Z. marina*.

Effect	SS	Degree of freedom	MS	F	P
Intercept	566.4868	1	566.4868	8985.591	<0.0001
Treatment	0.1907	2	0.0954	1.513	0.2714
Error	0.5674	9	0.0630		
Month	12.6256	2	6.3128	180.978	<0.0001
Month*Treatment	0.3274	4	0.0819	2.347	0.0935
Error	0.6279	18	0.0349		

Heatwave treatment was used as between-measures factor and sampling month was used as within-measures-factor.

bacterial isolates differed, and the sensitivities of single isolates varied in most cases with sampling time and – to a lesser degree – with heatwave treatment (Supplementary Figure S2 and Supplementary Table S1). Four of the five isolates exhibited significantly lesser sensitivity toward extracts sampled at the end of the experiment than toward extracts sampled earlier (Supplementary Figure S2 and Supplementary Table S1). Correspondingly the mean sensitivity of all five isolates was significantly lower at the end of the experiment, while the highest mean sensitivity was detected in samples from July (Figure 3 and Table 2). At the end of the experiment both heatwave treatments had a significant influence ( $p = 0.0470$ , Figure 3 and Table 2) on the defense capacity of *Z. marina* (mean activity strength against all five tested strains) and samples from eelgrass experiencing those treatments were more deterrent toward bacterial settlement than samples from control tanks.

The concentration of Rosmarinic acid on *Z. marina* surfaces from different heatwave treatments exhibited no significant differences ( $p = 0.358$ , Figure 4 and Table 3). However, the mean Rosmarinic acid concentration (pooled over the three treatments



**TABLE 2 |** Two-way-repeated measures ANOVA of mean antifouling defense strength of *Z. marina* against five bacterial strains at 1-fold natural concentration.

Effect	SS	Degree of freedom	MS	F	P
Intercept	6.0698	1	6.0698	4644.334	< 0.0001
Treatment	0.0114	2	0.0057	4.377	0.0470
Error	0.0118	9	0.0013		
Month	0.2725	2	0.1363	47.371	< 0.0001
Month*Treatment	0.0497	4	0.0124	4.318	0.0127
Error	0.0518	18	0.0029		

Heatwave treatment was used as between-measures factor and sampling month was used as within-measures-factor.

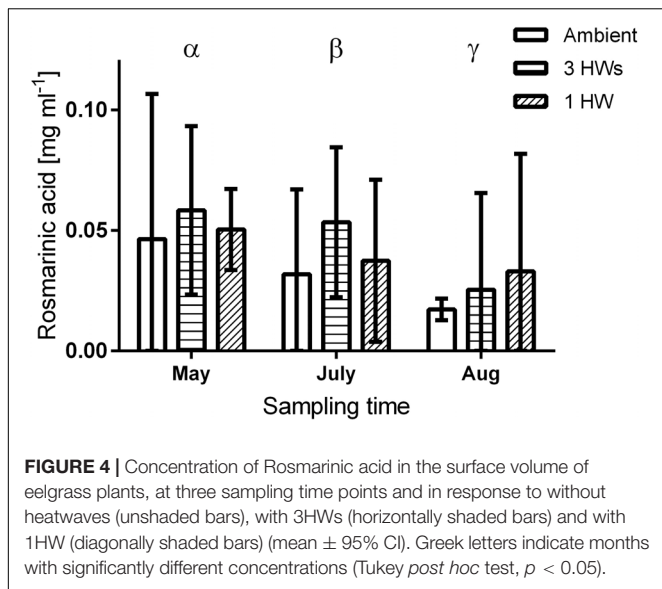
for each month) differed among sampling times and it decreased significantly from May to August.

## Correlations Among Factors

Both antifouling activity and Rosmarinic acid concentration varied between months, but were not significantly correlated for May or July (Figures 5A,B). However, a positive correlation of the two variables existed in August ( $p < 0.0176$ , Figure 5C). Bacterial abundance on eelgrass correlated with antifouling defense activity within the August samples ( $p < 0.0537$ , Figure 5F), but not for May or July samples (Figures 5D,E). Rosmarinic acid concentrations also correlated with the number of settled bacteria on eelgrass in August ( $p < 0.014$ , Figure 5I), but not in May or July (Figures 5G,H).

## DISCUSSION

The general design of our experiment allows for a comparison of heatwave effects and the effects of seasonal warming. During



**TABLE 3 |** Two-way-repeated measures ANOVA of Rosmarinic acid concentration on the surface of *Z. marina*.

Effect	SS	Degree of freedom	MS	F	P
Intercept	46651.13	1	46651.13	90.2616	<0.0001
Treatment	1193.07	2	596.53	1.1542	0.358
Error	4651.60	9	516.84		
Month	4335.65	2	2167.83	3.7621	0.0431
Month*Treatment	633.45	4	158.36	0.2748	0.8904
Error	10372.06	18	576.23		

Heatwave treatment was used as between-measures factor and sampling month was used as within-measures-factor.

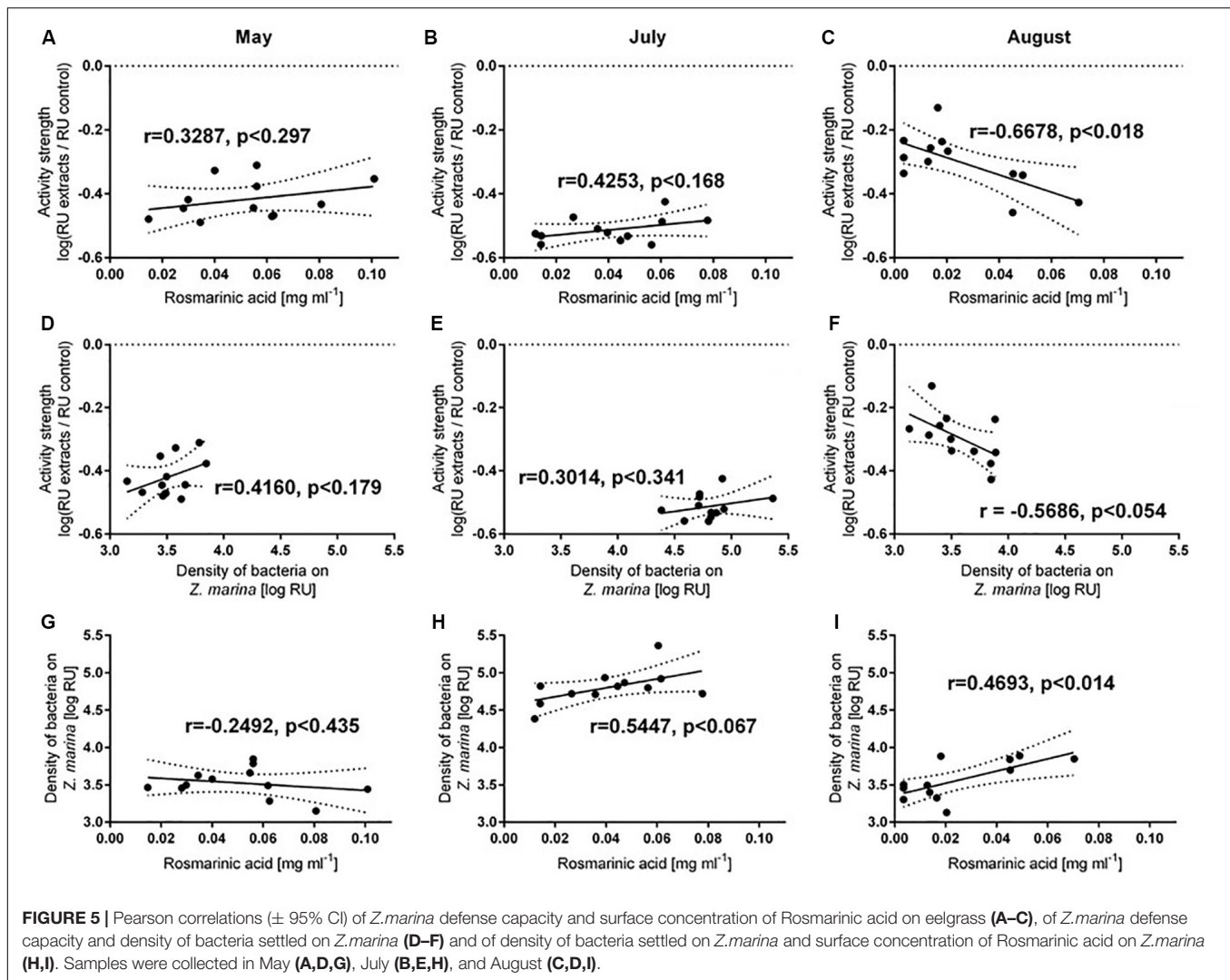
incubation in the benthocosms under control conditions with no heatwave, the *Z. marina* plants experienced a relatively rapid temperature increase from 9.5°C in May to 18.3°C in July, followed by a moderate increase to 19.4°C in August. The applied heatwaves, in contrast, were characterized by a much more rapid increase of 5.2°C within only a few days and they were therefore generally expected to cause more pronounced effects than seasonal warming. However, the seasonal effects were overall more pronounced and marked by a peak in bacterial settlers on *Z. marina* in July. A similar pattern with higher numbers of bacterial settlers on eelgrass in summer than spring and autumn was also observed in a field study conducted in the Kiel Fjord, where it reflected increased pressure by microbial settlers (Guan et al., 2019). In general bacteria tend to colonize aquatic plants in higher numbers at high temperature, either because the bacteria are more active under such condition (Case et al., 2011) or because the hosts become more susceptible when they are weakened by heat stress (Egan et al., 2013). The July peak in our experiment corresponded with a peak in defense activity strength against microbial settlers. This – together with the limited effects of different temperature treatments on the overall performance of *Z. marina* in our experiment (Saha

et al., 2019) and the circumstance that settler densities peaked before autumn – strongly suggests that seasonal fluctuations in settlement pressure rather than heat stress effects on eelgrass were the primary reason for fluctuating settler densities. The peak in defense activity strength in July also suggests that *Z. marina* has a general capacity to regulate its defense activity against settlers according to demand, similar to certain seaweeds (Saha and Wahl, 2013; Wang et al., 2018). The chemical defense of plants is in most cases dynamic and selective (Lankau, 2007) and only microbes that are resistant to the fluctuating chemical defense of *Z. marina* can be expected to colonize the plant.

Despite their more rapid impact heatwaves affected the defense capacity of eelgrass less than seasonal warming. There was no significant difference in defense capacity of *Z. marina* against bacteria among the HW treatments and the controls in May and July, but in August this capacity was relatively increased in samples that experienced heatwave treatments, i.e., 1HW and 3HW. Under control conditions the defense capacity of *Z. marina* significantly decreased from July to August by 65.6%, but this reduction was less pronounced when heatwaves were applied. Application of three heatwaves resulted in reduction of the defense activity strength by 37%, while the 1HW treatment prevented/had a significant decrease of the defense capacity from July to August. Thus, although not significantly different from the 3HW treated samples at the last sampling in August, plants that experienced their first heatwave directly prior to this last sampling exhibited a tendency toward stronger maintenance of defense capacity. This indicates that a single HW treatment causes certainly not a weakened, but rather an increased defense capacity against microfoulers as compared to repeated heatwaves. Thus, preconditioning by repeated heatwaves may not result in a stronger, but in tendency in a weaker defense of *Z. marina* against bacterial colonizers than one single heatwave at the warmest time of the year. This could also explain the fact that no difference in the defense capacities of differently treated *Z. marina* plants was observed in July, when plants from the 3HW treatment had just been exposed to the second (and thus to a repeated) HW, while the other two groups had received no HW yet.

The differences in defense activity strength in August were not reflected in differences in the densities of microbial settlers on *Z. marina*, which can be due to various reasons. One possible explanation can be direct heatwave effects on the microbial settler community. For example, an increase in bacterial settlement pressure may have compensated the decrease in settlers resulting from increased defense capacity (or vice versa). Alternatively, our sampling in August possibly happened too early or too late after the upregulation. In the first case (i.e., sampling directly after the upregulation) its effects on the microbiota had possibly not yet happened. In the second case (i.e., sampling too late) settler communities resistant to the new defense activity may already have been selected after the effects occurred.

Promoting effects of temperature increase on the antifouling defenses of marine macrophytes have been described before. For instance, surface concentrations of the deterrents Fucoxanthin and DMSP on the brown seaweed *Fucus vesiculosus* were higher at an increased temperature of 25°C (Saha et al., 2014). Thus, increased defense activity for heatwave treated samples when



compared to control samples could be a result of increased production of other (unidentified, Guan et al., 2017) defense chemicals for *Z. marina* at higher temperature.

Concentrations of the identified deterrent compound Rosmarinic acid changed steadily with the seasonal temperature increase, however, there was no significant change in Rosmarinic acid concentrations among different treatments. Rosmarinic acid concentration only differed among different months and showed a decreasing trend with the seasonal increase in water temperature from May through July to August. This strongly suggests that additional (unidentified) deterrent compounds that underwent other seasonal concentration dynamics must have been active and it confirms our previous conclusion that Rosmarinic acid cannot be the only compound that deters microsettlers from the surface of *Z. marina* (Guan et al., 2017; Papazian et al., 2019). Likewise, the differences in deterrence observed after different heatwave treatments cannot be fully explained with changes in Rosmarinic acid concentrations. In August, antifouling defense was strongest after the application of 1HW and 3HWs and the weakest under

control conditions. Mean Rosmarinic acid concentrations also decreased in this order, although there was no significant difference among different treatments and controls. However, heatwave treatments in August had no significant effect on Rosmarinic acid concentrations and correlations between Rosmarinic acid concentration and antifouling activity were not detected in May and July. A positive correlation was detected in August between the abundance of bacteria settled on *Z. marina* plants and the Rosmarinic acid surface concentration, suggesting that the increase in antifouling defense in the presence of high numbers of settlers in August could be possibly due to an increase in Rosmarinic acid. It remains unclear whether absence of a significant effect in July after the application of two heatwaves was due (i) to the generally increased defense capacity of *Z. marina* in July, or (ii) due to the fact that the heatwave applied in July was a second heatwave (perhaps resulting in a less pronounced effect) or (iii) to the fact that other compounds than Rosmarinic acid (that were, perhaps, simply not affected by the heatwave treatment) apparently dominated the deterrent surface chemistry of *Z. marina*.



## CONCLUSION

In conclusion, the application of a single heatwave in August during the warmest period caused a significant increase of anti-settlement activity when compared to control treatments, while this increase was less pronounced and non-significant in plants that had experienced preceding heatwaves. Since both, the 1HW and 3HWs treated plants had significantly increased defense capacity at the end of the experiment we conclude that a regulation of chemical defense capacity to heat stimulation must have taken place. Thus, even after transient exposure to water temperatures of 25°C, *Z. marina* from the Baltic Sea seems capable of a dynamic management of its surface defense chemistry; even though increased mortality and reduced growth were previously observed with plants from the same region that were maintained for 6 weeks at this temperature (Nejrup and Pedersen, 2008). In this light a collapse of *Z. marina* resistance to epibionts will hardly be weakened by such extreme heat events, provided the heatbursts do not occur over prolonged time periods.

## DATA AVAILABILITY STATEMENT

The content of this publication is part of the doctoral thesis of CG (Guan, 2017). The datasets generated for this study are available at PANGAEA, doi: 10.1594/PANGAEA.918682.

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## AUTHOR CONTRIBUTIONS

CG and MS performed the experiments. CG and FW analyzed the data. CG, MS, and FW conceived and designed the experiments and wrote the manuscript. All authors contributed to the article and approved the submitted version.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fmars.2020.00463/full#supplementary-material>

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**Conflict of Interest:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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