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The role of lag phases between real-term marine heatwaves in the trait responses of two macrophyte species

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ABSTRACT

Coastal marine macrophytes are critical ecosystem engineers providing valuable ecosystem services. However, they experience detrimental impacts from climate change-induced stresses such as marine heatwaves (MHW), which are becoming more intense and frequent. This study investigated trait responses in real-term heatwaves, Continuous (1MHW) and Consecutive (with a 4-day lag phase, 2MHW), on two key macrophytes, the seaweed *Fucus vesiculosus* and seagrass *Zostera marina*. Our results showed very few negative effects on traits from both temperature treatments. Physiological traits indicated that both macrophytes were not stressed by the treatments. *Fucus vesiculosus* showed little response to changes in temperature and the 2MHW treatment, which considered the lag phase, showed larger changes in frond area compared to the 1MHW treatment. In *Z. marina*, leaves presented statistically significant higher carbon content in the 1MHW treatment than in the control. Significantly higher leaf elongation rates and leaf width were also observed in *Z. marina* for the 2MHW treatment in comparison to the control. *Fucus vesiculosus* showed high acclimatization to changes in temperature, likely because it is a species adapted to grow in dynamic intertidal habitats. Contrary, *Z. marina* appeared to be more sensitive to the 1MHW treatment, as more significant changes were observed, however, the lag phase seemed not to be important in *Z. marina* as there was no change in trait response. Exploring the role of lag phases of different duration in the context of real-term MHW predictions is an important research direction and has relevance for ecosystem resistance that will ultimately affect the resilience of marine macrophyte populations.

1. Introduction

Coastal marine macrophytes, seaweeds and seagrasses, are valued for the ecosystem services they provide (Barbier et al., 2011; Cotas et al., 2023). These services include, among others, their role in mitigating anthropogenic CO_2 emissions through “blue carbon” sequestration and storage and fueling of food webs by supplying a combination of food and shelter, including commercially important species (de los Santos et al., 2020; UNEP, 2023). Despite their global significance, there are still gaps of knowledge about how ecological functions and subsequent services sustained by these macrophytes are affected by global climate change in many parts of the world.

Seaweed and seagrass ecosystems are facing multiple stressors from global change that adversely affect their biodiversity, resilience and functioning. Among these stressors, one of the greatest threats is the increase in seawater temperature due to global warming (Smith et al., 2023). Mean seawater temperature is expected to continue to increase over the next century, while more frequent and longer marine heatwaves (MHWs), that have already increased their geographical extension to higher latitudes, are also predicted (Oliver et al., 2018). MHWs are anomalous warm periods with a duration of at least five days exceeding the 90th percentile of historic temperatures (Hobday et al.,

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2016). As temperature is one of the main drivers of biochemical reactions, coastal macrophytes exposed to MHWs might show higher energetic costs to maintain growth, photosynthesis, reproduction, and, hence, survival (e.g. Piñeiro-Corbeira et al., 2018; Zayas-Santiago et al., 2020). In particular, field-based observational studies on natural coastal marine ecosystems worldwide have already shown population shifts and changes in distribution due to MHWs (Arias-Ortiz et al., 2018; Izquierdo et al., 2022). Meanwhile, controlled manipulative experiments have increased in recent decades to understand the cause-effect relationships of temperature on marine macrophytes observed in the field. These experimental studies have shown that before population-level changes occur, the study of the response of traits, namely biochemical, morphological and growth, and physiological traits, could serve as early indicators (Roca et al., 2016; Mauffrey et al., 2020) and help to provide a mechanistic understanding of the isolated effects of MHWs. Moreover, traits will determine the responses of the whole ecosystem to stressors and the ecosystem services they maintain (Gillis et al., 2023).

The knowledge accumulated on the effects of temperature on coastal marine macrophytes through these studies is extensive and has laid a foundation about their responses to high seawater temperature. We now know that, while elevations in temperature within the optimum photosynthetic range might be positive for photosynthetic performance, and thus growth, individual fitness is lost and survival rates decrease once a temperature threshold is exceeded (Marín-Guirao et al., 2016; Bennett et al., 2022). However, different marine macrophyte species or the same species inhabiting different geographical regions might not react in a uniform manner under MHWs due to their differences in adaptive processes and physiological and ecological characteristics, which will determine their responses, resistance and resilience (King et al., 2018; Bennett et al., 2022; Stipcich et al., 2022).

Most experimental investigations on temperature have aimed to study the effect of a sustained high average temperature on a single species (e.g., Nguyen et al., 2021) with few studies considering MHW with different properties, such as duration or frequency, that better define real-term MHW scenarios (i.e. MHWs that can already be experienced). Among these factors, the role of lag phases (i.e. a period with the reference temperature condition between successive MHWs) that differentiate continuous and consecutive MHWs have been little studied (Saha et al., 2020; Ducker et al., 2023; Stipcich et al., 2024). Information regarding how these periods affect different ecosystem stability properties, including resistance (i.e. the differential ability to resist changes in response to a perturbation, which is inversely related to the degree of change following a perturbation) in ecosystem characteristics, such as trait responses, is still very scarce (Smith et al., 2023; Hatum et al., 2024). This information is needed to make predictions about marine macrophyte resilience (i.e. the ability of the system to absorb changes of state variables and parameters, sensu Holling, 1973) to climate-change scenarios that will ultimately shape populations. For instance, Hemraj et al. (2020) observed that the capacity of certain marine invertebrates to recover physiologically in the period following MHWs is as important to the resilience as the responses during the MHW. However, to date, few studies with marine macrophytes have considered these aspects, and information about the resistance of different species, or what makes individuals resistant are very scarce (Nguyen et al., 2020; Saha et al., 2020).

This study aims to investigate the effect of MHWs and the role of a lag phase between two MHWs in the trait responses of two coastal marine macrophytes, the seaweed *Fucus vesiculosus* Linnaeus and the seagrass *Zostera marina* Linnaeus. With this aim, we exposed adult individuals of the selected species to simulated MHWs under experimental mesocosm conditions and investigated a suite of traits. We hypothesized that the inclusion of a lag phase (i.e. reference temperature conditions) between two MHWs (2MHW treatment) would reduce the stress impacts in comparison to a MHW of the same duration but with the absence of a lag phase (1MHW), on biochemical, morphological and biomass, growth, and physiological traits of both macrophyte species.

2. Materials and methods

2.1. Study area and marine heatwave categorization

The experiment was conducted in the context of the Baltic Sea, thus exposing selected macrophyte species to MHWs similar to those occurring in the area within the last years and with control temperature and salinity conditions similar to the ones recorded in the sampling area during the experiment. The Baltic Sea is a semi-enclosed micro-tidal brackish inland sea that is characterized by strong gradients in salinity and temperature. Most organisms occurring here are either of marine or freshwater origin that have adapted to these wide ranges of salinity and temperatures (Bonsdorff, 2006). The Baltic Sea is rapidly heating up due to ongoing climate warming. Long-term monitoring (>100 yrs) of water temperature in the northern part of the Baltic Sea, close to Tvärminne Zoological Station (University of Helsinki, Finland), show that the annual average temperature of the surface water has increased by 1.8 °C in the last 90 years and that periods of abnormally warm water events are now more frequent (Goebeler et al., 2022). In this study, we used surface water temperature data from this long-term monitoring series from 1990 to 2020, specifically collected during this period with a CTD probe every ten days (Goebeler et al., 2022), to determine the temperature used in the experiment and to categorize the heatwave. Temperature values for climatological mean and 90th percentile for the experimental period (June) were extracted from the long-term climatology dataset published by (Göbeler et al., 2022). The different MHW categories were calculated by multiples of the difference between the climatological mean and the climatological 90th percentile, which is the threshold used to identify MHWs. This results in four different categories, namely moderate (1-2x), strong (2-3x), severe (3-4x) and extreme (>4x) according to Hobday et al. (2016). The target temperature of the MHWs in this experiment corresponded to a moderate/strong category scenario. (Fig. 1A).

2.2. Biological material collection

Fucus vesiculosus and *Z. marina* were hand-picked by snorkeling and SCUBA diving between the 27th May and the 3rd of June 2022 from two sites at the entrance to the Gulf of Finland, Baltic Sea, in the proximity to Tvärminne Zoological Station, Finland. *Fucus vesiculosus* individuals and small rocks to be used as substratum were collected at Furuskär (59.83271°N, 23.26188°E) at a depth of ca. 1.5 m on the 27th of May, the 2nd of June and the 3rd of June. *Zostera marina* individuals and sediments were collected at Klobbarn (59.82947°N, 23.1792°E) at a depth of ca. 3.5 m on the 1st of June. A mean seawater temperature of 10 °C and a salinity of 6 were observed during the collection period (MONICOAST network of measurement buoys; www.helsinki.fi/monicoast). Collected individuals of *F. vesiculosus* and *Z. marina* had no visible imperfections. The permit for the collection of the study organisms was granted by Tvärminne Zoological Station (University of Helsinki). Both *F. vesiculosus* and *Z. marina* individuals were transported in a container of seawater from the collection sites to the station (<1 h) and placed into aerated tanks in a sheltered outdoor facility prior to the experimental set-up and pre-experimental measurements. The aerated tanks received a flow-through of unfiltered seawater from 7-m depth just outside the station and were exposed to a natural light cycle. The temperature of the incoming seawater followed *in situ* temperature in the field.

2.3. Experimental design and set-up

The mesocosm experiment was conducted in a sheltered outdoor facility and was set up with four tanks (dimensions 120 cm long, 80 cm wide, 86 cm height) which were filled to a volume of 400 L with a continuous flow of non-filtered seawater pumped directly from the sea (Fig. 2). The tubes transporting the water into the tanks were attached to

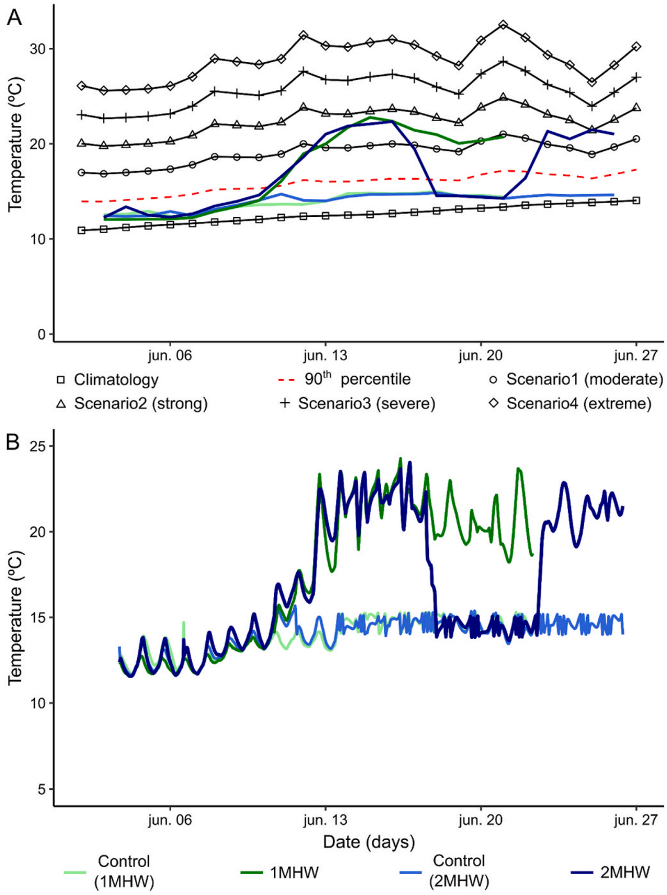

Fig. 1. (A). The climatology (1990–2020, Göbeler et al., 2022) from the study area (Tvärminne Storfjärden surface water) (square symbols) and upper limits for the different marine heatwave (MHW) categories were calculated by multiples of the difference between the climatological mean and the climatological 90th percentile during the month of June, when the experiment was performed (following categorization by Hobday et al., 2016). The green and blue lines show the daily mean temperatures in the experimental tanks of both heated treatments (1MHW and 2 MHW), resulting in a categorization as moderate to strong MHW. Data: University of Helsinki, Tvärminne Zoological Station long-term monitoring program. (B) Enlarged figure of the observed temperatures recorded every 30 min in the experimental tanks during the mesocosm experiment. Note that the y-axis of both panels has different scales. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

their sides to ensure there was minimal disruption from small-scale turbulence caused by the tubes to the pots where *F. vesiculosus* and *Z. marina* were placed. The water in the tanks was heated with TECO TK2000H heaters and an EHEIM Type 1250 pump pumped the water into an external filter (Fluval FX4, capacity 1200 L h^{-1}) which avoided the heaters being clogged from organic debris. Each tank was fitted with two AQUAMEDIC Aquarius 120 LED lights set to mimic the natural light cycle at the time of the experiment with sunrise at 05.00 a.m. and sunset at 11.00 p.m., simulating the normal photoperiod cycle in June in Finland (6:18, light to dark ratio). The lights had six different channels for different colors (red, blue, pink, green, purple and UV) and the photo-synthetic active radiation (PAR) was 131.33 ± 39.22 μmol photons m^{-2} s^{-1} (LI-193SA, LICOR).

Six plastic pots (individual volume approximately 7 L) were placed onto crates within each of the four tanks (total of 24 pots), ensuring that they were homogeneously exposed to the light system. The pots for *F. vesiculosus* were filled with sand from the vicinity of the marine station, while the pots for *Z. marina* were filled with sand (~5 cm height) collected from the same site as the seagrass individuals (Klobbarn). After the pre-experimental measurements (section *Overview of the trait measurements*), three healthy units (defined as experiment individuals) of *F. vesiculosus* and eight healthy units of *Z. marina* were randomly placed in each pot ensuring there were 3 pots of each species in each of the four tanks. *Fucus vesiculosus* units were composed of a whole frond and its holdfast (averaged 10.2 ± 0.3 cm long and 19.7 ± 9.1 g fresh weight, FW) that was attached to a small rock with tape and placed onto the sand of the pots. Each *Z. marina* unit was composed of 1 shoot with 4–6 green leaves (averaged 15.5 ± 4.6 cm long and 1.08 ± 0.29 g FW), along with a portion of rhizome (averaged 5.9 ± 1.4 cm long with 4 internodes) and corresponding roots, and were carefully planted into the sand. Altogether, we had 12 pots each for *F. vesiculosus* and *Z. marina*, with 36 tagged individual units with a unique ID for each species. In the *Z. marina* pots there were also 5 additional units with no tag which were to ensure enough material was available for biochemical analysis. Therefore, while *F. vesiculosus* had 3 units per pot, *Z. marina* pots contained 8 units.

Individuals were acclimated for a minimum of 8 days before the start of the experiment under the same conditions as the controls during the experiment (temperature 12.82 ± 0.85 °C, salinity 6.03 ± 0.05, and dissolved oxygen, DO, of 11.28 ± 0.24 mg L^{-1}). Two of the tanks were then assigned as treatments and heating was applied on June 11: one tank under a MHW of ca. 20 °C for 10 days (1MHW treatment, tank 3), and another tank under two consecutive MHWs of 5 days each at ca. 20 °C, interrupted by a 4-day lag phase (i.e. reference temperature condition) (2MHW treatment, tank 4) (Fig. 1B and 2). The other two tanks were used as respective controls for each of the heatwave treatments, called Control (1MHW) (tank 1) and Control (2MHW) (tank 2) from now on. This differentiation was done to assure the same environmental exposure conditions of controls, as the inclusion of a lag phase meant that the end of each treatment occurred at different times. During the experiment, natural diurnal oscillations in temperature in both controls and heatwave treatments were allowed. However, no overlap in the temperature ranges between the two conditions were observed: in the heatwave treatments, the temperature oscillated between 20 and 24 °C, corresponding to a moderate/strong MHW (Fig. 1A), while in the controls, temperature ranged from 12 to 16 °C (Fig. 1). Both control tanks had the same experimental temperature exposure and conditions as described above for the acclimation period (Fig. 1B and 2). In the different phases of the experiment, it took a day to warm the tanks up (i.e. 20 °C) and to cool down to the control temperature. In every case the new phase started once the average target temperature was reached.

2.4. *Seawater measurements*

Water samples of 25 mL were taken five times over the course of the experiment after the acclimatization period at 3-day intervals (day 0, 3, 6, 8 and 10), from the middle of the water column of each tank filtered through a GF/F filter and frozen (−20 °C) until analysis. These samples were used for determination of dissolved nutrient concentration of nitrate (NO_3^-, μg L^{-1}), nitrite (NO_2^-, μg L^{-1}) and phosphate (PO_4^{3-}, μg L^{-1}) using an autoanalyzer (Thermo Scientific Aquakem 250) and ammonium (NH_4^+, μg L^{-1}) with a manual spectrometric method (Hitachi U-1100). Dissolved oxygen (mg L^{-1}), temperature (°C), and conductivity/salinity were monitored at 30-min intervals with Onset loggers U26-001 (DO and temperature) and U24-001 (conductivity/salinity and temperature). The data registered in the loggers were calibrated at retrieval against values measured with a YSI ProSolo ODO/CT handheld multiparameter logger. Salinity, DO and seawater nutrient concentrations are detailed in Table S1 while seawater temperature through the experiment is detailed in Fig. 1B.

2.5. *Overview of the trait measurements*

We measured a suite of traits in the *F. vesiculosus* and *Z. marina* units

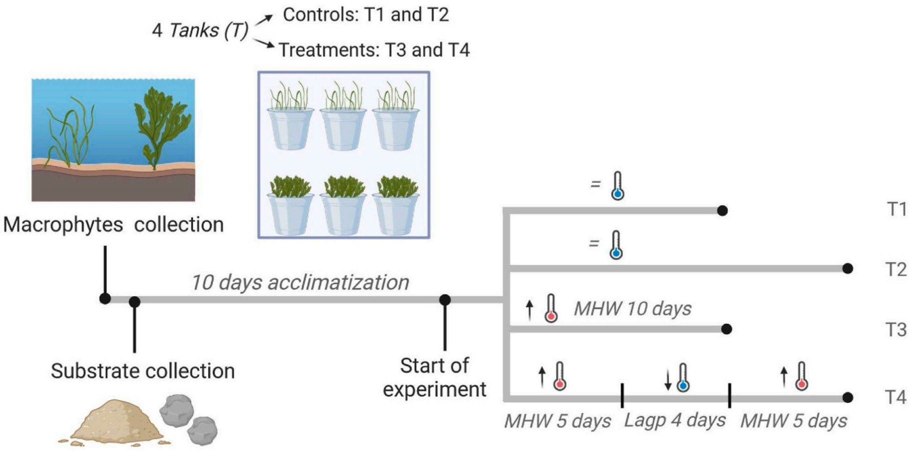

Fig. 2. Conceptual diagram illustrating the timeline of the mesocosm experiment performed at Tvärminne Zoological Station (University of Helsinki, Finland). T: Tank, MHW: marine heatwave. Lagp: lag phase between MHWs. Figure created by BioRender.

belonging to four categories: biochemical, morphological and biomass, growth, and physiological. Before the acclimatization period, a set of non-destructive traits were measured on the tagged *F. vesiculosus* units (total FW and total frond area) and the tagged *Z. marina* units (total FW, rhizome length, number of leaves per shoot, and shoot length). At the end of the experiment, we removed the units (tagged *F. vesiculosus* and *Z. marina*) from the aquaria, and recorded the FW. Afterwards, the same non-destructive traits as at the beginning were measured in *F. vesiculosus*. At the same time, *Z. marina* tagged units were separated into the different components and, then, the above-mentioned traits were also measured. On both *F. vesiculosus* and *Z. marina* units, fluorescence measurements were performed on the fronds and leaves of *F. vesiculosus* and *Z. marina*, respectively. Finally, non-tagged *Z. marina* units were separated into components for measurements of biochemical traits. Due to the differences in the growth pattern and morphology of the two species, some traits were species-specific.

2.6. Biochemical traits

At the end of the experiment, *F. vesiculosus* fronds were separated from the holdfast from all units within each pot (n = 3) and the material pooled. For *Z. marina*, we collected material from young leaves and new rhizomes from five units per pot which were not used for the growth estimation (i.e. tagged units). The plant and algal material were then frozen (−20 °C) and freeze-dried (−55 °C, ≥24 h) (Alpha 1–2 LD plus, Martin Christ Gefriertrocknungsanlagen GmbH, Germany), and ground to a fine powder with a ball mill (Retsch MM 400). The sub-samples were analyzed for C (carbon) and N (nitrogen) content (% of dry mass, DW) using an elemental analyzer (Elementar, Vario EL III) in the Centre of Marine Sciences of Algarve (Faro, Portugal) calibrated with Nicotinamide as a primary standard. Detection limits and precision for C were 1.11 % and 0.44 %, and for N 0.94 % and 0.07 %, respectively.

2.7. Morphological and biomass traits

Frond thickness and width of *F. vesiculosus* were measured with a caliper (mm, ±0.1 mm) before and after the experiment. Fronds were blotted dry and the fresh biomass weight (g FW, ±0.1 mg) of the entire units were measured before the treatments. At the end of the experiment, the frond thickness and width, fresh weight and dry weight (g DW, ±0.1 mg; 60 °C, 48 h) of the whole units were measured.

Before the acclimation period, we measured *Z. marina* morphological traits of the three tagged units. The leaf blade, leaf sheath, and rhizome length were measured with a ruler and the leaf thickness and leaf width with a digital caliper, and the number of leaves and roots were counted. Leaf traits were measured in all the leaves of a shoot. Before the units were placed in the experimental tanks, *Z. marina* units were blotted dry and their total fresh weight was measured. At the end of the experiment, the units were removed from the tanks and the FW and morphological traits were measured again. Then, the plant components of the unit (leaf, rhizome, and root) were separated, oven dried (60 °C, 48 h), and the DW of each component (leaf, rhizome and root) of all units were noted. Epiphytes were removed by gently scraping the leaves or fronds with a blade before any measurement.

2.8. Growth traits

The growth of *F. vesiculosus*, was measured as the daily net growth (DNG, g FW day^{-1}) that was calculated following equation (1) (Eq. (1)).

$$DNG\ (g\ FW\ day^{-1}) = \frac{FW_f - FW_0}{t_f - t_0} \tag{1}$$

DNG was defined as the gains or losses in biomass of the *F. vesiculosus* unit per unit of time, as the difference of the total fresh biomass of each unit between the day of transplantation into the pots and the harvest day ($FW_f - FW_0$), divided by the experimental time in days ($t_f - t_0$).

The total frond area of *F. vesiculosus* units was measured at the start of the acclimation period and after the experiment. After being gently cleaned of epiphytes, the fronds were laid flat onto a white, waterproof 5 mm squared graph paper and pressed down with a transparent Perspex sheet, ensuring that branches were carefully spread out and not bent. A photograph was taken perpendicularly above the *F. vesiculosus* units. The frond area was measured by processing the photographs with ImageJ (Schneider et al., 2012). Then, the frond growth in terms of total area was estimated as the difference of the total frond area (A, cm^2) of each unit between the day of transplantation into the pots and the harvest day ($A_f - A_0$), divided by the experimental time in days ($t_f - t_0$) (Eq. (2)).

$$Change\ in\ area\ (cm^2\ day^{-1}) = \frac{A_f - A_0}{t_f - t_0} \tag{2}$$

For *Z. marina*, the youngest leaf within the apical shoot of each tagged plant was hole punched 1 cm above the sheath at the beginning of the experiment to monitor the leaf growth and leaf appearance (Peralta et al., 2000). At the end of the experiment, each tagged shoot was retrieved, the leaves counted, the length of all leaves measured, and the position of the initial marked leaf within the shoot was recorded as a reference to separate new and old leaves. Then, the leaf elongation rate (LER, cm leaf $shoot^{-1}$ day^{-1}) was calculated as the total increase in leaf length per unit of time, computed from the length of the new leaves in the shoot and the increase in length of the old leaves (Eq. (3)).

$$LER\ \left(cm\ leafshoot^{-1}day^{-1}\right) = \frac{\sum_{i=1}^{n}\left[\left(L_{i,f} - L_{i,0}\right) > 0\right]}{t_f - t_0} \tag{3}$$

where $L =$ leaf length (cm), subscript i refers to the ith leaf within the shoot, subscript f and 0 refer, respectively, to final and initial conditions in days ($t_f - t_0$).

The leaf appearance rate (LAR, leaves shoot d^{-1}) was calculated as the new leaves in the shoot divided by the time from punching to harvest (Eq. (4)).

$$LAR\ \left(leaves\ shoot^{-1}day^{-1}\right) = \frac{(n-m)}{t_f - t_0} \tag{4}$$

where n is the total number of leaves at the end of the experimental period, m is the number of old leaves at the end of the experimental period, and $t_f - t_0$ is the experimental duration (days).

Daily net rhizome growth (NRG, cm rhizome day^{-1}) was calculated following Eq. (5).

$$NRG\ \left(cm\ day^{-1}\right) = \frac{R_f - R_0}{t_f - t_0} \tag{5}$$

as the difference in rhizome length at the end and start of the experiment (R_f - R_0), divided by the experimental period in days ($t_f - t_0$).

We also determined the daily net growth (DNG, g FW d^{-1}) as done with *F. vesiculosus* following Eq. (1).

2.9. Physiological traits

The photosynthetic performance of *F. vesiculosus* and *Z. marina* was measured through pulse amplitude modulated (PAM) chlorophyll fluorescence using rapid light curves (RLCs) by using a Diving-PAM fluorometer (Walz GmbH, Germany). RLCs were performed in the growing tips of *F. vesiculosus* and above the meristem of the second leaf in *Z. marina* in all units within the pots. First, the macrophytes were dark-adapted for 30 min and exposed to a single radiation pulse to measure the maximum photosynthetic efficiency or maximum quantum yield of PSII (Fv/Fm) following Genty et al. (1989). Then, RLCs were performed on dark-adapted tissues using clips that helped to attach and hold the optical cable of the PAM at 3 mm distance from the tissue. The RLCs consisted of 9 light saturating pulses where the PAR in each pulse increased from 0 to 2000 μmol photons $m^{-2}\ s^{-1}$. Irradiance pulses lasted 10 s and the effective quantum yield (ΔF/Fm') was determined at the end of each irradiance step. All *F. vesiculosus* and *Z. marina* units were immersed in seawater during dark-adaptation and RLCs performance to avoid tissue desiccation.

The relative electron transport rate (rETR, μmol $e^{-}\ m^{-2}\ s^{-1}$) was calculated for each step of the curve following the equation by Genty et al. (1989) [$F_v/F_m = (F_m - F_o)/F_m$], where F_m is the maximum dark-adapted fluorescence and F_o is the minimal fluorescence from a dark-adapted sample. rETR values were then plotted against PAR and the curve was fitted by using a second order polynomial regression. From these curves, several parameters were estimated including the maximum rETR (rETRmax, μmol $e^{-}\ m^{-2}\ s^{-1}$), which is the inflection point of the fitted rETR curve, the slope of the light limited part of the curve (α), and light saturation coefficient (Ek, μmol photons $m^{-2}\ s^{-1}$) following Eq. (6) (Touchette and Burkholder, 2000).

$$Ek = \frac{rETRmax}{Alpha} \tag{6}$$

2.10. Statistical analysis

Considering *F. vesiculosus* and *Z. marina* have notable biological differences, the analyses were performed separately for each macrophyte. All the biochemical, morphological and physiological trait responses were assessed with different one-way ANOVAs for each experimental heating by using Treatment (2 levels) as a fixed factor (i.e. 1MHW vs Control or 2MHW vs Control). Additionally, differences in the trait responses of both heating treatments were also tested with a one-way ANOVA (i.e. 1MHW vs 2MHW). Results of the one-way ANOVAs were reported by the F statistic with degrees of freedom as subscripts and the associated p-value. Normality and homoscedasticity assumptions were checked with Shapiro–Wilk tests and residual plots, respectively. Data were presented as means ± standard deviation (SD) and the significance level was established at $p < 0.05$. The analyses were completed using R software, version 4.0.5 (R Core Team, 2021), and all data were processed and plotted using the *tidyverse* package (Wickham et al., 2019) while the statistical analyses were conducted with the *car* package (Fox and Weisberg, 2019).

3. Results

3.1. Fucus vesiculosus trait responses

No significant statistical differences were observed for the carbon and nitrogen content of *F. vesiculosus* between the 1MHW treatment and its control, and between the 2MHW treatment and its control, nor between treatments (1MHW vs 2MHW) (Table 1). This was caused by the low variability between the treatments (Fig. 3A and B) (C content 37.4 ± 0.27 % DW, N content 1.4 ± 0.15 % DW). Growth traits (daily net growth and change in total frond area) of *F. vesiculosus* showed differing responses between heated treatments and their controls (Fig. 3C and D, Table 1), but did not show a significant difference between 1MHW and 2MHW treatments (Table 1). Daily net growth was generally positive but did not differ significantly treatments and their respective controls. However, change in total frond area differed significantly between the 2MHW treatment and its associated control (one-way ANOVA, $F_{1,11} = 1.07$, $p < 0.01$, Table 1), but not between the 1MHW treatment and its control. There was a greater change in frond area for *F. vesiculosus* during the 2MHW treatment (0.47 ± 0.32 cm^2) compared to its control (−0.23 ± 0.45 cm^2), in which the *F. vesiculosus* units presented a loss in the area (Fig. 3D).

No significant difference was found between 1MHW and 2MWH treatments for physiological traits (Fig. 3E, F, G and G, Table 1). A significant difference was seen between the 1MHW treatment and its associated control (one-way ANOVA, $F_{1,9} = 14.99$, $p < 0.01$, Table 1) for

Table 1

Results of one-way ANOVA performed to test differences across biochemical, growth and physiological traits of *Fucus vesiculosus* in experimental treatments (i.e. 1MHW vs Control, 2MHW vs Control, and 1MHW vs 2MHW). Significant differences (p < 0.05) are shown in bold. MHW: Marine heatwave.

Trait		Control (1MHW) - 1MHW		Control (2MHW) - 2MHW		1MHW - 2MHW	
	df	F	p	F	p	F	p
Carbon content	1,10	2.04	0.18	0.15	0.71	0.55	0.47
Nitrogen content	1,10	0.72	0.42	0.06	0.81	0.05	0.82
Daily net growth	1,13	0.11	0.75	0.64	0.44	0.05	0.81
Change in total frond area	1,11	1.07	0.32	12.14	**< 0.01**	1.37	0.26
Maximum quantum yield (Fv/Fm)	1,9	14.99	**< 0.01**	1.24	0.29	3.16	0.11
Maximum relative electron transport rate (rETRmax)	1,9	0.96	0.35	1.82	0.21	0.14	0.71
Initial slope of the rapid light curve (α)	1,9	0.46	0.51	1.61	0.24	0.02	0.89
Minimum saturating irradiance (Ek)	1,9	6.02	**0.03**	0.21	0.66	0.01	0.99

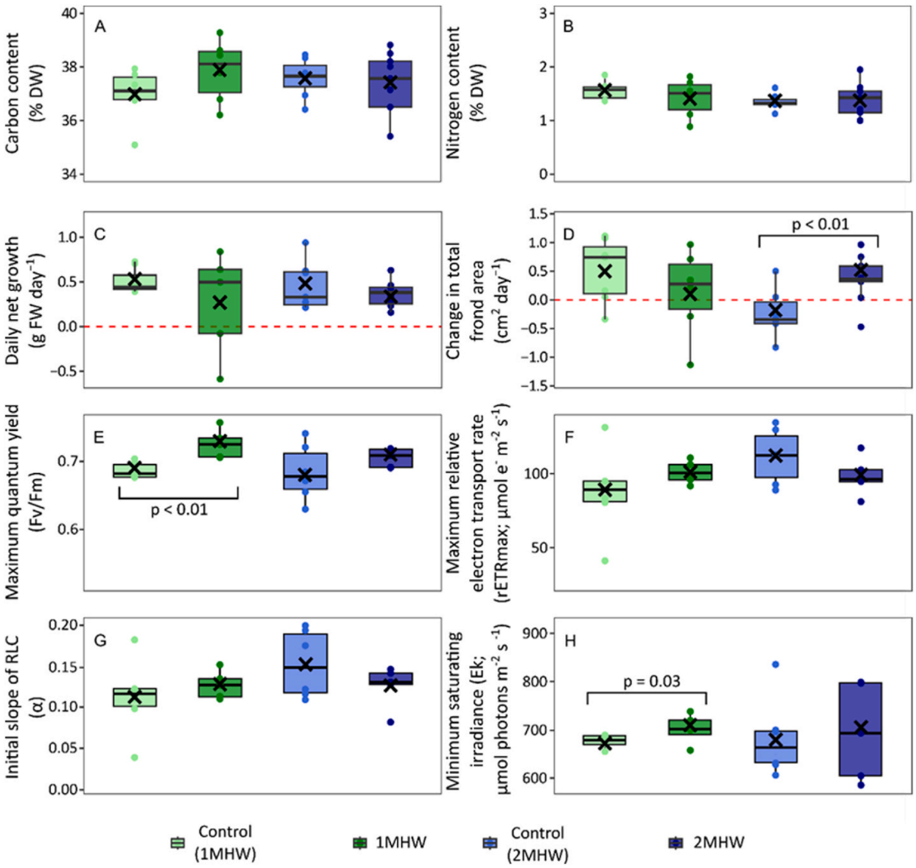

Fig. 3. Boxplots showing biochemical (A, B), growth (C, D) and physiological (E, F, G, H) trait responses of *Fucus vesiculosus* at the end of the experiment under the different treatments: Control (1MHW), 1MHW, Control (2MHW), and 2MHW. The dashed red line in panels C and D represent the growth 0. The boxes represent the interquartile range, the line within the box represents the median value, cross indicates means, and the whiskers represent the minimum and maximum values. The circles that fall out of the boxes represent individual data points at least 1.5 times lower or higher than the interquartile range. P-values on the panels are shown when significant differences between the MHW-treatment and its control were observed (one-way ANOVA, $p < 0.05$). No differences between the MHW treatments were observed. MHW: Marine heatwave, RLC: Rapid light curve. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

maximum quantum yield (Fv/Fm) values (Fig. 3E, Table 1), the 1MHW treatment had higher values for Fv/Fm compared to its control. The relative maximum electron transport rate (rETRmax) did not significantly respond to any heating treatment (Fig. 3F). The α-values in turn, did not differ between heated treatments and their controls (Fig. 3G). The minimum saturation irradiance (Ek) values also showed significant differences between treatments, where the control (677.17 ± 16.80 μmol photons m^{-2} s^{-1}) had lower values than the 1MHW treatment (713.28 ± 31.19 μmol photons m^{-2} s^{-1}) (one-way ANOVA, $F_{1,9} = 6.02$, $p = 0.03$) (Fig. 3H, Table 1).

3.2. *Zostera marina* trait responses

Leaf carbon content in the 1MHW treatment (39.91 ± 0.55 % DW) showed statistically significant higher values than in the control (38.85 ± 0.77 % DW) (one-way ANOVA, $F_{1,4} = 5.26$, $p = 0.05$, Table 2), but not for the 2MHW treatment. In contrast, no significant differences were observed for rhizome carbon content in either of the heated treatments since they presented a high variability (Table 2). Nitrogen content did not show significant differences between MHW-treatments and control in any of the tissues considered (Fig. 4B, Table 2).

In terms of growth traits, daily net growth of the plant showed no significant differences between heated treatments and their controls but higher growth rates were observed in plants under the 1MHW than in the 2MHW treatment (Fig. 4C). Similarly, the daily net rhizome growth showed no differences between treatments, being the overall average value 0.12 ± 0.02 cm day^{-1} (Fig. 4D). Positive values were also observed in terms of leaf appearance rate, with an average value of 0.1 leaves shoot^{-1} day^{-1} for all treatments (Fig. 4E). Significant differences were detected between treatments and their controls in the leaf elongation rate (Fig. 4F). In particular, significantly higher leaf elongation rates were observed in the 2MHW treatment in comparison to its control (one-way ANOVA, $F_{1,16} = 6.71$, $p = 0.02$, Table 2) which also showed the highest average values (1.82 ± 0.14 cm shoot^{-1} day^{-1}), but with no significant differences with the 1MHW treatment (Table 1). Similarly to growth, morphological traits including leaf thickness and width, and final number of roots and maximum root length, just showed significant differences between heated treatments and their controls for the AG tissues (Fig. S1). In particular, wider leaves (2.68 ± 0.55 cm) were observed under the 2MHW treatment in comparison to its control (2.21 ± 0.22 cm) (one-way ANOVA, $F_{1,95} = 21.43$, $p < 0.01$, Table S2). No significant differences were observed for the other heated treatment or traits considered.

The maximum quantum yield (Fv/Fm) values ranged between 0.54 and 0.68 showing no differences between treatments (Fig. 5A). The relative maximum electron transport rate (rETRmax) significantly responded to both heated treatments (Fig. 5B), with lower values both under the 1MHW (one-way ANOVA, $F_{1,16} = 9.34$, $p < 0.01$) and the 2MHW (one-way ANOVA, $F_{1,16} = 17.46$, $p < 0.01$) treatments in comparison to their controls (Table 2). In this way, the largest difference with its control was observed in the 2MHW treatment (72.78 ± 7.19 and 39.79 ± 11.44 μmol e$^-$ m^{-2} s^{-1} in the control and treatment,

Table 2

Results of one-way ANOVA performed to test differences across biochemical, growth and physiological traits of *Zostera marina* in experimental treatments (i.e. 1MHW vs Control, 2MHW vs Control, and 1MHW vs 2MHW). Significant differences ($p < 0.05$) are shown in bold. MHW: Marine heatwave.

Trait		Control (1MHW) - 1MHW		Control (2MHW) - 2MHW		1MHW - 2MHW	
df	F	p	F	p	F	p	
Carbon content in leaves	1,4	5.26	**0.05**	3.51	0.13	0.68	0.45
Carbon content in rhizomes	1,4	0.44	0.55	1.68	0.26	0.01	0.99
Nitrogen content in leaves	1,4	1.2	0.33	1.93	0.24	0.07	0.79
Nitrogen content in rhizomes	1,4	2.62	0.21	1.94	0.23	0.09	0.77
Daily net growth	1,16	2.05	0.17	0.03	0.87	5.57	**0.03**
Daily net rhizome growth	1,16	0.01	0.98	0.02	0.91	0.02	0.88
Leaf appearance rate	1,16	1.87	0.19	1.06	0.32	0.06	0.81
Leaf elongation rate	1,16	1.75	0.21	6.71	**0.02**	0.41	0.53
Maximum quantum yield (Fv/Fm)	1,15	0.08	0.77	0.92	0.35	0.02	0.91
Maximum relative electron transport rate (rETRmax)	1,16	9.34	**< 0.01**	17.46	**< 0.01**	7.17	**0.02**
Initial slope of the rapid light curve (α)	1,16	14.44	**< 0.01**	23.64	**< 0.01**	8.95	**< 0.01**
Minimum saturating irradiance (Ek)	1,15	2.24	0.16	0.41	0.54	8.81	**0.01**

respectively). The lowest rETRmax was observed in the 1MHW treatment (25.89 ± 8.89 μmol e^{-} m^{-2} s^{-1}) that showed significant differences with the 2MHW treatment (Fig. 5B, Table 2). The slope of the light-limited part of the curve, α-values, also showed significant differences between treatments, following the same pattern observed with rETRmax, with lower values in both heated treatments than in their controls, as well as lower values for the 1MHW than for the 2MHW treatment (Fig. 5C). The α-values in the 1MHW (one-way ANOVA, $F_{1,16} = 14.44$, $p < 0.01$) and the 2MHW (one-way ANOVA, $F_{1,16} = 23.64$, $p < 0.01$) treatments were 0.02 and 0.03 lower in the treatments than in their corresponding controls, respectively. Finally, the minimum saturation irradiance (Ek) did not show significant differences for any of the comparisons considered (Fig. 5D) probably due to the high variability observed under both heated treatments.

4. Discussion

While most studies in coastal marine macrophyte species have aimed to evaluate stress responses to temperature, the number of studies investigating how the form of MHWs in terms of duration and magnitude affect trait responses is increasing. Therefore, in this study, we were able to identify trait responses in two foundation macrophyte species in the northern Baltic Sea, *F. vesiculosus* and *Z. marina*, subjected to MHWs. These results may further have ecological significance for ecosystem resistance in real-time scenarios, that will ultimately affect resilience of populations. We expected that when including a lag phase, the stress impacts of a MHW on macrophyte trait responses would be reduced. In the case of *F. vesiculosus* no significant differences were found between MHW treatments. However, for *Z. marina*, higher growth rates were observed in 1MHW compared to 2MHW treatment, and yet, physiological trait responses showed higher values with 2MHW treatment compared to 1MHW treatment. Overall, neither of the studied macrophyte species were detrimentally impacted by the MHWs with and without a lag phase. In fact, *F. vesiculosus* and *Z. marina* showed little variation between MHW-treatments and controls for Fv/Fm which was above 0.7 for both treatments, a threshold that informs about the presence of stress in macrophytes due to not capturing light energy

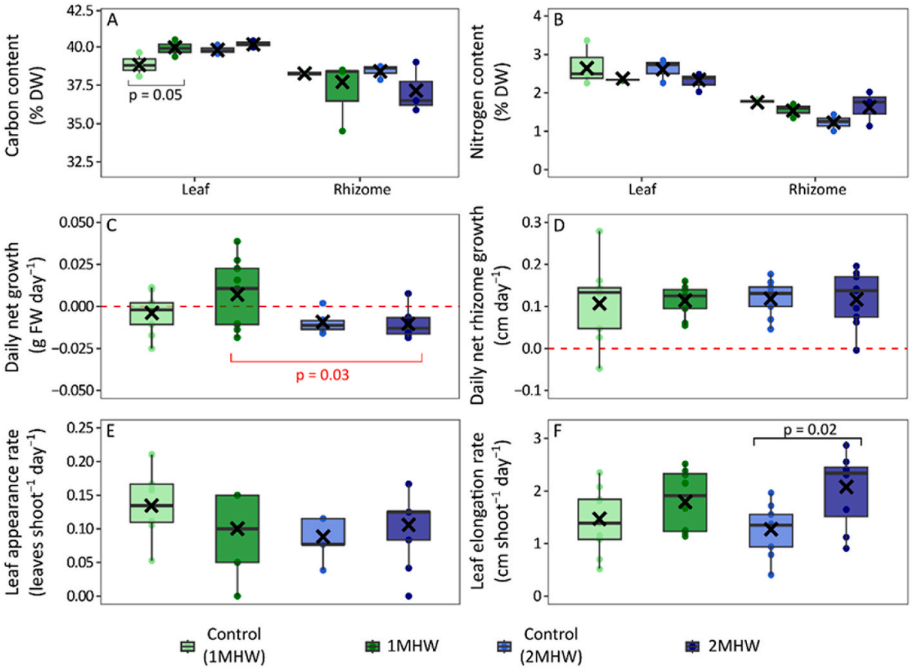

Fig. 4. Boxplots showing biochemical (A, B) and growth (C, D, E, F) trait responses of *Zostera marina* at the end of the experiment under the different treatments: Control (1MHW), 1MHW, Control (2MHW), and 2MHW. The dashed red line in panels C and D represent the growth 0. The boxes represent the interquartile range, the line within the box represents the median value, cross indicates means, and the whiskers represent the minimum and maximum values. The circles that fall out of the boxes represent individual data points at least 1.5 times lower or higher than the interquartile range. P-values on the panels are shown when significant differences between the MHW treatment and its control (black values) or between both MHW treatments (red values) were observed (one-way ANOVA, $p < 0.05$). MHW: Marine heatwave. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

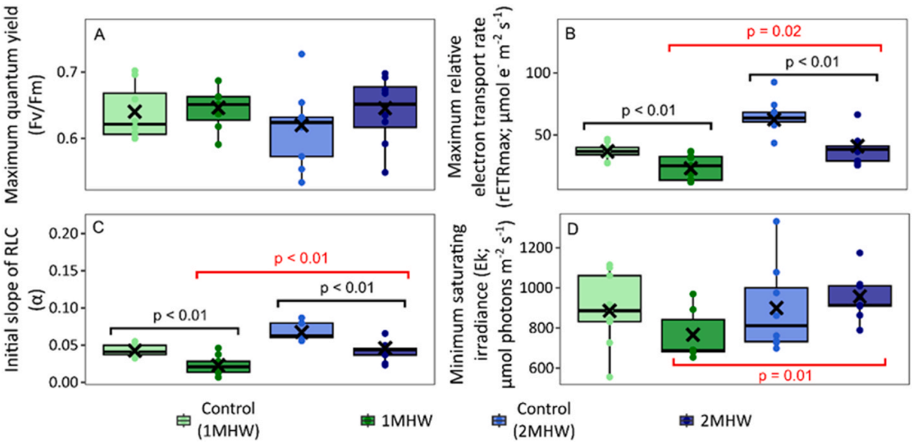

Fig. 5. Boxplots showing physiological trait responses of *Zostera marina* (A, B, C, D) at the end of the experiment under the different treatments: Control (1MHW), 1MHW, Control (2MHW), and 2MHW. The boxes represent the interquartile range, the line within the box represents the median value, cross indicates means and the whiskers represent the minimum and maximum values. The circles that fall out of the boxes represent individual data points at least 1.5 times lower or higher than the interquartile range. P values on the panels are shown when significant differences between the MHW treatment and its control (black values) or between both MHW treatments (red values) were observed (one-way ANOVA, $p < 0.05$). MHW: Marine heatwave, RLC: Rapid light curve. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

efficiently. These results indicate that, potentially, the temperatures used in this experiment, corresponding to a moderate/strong MHW, were lower than the maximum tolerance limits. However, the two macrophyte species showed contrasting trait responses to the studied MHWs (Figs. 3–5).

Growth rates in *F. vesiculosus* in terms of changes in total frond area were positively affected by the 2MHW treatment (Fig. 3D and 6). Growth rate encompasses various physiological processes and it can serve as a reliable estimate of the collective effect on the fitness of individuals. Previous studies have found that *F. vesiculosus* from the Baltic Sea can survive over a range of temperatures (0–26 °C) and that it only experiences mortality and tissue necrosis at 28 °C (Graiff et al., 2015; Takolander et al., 2017). Takolander et al. (2017) found that Baltic Sea *F. vesiculosus* have a growth maximum between 15 and 20 °C and a growth optimum at 20 °C. Furthermore, Baltic *F. vesiculosus* has also been shown to have a high thermal tolerance in part due to the high physical dynamics of the Baltic Sea i.e. changes in temperature (Graiff et al., 2015; Takolander et al., 2017; Saha et al., 2020). Our results indicated that at moderate to strong temperatures of 20–25 °C, *F. vesiculosus* could have been positively impacted by the short lag phase within the 2 MHWs (Fig. 6). Past experiments have shown that *F. vesiculosus* have down-regulated metabolism at lower temperatures, reducing growth (Kraufvelin et al., 2012), and then up-regulated growth at optimum temperatures (Graiff et al., 2015). It is also possible that the longer timing of the 2MHW treatment may have been sufficient acclimation time for the *F. vesiculosus* to adjust its growth as theorized previously (Graiff et al., 2015). As stated, Fv/Fm values for *F. vesiculosus* were close to 0.7 (mostly above) (Fig. 3E), indicating that the macrophytes were not stressed and potentially in the future could withstand moderate to strong increases in temperature. Further physiological traits (rETRmax) also confirmed this assessment. Lower Ek values may indicate increased photoacclimation mechanisms to maximize C fixation, but in this study, *F. vesiculosus* showed significantly higher Ek values in the 1MHW treatment compared to its control. This indicates that the macrophyte ability to fix C was not affected by the 1MHW treatment, confirmed by frond C content not showing differences between the two MHW treatments (Fig. 6). Neither morphological or biochemical traits in *F. vesiculosus* showed any significant differences between treatments (Table 1). This is possibly due to the short intensity, corresponding to a moderate/strong MHW, of the tested MHWs. For changes in morphological or biochemical traits of *F. vesiculosus* to manifest, the MHW may need to be longer in duration or more intense.

The majority of studies for temperate and tropical seagrass species have found that higher than optimum temperatures do not lead to reduced seagrass photosynthesis rates (Hendriks et al., 2017; Perry et al., 2019; Artika et al., 2020; Costa et al., 2021), but higher temperatures can cause a decrease in growth rates (Lee et al., 2007; Hendriks et al., 2017). Optimum temperatures for seagrass growth are usually lower than those for photosynthesis (Lee et al., 2007). In our study, we found that induced MHWs decreased rETRmax of *Z. marina* (i.e. a proxy of photosynthetic capacity) but this physiological effect was not reflected in lower growth (Fig. 6), at least considering the average daily rate during the whole duration of the experimental period. However, when comparing both heated treatments, more intense trait responses were observed in the 1MHW than in the 2MHW treatment (Table 2). This could be because respiration rates might have increased at higher temperatures, causing the discrepancy between photosynthesis and growth (Collier and Waycott, 2014). Instead, higher growth and enhanced morphology in terms of leaf elongation rate and leaf width were observed in plants under the 2MHW treatment than in the control. This could be a plant mechanism to increase potential photosynthetic material within the leaves and minimize boundary layer thickness for gas exchange, which might ultimately increase its thermal tolerance (Collier et al., 2018; Viana et al., 2020). Additionally, significantly lower α-values for both heatwave treatments compared to their controls followed the same pattern observed with rETRmax, indicating higher photosynthetic efficiency (Viana et al., 2020) and supporting the above-mentioned hypothesis (Fig. 6). Leaf carbon content was significantly higher in the 1MHW treatment compared to its control (Fig. 4), this could be due to carbon was not being used for growth and stored in the AG plant tissue (Costa et al., 2021), a strategy observed in other seagrass species.

Similar to *F. vesiculosus*, it is possible that the range of conditions that *Z. marina* experiences in the Baltic Sea may give it greater plasticity, increasing its thermal tolerance, as previously seen under heated conditions (Winters et al., 2011). The lag phase appeared to be sufficient for *Z. marina* plants to restore some of their physiological processes, such as rETRmax, Ek and α, which were significantly higher in the 2MHW treatment compared to the 1MHW treatment (Fig. 5). But as outlined above, the hypothesized higher respiration might disguise other negative processes to plant fitness. In other traits, growth did not increase with the lag phase of the 2MHW treatment, indicating that plants were still impacted by the heatwave. Additionally, plants in the 2MHW treatment showed a significantly lower daily net growth compared to

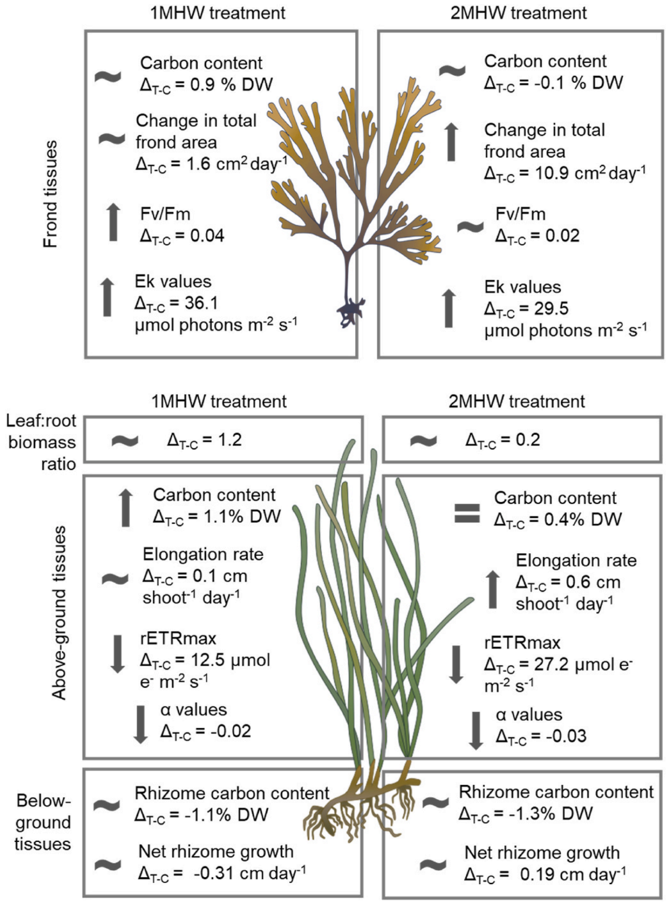

Fig. 6. Schematic representation of the main trait responses of *Fucus vesiculosus* (upper panel) and *Zostera marina* (lower panel) at the end of the experiment. Significant trait responses between MHW treatments and their controls (one-way ANOVA, $p < 0.05$) are represented with upward (higher values in the heated treatments, T, than in its corresponding control, C) and downward arrows (lower values in the heated treatments, T, than in its corresponding control, C). The difference (Δ) between the average values in the heated treatment (T) and its control (C) are shown. Negative values are depicted when average values in the control were higher than in the treatments. Macrophyte drawings are courtesy of the Integration and Application Network (www.ian.umces.edu/symbols/). MHW: marine heatwave.

the plants in the 1MHW treatment, indicating a need for more photosynthetic material for the plant (Table 2).

Finally, it is important to keep in mind that the results of this study were obtained from a mesocosm experiment focusing only on two treatments, with the same temperature intensity, and considering just a lag phase duration. Moreover, the different mesocosms tanks used, that were considered replicates, shared seawater from the same heating systems. In this sense, the results should be extrapolated to natural conditions cautiously even though they were aimed to reflect real-time scenarios.

5. Conclusions

Fucus vesiculosus showed a high acclimatization to changes in temperature, likely because it is a species adapted to grow in dynamic intertidal habitats, adding a suite of characteristics to its potential adaptation to intertidal areas. The lag phase seemed to be more important for the performance of *F. vesiculosus*, for example, higher changes in frond area were observed under the 2MHW treatment than under the 1MHW treatment (Fig. 3). Contrary to *F. vesiculosus*, *Z. marina* appeared to be more sensitive to the 1MHW treatment, as more significant changes were observed in the suite of traits used (Figs. 3–5). Possibly, exploring other MHWs parameters, such as a lag phase of a different duration (i.e. longer or shorter) or more intense MHWs (i.e. higher temperature) would show stronger differences between trait responses. Exploring the role of lag phases of different duration between more frequent heatwaves is an important direction of investigation for both macrophyte species, especially given we observed growth responses such as leaf elongation rate and change in frond area (Fig. 6). Morphological responses can have varied impacts on coastal macrophyte ecosystems such as changing the habitat suitability for various invertebrates and fish, which may impact the ecosystem service of habitat provisioning. An important research direction could be to unravel how site-specific variations may also influence the macrophyte response to stress, e.g. organisms occurring at sites which historically have been dynamic environments with frequent changes in temperature may show greater resilience to climate change-induced stress. Additionally, seagrass in different regions such as the Baltic Sea may also be more vulnerable to fast changing conditions (Ries et al., 2023), because they primarily reproduce clonally and may, hence, show lower resistance and recovery to MHWs (Ries et al., 2023). Moreover, to obtain even more realistic responses of these macrophyte species, future studies should also focus on the interactive effects of temperature and other stressors such as nutrient enrichment or turbidity, that might act together with increasing temperature in shaping coastal ecosystems.

CRediT authorship contribution statement

Lucy G. Gillis: Writing – original draft, Visualization, Investigation, Funding acquisition, Conceptualization. **Salvador Román:** Writing – original draft, Visualization, Investigation, Formal analysis. **Camilla Gustafsson:** Writing – review & editing, Resources, Investigation, Conceptualization. **Laura Kauppi:** Writing – review & editing, Resources, Methodology, Conceptualization. **Carmen B. de los Santos:** Writing – review & editing, Visualization, Formal analysis, Conceptualization. **Zulema Varela:** Writing – review & editing, Visualization, Investigation, Conceptualization. **Inés G. Viana:** Writing – original draft, Visualization, Investigation, Funding acquisition, Conceptualization.

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.marenvres.2024.106894.

Data availability

Data will be made available on request.

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