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Successive stressors alter microbiome composition and reduce resilience in the eelgrass *Zostera marina*

Luis G. Egea [a], Rocío Jiménez-Ramos [a,*], Lucía Rodríguez-Arias [b], Eduardo Infantes [c,d]

[a] Departamento de Biología, Facultad de Ciencias del Mar y Ambientales, Instituto Universitario de Investigación Marina (INMAR), Campus de Excelencia Internacional del Mar (CEI·MAR), Universidad de Cádiz, Puerto Real, Cádiz, Spain
[b] Centre d'Estudis Avançats de Blanes (CEAB-CSIC), Catalonia, 17300, Spain
[c] Department of Biological and Environmental Sciences - Kristineberg, University of Gothenburg, Fiskebäckskil, 45178, Sweden
[d] Department of Biology, University of Las Palmas de Gran Canaria, Canary Islands, 35017, Las Palmas, Spain

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ABSTRACT

Seagrass meadows are among the most threatened ecosystems worldwide, facing multiple anthropogenic stressors that often occur in succession. While plant-physiological responses to multiple stressors are well documented, the role of microbial symbionts in mediating consecutive stressors events remains poorly understood. Using a mesocosm experiment, *Zostera marina* (eelgrass) was exposed to sequential stressors: nutrient enriched sediments (NE; 70 mg of total N per 100 gDW sediment^{-1} for 28 days), followed by a simulated marine heatwave (MHW, 23.3 °C for 15 days) and subsequent storm event (25 cm/s flow, 12 days). Nutrient enrichment resulted in a microbiome shift, specifically a 49.2-fold enrichment of sulfur-oxidizing Arcobacteraceae and a 4.7-fold increase in Sulfurimonadaceae, suggesting possible microbiome-mediated responses mitigating sulfide toxicity. In contrast, warming responses were primarily physiological: aboveground biomass increased by 41.5 % and net production increased by 37.1 % (mg FW shoot^{-1} day^{-1}), with synergistic effects under combined enrichment and heat stress (up to 175 % higher production), indicating that temperature outweighs nutrient stress. Storm exposure triggered a 114 % increase in belowground biomass via root elongation, which increases the resilience of these plants to higher flow velocities, but this acclimation was diminished by 51 % in plants previously exposed to the MHW, indicating environmental legacy effects. Our results demonstrate that eelgrass resilience depends critically on stressors sequence, where legacy effects alter both plant-microbe interactions and physiological responses. These findings emphasize the need to incorporate both a consecutive-stressor approach and microbiome dynamics into seagrass research and conservation strategies under climate change.

1. Introduction

Seagrasses are marine ecosystem engineers that form extensive meadows in coastal regions worldwide, supporting unique biodiversity and providing critical ecosystem services such as carbon sequestration (Short et al., 2011; Apostoloumi et al., 2021). Despite their ecological and economic value, global seagrass coverage has declined by 19.1 % due to anthropogenic stressors such as eutrophication, marine heatwaves (MHWs), and intensified storms (Unsworth et al., 2019; Dunic et al., 2021). These losses reduced ecosystem functions, as degraded meadows diminish key services, including biodiversity support and carbon storage (Jiménez-Ramos et al., 2019; Riera et al., 2025), potentially turning them into carbon sources (Moksnes et al., 2021; Egea et al., 2023a; Yamuza-Magdaleno et al., 2024). Understanding seagrass stress responses is critical for effective conservation and restoration strategies, particularly as climate change amplifies multiple, co-occurring disturbances (Maxwell et al., 2017).

In recent years, multiple-stressors experiments have gained relevance because these designs are more representative of real-world conditions. However, most seagrass studies still evaluate multiple stressors under static (i.e., constant) conditions and simultaneously (e.g., Egea et al., 2018a; Ostrowski et al., 2023) whereas the effects of consecutive stressor combinations remain poorly understood. While the responses to multiple-stressors conditions often result in antagonisms and synergisms (Ostrowski et al., 2025) or are largely determined by the most severe stressor (Egea and Jiménez-Ramos, 2025a), the order of

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* Corresponding author.
E-mail address: rocio.jimenez@uca.es (R. Jiménez-Ramos).

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stress exposure also matters for plants (Zandalinas et al., 2024; Jiang et al., 2025). For instance, Yaffar et al. (2021) found a significantly lower root biomass and production in three tropical trees exposed to storms, particularly when they were previously exposed to warming. Although the consecutive stressor combination in plants is still in an early stage of research, studies in terrestrial plants reported that when plants experience consecutive stress combinations, the first stress exposure, even if mild, alters plant responses to subsequent stressor (Zhang and Sonnewald, 2017). This can be attributed to a combination of energy reserves depletion that compromises subsequent stress-response mechanisms (Jeandet et al., 2022) and the trade-off between growth and stress responses that reduces plant size (Lee, 2024), which may reduce resilience to subsequent challenges (Brewton and Lapointe, 2023). In seagrass research two key gaps persist: 1) the legacy effect of successive stressors, here defined as an environmental legacy effect on plants, where prior stress exposure alters subsequent responses (Pazzaglia et al., 2020; Egea et al., 2024), and 2) post-stress recovery dynamics (Jiménez-Ramos et al., 2023). An environmental legacy represents the lasting effects of stressors after their cessation, which can modify plant responses to future stress events (Kaisermann et al., 2017). Such legacy effects may be amplified or dampened depending on the stress sequence (Crain et al., 2008), and in seagrasses, they may arise through microbiome-mediated acclimation or host physiological exhaustion.

Seagrasses function as holobionts, hosting diverse symbiotic and non-symbiotic microorganisms known collectively as the microbiome (Zilber-Rosenberg and Rosenberg, 2008). Seagrasses host microorganisms in the phyllosphere (leaves) and rhizosphere (roots) that differ markedly from surrounding water and sediments (Vogel et al., 2021; Wang et al., 2021). Previous studies have demonstrated the importance of seagrass's microbiome in mediating seagrass health by increasing nutrient availability (Tarquinio et al., 2018), alleviating sulfide toxicity in anoxic sediments (van der Heide et al., 2012) and providing protection against pathogens (Mendes et al., 2011). Additionally, seagrasses have been shown to alter their microbiome via root exudates (Sogin et al., 2022), particularly to cope with stressful conditions. For instance, nutrient over-enrichment was correlated with enhanced abundances of putatively beneficial microbial taxa in *Zostera muelleri* (Fuggle et al., 2023). However, some stressors have been also shown to alter seagrass microbiome reducing pathogen protective taxa (e.g., under antibiotic pollution; Egea and Jiménez-Ramos, 2025b) or hampering the contribution of seagrasses to the blue carbon, for example under sunscreens (Vilaplana et al., 2025) or microplastic pollution (Egea et al., 2026). In recent years, the understanding of how seagrass and microbes interact under singular environmental factors has markedly advanced, nevertheless, the impact of consecutive stressors remains unclear in seagrass microbiome assemblage. In terrestrial plants, recent studies reported that plant-beneficial microbes can mediate consecutive and non-consecutive stressor events (Ali et al., 2023 and references therein). We therefore hypothesize that, just as the order of stress events can shape plant physiological and growth responses, it can also lead to substantial alterations in the microbiome, which might mediate the resistance of the host plant.

Nutrient enrichment highlights among one of the major environmental stressors for seagrasses (Burkholder et al., 2007). Excess nutrients can reduce seagrass biomass by promoting competition with opportunistic algae (Han et al., 2016), inducing ammonium toxicity (Govers et al., 2014; Jiménez-Ramos et al., 2022) or increasing herbivore pressure due to more palatable leaves (with higher N content or epiphytes load) (Jiménez-Ramos et al., 2018) or necrosis (Egea and Jiménez-Ramos, 2025a). At community level, nutrient enrichment and eutrophication can shift seagrass meadows from net autotrophy to heterotrophy (where community respiration exceeds primary production), thereby reducing the release of dissolved organic carbon (DOC) to surrounding waters (Zhang et al., 2022; Jiménez-Ramos et al., 2024a). Recent work highlights nutrient-driven shifts in microbe-plant mutualisms. For instance, sulfide-oxidizing bacteria, which mitigate phytotoxin sulfide accumulation (Lamers et al., 2013), can be modified under nutrient enrichment conditions (Fuggle et al., 2023), while some microbial pathogen taxa can spread by increased nutrient loads (Liu et al., 2018; Vilaplana et al., 2025). Thus, nutrient-induced microbiome changes require further study, particularly under sequential stressors.

While nutrient enrichment tends to have long-lasting effects on seagrass beds (Burkholder et al., 2007), marine heatwaves (MHWs) and storms are more seasonal stressors in temperate regions. MHWs typically occur in summer, whereas storms are more common in winter, and both have short-term but intense impacts. Warming, especially via marine heatwaves (MHWs), further threatens seagrasses (Safonova et al., 2024). Severe or prolonged MHWs can drive widespread mortality, as observed in temperate and subtropical meadows (Thomson et al., 2015; Serrano et al., 2021). Warming also enhanced growth of opportunistic algal species that compete with seagrass by space and resources (Noisette et al., 2020; Egea et al., 2023b). Thermal extremes can also indirectly stress seagrasses by altering their microbiome assemblages. For instance, warming can promote sulphate reducing bacteria, particularly under elevated nitrogen availability (Koch et al., 2007), resulting in accumulation of phytotoxic sulfides in sediments. Furthermore, MHWs can produce legacy effects that modulate seagrass responses to subsequent stressor events. For instance, Reynolds et al. (2016) showed that *Z. marina* exposed to experimental heatwaves initially increased shoot production and above-ground biomass but later exhibited reduced photosynthetic performance and shoot density during the recovery phase, demonstrating both short term stimulation and delayed negative (legacy) effects of thermal stress. Similar transient positive responses under moderate MHWs have also been reported by Breiter et al. (2024). Moreover, in a recent study, eelgrass exposed to a MHW followed by a pathogen challenge with *Labyrinthula zosterae*, showed a significantly reduced leaf biomass when plants had previously experienced the simulated MHW (Egea et al., 2024). These morphological adjustments may create environmental legacies that influence responses to subsequent stressors, such as storms.

The frequency and energy of storms is expected to increase due to climate change (Reguero et al., 2019), which can physically erode seagrass beds and shift their depth distribution (Infantes et al., 2009, 2022). Hydrodynamic forces modulate nutrient and CO_2 uptake by disrupting the diffusion boundary layer around the leaves, allowing greater supply of CO_2 (Egea et al., 2018b; Yamuza-Magdaleno et al., 2025) and nutrients (including ammonium; Morris et al., 2013; Gillis et al., 2017). Seagrasses typically acclimate to increase flow through morphological adjustments and biomass allocation. For instance, through shorter leaves and more roots under higher flow conditions (de los Santos et al., 2010). We hypothesize that these morphological adjustments can be enhanced or counteracted by previous morphological adjustments caused by MHWs. Thus, lower aboveground to belowground biomass ratio resulting from MHW exposure (Egea et al., 2024) might pre-adapt plants to subsequent storms, whereas longer and wider leaves Breiter et al. (2024) could cause the opposite effect. Then, the environmental legacy of MHWs in shaping seagrass responses to storms needs to be rigorously assessed to understand seagrass adaptive mechanisms to ongoing environmental perturbations.

To address these knowledge gaps, we conducted a controlled mesocosm experiment to answer the following questions: (1) How successive stressor events (MHW followed by a storm) interact with nutrient enrichment to affect eelgrass morphology, growth and productivity? (2) Does prior stress exposure create legacy effects that alter responses to subsequent stressors? (3) How do consecutive stressor combinations reshape the seagrass microbiome and influence plant-microbe interactions? To answer these questions, we exposed *Zostera marina* L. (eelgrass) to fertilized sediments containing slow-release fertilizer (70 mg total N per 100 g DW sediment) for 55 days, during which a simulated MHW (23.3 °C, 15 days) initiated on day 28, followed by a storm event (25 cm/s of orbital flow velocities, 12 days) during the final Phase of the experiment.

2. Material and methods

2.1. Sediment and plant collection

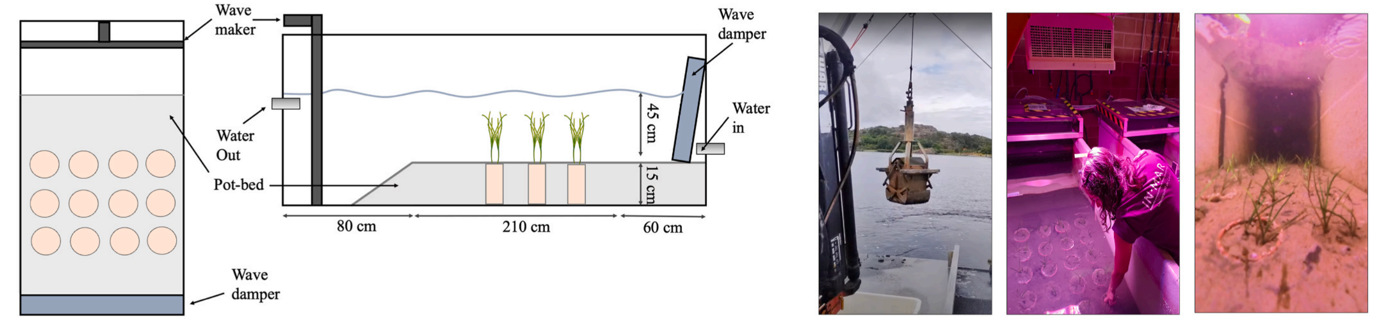
Zostera marina L. (eelgrass) and sediment were collected at Bökevik bay, in the Güllmar Fjord (58°14′N, 11°26′E), Swedish Skagerrak coast. In the bay, eelgrass forms dense patches between 1 and 5 m depth (Österling and Pihl, 2001) on sandy-silty sediments containing 0.5–12 % organic matter (Gibson et al., 1998; Egea et al., 2023a). Summer water temperatures ranged 14–20 °C, with salinity between 24 and 32 ‰ and minimal tides of ~0.2 m (Infantes et al., 2016). The bay is sheltered from prevailing south-westerly wind but experiences storms from the north-east, generating 0.5 m waves (0.24 m s^{-1} orbital velocities; Infantes et al., 2021).

To provide a natural and standardized substrate for the mesocosm experiment bare sediment was collected from 8 to 10 m depth using a vessel and a 0.35 m × 0.35 m box corer (Fig. 1a). The upper ~20 cm of sediment was homogenized and sieved to remove coarse material and macrofauna, ensuring uniform sediment conditions across treatments. *Z. marina* shoots and intact rhizome-root systems were collected at 1 m depth by snorkelling. After harvesting, plants were standardised by trimming rhizomes to 4 cm (3–4 internodes) and roots to 4 roots per shoot (2 cm length each), resulting in uniform shoots averaging 0.69 ± 0.014 g fresh weight per shoot and 13.25 ± 0.21 cm leaf length.

2.2. Experimental design

The experiment was conducted in indoor mesocosms at the Seagrass Ecology Lab facilities in Kristineberg Center for Sea Research and Innovation (Sweden) during July–Sept 2024. We established 48 experimental units (10 cm diameter x 12.7 cm height, 1 L volume), each containing sediment and two standardized shoots (see Section 2.3). Nutrient enrichment (NE) was achieved by adding slow-release Osmocote™ fertilizer (Substral®; 22:7:14; N:P:K) to half of the pots (430 mg TN g DW $sediment^{-1}$; 0.07 % DW), simulating nitrogen levels in high fertilized seagrass sediments (Peralta et al., 2003; Egea et al., 2020), while the remaining pots served as unfertilized controls. Pots were randomly distributed across four wave flumes (12 pots per flume; 50 % fertilized).

The flume tanks (350 x 80 × 80 cm) were equipped with electronic piston wave generators (0.5 Hz frequency) and a synthetic fiber absorber (20° slope) to minimize wave reflections (see technical details of flume tanks in Infantes et al., 2021, Fig. 1). Flow velocities were measured with an acoustic Doppler velocimeter, ADV (Nortek, Vectrino), with a 25 Hz sampling rate and 7 mm of sampling volume, positioned 10 cm above the bottom and 5 cm in front of the pots to avoid leaf interference (Luhar



(a) Top and side view of a hydraulic flume

(b) Pictures of the experiment assembly

(c) Treatments established throughout the experimental period

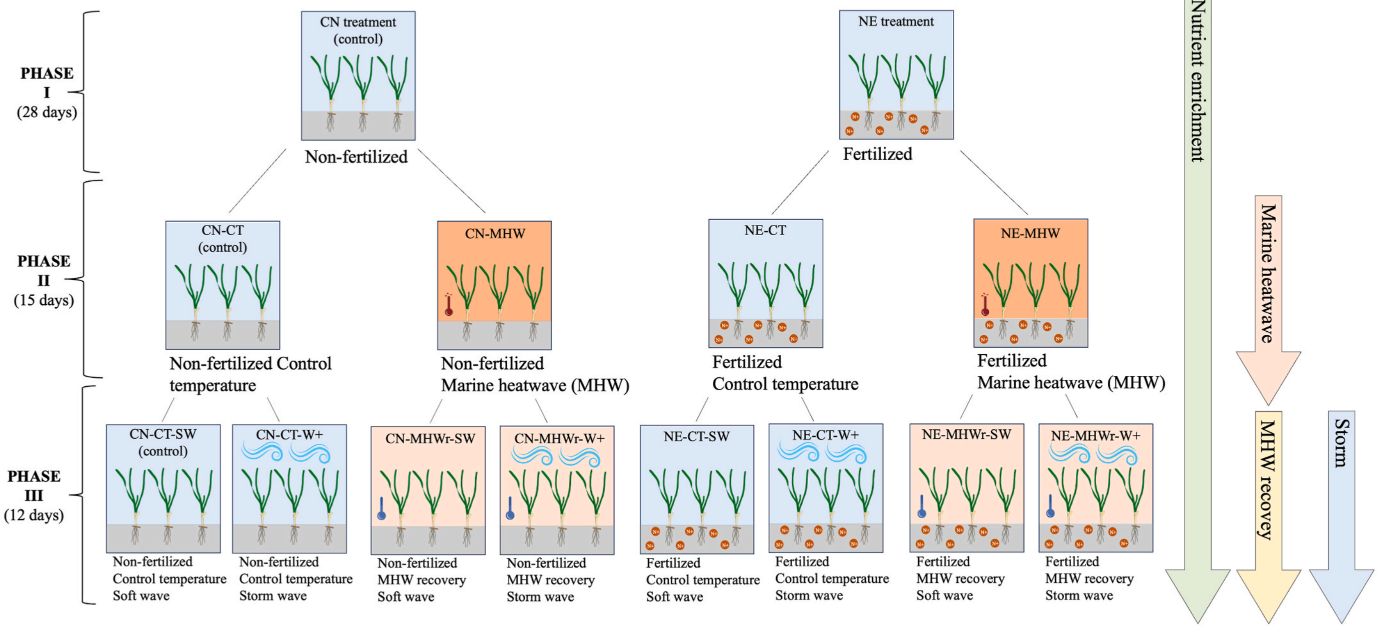


Fig. 1. Experimental setup for *Zostera marina* stressor exposure. (a) Schematic of the hydraulic flume with key components labelled; (b) images showing (from left-right) the box-corer in the vessel, the "pot-bed" within a flume tank and the running of the experiment; (c) Treatment timeline showing nutrient enrichment (Phase I: days 0–28), marine heatwave (Phase II: days 28–43), and storm simulation (Phase III: days 43–55).

et al., 2013). The water level was h = 45 cm, and the test section (240 x 80 contained a PVC “pot bed” module with 12 equidistant 10 cm diameter holes for pot placement, beginning 80 cm from the generator.

The mesocosms maintained natural conditions using flow-through seawater system (~5 L min^{-1}) directly from the Güllmars Fjord (23.9 PSU salinity, pH 8, 18.2 ± 1 °C). Gentle background waves (5 cm/s) and controlled lighting (HelioSpectra lamps; 140 μmol photon m^{-2} s^{-1} for 14 h daily; 7.4 mol photon m^{-2} d^{-1}) ensured optimal growth of eelgrass (Olesen and Sand-Jensen, 1993). Nutrient concentrations in seawater remained stable during the experiment (*t*-test on measurements in each flume tank, df = 11, $p > 0.05$ for all nutrients). The mean concentrations throughout the experiment were 0.3 ± 0.17 μM NH_4^+, 1.13 ± 0.87 μM NO_3^- and 0.22 ± 0.04 μM PO_4^{3-} (mean ± standard deviation; n = 32).

The 55-day experiment progressed through three sequential phases (see Fig. 1) in which nutrients slowly dissolved throughout the whole experiment. The slow-release fertilizer (Osmocote™) used for nutrient enrichment was designed to provide gradual nutrient release over several weeks, and nutrient availability was therefore expected to persist throughout the experiment. Particularly, in **Phase I** (Days 0–28) nutrient enrichment effects were evaluated by comparing control (CN; i.e., non-fertilized sediments) and nutrient-enriched (NE) sediment. During **Phase II** (Days 28–43) a simulated marine heatwave (MHW) event was introduced by warming the incoming seawater in two flumes to 23.3 ± 0.8 °C, while maintaining two control temperature flumes at ambient summer temperature (18.2 ± 1 °C). This resulted in four total treatment combinations (see Fig. 1). The MHW treatment simulated a summer heatwave according to the definition of Hobday et al. (2016) (i.e., anomalously warm water event features by values higher than the 90th percentile of the local long-term climatological observations during at least five days). To assess the similarity of the temperature reached in the MHW treatments to the natural MHW, the occurrence and characteristics of oceanic MHWs in the area were evaluated using the Marine Heatwaves Tracker app (Schlegel, 2024). Thus, the MHW treatments reached mean temperatures corresponding to severe MHWs in the area for the last 5 years (Fig. S1 in Supplementary Material). **Phase III** (Days 43–55) assessed the MHW recovery under two wave regimes. All flumes returned to ambient temperature while two (one previously under MHW and one control temperature) were subject to high wave energy (flow 25 cm/s), representing natural storm conditions (Liljebladh and Thomasson, 2001), with the remaining flumes maintaining gentle waves (5 cm/s) (the features of wave conditions are shown in Table S1 in Supplementary Material). This resulted in eight total treatment combinations (see Fig. 1).

To assess changes in plant dynamic properties (see section 2.3) and in the microbiome abundance and richness (see section 2.4) two pots were collected (from CN and NE treatments) from each flume tank at the end of Phase I, establishing flume tanks as replicates of sediment nutrient load. At the end of Phase II, four pots were collected (two CN and two NE treatments) from each flume tank, establishing flume tanks as replicates of seawater temperature conditions (i.e., MHW or control temperature) and pots within each flume tank as replicates of sediment nutrient load. At the end of Phase III, the remaining six pots in each flume tank were collected (three CN and three NE treatments), establishing flume tanks as replicates of MHW recovery under soft/storm wave condition and pots within each flume tank as replicates of sediment nutrient load. We acknowledge that this design involves some pseudo-replication. Nonetheless, we are confident that the observed effects reflect the experimental factors rather than potential confounding effects among flume tanks. The four indoor flume tanks were identical in size, material and wave-generation components, and were maintained under constant ambient conditions (e.g., artificial light, temperature) to simulate the environmental conditions of the donor meadows (light, temperature and salinity). In addition, they received seawater directly from the Güllmars fjord (where temperature was controlled for the second Phase) in a constant and identical flow of 5 L min^{-1} among them throughout the experimental period. Seawater temperature, salinity, pH and hydrodynamic values were daily monitored at the edges and center (three points per edge and center) of the pot-bed of each flume indicating no detectable confounding effects among flume tanks but only those produced by changes in the factors evaluated. In addition, sediments and plants were placed in 48 independent pots distributed among flume tanks. No significant differences in light and temperature were detected among pots within each flume tank. Thus, to control for pseudo-replication effect, multiple data per tank were amalgamated using single CN and NE pots within each tank as replicates of assessed factors in Phase II and III. Therefore, four truly replicates were taken for the two treatments in Phase I, four replicates (two truly replicates and two pseudo-replicates) were taken for the four treatments in in Phase II, and three replicates (pseudo-replicates) were taken for the eight treatments in Phase III.

2.3. *Measurement of response variables in plants*

At the start of the experiment, a small hole above the ligule, in the leaf base, to estimate the leaf growth rate following a modified Zieman method widely used to estimate seagrass growth (Jiménez-Ramos et al., 2024b). This process was repeated on day 31 to facilitate growth rates calculations for Phase II and III. At the end of each experimental Phase, plants were collected from pots carefully to keep rhizomes and roots intact. Plant growth was calculated as the incremental growth between the leaf base and the hole when they were harvested. Then, aboveground and belowground tissues of plants were individually weighed (FW) and morphometric measurements were carried out to estimate plant growth dynamics and biomass following established protocols with specific calculations detailed in Table 1. Specifically in this study, net production rate (NPR; mg FW shoot^{-1} d^{-1}) was obtained by the difference between the fresh biomass at the collecting time and the initial fresh biomass of each shoot, divided by the elapse time.

Root system analysis was performed following established methods adapted from terrestrial plant studies (e.g., Ziegler et al., 2023). Each shoot root system was scanned at 600 dots per inch (DPI) using an Epson XP-2200 scanner. The resulting high-resolution images were analyzed using Image-J software with the SmartRoot plugin (Lobet et al., 2011), a semi-automated tracing algorithm specifically designed for root architecture quantification. Prior to analysis, each image was calibrated using a reference scale scanned simultaneously with the root sample to ensure accurate pixel-to-millimeter conversion. Each root in the root system was manually traced and automatically measured with SmartRoot to calculate the total roots length. This approach provided precise, reproducible measurements of root system morphology while maintaining compatibility with established seagrass research methodologies.

Table 1
Morphometric and physiological traits quantified for *Zostera marina* shoots. Subscript i = 1 … n: each root in an individually shoot. Subscripts f and 0: final and initial conditions, respectively. $t_f - t_0$: elapse time. MLL_o and MLL_f are the length of the marked leaf (small hole) at the beginning and at the end of the study period.

Traits	Units	Formula
Net Production Rate	mg FW · shoot^{-1} · d^{-1}	$\text{NPR} = \frac{Biomass_f - Biomass_0}{t_f - t_0}$
Leaf growth rate	cm · shoot^{-1} · d^{-1}	$\text{LGR} = \frac{MLL_f - MLL_0}{t_f - t_0}$
Leaves loss rate	n° · shoot^{-1}	$LL = \frac{Leaves\ loss}{shoot}$
New shoots appearance	n° new shoots · shoot^{-1}	$NSA = \frac{New\ shoots}{initial\ shoot}$
Increase in rhizome length	cm · shoot^{-1}	$\Delta RL = \frac{Rizhome\ length_f - Rhizome\ length_0}{t_f - t_0}$
Total roots length	cm · shoot^{-1}	$TRL\left(cm \cdot shoot^{-1}\right) = \frac{\sum_{i=1}^{n} Roof\ length}{shoot}$

2.4. Sample collection and measurement for microbiome

Microbiome samples were collected following established methods (e.g., Wang et al., 2021; Vilaplana et al., 2025) at the end of Phase I and II. The complexity of the experimental design (i.e., logistical and budget) did not allow the collecting of these samples at end of Phase III. For each treatment, three replicate shoots were aseptically extracted using gloves to minimize contamination. Roots and leaves were separated and placed in sterile 50 mL vials for processing. For phyllosphere microbiome analysis, an 8-cm segment of the second youngest leaf from each plant (n = 3) was taken out and gently rinsed three to five times with sterile seawater to remove loosely associated microorganisms. Rhizosphere samples were obtained manually by shaking roots to dislodge loose sediment, followed by washing the roots with 0.2 μm filtered seawater to capture any sediment that was still adhered to the roots. All rhizosphere samples were taken before root scanning to avoid contamination. All samples were immediately preserved in sterile DNA/RNA Shield (Zymo Research, CA, USA) using DNA LoBind tubesand stored at −80 °C. Sequencing and bioinformatic processing were conducted at Novogene GmbH (Munich, Germany). DNA concentrations were standardized across samples. The V4-V5 hypervariable region of the 16S rRNA gene was then amplified using the primers 515F (GTGCCAGCMGCCGCGG-TAA) and 907R (CCGTCAATTCCTTTGAGTTT) (Westermann et al., 2017), and library preparation was performed using the NEBNext® Ultra™ II DNA Library Prep Kit with index adapters synthesized in-house by Novogene. Qubit and real-time PCR were used to quantify the resultant libraries, and a bioanalyzer was used to evaluate the fragment size distribution. Libraries were then sequenced on the Illumina NovaSeq 6000 SP platform (Caporaso et al., 2012) with 150 bp paired-end reads, which yielded 5.84 million read-pairs. The chimeric sequences in Clean Tags were detected and removed, leaving the Effective Tags which were used for subsequent analysis. Reads were clustered into operational taxonomic units (OTUs) at 97 % similarity using the default Opti clustering method implemented in mothur.

2.5. Data and statistical analyses

All statistical analyses and plots were performed using R 4.4.1 (R Core Team, 2024). Treatment effects on response variables were assessed separately for each experimental Phases using generalized linear models (GLMs). Model assumptions were verified through Shapiro-Wilk and Levene's tests for normality and homoscedasticity. All response variables were initially modeled with a Gaussian distribution and identity link, but if the residuals of a Gaussian GLM did not meet the assumption of normality, we used a GLM with a Gamma error distribution and reciprocal link function. Post hoc comparisons between treatment combinations were performed using estimated marginal means with Bonferroni correction ("emmeans" R package; Lenth, 2024). The significance level (α) set for all tests was 0.05. Letters above bars in plots indicate significant differences among treatments for each experimental Phase.

Regarding the microbiome analysis, we first plotted OTUs grouped into phylum (OTU reads assigned to chloroplast, mitochondria and others rated as low-quality were removed either in the plots and in the subsequent analyses). Changes in bacterial diversity (i.e., the number of different OTUs observed) and abundance (i.e., the number of reads per OTU) was evaluated by bacterial families. To identify compositional differences in bacteria assemblage between seagrass compartments (i.e., phyllosphere and rhizosphere) and among treatments, non-metric multidimensional scaling (NMDS) based on the Bray-Curtis distance were applied (Oksanen et al., 2024). Additionally, we tested for significant differences among obtained clusters using a permutational multivariate analysis of variance (PERMANOVA) with 999 permutations implemented using the adonis function from the 'vegan' package. The taxa which most contributed to the observed dissimilarity among treatments (observed only in rhizosphere) were reported by using similarity percentage analysis (SIMPER) with 999 permutations. The top-ranked OTUs in this analysis (i.e., those with the highest contribution to dissimilarity) were overlaid on the NMDS graph with the 'envfit' function (a list of all OTUs obtained from 'envfit' results is provided as Table S2 in the Supplementary Material). In addition, to illustrate the differences in the rhizosphere bacterial assemblage among treatments in Phase II, the top OTUs with higher (|>0.5 %|) average difference in relative abundances between treatments and control counterpart were plotted.

3. Results

3.1. Effects on Z. marina plants

Nutrient enrichment (NE) reduced both aboveground (−9.2 %; Fig. 2a) and belowground biomass (−26.6 %; Fig. 2b) compared to control (C) by the end of Phase I (28 days), though these differences were not significant (Table S3 in Supplementary Material). This biomass reduction corresponded to a 23.5 % decrease in net production rate (NPR) (Fig. 3a; Table S4 in Supplementary Material). These negative effects became more pronounced in subsequent phases, with significant reductions in belowground biomass and NPR emerging in Phase II. Fertilized sediments also induced significant physiological changes, including increase in leaf loss (42.9 %; Fig. 2d), reduced new shoots appearance (80 % reduction; Fig. 3c) and shorter total roots length (−51.1 %; Fig. 3d) at the end of Phase I, which was further observed throughout the succession of experimental phases.

The simulated marine heatwave (MHW) during Phase II) significantly enhanced aboveground biomass (41.5 %; Fig. 2a), leaf growth rate (30.9 %; Fig. 2c), and net production rate (37 %; Fig. 3a). These enhancements were more pronounced when compared the fertilized–control temperature (NE-CT) and the fertilized–MHW (NE-MHW) treatments. For instance, plants showed a 10.4 % higher NPR in NE-MHW when compared to non-fertilized–control temperature (CN-CT) but a 175.2 % higher NPR when compared to NE-CT treatment. In contrast, MHW exposure reduced belowground investment, leading to an 18.6 % reduction in belowground biomass (Fig. 2b), rhizome length (22.2 % reduction; Fig. 3b), and total roots length (29.7 % reduction; Fig. 3d). All stressed treatments showed substantially lower shoot recruitment (50 %–70 % reductions; Fig. 3c), indicating a systematic reallocation of resources from vegetative reproduction to leaf production under elevated temperatures.

Following the return to ambient temperature (Phase III), differences in biomass and growth between previously warmed and control plants diminished. Storm exposure significantly increased belowground biomass (114 %; Fig. 2b) due to an increase in total roots length (78.3 %; Fig. 3d), particularly in wave exposed treatments, regardless of nutrient load, leading to higher net production rate (NPR; Fig. 3a). However, these positive responses were reduced in plants that had experienced prior MHW stress. For instance, belowground biomass and total roots length were diminished by 51 % and 59 %, respectively, in plants exposed to only storm waves (CN-CT-W+) compared to plants exposed to MHW prior to those storm waves (CN-MHWr-W+). Rhizome length remained reduced across all stress treatments compared to controls, with the most pronounced reduction (41.4 %; statistically significant) occurring in the NE-MHWr-W+ treatment (nutrient enriched, post MHW and storm exposure; Fig. 3b).

3.2. Effects on microbiome

After filtering low-quality, unidentified chloroplast, and mitochondria reads, 1,609,395 operational taxonomic units (OTUs) were identified among the experimental samples. The bacterial OTUs were classified into 462 families (see repository dataset). Among them, 300 were found in leaves and 412 in roots, with 250 families found in both plant compartments. The phyla with the most abundant families were

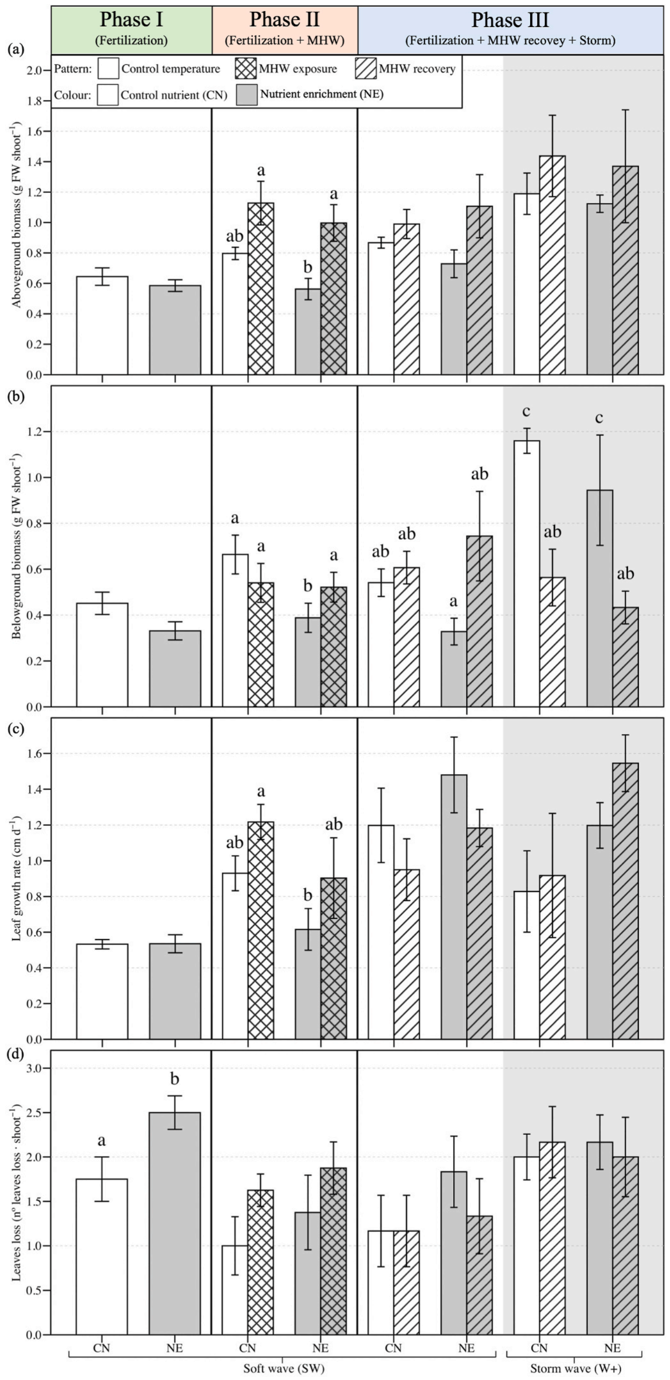


Fig. 2. Temporal changes in *Zostera marina* growth metrics across experimental Phases (separated by vertical lines): (a) aboveground biomass (g FW shoot^{-1}), (b) belowground biomass (g FW shoot^{-1}), (c) leaf growth rate (LGR, cm day^{-1}), and (d) number of leaves loss. Values represent means ± SE (n = 4). Different letters indicate significant differences among treatments within Phases ($p < 0.05$, GLM with Bonferroni correction. CN: control nutrient; MHW: Marine heatwave; NE: Nutrient enrichment.

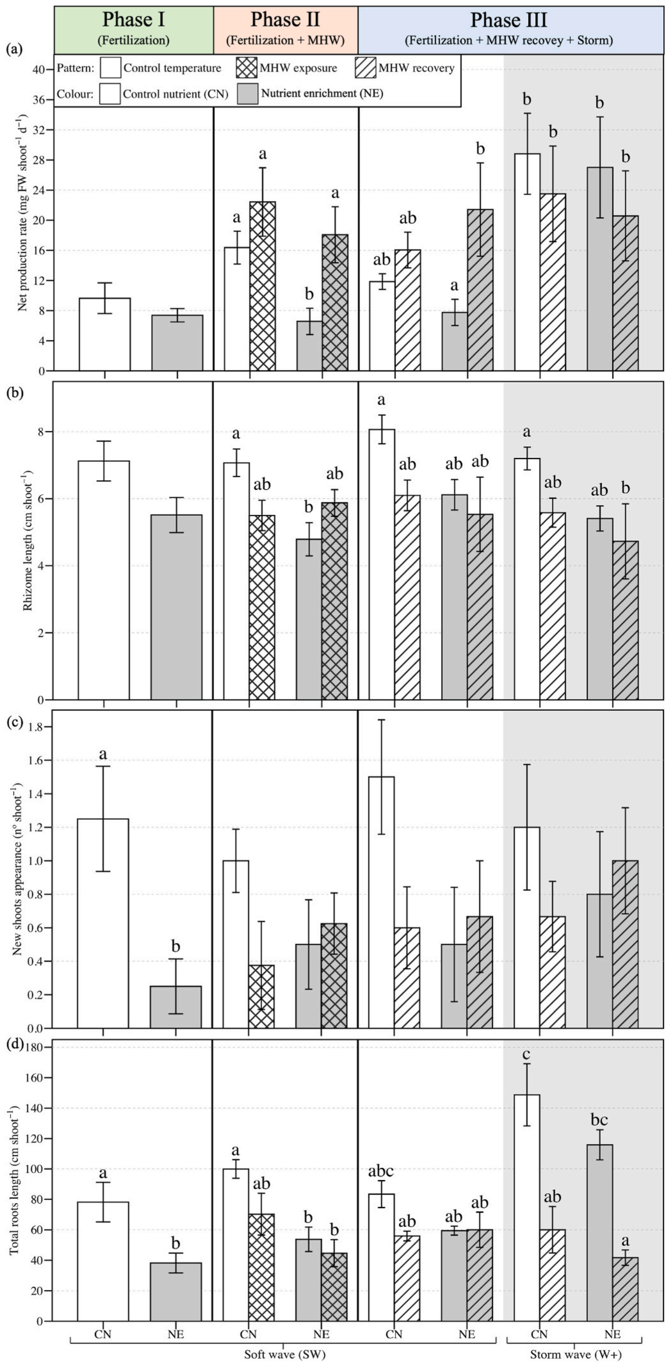


Fig. 3. Temporal dynamics of *Zostera marina* productivity and belowground development across experimental Phases (delineated by vertical lines): (a) net production rate (NPR, mg FW shoot^{-1} day^{-1}), (b) final rhizome length (cm shoot^{-1}), (c) shoot recruitment and (d) the total roots length (cm shoot^{-1}). Data represent treatment means ± SE (n = 4). Letters denote statistically distinct groups ($p < 0.05$, GLM with post-hoc testing). CN: control nutrient; MHW: Marine heatwave; NE: Nutrient enrichment.

Proteobacteria and Bacteroidota in phyllosphere, and Bacteroidota, Proteobacteria, Campylobacterota, Desulfobacterota and Chloroflexi in rhizosphere (Fig. 4). Proteobacteria and Bacteroidota overall were the most abundant phyla in both, phyllosphere and rhizosphere. These phyla often dominate in the microbiome of seagrasses such as *Z. marina*, *Z. noltii*, and *Cymodocea nodosa* (Ling et al., 2021). Some families of these phyla are crucial in seagrass health by contributing to nutrient cycling, antioxidant activity and protecting against pathogens (Miyamoto et al., 2023).

The top 60 families in phyllosphere and rhizosphere (20 % and 14.6 % of total families, respectively) represented, at least, the 92.3 % and 92.7 % of the bacteria abundance in samples, respectively (Fig. S2 in Supplementary material). In distance-based analyses across all samples, microbial communities were most strongly clustered based on seagrass compartment (i.e., phyllosphere versus rhizosphere compartments; PERMANOVA, F = 31.90, p-value <0.001, and $R^2 = 0.55$) (Fig. S3 in

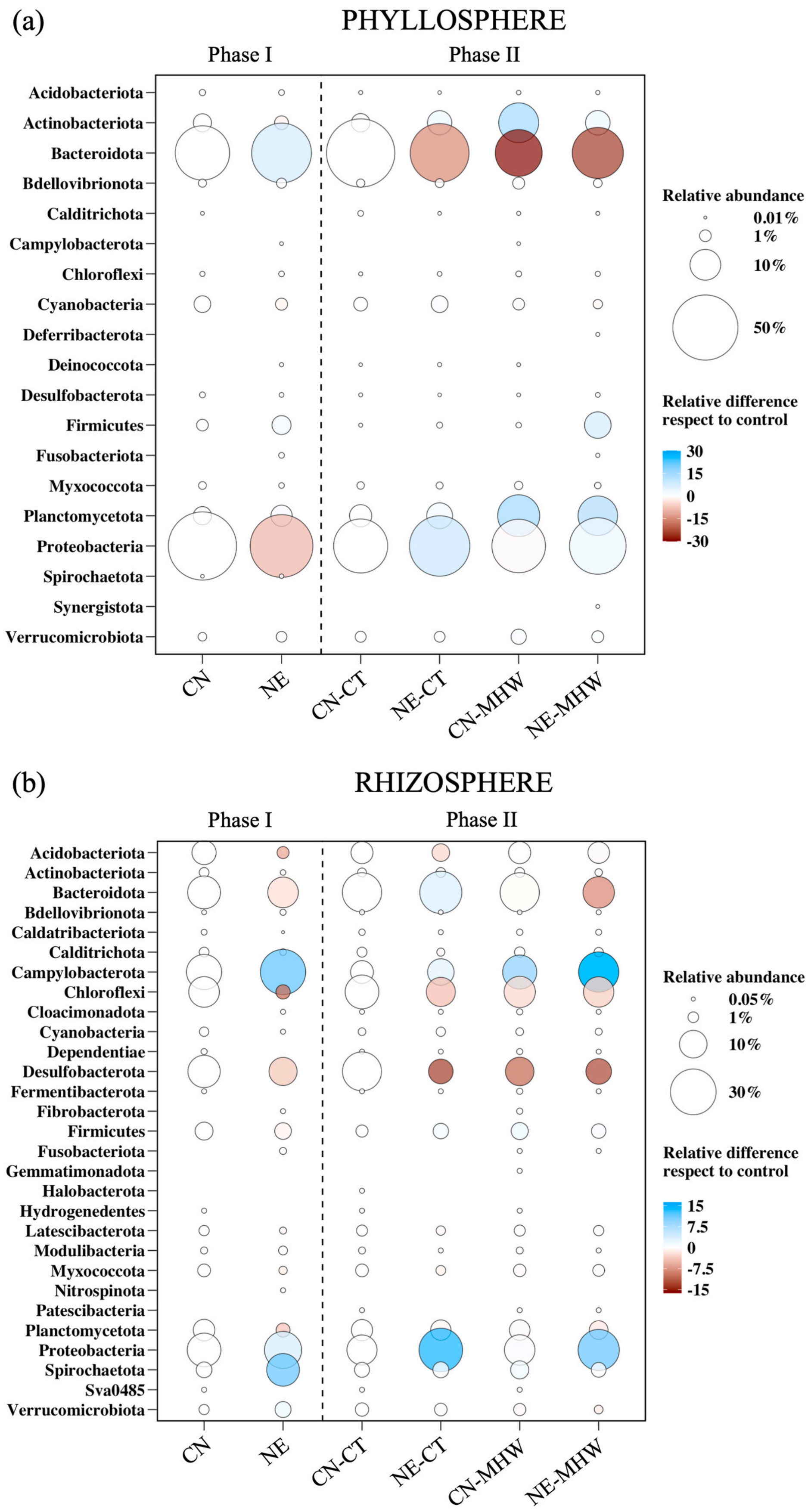


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Fig. 4. Differential abundance of bacterial phyla in (a) phyllosphere (>0.01 % relative abundance) and (b) rhizosphere (>0.05 % relative abundance) microbiomes. The thresholds were set to focus on the main phyla among the 32 detected in phyllosphere and the 51 in rhizosphere. Bubble size represents the total range (min-max) of mean OTUs relative abundance (%) across all treatments and experimental phases allowing comparisons of trait variability, while color indicates fold-change relative to the phase-specific control (blue: increase; red: decrease relative to CN [Phase I] or CN-CT [Phase II] treatments). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Supplementary material). However, when phyllosphere and rhizosphere samples were analyzed separately, we found that, in rhizosphere, NE treatment in Phase I was clustered forming a separated branch while all the other treatments forming another separated branch (PERMANOVA, F = 4.69, *p*-value = 0.0031, and $R^2 = 0.28$) (Fig. 5a and b and Fig. S4 in Supplementary Material). The main taxa caused this differentiation on NE treatment were the increase in Arcobacteraceae, Spirochaetaceae, Sulfurimonadaceae, Nitrincolaceae and Desulfocapsaceae and the decrease in Sulfurovaceae, Flavobacteriaceae, AB-539-J10, Anaerolineaceae and Thermoanaerobaculaceae (Fig. 5c and Fig. S5 in Supplementary Material). In particular, we observed significant increase in sulfur-oxidizing bacteria such as Arcobacteraceae (from 0.5 ± 0.3 % to 23.1 ± 9.0 % in relative abundance, 49.2-fold higher) and Sulfurimonadaceae (from 0.9 ± 0.4 % to 4.1 ± 0.7 % in relative abundance, 4.7-fold higher) in NE treatment compared to control in Phase I. In addition, the distance-based analyses grouped most of samples with fertilized sediments in Phase II (i.e., NE-CT and NE-MHW treatments). In contrast, no clear trends were observed in the phyllosphere microbiome.

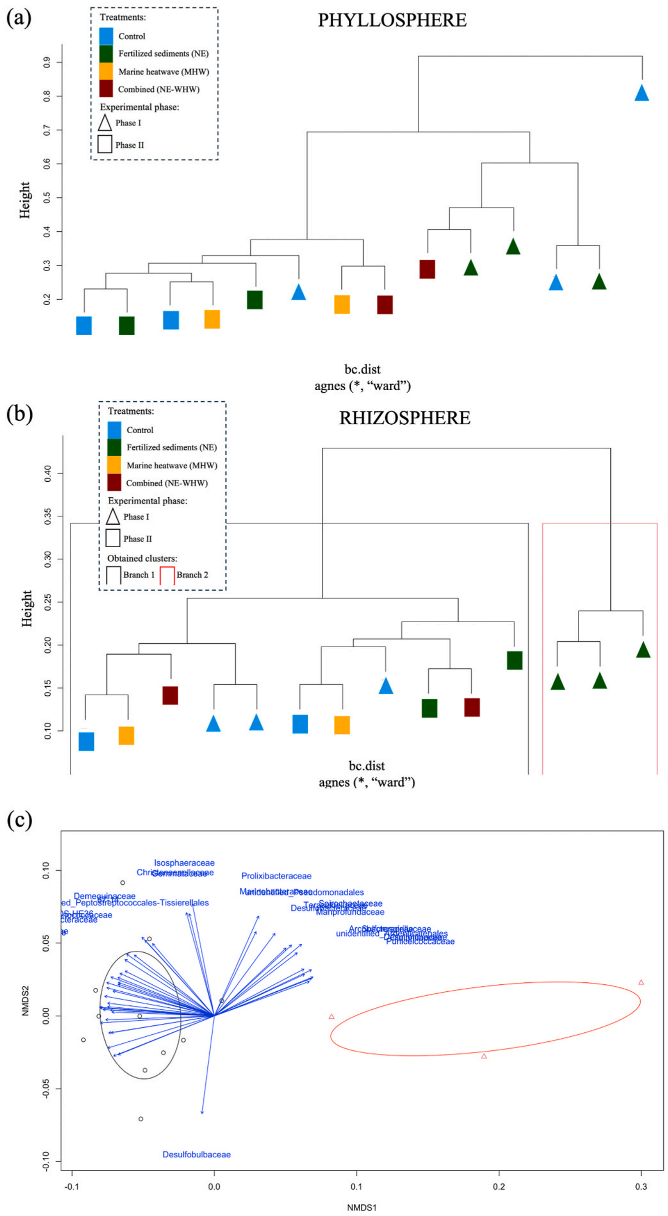

Fig. 5. Microbial community dissimilarity analysis: Hierarchical clustering of (a) phyllosphere and (b) rhizosphere communities based on Bray-Curtis distances. (c) Non-metric Multi-dimensional Scaling (NMDS) ordination of rhizosphere microbiome with a 95 % confidence ellipses for identified clusters. Vector arrows represent OTUs significantly contributing to community dissimilarity (envfit; $p < 0.001$, SIMPER pairwise tests), with complete taxonomic identities provided in Table S2.

Higher microbiome abundance was found in both the phyllosphere and rhizosphere, under nutrient enrichment (NE) in Phase I, with a statistically significant increase only in the rhizosphere (GLM, Std. E. = 3.31E-06, t-value = −2.58, *p*-value = 0.032; Table S5 in Supplementary Material). This difference between NE and control (CN) treatments was reduced in Phase II. A notable increase in microbiomal abundance was observed in the NE-MHW rhizosphere, showing a 1.72-fold increase compared to the CN treatment (Fig. 6a). In contrast, neither distance-based nor GLMs analyses indicated significant overall changes in bacterial communities between warmed and ambient conditions. Nonetheless, the relative abundance of some phyla increased in CN-MHW treatment (Figs. 4 and 6b) such as Campylobacterota (driven by Sulfurovaceae), Proteobacteria (notably Sedimenticolaceae and Methylophagaceae, though Rhizobiaceae and Rhodobacteraceae decreased), and Spirochaetota (Spirochaetaceae). Conversely, Desulfobacterota (mainly Desulfobulbaceae) and Chloroflexi (mainly due to AB-539-J10) decreased under warming, despite some increases in other taxa like Desulfocapsaceae.

4. Discussion

Our study demonstrates that the sequence of stress events plays a critical role in determining seagrass responses to multiple-stressor, as important trade-offs emerged when stressors were applied sequentially. These trade-offs involved both microbial and physiological adjustments. For example, microbial shifts resulted in the enrichment of sulfur-cycling bacteria. However, these changes should be interpreted as potential indicators of microbiome restructuring rather than confirmed buffering effects, since direct causal links cannot be established without sterile or inoculated controls. Most notably, prior MHW exposure reduced the plant's capacity to acclimate to subsequent storm disturbances, highlighting environmental legacy effects that may shape seagrass resilience. Together, these findings advance our understanding of cumulative stress impacts in coastal ecosystems, emphasizing the need to integrate microbiome dynamics and environmental legacy effects into seagrass conservation and management strategies.

4.1. *Legacy effects and ecological memory*

Our results contribute to the growing body of evidence that stressor sequence, rather than mere coexistence, mediates ecological responses through both physiological and microbial pathways. While traditional legacy effects refer to the persistence of soil microbial communities that influence the growth of subsequent plant generations after the original plant has died (e.g., plant-soil feedback theory; Bever et al., 2012), our study focuses on legacy effects of environmental change mediated by shifts in the microbiome, whereby prior stress events alter the living plant-microbiome system and shape its response to subsequent stressors. This phenomenon can be interpreted within three complementary

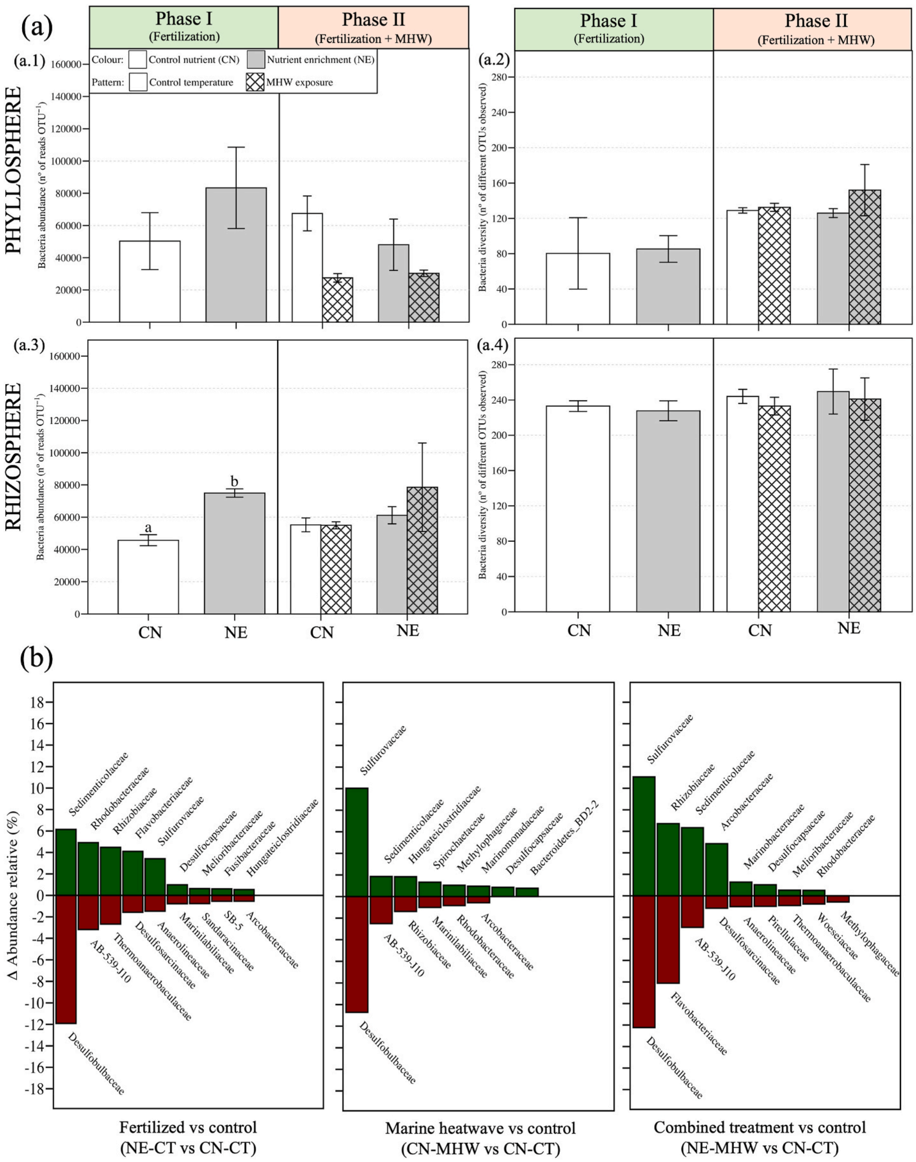

Fig. 6. Microbial diversity patterns: (a) Average bacteria abundance and diversity in phyllosphere (a.1 and a.2) and rhizosphere (a.3 and a.4) across treatments in phase I and II. (b) The top OTUs showing the highest average difference in relative abundances (>0.5 %) in rhizosphere between treatments and the control in Phase II (positive values indicates enriched; negative values indicates depleted).

frameworks: First, *ecological memory*, where prior stress modifies system responsiveness (Ogle et al., 2015). Second, *stress priming*, involving microbial-mediated preconditioning that alters subsequent tolerance (Hilker and Schmülling, 2019). Third, *metabolic hysteresis*, referring to persistent feedbacks between physiological and microbial processes that influence recovery trajectories (Classen et al., 2015). Together, these frameworks help explain how early-stage stress exposure leaves physiological and microbial legacies that determine future performance under additional stress.

In this study, prior MHW exposure diminished root investment during subsequent storms (59 % reduction; Fig. 3d), reflecting an energy allocation trade-off in which above-ground production was prioritized at the expense of capacity for hydrodynamic acclimation. A similar pattern has been observed in terrestrial plants, where warming followed by storms reduced root biomass and production in tropical tree species (Yaffar et al., 2021). These results suggest that energy and carbon reserves used to cope with initial thermal stress may limit subsequent morphological plasticity, constraining recovery under high-flow conditions.

Notably, environmental legacy effects manifested asymmetrically across stressors. Nutrient enrichment primarily influenced the microbiome, suggesting a role for microbial buffering mechanisms, whereas thermal stress induced stronger physiological effects with comparatively minor microbial restructuring. This asymmetry suggests that stressor sequences may produce distinct host-microbe strategies. While physiological plasticity might determine thermal responses, microbiome-mediated buffering might dominate under nutrient stress. However, these hypotheses require further validation through experiments conducted under sterile conditions or controlled inoculation to clarify these mechanisms and to better evaluate seagrass resilience as a dynamic interplay of sequenced stressors, microbial memory, and metabolic trade-offs.

4.2. *Nutrient enrichment and thermal stress*

Nutrient-enrichment produced only modest, non-significant reductions in above-ground biomass (−9.2 %) and net production rate (−23.5 %) in Phase I (Figs. 2a and 3a), despite the well-known risks of eutrophication. While elevated nitrogen and phosphorus typically enhance microbial respiration leading to sediment anoxia and sulfide toxicity (Bulseco et al., 2024), our results suggest that eelgrass avoided these detrimental effects through two mechanisms: (1) selective enrichment of sulfur-cycling bacteria (Arcobacteraceae, Sulfurimonadaceae and Desulfocapsaceae Fig. 5c) that mitigate sulfide accumulation (Zhang et al., 2020; Sogin et al., 2022) and (2) maintenance of root oxygenation under our high-light conditions, creating an oxic microzone around roots that further limited sulfide intrusion (Pedersen et al., 2004). This microbially mediated buffering, evidenced by strong rhizosphere community restructuring (Fig. S5), suggests that seagrass-microbe interactions can stabilize plant performance under nutrient stress (Haviland et al., 2022). Such resilience underscores the importance of considering both microbial symbionts and environmental context when predicting nutrient effects on seagrass ecosystems.

Thermal stress produced more pronounced physiological than microbial responses. The simulated MHW triggered increases in net production rate (NPR) and aboveground biomass in nutrient enriched treatments, but reduced belowground biomass, shoot appearance and rhizome length. These patterns were mainly driven by accelerated leaf growth rate (LGR), consistent with previous reports of short-term stimulation under moderate warming (Reynolds et al., 2016; Beca-Carretero et al., 2018; Breiter et al., 2024). Our findings align with previous studies showing that marine heatwaves can initially stimulate seagrass productivity but often lead to delayed and detrimental effects during recovery (Reynolds et al., 2016). However, by incorporating nutrient enrichment, our study extends this understanding to demonstrate how compounded stressors can modify both the magnitude and persistence of MHW effects on eelgrass physiology and resilience. *Z. marina* exposed to ∼17–18 °C for 5 weeks (+4.5 °C relative to ambient temperature) showed enhanced productivity, but at higher temperatures above 20 °C experienced thermal stress impairing photosynthesis and carbon fixation (Winters et al., 2011; Egea et al., 2024). The positive responses observed here likely reflect a combination of moderate warming and high light, conditions that can promote carbon fixation and growth when algal competition is limited (Kaldy, 2014; Moreno-Marín et al., 2018). Our results align with Reynolds et al. (2016), who documented both short-term stimulation and delayed negative effects following heat exposure. We similarly observed that differences in biomass and growth between previously warmed and control plants diminished during experimental Phase III, the recovery phase. The absence of pronounced delayed decline in the experiment might reflect its shorter recovery duration compared to previous studies.

4.3. Microbiome responses under warming and hydrodynamic acclimation

Under MHW conditions, *Z. marina* showed only modest shifts in microbial composition, characterised by an increase in organic matter-degrading (Spirochaetaceae) and sulfur-related taxa (Sulfurovaceae, Sedimenticolaceae, Desulfocapsacease), consistent with previous studies (Aires et al., 2024). This limited restructuring contrasts with the pronounced microbial changes observed under nutrient enrichment and is consistent with prior work showing minimal microbiome response to moderate warming (Zhang et al., 2023; Walker et al., 2024). Our results support the growing evidence that moderate warming alone has limited impact on the seagrass microbiome, and that significant changes in microbiome typically occur when warming is combined with other stressors like ocean acidification (Zhang et al., 2023), light reduction (Vogel et al., 2021), or nutrient enrichment (this study). These findings suggests that the eelgrass microbiome may play a more relevant role in resisting nutrient stress rather than in adapting to warming.

Under stress, seagrass can release root exudates containing sugars, amino acids and other metabolites, a “crying-for-help” adaptative strategy that promotes beneficial bacterial groups (Crump et al., 2018; Wang and Song, 2022). The quality and quantity of exudates create favourable conditions for specific bacteria groups, for instance sulfur-cycling bacteria (Zhang et al., 2020; Sogin et al., 2022), thereby altering the composition and diversity of microbial community to benefit their host plant. Particularly, we found a notable increase of Arcobacteraceae and Sulfurimonadaceae in NE treatments, which are known for their roles in sulfide oxidation reducing the possible sulfide toxicity in the plant host (Martin et al., 2022; Randell et al., 2023).

In contrast to nutrient stress, under warming, resistance appears to be mainly based on plant-physiological responses rather than microbial-based strategies. For example, we observed an increase in the above-to belowground biomass ratio (AG:BG ratio; from 1.4 to 2.1 in control and MHW treatments, respectively; Fig. S6), likely reducing the respiratory burden associated with maintaining belowground biomass (Ralph et al., 2007). Similar shifts towards higher AG:BG ratios under moderate warming have been reported for *Z. marina* and other aquatic macrophytes, reflecting an adaptive reallocation of resources to sustain photosynthetic tissues when temperature accelerates metabolic demand (Wong and Dowd, 2023; Olesen and Sand-Jensen, 1993). Comparable patterns are also observed in terrestrial plants exposed to heat or drought, where carbon balance constraints favor above-ground growth to maintain productivity (Poorter et al., 2012). Additionally, the observed increase in leaf loss (Fig. 2d) suggests a potential bottom-up control of epiphyte loading, whereby faster leaf turnover may reduce epiphyte accumulation by shedding older heavy colonized leaves more rapidly (Borum, 1987; Peterson et al., 2007). Together, these results indicate that under warming, eelgrass resilience relies largely on physiological plasticity, via resource reallocation and accelerated leaf turnover, rather than on microbiome-mediated buffering mechanisms.

After temperatures returned to ambient levels (Phase III), differences in biomass between warmed and control plants diminished. Wave exposure significantly increased belowground biomass, primarily due to longer roots (Figs. 2b and 3d). While hydrodynamic effects on aboveground structure are well documented (Peralta et al., 2006), impacts on root architecture remain understudied, with more research focused on seedlings (Infantes et al., 2011; Zenone et al., 2022). Our findings extend those of de los Santos et al. (2010), showing that even short-term hydrodynamic events can induce belowground acclimation in adult *Z. noltii*. However, this response was significantly weakened in individuals that had previously experienced MHWs, reflecting an environmental legacy effect. We suggest that carbon and energy reserves used to cope with the earlier heat stress (e.g., sucrose depletion; Moreno-Marín et al., 2018) may have limited the capacity to respond fully to a subsequent hydrodynamic event. Such depletion of storage compounds could reduce the resources available for root regeneration and anchorage, weakening resistance to mechanical disturbance and impairing recovery after consecutive stress. Consequently, these results indicate that successive stress events may compromise the long-term resilience of eelgrass, even when plants show initial physiological acclimation.

4.4. Ecological implications and limitations

The observed responses of *Zostera marina* to the consecutive stressors have important implications for seagrass conservation, management and ecosystem functioning. Although moderate nutrient enrichment alone may cause limited physiological stress, its combination with thermal and hydrodynamic events can substantially reduce seagrass performance. The reallocation of biomass toward shoots at the expense of roots under MHWs could compromise sediment stability and carbon storage, threatening the role of seagrass meadows as blue carbon sinks (Duarte et al., 2013). Reduced belowground reserves may further decrease resilience to additional stressors, such as light limitation (Lapointe et al., 2020), herbivory (Jiménez-Ramos et al., 2024a) or storms (this study). Although we observed some hydrodynamic acclimation via root elongation (Fig. 3d), this adaptive capacity was impaired by prior MHW exposure, suggesting that cumulative stressors may erode the "anchoring resilience". Such biomechanical vulnerability (Jiménez-Ramos et al., 2017a) could trigger feedback loops where uprooted shoots create bare patches susceptible to sulfide intrusion, further compromising neighboring shoots (Bulseco et al., 2024).

While these results highlight key mechanisms underlying seagrass resilience, they should be interpreted with caution given the inherent limitations of mesocosm studies. Our experimental design simplified natural variability by controlling for light, temperature and nutrient loads, and did not account for sediment heterogeneity, episodic hydrodynamic forcing, or biotic interactions such as grazing and algae competition (Jiménez-Ramos et al., 2017b; de la Hoz et al., 2025). Moreover, the short duration (55 days) may not capture long-term microbial succession or carbon budget changes (Soissons et al., 2016; Mishra et al., 2025). Finally, although we minimized pseudo-replication by aggregating pot-level data within flumes and standardizing flume conditions, subtle tank effects cannot be fully excluded. Despite these constraints, the mesocosm approach provided valuable mechanistic insights into stressor-sequence effects on seagrass–microbiome–physiology linkages. Future work should test these mechanisms under natural conditions across seasons and environmental gradients, and incorporate manipulative microbiome experiments to isolate causal pathways. Such studies will be essential to predict how seagrass ecosystems respond to increasingly frequent and sequential climate disturbances.

5. Conclusions

This study demonstrates that *Zostera marina* responds to successive stressors through distinct but interacting mechanisms. Under nutrient enrichment, microbial shifts, particularly the enrichment of sulfur-cycling bacteria, suggest potential microbiome-mediated buffering against sulfide stress. In contrast, marine heat waves (MHW) indicated primarily physiological responses, with increased above-to-below-ground biomass ratios indicating resource reallocation to sustain productivity under elevated temperatures. Crucially, stressor sequence dictated outcomes. Prior MHW exposure reduced subsequent storm acclimation by 51 %, revealing a clear environmental legacy effect. While northern populations showed thermal tolerance (23–25 °C), trade-off in below-ground reserves highlights their vulnerability to repeated disturbances. Together, these findings advance current resilience frameworks by showing that the order and combination of stressors, rather than their individual magnitude, influences seagrass performance. They emphasize the need of integrating sequential-stressor approaches and microbiome dynamics into experimental designs, conservation planning and restoration strategies for these climate-threatened coastal ecosystems.

CRediT authorship contribution statement

Luis G. Egea: Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Rocío Jiménez-Ramos:** Writing – review & editing, Writing – original draft, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Lucía Rodríguez-Arias:** Writing – review & editing, Investigation. **Eduardo Infantes:** Writing – review & editing, Project administration, Methodology, Investigation, Funding acquisition, Data curation, 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.2025.107748.

Data availability

The dataset for this article is open access in the Zenodo repository: https://doi.org/10.5281/zenodo.15516836

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