

Degradation, conservation and ecological restoration of seagrass beds under intensifying global changes

Edited by

Shaochun Xu, Juan D. Gaitan-Espitia and
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Degradation, conservation and ecological restoration of seagrass beds under intensifying global changes

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Editorial: Degradation, conservation and ecological restoration of seagrass beds under intensifying global changes

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Editorial on the Research Topic

[Degradation, conservation and ecological restoration of seagrass beds under intensifying global changes](#)

Global changes (including warming, ocean acidification, and intensified human disturbances) have profoundly impacted marine environments, threatening foundational species and their critical habitats (Hoegh-Guldberg and Bruno, 2010). Addressing these compounded pressures requires an enhanced understanding of marine ecosystems alongside intensified protection and restoration efforts.

Seagrasses, a group of foundational marine species, are widely distributed along tropical and temperate coastlines, where their meadows support essential ecosystem functions (Unsworth et al., 2019b). These meadows, one of the three primary nearshore ecosystems, provide habitat, food, and nurseries to numerous marine species (Costanza et al., 1997). Moreover, they contribute to water quality by enhancing sediment deposition and removing excess nutrients (Dennison et al., 1993), while their role as carbon sinks helps mitigate climate change (Fourqurean et al., 2012). It is estimated that 19% of the surveyed seagrass area has been lost since 1880, with individual meadows declining globally at a rate of 1–2% per year (Dunic et al., 2021), driven by both natural and anthropogenic factors, including physical disturbances, sediment and nutrient runoff, invasive species, algal overgrowth, and warming waters (Waycott et al., 2009). These threats have catalyzed a global push to conserve seagrasses, aiming to reduce their loss and strengthen their ecosystem functions. The success of the efforts relies on the knowledge exchange and integration of scientific research into conservation/restoration practices, aiming to develop

more effective restoration techniques that promote the resilience of seagrass ecosystems and their services.

Our aim for this Research Topic in *Frontiers in Marine Science* was to bring together studies that improve the understanding of the mechanisms underpinning seagrass degradation and ecological responses to environmental change, as well as the technological approaches that can enhance restoration success. This Research Topic includes nine scientific papers (one review and eight original research articles) covering diverse species from temperate to tropical waters. The main focus was on the assessment of ecological impacts of environmental stressors, adaptive physiological responses to climate change, advances in restoration techniques, ecological restoration benefits, and microbial contributions to restoration success.

Seagrass degradation drivers

Despite their critical ecological roles sustaining biodiversity and ecosystem functions, seagrasses are experiencing significant global degradation (Duarte et al., 2025; Jones et al., 2025). Long-term observations highlight anthropogenic activities as primary drivers. While some of these activities (e.g., oyster aquaculture) may have minor effects on seagrass meadows, at least in the short-term and at small geographic scales (Rubino et al.), others can result in detrimental changes of the seagrass ecosystems in the long-term (Green et al., 2021). For example, in Bohai Bay, Tangshan, extensive alterations in *Z. marina* meadows occurred between 1974 and 2019 due to land reclamation. Partial recovery followed the construction of artificial “longshore sandbanks” after 2012, but meadows remain threatened by fishing, dredging, and aquaculture activities (Xu et al., 2021b). Similarly, the increase in anthropogenic heavy metal pollution (e.g., copper and cadmium) has negative effects on seagrass meadows by suppressing the growth and photosynthesis of these marine plants. This effects can vary among species and populations depending on their specific sensitivities and tolerances (e.g., *Z. marina*: copper $EC_{50} = 28.9 \mu M$; *Ruppia sinensis*: copper $EC_{50} = 50 \mu M$), and the interactions with other environmental stressors (e.g., elevated temperatures) (Gu et al., 2021; Qiao et al., 2022).

Natural environmental changes can also represent ecological challenges that further compromise meadow stability (Unsworth et al., 2012). For instance, natural biochemical changes in the sediments of seagrass meadows (e.g., accumulation of sulfides), can interact with temporal changes in other environmental drivers (e.g., elevated temperature), impacting the performance (e.g., photosynthesis and growth) of seagrasses. While short-term removal of the stress allows recovery, the prolonged exposure can result in irreversible mortality (Zhang et al., 2024). Similar negative effects can be observed in response to natural environmental changes in oxygen availability and irradiance, in which low oxygen conditions under low light can trigger severe photosynthetic impairment and metabolic alterations (Zhang et al., 2021). The scale of the effects of natural environmental changes on seagrass meadows depends on the magnitude and intensity of the changes. For example, natural extreme events

such as the super Typhoon Lekima, nearly eradicated 1031.8 ha of *Z. japonica* in the Yellow River Delta, accompanied by substantial losses of organic carbon (>35%) and nitrogen (>65%) in the top 35 cm of sediments. The long-term detrimental effects of this extreme event together with the absence of seeds and overwintering shoots prevented natural recovery of the seagrass populations (Yue et al., 2021a).

Anthropogenic-driven climate change is another major threat to seagrass ecosystems, altering their ecological dynamics at a global scale. For instance, ocean warming, which is associated to the increase in atmospheric CO_2 , has shifted the geographic distribution of seagrasses (*Z. marina*) in the Northeast Pacific (eastern China). Such ecological alterations are further exacerbated by the influence of anomalous extreme temperatures (e.g., marine heatwaves), resulting in drastic declines in the survival and functioning of these marine plants (Xu et al., 2022a; Pei et al.), despite their short-term capacity to regulate the oxidative stress associated to elevated temperatures (Pei et al.). Elevated atmospheric CO_2 can also alter the carbonate chemistry of the seawater resulting in ocean acidification. Contrary to ocean warming, ocean acidification has shown positive effects promoting seagrass growth. However, these beneficial effects cannot offset the functional impacts of heat-induced stress (Wang et al.).

Indirect anthropogenic environmental changes can also result from biological sources in which biotic stressors, such as algal blooms and invasive species, represent important ecological challenges that threaten the stability and distribution of seagrasses. In the Swan Lake lagoon, large meadows of *Z. marina* (199–232 ha), have been impacted by the rapid expansion of green tides of *Chaetomorpha linum*, enhancing the ecological competition for resources (e.g., space and light) (Xu et al., 2019a). Similar competition with invasive plants (e.g., *Spartina alterniflora*) has resulted in the suppression of seagrass growth and the contraction of *Z. japonica* habitats in the Yellow River Delta (Yue et al., 2021b).

Collectively, seagrass degradation results from anthropogenic pressures, natural disturbances, biological invasions, and climate change, involving light limitation, physical disturbance, chemical stress, and metabolic imbalance, which often act synergistically to accelerate decline and reduce resilience (de Fouw et al.). Therefore, future management must simultaneously alleviate local stress and adapt to global change to maintain ecosystem resilience.

Seagrass restoration

Restoration predominantly relies on transplantation and seed-based approaches. Transplantation, while potentially damaging donor beds and resource-intensive, offers relatively high survival and remains the most widely applied method. However, with restoration scaling to hundreds of hectares in China, overreliance on donor beds creates a “destructive-restorative” paradox. Seed-based restoration minimally impacts donor populations and enhances genetic diversity, making it a promising future strategy. Seed- and seedling-based approaches have emerged as a key focus for sustainable, large-scale restoration.

Seed-based methods offer potential for large-scale implementation but face high failure rates and technical bottlenecks (Unsworth et al., 2023; Unsworth and Rees, 2025). Seed selection and storage are critical: high-quality *Z. marina* seeds with >72% integrity can be obtained via saline flotation (>1.20 specific gravity) and maintained at 0°C with nanosilver or copper sulfate treatment, preserving 80–90% viability (Xu et al., 2019b). Long-term storage at 0°C and 40–50 psu salinity can potentially extend viability up to 17 months, providing a foundation for seed banks (Xu et al., 2020).

Environmental conditions (including burial depth, water depth, salinity, and temperature) critically influence germination and seedling establishment. Field experiments show optimal emergence for *Z. marina* at 2 cm burial depth ($21.33 \pm 9.30\%$), while 10 cm prevents germination (Xu et al., 2021a). Freshwater favors germination (up to 88.67%) but compromises seedling morphology and survival; salinity >20 psu is required for stable establishment (Xu et al., 2016). Various techniques, such as mudball sowing (Xu et al., 2022b), bag fixation (Unsworth et al., 2019a), nutrient supplementation (Unsworth et al., 2022), and seed injection (Govers et al., 2022), demonstrate practical potential. For example, bag sowing achieved 94% mature plant emergence, though storm-induced sediment burial poses risks (Unsworth et al., 2019a).

Regional applications reveal species- and site-specific differences. In the Yellow River Delta, optimal burial depth varies with sediment type (sand 4 cm, silt 2 cm), and *Z. japonica* seed source affects performance (Yue et al., 2024). Transplantation studies on *Z. pacifica*, *T. hemprichii*, and *E. acoroides* further illustrate interspecific and regional variability. For instance, *E. acoroides* in Hainan exhibits 90% survival, outperforming *T. hemprichii* (Shen et al.), while *Z. pacifica* restoration on the US west coast is highly dependent on light, wave exposure, and dissolved oxygen thresholds (Sanders et al.). Rhizosphere microbial communities also influence seedling resource allocation, highlighting the need to integrate microbial factors into restoration planning (Randell et al.).

Ecological benefits

Restored meadows rapidly enhance benthic biodiversity, approaching natural meadow levels (Gräfnings et al.). Long-term monitoring indicates gradual increases in sediment carbon storage and microbial diversity, although short-term carbon sequestration gains are modest (Xu et al., 2025). Across 228 Southeast Asian restoration cases, moderate depths (2–4 m), adequate light, and low-to-moderate energy environments optimize success, with vegetative transplantation yielding the highest survival rates (Thorhaug et al.).

Future directions

With the increasing frequency, magnitude, and duration of global change, research on seagrass degradation mechanisms, management, restoration, and ecosystem function assessment is increasingly urgent. The studies summarized herein contribute

critical insights into seagrass ecology, identify knowledge gaps, and highlight new directions. Future research should integrate climate change and anthropogenic pressures, advance precision management, innovate restoration techniques—including mechanization to improve efficiency—quantify carbon storage and ecosystem services, and leverage interdisciplinary approaches and predictive modeling to support sustainable management. We anticipate that this field will attract broad scientific interest, uniting researchers with diverse and complementary expertise to collectively advance seagrass conservation and restoration.

Author contributions

SX: Writing – original draft. RU: Writing – review & editing. JG-E: Writing – review & editing. YZ: Writing – review & editing.

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Seasonal temperature variation in *Zostera marina* seedlings under ocean acidification

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Objective: To investigate the responses of *Zostera marina* seedlings to the individual and combined stresses of seasonal temperature increase and ocean acidification (OA) caused by global climate change and anthropogenic factors. This data will help in efforts to protect and restore seagrass beds in temperate coastal zones of China.

Methods: A mesoscale experimental system was utilized to analyze stress response mechanisms at multiple levels - phenotype, transcriptome, and metabolome - during the seedling stage of *Z. marina*, a dominant temperate seagrass species in China. The study monitored the seedlings under varying conditions: increased seasonal temperature, OA, and a combination of both.

Results: Findings revealed that under high-temperature conditions, carotenoid biosynthesis was stimulated through the upregulation of specific metabolites and enzymes. Similarly, the biosynthesis of certain alkaloids was promoted alongside modifications in starch, sucrose, and nitrogen metabolism, which improved the plant's adaptation to OA. Unique metabolic pathways were activated under OA, including the degradation of certain amino acids and modifications in the citric acid cycle and pyruvate metabolism. When subjected to both temperature and OA stresses, seedlings actively mobilized various biosynthetic pathways to enhance adaptability and resilience, with distinct metabolic pathways enhancing the plant's response under diversified stress conditions. In terms of growth, all treatment groups exhibited significant leaf length increase ($p < 0.05$), but the weakest growth index was observed under combined stress, followed by the thermal treatment group. Conversely, growth under OA treatment was better, showing a significant increase in wet weight, leaf length, and leaf width ($p < 0.05$).

Conclusion: Seasonal temperature increase was found to inhibit the growth of *Z. marina* seedlings to some extent, while OA facilitated their growth. However, the positive effects of OA did not mitigate the damage caused by increased seasonal temperature under combined stress due to seedlings' sensitivity at this stage. Our findings elucidate differing plant coping strategies under varied stress conditions,

contingent on the initial environment. This research anticipates providing significant data support for the adaptation of *Z. marina* seedlings to seasonal temperature fluctuations and global oceanic events like OA, propelling the effective conservation of seagrass beds.

KEYWORDS

seagrass beds, *Zostera marina* seedling, ocean acidification, seasonal temperature variation, growth, restoration

1 Introduction

As marine angiosperms, seagrasses act as physical barriers to reduce waves impacts, such as turbulence (Granata et al., 2001; Koch et al., 2007). Seagrass beds are recognized as carbon sinks (Fourqurean et al., 2012), effective in accumulating subsurface carbon deposits (McLeod et al., 2011; Fourqurean et al., 2012) and capturing carbon (Duarte et al., 2005; Barbier et al., 2011), and in addition, seagrass beds can recycle nutrients (Hemminga et al., 1991), provide habitat for many epiphytes and animals (Coll et al., 2011), are nurseries for many fish species, and have high primary productivity (Espino et al., 2011). Seagrass beds can also play a key role in biogeochemical cycling in nearshore waters with these characteristics (Hemminga and Duarte, 2000; Celia and Fernando, 2023). Therefore, seagrass beds with considerable conservation value deserve attention and protection (Barbier et al., 2011).

It has been found that some seagrasses grow rapidly with an annual reproduction pattern (Orth et al., 2006a). The vitality of these seagrasses has a distinct seasonal character (Marbà et al., 1996). *Zostera marina*, a common seagrass species in the temperate zone of the Northern Hemisphere (Olsen et al., 2016), is a typical dominant species of temperate seagrasses in China, forming a large number of *Z. marina* meadows along the coast of the Yellow Sea and Bohai Sea in China (Xu et al., 2019). Yan et al. (2023) found that the sea surface temperatures (SST) in the Yellow Sea and Bohai Sea in China over the past 20 years were highly seasonal. It was shown that such seasonal temperature variations affect the growth and productivity of vegetation in marine environments (Sand-Jensen, 1975; Orth and Moore, 1986; Watanabe et al., 2005). And the growth of coastal seagrasses is greatest in summer, with increases in biomass, shoot density and canopy height (Boström et al., 2003). As winter temperatures drop, seagrass leaves virtually disappear, reducing aboveground biomass and eventually leaving only belowground rhizomes to acclimatize to cold temperatures at the lowest of growth (Baden and Pihl, 1984; Burke et al., 1996).

Seasonal changes in the local environment of coastal areas imply not only SST changes but also in other abiotic variables. For example, SST, light, pH, and sediments can regulate seagrass growth (Hemminga and Duarte, 2000; Lee et al., 2005). Correlations exist between environmental variables (Celia and Fernando, 2023). Specifically, there are spatial pattern correlations across latitudinal

gradients among seawater temperature, nutrients concentrations, and plants primary productivity (Parmesan and Yohe, 2003; Lemos and Pires, 2004; Tuya et al., 2012; Celia and Fernando, 2023). Notably, the impacts of those abiotic variable stressors vary by different seagrass species (Marbà et al., 1996; Lee et al., 2005; Celia and Fernando, 2023).

It is well known that atmospheric CO₂ levels are increasing under human influence, which pose many problems for marine ecosystems (Wang et al., 2022). The oceans continue to absorb CO₂ from the atmosphere and lead to a decline in ocean pH, changes in the carbonate system, and ocean acidification (OA) (Caldeira and Wickett, 2003), as a major disturbance in modern ocean events (Zhong et al., 2021). Environmental changes caused by OA will directly alter the function and structure of marine ecosystems, such as seagrasses (Gattuso et al., 2015; Zhong et al., 2021). By the end of the century, sea surface pCO₂ are projected to exceed 1000 µatm (IPCC, 2013). Currently, many studies have shown the variation of CO₂ concentration and temperature can produce a combined response in marine organisms (Nagelkerken and Munday, 2015; Watson et al., 2018; Brandenburg et al., 2019; Gao et al., 2019; Van Colen et al., 2020; Zhong et al., 2021). However, little is known about the adaptive responses of seagrasses to the OA and seasonal temperature fluctuations at different life stage. In recent decades, seagrass meadows due to human activities and climate change is a global concern, and their restoration has received much attention (Orth et al., 2006a). Seagrass seedling transplantation has been a useful tool for seagrass restoration (Pereda-Briones et al., 2018) due to its low damage on donor seagrass beds (Seddon, 2004; Orth et al., 2006b) and its ability to ensure the genetic diversity of seagrasses (Waycott et al., 2009). Therefore, it is important for seagrass conservation to explore the mechanisms by which seagrass seedlings respond to seasonal temperature increases and OA.

In the study, we studied the acclimatization response of *Z. marina* seedlings to OA and seasonal temperature changes. Under mesoscale experiments, this study investigated how *Z. marina* seedlings responded to (1) a large increase in seasonal temperature; (2) the occurrence of OA events; (3) OA events and seasonal temperature alternation, as assessed by growth, transcriptomics and metabolomics. This study aims to understand how seagrass seedlings adapt to seasonal temperature differences and global OA events in order to facilitate effective methods of seagrass conservation.

2 Materials and methods

2.1 Sample collection

A total of 120 *Z. marina* were collected in March 2022 at the sea area of Caoheidian (39.1131°N, 119.120°E), and 10 meters were spaced between each sample point to avoid collecting the same plants (Watanabe et al., 2005). All plants were in dark plastic boxes containing *in situ* seawater to the laboratory within 1 h. The leaves, roots and stems of each plants were gently brushed and rinsed with tap water. Then, put them in a seawater recirculation tank for 7 days as a pre-culture stage. The transient condition was achieved in a recirculating seagrass culture system with a temperature of 14°C, a salinity of 32, a water flow rate of 800 mL min⁻¹, a light intensity of 240 μmol photons m⁻², and a 12:12 h light/dark ratio.

2.2 Experimental design

To setup temperature of each treatment, we used the data from the National Oceanic and Atmospheric Administration optimized interpolated sea surface temperature (OISST) (<https://www.ncei.noaa.gov/metadata/geoportal/rest/metadata/item/gov.noaa.ncdc:C00844/html>) (Richard et al., 2008), which spatial grid resolution is 0.25 degrees and temporal resolution is 1 day. The SST daily climatology of the Bohai Yellow Sea (34°N–42°N, 115°E–127°E) was extracted and calculated using the 20-year (2003–2022) SST mean values. All data were calculated in Ferret Version 7.6.0 and plotted in MATLAB software.

Four groups were set up with 30 well-grown, similarly growing plants in each group. Each group had a separate recirculation system consisting of a tank, a light system, a circulation system, a filtration system, a temperature control system and a monitoring system (more details in the Methods of Yan et al., 2023). Meanwhile, *in situ* seawater was used to maximize the simulation of the natural environment for *Z. marina* growth. The average SST of May is 14.03°C and the average SST of Jun is 19.02°C (Figure 1). To predict *Z. marina* response to OA, we choose the pCO₂ of the end of the century (1000 μatm) was used as the future OA condition. The control group (CK) was with the seawater

temperature in May (14 ± 1°C) represented the spring season (Figure 1A), and the CO₂ concentration was that of the current atmospheric pCO₂ (410 μatm); the second group (OA) was the acidification group, in which the seawater temperature was 14 ± 1°C, and the CO₂ concentration was that of the future atmospheric pCO₂ (1000 ± 50 μatm); the third group (OW) was the SST temperature with seasonal changes treatment group, which has a current CO₂ concentration of 410 μatm and June temperature of 19 ± 1°C (Figure 1B); and the fourth group (AW) was the treatment group with composite stressors, i.e. extreme CO₂ concentration of 1000 ± 50 μatm and early summer temperature of 19 ± 1°C. During the experiment, the CO₂ flux of each group was controlled and monitored by a CO₂ enricher (SFO-E, China), which can pass CO₂ with a set concentration or air directly into the seawater tank (Yan et al., 2023). Water temperature, pH, and TDS were monitored in real-time using a temperature monitor (Caperplus Q2-PH/TDS, China) and a multiparameter water quality tester (ProQuatro; YSI, Yellow Springs, Ohio, USA) to make sure constant water quality. The whole treatment was 14 days.

2.3 Plant growth

Nine samples were selected from each group. Since the plants did not have a very long leaf length (10 cm on average), a uniform de-averaging of the leaf length was not done. Leaf length (cm) was measured from the base of the meristematic tissue, leaf width (cm) was from the middle part of the leaf, and wet weight (g) was of the whole plant at the day 0 and 14 of the experiment. Before measurement, tap water was used to gently washed each sample, and then three growth index were measured to analyze their changes.

2.4 RNA extraction and transcriptome analysis

Three samples per treatment group were selected for transcriptomics sequencing. First, total RNA was extracted using the Trizol kit (Invitrogen, Carlsbad, CA, USA) according to the

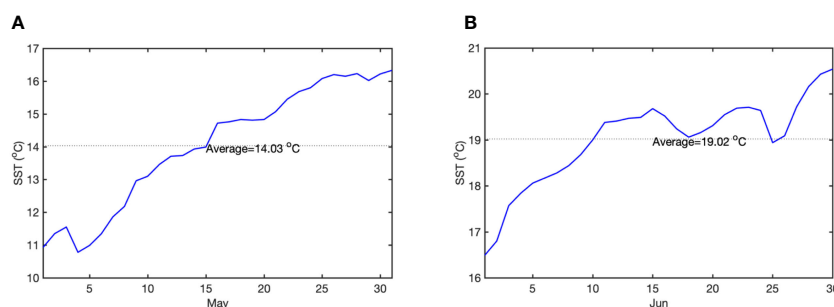


FIGURE 1

Sea surface temperature daily climatology of May and June. (A) daily SST of May; (B) daily SST of June. The dashed black line denotes the average SST (blue line).

method provided by the manufacturer. RNA quality was assessed on an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA) and assayed using an RNase-free RNA quality was assessed on an Agilent 2100 Bioanalyzer (Agilent Technologies Palo Alto, CA, USA) and detected using RNase-free agarose gel electrophoresis. After extraction of total RNA, mRNA was enriched with Oligo(dT) beads, purified, and amplified by polymerase chain reaction. The resulting cDNA library was sequenced at Illumina Novaseq6000 to obtain all the raw data. To obtain high-quality clean data, the reads were first filtered using fastp V. 0.18.0 (Chen et al., 2019). Next, the reads were mapped using Bowtie2 V. 2.2.8 (Langmead and Salzberg, 2012), followed by removing the rRNA-mapped reads. Then, the remaining clean data were mapped to the reference genome of *Z. marina* using HISAT2. The reference gene from ENSEMBL (<http://www.ensembl.org/index.html>) was sequenced in 2016 and renewed their genome annotation in 2017 (Olsen et al., 2016). Fragments per kilobase of transcript per million mapped reads (FPKM) values were calculated using the RSEM software (Li and Dewey, 2011) to compare gene expression differences among samples. Principal component analysis (PCA) was performed using the R package gmodels (<http://www.rproject.org/>) to reveal relationships between samples. Differential expression analysis (Love et al., 2014) was performed on RNAs between two different groups using DESeq2 software (Robinson et al., 2010). Genes with a false discovery rate (FDR) of less than 0.05 and an absolute fold change of ≥ 2 were considered differentially expressed genes (DEGs). All DEGs were mapped to GO terms in the Gene Ontology database (<http://www.geneontology.org/>) to identify the major biological functions performed by DEGs. Kyoto Encyclopedia of the Genome (KEGG) metabolic pathway enrichment analysis was performed to identify major metabolic pathways in which DEGs were involved (Kanehisa and Goto, 2000).

2.5 Metabolite extraction and metabolome analysis

Six samples from each group were selected for metabolomics analysis. Aliquots of samples were prepared as quality control (QC) (Want et al., 2013). For metabolite extraction, we first ground the leaves (100 mg) individually in liquid nitrogen and then resuspended the homogenate in 500 μ l of precooled 80% methanol by vortexing thoroughly. The samples were incubated on ice for 5 min and then centrifuged at 15,000 g for 20 min at 4°C. The supernatant was analyzed by LC-MS grade. The supernatant was diluted with LC-MS grade water to a final concentration of 53% methanol. The sample was then transferred to a new Eppendorf tube and centrifuged at 15,000 g for 20 minutes at 4°C. The supernatant was injected into the LC-MS sample. Finally, the supernatant was injected into the LC-MS/MS system for analysis (Dunn et al., 2011).

The metabolites were detected in positive ion mode (POS) and negative ion mode (NEG). Positive and negative ion modes were subsequently analyzed separately. Data were z-score normalized (Kolde, 2015). Orthogonal projection to latent structures-discriminant analysis (OPLS-DA) was performed on six

comparison groups (CK-vs-AW, CK-vs-OW, OA-vs-OW, CK-vs-OA, OW-vs-AW, OA-vs-AW) using R package models (<http://www.r-project.org/>) (Bylesjö et al., 2006). The OPLS-DA model was further validated by cross-validation and permutation tests (Saccenti et al., 2014). The variable importance (VIP) score was applied to rank the most distinguishable metabolites between the two groups. The *p* values were calculated using R (Rao et al., 2016), and correlation heatmaps were plotted by the R corrplot software package (Wei et al., 2017). In addition, a t-test was used to screen for differentially expressed metabolites (*p* value < 0.05 and VIP ≥ 1). The abundance of differentially expressed metabolites in the same group was normalized by z-value using R, and the VIP scores from OPLS-DA were used to map the volcano plots (Yoon et al., 2020). Finally, metabolites were mapped to KEGG metabolic pathways enrichment analyses (Kanehisa and Goto, 2000). The *p* values were corrected for false discovery rate (FDR), and metabolic pathways with an FDR of ≤ 0.05 were screened as metabolic pathways significantly enriched for differentially expressed metabolites.

2.6 Statistical analysis

The data of growth indexes of each treatment were expressed as mean $\pm$ standard deviation (SD) and statistically analyzed. One-way analysis of variance was performed using IBM SPSS Statistics 27.0 (IBM SPSS, Chicago, IL, USA). Data were visualized using Origin 2021 (Originlab, Northampton, MA, USA).

3 Results

3.1 Plant growth

After 14 days, CK and OA tended to increase in wet weight at 14°C, and the increase was higher in the OA group; at 19°C, there was no significant change in OW and AW (Figure 2A). In addition, leaf length increased in all four groups (*p* < 0.05, Figure 2B). The increase was smaller in OA, OW, and AW groups than in CK group. OW group had the longest leaf length, which showed the largest increase, while the AW group showed the smallest. Similarly, leaf width increased significantly in all four groups (Figure 2C, *p* < 0.05), with the largest increase in CK group, followed by OA group, and the smallest increase in AW group.

3.2 Transcriptome results

A total of 12 leaf tissue samples (three replicates from each group) were sequenced and got a 9.55 Gb high-quality reads with an average of 4.96 Gb. The percentage of bases filtered to Q30 in clean data ranged from 92.56% to 94.53%. The GC ratios of filtered sequence bases ranged from 44.79% to 45.63%. Comparison of the clean data with the *Z. marina* reference genome (Olsen et al., 2016) revealed a rate of 90.25–94.57%.

Based on transcriptome analysis, we found that the number of DEGs varied among different comparisons (Figure S1). The greatest

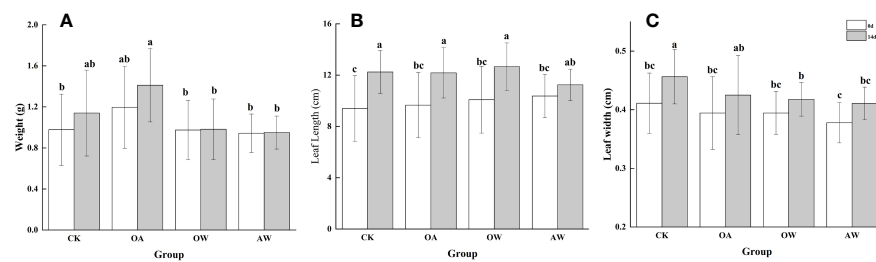


FIGURE 2

(A) Wet weight, (B) leaf length, and (C) leaf width for different groups at the beginning and end of the experiment. CK means the group control with 14°C, and current atmospheric $p\text{CO}_2$ (410 μatm); OA is with seawater temperature of 14°C, and the future atmospheric $p\text{CO}_2$ (1000 μatm); OW is with a current CO_2 concentration (410 μatm) and June temperature of 19°C; AW is with an extreme CO_2 concentration of 1000 μatm and early summer temperature of 19°C.

number of DEGs were found between CK and AW. This was followed by CK-VS-OW, OA-VS-OW, CK-VS-OA, OW-VS-AW, and the least was OA-VS-AW.

A total of 1,290 DEGs were identified in AW compared to CK, of which 763 were upregulated genes (Figure 3A). GO enrichment analysis showed that DEGs were mainly enriched in biological process, cellular process, metabolic process, and so on. The top 10 terms in biological process were flavonoid biosynthetic process, asparagine biosynthetic process, asparagine metabolic process, flavonoid metabolic process, glutamine family amino acid metabolic process, single-organism process, hyperosmotic response, response to chemical ($q < 0.01$). These DEGs were

involved in 107 KEGG pathways (Figure S2A). Among them, biosynthesis of secondary metabolites; flavonoid biosynthesis; alanine, aspartate and glutamate metabolism; glycine, serine and threonine metabolism; biosynthesis of amino acids, starch and sucrose metabolism; and galactose metabolism showed significant enrichment ($p < 0.01$, Figure S2A), with the following valine, leucine and isoleucine biosynthesis; arachidonic acid metabolism; DNA replication; plant-pathogen interaction; betalain biosynthesis; circadian rhythm-plant, 2-oxocarboxylic acid metabolism; and vitamin B6 metabolism ($p < 0.05$, Figure S2A).

In OW compared to CK, 1,220 DEGs were identified with 701 upregulated genes (Figure 3B). GO enrichment analysis showed

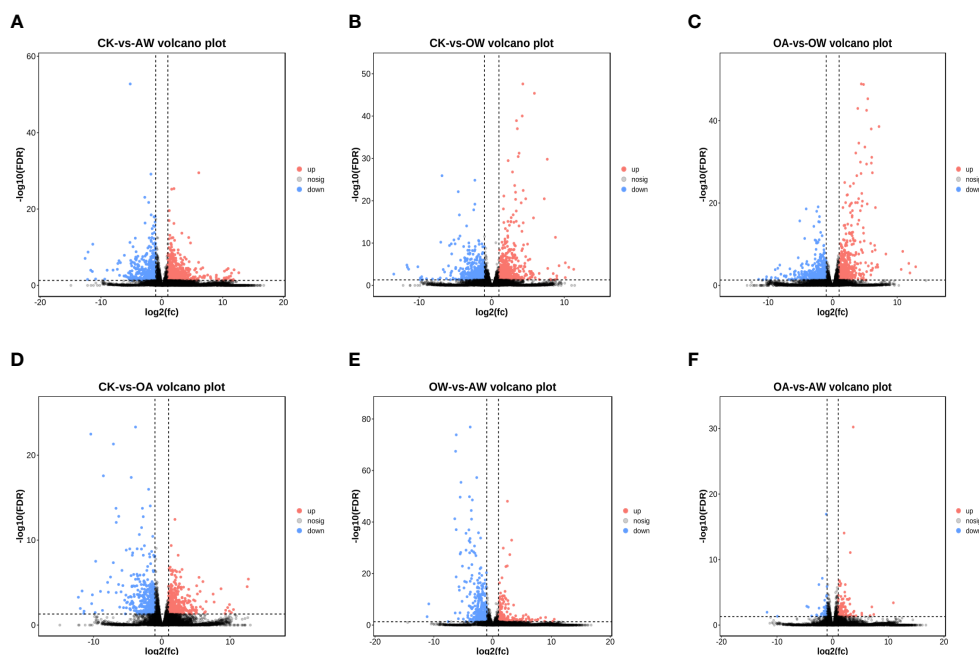


FIGURE 3

Volcano plot of differentially expressed genes (DEGs) in different treatments. DEGs in (A) CK-vs-AW, (B) CK-vs-OW, (C) OA-vs-OW, (D) CK-vs-OA, (E) OW-vs-AW, (F) OA-vs-AW. Each dot is a gene. The horizontal coordinate is $\log_2$ fold-change, and the vertical coordinate is $-\log_{10} q$ values. Red, significantly upregulated genes; blue, significantly downregulated genes; gray, no significant differences. All DEGs are with $q < 0.05$ and $|\log_2 FC| > 1$. The closer the genes are to the ends, the greater the degree of difference. CK means the group control with 14°C, and current atmospheric $p\text{CO}_2$ (410 μatm); OA is with seawater temperature of 14°C, and the future atmospheric $p\text{CO}_2$ (1000 μatm); OW is with a current CO_2 concentration (410 μatm) and June temperature of 19°C; AW is with an extreme CO_2 concentration of 1000 μatm and early summer temperature of 19°C.

that the DEGs significantly enriched in biological processes were response to stress, chemical, organic substance, cellular response to UV ($q < 0.005$), response to stimulus and acid chemical ($q < 0.05$). KEGG pathways revealed these DEGs were enriched in 103 pathways (Figure S2b). The most significant pathways were metabolic pathways, amino sugar and nucleotide sugar metabolism, starch and sucrose metabolism, plant-pathogen interaction ($p < 0.01$, Figure S2B), followed by biosynthesis of secondary metabolites, arachidonic acid metabolism, photosynthesis-antenna proteins, galactose metabolism, nitrogen metabolism, flavonoid biosynthesis, DNA replication, fructose and mannose metabolism, ascorbate and aldarate metabolism, valine, leucine and isoleucine biosynthesis ($p < 0.05$, Figure S2B). In particular, NCED (9-cis-epoxycarotenoid dioxygen) was significantly upregulated in carotenoid biosynthesis.

A total of 1146 DEGs were identified in OW compared to OA, of which 555 were upregulated and 591 were downregulated (Figure 3C). GO enrichment analysis showed that response to organic substance, stimulus, acid chemical, chemical, endogenous stimulus, stress, generation of precursor metabolites and energy, response to organonitrogen compound, photosynthesis, light reaction, photosynthesis, response to hormone, asparagine biosynthetic process, cellular response to acid chemical, and immune system process were significantly enriched in biological processes ($q < 0.005$). There were 92 significant KEGG pathways (Figure S2C). Among them, metabolic pathways were the most significant ($p < 0.01$ and $q < 0.005$), followed by biosynthesis of secondary metabolites; photosynthesis-antenna proteins; glutathione metabolism; carbon fixation in photosynthetic organisms; pentose phosphate pathway; photosynthesis; MAPK signaling pathway-plants; alanine, aspartate and glutamate metabolism; and biosynthesis of amino acid ($p < 0.01$).

A total of 836 DEGs were identified in OA compared to CK, of which 442 were upregulated (Figure 3D). In biological processes, DEGs significantly enriched in, firstly, asparagine biosynthetic process, asparagine metabolic process, response to organonitrogen compound, aspartate family amino acid biosynthetic process, response to chemical, glutamine family amino acid metabolic process, response to stimulus, and acid chemical ($q < 0.001$), followed by response to stress, cell communication, response to light intensity, cellular response to starvation, response to starvation, cellular response to nutrient levels, hyperosmotic response, alpha-amino acid biosynthetic process, response to nutrient levels, cellular response to extracellular stimulus, cellular response to external stimulus, response to extracellular stimulus ($q < 0.005$). These DEGs were involve in 98 KEGG pathways (Figure S2D). Specifically speaking, biosynthesis of secondary metabolites; alanine, aspartate and glutamate metabolism; biosynthesis of amino acids; carbon metabolism; carbon fixation in photosynthetic organisms; arachidonic acid metabolism; glycine, serine and threonine metabolism; linoleic acid metabolism; and phenylalanine, tyrosine and tryptophan biosynthesis showed the significance ($p < 0.01$, Figure S2D), followed by starch and sucrose metabolism, glyoxylate and dicarboxylate metabolism, TCA cycle, cyanoamino acid metabolism, vitamin B6 metabolism, flavonoid biosynthesis, alpha-linolenic acid metabolism, and galactose

metabolism ($p < 0.05$, Figure S2D). In tropane, piperidine and pyridine alkaloid biosynthesis, hisC (histidinol-phosphate aminotransferase) was significantly upregulated. In starch and sucrose metabolism, eight genes showed strong up or downregulation trends. For example, *pgm* (phosphoglucomutase) and *glgA* (starch synthase) showed upregulation, and *INV*, *sacA* (beta-fructofuranosidase), and *SUS* (sucrose synthase) were downregulated.

A total of 591 DEGs were identified in AW compared to OW, of which 380 were upregulated genes (Figure 3E). Top 15 GO terms in biological process were response to stimulus, chemical, organic substance, acid chemical, endogenous stimulus, organonitrogen compound, signal transduction, hormone, other organisms, biotic stimulus, external biotic stimulus, stress, cellular response to stimulus, signaling, and single-organism signaling ($q < 0.001$). KEGG results showed that DEGs were enriched in 78 metabolic pathways. Biosynthesis of secondary metabolites; amino sugar and nucleotide sugar metabolism; phenylpropanoid biosynthesis; flavonoid biosynthesis; phenylalanine, tyrosine and tryptophan biosynthesis; vitamin B6 metabolism; MAPK signaling pathway-plant, ascorbate and aldarate metabolism; glycine, serine and threonine metabolism; betalain biosynthesis; and tyrosine metabolism expressed significantly ($p < 0.01$, Figure S2E). Isoquinoline alkaloid biosynthesis; starch and sucrose metabolism; cutin, suberine and wax biosynthesis; linoleic acid metabolism; and biosynthesis of amino acids likewise showed a significance ($p < 0.05$, Figure S2E).

There were 172 DEGs in AW compared to OA, of which 132 were upregulated (Figure 3F). Protein complex subunit organization and protein oligomerization were significantly enriched in biological processes ($p < 0.05$). DEGs were enriched in 47 metabolic pathways. DNA replication, photosynthesis-antenna proteins, RNA degradation, sesquiterpenoid and triterpenoid biosynthesis, and carbon fixation in photosynthetic organisms showed significant enrichment ($p < 0.05$, Figure S2F).

3.3 Metabolomic results

We selected 24 samples (6 samples per group) for metabolomics analysis. Two ion modes, positive ion mode (POS) and negative ion mode (NEG), were combined, and all data were analyzed separately in each mode. A total of 25,871 metabolites were identified and 6,028 were named metabolites. Under POS mode, 3,723 named metabolites were identified, mainly including glycerolipids (15%), carboxylic acids and derivatives (9%), and organooxygen compounds (8%). A total of 2,305 named metabolites composed of glycerolipids (17%), glycerophospholipids (9%), and fatty acyls (8%) were identified under NEG mode. There were differences among different comparisons (Figure 4). The prediction rates of the OPLS-DA models were 0.413-0.794 (Table S2), which means models were reliable (Figure S3).

There were differences in the differentially expressed metabolites between different comparisons (Figure S4). In AW compared with CK, 114 differentially expressed metabolites were identified in POS mode, 69 were upregulated, and the top five were

kojibiose, D-maltose, 4-hydroxy-2-butenic acid gamma-lactone, 1,2,3-trihydroxybenzene, and 3-hydroxyadipic acid 3,6-lactone (Figure 5). In NEG mode, 35 differentially expressed metabolites were identified, of which 28 were upregulated (Figure S4). Among them, 1-deoxy-a-(N6-lysino)-D-fructose, 2-Ethyl-2-hydroxybutyric acid, malonic acid, L-glutamic acid, and itaconic acid were the top five, and only N-acetylglutamic acid was downregulated (Figure 5). KEGG results revealed 67 pathways were significantly enriched (Figure 6A), of which biosynthesis of plant secondary metabolites and biosynthesis of terpenoids and steroids were the most highly enriched ($p < 0.01$), followed by the butanoate metabolism; catecholamine transferase inhibitors; propanoate metabolism; biosynthesis of alkaloids derived from ornithine, lysine and nicotinic acid; C5-Branched dibasic acid metabolism; sulfur metabolism; and biosynthesis of alkaloids derived from shikimate pathway ($p < 0.05$).

A total of 109 differentially expressed metabolites were identified in OW compared with CK under POS mode, including 71 upregulated. Among them, L-glutamine (downregulated), kojibiose, 4-hydroxy-2-butenic acid gamma-lactone, 1,2,3-Trihydroxybenzene, and D-maltose were the top five (Figure 5). In NEG mode, there were 27 differentially expressed metabolites with 22 upregulated and five downregulated (Figure S4). And cis, cis-Muconic acid, trehalose, 2-ketobutyric acid, succinic acid, malonic acid, and D-ribose were differentially expressed metabolites, and all were upregulated (Figure 5). KEGG pathway enrichment analysis revealed 65 noteworthy signaling pathways

(Figure 6B), among which plant hormone signal transduction carotenoid biosynthesis was most highly enriched ($p < 0.01$), followed by aminoacyl-tRNA biosynthesis; alanine, aspartate and glutamate metabolism; catecholamine transferase inhibitors; biosynthesis of plant secondary metabolites; and biosynthesis of terpenoids and steroids ($p < 0.05$). Beta-cryptoxanthin, zeaxanthin, and abscisate were significantly upregulated in carotenoid biosynthesis.

In OW compared with OA, 131 differentially expressed metabolites were identified in POS mode, of which 82 were upregulated (Figure S4), and L-glutamine, L-isoleucine, 6-deoxyfagomine, and kojibiose (downregulated) were the top five (Figure 5). In NEG mode, 48 were identified, of which 29 were upregulated (Figure S4), and cis,cis-Muconic acid; 3,4-Dihydroxybenzaldehyde; 1-deoxy-1-(N6-lysino)-D-fructose (only downregulated); succinic acid; 2-ketobutyric acid; and caffeic acid were top five (Figure 5). KEGG analysis revealed 86 significant pathways (Figure 6C), of which aminoacyl-tRNA biosynthesis; valine, leucine, and isoleucine biosynthesis; biosynthesis of plant secondary metabolites; glucosinolate biosynthesis; ABC transporter ($p < 0.01$); propanoate metabolism; biosynthesis of amino acid; glycine, serine and threonine metabolism; and catecholamine transferase inhibitors ($p < 0.05$) were significantly enriched.

In OA compared with CK, 65 differentially expressed metabolites were identified in POS mode, of which 37 were upregulated (Figure S4). The top five were L-glutamine, 6-deoxyfagomine, beta-leucine, betaine, and piperidine and all of

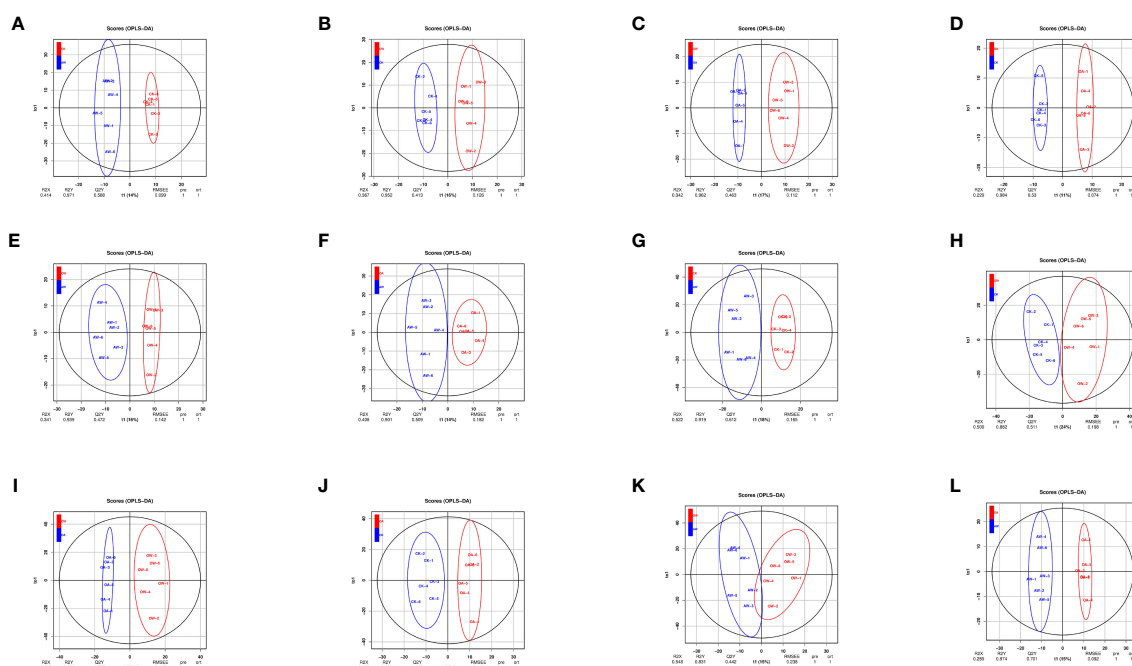


FIGURE 4

Score plot of orthogonal partial least-squares discriminant analysis (OPLS-DA). (A-F): POS mode; (G-L): NEG mode. (A, G): CK-vs-AW; (B, H): CK-vs-OW; (C, I): OA-vs-OW; (D, J): CK-vs-OA; (E, K): OW-vs-AW; (F, L): OA-vs-AW. The horizontal coordinate represents the predicted principal component (PC), which can be used to directly distinguish differences between different treatments; the vertical coordinate represents the orthogonal PC, which can reflect the variation within one group. CK means the group control with 14°C, and current atmospheric pCO₂ (410 μatm); OA is with seawater temperature of 14°C, and the future atmospheric pCO₂ (1000 μatm); OW is with a current CO₂ concentration (410 μatm) and June temperature of 19°C; AW is with an extreme CO₂ concentration of 1000 μatm and early summer temperature of 19°C.

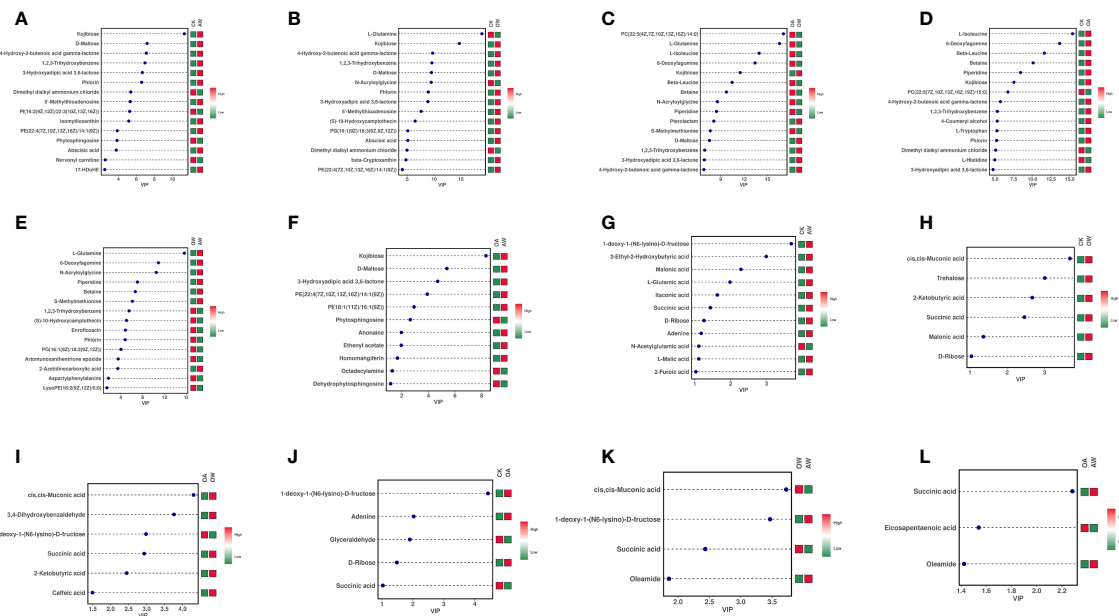


FIGURE 5

Variable importance in projection (VIP) plots of differentially expressed metabolites in different treatments. (A–F): POS mode; (G–L): NEG mode.

(A, G): CK-vs-AW; (B, H): CK-vs-OW; (C, I): OA-vs-OW; (D, J): CK-vs-OA; (E, K): OW-vs-AW; (F, L): OA-vs-AW. The horizontal coordinate is the VIP value, and the vertical coordinate is the top 15 differentially expressed metabolites; the abundance of each metabolite in each group was averaged, and then z-score analysis was performed for each group. The colors on the right side indicated the abundance of the metabolite in different groups, red indicated upregulation, and green indicated downregulation. The higher the value of VIP, the greater their contribution to the differentiation of samples. The metabolites with default VIP greater than 1 had a significant difference. CK means the group control with 14°C, and current atmospheric pCO₂ (410 μatm); OA is with seawater temperature of 14°C, and the future atmospheric pCO₂ (1000 μatm); OW is with a current CO₂ concentration (410 μatm) and June temperature of 19°C; AW is with an extreme CO₂ concentration of 1000 μatm and early summer temperature of 19°C.

them showed upregulation (Figure 5). A total of 21 were identified in NEG mode, of which 12 were upregulated (Figure S4), and 1-deoxy-1-(N6-lysino)-D-fructose, adenine, glyceraldehyde (downregulated), D-ribose, succinic acid (downregulated) were

the highly significant differentially expressed metabolites (Figure 5). KEGG pathway analysis of differentially expressed metabolites identified 61 significant pathways (Figure 6D), such as glucosinolate biosynthesis; plant hormone signal transduction;

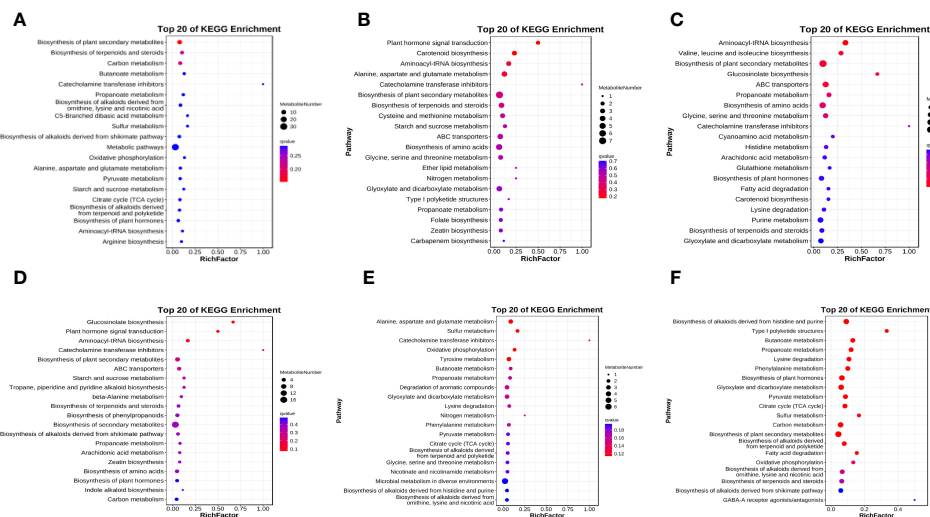


FIGURE 6

Bubble diagram of metabolic pathways. Top 20 pathways with the smallest q values were shown. The vertical coordinate is the pathway name, the horizontal coordinate is the enrichment factor, and the circle size indicates how many metabolites in the pathway. The color shows the significance, and the redder the color, the smaller the q value. CK means the group control with 14°C, and current atmospheric pCO₂ (410 μatm); OA is with seawater temperature of 14°C, and the future atmospheric pCO₂ (1000 μatm); OW is with a current CO₂ concentration (410 μatm) and June temperature of 19°C; AW is with an extreme CO₂ concentration of 1000 μatm and early summer temperature of 19°C.

aminoacyl-tRNA biosynthesis; catecholamine transferase inhibitors ($p < 0.01$); biosynthesis of plant secondary metabolites; ABC transporters; starch and sucrose metabolism; tropane, piperidine and pyridine alkaloid biosynthesis ($p < 0.05$). Among them, L-isoleucine and azacyclohexane were significantly upregulated in tropane, piperidine, and pyridine alkaloid biosynthesis. In starch and sucrose metabolism, maltose showed upregulation, and trehalose 6-phosphate showed downregulation.

In AW compared with OW, 63 differentially expressed metabolites were identified in POS mode, of which 29 were upregulated metabolites; and 26 were identified in NEG mode, of which 14 were upregulated metabolites (Figure S4). In POS mode, L-glutamine, 6-deoxyfagomine, N-acryloylglycine, piperidine, and betaine were the top five, and all of them showed upregulation (Figure 5). In NEG mode, cis,cis-muconic acid (downregulated), 1-deoxy-1-(N6-lysino)-D-fructose, succinic acid (downregulated), and oleamide were highly significant (Figure 5). There were 41 significant KEGG pathways (Figure 6E), such as alanine, aspartate and glutamate metabolism; sulfur metabolism ($p < 0.01$); catecholamine transferase inhibitors, oxidative phosphorylation; tyrosine metabolism; butanoate metabolism; propanoate metabolism; degradation of aromatic compounds; glyoxylate and dicarboxylate metabolism; lysine degradation; nitrogen metabolism; and phenylalanine metabolism ($p < 0.05$). L-glutamine was significantly upregulated in nitrogen metabolism.

In AW compared with OA, 64 differentially expressed metabolites were identified in POS mode, of which 36 were upregulated, and 26 were identified in NEG mode, of which 14 were upregulated, (Figure S4). In POS mode, kojibiose, D-maltose, and 3-hydroxyadipic acid 3,6-lactone were the top three, and all of them showed upregulation (Figure 5). In NEG mode, succinic acid, eicosapentaenoic acid (downregulated), and oleamide were highly significant differentially expressed metabolites (Figure 5). KEGG results identified 59 noteworthy signaling pathways (Figure 6F), among which biosynthesis of alkaloids derived from histidine and purine, type I polyketide structures, butanoate metabolism, propanoate metabolism, lysine degradation, phenylalanine metabolism, biosynthesis of plant hormones, glyoxylate and dicarboxylate metabolism, pyruvate metabolism, TCA cycle, sulfur metabolism, carbon metabolism, and biosynthesis of plant secondary metabolites were the most enriched ($p < 0.01$), followed by biosynthesis of alkaloids derived from terpenoid and polyketide; fatty acid degradation; oxidative phosphorylation; biosynthesis of alkaloids derived from ornithine, lysine and nicotinic acid; biosynthesis of terpenoids and steroids; biosynthesis of alkaloids derived from shikimate pathway; GABA-A receptor agonists/antagonists; valine, leucine and isoleucine degradation; and degradation of aromatic compounds ($p < 0.05$). Significant upregulation of acetyl-CoA and succinate occurred in TCA cycle.

4 Discussion

It is well known that seagrass beds have important conservation values (Barbier et al., 2011). However, the response of *Z. marina* to the single and synergistic effects of seasonal warming and OA,

especially at the seedling stage, is limited. In this study, we explored the mechanisms and response differences in different environmental combinations (OW, OA, AW) to *Z. marina* seedling growth (leaf length, leaf width, wet weight), RNAs and metabolites from multiple perspectives using phenomics, transcriptomics and metabolomics.

4.1 Seasonal temperature increase could limit the growth of *Zostera marina* seedlings

The results of metabolomics and transcriptomics showed that under suitable temperature, *Z. marina* seedlings activate the upregulation of beta-cryptoxanthi, zeaxanthin, and abscisate upregulated by NCED and then ultimately promotes the biosynthesis of carotenoid biosynthesis to adapt to rapid seasonal temperature increases (Figure 7). Notably, this pathway was specific to such a condition (Figure 7). Previous studies have shown that several carotenoids at different depths exhibit stimulatory properties in *Posidonia sinuos* (Collier et al., 2008). The process of epoxidation of zeaxanthin requires oxygen and energy consumption (Janhs et al., 2009), which may lead to reduced photosynthetic efficiency and competition for energy with other metabolic processes (Silva et al., 2013). This is detrimental to *Z. marina* seedlings under seasonally rapid SST warming. Also, the results of the multiple-omics may correspond to the results of growth. After 14 days, there was no significant increase in wet weight in OW group. Although the leaf length and width increased, the growth in OW was not as good as that of CK (Figure 3). This also suggests that, for seedlings in group OW, although seasonal temperature increases over a short period are not fatal to individual plants, they can still limit the growth of *Z. marina* seedlings for a certain period. Although many studies have shown that summer is the peak growth season for *Z. marina* (Nguyen et al., 2021; Azcarate-Garcia et al., 2022), this is different from results of this study. This may be due to the fact that our study was the result of intense stress for a short period. Since temperature increase occurred over a longer period, *Z. marina* seedlings gradually adapted to the change in temperature. In addition, as *Z. marina* grow from seedlings to adults, their ability to adapt to temperature increases. At this point, the temperature of 19°C is no longer a stressor that inhibits the growth of *Z. marina* adult (Nguyen et al., 2021; Azcarate-Garcia et al., 2022; Yan et al., 2023).

4.2 Ocean acidification may promote the growth of *Zostera marina* seedlings under suitable temperature

Under OA, by upregulating L-isoleucine and azacyclohexane, *Z. marina* seedlings promote the biosynthesis of tropane, piperidine, and pyridine alkaloids (TPPA). Meanwhile, starch and sucrose metabolism were promoted through the coordination of several genes, such as upregulation of *glgA* and downregulation of sucrose synthase, and through the regulation of some metabolites, such as

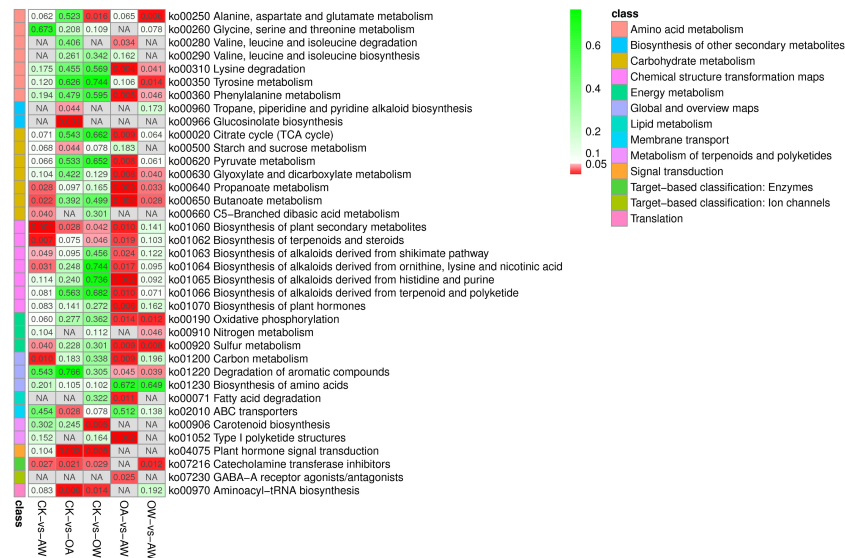


FIGURE 7

Kyoto encyclopedia of genes and genomes pathways based on p -value. Different classes are shown in different colors. The redder the color, the smaller the p -value. CK means the group control with 14°C, and current atmospheric $p\text{CO}_2$ (410 μatm); OA is with seawater temperature of 14°C, and the future atmospheric $p\text{CO}_2$ (1000 μatm); OW is with a current CO_2 concentration (410 μatm) and June temperature of 19°C; AW is with an extreme CO_2 concentration of 1000 μatm and early summer temperature of 19°C.

the upregulation of maltose and downregulation of trehalose 6-phosphate (Figure 7). Finally, a different response mechanism from the other stress environments was realized (the two pathways mentioned above were not significant in the other groups) (Figure 7). During the growth of *Z. marina* seedlings, leaf length, leaf width and wet weight increased significantly, with the highest increase in wet weight compared to the other comparisons. This suggests that OA did not inhibit the growth of *Z. marina* seedlings at suitable temperatures and even played a role in promoting it (Invers et al., 2002; Jiang et al., 2010; Russell et al., 2013; Martínez-Crego et al., 2014; Campbell and Fourqurean, 2018; Guerrero-Meseguer et al., 2020). In many higher plants, low molecular weight chemicals such as alkaloids are produced, and these secondary metabolites provide immunity to the body against pathogens (Yamada and Sato, 2013). The TPPA biosynthetic pathway, which can be used to modulate alkaloids with pharmacological properties such as anti-inflammatory and antimicrobial properties, has been frequently reported in previous studies (Rischer et al., 2006; Huang et al., 2019). We hypothesized that biosynthesis of TPPA, as well as starch and sucrose metabolism, play distinct roles in metabolic capacity from the other comparisons (Figure 7), thereby increasing the resistance of *Z. marina* seedlings and enhancing their adaptation to OA.

In summary, under suitable temperatures, both in the face of increased temperature and OA, biosynthesis of plant secondary metabolites, plant hormone signal transduction, catecholamine transferase inhibitors, aminoacyl-tRNA biosynthesis, all of which enhanced the biosynthesis of plant secondary metabolites in *Z. marina* seedlings (Figure 7). Among them, plant hormone signal transduction and aminoacyl-tRNA biosynthesis were unique when *Z. marina* seedlings responded to temperature increases or OA (Figure 7).

4.3 Sequential or concurrent occurrence of seasonal temperature increase and ocean acidification differentially affect *Zostera marina* seedlings

In OW-VS-AW, nitrogen metabolism showed unique significance compared with other metabolic pathways (Figure 7). That is, under high SST caused by seasonal change, *Z. marina* seedlings increase the ability to adapt to OA by upregulating L-glutamine to accelerate nitrogen metabolism in such a unique metabolic pathway. In OA-VS-AW, we also found significant enrichment of metabolic pathways different from the other treatments, including valine, leucine and isoleucine degradation; TCA; pyruvate metabolism; biosynthesis of alkaloids derived from histidine and purine; biosynthesis of alkaloids derived from terpenoid and polyketide; and biosynthesis of plant hormones (Figure 7). Notably, TCA cycle can link almost individual metabolic pathways (Akram, 2014; Lin et al., 2016; Zhang et al., 2017), which is an important central pathway can energize organisms and produce essential metabolites for energy production and biosynthetic reaction (Arnold et al., 2022). TCA cycle did not show a significant effect in CK-VS-OW, although they both increased the temperature (Figure 7). This suggests that the stress mechanisms of *Z. marina* seedlings are different even if they later encounter the same environmental stress. Under OA, *Z. marina* seedlings respond to seasonal temperature surges by a unique metabolic pathway that promotes TCA cycle through the upregulation of acetyl-CoA and succinate (Arnold et al., 2022). In addition to these treatment-specific KEGG pathways, there are co-existing metabolic pathways that counteract high temperatures and OA, such as lysine degradation, phenylalanine metabolism,

glyoxylate and dicarboxylate metabolism, oxidative phosphorylation, and degradation of aromatic compounds.

These results suggested *Z. marina* seedlings respond to environmental changes by accelerating common metabolic pathways when faced with SST increase by seasonal change and OA. However, it is worth noting that the sequencing of these two events had different effects on *Z. marina* seedlings. In addition, OW-VS-AW and CK-VS-OA, and OA-VS-AW and CK-VS-OW also shared the same metabolic pathways but with many differences, which means although *Z. marina* responds to the same oceanic events, their initial growth environment has some influence on their response mechanisms.

Metabolomics results showed that *Z. marina* seedlings responds to the dual stress conditions of seasonal SST increases and OA simultaneously by actively mobilizing nine KEGG pathways, such as the biosynthesis of plant secondary metabolites, biosynthesis of terpenoids and steroids, carbon metabolism, butanoate metabolism (Figure 7). Among them, propionate metabolism, butyrate metabolism, and sulfur metabolism showed enrichment under different stress conditions (CK-VS-AW, OA-VS-AW, and OW-VS-AW) (Figure 7). This suggests that, regardless of whether the increases in temperature and OA occurred sequentially or concurrently, *Z. marina* increased these three metabolisms in order to improve their adaptive capacity to environmental changes. In terms of growth of *Z. marina* seedlings, AW had the lowest growth rate compared with other treatments, and the wet weight did not increase basically. Compared with single stress, OA had the least effect on the growth, followed by OW; AW had the most inhibitory effect. This result is inconsistent with the results of Yan et al., who believed that the addition of OA would reduce the effect of temperature increase on the growth of *Z. marina* (Yan et al., 2023). We think the reason for this difference is that the materials selected for the two studies had different growth periods of *Z. marina*. We selected the period of seedling, during which the plants are more sensitive to changes in environmental factors (Gogna and Bhatla, 2020). Transcriptomics and metabolomics were combined to show that the sequence of environmental factors greatly affected the response mechanism and adaptive ability of seagrass plants.

5 Conclusion

In this study, we investigated the response of *Z. marina* seedlings to seasonal warming and OA using phenomics and multi-omics (transcriptomics and metabolomics). Different combination or occurrence order of environmental changes prompted different responses for *Z. marina* seedlings. Leaf length, leaf width, and wet weight increased significantly under different stress, with overall plant growth reaching an optimum under OA, but OW and AW were lower than the control. This indicated that OA did not inhibit the growth of plant seedlings and even promoted their growth. The short-term seasonal temperature increase did not cause fatal damage but still limited their growth to some extent. Multi-omics results showed that at appropriate temperatures, *Z. marina* seedlings collectively promote carotenoid biosynthesis

through the upregulation of beta-cryptoxanthin, zeaxanthin, and the upregulation of NCED, which in turn mobilizes the upregulation of abscisate, ultimately differentiating the treatment from other treatments for resistance to the stress mechanisms against seasonal warming. Under the same conditions, the plants promoted tropane, piperidine and pyridine alkaloid biosynthesis through the upregulation of L-isoleucine azacyclohexane. The different genes (upregulation of *glgA* and downregulation of sucrose synthase) and the differentially expressed metabolites (upregulation of maltose and downregulation of trehalose 6-phosphate) were used to promote starch and sucrose metabolism, and finally, the specific mechanism to respond to the stress factors was realized to enhance the adaptive capacity to OA. When *Z. marina* seedlings are already under some environmental stress, it responds differently to seasonal warming and OA. Under high temperatures, when *Z. marina* seedlings respond to OA, they accelerate nitrogen metabolism by upregulating L-glutamine. In OA, nine unique metabolic pathways, including TCA cycle and pyruvate metabolism, work together to respond to environmental changes when ambient temperatures rise again. In response to two stressors at the same time, plants mobilize biosynthesis of plant secondary metabolites, carbon metabolism, and other metabolic pathways to increase their resistance. Based on these results, we found that although plants face the same factors, their initial survival environment leads to different response mechanisms. In addition to the specific pathways described above for each treatment, there are common pathways that stimulate plant metabolism and sulfur metabolism in response to seasonal temperature rise or/and OA, such as propionate metabolism, butyrate metabolism, and sulfur metabolism.

Data availability statement

The transcriptome data can be found in Bioproject accession number: PRJNA1031782. Further inquiries can be directed to the corresponding authors.

Author contributions

ZW: Formal Analysis, Methodology, Writing – original draft, Writing – review & editing, Data curation, Visualization. YP: Formal Analysis, Methodology, Writing – original draft, Data curation. WY: Conceptualization, Supervision, Writing – review & editing, Writing – original draft. LL: Conceptualization, Methodology, Writing – original draft. BZ: Conceptualization, Funding acquisition, Supervision, Writing – review & editing.

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Conflict of interest

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

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Supplementary material

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fmars.2023.1304132/full#supplementary-material>

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The root of influence: root-associated bacterial communities alter resource allocation in seagrass seedlings

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Introduction: Seagrass roots harbour diverse assemblages of microorganisms that likely benefit the growth and survival of meadows. Yet, restoration efforts rarely consider their effect on developing seagrass seedlings. Sediment origin should determine the types of rhizosphere and root-colonising (rhizoplane) microorganisms and thus the performance of seedlings during restoration, particularly for slow growing climax species like *Posidonia*. Recent *Posidonia* restoration attempts in South Australia used commercially sourced 'play pit sand' for seedling propagation and planting, but have been impacted by high mortality. More natural substrates like seagrass meadow sediment have not been previously investigated for suitability over conventional substrates with regard to seedling growth and survival.

Methods: To assess the relevance of seagrass associated microorganisms in the growth of *Posidonia angustifolia* seedlings, we investigated the bacterial communities from tank-raised seedlings propagated in autoclave treated and untreated 'play pit sand' and meadow mix (comprising a 1:3 ratio of natural meadow sediment and beach sand) over a 12-week period. Autoclave treatment was adopted in order to diminish the bacterial load prior to planting and thus inform their contribution (if any) on early seedling growth. Samples for bacterial community analysis and seedling growth measurements (numbers and total length of roots/leaves, root diameter, seedling weight, starch reserves) were taken at 4 and 12 weeks. Bacterial assemblages were surveyed from DNA extracts from bulk and rhizosphere sediments and root tissues, as well as from swabs of *P. angustifolia* fruit, established meadow sediment and water samples prior to seedling propagation, by constructing Illumina 16S rRNA gene libraries.

Results: While most growth measurements did not vary significantly between sediment type or treatment, proportional growth of roots versus leaves (as expressed as a pseudo root:shoot ratio) was significantly related to treatment,

sediment type and seed length. Seedlings from meadow mix invested more in leaves, regardless of treatment, when compared to play sand. Autoclave treatment increased investment in roots for play sand but increased the investment in leaves for meadow mix. Bacterial communities differed significantly between sediments and between sample types (bulk, rhizosphere and roots), with the roots from meadow mix seedlings containing an increased abundance of various potentially beneficial bacterial taxa.

Discussion: While such changes appear to affect the early development of seedlings, bacterial community dynamics are also likely coupled to changes in nutrient availability. Further research is thus required to disentangle host seedling growth-nutrient-bacterial community dynamics with the view to identifying microbes that may support the growth and vigour of seedlings under different nutrient conditions as part of future restoration efforts.

KEYWORDS

seagrass restoration, *Posidonia*, bacterial communities, meadow sediment, rhizosphere, rhizoplane, seed starch, seedling growth

1 Introduction

Seagrass meadows are one of the most productive systems on earth (Nordlund et al., 2016), and are fundamental to the broader dynamics of marine ecosystems. They provide important nursery grounds for a multitude of fish species (Blandon and zu Ermgassen, 2014), are highly efficient carbon sequesters (Fourqurean et al., 2012; Duarte and Krause-Jensen, 2017), and protect coastlines from storm surges and erosion (Paul, 2018). Seagrass meadows also harbour diverse, complex assemblages of sedimentary microorganisms (Jankowska et al., 2015; Sun et al., 2015; Cúcio et al., 2016) that, like in terrestrial soils, underpin the health and critical services of these ecosystems by supporting plant growth and vigour. Central to this supporting function are microbes that occur in close proximity to seagrass roots (rhizosphere), or are more intimately associated with root surfaces (rhizoplane) or underlying tissues (endosphere), where they form important components of the 'seagrass holobiont' (Ugarelli et al., 2017). Bacteria are thought to be dominant constituents of the seagrass microbiome where, among other roles, they act to fix nitrogen, solubilise essential nutrients like phosphorus (Jose et al., 2014), and detoxify the surrounding sediment by oxidising sulfide (Garcias-Bonet et al., 2012; Sun et al., 2015; Martin et al., 2019). In certain seagrasses, like species of *Posidonia* that form extensive meadows and have a long evolutionary history with coastal waters (Aires et al., 2011), root-associated bacteria have been shown to be highly diverse and more efficient at nitrogen fixation (by as much as 10x) than bacterial communities associated with the leaves (phyllosphere) (Garcias-Bonet et al., 2012; Lehnert et al., 2016). The seagrass holobiont may include endosymbionts thought to improve the capacity of seagrasses to thrive in nutrient poor environments (Mohr et al., 2021). Furthermore, regressing *P. oceanica* seagrass meadows have

been linked to declining bacterial communities in sediments (García-Martínez et al., 2009), suggesting an important role for microorganisms in seagrass growth and survival.

Seagrass-bacterial community dynamics are thought to be shaped by a range of different plant-host and environmental factors. Alongside changes in the surrounding environmental conditions (e.g. light availability, meadow health, geographical location) (García-Martínez et al., 2009; Bourque et al., 2015; Martin et al., 2017), this may include the emission of oxygen and exudates from the root tips which create microhabitats for a plethora of both aerobic and anaerobic bacteria (Jensen et al., 2007; Kilminster and Garland, 2009; Martin et al., 2019). In terrestrial plants, root exudates produced throughout the plant life cycle have been suggested to influence the occurrence of different subsets of microbes with specific functions (Chaparro et al., 2014), leading to the formation of juvenile and adult plant-specific assemblages (Edwards et al., 2018). Modulation of these communities through the inoculation of roots with bacteria that possess health- and growth-promoting traits (Hayat et al., 2010), has been reported to increase seedling growth, survival and nutrient availability with potential benefits for agriculture and revegetation projects (Reed et al., 2005; Thrall et al., 2005; Wang et al., 2017). Though an intriguing prospect yet to be realised for seagrasses, stimulation of sediment bacterial activity through organic matter enrichment and remineralisation has been reported to increase belowground biomass of *P. australis* seedlings, enhancing their root branching and stability in sediments (Fraser et al., 2015). Furthermore, the inoculation of experimental mesocosms with meadow sediment (presumably containing beneficial microbes) has been anecdotally noted to increase stem density compared to controls in Eelgrass (*Zostera marina*) seedlings (Ort et al., 2014). Observations such as this imply that improving our understanding

of the seagrass microbiome and its role in seedling establishment may provide useful insights into their role in supporting restoration attempts.

Indeed, with the mounting loss of seagrass meadows occurring throughout the world due to anthropogenic disturbance (Waycott et al., 2009), there has been a significant investment in restoration efforts in recent years. For this, seeds, seedlings or cuttings are either placed directly into the sediment (Van Katwijk et al., 2016; Valdez et al., 2020) or, in turbulent areas, anchored to the sediment using various means, e.g. sediment filled hessian sacks (Irving et al., 2010; Zhang et al., 2015; Unsworth et al., 2019). Thus far, restoration attempts have had varied success, with notable challenges arising from, among others, propagule supply and survival (Tan et al., 2020; Vanderklift et al., 2020; Boudouresque et al., 2021). For environmentally and economically important seagrass meadow systems in Australia, those occurring off the Adelaide metropolitan coast in South Australia, (which have seen losses of ~6,200 ha since 1949) have been the focus of recent efforts (Tanner et al., 2014). Compared to most local seagrasses, the genus *Posidonia* has suffered a greater reduction in its Adelaide distribution, with only *Amphibolis* displaying greater susceptibility (Bryars et al., 2006a). This is concerning because *Posidonia* meadows are climax communities that can take many decades to recover naturally and require an absence of ocean swell for seedlings to establish (Bryars and Neverauskas, 2004). Currently, for restoration attempts employed in this region, fruit are collected and stored in onshore tanks until the seedling (germinated seed) is released. The seedlings are then transplanted offshore into sand-filled hessian sacks by divers (Tanner, 2015). While initial findings have been promising, there is high plant mortality (~86%) within 3 to 4 years (Tanner and Theil, 2016). Improving our understanding of the early development of *Posidonia* seedlings is critical for enhanced restoration outcomes. Recent efforts have primarily focused on investigating sediment composition and nutrient addition effects on growth and survival (Statton et al., 2013; Statton et al., 2014; Fraser et al., 2015; Tanner and Theil, 2016; Tanner and Theil, 2019; Tanner, 2023). Further advances, however, may be gained by improving our understanding of the microbiome and the identification of microbes that may support early vigour. However, such knowledge is currently limited for species like *Posidonia* in Australia (Ugarelli et al., 2017; York et al., 2017).

Here, we examine the rhizosphere and root-associated bacterial community assemblages of *Posidonia angustifolia* seedlings, a dominant and ecologically significant meadow species endemic to Australian temperate waters that typically inhabits sandy sediments, and which has suffered widespread loss (Cambridge and Kuo, 1979; Carruthers et al., 2007; Tanner et al., 2014). Specifically, we sought to compare the bacterial community diversity of seedlings grown in a commercial 'play pit sand' currently used in restoration efforts, with a mixture that included locally sourced seagrass meadow sediment. Bacterial communities and potential effects on seedling growth (as assessed using an Illumina 16S rRNA gene deep-sequencing approach, and changes in root and leaf length metrics respectively) were studied through comparisons between autoclave treated and untreated sediments. This study is one of the first to investigate the potential influence of the microbiome on seagrass

seedlings, where such knowledge could be used to identify potentially beneficial bacterial species that may support future restoration programs by improving seedling growth.

2 Materials and methods

2.1 Collection of seagrass meadow sediment, fruits and samples

Meadow sediment required for seedling propagation (~20 kg) was freshly collected prior to the experiment on the 23rd of December 2018 from the edge of the intertidal and subtidal zone of Lady Bay, Normanville, South Australia (35°28'10.07"S, 138°17'27.41"E). The meadow here is protected by a shore platform of limestone conglomerate and consists largely of *P. angustifolia* with scattered *P. sinuosa* and a patch of *Amphibolis* sp. (Figure 1A). The sediment was collected above the *Posidonia* rhizomes, sieved to remove molluscs (> 4 mm), and then submerged in a flow-through ~50 cm deep seawater tank until required. Any remaining molluscs that may predate on the seagrass seedlings were opportunistically removed during a two-week holding period prior to the commencement of the experiment. To characterise the bacterial assemblages associated with the native meadow sediment, bulk sediment samples (n = 3) were also collected for DNA extraction from just above the *P. angustifolia* rhizomes (to a depth of ~5 cm) at Lady Bay using sterile 6 ml syringes with the ends cut off. In addition, to identify the variation (if any) in the root-associated bacterial community composition between mature plants from the meadow and tank-raised seedlings grown in different substrates, root samples were also obtained from six adult *P. angustifolia* by carefully removing the plants from the sediment and trimming the tips of the primary and secondary roots (~1 cm) using scissors cleaned with 70% ethanol solution. Ten randomly selected root cuttings were rinsed thoroughly in 0.22 µm filter sterilised seawater to remove any sediment particles and placed into sterile centrifuge tubes. A seawater sample (1 L) was also collected from between the leaves of the *P. angustifolia* to control for the surrounding environmental bacterial communities. All samples were placed on ice for transport (<1 hr), and then the sediment and root samples were stored at -20°C and the water at 4°C until DNA extraction.

In the absence of mature *P. angustifolia* fruits at Lady Bay, seedlings required for the experiment were obtained from beach-cast fruits collected from West Beach (34°57'2.01"S, 138°30'10.62"E) near Adelaide, South Australia on the 28th of December 2018, where large *P. angustifolia* meadows are dominant offshore (Cambridge and Kuo, 1979; Bryars et al., 2006b). To identify the role (if any) of fruit-associated bacteria in seedling root colonisation, 10 randomly collected fruits were rinsed thoroughly with 0.22 µm filter sterilised seawater and the entire surface swabbed using FLOQSwabs® (Copan Diagnostics, Murrieta, CA, United States). Swabs were placed into sterile centrifuge tubes on ice, and stored at -20°C until DNA extraction. Fruits collected for seedling propagation were placed in a semi-submersible mesh tray in the same seawater tank used to hold the meadow sediment to dehisce (open) before seedlings could be separated and planted.

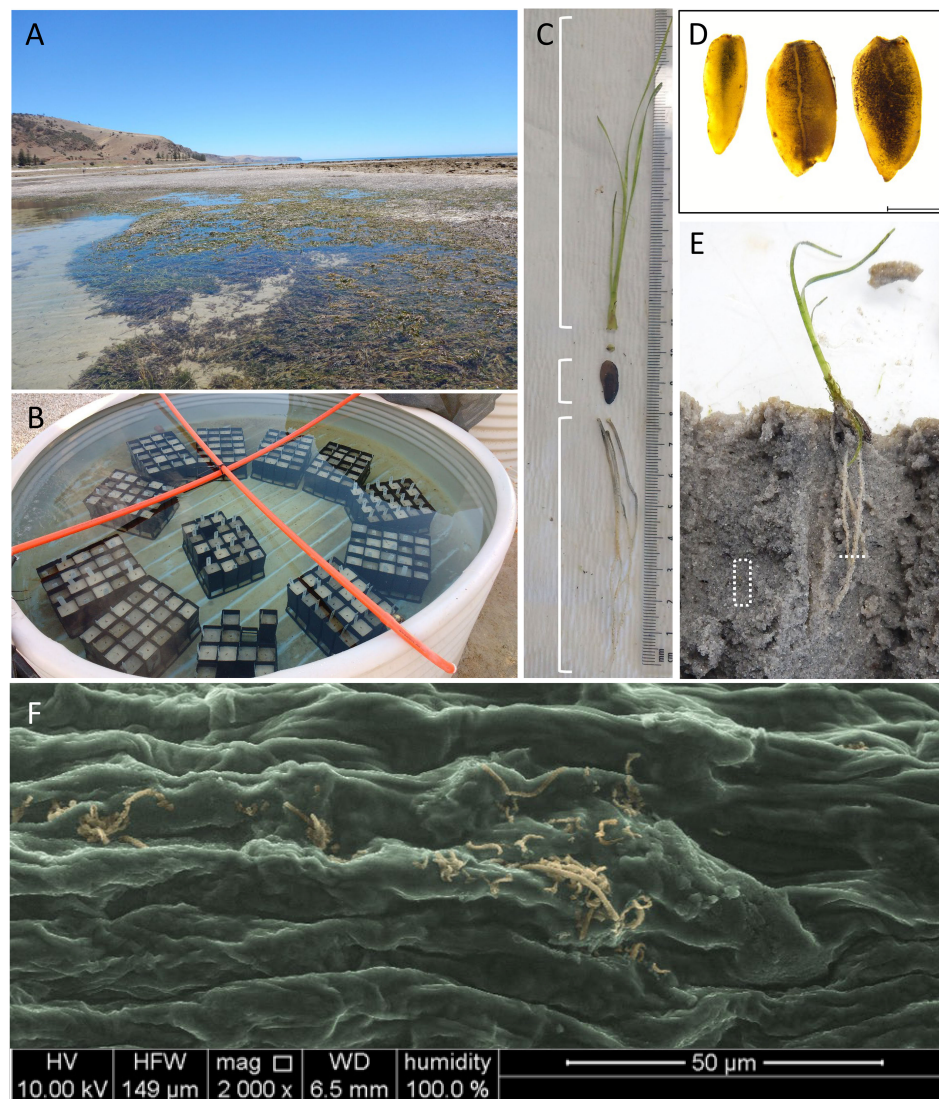


FIGURE 1

Study site and sampling method. A particularly low tide at (A) Lady Bay, South Australia, has exposed *Posidonia* seagrass in the background near the rocky platform, in the foreground a transition from *Amphibolis* to the *Posidonia*, where some samples were taken, is shown. The circulating sea water (B) tank with potted seedlings. (C) Seedling for growth measurements divided into leaves, seed and roots. (D) Microscope image of seed starch stained with iodine. Scale bar = 5 mm. (E) Example seedling sampled for bacterial community analysis, line indicates portion of a root used for rhizosphere and root sampling, rectangle shows approximate location for bulk sediment sample. (F) A colourised Environmental Scanning Electron Microscope (ESEM) image of microorganisms found adhering to the root (~0.2mm from the tip) of a seedling grown in untreated meadow mix.

2.2 Experimental design and propagation of seedlings

The effects of two propagating substrates and their associated bacterial assemblages on *P. angustifolia* seedling growth were investigated over a 12-week period during summer (January–April 2019). One substrate was a commercial 'play pit sand' (PS) ('Play Sand' 20 kg bags, Richgro Garden Products, Jandakot, WA, Australia) of a coastal, yet terrestrial origin (Richgro pers. comms.) which was similar to sand currently used in local restoration programs in the region. The other substrate, a 'meadow mix' (MM), consisted of a 1:3 ratio of seagrass meadow sediment and beach sand obtained from a local beach (West Beach, SA, Australia). Beach sand was incorporated into this mix in order

to avoid minimise the logistical difficulties and impacts on the collection of large quantities (~80 kg) of sediment from the meadow. To elucidate the contribution of sediment bacterial communities in early seedling growth, half of each of the propagating substrates were autoclaved at 121°C for 52 min to diminish the bacterial load prior to planting. Propagating substrates (representing four treatments: MM autoclave treated, MM untreated, PS autoclave treated and PS untreated) were evenly distributed into fine mesh-lined 575 ml super tube plant pots (DanBar Plastics, Horsham, VIC, Australia) as used elsewhere (Statton et al., 2013; Statton et al., 2014). Undamaged dehiscent seedlings (n = 230), regardless of size, were randomly allocated and planted into the individual experimental pots, which were then randomly placed in trays within a flow-through ~50 cm deep

seawater tank (Figure 1B), with unfiltered water sourced directly from the adjacent Gulf St Vincent (the same water body from which the seagrass fruits were obtained). A flow rate of $\sim 10 \text{ L min}^{-1}$, giving a turnover time of $\sim 3 \text{ h}$, was maintained throughout the experiment. A single tank was used to avoid tank effects which can occur when samples are separated into multiple tanks (Statton et al., 2014). The tank was covered with 75% shade cloth. This shading mimics the light levels at $\sim 8 \text{ m}$ depth, which is within the depths *P. angustifolia* is known to occur naturally and reduces water temperature in the shallow tanks, helping to slow the rate of algal growth. The trays were moved a quarter clockwise around the tank weekly to reduce any further bias that may arise from variable light and water circulation. Seedlings were regularly cleaned carefully by hand to reduce epiphyte growth. At the time of planting, three untreated meadow mix samples were collected (pre-potting) for bacterial comparison with the native meadow sediment. A pre-experiment seawater sample (1 L) from the middle of the tank was also collected to control for the surrounding environmental bacterial communities. Sediment samples were stored at -20°C and the water at 4°C until DNA extraction.

2.3 Measurements of seedling growth and seed starch reserves

Seedling growth measurements (total leaf and root length/count, lateral root count, root diameter, and seedling weight) were taken destructively in weeks 4 and 12. A total of 37 seedlings were evaluated for each substrate/treatment (17 in week 4, and 20 in week 12), except for the autoclaved PS, where $n = 32$ due to plant loss (17 in week 4, and 15 in week 12). Seed length was also measured as a covariate. For measurements, seedlings were removed in small batches (to reduce dehydration shrinkage), cleaned of loosely adherent sediment by rinsing in water, and gently dried with absorbent tissue. Seedlings were then weighed and separated into component parts (i.e. seed, leaves and roots), which were measured lengthwise (Figure 1C). The primary root diameter was determined $\sim 1 \text{ cm}$ from the base, where the root meets the stem, using callipers. To explore the treatment effects on above and belowground resource allocation, the ratio of total root length to total leaf length (herein termed the pseudo root:shoot ratio, PRSR) was estimated. Furthermore, at the end of the experiment, microscopic observations of surplus seedling roots (as directly removed from the pots without rinsing in water) were undertaken using a Nikon SMZ745T dissecting microscope to examine the impacts of treatment on gross morphology, with representative images captured using a Nikon DS-Fi2 digital camera (Supplementary Figure S1: Datasheet 2).

To investigate the usage of seed starch reserves by the seedlings, the seeds were halved longitudinally at the end of week 12 and one half was stained (1:5 ratio Lugol's iodine to water) to highlight the remaining endosperm, which was photographed using a Nikon SMZ745T dissecting microscope and Nikon DS-Fi2 digital camera (Figure 1D). ImageJ software (version 1.52i) (Schindelin et al., 2012) was used to measure total area (mm^2) of the seed and remaining starch area (mm^2), as indicated by a dark brown-black colouration.

Seedling growth was investigated using multiple regressions for: 1) total biomass (seedling weight); 2) resource allocation into above- and below-ground compartments (PRSR); and 3) degree of establishment (number of lateral roots). Each regression was implemented with the "lm" command in R, version 4.0.2 (R Core Team, 2020) and included substrate type (PS or MM), treatment (autoclaved or untreated), seed length (mm, log10 transformed), growth stage (week 4 or 12), and their interactions as explanatory variables; i.e., Response Variable $\sim$ Sediment Type * Treatment * log (Seed Length) * Growth Stage. Growth stage was excluded as a variable for lateral roots, as lateral roots had not yet developed in week 4. Models including all variables and interactions were simplified by removing components that were not significant ($p > 0.05$). The assumptions of normally distributed and homogeneous residuals were verified by visual inspection of Q-Q plots and residuals plotted against fitted values for the final models (including only significant variables) (Supplementary Figures S2–S4: Datasheet 2).

To test whether parameters measuring plant growth differed between weeks 4 and 12, we used mixed linear models with week as the fixed effect and treatment and sediment as random effects, implemented using the lme4 (Bates et al., 2015) and lmerTest (Kuznetsova et al., 2017) packages. If the response variable consisted of count data, we implemented generalised mixed models with a Poisson error distribution using the 'glmer' command.

2.4 Sediment analysis

Autoclave treatment of sediment has been previously reported to influence nutrient levels and grain size (Lotrario et al., 1995; Otte et al., 2018). Therefore, at the end of the seedling growth experiment, five replicates from each substrate treatment group were analysed for calcium carbonate, organic carbon, nitrogen and phosphorus. For this, sediment was collected from the centre of the pot (excluding the top and bottom 2 cm) after removal of seedling, to best represent nutrients around the roots. The nitrogen, total carbon and organic carbon were assessed using Dumas high temperature combustion, rapid titration was used for inorganic carbon and equivalent calcium carbonate, and reverse aqua regia microwave assisted digestion for phosphorus (Rayment and Lyons, 2011). Tests were conducted by the Australian Precision Ag Laboratory (APAL), Hindmarsh, South Australia. Individual nutrient types (calcium carbonate, organic carbon, nitrogen and phosphorus) were compared between sediment and treatment by two-way analysis of variance (ANOVA) tests.

Particle size was determined using a Malvern Mastersizer 3000 (Malvern Panalytical Ltd., Malvern, UK) with water as the dispersant at the University of South Australia, Mawson Lakes, South Australia. Three samples from each sediment treatment were used to confirm particle size. The output was classified using the Wentworth (1922) scale in order to describe sediment characteristics. Grain particle size was compared with a permutational multivariate analysis of variance (PERMANOVA) with 9999 permutations under a reduced model, allowing for type

III (partial) sums of squares and a fixed effects sum to zero for mixed terms.

2.5 Collection of seedling samples for bacterial community analysis

To assess changes in the microbiome (bacterial communities) of the seedlings, five seedlings from each substrate treatment group were randomly selected at each growth stage (in week 4 and 12; $n = 10$ in total per treatment group). Roots were sampled by carefully removing the plants from the sediment and trimming the root tip (~ 2 cm) using scissors cleaned with 70% ethanol (Figure 1E). For some seedlings where the roots extended through the bottom of the pots at week 12, the tips were removed and a portion of the roots still in contact with the sediment was collected. To distinguish the rhizosphere from the more intimately associated rhizoplane (or endosphere) constituents, the root tips ($n=10$) were first placed in individual Lysing Matrix E tubes (MP Biomedicals) containing 978 μ l sodium phosphate buffer and manually agitated with sterilised forceps to collect the loosely adhering sediment (rhizosphere) for downstream DNA extraction. The washed root tips (comprising the rhizoplane/endosphere constituents) were then cut into smaller pieces using a sterile scalpel, and the fragments from each placed into individual Lysing Matrix E tubes (MP Biomedicals) containing 978 μ l sodium phosphate buffer for DNA extraction. The occurrence of adherent (rhizoplane-associated) communities was verified by the direct visualisation of fresh roots using the FEI Quanta 450 FEG Environmental Scanning Electron Microscope (ESEM) through Adelaide Microscopy (University of Adelaide, South Australia) (Figure 1F; [Supplementary Materials: Datasheet 2 and Methods](#)).

To distinguish the root-associated (rhizosphere and rhizoplane/endosphere) communities from the surrounding sediment, bulk sediment was also collected from three seedlings for each treatment, which was taken ~ 1.5 cm away from, but horizontal to, the root tips (Figure 1E). Aliquots of ~ 250 mg of bulk sediment were placed into individual Lysing Matrix E tubes (MP Biomedicals) for downstream DNA extraction. In addition, a seawater sample (1 L) was collected from the middle of the tank in weeks 4 and 12 to assess the contribution of the environmental bacterial assemblages. Rhizosphere and seawater samples were stored at 4°C and the bulk sediment and root samples at -20°C prior to DNA extraction.

2.6 DNA extraction, PCR amplification, and Illumina sequencing

To analyse the global bacterial community structure, DNA was extracted from propagating substrates and root samples using the FastDNA™ Spin Kit for Soil (MP Biomedicals) according to the manufacturer's instructions. To ensure efficient lysis, a further bead-beating step was performed for all root samples using the same default Fast Prep-24™ 5G Instrument settings (MP Biomedicals). In addition, to evaluate the surrounding environmental bacterial assemblages, DNA was extracted from the seawater samples

following filtration onto 0.22 μm Nalgene™ Rapid-Flow™ filters (ThermoFisher Scientific) using the same kit according to the manufacturer's instructions. Sample DNA was re-eluted in 100 μ l of DES (MP Biomedicals), quantified using the NanoDrop™ 2000 spectrophotometer (ThermoFisher Scientific) and stored at -20°C prior to down-stream library preparation.

The V1-V2 hypervariable region of the 16S rRNA gene was amplified from DNA extracts using a multi-step PCR approach, with pre-enrichment using universal eubacterial primers 27F and 338R as described previously ([Camarinha-Silva et al., \(2014\)](#)). More specifically, for Illumina library generation, ~ 25 ng of each DNA sample was first subjected to 20 cycles of PCR, whereby 1 μ l of this reaction was used as the template in a second 15 cycle PCR containing individual sample 6 nt barcodes and Illumina specific adaptors. One microlitre of this reaction was subsequently used in a final 10 cycle PCR for incorporating the Illumina multiplexing sequencing and index primers. The resultant PCRs were visualised via agarose gel electrophoresis and products of the expected size (~ 438 bp) were purified using Agencourt AMPure XP beads (Beckman Coulter). Samples were quantified using the Quanti-iT™ Picogreen® dsDNA kit (Life Technologies) following the manufacturer's instructions. All samples ($n = 133$) were then pooled in equimolar ratios and sequenced by the Australian Genome Research Facility (AGRF, North Melbourne, VIC, Australia) on the Illumina MiSeq platform using 250 nt paired-end sequencing chemistry. Amplicons generated from a single bacterial species (*Lactobacillus reuteri*) were also sequenced alongside the samples as controls.

2.7 Bioinformatics and statistical analysis of sequence data

Approximately 16 million raw sequence reads were obtained from a total of 133 samples comprising: 23 seagrass meadow 'environment' samples (1x seawater, 3x sediment, 9x fruit swabs, and 10x seagrass roots); and 110 experimental samples (3x seawater, 27x bulk sediment, 40x rhizosphere and 40x roots) ([Supplementary Table S1](#)). Reads were assembled using PEAR (version 0.9.5; [Zhang et al., 2014](#)), and the primers identified and removed. Trimmed sequences were processed using Quantitative Insights into Microbial Ecology (QIIME version 1.8; [Caporaso et al., 2010](#)), USEARCH (version 8.0.1623; [Edgar, 2010](#)), and UPARSE software ([Edgar, 2013](#)). Using USEARCH tools, sequences were quality filtered to remove low-quality reads, full-length duplicate sequences and singletons. Sequences were clustered into operational taxonomic units (OTUs) at a minimum identity of 97%, with putative chimeras removed using the RDP-gold database as a reference ([Cole et al., 2014](#)).

A total of 6,475,702 high quality, paired-end reads were clustered into 37,674 OTUs (mean = $48,689 \pm 15,968$ reads/sample; min = 20,261; max = 105,995). These OTUs were further filtered as conducted previously ([Legrand et al., 2018](#)) where only those that contributed to $> 0.01\%$ dataset were retained. The resultant OTUs were interrogated against the RDP and SILVA databases ([Wang et al., 2007](#); [Quast et al., 2013](#)), whereby

taxonomic lineages based on the SILVA taxonomy and the best hit from RDP were assigned for each OTU (Supplementary Datasheet 1). A further 55 chloroplast and mitochondrial OTUs were removed, leaving a total of 1,532 OTUs for downstream analysis. Rarefaction curves were used to assess (retrospectively) sampling depth (Supplementary Figure S5: Datasheet 2).

In order to explore for patterns across the global bacterial communities, a data matrix comprising the percent standardised abundances of 1,532 OTUs across all 133 samples was constructed, where samples were then ordinated using non-metric multidimensional scaling (nMDS) with 50 random restarts (Clarke and Warwick, 2001) and principal co-ordinates analysis (PCoA) with 2 axes using the Bray-Curtis algorithm (Bray and Curtis, 1957). Multivariate dispersion indices (MVDISP) were calculated in Primer-E to gauge the degree of variation among replicate samples within sample groups (i.e. MM and PS bulk sediment, rhizosphere and roots). Groups of samples were evaluated for significant differences using both one-way and two-way permutational multivariate analysis of variance (PERMANOVA) with 9999 permutations, allowing for type III (partial) sums of squares, fixed effects sum to zero for mixed terms, and exact p-values generated using unrestricted permutation of raw data (Anderson, 2001). Groups of samples were considered significantly different if the p-value falls < 0.05 . Pairwise tests in PERMANOVA were used to determine which predefined categories were significantly different (using unadjusted p-values). The multivariate analyses, bacterial class plots and rarefaction curves were generated using PRIMER (v.7.0.11), PRIMER-E, Plymouth Marine Laboratory, UK (Clarke and Warwick, 2001). Venn diagrams were used to visualise shared and unique OTUs between sample groups (i.e. bulk vs rhizosphere vs roots) for untreated MM and PS, and between the two substrates for each sample group (i.e. bulk MM vs bulk PS; rhizosphere MM vs rhizosphere PS; root MM vs root PS). The entire sampling period (i.e. weeks 4 and 12) was considered in determining the numbers of unique and shared OTUs among sample groups, with those unique to untreated MM and PS roots reported in tables.

Measures of species diversity were calculated using algorithms for OTU richness (S), Pielou's evenness (J'), Shannon diversity (H') and Simpson diversity ($1-\lambda$), along with algorithms of taxonomic distinctness for diversity (delta+) and evenness (lambda+), using PRIMER (v.7.0.11) (Clarke and Warwick, 2001). These indicators of diversity (S, J', H', $1-\lambda$, delta+, lambda+) were compared between groups of samples using one- and two-way ANOVA (i.e. fruit swabs vs bulk meadow sediment vs bulk meadow mix vs roots; and sample type [bulk/rhizosphere/root] vs sediment type [MM/PS] respectively) (Prism v. 7.01, Graphpad Software Inc.). To determine the impact of autoclave treatment on the bacterial communities, comparisons of OTU richness and average taxonomic distinctness (delta+) between autoclave treated/untreated substrates (MM/PS) and sampling time (growth stages: weeks 4 and 12) were also assessed using two-way ANOVA. Tukey's *post-hoc* multiple comparisons was also performed on significantly different ($p < 0.05$) variables in Prism. Diversity indicators were plotted in PRIMER.

Differential abundance analysis based on Linear Discriminant Analysis (LDA) Effect Size (LEfSe) was also conducted in MicrobiomeAnalyst (Dhariwal et al., 2017) to discern the top 20 significant families and OTUs contributing to the observed differences among treatments; as determined using the Kruskal-Wallis rank test (adjusted p-value cut off = 0.001), with the Log LDA Score value adjusted to 2.0 and significant taxa/OTUs given in descending order from the highest to lowest LDA score.

2.8 Data deposition

The OTU table and seedling growth data used for the associated analyses is presented in Supplementary Datasheet S1 and Supplementary Table S2, respectively. Sequences from individual samples were deposited within the NCBI SRA repository under accession numbers SAMN28854748 – SAMN28854880.

3 Results

3.1 Sediment characteristics

PS was calcareous and unsorted, consisting primarily of fine (grain size = $> 125 - 250 \mu\text{m}$; 37%), medium ($> 250 - 500 \mu\text{m}$; 44%) and coarse ($> 0.5 - 1 \text{ mm}$; 15%) sand. MM was siliceous and moderately well sorted with fine (60%) and medium (35%) sand representing the majority of the sediment grains. Grain size was not changed by autoclave treatment (one-way PERMANOVA: pseudo- $F = 1.38$, $p = 0.298$ for PS; pseudo- $F = 0.88$, $p = 0.607$ for MM; Supplementary Figure S6: Datasheet 2). PS had higher concentrations of phosphorus (two-way ANOVA: $P - F_{1,16} = 855.98$, $p < 0.001$), nitrogen ($N - F_{1,16} = 64.29$, $p < 0.001$), inorganic carbon (inorgC - $F_{1,16} = 3978.12$, $p < 0.001$), calcium carbonate ($\text{CaCO}_3 - F_{1,16} = 14457.58$, $p < 0.001$) and total carbon ($C - F_{1,16} = 855.98$, $p < 0.001$) compared to MM (Table 1). Autoclave treatment did not significantly alter nutrient levels ($P - F_{1,16} = 1.34$, $p = 0.264$; $N - F_{1,16} = 0.29$, $p = 0.600$; inorgC - $F_{1,16} = 0.47$, $p = 0.502$; $\text{CaCO}_3 - F_{1,16} = 2.89$, $p = 0.108$; $C - F_{1,16} = 1.34$, $p = 0.265$).

3.2 Seedling growth

A total of 30 seedlings, six in autoclaved treated MM and eight in each of the other treatments, failed to thrive (rotten or damaged seed) and were excluded from analyses. Summary statistics for key seedling growth parameters at the end of the experiment (week 12) are given in Supplementary Table S2. Overall, leaf count declined (mean 4.63 ± 0.66 to 3.34 ± 0.53 SD; $z = -3.84$) and combined length of leaves increased significantly (mean 237.41 ± 44.17 to $335.22 \text{ mm} \pm 77.22$ SD; $t = 9.34$, $p < 0.001$) from week 4 to 12. Limited change in root count was observed (3.07 ± 0.71 to 2.80 ± 0.82 ; $z = -0.95$, $p = 0.340$) but combined length of roots increased (186.13 ± 68.38 to $279.86 \text{ mm} \pm 92.67$ SD; $t = -1.39$, $p = 0.170$), with some roots restricted by the size of the pot by week 12. Root diameter showed

TABLE 1 Nutrients and properties of sediments and treatments.

Properties	Sediment type			
	Play sand	Play sand (T)	Meadow mix	Meadow mix (T)
Total P (ppm)	264.4 ± 25.77	256 ± 19.68	44.6 ± 8.88	35.6 ± 1.14
Total N (%)	0.082 ± 0.004	0.08 ± 0	0.064 ± 0.005	0.068 ± 0.004
Total inorganic C (%)	11.4 ± 0.55	11.6 ± 0.55	0.55 ± 0.01	0.59 ± 0.02
CaCO ₃ equivalent (%)	93.4 ± 3.21	95.6 ± 0.89	4.59 ± 0.01	4.9 ± 0.02
Total C (%)	11.3 ± 0.50	11.5 ± 0.1	0.744 ± 0.09	0.698 ± 0.09
Total organic C (%)	< 0.25	< 0.25	< 0.25	< 0.25
Very fine sand > 63 – 125 µm (%)	2.187 ± 0.015	1.93 ± 0.391	2.54 ± 0.583	2.627 ± 0.158
Fine sand > 125 – 250 µm (%)	37.477 ± 0.411	36.913 ± 1.133	60.813 ± 0.570	59.857 ± 0.142
Medium sand > 250 – 500 µm (%)	44.09 ± 0.298	45.087 ± 1.128	35.763 ± 1.106	36.427 ± 0.301
Coarse sand > 0.5 – 1 mm (%)	16.227 ± 0.524	16.07 ± 0.399	0.897 ± 0.090	1.09 ± 0.01
Very coarse sand > 1 – 2 mm (%)	0.02 ± 0.035	0	0	0

Means and standard deviations are reported for most measurements. For total organic C the mode (most common value) is reported, as for 85% of the samples results were recorded as <0.25%. (T) indicates autoclave treated sediments.

negligible change between the sample periods (0.78 ± 0.14 to $0.75 \text{ mm} \pm 0.11 \text{ SD}$). Root hairs in PS seemed to be longer, more abundant, and entwined with sediment particles (Supplementary Figures S1A, B: Datasheet 2), while the number of lateral roots was greater in MM. The elongation zones (area free of root hairs where young cells increase in size before becoming part of the maturation zone) of MM roots were often notably longer than PS. Rust coloured root surfaces were observed for the untreated MM, though were absent from the autoclaved treatment groups (Supplementary Figures S1C, D: Datasheet 2). Mean seedling weight increased by 62% from week 4 ($0.29 \text{ g} \pm 0.08 \text{ SD}$) to 12 ($0.47 \text{ g} \pm 0.14 \text{ SD}$; $t = 8.94$, $p < 0.001$).

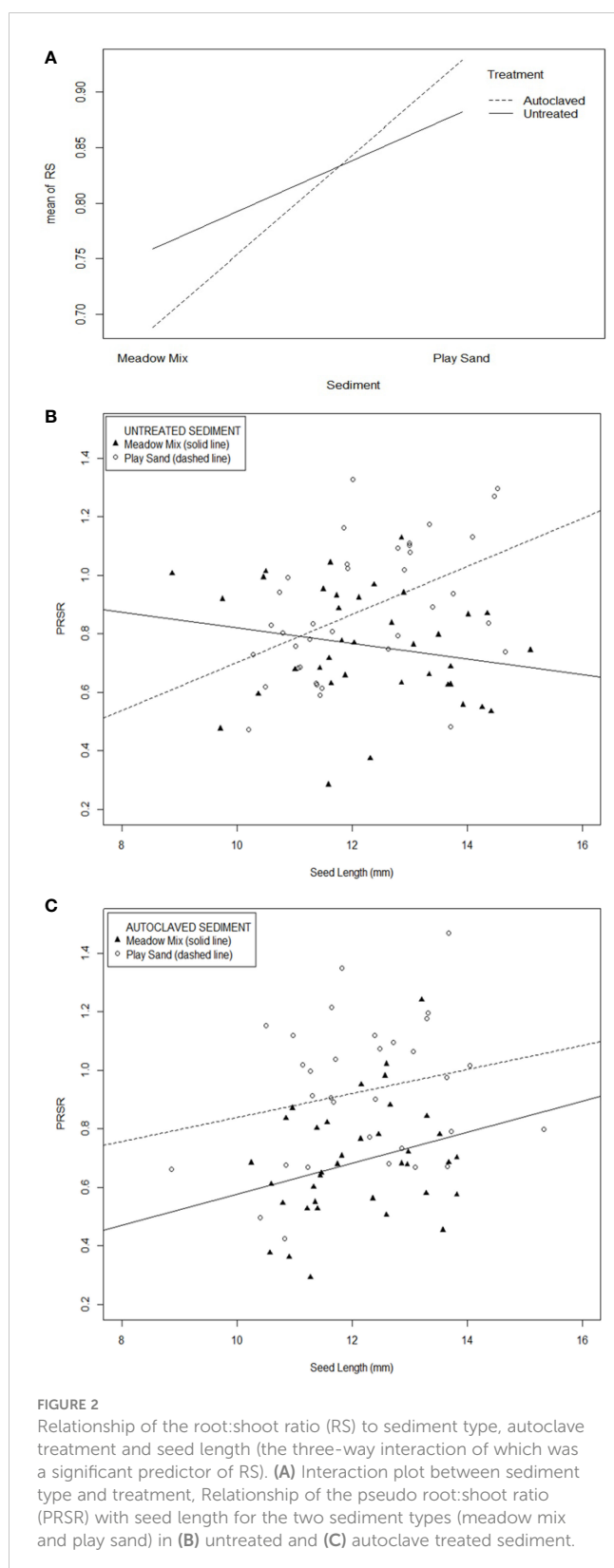
Multiple regressions suggested that total biomass (seedling weight) had a linear and positive relationship with age (weeks 4 and 12; $t = 11.27$, $p < 0.001$) and the size of the seed ($t = 7.33$, $p < 0.001$). No other factors (treatment and sediment type) or interactions were significant. A three-way interaction between treatment, sediment and seed length was a significant predictor of the pseudo roots:shoot ratio (PRSR; ratio of total root to total shoot length; $t = 2.16$, $p = 0.032$), with PRSR being larger in PS than in MM, especially for autoclaved sediment (Figure 2A). In addition, seed length had a significant positive linear relationship with PRSR in PS ($r = 0.35$, $p = 0.003$) but not in MM ($r = 0.01$, $p = 0.910$) (Figure 2B), and in autoclaved ($r = 0.24$, $p = 0.045$) but not untreated ($r = 0.13$, $p = 0.270$) sediment (Figure 2C). Growth stage had no influence on PRSR, either individually or interacting with other terms. The interaction between sediment type and seed length was a significant ($t = 2.90$, $p = 0.004$) predictor of the number of lateral roots, with the number of lateral roots increasing significantly with seed size in PS ($r = 0.50$, $p = 0.002$) but not in MM ($r = 0.14$, $p = 0.370$; Supplementary Figure S7A: Datasheet 2). Treatment did not influence the number of lateral roots. The remaining starch area was positively and significantly related to

the total seed area and was not influenced by sediment type or treatment (multiple regression: $p < 0.001$) (Supplementary Figure S7B: Datasheet 2).

3.3 Bacterial communities of the seagrass meadow environment

To establish the bacterial communities associated with the seagrass 'environment', from which inferences could then be drawn from the seedling growth experiment, the diversity of the Lady Bay *Posidonia* meadow sediment, seawater, adult *P. angustifolia* roots and beach cast fruit, and the MM prepared for the experiment were first evaluated. Bacterial assemblages of the Lady Bay *Posidonia* meadow sediment, MM (pre-potted), seawater, mature seagrass roots and beach-cast fruit clustered according to their sources (Figure 3A). This was confirmed by a one-way PERMANOVA (pseudo- $F = 13.02$, $p < 0.001$), with pairwise comparisons revealing significant differences among all sample types ($t = 2.58 - 4.52$; $p < 0.001$) except for bulk meadow sediment and MM ($t = 3.30$; $p = 0.096$). Overall, samples largely comprised taxa belonging to seven bacterial classes (representing ~89% of the total sequence reads across all samples), with a further 47 ('Other') classes accounting for the remaining ~11%. Proteobacteria appeared to be a dominant component of all samples, with δ -proteobacteria notably abundant in roots and (albeit to a lesser extent) in sediment samples (Figure 3B). In contrast, α -proteobacteria dominated the bacterial communities in seawater and on fruit. Roots also appeared to have a lower abundance of γ -proteobacteria and Acidimicrobiia (Actinobacteria), and a greater abundance of Anaerolineae (Chloroflexi) compared to all other sample types.

Measures of diversity (OTU richness, Shannon's and Simpson's diversity, Pielou's evenness) all followed a similar trend, with sediment samples (bulk meadow sediment and pre-potted MM)



having higher values and fruit swabs the lowest (Supplementary Figure S8A: Datasheet 2). For each diversity metric, significant differences were observed between all sample groups (one-way ANOVA: $df = 3$, $F = 11110 - 40.82$, $p < 0.001$) except for between MM and meadow sediment (all diversity measures), and

between MM/sediment and roots (Simpson's index of diversity only). In plotting average taxonomic distinctness ($\Delta+$) as a function of variation in taxonomic distinctness (Δ), greater taxonomic breadth (based on higher values of Δ) were observed for meadow sediment, pre-potted MM, and roots. Furthermore, while both meadow sediment and pre-potted MM also had a more even distribution of OTUs representing each of the bacterial lineages (based on lower values of Δ) roots were more varied and comprised samples that were dominated by a few distinct bacterial lineages while still being taxonomically diverse (based on higher values for Δ). In contrast, fruit samples were less taxonomically diverse with a more taxonomically uneven distribution of OTUs among lineages, indicating the presence of select bacterial taxa (Supplementary Figure S8B: Datasheet 2). While this was confirmed by one-way ANOVA ($df = 3$; $F = 10.24 - 42.64$; $p < 0.001$), pairwise differences using Tukey's *post-hoc* test were observed only between fruit swabs and all other samples ($p < 0.001$). Differential abundance analysis revealed several significantly different bacterial families and OTUs associated with the differences among sample groups ($p < 0.001$) (Supplementary Figures S9A, B: Datasheet 2). The most notable included *Desulfobacteraceae* (OTUs 82, 653, 131 and 2488: *Desulfatitalea* sp.), *Sedimenticolaceae*, and *Spirochaetaceae* in roots; *Bacteroidetes* BD2-2, *Thiotrichaceae* (OTU_98: unclassified sp.), uncultured Actinomarinales, and *Desulfobulbaceae* (OTU_118: unclassified sp.) in meadow sediment; and *Rhodobacteraceae* (OTU_2: *Thalassococcus* sp.; OTU_73 unclassified sp.), unclassified Chloroflexi P2-11E (OTU_15), *Methylophilaceae* (OTU_50: *Methylophilus* sp.), and *Burkholderiaceae* (OTU_81: *Burkholderia* sp.) in fruit. The addition of beach sand to meadow sediment (which created the MM used in the growth experiment) did have some effect on particular families/OTUs, with those from the meadow sediment apparent but less pronounced in the MM. This was also accompanied by a greater abundance of *Flavobacteriaceae* (OTU_29: *Muriicola* sp.), *Woeseiaceae* (OTU_45: *Woeseia* sp.), *Idiomarinaceae* (OTU_311: *Idiomarina* sp.) and γ -proteobacteria BD7 8 (OTU_13: unclassified sp.) in the MM compared to the meadow sediment. For seawater, particular SAR clades (namely α -proteobacteria SAR11 1a and Puniceispirillales SAR116) appeared to be dominant features, and were more evident from fruit compared to the other sample groups. Interestingly, a number of taxa that were predominant from fruit (namely *Methylophilaceae* and unclassified Chloroflexi P2-11E), were also more abundant in roots compared to the other samples.

3.4 Seedling bacterial communities within different propagating substrates

In order to determine the differences in the contribution of the surrounding environmental microbial consortia on the establishment of seagrass specific associations during early growth, we evaluated the bacterial communities from the bulk sediment, rhizosphere and roots of seedlings grown in two different propagating substrates (PS and MM). Seedlings grown in untreated MM separated from those grown in PS, with bulk sediment, rhizosphere and roots clustering independently of one

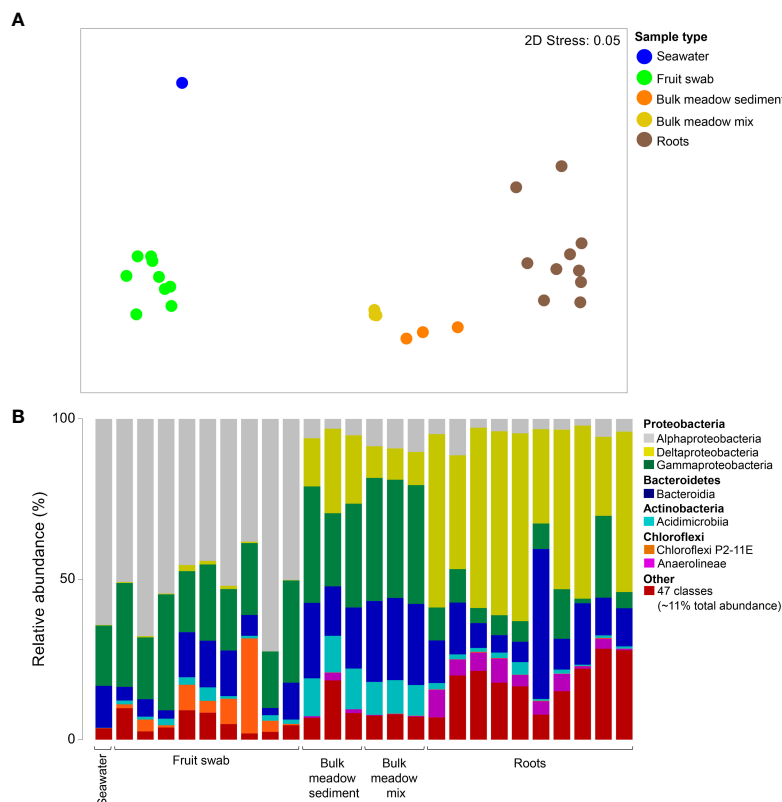


FIGURE 3

Differences in the bacterial assemblages of the seagrass environment. **(A)** Ordination plot representing the global bacterial community structure from field-collected seawater, seagrass fruit swabs, bulk meadow sediment and mature seagrass root samples; and bulk (pre-potted) meadow mix used in the growth experiments, as assessed by non-metric multidimensional scaling (nMDS) using Bray–Curtis dissimilarity. **(B)** Mean relative abundances of the predominant 7/54 bacterial classes (accounting for 89% of the total sequence reads) detected from the samples, where “Other” represents the remaining 47 classes (accounting for 11% of the total sequence reads).

another (Figure 4A). Highly significant differences were observed between sample types (bulk sediment, rhizosphere and roots) and substrates (MM and PS) (pseudo- $F = 7.18$, $p < 0.001$, pseudo- $F = 30.09$, $p < 0.001$, respectively). Despite a similar trend across sample types (where pairwise differences were observed between all groups, $p < 0.001$), there was a significant interaction effect (pseudo- $F = 6.87$, $p < 0.001$), indicating that the changes were substrate specific. The greatest variation among groups of samples (as indicated by higher MVDISP values) was observed for MM compared to PS, whereby variation among samples increased from bulk sediment to rhizosphere to roots (Figure 4A, inset key table). Overall, OTUs were comprised of at least 32 phyla, 53 classes, 162 orders, 265 families, and 513 genera (Supplementary Datasheet 1). However, samples were largely represented by taxa belonging to five phyla and nine bacterial classes (representing ~94% of the total sequence reads across all samples), with a further 44 (“Other”) classes accounting for the remaining ~6% (Figure 4B). In general, like the meadow environment, samples from seedlings grown in different propagating substrates were largely dominated by Proteobacteria. Compared to MM, PS had a higher abundance of γ - and α -proteobacteria and Actinobacteria and a lower abundance of Epsilonbacteraeota (Campylobacteria) and Bacteroidetes, while MM had higher abundances of δ -proteobacteria, Bacteroidetes (Bacteroidia) and Acidimicrobiia. Rhizosphere and root samples

were more similar than either was to bulk sediment for both substrates, with those from MM having notably higher abundances of Campylobacteria and, to a lesser extent, Ignavibacteria (Bacteroidetes) and Spirochaetia (Spirochaetes) compared to PS.

Both substrates comprised a large number of common (core) OTUs, with > 50% of the total OTUs shared between the different sample types for both MM (659/1182) and PS (564/1065) (Figure 5A). Similar numbers of unique OTUs were also apparent among the different sample types for both substrates, with the largest numbers occurring within the rhizosphere (170 in MM and 125 in PS) and the least in the roots (46 in MM and 42 in PS). For both substrates, rhizosphere and root samples shared a greater number of OTUs compared to bulk sediment. Of the OTUs found to be specifically associated with each sample type (i.e. either in bulk sediment, rhizosphere or roots), only a small number were unique to either MM or PS (Figure 5B). For the rhizosphere, this included 35 from MM and 41 from PS (Supplementary Tables S3 and S4), and for the roots 14 from MM and 15 from PS (Supplementary Tables S5 and S6). As many as ~60% of these unique OTUs were also detected in the seagrass meadow environmental samples, with several being abundant or exclusively associated with mature roots or fruit. Most notably, across both substrates this included several OTUs belonging to the α -proteobacteria (namely *Rhodobacteraceae*

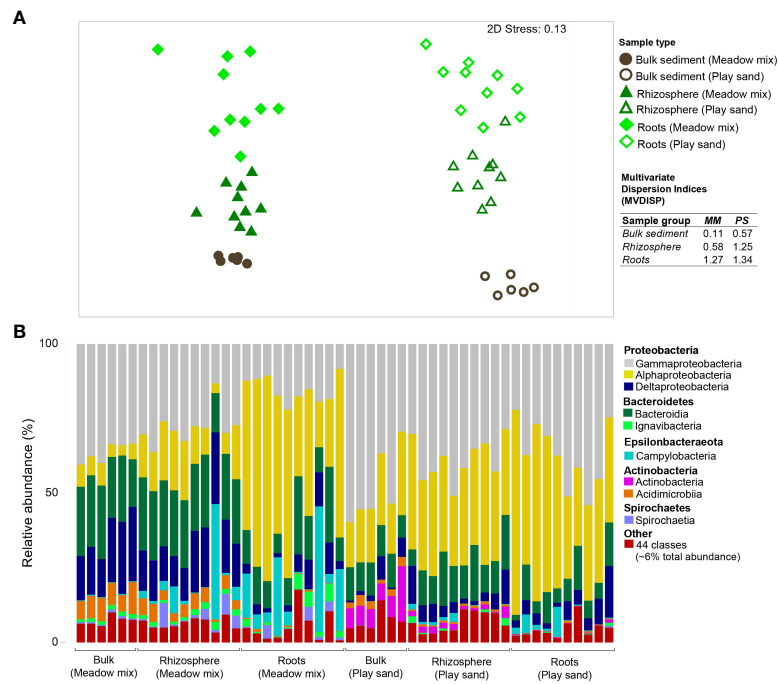


FIGURE 4 Differences in the bacterial communities of seagrass seedlings grown in different propagating substrates. **(A)** Ordination plot representing the global bacterial community structure from bulk sediment, rhizosphere and seagrass root samples collected from the untreated experimental meadow sediment mix and play sand used in the growth experiments, as assessed by non-metric multidimensional scaling (nMDS) using Bray–Curtis dissimilarity. Corresponding multivariate dispersion indices (MVDISP) representing the global variation in the bacterial community composition among replicate samples are given for each sample group (inset key table), where higher values represent greater variation and lower values less variation among samples. **(B)** Mean relative abundances of the predominant 9/53 bacterial classes (accounting for 94% of the total sequence reads) detected from the samples, where “Other” represents the remaining 44 classes (accounting for 6% of the total sequence reads).

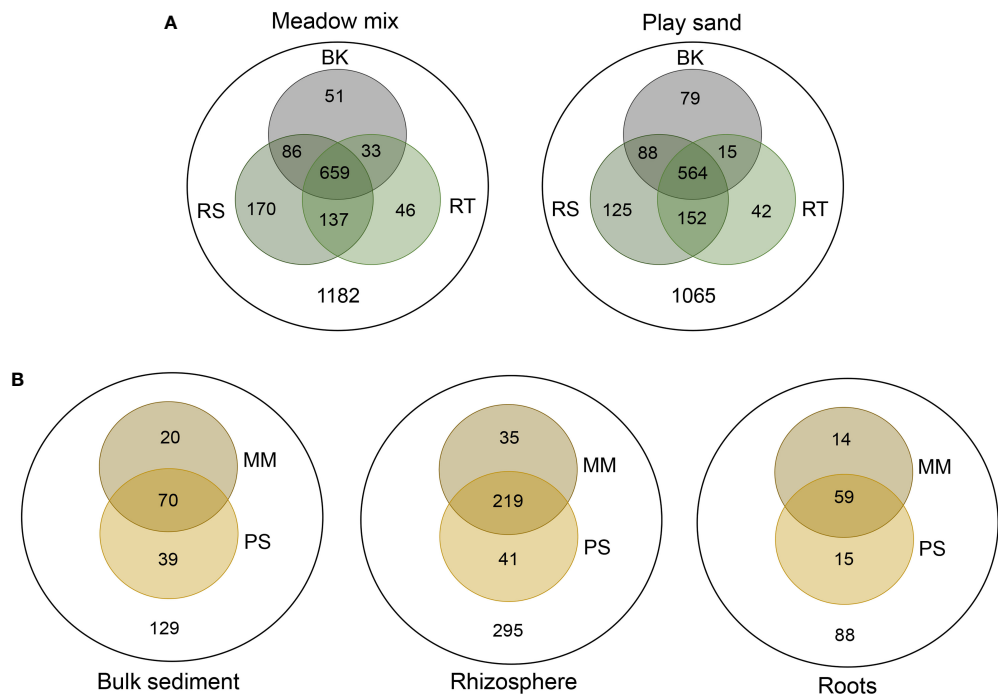


FIGURE 5 Venn diagrams depicting the distribution of shared and unique OTUs in **(A)** bulk (BK), rhizosphere (RS) and root (RT) samples from meadow mix and play sand; and **(B)** meadow mix (MM) and play sand (PS) from each sample group. Total OTUs are denoted in the outer circles.

and *Hyphomonadaceae*) that were largely abundant in fruit. Alongside this were other fruit associated OTUs that were detected solely in the roots of either PS or MM. Within MM this included OTU_215 *Marinobacterium* (*Nitrincolaceae*; γ -proteobacteria) and OTU_570 *Spirochaeta* 2 (*Spirochaetaceae*; Spirochaetes), and in PS OTU_910 uncultured *Micavibrionaceae* (α -proteobacteria), OTU_8302 *Methylothera* (*Methylophilaceae*; γ -proteobacteria) and OTU_8835 unclassified *Babeliales* UBA12409 (*Dependentiae*) ([Supplementary Tables S5 and S6](#)).

Overall, the highest species (OTU) richness was observed for MM, particularly within the rhizosphere ([Figure 6A](#)). For roots, however, richness and other measures of diversity (Shannon's and Simpson's diversity, Pielou's evenness) were substantially reduced.

For each diversity metric, significant differences were observed between all three sample types (i.e. bulk sediment, rhizosphere and roots) (two-way ANOVA: $df = 2$, $F > 13$, $p < 0.001$). There were also significant differences in species richness, Shannon's diversity and taxonomic distinctness metrics between substrates (i.e. MM and PS) (two-way ANOVA: $df = 1$, $F > 8$, $p < 0.005$). There was an interaction effect in Pielou's evenness, Shannon's diversity and variation in taxonomic distinctness (two-way ANOVA: $df = 2$, $F > 7$, $p < 0.004$), indicating that the changes across sample types (from bulk to rhizosphere to roots) were substrate specific.

In plotting average taxonomic distinctness ($\delta+$) as a function of variation in taxonomic distinctness ($\lambda+$), all MM samples comprised greater taxonomic breadth (based on

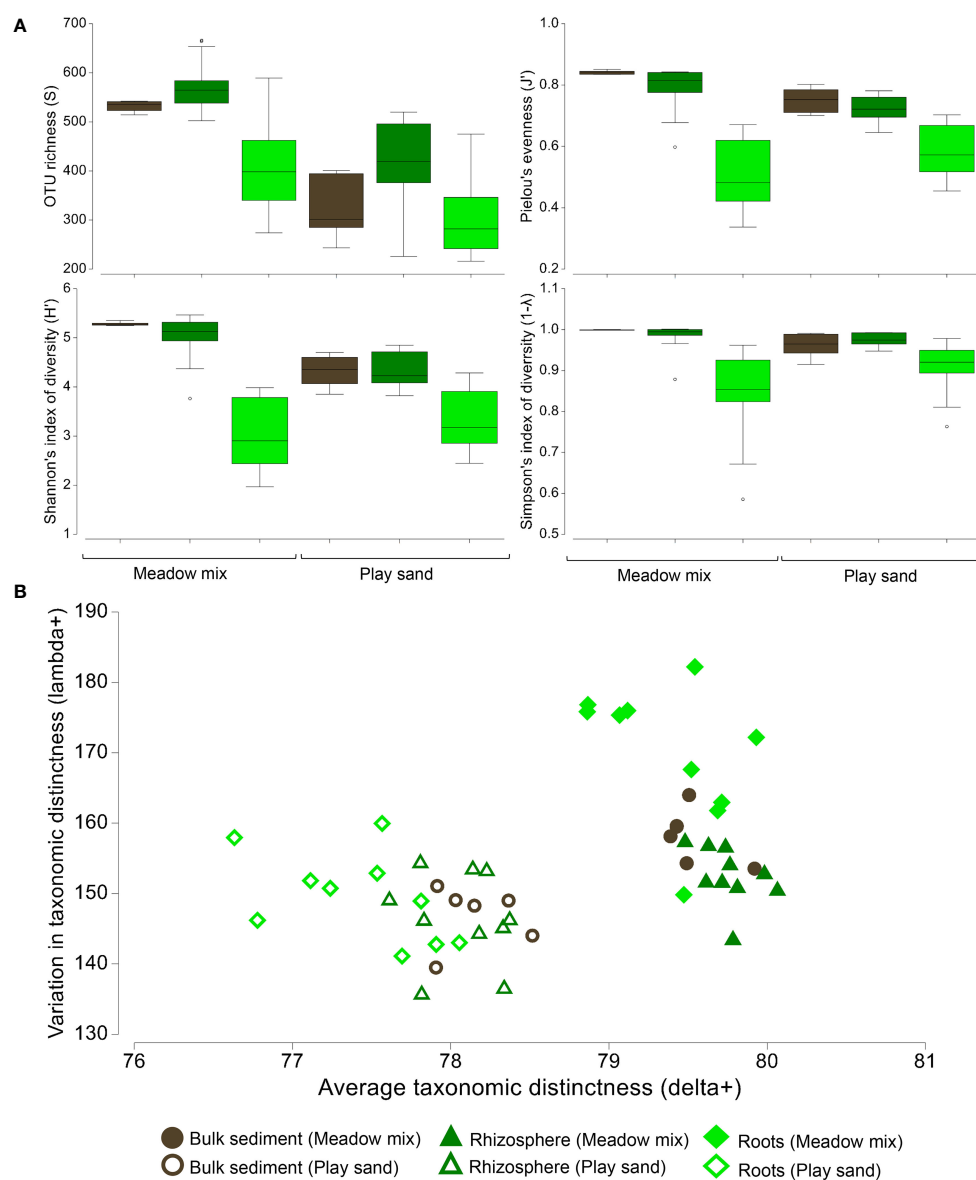


FIGURE 6

Measures of diversity from seagrass seedlings grown in different substrates. (A) Box plots representing the median, interquartile ranges (IQR) and distribution of measures of OTU richness, Shannon's and Simpson's indices of diversity and Pielou's evenness from bulk sediment, rhizosphere and seagrass root samples collected from the untreated meadow mix and play sand used in the growth experiments. (B) Scatter plot charting the average taxonomic distinctness ($\delta+$) as a function of variation in taxonomic distinctness ($\lambda+$) for each of the sample groups.

higher values of delta+) compared to PS (Figure 6B). Furthermore, bulk sediment and rhizosphere samples of the MM had a more even distribution of OTUs representing each of the different bacterial lineages (based on lower values of lambda+), while the roots were dominated by a few distinct bacterial lineages while still being taxonomically diverse. In contrast, PS samples were less taxonomically diverse with a more taxonomically even distribution of OTUs among lineages. The roots of PS established the lowest level of bacterial taxonomic diversity. Differential abundance analysis revealed several significantly different bacterial families and OTUs associated with the differences among sample groups ($p < 0.001$) (Supplementary Figures S10A, B: Datasheet 2). MM samples were enriched with *Flavobacteriaceae*, *Desulfobacteraceae*, *Woeseiaceae*, uncultured Actinomarinales, *Desulfobulbaceae*, *Thiovulaceae*, *Marinilabiliaceae*, and *Chromatiaceae*, while PS samples were enriched with *Solimonadaceae*, *Burkholderiaceae*, *Micrococcaceae*, *Xanthomonadaceae*, *Marinomonadaceae*, and *Tenderiaceae*. Most notable in the rhizosphere and/or roots of seedlings grown in MM were *Rhizobiaceae* (OTU_5: *Lentibacter* sp.), *Arcobacteriaceae* (OTU_32: *Arcobacter* sp.), *Marinilabiliaceae* (OTU_9: *Labilibacter* sp.), *Magnetospiraceae* (OTU_59: *Magnetovibrio* sp.), and *Methylophagaceae* (OTUs 163 and 2764: *Methylophaga* sp., unclassified sp.); and in PS *Rhodobacteraceae* (OTUs 2 and 4: *Thalassococcus* sp., unclassified sp.), *Methylophagaceae* (OTU_3: *Methylophaga* sp.); *Rhizobiaceae* (OTU_21: *Lentibacter* sp.), and *Devosiaceae* (OTU_40: *Devosia* sp.).

3.5 Seedling bacterial communities at different growth stages

Given that root samples likely represent the closest relationships with seagrass seedlings and the surrounding microbial consortia, we investigated these bacterial communities at the different growth stages (weeks 4 and 12) in untreated samples only (Figure 7A). Significant differences between groups were evaluated using a two-way PERMANOVA which crossed substrate type (MM and PS) with sampling period (week 4 and week 12), indicating that there were highly significant differences between substrates and between sampling periods (pseudo- $F = 11.10$, $p < 0.001$, pseudo- $F = 3.50$, $p = 0.001$, respectively). However, there was a significant interaction effect (pseudo- $F = 2.67$, $p = 0.006$), indicating that the changes across growth stages were substrate specific, with pair-wise differences revealing only a significant change in the PS roots across growth stages ($t = 2.26$; $p = 0.007$). While ordination of the seedling root samples with those obtained from mature (adult) meadow roots from Lady Bay revealed a distinct separation between groups, MM roots were closer to the Lady Bay samples than those from PS (Figure 7A). In evaluating the diversity metrics among sample groups, no significant differences were apparent for the MM roots across growth stages or with the adult meadow roots (Figure 7B). However, PS roots differed significantly for Pielou's evenness and Shannon's diversity, with both measures increasing substantially between weeks 4 and 12 (one-way ANOVA: $df = 4$; $F = 3.43 - 49.12$; $p < 0.023$) (Supplementary Table S4D). Furthermore,

unlike MM and adult roots, PS roots also had a significantly reduced, though slightly more even, distribution of OTUs across bacterial lineages (based on lower values for delta+ and lambda+, respectively).

Differential abundance analysis revealed several significantly different bacterial families and OTUs associated with the differences among sample groups ($p < 0.001$) (Supplementary Figures S11A, B). Unlike PS, bacterial root communities of seedlings grown in MM were somewhat more similar to adult root communities, with several that were predominant in MM also having the correspondingly highest abundance in adult roots. Most notably, this included *Desulfobacteraceae* (OTUs 82, 653 and 131: *Desulfatitalea* sp.), *Bacteroidetes* BD2-2, *Sedimenticolaceae*, *Spirochaetaceae*, and uncultured *Ardenticatenales*. However, a number of other families/OTUs that were also predominant in MM roots had instead the lowest abundance in adult roots, including *Rhizobiaceae*, *Arcobacteraceae* (OTUs 16 and 32: *Arcobacter* sp.), *Flavobacteriaceae*, *Magnetospiraceae*, and *Marinilabiliaceae* (OTU_9: *Labilibacter* sp.). PS roots were enriched by taxa that generally occurred in the least abundance in adult roots (e.g. *Rhodobacteraceae* and *Methylophagaceae*) (Supplementary Figure S11A: Datasheet 2).

3.6 The effect of autoclave treatment on seedling bacterial communities

To better gauge the contribution of the surrounding microbial consortia on the early growth of seagrass seedlings and the establishment of the more intimate root-associated (rhizoplane) communities, we also investigated the bacterial assemblages from the roots of seedlings grown in autoclave treated and untreated MM and PS. Significant differences between groups were evaluated using two-way PERMANOVA which crossed treatment (autoclave treated and untreated sediment) with sampling period (week 4 and week 12). While autoclave treatment had a significant impact on the root bacterial communities of both MM (pseudo- $F = 6.65$, $p < 0.001$) and PS (pseudo- $F = 2.03$, $p = 0.046$), the shift in the bacterial communities was much more pronounced in the MM compared to the PS (Figures 8A, B). More specifically, in the MM, greater separation occurred between autoclave treated and untreated samples, followed by sampling periods, while for PS there was a greater separation between sampling periods, followed by treatment. The pronounced change in beta diversity of MM samples with treatment was also reflected in significant changes in alpha diversity, such as species (OTU) richness (two-way ANOVA: $F = 31.03$, $p < 0.001$) and average taxonomic distinctness (delta+) ($F = 14.40$, $p = 0.002$). Most notably, as much as ~50% of the OTUs were lost from the MM root samples following autoclave treatment at week 4, with a notable reduction in the distribution of OTUs across bacterial lineages (based on lower values for delta+) at week 12. For PS, significant differences in these measures were only observed between sampling periods rather than treatment (richness: $F = 9.59$, $p = 0.007$; delta+: $F = 6.66$, $p = 0.020$), with an increase in richness and a reduction in the distribution of OTUs across

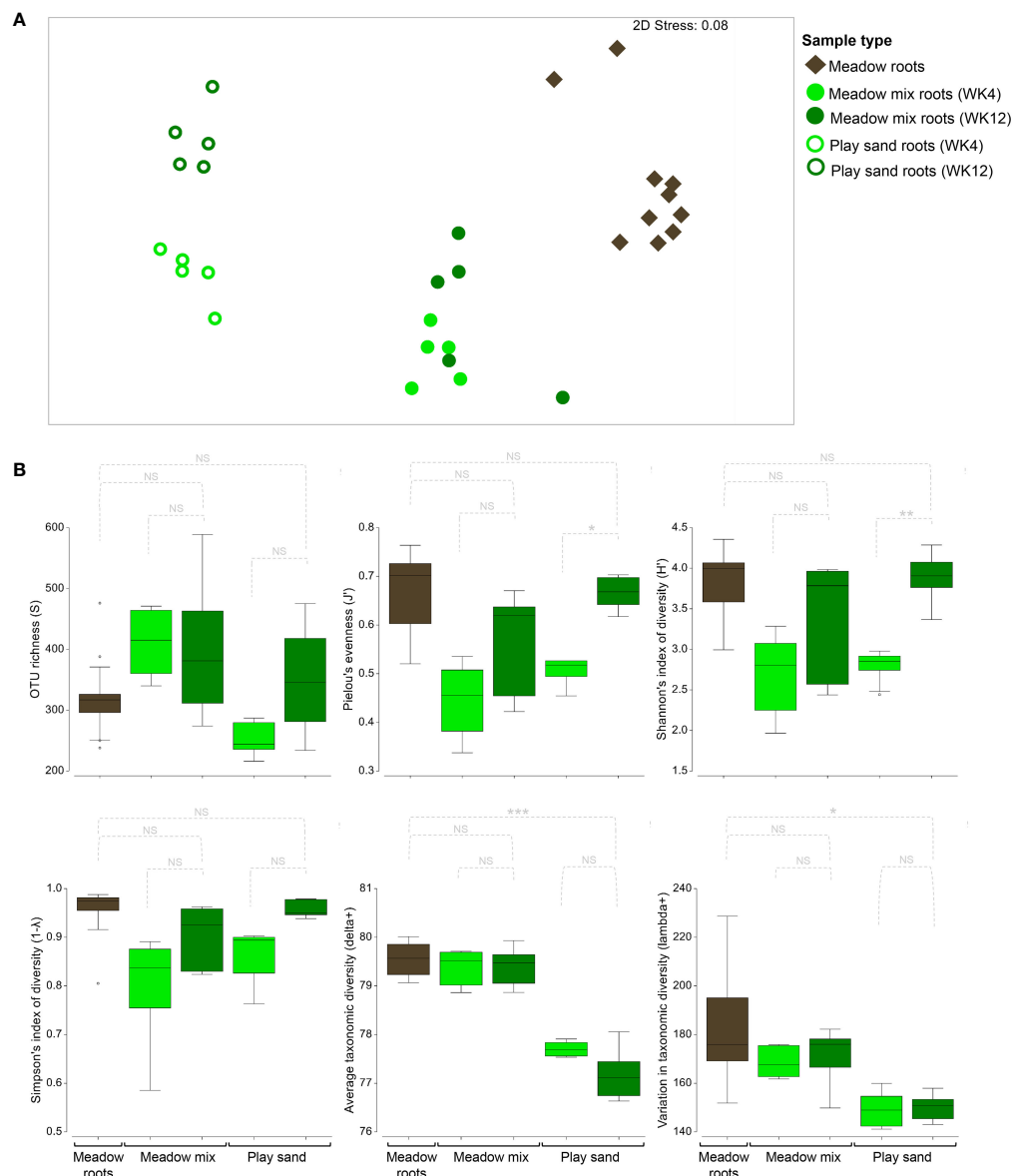


FIGURE 7

Differences in the root bacterial communities of seagrass seedlings across growth stages. **(A)** Ordination plot representing the global bacterial community structure from mature seagrass roots obtained from Lady Bay (South Australia) and seagrass seedling roots grown in untreated meadow mix and play sand at weeks 4 and 12 (WK 4, WK12), as assessed by non-metric multidimensional scaling (nMDS) using Bray–Curtis dissimilarity. **(B)** Box plots representing the median, interquartile ranges (IQR) and distribution of measures of OTU richness, Shannon's and Simpson's indices of diversity, Pielou's evenness, and average and variation in taxonomic distinctness (delta+, lambda+) from each of the sample groups.

bacterial lineages (based on lower values for delta+) from week 4 to week 12.

Differential abundance analysis revealed several significantly different bacterial families and OTUs associated with the differences among sample groups ($p < 0.001$) (Supplementary Figures S12A, B; Datasheet 2). Most notable in MM was a decrease in abundance of *Rhizobiaceae* (OTU_23517: *Lentilitoribacter* sp.), *Thiovulvaceae*, Bacteroidetes BD2-2 (OTU_97: unclassified sp.), *Magnetospiraceae* (OTU_59: *Magnetovibrio* sp.), *Melioribacteraceae*, *Sedimenticolaceae* (OTU_138: *Candidatus* Thiodiazotropha), and *Calditrichaceae*, and an increase in *Rhodobacteraceae* (OTU_4: unclassified sp.), *Cellvibrionaceae*, *Arcobacteraceae* (OTUs 23 and 32: *Arcobacter*

sp.), *Desulfobacteraceae*, and *Desulfovibrionaceae*. While changes in PS roots were generally not as pronounced, certain taxa predominant in untreated MM became enriched or decreased in abundance following treatment (namely *Magnetospiraceae* [OTU_59 *Magnetovibrio* sp.] and *Thiovulvaceae*, respectively).

4 Discussion

Our findings illustrate the complexity of the microbiome (bacterial assemblages) of seagrass seedlings and that manipulation of the microbiome could potentially lead to better

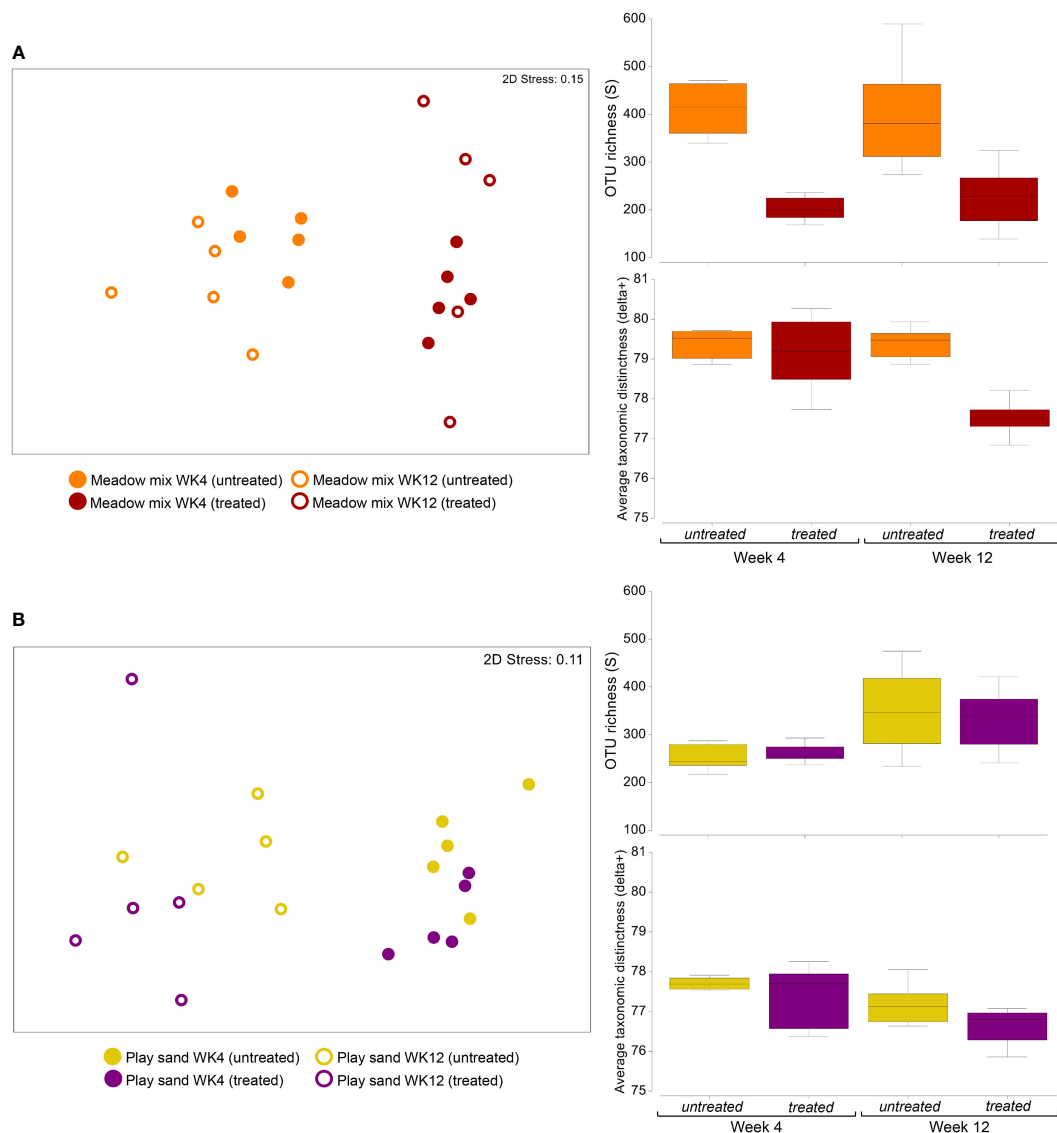


FIGURE 8

Differences in the root bacterial communities of seagrass seedlings following autoclave treatment of propagating substrates. Ordination (nMDS) plots based on Bray-Curtis dissimilarity representing the global bacterial community structure between autoclave treated and untreated root samples at weeks 4 and 12, and corresponding box plots representing the median, interquartile ranges (IQR) and distribution of measures of OTU richness and average taxonomic distinctness from each of the sample groups for (A) meadow mix and (B) play sand.

outcomes for *Posidonia* restoration. The bacterial communities of seedlings differed significantly between meadow mix – MM (a more 'natural' propagating substrate) and the predominantly used play sand - PS, with the composition of taxa in the rhizosphere and roots being unique for each propagating substrate. The meadow mix contained a larger diversity of bacteria compared to play sand, suggesting that seagrass seedlings in this substrate likely have a greater 'pool' of potentially beneficial microbes that could be selected to support growth (Ugarelli et al., 2017; Conte et al., 2021). Growth measurements offered little support for the hypothesis that bacterial communities influence the growth rate of seagrass seedling. However, when considering the pseudo root: shoot ratio (PRSR), seed length had an effect as well as substrate type and autoclave treatment, suggesting that the seedlings may be

responding to differences in the microbiome from as early as four weeks after planting.

4.1 Sediment-associated niche variation in seagrass bacterial communities

We observed marked differences in the bacterial communities of samples collected from the natural *Posidonia* meadow environment and the untreated propagating substrates used in the growth experiment. Meadow sediment and root communities were distinct to those observed from the seawater and fruit, and when meadow sediment was incorporated with beach sand (in preparing the meadow mix used in the growth experiment), it yielded a

substrate that was considerably more diverse and comprised a greater proportion of δ -proteobacteria, Bacteroidia (Bacteroidetes) and Acidimicrobia (Actinobacteria) compared to play sand. Most notable in the meadow mix was the enrichment of taxa previously reported from other seagrass meadows that are important constituents in biogeochemical cycling of sulfur (Jørgensen et al., 2019). This included the sulfate reducing δ -proteobacteria families *Desulfobacteraceae* and *Desulfobulbaceae* (Kuever, 2014a; Kuever, 2014b), as well as the sulfide oxidising families *Thiovulaceae* (Campylobacteria) (Waite et al., 2017), *Thiotrichaceae* (γ -proteobacteria) (Garrity et al., 2005) and *Chromatiaceae* (purple sulfur bacteria) (Imhoff, 2014). In addition, alongside other ubiquitous marine sediment associated taxa like *Woesiaceae* (which are purported denitrifiers) (Mußman et al., 2017), the Bacteroidetes (namely *Flavobacteriaceae* and *Bacteroidetes* BD2-2), a group assumed to contribute to the cycling of carbon through the breakdown of complex polysaccharides of algae and accumulated plant deposits (Fernández-Gómez et al., 2013; Gavrilidou et al., 2020), occurred only in substrates that included meadow sediments. In contrast, play sand (despite sharing a large proportion of OTUs with meadow mix) was less diverse and had notably higher abundances of γ - and α -proteobacteria and Actinobacteria (Actinobacteria) and less Bacteroidetes. This included the occurrence of certain families such as *Solimonadaceae* that are typically associated with soils and other aquatic ecosystems (Zhou et al., 2014), and likely reflects the coastal, yet terrestrial, source of the play sand used here. The types of bacterial assemblages observed thus appear to be primarily a function of the origins of the propagating substrates, where the incorporation of meadow sediment into beach sand is more likely to support assemblages represented in the natural environment, which may promote greater resilience and better growth. For example, communities with a high ratio of Proteobacteria and/or Actinobacteria compared to Bacteroidetes may reduce a plant's resistance to environmental stresses (Pérez-Jaramillo et al., 2018), with a reduction of sediment associated Bacteroidetes thought to be a precursor to a decline in *Posidonia oceanica* meadows (García-Martínez et al., 2009). Furthermore, at least in terrestrial systems, Bacteroidetes have also been attributed to pathogen suppression and the mobilisation of other essential and limited nutrients like phosphorous which are important for metabolism in seagrasses (Touchette and Burkholder, 2000; Lidbury et al., 2021). Given the relatively low concentrations of total phosphorous in the meadow mix compared to the play sand here (by at least an order of magnitude), higher proportions of Bacteroidetes in the meadow mix may be of particular benefit.

Despite the presence of a large number of shared OTUs, partitioning of the communities between the bulk sediment, rhizosphere and roots was evident for both untreated substrates, with the rhizosphere and roots being more similar to one another than the bulk sediment, a trend that is widely reported among angiosperms (Fitzpatrick et al., 2018). This suggests that the rhizosphere and roots constitute distinct niches that are strongly influenced by the surrounding sediment. In seagrasses, these microenvironments are suggested to be driven by root exudates (Martin et al., 2017; Martin et al., 2019). For *Posidonia oceanica*,

such exudates occur primarily as sucrose, which is secreted in large (μ M) concentrations into the rhizosphere together with inhibitory phenolic compounds which, alongside its role in balancing local osmolarity, is thought to regulate the surrounding bacterial communities and attract species that may support the health of the plant (Sogin et al., 2022). Interestingly, of the select few rhizosphere constituents reported by Sogin et al., (2022) to represent putative sucrose specialists with the capacity to breakdown otherwise inhibitory phenolic compounds (based on metagenome-assembled genomes and sediment metatranscriptomes), OTUs associated with two orders (namely *Desulfobacterales* and *Xanthomonadales*) were observed here. Specifically, in the meadow mix the *Desulfobacterales* families *Desulfobacteraceae* and *Desulfobulbaceae* were enriched in the bulk sediment and rhizosphere, while in the play sand we observed the enrichment of the *Xanthomonadales* family *Xanthomonadaceae*. The *Desulfobacteraceae* and *Desulfobulbaceae* are typically anaerobic sulfate reducers, though some species are also capable of nitrogen fixation (Kuever, 2014a; Kuever, 2014b), and are frequently reported from meadow sediments and the rhizosphere of seagrasses (Jensen et al., 2007; García-Martínez et al., 2009; Cúcio et al., 2016; Hurtado-McCormick et al., 2019; Markovski et al., 2022). Supported by the highly reduced, sulfate rich conditions typical of these systems, particularly in sediments that are naturally composed of finer particles (Martinez-Garcia et al., 2015), such taxa not only play an important role in the decomposition of organic matter, but in supporting seagrass productivity as potential diazotrophs (Herbert, 1999; Welsh, 2000; Sun et al., 2015). In contrast, the *Xanthomonadaceae* are obligately aerobic and are one of the largest groups of phytopathogens and endophytes (Saddler and Bradbury, 2015; Assis et al., 2017). Given their reliance on oxygen for respiration, their enrichment in play sand, rather than meadow mix, suggests that the surrounding conditions of the play sand are likely to have been at least partially oxic (despite the rhizosphere of seagrasses usually being anoxic) (Borum et al., 2006). Though oxygen concentrations were not measured here, grain size strongly influences the permeability and movement of oxygen into the sediment, with more coarse sediments associated with increased pore water advection (Ahmerkamp et al., 2017). Of course, while other factors cannot be excluded (e.g. radial diffusion of oxygen from the roots) (Borum et al., 2006; Brodersen et al., 2018), the majority (~60%) of the play sand was made up of medium-coarse grains (unlike the meadow mix which was largely fine grained), and thus it seems plausible that varied conditions arising from differences in grain size may have led to the enrichment of specific assemblages in these substrates. Indeed, in other seagrasses like *Enhalus acoroides* it has also been speculated that differences in grain size may have an impact on the rhizosphere communities, with fine sediments supporting a stronger "rhizosphere effect" due to the limited diffusion of exudates away from the root (Zhang et al., 2022). However, given the clear partitioning of the communities among the microenvironments of both substrates, grain size does not appear to be the sole contributing factor.

Interestingly, alongside CO_2 fixation, the *Xanthomonadaceae* also have the capacity to metabolise H_2 (Sogin et al., 2022), a

product generated from nitrogen fixation (Teng et al., 2019) and fermentation by ubiquitous sedimentary bacterial taxa like *Woeseiaceae*, which is reported to accumulate in high concentrations in the pore water of permeable (sandy) seagrass sediments (Kessler et al., 2019). With other nitrogen fixers (e.g. *Rhizobiaceae*) (Carareto Alves et al., 2014) and fermentative bacteria (*Woeseiaceae*) also occurring in the play sand (albeit in lower abundances compared to meadow mix), the generation of H_2 required for supporting such populations is likely, and may be further supported here by the enrichment of other aerobic H_2 utilising organisms in the play sand.

Various *Burkholderiaceae* (β -proteobacteria) genera, such as *Acidovorax*, *Hydrogenophaga*, and *Polaromonas*, all of which comprise species capable of the oxidation of hydrogen (Willems et al., 1989; Willems et al., 1990; Sizova and Panikov, 2007), were detected somewhat unexpected as the *Burkholderiaceae* are typically reported from the leaves rather than the rhizosphere of seagrass (Hurtado-McCormick et al., 2019). Generally, such taxa have been observed in association with sediments contaminated with polycyclic aromatic hydrocarbons (PAHs). For example, *Hydrogenophaga* was found to be enriched in PAH-treated sediments of *Halophilis ovalis* (Ahmad et al., 2021). Intriguingly, alongside other β -proteobacteria like *Acidovorax* and *Polaromonas*, the Xanthomonads are also well known degraders of petroleum hydrocarbons (Chang and Zylstra, 2010; Tan and Parales, 2019). In Australia, the occurrence of PAHs in nearshore marine sediments is well documented and likely arises from industrial discharge, bushfires, and urban, recreational and agricultural activities (Maher and Aislabie, 1992). Their impact on seagrasses is varied, but in sub-lethal concentrations they can be incorporated into the tissues and reduce their tolerance to other stressors (Zieman et al., 1984; Runcie et al., 2005). For *Posidonia australis*, a decrease in photosynthetic oxygen production has been reported in the presence of PAHs together with other chemical dispersants (Hatcher and Larkum, 1982). While the occurrence of such species (particularly in the play sand) thus raises some concerns regarding the possibility of PAH contamination, evidence from terrestrial and other marine plants indicates a possible role for such constituents in the broader plant functioning to include the rhizoremediation of petroleum contaminants (Kotoky et al., 2018; Chen et al., 2019; Sampaio et al., 2019).

4.2 Seagrass root bacterial communities

Differences in the bacterial communities of the root (rhizoplane) samples were also observed and included the varied enrichment of specific taxa and small numbers of unique OTUs in both of the untreated substrates. As reported for other plant species and in seagrasses, the rhizoplane is considered an exclusive niche that comprises a smaller subset of taxa that are selected for in a stepwise manner from the surrounding sediment and rhizosphere, leading to the formation of more intimate associations likely fostered by the host plant (Edwards et al., 2015; Zhang et al., 2022). In our study, greater intimacy was reflected by microbial cells

adhering tightly to the root surfaces of *P. angustifolia* seedlings (as observed by ESEM) and by considerably reduced species (OTU) richness and diversity in the roots compared to the rhizosphere and bulk sediment. Though the rapid dehydration of seedling Martinez-Garcia root specimens prior to ESEM visualisation impeded Martinez-Garcia's ability to obtain a detailed assessment of microbial cell densities, cell coverage was sparse and is consistent with the lower rates of colonisation observed previously on the roots of the closely related Australian species *P. australis* and *P. sinuosa*, compared to the Mediterranean species *P. oceanica* (García-Martínez et al., 2005). This may be further exemplified by the notable variation observed among root samples compared to the rhizosphere or bulk sediment, particularly in the meadow mix. Unlike play sand, bacterial root communities of seedlings grown in meadow mix were somewhat more similar to adult roots and were represented by OTUs that, despite covering a considerable breadth of taxa (based on high values for delta+), were dominated by a few distinct bacterial lineages (based on high values for lambda+). This higher taxonomic diversity (delta+) of the meadow mix seedlings may afford greater resilience to disturbances that affect their bacterial communities, as increased diversity improves the resilience and recovery time of a natural system after disturbance (Hector and Bagchi, 2007; Cardinale et al., 2012). However, phylogenetic conservatism in bacterial lineages indicates that many related OTUs may have similar functional traits (nitrogen fixation, sulfate-reduction etc) (Martiny et al., 2013), implying that functional diversity should also be considered using complementary omics-based approaches (e.g. metatranscriptomics) or assays that directly measure specific activities (e.g. nitrogen fixation).

The high productivity of seagrass meadows is linked to their inability to resorb nutrients before discarding aging foliage (Hocking et al., 1981; Hemminga et al., 1999). Consequently, seagrasses have high nutrient (nitrogen, phosphorus etc) demands and a strong reliance on microbes, associated with both the leaves and roots, to sequester nutrients in the often nutrient poor marine environment (Ugarelli et al., 2017; Tarquinio et al., 2019). For example, *P. oceanica* root bacteria can be up to 10 times more efficient at nitrogen fixation than those found on the leaves (Lehnen et al., 2016). Several bacterial taxa known to be important for nutrient acquisition were particularly common in meadow mix samples. Alongside the occurrence of saccharolytic bacteria like *Flavobacteriaceae* and *Spirochaeta* (Leschine et al., 2006; Breznack and Warnecke, 2008; Gavrilidou et al., 2020), which likely play a role in the remineralisation and processing of organic carbon (e.g. from decaying plant matter or exudates), we also observed taxa associated with nitrogen cycling. These taxa included *Rhizobiaceae* (*Lentilitoribacter* spp.) and *Arcobacteraceae* (*Arcobacter* spp.), which are commonly associated with nitrogen fixation (Crump et al., 2018; Tedersoo et al., 2018), and anaerobic sulfate-reducing bacteria, which provide seagrasses with a significant portion of their required nitrogen (Welsh, 2000; Tarquinio et al., 2019). Most notably, like in the rhizosphere (as stated above), high abundances of *Desulfobacteraceae* (*Desulfatitalea* spp.) and *Desulfobulbaceae* were observed and these taxa can, in addition to nitrogen fixation, also remove toxic methanol (Dekas et al., 2014; Crump et al., 2018), a potential exudate of the growing seedling

(Abanda-Nkpawatt et al., 2006). Interestingly, other γ -proteobacteria capable of utilising toxic methanol were also observed in the roots of seedlings (though in both substrates) and included the methylotrophic lineages *Methylophagaceae* (*Methylophaga* spp.) and *Methylophilaceae* (*Methylotenera* spp.). Alongside their role in mitigating abiotic stresses in plants, they also contribute to nitrogen fixation (albeit indirectly through ammonia production) and secrete phytohormones, like auxins, that are required for seed germination and plant root growth (Trotsenko et al., 2001; Kumar et al., 2019).

Intriguingly, *Methylophilaceae* were also notably abundant in the fruit swab samples as well as the adult roots in Lady Bay, and together with *Methylophaga* species have been previously reported from the fruits of *Halophila ovalis* (Tarquinio et al., 2021). Given the distinct sources of the substrates used for the propagation of seedlings, the occurrence of such taxa in the roots of both play sand and meadow mix samples may reflect their common origins, and may be indicative of a prospective role for the reproductive tissues in supplying microbes that may support early seedling establishment as potential phytosymbionts. Nevertheless, other pertinent fruit associated taxa observed here and reported from the fruits and leaves of *H. ovalis* (namely *Rhodobacteraceae*) (Tarquinio et al., 2021), were predominantly associated with the roots of seedlings grown in play sand rather than meadow mix, suggesting that other factors likely contribute to their selection. The *Rhodobacteraceae* are a group of largely aerobic bacteria that exhibit diverse nutritional strategies (as photo- or chemoheterotrophic, or purple non-sulfur bacteria), colonise surfaces (as epiphytes) and may form symbioses with various hosts (Pujalte et al., 2014). Of the *Rhodobacteraceae* associated OTUs that were detected, *Thalassococcus* sp. (*Roseobacter* clade) was previously isolated from corals and can reduce nitrate to nitrite (Lee et al., 2007). Because sandy (permeable) sediments provide improved penetration of oxygen and nutrients that favour aerobic denitrification (Gao et al., 2009; Marchant et al., 2014), the occurrence of such organisms in the play sand (given its larger particle sizes compared to meadow mix) was not surprising. Nitrate reduction is an important process in terrestrial and marine ecosystems, and though often associated with N loss, is also important in N-recycling and assimilation by plants (through the conversion of nitrate to ammonium) (Herbert, 1999; Liu et al., 2022). Seagrasses obtain their nitrogen through leaf absorption and root uptake from the sediment pore water, whereby nitrate reductases catalyse the important first step in its assimilation (Touchette and Burkholder, 2000; Alexandre et al., 2004). Interestingly, in semiaquatic plants, like rice, the inoculation of seeds with denitrifying bacteria has been associated with improved growth and nitrogen use efficiency, and was attributed to the nitrate reductase activity of the bacteria (Wang et al., 2017). Given the low nutrient concentrations in *Posidonia* meadows (Cambridge and Hocking, 1997; Gobert et al., 2002), the relevance of such organisms in supporting the nitrogen demands of seagrass is thus intriguing but requires further investigation.

The occurrence of other putative denitrifying bacteria was also evident in the roots from seedlings grown in meadow mix. This included OTUs associated with the *Ignavibacteria* (Bacteroidetes), a metabolically versatile group that are facultatively anaerobic and, as

revealed from the analysis of environmental MAGs, are capable of dissimilatory nitrate reduction to ammonium (DNRA) and the reduction of N_2O to N_2 (Bei et al., 2021). Widely distributed in paddy soils and reported from marine sediments surrounding *H. ovalis* (Tarquinio et al., 2021), the *Ignavibacteria* have been suggested to thrive in N-cycling consortia, contributing to their denitrifying capacity (Liu et al., 2012) and potentially supporting the growth of N_2 fixing bacteria around the root zone. In addition, the *Ignavibacteria* are also reported to decompose complex polysaccharides (e.g. cellulose and hemicellulose), degrade phenylacetate and synthesise trehalose (Bei et al., 2021). Interestingly, phenylacetic acid (PAA - the conjugate acid of phenylacetate) is a growth promoting plant hormone and antimicrobial (Cook, 2019), which naturally occurs in the shoots and on the fruits and seeds of higher plants (Wightman and Lighty, 1982). When added exogenously, it induces root hair growth and upregulates the expression of pathogen defence and growth-related genes (Sumayo et al., 2018).

We observed marked differences in the root architecture of seedlings grown in different substrates. Roots from seedlings grown in play sand had strikingly longer root hairs that adhered tightly to the sediment particles, while those from meadow mix had less root hairs but had greater numbers of lateral roots and elongation zones that were noticeably larger. The formation of extensive root hairs has been previously reported for *P. oceanica* and suggested to represent an early anchoring mechanism on rocky surfaces, despite being also observed in sandy substrates (Badalamenti et al., 2015). Like root hairs, lateral root formation is also evident in *Posidonia*, with *P. australis* forming extensive root systems with long laterals that make up the bulk of the root length and are suggested to act as important anchoring mechanisms, particularly in response to hydrodynamic exposure (Hovey et al., 2012). The processes governing lateral root development in plants is complex, and is tightly coupled to local nitrate concentrations and hormonal (e.g. auxin and abscisic acid) signalling pathways, whereby the presence of nitrate stimulates lateral root elongation, while its accumulation at the root (shoot) interface reduces their formation (Zhang H. et al., 1999; Mantelin and Touraine, 2004). Mantelin and Touraine (2004) proposed that the occurrence of prospective plant growth promoting (denitrifying) bacteria may interfere in these mechanisms by reducing the local nitrate pool sufficiently enough to increase the numbers of lateral roots while limiting the effects on their elongation.

In rice field soils, the phenylacetate degrading capacity of *Ignavibacteria* is induced together with the synthesis of trehalose under micro-oxic conditions, which is thought to act to protect the cells against osmotic stress (Paul, 2018; Bei et al., 2021). This may be particularly important in the root zone of *Posidonia* where high amounts of plant exudates like sucrose may accumulate (as stated above) (Sogin et al., 2022), and are less likely to diffuse away from the roots in finer sediments (Gupta and Mukerj, 2002). Hypothetically, in the case of *Ignavibacteria*, its putative denitrifying capacity would thus also help to support lateral root branching (as needed for improved seagrass anchorage) in exchange for an increased supply of phenylacetate and other carbon sources needed to support its populations. Furthermore,

given that PAA may also be synthesised by other bacteria and fungi as a defence mechanism (for a review see Cook, 2019), its relevance as a phenylacetate degrader in the control of other microbial populations in the root microenvironment may also be relevant.

Enriched in the roots of seedlings were also OTUs representing taxa associated with other biogeochemical processes that are important for supporting conditions that promote seagrass growth and productivity, including the cycling of sulfur and iron. Those connected to the sulfur cycle included representatives of the sulfate-reducing bacteria (as detailed above), as well as known or putative sulfur-oxidising bacteria, such as *Arcobacteraceae*, *Thiovulaceae* and *Sedimenticolaceae* (Waite et al., 2017; Crump et al., 2018; Martin et al., 2019). Reported from the roots of *Zostera marina* (Wang et al., 2021), sulfur-oxidising bacteria are thought to support seagrasses by reducing the toxic sulfide that accumulates from sulfate-reducing bacteria activity and is common in vegetated marine sediments (Borum et al., 2005; Lamers et al., 2013). While play sand roots also contained *Arcobacteraceae* and *Thiovulaceae* (albeit to a lesser extent), the *Sedimenticolaceae* were largely observed in roots from seedlings grown in meadow mix. Moreover, unlike the other sulfur-oxidising bacteria, this family was abundant in the mature roots from Lady Bay. Represented by an OTU most closely associated with *Ca. Thiodiazotropha*, these bacteria are symbionts of lucinid bivalves and have been reported from other seagrasses, including *P. australis* (Martin et al., 2020). Its association with roots is attributed to the close affiliation lucinids have with seagrasses (Van Der Geest et al., 2020) and its capacity to survive outside the host (Martin et al., 2020). Lucinid bivalves occur in *P. australis* meadows around Australia (Barnes and Hickman, 1999) and, although any obvious molluscs were removed from the collected sediment, their occurrence suggests that they were introduced directly from the meadow sediment when preparing the meadow mix, which is further supported by their absence from the play sand.

The colonisation of the roots by sulfur-oxidising bacteria is thought to be influenced by radial oxygen loss (ROL) from primarily young, actively growing root tips, which create oxic-anoxic microzones that also favour the growth of filamentous cable bacteria (Martin et al., 2019) – as likely represented here by the detection of *Ca. Electrothrix*, an organism originally detected from marine sediments (Trojan et al., 2016). Cable bacteria perform electrogenic sulfur oxidation (e-SOx) which not only mediates the impacts of toxic sulfide, but also increases the availability and mobilisation of essential nutrients like iron and phosphorous through dissolution of minerals like FeS from sediment acidification (Brodersen et al., 2017; Martin et al., 2019). Interestingly, *Ca. Electrothrix* was also recently reported from the root surfaces of *P. australis* seedlings and other aquatic plants (potentially as endophytes) where, in oxygen-releasing seedling shoots, it was accompanied by the formation of red iron oxide precipitates on the root surfaces (Scholz et al., 2021). This is consistent with our findings, where similar red deposits were observed on the seedling root surfaces, though only on those from untreated meadow mix. These iron 'plaques' have also been reported from rice and other aquatic plants, and act as a buffer against the uptake of toxic sulfide and heavy metals like cadmium

and arsenic, and as a sink for phosphorous (Zhang X. et al., 1999; Liu et al., 2004; Seitaj et al., 2015; Fu et al., 2018; Scholz et al., 2021). Iron redox changes in the rhizosphere environments of rice and wetland plants are also suggested to create niches for iron bacteria (Emerson et al., 1999; Maisch et al., 2019), and may be reflected here by the detection of OTUs associated with particular magnetotactic bacteria belonging to the family *Magnetospiraceae* (namely *Magnetovibrio* sp.). As organisms that use soluble forms of iron (i.e., Fe^{2+} and Fe^{3+}) for the synthesis of their magnetosomes (Lefèvre and Bazylinski, 2013), their activity is likely coupled to the acidic dissolution activity of FeS by e-SOx bacteria (Sulu-Gambari et al., 2016). While iron is important for growth of plants, it may also be toxic at high concentrations (Mehraban et al., 2008). Although there is no evidence to support this, the uptake of excess dissolved iron by magnetotactic bacteria may ameliorate potential toxicity. Furthermore, given that some *Magnetovibrio* species may support nitrogenase activity (Bazylinski et al., 2013), they may support seedling growth by fixing nitrogen. Given the benefits of iron additions on seagrass survival and growth rates of shoots (Marbà et al., 2007), these and other bacteria contributing to the cycling of iron and other nutrients are potentially important for seagrass health.

4.3 Seedling root bacterial communities with growth and treatment

Despite the limited duration of the growth experiment (12 weeks), changes in the root bacterial assemblages of seedlings were evident between the sampling periods (weeks 4 and 12), indicating that these communities are dynamic during early growth. The most pronounced changes occurred in those grown in play sand, where a marked increase in species (OTU) diversity and evenness was observed between weeks 4 and 12. In contrast, the meadow mix root communities did not change significantly over the sampling period, but instead remained more similar to those belonging to the adult meadow plants, where OTUs represented a greater breadth of bacterial lineages (based on measures of delta+) compared to play sand. As mentioned above, given the differences in the origins of the substrates, such a finding was not unexpected, and likely reflects the differences in the richness and diversity of their respective bacterial 'pools' from which taxa may be sourced for supporting seedling growth. For play sand, where community diversity was already low compared to meadow mix, such marked changes in diversity were likely caused by the introduction and/or enrichment of various taxa following propagation and extended submersion of the potted medium in seawater. This may include organisms like *Rhodobacteraceae* and *Methylophagaceae* that, as discussed above, are known to be surface colonisers of the fruits and/or leaves of other seagrasses like *H. ovalis*, and could have established on the seedlings either before or shortly after release from the fruit (Tarquinio et al., 2021). Their marked enrichment in the play sand samples shortly following propagation (week 4), particularly when compared to the meadow mix samples, may be reflective of this and may be linked to reduced niche competition due to lower initial bacterial species richness (Chu et al., 2021).

Interestingly, while there was a lack of significant changes in measures of diversity in the meadow mix samples, prominent shifts in the abundance of various taxa were observed over the sample period. Alongside the enrichment of certain taxa in week 12 that were also predominant features in the adult meadow roots (namely *Desulfobacteraceae*, Bacteroidetes BD-2, *Sedimenticolaceae*, *Spirochaetacea* and uncultured Ardentcatenales), this included a number of taxa that were conversely less abundant in the adult roots (namely *Flavobacteriaceae*, *Magnetospiraceae*, *Thiovulaceae* and *Rhizobiaceae*). Together, these observations support the notion of seagrasses having transitional bacterial communities dependent on life stage and plant needs, as has already been shown in terrestrial plants (Chaparro et al., 2014; Edwards et al., 2018). For example, during early growth, the enrichment of important nitrogen fixing taxa like *Rhizobiaceae* may be important for the generation of above ground (leaf) tissues, which then generate a sustained supply of energy from photosynthesis, supporting both these early microbial colonisers and its further growth and development. As the plant grows, increases in organic carbon deposition from e.g. root exudation, may lead to changes in the surrounding sediments that stimulate sulfate reduction and the colonisation of the root tissues by sulfate-reducing bacteria, like *Desulfobacteraceae*, which, as stated above, may also serve as potential diazotrophs to further support seagrass productivity.

Indeed, while both sediment types favoured roots over shoots and displayed ratios like those reported for other *Posidonia* seedlings using traditional root:shoot biomass ratios (Statton et al., 2014), seedlings grown in meadow mix showed greater investments in leaf growth (based on lower pseudo root:shoot ratios) compared to those in play sand, which instead allocated more energy to growing roots. Despite both strategies being advantageous, where greater resource allocation to the roots may increase seedling anchorage and access to sedimentary nutrients, more leaves provide greater photosynthetic potential and increased access to water soluble nutrients. Nonetheless, the occurrence of such varied growth strategies was somewhat unexpected given that seed size was the most important predictor of growth in our study; a feature that likely reflects the reliance of *Posidonia* seedlings on initial seed reserves, as reported elsewhere (Hocking et al., 1981; Statton et al., 2013; Statton et al., 2014). Accordingly, it is tempting to postulate that the mode of growth during early establishment is thus likely driven by other substrate type and the associated microbial consortia. This may include the enrichment of nitrogen fixing bacteria together with other taxa that may support metabolic activity (e.g. the iron bacteria *Magnetospiraceae*, as stated above). Indeed, given the comparably reduced abundance of these and other putatively beneficial taxa (e.g. Bacteroidetes) from the roots of seedlings grown in the autoclave treated meadow mix (and a concomitant shift towards greater investment in leaves rather than roots in this treatment group), such a prospect is intriguing and requires further elucidation. This follows the ability of autoclaving to alter community composition by reducing the number of viable bacterial cells and their activity in marine sediments (Otte et al., 2018). However, given that autoclaving can also affect nutrient bioavailability (despite the lack of obvious

impacts on macronutrients here), and is intrinsically coupled with the surrounding bacterial consortia (Hayat et al., 2010), further efforts are required to disentangle the mechanisms that underpin the adoption of a specific growth strategy. This may include assessing whether *Posidonia* seedlings potentially 'nurture' the selection and enrichment of certain microbes that may support growth under altered nutrient conditions.

4.4 Relevance for restoration

Our two sample periods showed a gradual, yet significant, change to the bacterial communities that was possibly driven by the individual seedling's exudates. There is growing evidence that the microbiome is highly dynamic during a plant's seedling phase and relatively stable when the plant reaches maturity (Chaparro et al., 2014; Edwards et al., 2018). This indicates the importance of early exposure to diverse or known beneficial marine bacteria in seagrass revegetation sediment and these bacteria could stay with the plant for life and vary in response to the plant's needs. Indeed, our results highlight important effects of adding sediment from natural seagrass stands on the microbiome of *P. australis* seedlings, increasing diversity and adding many potentially beneficial taxa. They also document clear impact on the growth patterns of the seagrass. However, these potentially positive effects should be carefully interpreted. Local *in situ* restoration trials in the Greater Adelaide metropolitan area over the last four years that added organic matter show that, while sediment type may influence growth, it did not affect growth or survival (Irving et al., 2010; Tanner and Theil, 2016). While it is possible that the sudden addition of carbon in these trials disrupted the microbes and forced a rapid change in the community structure that was detrimental to the developing seedlings, this highlights that further experiments are needed to confirm the relevance of adding sediment from natural seagrass stands for conservation success. Such experiments should move beyond the 12-week stage of seedlings. Furthermore, *Posidonia* seedlings naturally recruit to restored *Amphibolis* patches (Tanner and Theil, 2016) and can grow well in the presence of macroalgae (Pereda-Briones et al., 2018). Hence, *Posidonia* spp. are climax species (Bryars and Neverauskas, 2004), i.e., arrive at the later stages of ecological succession, and setting root among other marine plants may allow species of this genus to gain parts of the bacterial community that has developed during the earlier successional stages. Therefore, it may be necessary to utilise the natural succession process during restoration. Nevertheless, with the findings from our limited growth experiment also pointing to the involvement of specific bacterial constituents in early growth, it is tempting to speculate on a future role for the direct application of such microbes (as potential plant probiotics) for supporting seedling vigour and improved resilience. However, given the limited cultivability of environmental microbes, renewed efforts will likely be required to overcome the inherent challenges associated with their isolation and maintenance (Kapinusova et al., 2023).

5 Conclusion

Modification of root bacterial assemblages, whether through changes in the propagation substrate or through alteration by autoclaving, appears to affect the growth of *P. angustifolia* seedlings. Bacterial communities therefore appear to be an important consideration for the restoration of this late-successional species. Our findings suggest that early exposure of seedlings to the bacteria found in a seagrass community may provide benefits for future *Posidonia* restoration. Seedlings grown in a more natural substrate that was amended with seagrass meadow sediment (a meadow mix) possessed communities that were highly diverse and more similar to the natural environment, with many taxa likely involved in essential nutrient cycling processes. In contrast, the less natural play sand sediment had lower diversity and comprised taxa that were not typical of marine systems, and which may have varied functions. Future restoration efforts therefore may benefit from using propagating substrates that incorporate natural microbial populations associated with healthy seagrass meadows to promote the formation of rhizobiomes that comprise beneficial bacteria that will support early seedling growth. Given the limited duration of our trial, the relevance of the early establishment of bacterial assemblages on seedling growth and vigour requires further elucidation (beyond 3 months) and should be supported by their performance under different experimental conditions (e.g. varied temperature, salinity) as well as in the natural environment following their transplantation as part of restoration trials. This includes the functional assessment of particular bacterial taxa in key nutrient cycling processes, with the view to identify those that may enhance restoration, perhaps in the form of an inoculum that could be added to the seedling or hessian sacks before deployment.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/[Supplementary Material](#).

Author contributions

AR: Conceptualization, Formal Analysis, Methodology, Writing – original draft, Writing – review & editing, Investigation. JT: Conceptualization, Methodology, Writing – review & editing, Funding acquisition, Project administration, Resources, Supervision. MW: Methodology, Writing – review & editing, Formal Analysis. SC: Formal Analysis, Writing – review & editing, Investigation. GK: Formal Analysis, Writing – review & editing, Methodology, Supervision. AO: Formal Analysis, Methodology, Supervision, Writing – review & editing,

Conceptualization, Data curation, Project administration, Resources, Writing – original draft.

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Conflict of interest

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

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Supplementary material

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fmars.2023.1278837/full#supplementary-material>

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Restoration performance of *Thalassia hemprichii* and *Enhalus acoroides* at Gaolong bay and Xincun lagoon, Hainan Island, China

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Introduction: Seagrasses play a key role in biodiversity maintenance, sediment stabilization, and carbon reduction. Due to the significant decline of seagrass populations worldwide, many projects to restore seagrass by transplantation have been undertaken in recent decades. However, effective restoration technologies required to rapidly restore seagrass on Hainan Island are yet to be developed.

Methods: In this study, an experiment was carried out to investigate methods for alleviating continuous degradation of seagrass beds on Hainan Island. Seagrass were transplanted by “single-plant-fixed-distance transplantation” method, survival rate, shoot formation rate, coverage and shoot density of transplanting plants were monitored for 23 months.

Results: Over 67% of *T. hemprichii* and 90% of *E. acoroides* were still alive, shoot density were over 11 shoots/m² and 15 shoots/m² respectively after almost two years, *E. acoroides* was more likely to survive than *T. hemprichii* both in Gaolong Bay and Xincun lagoon. Because of the damage to below-ground tissues, the survival rate, shoot formation rate, coverage and shoot density decreased during the first one year after transplantation, then increased gradually.

Discussion: We considered transplantation established successfully of *T. hemprichii* and *E. acoroides* in the new site was about 12 months. In addition, *T. hemprichii* was suitable for transplanted in Xincun lagoon, and *E. acoroides* was suitable for transplanted in Gaolong bay. The results demonstrated that the growth of transplanted seagrass was greatly affected by suspend solids and DIN. The results of this study may provide data support and scientific basis for seagrass transplantation in South China.

KEYWORDS

Thalassia hemprichii, *Enhalus acoroides*, Gaolong bay, Xincun lagoon, seagrass restoration

1 Introduction

Seagrasses are flowering plants that grow predominantly in coastal areas, such as bays and lagoons. They play an important role in marine ecosystems (Duarte and Krause-Jensen, 2017). The seagrass can reduce water flow, stabilize sediment. Seagrass in coastal waters provide habitat and nursery for fish and invertebrates (Duarte and Chiscano, 1999), supply food for large herbivores (Klumpp et al., 1993). Also, seagrass play an important role in the nutrition transfer, nitrogen fixation and maintain the physical and chemical properties of marine organisms (Van de Koppel et al., 2015; Lee et al., 2016; Nordlund et al., 2016; Jahnke et al., 2019).

However, seagrass beds can be changed by both natural and anthropogenic influences (Tan et al., 2020). Physical disturbance, bitten by herbivore, intraspecific competition in seagrass, pollution of nutrients and flood waters with sediment may influence the biomass, distribution area and species composition of seagrass bed (Rasheed, 2004; Rasheed et al., 2014). Anthropogenic influences, such as coastal development, runoffs from coastal urban and agricultural, discharge of untreated sewage and industrial wastewater, have led to extensive losses of seagrasses shallows located near estuaries and coasts (Huang et al., 2006; Waycott et al., 2009; Arias-Ortiz et al., 2018). This reinforces the need for seagrass restoration that would lead to a reduction in the loss of seagrass and an enhancement of biodiversity in these important coastal ecosystems.

Various of seagrass habitat restoration methods have been investigated in the past years, dependent on species (Fonseca et al., 1983). The common restoration methods include habitat restoration (Tan et al., 2020), seed dispersal, and transplantation. Habitat restoration may be a very slow process, taking a long time. Since the high survival rate and growth rate, transplantation has been considered as an effective way to slow down the degradation of seagrass (Davis and Short, 1997; Orth et al., 1999; Fishman et al., 2004; Paling et al., 2007; Park and Lee, 2007; Bastyan and Cambridge, 2008). In the past few decades, transplantation have been developed and attempted in many countries (Li et al., 2010), and several transplantation have achieved successful, including transplantation of “sod”, “plug” and “peat pots” (all the plant materials and surrounding sediment were put into a peat pot, then planted into sediment with an artificial supporting medium or not), “staples” or “sprigs” (a length of rhizome including roots and shoots, fixed into the sediment by a wire staple) (Keulen et al., 2010). These seagrass transplantation methods have also been tried on *Zostera marina* (Short et al., 2002), *Posidonia oceanica* (Calvo et al., 2021; Mancini et al., 2021), *Zostera noltii* (Van Katwijk et al., 2009), *Thalassia testudinum* (Thorhaug, 1985), *Halodule wrightii* (Phillips, 1974), *Syringodium filiforme* (Thorhaug, 1985), *Posidonia sinuosa* (Paling et al., 2001), *Amphibolis griffithii* (Paling et al., 2001), *Posidonia australis* (Bastyan and Cambridge, 2008), *Halophila ovalis* (Kirkman, 1998) in the United States, Western Australia and North America. In Korea, *Zostera marina* become the most widely distributed seagrass species after transplantation (Park and Lee, 2007; Lee and Park, 2008). A few seagrass restorations have been trialed in China recent years. The restoration species include

H. ovalis, *Zostera japonica*, *Z. marina* in Shandong province (Zhang et al., 2020). In Guangxi Zhuang Autonomous Region, the most suitable species for seagrass transplantation are *Z. japonica*, *H. ovalis* and *Halophila beccarii*, and the plug of transplantation method is more practical in the intertidal zone of Guangxi because of the higher survival rate (Qiu et al., 2014).

The total seagrass area of China (except Sansha city) is 11113.4 ha. The seagrass meadow in Hainan Province covers an area of 4457.0 ha, accounting for 40.0% of the total area (Li et al., 2022). It is mainly distributed in the eastern and southern coast of Hainan Province, such as Wenchang, Qionghai, Lingshui, and Sanya (Huang et al., 2006; Jiang et al., 2017). Due to human activities, such as artificial island construction, aquaculture and fishing, eutrophication of sea water (Ruiz et al., 2001; Moksnes et al., 2008; Fang et al., 2018; Cui et al., 2021), the meadows have been declining over the last year (Jiang et al., 2017). However, few studies focus on the transplantation methods and continuous measurement of seagrass in Hainan province.

In this study, *Thalassia hemprichii* and *Enhalus acoroides* shoots were transplanted into Gaolong bay and Xincun Lagoon in May 2019 to establish seagrass transplantation in Hainan province. The physiological status of the transplanting were monitored every 4 months for a year. The survival rate, shoot formation rate, coverage and shoot density of transplants were monitored every 3 months for 23 months. The successful application of the transplants techniques not only provide practical guide for the meadow restoration in South China, but also provide valuable references for other seagrass species restoration.

2 Materials and methods

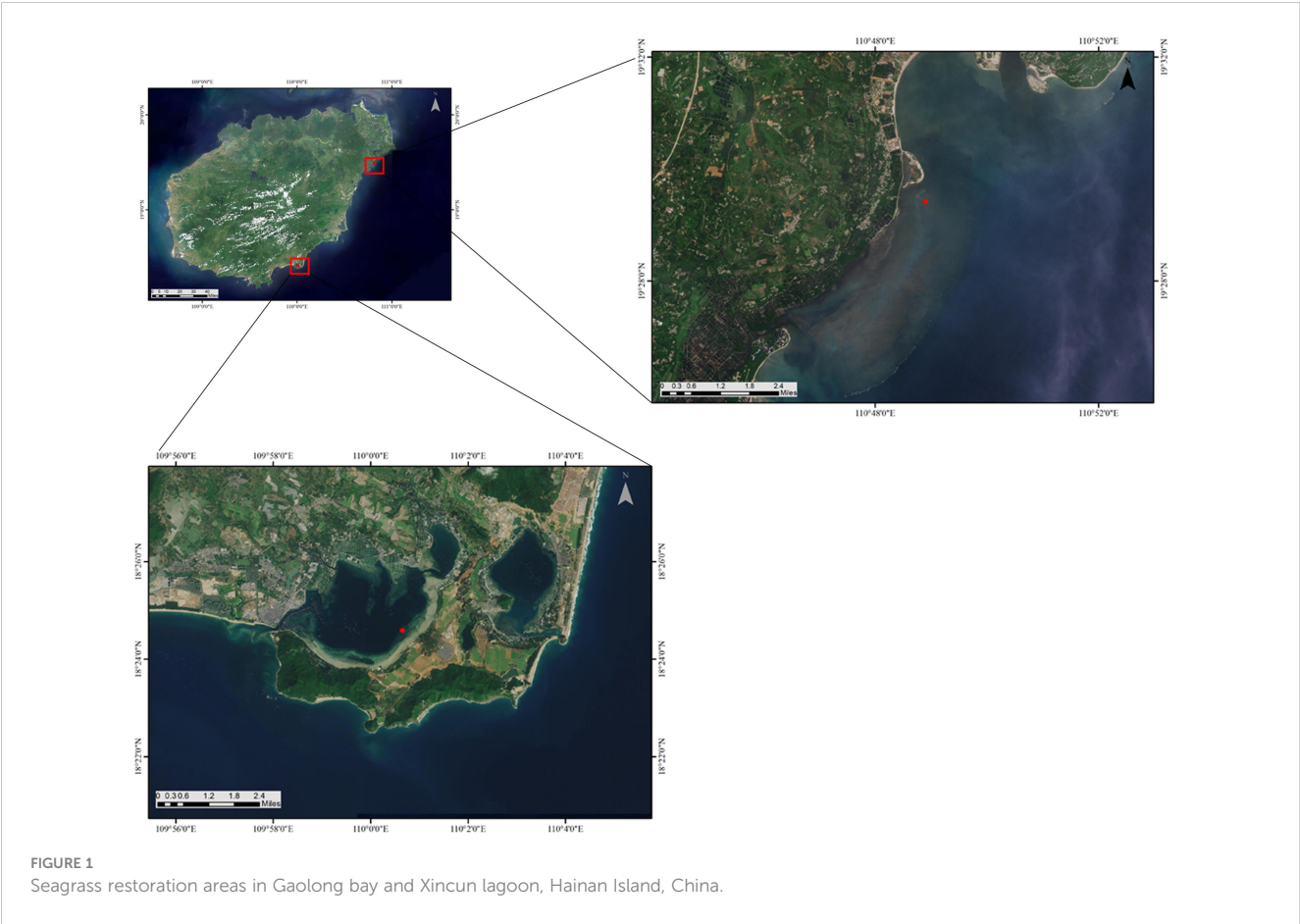
2.1 Study area

Gaolong bay (19°29′24.91″N, 110°48′53.66″E) is an open shallow water area with high salinity which located off the northeast coast of Hainan Island, China. The water exchanges well, and with a good self-purification ability. The bottom materials of Gaolong bay are predominantly coral debris, gravel, and sand (Zhou et al., 2012). The mainly human activities are fishing and building artificial islands.

Xincun lagoon (18°24′35.41″N, 110°0′38.90″E) is located on the southeast coast of Hainan Island. Relatively, the water of Xincun lagoon has low salinity and poor self-purification ability. The bottom material is sand-mud (Moksnes et al., 2008). Fishing, cage culture and ship anchoring are common in Xincun lagoon (Figure 1; Table 1).

2.2 Collection of transplanting shoots

The branching rate of rhizomes is an important indicator of seagrass cloning ability, a high branching rate indicating strong seagrass cloning ability (Sintes et al., 2005). The branching and rhizome elongation rates of *T. hemprichii* are higher than *E. acoroides* (Figure 2) (Marbà and Duarte, 1998). Although *T.*



hemprichii and *E. acoroides* were not the only dominant seagrass species in Gaolong bay or Xincun lagoon, they possess strong adaptability and high survival rates. Therefore, they were selected for seagrass restoration in this study.

In order to reduce the destruction of seagrass original habitat, meadows with areal extent more than 30 m² and coverage greater than 80% were selected for donating shoots (Fang et al., 2018). All the donor shoots were collected from a healthy seagrass bed located

in Lingshui Xincun lagoon seagrass special protected area (18°24' 42''N, 109°57'58''E), Hainan, China. The shoots were collected individually manually to reduce damage to the seagrass bed. Each transplanting shoot had three to five rhizome nodes and healthy leaves. The collected transplanting shoots were temporarily stored in large boxes with small quantity of cold seawater during transport to the transplanting location, preventing shoots desiccation. All the transplanting shoots were transplanted within 24 h.

TABLE 1 Comparison of habitats in Gaolong bay and Xincun lagoon.

Research area	Bodies of water	Sediment	Water quality Dominant seagrass	Aquaculture	Number of fishing vessels in the area	Recreational activities	Land reclamation	Pollution emission
Gaolong bay	open Bay	coral debris, gravel, sand	<i>Thalassia hemprichii</i> , Class I sea water and <i>Enhalus acoroides</i>	fish, shrimp, crab	2517 ships	beach excursions	construction of artificial island	aquaculture sewage
Xincun lagoon	coastal lagoon	mud- sand	<i>Thalassia hemprichii</i> , Class II sea water and <i>Enhalus acoroides</i>	fish, shrimp, mussels, <i>Eucheuma muricatum</i> , crab	1545 ships	Monkey Island equipped with a marina	/	aquaculture sewage, sewage and household wastes

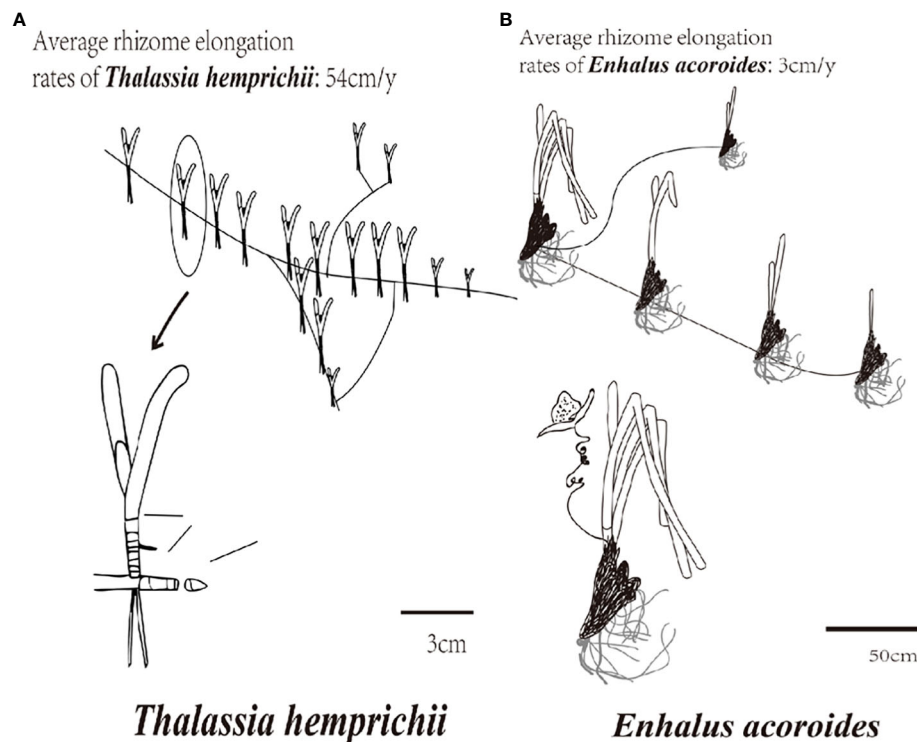


FIGURE 2

Branching rate and rhizome elongation rate of *T. hemprichii* and (*E. acoroides*). (A) *T. hemprichii*; (B) (*E. acoroides*).

2.3 Transplantation method

To compare the restoration performance of *T. hemprichii* and *E. acoroides* in Gaolong bay and Xincun lagoon, we selected the transplant sites with similar habitat conditions.

The transplanting shoots were planted manually by scuba divers through the frame method. We designed a fixing technique represented by an iron transplanting frame specifically. The iron transplanting frame was 1 × 1 m, and divided internally into sixteen small squares evenly, one transplanting shoots in each square. A layer of fishing nets was attached on the surface of the transplanting frame by cable ties. Four 15 cm triangular irons legs were welded to the bottom of the transplanting frame at each of the corners. The transplant frame was inserted into bottom materials to make sure the transplanting shoots were anchored to the sediment (Figure 3).

Sixteen transplanting shoots were planted in each 1 × 1 m transplanting frame, named transplanting unit. The transplanting unit was laid out in regular patterns, with the distance was 50 cm. We called this “single-plant-fixed-distance transplantation”, which was easy to conduct and less damage to surrounding habitats (Piazzi et al., 2021).

From March to April 2019, 90 transplanting units of *T. hemprichii* (including 1,440 transplanting shoots) and 10 transplanting units of *E. acoroides* (including 160 transplanting shoots) were transplanted in Gaolong bay, while 40 transplanting units of *T. hemprichii* and 60 transplanting units of *E. acoroides* were transplanted in Xincun lagoon, including 640 and 960 transplanting shoots respectively.

After transplantation, each transplanting unit was marked with serial numbers, transplantation time and other information were recorded. All the transplanting units placed were surrounded with plastic garden mesh (5 × 5 cm mesh size).

2.4 Environmental parameters

Water temperature was monitored by Pen Type Water Quality Meter (C-600, AZ Instrument Corp., Taiwan, China). To determine water salinity, suspended solids, dissolved inorganic nitrogen (DIN = nitrate + nitrite + ammonium) and dissolved inorganic phosphate (DIP), three replicate surface water samples were collected every 4-months by 200ml bottles. Water samples were stored on ice, then transport to the laboratory. Each water sample was filtered through Whatman GF/F filters (0.7 μm pore size) immediately. The content of suspended solids in the water was determined by weighing method. The DIN and DIP contents of the seawater samples were measured using standard colorimetric techniques by a spectrophotometer (TU-1900, Metash Instruments Co., Ltd, Shanghai, China). Nitrate, nitrite, ammonium and DIP were measured by zinc cadmium reduction method, hydrochloride naphthodiamide method, hypobromite oxidation method, and phosphorus molybdenum blue spectrophotometry, respectively (General Administration of Quality Supervision, 2008). Salinity was monitored using a Lab Salinometer (SYA2-2, National Ocean Technology Center, Tianjin, China).

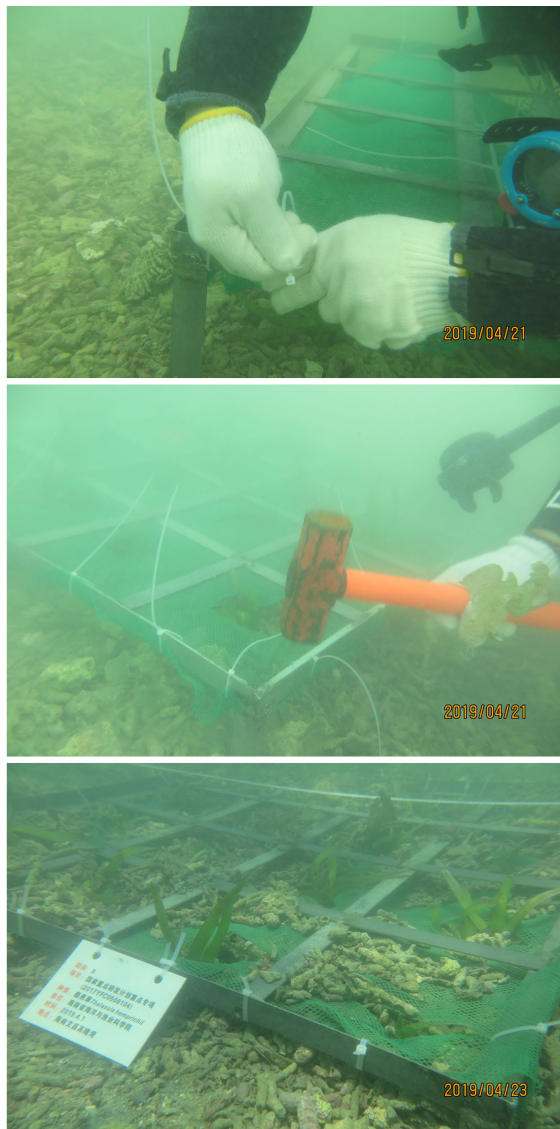


FIGURE 3
The “single-plant-fixed-distance transplantation” method and its underwater application.

2.5 Monitoring of transplantation

Statistical analyses were performed on monitoring the survival rate, shoot formation rate, coverage and shoot density of transplantation every 2 or 3 months for 23 months, from May 2019 to Mar 2021. Due to the Covid emergency, there was no monitoring in Dec 2020.

Survival rate (Renouard and Nisand, 2006): The percentage of living shoots after transplanting establishment. The survival rate of transplantation, which is the average of each transplanting unit, was calculated as follows:

$$L = \sum_{i=1}^i \frac{1}{i} (U_i/U) \times 100 \%$$

In formula: L is seagrass survival rate (%), U_i is the number of surviving shoots in the i th transplanting unit, and U is the total number of transplanting shoots in each transplanting unit.

Shoot formation rate: The percentage of transplanting rhizomes with newly shoots. The shoot formation rate was estimated by counting the number of transplanting rhizomes with newly shoots or not respectively in each transplanting unit. The specific calculations were as follows:

$$B = \sum_{i=1}^i \frac{1}{i} (N_i/U) \times 100 \%$$

where, B is the seagrass propagation rate (%), N_i is the number of transplanting rhizomes with newly shoots in the i th transplanting unit, and U is the total number of shoots in each transplanting unit. **Coverage:** The percentage of vertical projected area of vegetation (including stems, branches and leaves) on the ground against the total area of the transplanting unit. The coverage was estimate through a photogrammetry-based Chinese patent “A method for determining vegetation coverage based on grb and gridlines”. Firstly, suit images of each transplanting unit were acquired by scuba divers (Figure 4A). The color value of each pixel was changed into Visible-band Difference Vegetation Index (VDVI) ($VDVI = (2 \times G - R - B)/(2 \times G + R + B)$), which is designed to distinguish green vegetation (Wan et al., 2018; Ventura et al., 2022). Depending on threshold setting to 0.25, an image object was determined whether belonged to cover class, then this pixel was defined “vegetation point” (Figure 4B). The suit image was added grid lines of 20×20 pixels (Figure 4C). To distinguish the vegetation from sediment, we set two threshold values. When the “vegetation point” coverage was over 80% of a grid, the grid would be defined “vegetation grid”. If the “vegetation point” coverage was less than 20%, the grid would be defined “background grid”. Finally, the coverage was estimated by calculating the ratio of “vegetation grids” to total grids (Figure 4D).

Shoot density (Mancini et al., 2021): The number of shoots/ m^2 . It was estimated by counting the number of surviving shoots in each transplanting unit (1×1 m).

2.6 Statistical analyses

All values were expressed as mean $\pm$ standard error. Microsoft office software was used to organize data and plot histograms. Statistical analyses were performed using SPSS 26.0 (SPSS Inc., Chicago, Illinois, USA). Data were tested for normality and homogeneity of variance to meet the assumptions of parametric statistics. Significant differences among sampling months were tested using a one-way analysis of variance (ANOVA) and least significant difference (LSD). Pearson correlation analysis was used to analyze the correlation of each parameter.

3 Results

3.1 Environmental parameters of transplanted site

The water temperature showed a clear temporal variation, it was high during summer and low during winter and spring. In Gaolong bay, the highest value was 30.12° in Aug 2019, and lowest value was

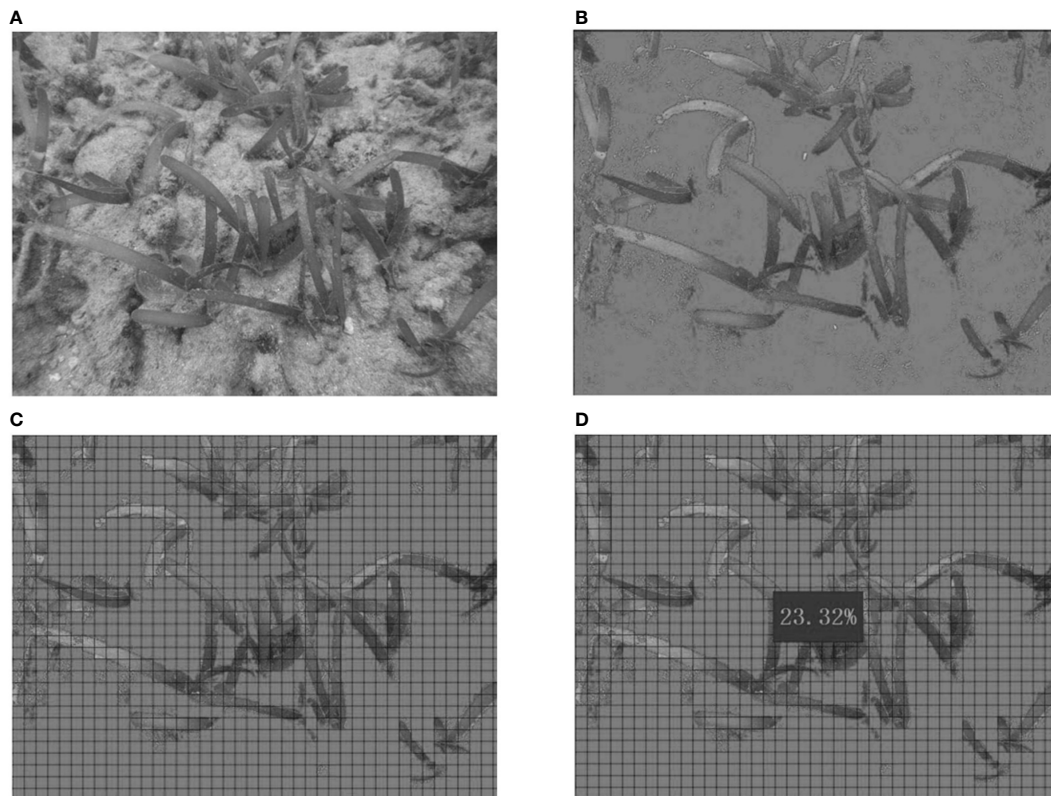


FIGURE 4

The method for determining vegetation coverage based on grb and gridlines. (A) the suit images of transplanting unit; (B) the color value of each pixel in the suit images was changed into VDI; (C) the grid lines of 20 × 20 pixels were added on image; (D) the coverage was estimated.

26.1° in Dec 2019. In Xincun lagoon, the highest value was 31.80° in Aug 2019, but the lowest value was 29.90° appeared in Apr 2020. The average of water temperature changed significantly ($P < 0.001$) both in Gaolong bay and Xincun lagoon with sampling every 4-months (Figure 5A; Supplementary Table S1).

The salinity was changed significantly ($P < 0.001$) in Gaolong bay, the lowest value was 32.5723 in Apr 2019 while the highest was 33.9659 in Aug 2019. The salinity in Xincun lagoon showed the same variation pattern, with the highest value of 33.3534 in Aug 2019, but the lowest value 31.7700 appeared in Apr 2020 (Figure 5B; Supplementary Table S1). Overall, the salinity in summer was slightly higher than in other seasons, due to the rainfall and temperature.

The contents of suspend solids were increased from spring to winter both in Gaolong bay and Xincun lagoon. The highest value

were 13.2733 mg/L and 8.5033 mg/L respectively in Gaolong bay and Xincun lagoon in Dec 2019, while the lowest values were 5.5000 mg/L in Gaolong bay in Apr 2019 and 4.7233 mg/L in Xincun lagoon in Apr 2020 (Figure 5C; Supplementary Table S1).

The water inorganic nutrients (DIN and DIP) concentrations were showed significantly ($P < 0.001$) temporal variation in Gaolong bay. DIN concentration was highest (0.1077 mg/L) in Aug 2019 and lowest (0.0120 mg/L) in Apr 2020 (Figure 5D; Supplementary Table S1). DIP concentration was highest (0.0081 mg/L) in Aug 2019 and lowest (0.0012 mg/L) in Apr 2019 (Figure 5E; Supplementary Table S1). The water inorganic nutrients in Xincun lagoon were changed significantly ($P < 0.001$) with sampling every 4 months, but did not show seasonal variation pattern (Figures 5D, E; Supplementary Table S1). In Gaolong bay, the higher water inorganic nutrients

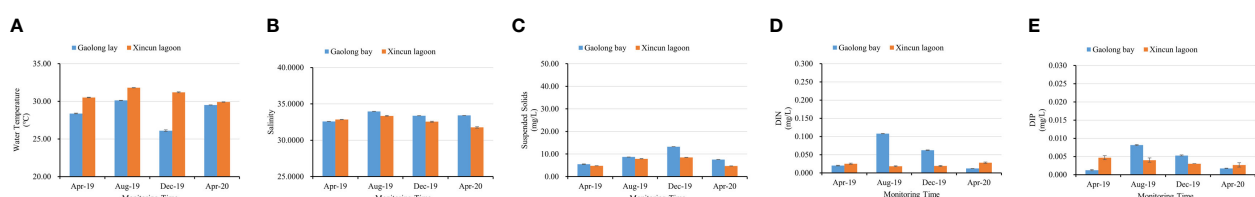


FIGURE 5

Seasonal changes in environmental parameters of transplanted site from Apr 2019 to Apr 2020. (A) water temperature; (B) salinity; (C) suspend solids; (D) DIN; (E) DIP.

concentrations in summer may be related to the water pollution caused by over culture fisheries.

3.2 Comparison of transplantation performance of *T. hemprichii*

The survival rate of *T. hemprichii* transplanted in Gaolong bay and Xincun lagoon showed the same variation pattern. It was decline sharply at the first 13 months, then increased gradually. The survival rate of *T. hemprichii* transplanted in Gaolong bay was highest (90.42%) in May 2019 and lowest (56.18%) in Jun 2020, the highest and lowest value were 99.53% and 51.25% respectively in Xincun lagoon. The survival rate of *T. hemprichii* approached 70% by Mar 2021 both in Gaolong bay and Xincun (Figure 6A).

The shoot formation rate of *T. hemprichii* decreased firstly, the lowest value appears in Nov 2019 in Gaolong bay and from Nov 2019 to Jun 2020 in Xincun lagoon. Then the shoot formation rate of *T. hemprichii* increased rapidly, especially transplanted in Gaolong bay. After 18 months, the shoot formation rate of *T. hemprichii* transplanted in Xincun lagoon decreased sharply in Oct 2020, and it was only 2.03% in Mar 2021, while the shoot formation rate of *T. hemprichii* transplanted in Gaolong bay was as high as 66.25% (Figure 6B).

Coverage and shoot density are important indicators of seagrass community monitoring (Hashemi et al., 2005). The coverage and shoot density of *T. hemprichii* transplanted in Gaolong bay and Xincun lagoon were changed slightly, and with no obvious variation trend. After 23 months, the coverage of *T. hemprichii* transplanted in Gaolong bay and Xincun lagoon were 5% and 8.1%, the shoot density was 11.26 shoots/m² and 14.9 shoots/m², respectively (Figures 6C, D).

The survival rate, shoot formation rate, coverage and shoot density changed significantly ($P < 0.001$) with sampling every 2 or 3 months (Supplementary Tables S2, S3).

3.3 Comparison of transplantation performance of *E. acoroides*

The survival rate of *E. acoroides* transplanted in Gaolong bay and Xincun lagoon showed a trend of slight decline and then rise. The survival rates were all above 90% except Apr 2020 in Gaolong bay. The survival rates were highest in Nov 2019 in Gaolong bay (100.00%) and in May 2019 in Xincun (99.17%), the lowest value appeared in Apr 2020 in Gaolong bay (88.75%) and in Jun 2020 in Xincun (90.63%) (Figure 6E).

The shoot formation rate was less than 5% until summer 2020 both in Gaolong bay and Xincun lagoon, then increased gradually. The highest value arose in Mar 2021, 14.38% and 11.35% respectively in Gaolong bay and Xincun lagoon (Figure 6F). The shoot formation rate changed significantly ($P < 0.001$) with samplings.

The coverage of *E. acoroides* transplanted in Gaolong bay declined slightly before Aug 2019, the value was 19.70%. Then the coverage increased gradually, maintain the coverage at 21.90% from Apr 2020. In Xincun lagoon, the coverage declined from 19.88% to 14.97% until Jun 2020, then increased to 16.18% in Mar 2021 (Figure 6G).

The shoot density of *E. acoroides* declined first, and then rises both in Gaolong bay and Xincun lagoon, and the mean shoot density remained around 15 shoots/m². In Mar 2021, the shoot density was 15.50 shoots/m² and 14.90 shoots/m² respectively in Gaolong bay and Xincun lagoon (Figure 6H).

The difference of survival rate, coverage and shoot density among samplings was not significant ($P > 0.05$) (Supplementary Tables S2, S3).

3.4 Analysis of significant differences between species in the same region

Independent sample t-test was used to analyze the survival rate, shoot formation rate, coverage and shoot density of *T. hemprichii* and *E. acoroides* after 23 months of transplantation.

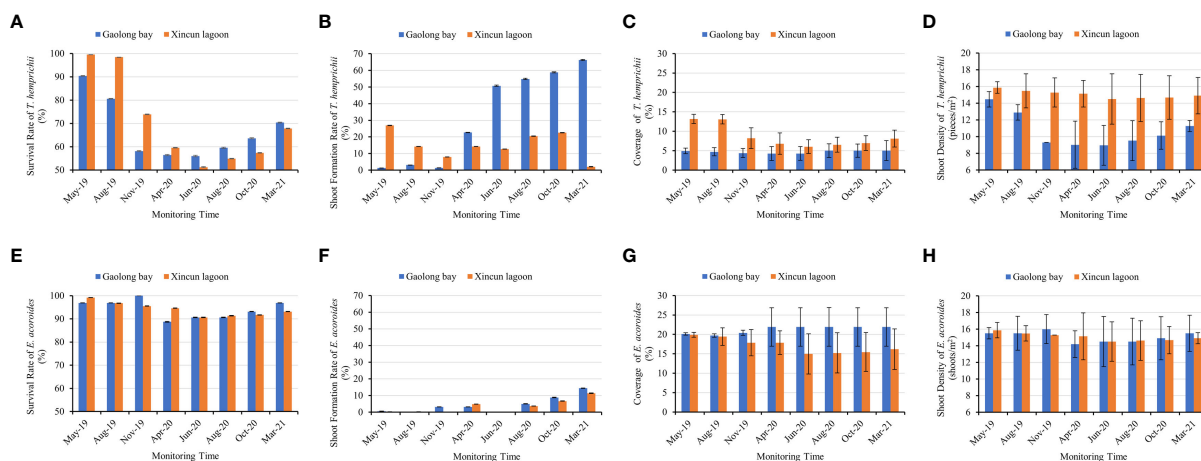


FIGURE 6

Comparison of the survival rate, shoot formation rate, coverage, and shoot density between *T. hemprichii* and *E. acoroides* in Gaolong bay and Xincun lagoon. (A) the survival rate of *T. hemprichii*; (B) the shoot formation rate of *T. hemprichii*; (C) the coverage of *T. hemprichii*; (D) the shoot density of *T. hemprichii*; (E) the survival rate of *E. acoroides*; (F) the shoot formation rate of *E. acoroides*; (G) the coverage of *E. acoroides*; (H) the shoot density of *E. acoroides*.

Both in Gaolong Bay and Xincun lagoon, the survival rate, coverage and shoot density of *E. acoroides* were significantly higher than that of *T. hemprichii* ($p < 0.0001$). The results indicating that *E. acoroides* was more likely to survive than *T. hemprichii* both in Gaolong bay and Xincun lagoon. Interestingly, *T. hemprichii* showed a stronger shoot formation ability, the shoot formation rate of *T. hemprichii* was significantly higher than that of *E. acoroides* ($t = 7.717$, $p < 0.001$).

3.5 Analysis of significant differences between regions of the same species

Independent sample t-test was used to analyze the survival rate, shoot formation rate, coverage and shoot density of in Gaolong bay and Xincun lagoon after 23 months of transplantation.

There was no significant difference in survival rate and shoot density of *T. hemprichii* between transplanted in Gaolong bay and Xincun lagoon ($p > 0.05$). But the shoot formation rate in Gaolong bay was significantly higher than in Xincun lagoon ($t = 8.601$, $p < 0.0001$), the coverage showed the opposite result ($t = -3.941$, $p < 0.0001$).

Similarly, there was no significant difference in survival rate, shoot formation rate and shoot density of *E. acoroides* between transplanted in Gaolong bay and Xincun lagoon ($p > 0.05$). Only the coverage of Gaolong bay was significantly higher than that of Xincun lagoon ($t = 0.042$, $p = 0.008$).

Considering the final effectiveness of transplantation, we thought that *T. hemprichii* was suitable for transplanted in Xincun lagoon, and *E. acoroides* was suitable for transplanted in Gaolong bay (Figure 7).

3.6 Correlation analysis

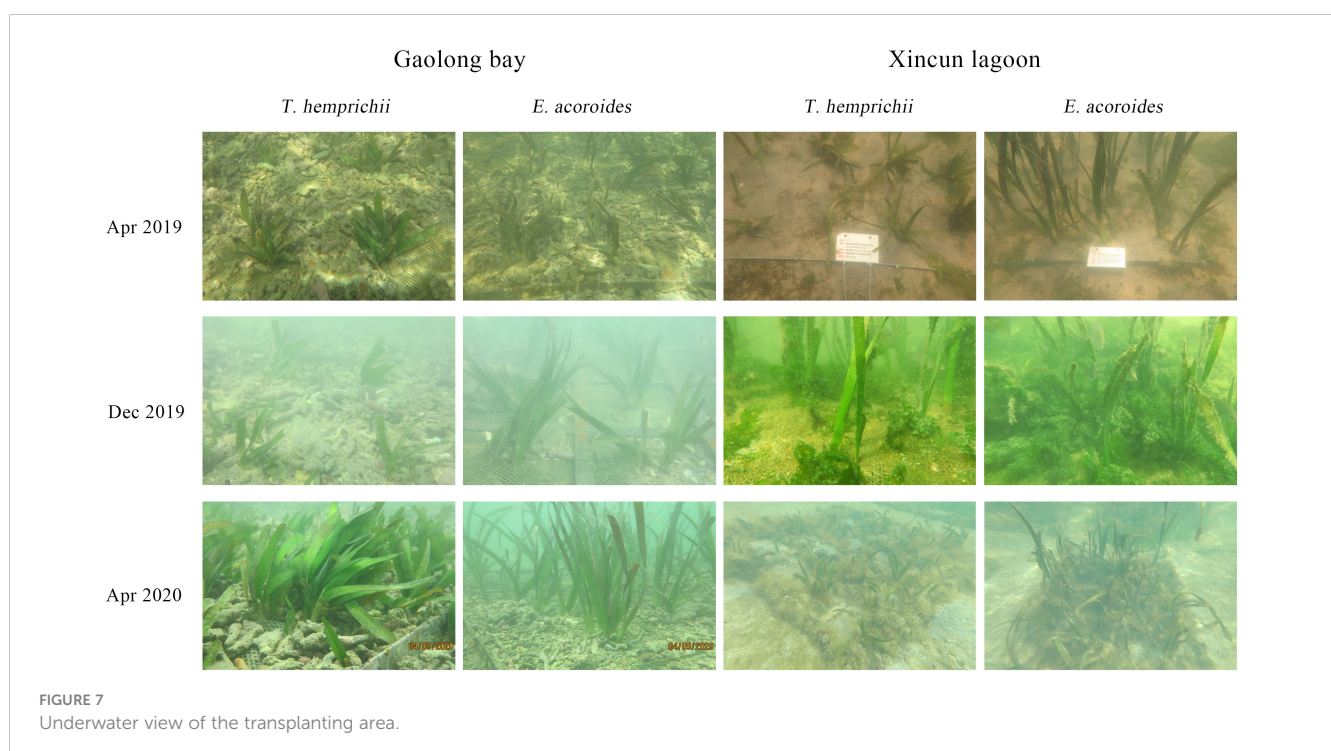
The survival rate, shoot formation rate, coverage and shoot density of transplantation were taken into pertinence analysis with environmental parameters. The results showed that, there was no relationship of contents of suspend solids, DIN, DIN, and N:P ratio with survival rate, coverage and shoot density of transplantation in Gaolong bay (Figure 8A). While in Xincun lagoon, content of suspend solids and DIN had both significant negative correlations with shoot density (Figure 8B).

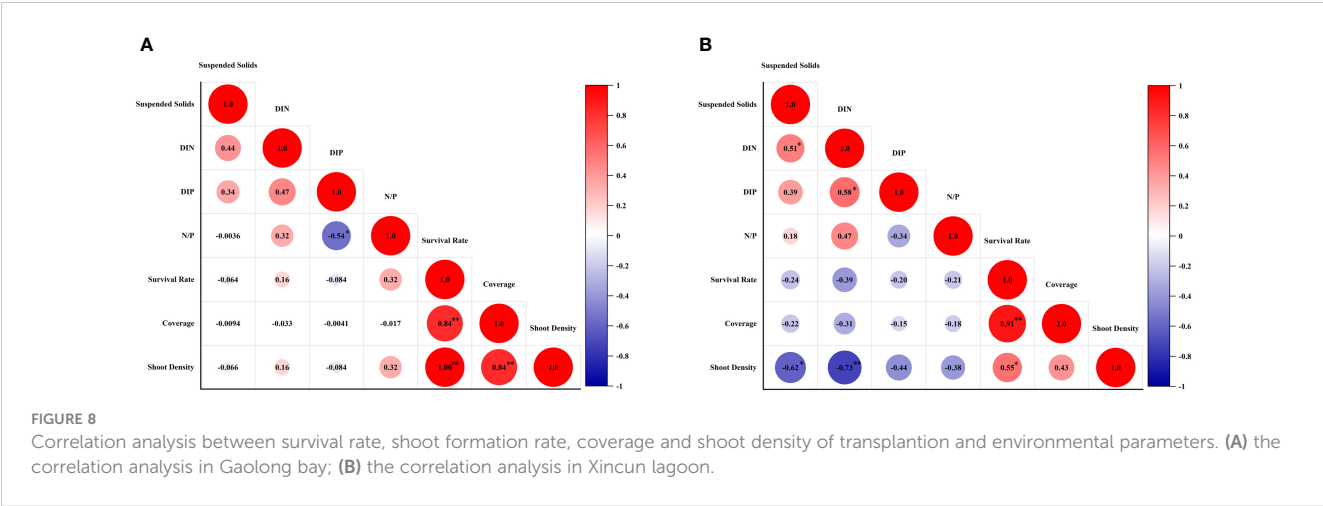
4 Discussion

4.1 The parameters in assessing success of seagrass transplantation

In order to promote effective restoration, there must be a clear definition of the success of seagrass transplantation. A number of criteria were used to evaluate the success of seagrass transplantation (Table 2). Usually, more than one parameter was considered to assess whether the ecological function of seagrass have been recovered after transplanted. Many parameters of habitat functions, such as abundance, composition, complexity of benthic organisms of the seagrass community and macroalgal were monitored in seagrass transplantation studies (Fonseca et al., 1996). We monitored the survival rate, shoot formation rate, coverage and shoot density to examine establishment of seagrass transplantation in this study.

The percentage of transplanting units surviving was the most common criteria (Fonseca et al., 1998), and was expressed as a percentage of the original number. When transplanting seagrasses,





it is inevitable to sever the root and rhizome tissues of the donor shoots, especially in bare-root transplanting method, such as the staple method, TERFS method, and shell method (Fonseca et al., 1996; Calumpong and Fonseca, 2001; Short et al., 2002; Lee and Park, 2008; Cambridge and Kendrick, 2009). Sediment-associated seagrass transplantation methods, such as plug, sod, and core methods, have less impact on transplanting during the first 1-2 months. In this study, the survival rate of transplantation declined rapidly in first one year after transplantation, especially for *T. hemprichii*, which may be due to damage to underground tissues. The initial stress caused by root and rhizome tissues damage may lead to physiological and morphological changes of transplantation shoots (Struve et al., 2000). It was reported that, the photosynthetic efficiency of *P. sinuosa* (Horn et al., 2009), and the leaf length and width of *T. testudinum* decreased (Tussenbroek, 1996) after transplanting for 1-2 months. However, in this study, we did not observe significant changes in the morphology of transplantation shoots. After almost two years, more than 65% of *T. hemprichii* and

90% of *E. acoroides* were still alive, thanks to the seagrass shoots immobilized into the sediment.

Fonseca et al. (1998) thought that, random samples should be taken to measure the number of new shoots, because the addition of new shoots was a more accurate way to assess asexual reproductive activity of transplanting. Compared with sexual reproduction, clonal reproduction is the main way to maintain the regeneration and stability of seagrass population (Harwell and Rhode, 2007). In this study, we calculated shoot formation rate in place of shoots number. The shoot formation rates of transplants were less than 5% during first 5-6 months after transplantation. The low shoot formation rates may be due to transplanting damage in the initial months. From Nov 2020, the shoot formation rates increased greatly, indicated that, the transplanted plants had spread to where they lived and had been colonized successfully. However, for the seagrass plants with interlaced rhizomes, shoot formation rate may not be a good indicator.

The coverage usually measured by a 50 × 50 cm quadrat which divided into 5 × 5 cm grids. The quadrat randomly placed on the transplanting unit. The total number of seagrass shoots in a 5 × 5 cm grids was converted to the coverage of per square meter. In this study, we reduced the time of scuba diving spent underwater by measuring the coverage using a photogrammetry-based method.

Due to the damage of transplantation shoots, the shoot density usually decreases at the initial stage of transplantation, and then increases with the formation of lateral shoots (Orth et al., 1999; Park and Lee, 2007; Bastyan and Cambridge, 2008; Lee and Park, 2008). Thus, the increase of shoot density was an indicator of successful establishment of transplanting. However, in this study, the shoot density of *T. hemprichii* transplanted in Xincun lagoon and the shoot density of *E. acoroides* transplanted in Gaolong bay and Xincun lagoon did not decline during the early period of transplantation. Thus, we considered that, transplant establishment success could not be evaluated by just one indicator. Finally, we monitored the survival rate, shoot formation rate, coverage and shoot density, prominent change in morphology and growth of shoots to examine whether e seagrass transplantation was established successfully.

It was often thought that different species of seagrass transplantations took different times to establish successful

TABLE 2 List of ten most used parameters in transplantation monitoring studies.

Monitoring Parameter	Definition
Monitoring frequency	Irregular time intervals were selected for monitoring after a transplanting location.
Survival rate	The survival rate of the transplanting unit.
Number of shoot	Direct count the number of shoots in the transplanting unit.
Shoot density	The density of the transplanting units.
Coverage	The coverage of the transplanting units.
Leaf length	Direct measure the length of seagrass leaf.
Leaf width	Direct measure the width of seagrass leaf.
Rhizome length	Direct measure the total length of living rhizome.
Direct mapping	A Photograph of the actual transplanting unit for the covered area.
Biomass	The weight of seaweed in a given area.

transplantation in new locations (Campbell and Paling, 2003; Horn et al., 2009). *Z. marina* transplantation generally required a shorter establishment time compared to other seagrass species (Paling et al., 2007; Park and Lee, 2007). Different transplanting methods led to different establishment time of the same seagrass species, even in the same location (Fonseca et al., 1996; Paling et al., 2001; Campbell and Paling, 2003; Horn et al., 2009). Park and Lee (2007) reported that, establishment time for *Z. marina* transplanting required 1.0–2.3 months by staple method, TERFS method took 2.2–2.6 months, and shell method took 3.2–3.7 months by. Based on the measurements of survival rate, shoot formation rate, coverage and shoot density, we concluded that transplantation established successfully of *T. hemprichii* and *E. acoroides* in the new site was about 12 months after transplantation. Because *T. hemprichii* and *E. acoroides* were hardly used in transplantation, we were unable to compare the results with other reports.

4.2 The influencing factors of seagrass transplantation

The survival and growth of transplanted seagrass plants were influenced by numerous factors, such as various environmental factors and human activities.

Water temperature is an important factor affecting the distribution and growth of seagrass (Robertson and Mann, 1984; Han et al., 2012). The heat tolerance of seagrass and the optimum temperatures of photosynthesis, respiration, and growth seagrass decided the direct effects of temperature changes. Under optimal growth temperature, water temperature was positive correlated to the shoot density, photosynthetic efficiency, and leaf growth rate of *T. hemprichii* strongly (Liu et al., 2020). With further rising temperature, the increase of leaf respiration rate was faster than the increase of photosynthetic rate, resulting in the continuous decline of photosynthetic respiration ratio, which destroyed the balance between photosynthetic rate and respiratory rate (Zimmerman et al., 1989). However, the correlation between seasonal water temperature and seagrass growth was weak in this study. We thought that, water temperatures during spring and autumn were within the optimum growth temperature range for *T. hemprichii* and *E. acoroides*, increased water temperatures may enhance seagrass productivities. However, the increased water temperature in summer exceeded the optimum temperature for seagrass growth, reduced the productivities of seagrass. Therefore, the seasonal variations between water temperature and seagrass productivity may cancel each other out. This conclusion is consistent with previous research (Barber and Behrens, 1985; Lee et al., 2005).

Salinity is a main environmental factor affecting the structure and function of seagrass community. Salinity change will have impact on seed germination, shoots formation, photosynthesis and respiration, growth and biomass distribution of seagrass, resulting distribution changes. At low salinities, although seagrass photosynthesis was maintained, productivity reduced by 20%. Higher than optimal salinity can also negatively affect seagrass. Under hypersaline conditions, production and biomass of *A. antarctica* decreased (Walker, 1985). Because salt stress may limit

growth by underexpanding cells and inhibiting the photosynthates and proteins. In our study, salinity of transplant sites was suitable for *T. hemprichii* and *E. acoroides*, no correlation between salinity and transplantation performance were observed.

The most immediate effect of an increase of suspend solids is a reduction in available light reaching the seagrass beds. Experimental studies had shown that, the decrease of light reduced plant size, leaves number of individual shoots, shoot density, leaf width and growth rate of seagrass, and increased in leaf length (Cambridge and Kendrick, 2009). In this study, when the content of suspend solids reached the highest value in Dec 2019, the survival rate, shoot formation rate and shoot density of *T. hemprichii* were at low level. Furthermore, we found a significant inverse correlation between the suspend solids and the shoot density in Xincun lagoon, meaning the higher suspend solids, the lower photosynthetic efficiency, ultimately led to lower shoot density.

The productivity of persistent seagrasses depends on the availability of nutrients, including water and sediment nutrients. It is usually considered that, sediment pore water is the main source of nutrients for seagrasses, because of the higher concentrations of nutrient compared to the surface water. However, studies have shown that seagrass can absorb nutrients through leaves and root tissue, seawater and sediment nutrients contributed equally (Iizumi and Hattori, 1982; Lee and Dunton, 1999). To nutrient limited plants, the typical responses to N and/or P supplying were increases of shoot size, productivities, and biomass (Bulthuis et al., 1992; Lee and Dunton, 2000). But seagrass growth is not always limited by the concentration of nutrients in the surrounding environment, as increased nutrient supply has a limited effect on seagrass growth sometimes (Dennison et al., 1987; Lee and Dunton, 2000). Seagrass production may have little relationship with water nutrients (Lee and Dunton, 2000). Stimulated by phytoplanktonic, epiphytic and macroalgal growth, elevated water nutrient levels may have indirect negative effect on seagrasses. Due to water column nutrient enrichment, the primary producers thrive, resulting in less light available to seagrasses. This conclusion was demonstrated by Burkholder et al. (1992) in *Z. marina*. For the same reason, DIN was significant negative correlations with shoot density in Xincun lagoon in this study.

Sediment types and grain-size characteristics have a great effect on the growth of seagrass (Eckrich and Holmquist, 2000). Park and Lee (2007) evaluated the feasibility and efficiency of *Z. marina* transplanting methods based on sediment type at different transplantation site. The results show that, the survival rate of shell method was higher in muddy (81.3%) and silty sediments (76.5%), and lower in sandy sediments (5.0%); While, both staple method and TERFS method had higher transplant survival rate. In this study, Gaolong bay was characterized by high coral debris, gravel and sand content in the sediment, whereas Xincun lagoon had mud and sand. Seagrass rhizomes transplanted by “singleplant-fixed-distance transplantation” method were inserted into the sediment firmly. There was no significant difference in survival rate and shoot density of seagrass between transplanted in Gaolong bay and Xincun lagoon after 23 months of transplantation. Our experiment shows that, an appropriate fixing technique is essential if transplantation is to be successful.

Over the last decade, seagrass beds are under a number of threats, particularly from human causes, such as shrimp and fish farming, shellfish harvesting, fishing through poisons, electricity and explosives, as well as trawling, pollution of aquaculture wastewater and dredging. Thankfully, this study was carried out without any human influence due to the plastic garden mesh set up around the transplanting area. The transplantation actions and protection measures for seagrass beds will play a positive role in reducing seagrass loss, optimizing biodiversity of seagrass bed, and enhancing ecosystem service (Possingham et al., 2015), especially for slow-growing species such as *T. hemprichii* and *E. acoroides*.

Data availability statement

The original contributions presented in this study are included in the article/Supplementary Material. Further inquiries can be directed at the corresponding authors.

Author contributions

JS: Data curation, Formal Analysis, Funding acquisition, Supervision, Writing – original draft, Writing – review & editing. LY: Writing – original draft. JZ: Investigation, Writing – review & editing. SJ: Writing – review & editing. YW: Writing – review & editing. DW: Writing – review & editing. ZC: Writing – original draft. CS: Funding acquisition, Writing – review & editing.

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Conflict of interest

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

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Supplementary material

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fmars.2023.1294779/full#supplementary-material>

SUPPLEMENTARY TABLE 1

The environmental parameters monitoring results.

SUPPLEMENTARY TABLE 2

The transplantation monitoring results in Gaolong bay.

SUPPLEMENTARY TABLE 3

The transplantation monitoring results in Xincun lagoon.

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Restored intertidal eelgrass (*Z. marina*) supports benthic communities taxonomically and functionally similar to natural seagrasses in the Wadden Sea

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Ecological restoration has become an important management-tool to counteract the widespread losses of seagrass meadows and their associated biodiversity. In the Dutch Wadden Sea, long-term restoration efforts have recently led to the successful restoration of annual eelgrass (*Zostera marina*) at high densities on a local scale. However, it is yet unknown if restored seagrass plants also lead to improved local biodiversity and ecosystem functioning in the intertidal zone. We therefore compared the macrozoobenthos communities of a small-scale restored meadow to 22 naturally occurring intertidal seagrass meadows. Using a taxonomic and trait-based approach we aimed to study 1) how intertidal seagrasses (*Zostera marina* and *Zostera noltii*) affect benthic communities and their functional trait distribution and 2) if a restored meadow facilitates benthic communities similar to natural meadows. We found that both natural and restored seagrasses increased abundances of benthic animals and the richness (both taxonomic and functional) of associated benthic communities compared to nearby unvegetated areas. Additionally, the presence of intertidal seagrass shifted benthic community composition both taxonomically and functionally, thus broadening the niche space for species inhabiting tidal flats. Seagrasses especially facilitated epifaunal species and traits associated with these animals. Surprisingly, our results indicate that the mere presence of seagrass aboveground structure is enough to facilitate benthic communities, as neither higher seagrass cover nor biomass increased benthic biodiversity in the intertidal zone. By studying the effect of seagrass restoration on benthic diversity, we found that the restored meadow functioned similarly to the natural meadows after only two years and that the success of our restoration efforts indeed led to local biodiversity enhancements. Our findings contribute to the understanding of the ecological functioning of intertidal seagrasses and can be used to define/refine conservation and restoration goals of these valuable ecosystems.

KEYWORDS

seagrass, ecological restoration, biodiversity, Wadden Sea, functional diversity, macrozoobenthos

1 Introduction

Seagrasses are marine flowering plants that form vast underwater meadows in coastal waters worldwide. In addition to being some of the most productive ecosystems in the world (Duarte and Chiscano, 1999), these meadows provide essential ecosystem services and contribute to climate change mitigation. For instance, seagrass meadows provide vital nursery habitats for commercially important species, offer coastal protection, and sequester large amounts of carbon (Nordlund et al., 2016). Additionally, seagrass meadows are generally regarded as diversity hotspots, serving as crucial habitats to a wide array of animals, from charismatic mega herbivores to microscopic critters. However, seagrasses are seriously threatened and during the last century an estimated ~29% of the global seagrass area was lost (Orth et al., 2006; Waycott et al., 2009).

Ecological restoration has become an important management-tool, supplementing traditional passive conservation efforts, to counteract declines of seagrasses and other coastal ecosystems. However, coastal ecosystem restoration is still in its infancy and often challenging in practice (Bayraktarov et al., 2016). The reasons hindering successful coastal restoration are many, ranging from practical methodological problems to how the public perceives marine ecosystem restoration (Suding, 2011; Bayraktarov et al., 2016; Abelson et al., 2020). One specific aspect that is often lacking is how restoration success is defined and measured (Abelson et al., 2020). Success is often defined solely based on how the restoration effort affects the target species (Suding, 2011). Although useful as a short-term indicator of success, this is generally an insufficient approach since ecological restoration is about restoring ecosystem integrity and associated ecosystem functions (Suding et al., 2015). Hence, there is a need for monitoring programs to also measure restoration success beyond the target species. To evaluate the success of a restoration project, The Society of Ecological Restoration (SER, 2004; Gann et al., 2019) recommends assessing nine ecosystem attributes including biodiversity, resilience and self-sustainability. Ideally, restoration projects would follow these guidelines, but in reality, monitoring all the proposed ecosystem attributes requires more funds and long-term commitment than restoration projects usually have available. Thus, measurements that combine feasibility with information about the overall performance of the restored ecosystems are urgently needed.

In the Wadden Sea, seagrasses experienced large declines during the 20th century. Vast subtidal eelgrass (*Zostera marina*) meadows vanished completely in the 1930s, due to the seagrass wasting disease and coastal development (den Hartog and Polderman, 1975; Giesen et al., 1990). Intertidal seagrass populations persisted, but were heavily diminished by increased eutrophication in the 1970s and 80s (van Katwijk et al., 2010). During the last decades, intertidal meadows have recovered in the northern Wadden Sea (Dolch et al., 2013), but in the south (Netherlands and Lower Saxony) recovery has remained almost entirely absent (Dolch et al., 2017). Today, only intertidal seagrasses, perennial dwarf eelgrass (*Zostera noltii*) and annual common eelgrass (*Z. marina*), persist in the Wadden Sea. The loss of seagrass meadows has affected the ecosystem functioning and biodiversity of the sea. For instance, seagrass declines have

altered sediment dynamics, increased water turbidity, led to the loss of commercially important nursery habitats and reduced food availability for herbivorous birds (Wolff, 1979; Ganter, 2000; Polte et al., 2005; van der Heide et al., 2007; Eriksson et al., 2010).

During the last decades, seagrass restoration efforts have been performed in the Dutch Wadden Sea (e.g., van Katwijk et al., 2009; van Duren and Van Katwijk, 2015; Govers et al., 2022), with the goal to counteract the widespread seagrass losses and to recover lost ecosystem functions. Recently, the persistent restoration efforts have started to pay off, as we have been able to restore high densities of *Z. marina* on relatively large areas (2664 m² in 2020 and 1 ha in 2021, Gräfnings et al., 2023 & unpublished results) using a newly developed seeding method (DIS-method; Govers et al., 2022; Gräfnings et al., 2022). However, to properly evaluate if the restoration has been successful, further monitoring is needed to confirm that the efforts have also enhanced ecosystem integrity beyond the target species.

In this paper we investigate how seagrass restoration affects benthic communities as an indicator of the degree of restoration success (Dolbeth et al., 2013; Lefcheck et al., 2017). Macrozoobenthic communities are the foundation of many ecosystem services in benthic systems (Snelgrove, 1999). Macrozoobenthic animals can affect their surroundings in multiple ways, from nutrient recycling and oxygenation of sediments to grazing and stabilization of sediments (see review by Levin et al., 2001). Furthermore, many benthic animals function as crucial food resources for higher trophic levels and can thus offer information about cross-ecosystem dynamics. Hence, studying benthic communities can offer valuable information about how restoration efforts affect local biodiversity and ecosystem functioning, especially if complementary taxonomic and functional diversity approaches are used in parallel (Henseler et al., 2019). Traditional taxonomic diversity approaches base their results on species identities, while functional diversity is usually measured through species traits (Violle et al., 2007) and is considered a good predictor of ecosystem function (Cadotte et al., 2011). However, before the effect of the restored seagrass on benthic communities can be properly evaluated in the Wadden Sea, accurate information is needed about how natural seagrass meadows affect benthic communities in the area. Although the effect of seagrasses on benthic diversity (both taxonomic and functional) is well studied (e.g., Orth et al., 1984; Boström and Bonsdorff, 1997; Lefcheck et al., 2017; Boyé et al., 2019), little is known about how intertidal seagrasses affect benthic diversity (especially functional diversity) in the Wadden Sea or in restored seagrass beds.

In this study, we assess 1) how intertidal seagrass (annual *Z. marina* and/or perennial *Z. noltii*) presence affect taxonomic and functional diversity (richness, evenness) and community structure (composition) of macrozoobenthos and 2) whether a recently restored *Z. marina* meadow affects the associated benthic community similar to natural meadows in the intertidal Wadden Sea. We expect intertidal seagrasses to generally facilitate biodiversity, but that the two seagrass species might affect benthic communities differently due to differences in structural attributes and life cycle. For instance, the yearly turnover of annual *Z. marina*

is expected to affect benthic community composition differently than more stable perennial seagrasses like *Z. noltii*. We hypothesize that mobile benthic species will benefit the most from the short life cycle of annual *Z. marina*. Additionally, only *Z. noltii* forms traditional seagrass ‘meadows’ in the Wadden Sea, while monospecific annual *Z. marina* populations spread out very sparsely (<1 plant/m²) over large areas (personal observation; Figure 1D). The ability of seagrass meadows to shelter organisms (from both predators and physical disturbance) is often considered one of the most important functions facilitating biodiversity (see review by Boström et al., 2006), and in the absence of a meadow-structure the positive effect of seagrasses on the benthic diversity might be reduced. Finally, we expect the restored seagrass to facilitate benthic biodiversity, but potentially to a lesser degree than natural seagrasses, due the short time span after restoration. With our findings we aim to explain the importance of intertidal seagrass ecosystems for associated benthic communities and their functionality, to evaluate seagrass restoration success beyond the target species, and to provide practical recommendations concerning the conservation and restoration of intertidal seagrasses in the Wadden Sea and globally.

2 Methods

2.1 Study area

The Wadden Sea is a temperate coastal sea that extends from the northwest Netherlands to the southwest coasts of Denmark. The Wadden Sea has a diurnal tidal cycle (range: 1.5 to 4 meters) and during low tide, approximately 50% of the seas total area (~8000 km²) emerges as intertidal flats. These tidal flats provide a hotspot for birds and marine biodiversity.

2.2 Seagrass restoration

Since 2018, eelgrass (*Z. marina*) restoration trials have been successfully performed northeast of Griend-island (Figure 1; N 53.2692, E 5.2949) in the Dutch Wadden Sea (Gräfnings et al., 2022; Gräfnings et al., 2023). Prior to restoration experiments in 2018, there was no eelgrass growing on the tidal flats surrounding the island. In March 2020, a 400 m² plot was seeded on the Griend sandflat with the DIS-method (Govers et al., 2022; Gräfnings et al., 2022). In the restoration plot, eelgrass seeds were injected to a depth of 3 cm, with ~3 seeds/injection and with a seeding density of 50 injections/m². In July 2020 on average 25.75 eelgrass plants/m² had been established in the 400 m² restoration plot. A year later in July 2021, on average 25 second generation eelgrass plants/m² were counted in the restoration plot.

2.3 Data collection and processing

In the summer (July– August) of 2018, 22 natural seagrass sites were sampled once during low tide across the international Wadden

Sea (Figure 1). At each site, we estimated the % cover of *Z. marina* and *Z. noltii* by walking three 30 m x 1 m belt transects. At 15 of the sites, we collected samples both from inside and outside (>20 m from the seagrass) of the seagrass. At the remaining 7 sites, samples were only collected inside the seagrass. At each site, three benthic macrofauna cores (top 30 cm & 15.5 cm diameter) were sampled. Cores sampled from within the seagrass meadow always contained seagrass. In the field, the samples were sieved (1 mm), after which any seagrass biomass in the samples was separated and pooled. Macrozoobenthos from the three cores were pooled and stored in 70% ethanol until identification. In July 2021, we sampled six benthic macrofauna cores (top 30 cm & 15.5 cm diameter) inside and outside the seagrass restoration plot at Griend (Figure 1E). Samples were sieved (1 mm) and stored in a 4% formaldehyde solution until identification. The twelve macrofauna cores from Griend were treated as independent samples. In the lab, all individuals were counted and identified to the finest taxonomic level possible under a dissecting microscope. The individuals were identified to 54 different taxonomic groups (35 at species-level, 11 at genus-level (e.g., *Eteone* sp.), 2 at family-level (e.g., *Polynoidae*), 3 at order-level (e.g., *Actinaria*), 2 at phylum-level (e.g., *Nematoda*) and 1 at class-level (*Bivalvia*). Once counted and identified, the biomass of individuals of the same taxonomic group was determined per sample. Samples were first dried for 48h at 60°C in a ventilated stove, after which dry weight was measured. Following this, the samples were incinerated for 5 hours at 560°C and then weighed again to obtain the ash free dry mass (AFDM). Seagrass samples were dried for 48 h at 60°C in a ventilated stove, after which the total dry weight of the plants was measured.

2.4 Macrozoobenthos traits

We chose to include traits explaining the size, morphology, mobility, feeding type, burrowing depth and reproduction of the benthic communities (Table 1). These traits were chosen to characterize the basic ecology of the benthic animals and to give an indication how intertidal seagrasses affect benthic ecosystem functioning. All traits were categorical and divided into several modalities (Table 1). The species-specific affinities to all modalities were fuzzy coded within a range from 0 to 3 (0= no affinity, 1= low affinity, 2= moderate affinity and 3= high association of taxon with the trait category). The fuzzy-coded trait values were extracted from two macrozoobenthic biological trait databases (Gusmao et al., 2022; Meijer et al., 2023). To standardize the data between traits with different numbers of modalities, we divided the individual modalities by the total number of modalities of the corresponding trait (see Henseler et al., 2019). As we were not able to identify some bivalves beyond class-level, these individuals were allocated trait information common to the entire class in the Wadden Sea.

2.5 Statistical analysis

All statistical analyses were performed in R version 4.0.3 (R Core Team, 2020). We investigated the effect of intertidal seagrasses

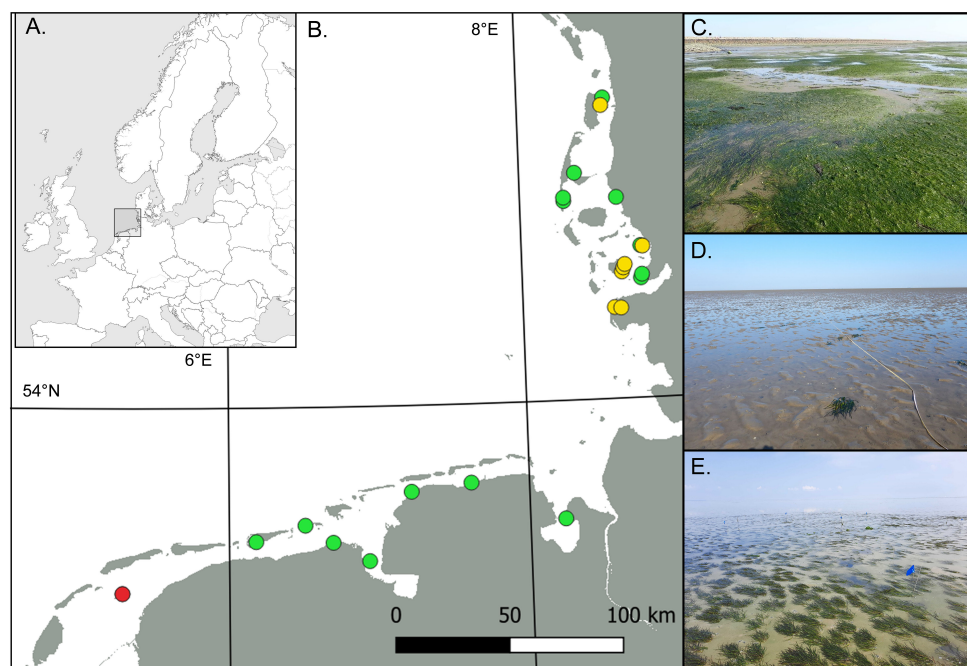


FIGURE 1

(A) Location of the Wadden Sea in Europe. (B) Locations of sampled seagrass sites in the Wadden Sea where green indicates sites macrozoobenthos was sampled both inside and outside the seagrass and yellow indicates sites macrozoobenthos was sampled only inside the seagrass. The red circle indicates the seagrass restoration site northeast of Griend-island. Pictures on the right show (C) a mixed *Zostera noltii* & *Zostera marina* meadow, (D) a monospecific *Z. marina* meadow and (E) the restoration plot at Griend.

on both taxonomic and functional diversity of macrozoobenthos. To describe taxonomic diversity, we calculated taxonomic richness, Shannon index and Pielou's evenness using the "vegan"- package (Oksanen et al., 2018). To describe functional diversity, we calculated three trait-based indices (functional richness, evenness and dispersion) corresponding to the previously mentioned taxonomic indices. Functional richness (FRic) expresses the amount of trait space that is occupied by the species of a community (Mason et al., 2005). Functional evenness (FEve), describes how evenly species abundances are distributed between the expressed trait categories (Mason et al., 2005). Functional dispersion (FDis) describes the abundance weighted mean distance of individual species to their weighted group centroid in the multidimensional trait space (Laliberté and Legendre, 2010) and thus measures the spread of the community within the trait space. Hence, functional dispersion can be considered a measure of functional diversity (Laliberté and Legendre, 2010). Additionally, community-level weighted means of trait values (CWM) were computed for each sample. CWMs express trait values weighted by species abundances and can be used to compare how communities differ functionally. Functional diversity indices were calculated with the "FD"-package (Laliberté and Legendre, 2010; Laliberté et al., 2014). We used count-data (individuals/m²) to calculate the different indices and community-level weighted means. Before calculating the trait-based indices, count-data was log (x+1) transformed to reduce the influence of dominant species without losing the abundance effect.

2.5.1 Analyzing the effect of natural intertidal seagrass on benthic diversity

Differences in benthic diversity indices and abundances (counts and AFDM) between natural seagrass and nearby bare areas were analyzed with linear mixed effect models using the lmer-function in R (package: "lme4"). In the models, we included data from sites where macrozoobenthos was sampled both inside and outside the seagrass (15 sites). No distinction between the two seagrass species was included in these models. "Site" was included as a random factor (random intercept: "1|Site") in each model. Residuals of the linear mixed effect models were checked for normality and, if necessary, the response variable was transformed to fit model assumptions (log-transformed: Taxonomic richness; sqrt-transformed: FEve & FDis). An ANOVA from the car package (Fox and Weisberg, 2011) was applied to conduct the Wald Chi-Squared test on model outputs.

To investigate if the two seagrass species affect benthic communities differently in the intertidal Wadden Sea, we first divided the sampled seagrass meadows into three categories: Mixed meadows (both seagrass species present; n=5), monospecific *Z. marina* (n=8) and monospecific *Z. noltii* (n=9). Data from all sampled seagrass sites (22 sites) were used for the analyses. One-way ANOVAs were performed to analyze if benthic diversity indices differed between seagrass meadow types, after controlling that the data met the assumptions of parametric tests. Additionally, linear regressions were performed to investigate if seagrass cover (%) and biomass (DW, g) affected benthic diversity indices.

TABLE 1 List of macrozoobenthos traits and their modalities used in functional trait analysis.

Trait	Modality	Label	Relevance
Bioturbation type	Epifauna	E	Habitat modification, sediment processing, nutrient cycling
	Surficial modifier	SM	
	Upward conveyor	UC	
	Downward conveyor	DC	
	Bio diffuser	BD	
	Regenerator	Re	
Burrowing depth, cm	Surface	Su	Space usage, bioturbation
	0-3	0-3	
	3-8	3-8	
	8-15	8-15	
	15-25	15-25	
	>25	>25	
Adult body size, mm	<5	5	Productivity, palatability
	5-10	5-10	
	10-20	10-20	
	20-40	20-40	
	40-80	40-80	
	80-160	80-160	
	>160	>160	
Feeding mode	Deposit feeder	DF	Food acquisition, trophic level
	Suspension feeder	SF	
	Scavenger/Opportunistic	OS	
	Grazer	G	
	Predator	P	
Longevity, years	<1	1	Life span, productivity
	1-3	1-3	
	3-6	3-6	
	6-10	6-10	
	>10	>10	
Adult movement type	Sessile	Se	Mobility, dispersal, ability to escape predation
	Swimmer/Floater	S/F	
	Crawler/Walker	C/W	
	Burrowing	Bu	
Reproduction	Asexual	A	Reproduction, productivity

(Continued)

TABLE 1 Continued

Trait	Modality	Label	Relevance
	Broadcast	Bc	
	Brooder/Egg layer	Br	
	Benthic	Be	
Skeleton	Soft	So	Palatability
	Calcified	Ca	
	Chitinous	Ch	

2.5.2 Analyzing the effect of restored intertidal seagrass on benthic diversity

Differences in benthic diversity indices (taxonomic richness, Shannon index, Pielou's evenness FRic, FEve, FDis) and abundances (counts and AFDM) between the restored seagrass plot at Griend (n=6) and nearby bare area (n=6) was analyzed with one-way ANOVAs. Prior to analysis, data were tested to meet the assumptions of parametric tests.

2.5.3 Analyzing the effect of intertidal seagrass on benthic community composition

To assess if benthic communities differed between seagrasses and nearby bare areas, we performed non-metric multidimensional scaling (nMDS) on the taxonomic (based on counts) and functional (based on CWM values) compositions. The replicates from the seagrass restoration site were pooled (seagrass and bare separately) and included in the analysis, to get an indication if restored seagrass affected benthic communities similarly as natural meadows. Bray-Curtis dissimilarity was applied for count-data and the Gower distance for CWM trait values. To statistically test for differences in taxonomic and functional compositions among habitats, we used permutational multivariate ANOVAs (PERMANOVA; 9999 permutations; package: "vegan"). Site was included as a random factor in the PERMANOVA analysis. Before PERMANOVAs were performed, we checked with a permutational test of multivariate dispersion (PERMDISP; package: "vegan") if the observations within groups were spread equally between the habitats. Furthermore, we used Multilevel pattern analysis (package: "indicspecies") to determine which species and modalities were driving differences between seagrass and bare communities. Multilevel pattern analysis was performed separately for natural meadows (n=15) and the restoration site (n=6).

3 Results

3.1 Effect of (natural) intertidal seagrass on benthic diversity

Benthic taxonomic richness was significantly increased by natural intertidal seagrasses ($\chi^2=7.47$, $df=1$, $p=0.006$), with seagrasses sheltering on average 33% higher taxonomic richness

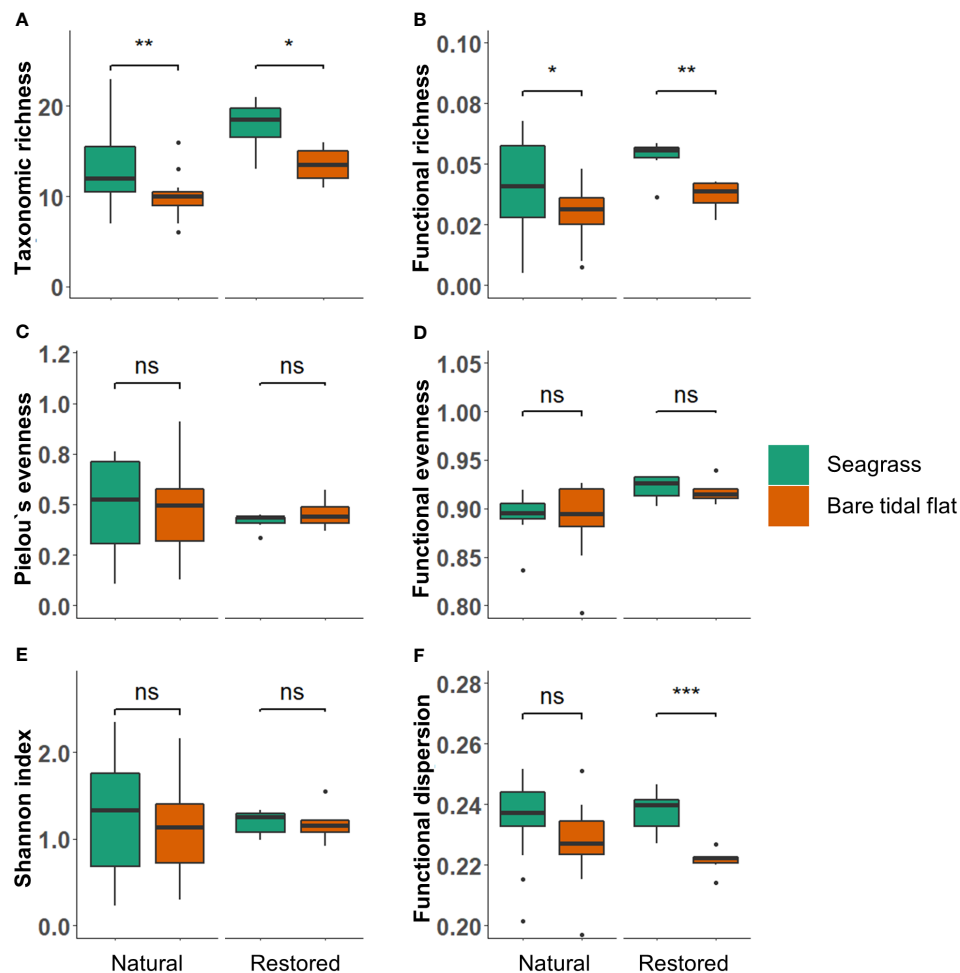


FIGURE 2

Taxonomic and trait-based indices inside and outside seagrass in natural meadows and in the restored meadow: (A) Taxonomic richness (B) Functional richness, (C) Pielou's evenness, (D) Functional evenness, (E) Shannon index and (F) Functional dispersion. Boxplots show median (line in box), upper and lower quartile (box), 1.5 x interquartile range (vertical line) and outliers (circle). The indices were calculated with count-data (individuals/m²). Stars indicate significance of $p < 0.001$ for ***, $p < 0.01$ for ** and $p < 0.05$ for *. Statistically non-significant results are indicated with ns.

than nearby bare areas (Figure 2A). The presence of seagrass on average doubled the amount of epifaunal richness ($\chi^2=13.26$, $df=1$, $p < 0.001$), while no significant differences were observed in endobenthic taxonomic richness (Supplementary Figure S1). However, interestingly seagrass presence did not significantly affect either Pielou's evenness or Shannon index (Figures 2C, E).

The patterns in functional diversity indices largely mirrored their taxonomic counterparts. Functional richness was significantly higher in the presence of intertidal seagrasses ($\chi^2=6.17$, $df=1$, $p=0.013$; Figure 2B), signifying that the number of expressed traits was increased by seagrass presence. However, neither functional evenness or dispersion of benthic communities differed significantly between seagrasses and bare areas (Figures 2D, F). On average, 170% more benthic individuals inhabited seagrasses than nearby bare areas ($\chi^2=4.87$, $df=1$, $p=0.027$, Figure 3A), while the animals' biomass did not differ significantly between the habitats (Figure 3B).

No significant differences were found in macrozoobenthic diversity indices between the three seagrass categories (Mixed meadows, monospecific *Z. marina* and monospecific *Z. noltii*; Figure 4).

Additionally, no correlation was found between site-wide seagrass cover % and benthic diversity indices (both taxonomic and functional; Supplementary Figures S2A, C, E, G, I, K). Similarly, no correlation was discovered between seagrass biomass (dry weight, g) and benthic diversity indices (both taxonomic and functional; Supplementary Figures S2B, D, F, H, J, L).

3.2 Effect of restored intertidal seagrass on benthic diversity

The recently restored *Z. marina* meadow at Griend (<2 years) had an almost identical effect on benthic taxonomic diversity as naturally occurring seagrasses in the Wadden Sea. Benthic taxonomic richness was 32% higher in the restored seagrass meadow compared to nearby bare area ($F(1,10) = 8.76$, $p = 0.014$; Figure 2A). Similar to natural meadows, the restored seagrass did not affect either Pielou's evenness or Shannon index significantly (Figures 2C, E). Additionally, the patterns in functional diversity indices largely mirrored their

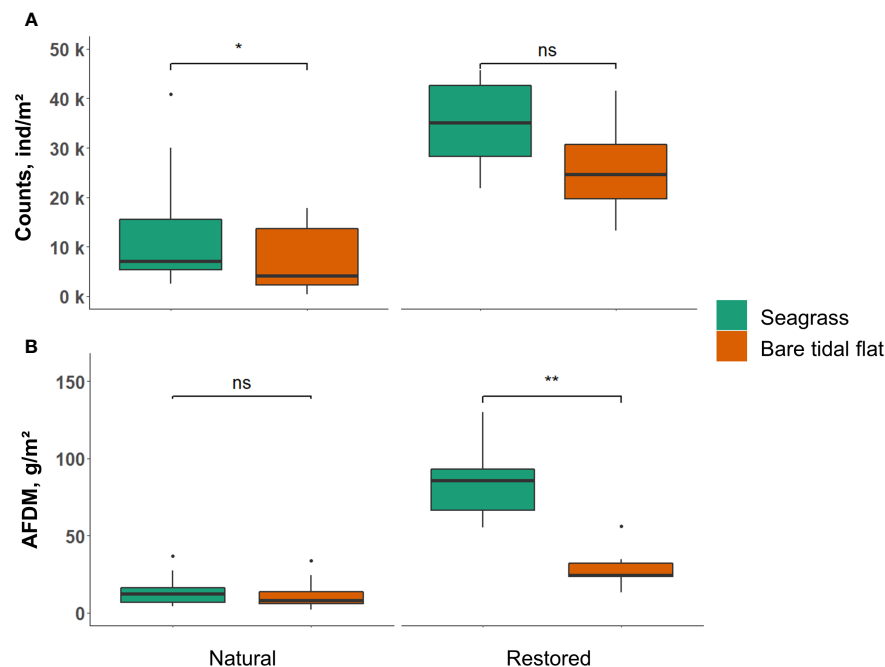


FIGURE 3

(A) Counts of individuals and (B) AFDM of macrozoobenthos inside and outside seagrass in natural meadows and at the restoration site. Boxplots show median (line in box), upper and lower quartile (box), 1.5 x interquartile range (vertical line) and outliers (circle). Stars indicate significance of $p < 0.01$ for ** and $p < 0.05$ for *. Statistically non-significant results are indicated with ns.

taxonomic counterparts. Similar to natural meadows, functional richness was also higher in the restoration plot compared to nearby bare area ($F(1,10) = 13.23$, $p = 0.004$; Figure 2B), signifying that the amount of expressed traits in the benthic community was increased through seagrass restoration. However, unlike in the natural meadows, the restored seagrass also significantly increased functional dispersion of the macrozoobenthos ($F(1,10) = 22.23$, $p < 0.001$; Figure 2F), signifying that the diversity of expressed traits was higher in the restoration plots compared to the nearby bare area. Functional evenness of the associated benthic communities did not significantly differ in the restoration plot and nearby bare area (Figure 2D). In contrast to natural seagrasses, we found no difference in animal counts between the restored seagrass and nearby bare area (Figure 3A), while biomass of the benthic community was almost 3x higher in the restored seagrass ($F(1,10) = 20.12$, $p = 0.001$; Figure 3B). Mainly three benthic species (mudsnail *Peringia ulvae*, common cockle *Cerastoderma edule* and ragworm *Hediste diversicolor*) were responsible for the higher biomass measured in the restored seagrass.

3.3 Effect of intertidal seagrass on benthic community composition

Benthic community composition was significantly different in the presence of seagrass compared to nearby bare areas (Figure 5), both taxonomically (PERMANOVA; $p = 0.04$) and functionally ($p = 0.001$). Interestingly, the restored seagrass meadow seemed to affect the benthic community similarly as natural meadows (Figure 5). Blue mussels (*Mytilus edulis*), isopods (*Idotea* spp.) and juvenile

shore crabs (*Carcinus maenas*) functioned as indicator species for seagrass meadows in the Wadden Sea (Multilevel pattern analysis, $p < 0.05$). At the restoration site, blue mussels (*Mytilus edulis*), mudsnails (*Peringia ulvae*), nematodes and ragworms (*Hediste diversicolor*) were indicative of benthic communities in the seagrass, while two polychaeta species (*Notomastus latericeus* & *Heteromastus filiformis*) functioned as indicator species for the bare area. Many trait modalities were driving the differences in functional composition between benthic communities in seagrass and bare sites, both on the Wadden Sea scale and at the restoration site (Figure 6). Trait modalities linked to epifaunal species (e.g., sessile, surface living and suspension feeding) were expressed more in seagrass, while the most significant trait modalities telling bare areas apart from seagrasses were: burrowing movement type, deposit feeding and soft skeleton (Figure 6).

4 Discussion

Our results show that seagrasses (*Zostera marina* and *Zostera noltii*) increase the richness of benthic diversity in the intertidal Wadden Sea. By facilitating epifaunal animals, seagrasses support distinct benthic communities that differ from unvegetated areas both taxonomically and functionally. Importantly, we found that seagrass restoration can quickly (within 2 years) facilitate benthic communities to be taxonomically and functionally similar to communities found in natural seagrass meadows. Hence, we show that the current intertidal seagrass restoration practice in the Dutch Wadden Sea not only leads to restored plants, but also aids in the

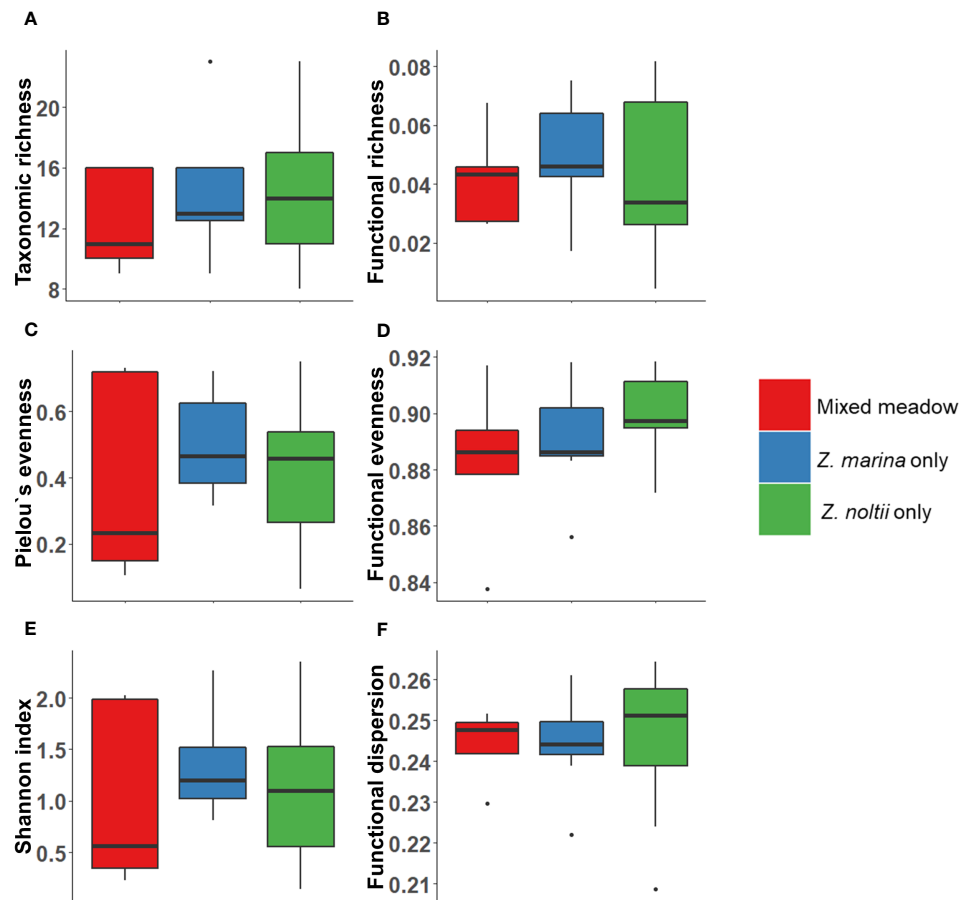


FIGURE 4

Taxonomic and trait-based indices of different seagrass meadow types [Mixed meadow (both seagrass species present; $n=5$), monospecific *Z. marina* ($n=8$) and monospecific *Z. noltii* ($n=9$)] in the Wadden Sea: (A) Taxonomic richness (B) Functional richness, (C) Pielou's evenness, (D) Functional evenness, (E) Shannon index and (F) Functional dispersion. Boxplots show median (line in box), upper and lower quartile (box), 1.5 x interquartile range (vertical line) and outliers (circle). The indices were calculated with count-data (individuals/m²). No statistical differences were detected.

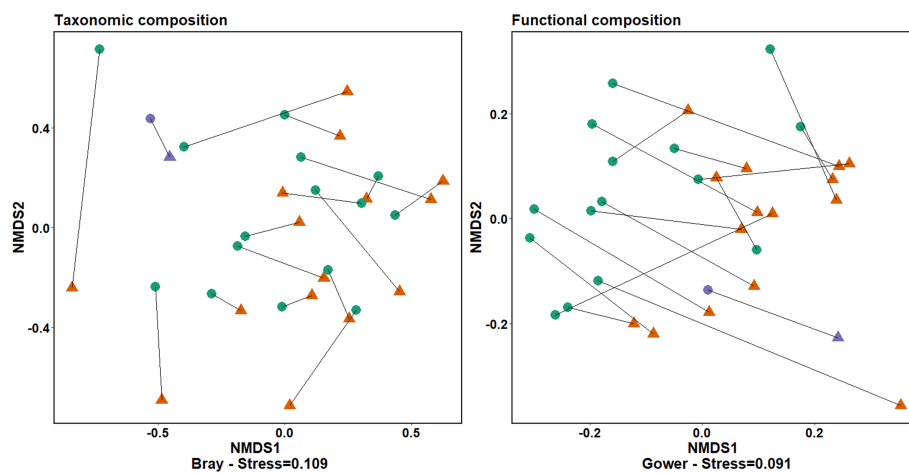


FIGURE 5

nMDS on taxonomic composition based on count-data and functional composition based on CWM values. Green circles indicate seagrass, orange triangles bare areas and the blue color indicates the two data points of the restoration site. Lines between points signify that data belongs to the same site.

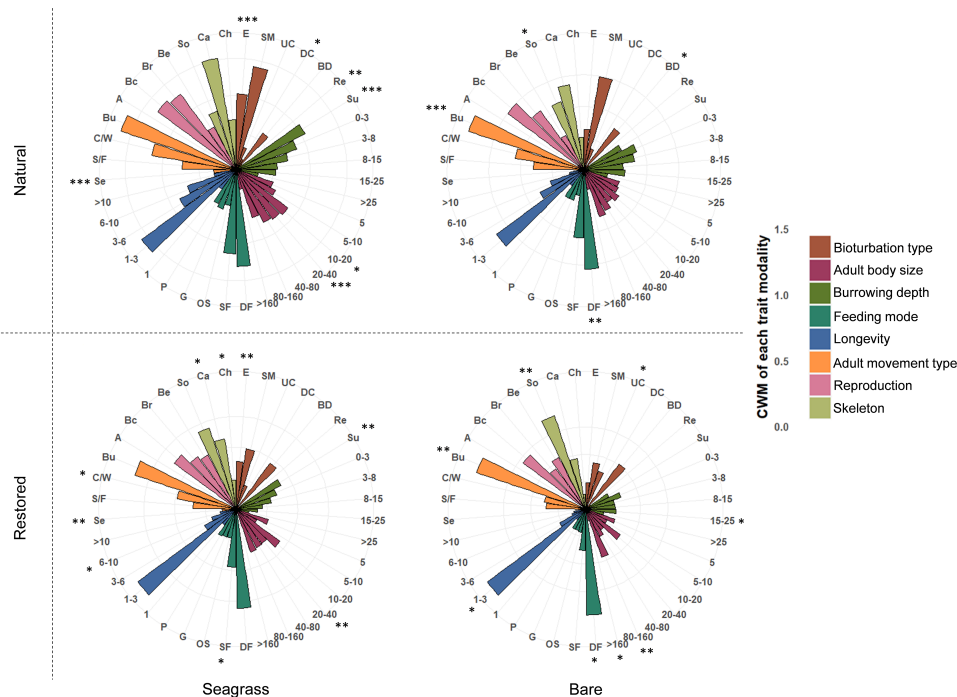


FIGURE 6

Mean CWM values for benthic communities at seagrass meadows and nearby bare areas in the Wadden Sea and at the restoration site. The stars indicate significant results of the Multilevel pattern analysis highlighting which modalities indicate either seagrass or bare areas. Stars stand for $p < 0.001$ for ***, $p < 0.01$ for ** and $p < 0.05$ for *. For label descriptions, see Table 1.

regeneration of ecosystem integrity through enhancing both taxonomic and functional diversity.

4.1 Intertidal seagrass enhances benthic biodiversity in the Wadden Sea

The effect of seagrasses on biodiversity is well studied and literature shows that the presence of seagrass usually increases the diversity and abundance of benthic fauna (e.g., Orth et al., 1984; Boström et al., 2006). We found that in the intertidal Wadden Sea, seagrasses also support richer (both taxonomically and functionally) and more abundant benthic communities. However, benthic taxonomic richness in the Wadden Sea was lower than what is generally measured in Atlantic seagrass meadows (species richness varying between 10–60; Orth, 1973; Stoner, 1980; Edgar et al., 1994; Blanchet et al., 2004 and references within). This may be the result of challenging living conditions that animals are subjected to in the intertidal Wadden Sea. Seagrasses in the Wadden Sea grow in the upper intertidal zone, which means that the plants and associated communities emerge over the water level for several hours (>5h) each tidal cycle. For most marine species, emergence (even short periods) is very stressful (increased desiccation/temperature) and thus only specialized or resistant benthic species inhabit the area. The ability of seagrasses to modify and ameliorate local conditions is crucial to support rich benthic life in seagrass systems. For instance, the ability of seagrass meadows to provide animals shelter, stabilize sediments, reduce hydrodynamics,

aerate sediments and recycle nutrients, are all processes that change living conditions on the bottom of the sea and facilitate macrozoobenthos (Orth et al., 1984; Boström et al., 2006; van der Zee et al., 2016). In the intertidal Wadden Sea, we suggest that seagrasses facilitated species richness by providing shelter (from predators/hydrodynamics/desiccation), food-provisioning and attachment structures to benthic animals.

Interestingly, intertidal seagrasses affected benthic diversity and community compositions very similarly across the Wadden Sea, despite the large spatial scale and differences in seagrass species composition. Worth highlighting is the fact that seagrass presence affected functional compositions more uniformly than their taxonomic counterparts (Figure 5), which suggests that seagrasses facilitate certain traits rather than specific macrozoobenthic species. Functional diversity, and more broadly ecosystem function, is then driven by density shifts in trait representation rather than the presence or absence of traits (e.g., Hewitt et al., 2008). Additionally, we found that higher seagrass cover % and biomass had no detectable influence on benthic diversity. This is somewhat surprising, as the ability of seagrasses to form meadows and modify their environment has previously often been linked with higher benthic richness (Boström et al., 2006 and references within). In our study, monospecific *Z. marina* meadows with very low cover % (< 1 plant/m²) supported similar benthic communities as dense perennial *Z. noltii* meadows. Hence our results suggest that the mere presence of seagrass, regardless of species or meadow structure, seems to facilitate benthic biodiversity in the intertidal Wadden Sea. However, seagrasses in the intertidal Wadden Sea

provide benthic animals only seasonal hotspots, as during the winter period most aboveground biomass is lost. Thus, benthic communities or at least epifauna need to recolonize the beds each spring. Interestingly, the only benthic species that was exclusively found in samples taken inside the seagrass meadows, was the economically and ecologically important blue mussel (*Mytilus edulis*). Thus, our results suggest that seagrasses can potentially provide a stepping stone/refuge for mussel spat, facilitating the dispersal of these valuable bivalves.

4.2 Seagrass restoration quickly facilitates benthic communities

Ecological restoration generally aims at restoring ecological integrity by regenerating high biodiversity and lost ecosystem functions. Here, after only two growing seasons a relatively small-scale restoration plot facilitated similar benthic diversity as natural meadows in the Wadden Sea. Our results are especially promising when considering that aquatic restoration efforts often fail to introduce diversity of associated communities to pre-disturbance levels (Rey Benayas et al., 2009). Successful recovery of diversity has most often been observed in systems that are dominated by fast-reproducing organisms with high dispersal ability (Jones and Schmitz, 2009; Duarte et al., 2015). This is most likely also the case in the Wadden Sea, where natural meadows need to be recolonized each spring by epifaunal animals (as discussed above), and one of the main reasons why diversity was able to rebound so quickly at the restoration site. Additionally, most benthic species in this study were found both in seagrass and on the adjacent bare flats, which suggests that intertidal seagrasses do not shelter unique species, but instead provide larger niche spaces with room for richer aggregations. Presumably, mainly by providing aboveground structure and protection, seagrasses facilitate epifaunal animals and traits associated with life above the sediment surface (e.g., sessile, surface living, suspension feeder, Figure 6). Importantly, by investigating the facilitated traits and overall functional diversity of the benthic communities, we could establish that the restored seagrass meadow also functioned very similarly to natural meadows. When comparing restoration efforts over large geographical scales, variation in species assemblages can obscure restoration success, whereas functional trait assemblages may provide a more general indicator of restoration success as they remain more stable over large distances (Bremner, 2008; Hewitt et al., 2008). We concur with previous studies (Dolbeth et al., 2013; Lefcheck et al., 2017) highlighting the usefulness of functional diversity measurements as predictors for seagrass restoration success and urge future restoration projects to incorporate this approach when evaluating restoration success.

4.3 Study limitations

It is important to note the limitations of the study. We only investigated one restoration site, restored with only one of the two native seagrass species and therefore the results of this study cannot be

generalized. However, as the included restoration site is the only site where seagrass restoration has been performed successfully in the whole Wadden Sea, we argue that this study should be seen as an important early indication of the value and potential of intertidal seagrass restoration in this area. Additionally, we only investigated the effect of seagrasses on benthic diversity and therefore further research is needed to investigate how the restoration of intertidal seagrass may affect higher trophic levels such as fish and birds. We expect that intertidal seagrasses can also indirectly benefit higher trophic levels (birds and fish) by increasing food availability. For instance, it has been shown that fish diversity is positively influenced by benthic species richness (Lebreton et al., 2012). In light of our results, we expect that especially predators of epifaunal species (e.g., sanderling *Calidris alba*) and herbivorous birds (e.g., brent geese *Branta bernicla*) can benefit from seagrass presence in the intertidal Wadden Sea.

4.4 Implications for seagrass conservation and restoration

Our results have important implications for the future of seagrass restoration in the intertidal Wadden Sea. We show that active restoration resulted in the recovery of a threatened marine habitat (intertidal seagrass), restoration of biodiversity and provisioning of habitat. Tangible deliverables like these, can be used to effectively engage stakeholders and to communicate the value of intertidal seagrasses and that restoration of these valuable habitats is possible. Additionally, we argue that dwarf eelgrass (*Z. noltii*) should be better incorporated in future restoration efforts in the Wadden Sea, as our results show that the smaller seagrass increases benthic diversity to a similar degree as *Z. marina*. Thus far restoration efforts in the Dutch Wadden Sea have primarily targeted *Z. marina*, but considering our results we argue that *Z. noltii* should also get a more visible role in conservation and restoration practices, due to the species ecological importance and ability to create stable perennial meadows.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

The manuscript presents research on animals that do not require ethical approval for their study.

Author contributions

MG: Conceptualization, Formal analysis, Investigation, Visualization, Writing – original draft, Writing – review & editing. IG: Formal analysis, Investigation, Writing – review & editing. SV: Investigation, Writing – review & editing. IF: Investigation, Writing – review & editing. TV: Conceptualization,

Funding acquisition, Supervision, Writing – review & editing. JH: Conceptualization, Investigation, Writing – review & editing, Funding acquisition. KM: Resources, Writing – review & editing. BE: Resources, Writing – review & editing. QS: Funding acquisition, Writing – review & editing. LG: Conceptualization, Funding acquisition, Investigation, Supervision, Writing – review & editing.

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Conflict of interest

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

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Supplementary material

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fmars.2023.1294845/full#supplementary-material>

SUPPLEMENTARY FIGURE 1

Epi- and endobenthic taxonomic richness inside and outside natural seagrass meadows and the restored meadow. Boxplots show median (line in box), upper and lower quartile (box), 1.5 x interquartile range (vertical line) and outliers (circle). Stars indicate significance of $p < 0.001$ for ***, $p < 0.01$ for ** and $p < 0.05$ for *.

SUPPLEMENTARY FIGURE 2

Relationships between seagrass cover % and benthic (A) taxonomic richness, (C) functional richness, (E) Pielou’s evenness, (G) Functional evenness, (I) Shannon index and (K) Functional dispersion in the intertidal Wadden Sea. Relationships between seagrass dry weight (g) and benthic (B) taxonomic richness (D) functional richness, (F) Pielou’s evenness, (H) Functional evenness, (J) Shannon index and (L) Functional dispersion in the intertidal Wadden Sea.

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Wave, light, and dissolved oxygen exposures drive novel coastal eelgrass (*Zostera pacifica*) transplant performance

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The high ecological and economic value of seagrass has been long recognized, with these foundational habitats providing myriad ecosystem services. Yet through cumulative anthropogenic impacts, seagrasses are exhibiting extensive declines globally. A litany of studies and active restoration trials have demonstrated practical methodologies to restore seagrass habitats and effectively return critical habitat functions to degraded coastal zone systems worldwide. Seagrass loss along the U.S. West Coast has precipitated decades of seagrass protection, conservation, and restoration efforts. Yet, mitigation transplanting efforts have prioritized *Zostera marina* (narrow-leaved eelgrass) in shallow, protected environments, while a dearth of information is available on species inhabiting offshore islands and exposed mainland coasts. In this study, we conducted a novel transplant of *Zostera pacifica*, a wide-leaved species found in depths of 7 – 20 m along the offshore islands and mainland coast of California. Transplants were conducted at three geographically distinct sites in Santa Monica Bay, coupled with continuous monitoring of biophysical parameters providing insight into physical drivers at transplant and donor sites. Utilizing *in situ* data, and environmental thresholds adapted from the literature for *Z. marina*, we performed exposure analyses to evaluate factors influencing *Z. pacifica* transplant performance. Exceedances of threshold values for environmental parameters, specifically, wave exposure and near-bed flow speeds ($H_{rms} > 0.59$ m and $U_{rms} > 0.1$ m s⁻¹), photosynthetically active radiation (< 3 and > 5 mol m⁻² day⁻¹) and dissolved oxygen (< 3 mg O₂ L⁻¹) exposure impacted transplant survivorship. These results suggest *Z. pacifica* persist in biophysically dynamic conditions and are sensitive to exceedances of thresholds, underlining the importance of pre-transplant site-selection processes to this species. These data represent the first holistic study of *Z. pacifica* transplanting on an exposed mainland coast, which provides a view into the baseline environmental envelopes within existing *Z. pacifica* habitat, and further, may serve as a model for investigating scalable open coast seagrass restoration for temperate regions.

KEYWORDS

eelgrass, transplant, *Zostera pacifica*, PAR, DO, wave, thresholds

1 Introduction

Seagrasses are a suite of submerged aquatic angiosperms found globally and constitute critical foundational species in near-shore, shallow water marine systems (Duarte, 2002; Short et al., 2007; Unsworth and Cullen-Unsworth, 2014). Seagrasses provide myriad ecosystem services, supporting diverse fish and invertebrate species (Hoffman, 1986; Irlandi et al., 1999; Allen et al., 2002; Pondella et al., 2003; Tanner et al., 2019) and buffering against coastal erosion (Hansen and Reidenbach, 2012; Duarte et al., 2013). Seagrasses act as ‘blue carbon’ systems, capable of mitigating local impacts of ocean acidification (Kapsenberg and Hofmann, 2016; Ricart et al., 2021), and contributing to biological carbon fixation (Fourqurean et al., 2012; Duarte and Krause-Jensen, 2017; Ward et al., 2021). Regardless of the ecological function, value, and importance of seagrasses (Barbier et al., 2011), these habitats are experiencing stark rates of decline at ~ 30% loss globally (Orth et al., 2006; Waycott et al., 2009; Krause-Jensen et al., 2020). The shallow coastal zones colonized by seagrasses are often jointly exposed to multiple local stressors (e.g., sedimentation, eutrophication, habitat fragmentation) (Obaza et al., 2015; Adams et al., 2016; Livernois et al., 2017) compounded by cumulative global pressures (e.g., elevated sea surface temperatures, sea level rise) (Turschwell et al., 2021; Unsworth et al., 2022). The vast benefits of seagrass ecosystems, coupled with ongoing threats to habitat, have precipitated diverse lines of inquiry into drivers of ecosystem structure, function, and the capacity for habitat restoration (Short and Wyllie-Echeverria, 1996; McKenzie et al., 2020).

Seagrass spatial coverage and habitat function are controlled by complex abiotic and biotic environmental factors (Munsch et al., 2023). Biological top-down controls can positively impact seagrass health (Hughes et al., 2013; Foster et al., 2021), or as is the case with herbivory, cause direct consumptive loss to the seagrass bed (Altstatt, 2003; Heck and Valentine, 2006; Jiménez-Ramos et al., 2021; Valentine and Heck, 2021). High ocean temperatures can change photosynthetic efficiencies, deteriorate disease resistance, alter community structure, and reduce growth and survival of seagrasses growing near species-specific thermal tolerances (Johnson et al., 2003; York et al., 2013; Moore et al., 2014; Jakobsson-Thor et al., 2020; Strydom et al., 2020). Likewise, dissolved oxygen is a vital factor influencing habitat function (Parnell et al., 2020; Tassone et al., 2022), with hypoxia and anoxia precipitating reduced growth and survival rates in seagrasses (Holmer and Bondgaard, 2001; Greve et al., 2003; Pulido and Borum, 2010). The two most consequential drivers influencing seagrass distribution at local scales are wave action and light attenuation (Zimmerman et al., 1997; Short and Coles, 2001; Duarte, 2002). Physical damage via wave exposure can break shoots and uproot plants, often setting the shallow limit of seagrass beds (Fonseca and Bell, 1998; Kopp, 1999; Koch, 2001; Short et al., 2002). Hydrodynamics can disrupt critical seagrass ecological processes, impacting nutrient uptake (Cornelisen and Thomas, 2004; El-Hacen et al., 2019; Dahl et al., 2020) and altering ‘blue carbon’ capabilities through POC and DOC resuspension (Egea et al., 2023). Light regimes influence the photosynthetic capabilities of seagrass, and often constrain the depth limit of successful colonization

(Ostenfeld, 1905; Phillips, 1964; Backman and Barilotti, 1976; Dennison and Alberte, 1982; Duarte, 1991; Nielsen et al., 2002; Adams et al., 2016; Beca-Carretero et al., 2021; Krumhansl et al., 2021; Jiménez-Ramos et al., 2023). Stochastic environmental events such as storms alter seagrass survival and can precipitate cascading declines in regional and local seagrass coverage (Rasmussen, 1977; Pollard and Greenway, 2013). The elucidation of species-specific biophysical limitations remains a critical component needed to enact appropriate and effective conservation measures and restorative actions (Aoki et al., 2020).

Along the Pacific Coast of North America, *Zostera marina* (L.) (narrow-leaved eelgrass) is the largest distributed seagrass species in the region inhabiting coastal estuaries and bays (Short et al., 2007; Blok et al., 2018). A second principal eelgrass species, the larger congener *Zostera pacifica* (wide-leaved eelgrass) (Watson, 1890), is present in just the Southern California Bight (SCB), inhabiting coastal shelf habitats and offshore islands in water depths of 7 – 20 m (Cottam and Munro, 1954; State Water Resources Control Board (SWRCB), 1979; Engle and Miller, 2005; Coyer et al., 2008; Olsen et al., 2014; Obaza et al., 2022). Seagrass habitats along the West Coast of the USA, especially in California, are subject to significant anthropogenic impacts that have precipitated substantial losses of *Zostera* spp. (National Oceanic and Atmospheric Administration, 2014; Kelly et al., 2019), in some cases as severe as 95% loss in a 10-year period (O’Leary et al., 2021). To combat these losses, regulatory agencies have enacted ‘no net loss’ guidelines which necessitate compensatory mitigation when coastal development degrades *Zostera* spp. habitat (Bernstein et al., 2011). As such, restoration efforts to alleviate the loss of *Zostera* spp. habitat have been ongoing for nearly 50 years (Phillips, 1964), and in the SCB, for more than 30 years (Ward and Beheshti, 2023). These compensatory mitigations and active restoration projects have remained relatively small and have utilized a multitude of transplant techniques, including single shoot, bundle shoot, transplant frames (TERFS), among others, and each project resulted in varying degrees of project success (Williams, 2001; Altstatt, 2003; Ward and Beheshti, 2023).

Restoration of estuarine and protected seagrass systems have garnered recognizable successes in restoring seagrass extent and habitat function (Orth et al., 2020; Beheshti et al., 2022; Gräfnings et al., 2023). Yet, global attempts at exposed open coast seagrass restoration are limited, with successful projects confined primarily to Western Australia (Paling et al., 2001; Paling et al., 2003; Paling et al., 2007; Tan et al., 2020), though additional examples of successful projects exists in Portugal and Tanzania (Paulo et al., 2019; Wegoro et al., 2022). Open coast seagrass habitats, especially on the West Coast of the U.S., are plagued by inconsistent, outdated, or entirely absent data; congruent with the challenges highlighted by global assessments seeking to gauge seagrass coverage more accurately (McKenzie et al., 2020). To date, nearly all seagrass restoration projects in the SCB have focused on *Z. marina* following impacts from coastal development projects occurring in depths from 1 to ~6 m in protected bays, lagoons, and estuaries (Altstatt, 2003; and e.g., Hoffman, 1986; Pondella et al., 2006; Merkel & Associates, Inc, 2010; Obaza et al., 2015; MBC Applied Environmental, 2016), a disparity evidenced in a meta-analysis

that identified 43 out of 44 transplant projects occurring in the SCB from 1989 to 2020 focused on *Z. marina* in protected embayments and estuaries (Ward and Beheshti, 2023).

Santa Monica Bay (SMB) constitutes the expanse from the rocky headland of Point Dume to the Palos Verdes Peninsula and includes much of the Los Angeles watershed (Figure 1). SMB comprises a vast diversity of intertidal and subtidal habitats that are critically important for the ecological, economic, and cultural wellbeing of the region, earning the designation as one of the USA EPA's 28 National Estuary Programs (NEP). Historic accounts show that along with vast sandy habitats as well as rocky reefs and kelp forests, *Zostera* spp. were prevalent within the SMB NEP boundaries (Egstrom, 1974). Urbanization (i.e., coastal development, eutrophication, and pollution) associated with the growth of Los Angeles contributed to widespread degradation of the local marine environment (North, 1963; State Water Resources Control Board (SWRCB), 1979; Wilson and North, 1983; Williams et al., 2021). Contemporary assessments, though scattered and incomplete, of *Zostera* spp. in SMB reveal patchy and ephemeral beds predominately along the north-western shores near Malibu (Egstrom, 1974; Bernstein et al., 2011), in addition to robust and stable beds on offshore islands (Engle and Miller, 2005). This paucity of information specifically regarding *Z. pacifica* habitat hinders conservation efforts (but see Engle and Miller, 2005;

Coyer et al., 2008; Altstatt et al., 2014; Olsen et al., 2014; Obaza et al., 2022).

The primary goals of this study were to apply prior successful *Zostera* spp. transplant techniques to a novel environment (i.e., exposed mainland coast) to advance methods for *Z. pacifica* restoration, and subsequently, assess the efficacy and scalability of open coast restoration in the SCB. In particular, the purpose of our study was to (1) track the temporal survivorship of transplanted *Z. pacifica* across three geographically distinct transplant sites within SMB, (2) quantify a suite of environmental parameters likely to be key to open coast seagrass survival, and (3) elucidate the conditions within extant open coast *Z. pacifica* beds. To evaluate causative factors impacting transplant performance, continuous *in situ* measurements of biophysical oceanographic metrics (temperature, wave characterizations, photosynthetically active radiation, and dissolved oxygen) were recorded at discrete transplant and extant donor sites from 2021 through 2023. These data can provide scientists, resource managers, and restoration practitioners a lucid view into the realized environmental niche of *Z. pacifica* and can substantially improve our ability to protect and restore this regionally significant foundational coastal habitat. And further, upscaling *Zostera* spp. restoration throughout an expanded scope of available habitat (i.e., open coast), allows for a greater range of improved habitat function. This study advances our foundational

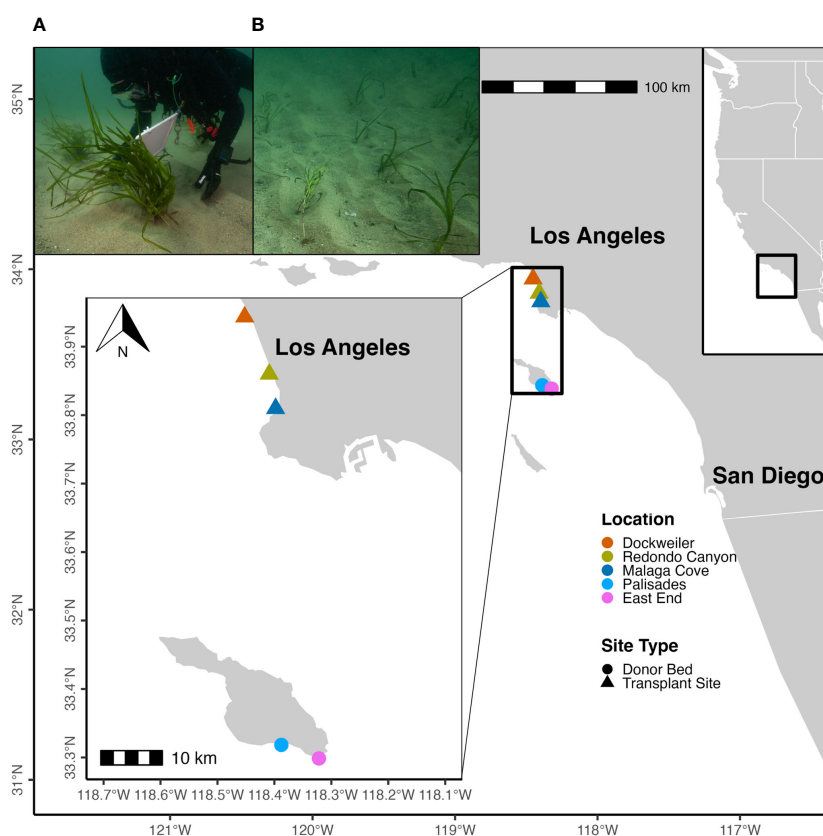


FIGURE 1

Locations of *Zostera pacifica* (wide-leaved eelgrass) transplant sites and donor bed sites within the Southern California Bight. Sites are indicated by color and site types are designated by shape. Photos from transplant sites of the (A) bundle shoot transplant method and (B) single shoot transplant method are displayed.

understanding of temperate open coast seagrass habitats by disentangling the biophysical oceanographic dynamics and species-specific requirements conducive to *Z. pacifica* proliferation, further aiding to inform effective restoration.

2 Materials and methods

2.1 Study sites

The Southern California Bight (SCB) spans ~1200 km of coastline from Ensenada, Mexico to Point Conception, California and consists of a multitude of subtidal habitats, including rocky reef, kelp forest, and seagrass habitats (Williams et al., 2022). This project was focused along the mainland coast of Santa Monica Bay (SMB) and offshore Catalina Island (Figure 1). Three transplant sites were within coastal SMB, while the two donor bed sites (two largest extant *Z. pacifica* beds in the region) were located ~40 km away on the south-eastern side of Catalina Island. Each site was ~0.5 km offshore of the local coastline (mainland or Catalina). All transplant sites were established at 10–12 m depth, typical of natal *Z. pacifica* beds within the SCB (Bernstein et al., 2011; Obaza et al., 2022). *Z. pacifica* transplanting at the three mainland sites occurred in July 2021, during the primary growing season (March through October) established by the 2014 National Oceanic and Atmospheric Administration California Eelgrass Mitigation Policy (CEMP). Donor material was collected at Catalina and transplanting occurred at Redondo Canyon on 20 July 2021, at Malaga Cove on 22 July 2021, and at Dockweiler on 27 July 2021. Dockweiler, Redondo Canyon, and Malaga Cove received 520, 486, and 526 shoots, respectively. A total of 1532 shoots were collected and transplanted to the three sites, constituting the largest *Z. pacifica* transplant reported to date.

2.2 Donor material collection

The donor shoots were collected from two robust extant beds on the windward side of Catalina Island, Palisades and East End, with areas and densities of 27.74 hectares and 112 shoots m⁻², and 10.48 hectares and 114 shoots m⁻², respectively (Obaza et al., 2022). The donor bed sites ranged from 11–22 m depth. While numerous extant *Z. pacifica* beds exist closer to the mainland within SMB, their ephemeral nature and small patchy distribution excluded them as adequate donor beds for transplant experiments. We instead selected sites at Catalina Island characterized by extensive and healthy *Z. pacifica* populations with decadal stability (Engle and Miller, 2005).

Material was collected from donor beds, for both the single shoot and bundle shoot transplant methodologies, in a systematic fashion, utilizing a thinning approach to avoid the creation of noticeable (> 0.5 m⁻²) bare patches, and minimizing fragmentation. For the single shoot method, divers selected individual shoots and gently maneuvered the shoot and rhizome out of the sediment such that at least three internodal segments (~100 mm) of rhizome were attached to the apical shoot (Altstatt,

2003). Rhizomes with a single shoot were selectively used for this method, but per Paling et al. (2007) sediment was not actively removed from the rhizomes. Material for the bundle shoot method was collected similarly, although rhizomes with several shoots attached were also included along with a small amount of sediment. Divers selected shoots from the shallow edge, middle, and deep edge of the site to minimize fragmentation impact on the donor bed, while likely increasing clonal genetic diversity following Olsen et al. (2014). To complete the bundle approach (Zhou et al., 2014), multiple shoots were connected with biodegradable twine on the research vessel during the transit to the transplant sites. Donor material was temporarily stored in large coolers with flow-through seawater and kept shaded for up to four hours between collecting and transplanting.

2.3 Transplant

Collecting donor material and transplanting for single shoot and bundle shoot methodology occurred on the same day for each transplant site to minimize exposure stress. At each transplant location, SCUBA divers established a 30 m line at 10 to 12 m depth with individual experimental plots dispersed on either side. Each transplant site consisted of seven experimental plots, four bundle shoot plots (each 12 m⁻²) and three single shoot plots (each 9 m⁻²), ensuring inter-site replication along a single depth strata. Material was planted in a grid pattern within each plot and meter sticks were used to maintain transplant spacing. For the bundle shoot method, between 10–13 bundles (consisting of 80–110 total shoots) were planted at 1 m intervals within the four replicate plots at each site (see Figure 1A). For each plot, divers excavated a small hole in the sediment, and the entire rhizome mass of the bundle was placed in the excavated hole along with a wooden tongue depressor, which was secured to the rhizome mass and positioned parallel to the substrate to act as an anchor. Single shoot planting was conducted using methods adapted from Altstatt (2003). Between 40–46 shoots were planted at 0.5 m intervals within the three replicate single shoot plots at each site (see Figure 1B). Divers used a trowel to excavate sediment and gently maneuvered a single rhizome into the hole, placing a small gardening stake over the rhizome to act as an anchor. Sediment was pushed back over the rhizome so that it was completely buried, leaving the shoot naturally situated above the sediment.

2.4 Biological monitoring

All biological monitoring was conducted via SCUBA. Fish and eelgrass structural surveys were conducted at donor beds prior to (June 2021), and after (August 2021, July 2022, March 2023), collection of transplant material. Fish surveys were conducted at each site, where divers swam < 1 m above the substrate and identified the species, counted the abundance, and determined the size of each fish within a 1 m high x 2 m wide survey window along the timed roving diver fish surveys (per Pondella et al., 2006; Looby and Ginsburg, 2021; Obaza et al., 2022). Collection of

morphometrics (length, width, and density) of *Z. pacifica* in donor beds followed methods in [McCune et al. \(2020\)](#) and [Obaza et al. \(2022\)](#).

Divers performed pre-transplant site surveys at each transplant location, and subsequently conducted monitoring to assess the biological characteristics of the transplants at intervals of 1-day, 1-week, 2-weeks, 1-month and quarterly thereafter through June 2023. Survivorship was defined as the number of remaining planting units (i.e., single shoots or bundle shoots) within plots ([Wegoro et al., 2022](#)). Inter-plot survivorship was assessed by enumerating single shoots and bundle shoots in each experimental plot at each transplant site during each timepoint ([Altstatt et al., 2014](#)). Divers temporarily placed flagging stakes next to each shoot to aid in accurate counts and to increase efficacy during monitoring events.

2.5 Environmental monitoring

The establishment of habitat and species-specific threshold values is a critical tool in ecosystem management regimes, as exceedances of threshold values can catalyze abrupt responses, often leading to habitat decline or loss ([Uhrin and Turner, 2018](#)). Threshold values of biophysical parameters impacting *Z. marina* have been expounded in numerous studies across its geographical distribution, both *ex situ* and *in situ* ([Short et al., 2002](#); [Lee et al., 2007](#); [Moore and Jarvis, 2008](#); [Thom et al., 2008](#)). No critical threshold tolerances have been investigated for *Z. pacifica*; thus, biophysical thresholds established in this study have been adapted either from data collected near *Z. pacifica* habitat or from applicable *Z. marina* literature.

We measured temperature, light, dissolved oxygen, and wave exposure at each site, as these biophysical parameters can result in adverse impact to seagrass health, function, and survival ([Fonseca and Bell, 1998](#); [Holmer and Bondgaard, 2001](#); [Johnson et al., 2003](#); [Duarte et al., 2007](#)). Temperature was measured from July 2021 – June 2023. Wave dynamics were measured across four deployments October 2020, July – August 2021, September – December 2021, and February – June 2022. Light and dissolved oxygen sensors were added from May 2022 – June 2023. The number of days with acceptable data in the time series varied among the sites and environmental metrics ([Table 1](#)).

2.5.1 Temperature

Following known adverse effects of elevated temperature on seagrass survival ([Hammer et al., 2018](#)), this study adapted a temperature threshold based on data collected near a *Z. pacifica* bed on Santa Cruz Island, California ([Kapsenberg and Hofmann, 2016](#)) and studies presented in a review by [Lee et al. \(2007\)](#). The upper temperature induced growth inhibition threshold for this study was set at 20°C.

In situ bottom temperature was measured with thermistor instruments deployed continuously throughout the project. HOBO Temperature/Light Data Logger (Onset UA-002-64 loggers) set to 10-minute sampling interval were deployed at < 1 m above the

substrate in the transplant sites from July 2021 – July 2022. HOBO loggers were phased out of the project and from May 2022 – June 2023, transplant and donor beds had bottom mounted (< 1 m above substrate) continuous temperature data recorded by miniPAR data loggers (Precision Measurement Engineering) set to 10-minute sample interval from May – October 2022 and 5-minute sample interval from November 2022 – June 2023).

2.5.2 Wave exposure

Wave action can cause dislodgment and physical damage to seagrass ([Koch, 2001](#)). Open Wave Height Loggers (OWHLs) are an inexpensive open-source design pressure transducer and digital recorder capable of continuous, multi-month deployment, with data quality comparable to commercially available products ([Lyman et al., 2020](#)). OWHLs are a cost-effective approach for site selection and optimization projects necessitating multi-site wave observations. OWHLs (4 Hz continuously recording) were deployed < 1 m from the substrate in each donor bed and transplant site across multiple seasons. An initial deployment in October 2020 ($n = 19$ days) was conducted at the Palisades donor site to characterize wave conditions at a donor bed. The second deployment occurred July – August 2021 ($n = 44$ days) at the three sites in SMB, providing data on inter-transplant site differences prior to and during the initial transplant period. Based on the findings from this deployment, notably that Dockweiler experienced significantly higher magnitude and longer sustained exposure to wave conditions than the other two transplant sites (see results below), the Dockweiler transplant site was excluded from subsequent OWHL deployments. Instead opting to reallocate the four OWHL sensors to concomitantly monitor both donor bed sites (Palisades and East End) and transplant sites (Redondo Canyon and Malaga Cove). The third ($n = 95$ days) and fourth ($n = 108$ days) OWHL deployments occurred from September – December 2021 and February – June 2022, respectively, in order to characterize wave conditions throughout the transplant and post-transplant time period to understand the role wave exposure might play in survivorship and persistence.

Wave spectra and derived quantities including root-mean-square wave heights, peak period, and wave orbital velocity at the bottom (seabed), were calculated from the OWHL pressure time series in MATLAB ([The MathWorks Inc, 2022](#)). First, to correct for attenuation of the pressure signal with depth, a pressure response factor (e.g., [Dean and Dalrymple, 1991](#)) was applied to the discrete Fourier transform calculated for each 30-min interval. From the corrected sea surface time series, the power spectral density (hereafter ‘the wave spectrum’), was then calculated every 30 minutes. The root-mean-square wave height (H_{rms}) is representative of the energy of the sea state (swell and wind waves) and is calculated as the square root of eight multiplied by the variance of the surface elevation. Note that in some studies wave energy is reported as a significant wave height, H_s , which is proportional to H_{rms} so that $H_s = \sqrt{2}H_{rms}$. The peak period (T_p) is the inverse of the frequency containing the highest wave energy found from the wave spectrum (e.g., [Holthuijsen, 2007](#)). Particle trajectories under a wave follow an orbital motion ([Dean and](#)

TABLE 1 Biophysical environmental data summary.

	Dockweiler	Redondo Canyon	Malaga Cove	Palisades	East End
Temperature					
Mean (°C)	15.66	15.30	16.03	16.46	16.50
SD (°C)	1.58	1.82	1.69	2.41	2.64
n (days)	535	598	515	338	369
Wave					
H _{rms} mean (m)	0.45	0.48	0.50	0.37	0.38
H _{rms} SD (m)	0.08	0.20	0.18	0.09	0.10
H _s (m)	0.64	0.67	0.71	0.53	0.54
H _s SD (m)	0.11	0.28	0.26	0.13	0.14
U _{rms} mean (m s ⁻¹)	0.12	0.11	0.13	0.09	0.11
U _{rms} SD (m s ⁻¹)	0.02	0.05	0.05	0.03	0.03
Tp mean (s)	14.80	8.55	12.20	14.70	14.10
Tp SD (s)	1.84	1.78	3.16	1.68	1.76
n (days)	44	237	237	195	195
PAR					
Mean (mol m ⁻² day ⁻¹)	0.87	1.44	1.78	3.02	4.01
SD (mol m ⁻² day ⁻¹)	0.92	1.00	1.09	2.51	2.88
n (days)	181	270	215	338	369
DO					
Mean (mg O ₂ L ⁻¹)	5.77	7.34	6.19	8.13	7.76
SD (mg O ₂ L ⁻¹)	2.95	1.04	2.30	0.37	0.55
n (days)	181	270	215	369	249
Depth of sensor (m)	11.4	11.6	11.1	12.0	11.6

Temperature data spans July 2021 – June 2023. Wave dynamics were measured across four deployments October 2020, July – August 2021, September – December 2021, and February – June 2022. Photosynthetically active radiation and dissolved oxygen sensors were deployed from May 2022 – June 2023. *Zostera pacifica* transplant occurred in July 2021.

Dalrymple, 1991), where the horizontal velocity U_{rms} was calculated following Svendsen (2005) and Monismith et al. (2013).

Short et al. (2002) established a threshold value for wave exposure limiting *Z. marina* transplant beds as the mean of the conditions at the donor bed(s) plus two standard deviations. In our study, data from each donor bed site across all deployments was aggregated to calculate mean + 2SD, resulting in a threshold H_{rms} value of 0.59 m and a U_{rms} value of 0.1 m s⁻¹.

2.5.3 Photosynthetically active radiation

Light availability is critical to seagrass growth and survival (Duarte, 1991). Photosynthetically active radiation (PAR) was measured continuously *in situ* as photosynthetic photon flux density (PPFD) using optical time-series instruments equipped with cleaning wipers deployed from May 2022 – June 2023. The fixed vertical sensor arrays were deployed < 1 m from the substrate. The miniPAR light sensor (Precision Measurement Engineering) sampled at 10-minute intervals from May – October 2022 and 5-minute intervals from November 2022 – June 2023, is equipped with

a LI-192 Underwater Quantum Sensor (manufactured by LI-COR), which can detect a wavelength range of 400 – 700 nm. To reduce the noise created by surface light flicker, the value measured by the miniPAR sensor is an average of 30 readings taken over a 5 second period. The anti-fouling cleaning wiper was set to wipe the sensor at 3-hour intervals throughout the deployments. The PPFD data (μmol m⁻² s⁻¹) were integrated over the course of each sampling day, creating daily integrated PAR (mol quantum m⁻² day⁻¹) following Dunic and Côté (2023).

Thom et al. (2008) conducted *ex situ* and *in situ* research on light requirements for *Z. marina* in the Pacific Northwest region of the U.S. and found 3 mol m⁻² day⁻¹ to be growth and survival limiting, while 5 mol m⁻² day⁻¹ was found to induce strong growth rates with long-term survival. This study has adapted these values as light thresholds.

2.5.4 Dissolved oxygen

Accelerated seagrass mortality and reduced growth rates are common impacts associated with low dissolved oxygen (DO)

conditions (Holmer and Bondgaard, 2001). DO was measured *in situ* using a submersible miniDOT instrument (Precision Measurement Engineering) sampling at 10-minute intervals from May – October 2022 and 1-minute sample interval from November 2022 – June 2023. The miniDOT contains an optode that detects oxygen concentrations in the water column across a sensing foil membrane and records values in $\text{mg O}_2 \text{ L}^{-1}$. Temperature is measured concurrently with DO values. A 33.50 ppt salinity correction factor, representative of the long-term average for the region (Carter et al., 2022), was applied to the raw data following the manufacturer's recommendation. Further, miniDOT instruments record a 'Q value' corresponding to the quality of the individual measurement, where a value > 0.7 is indicative of proper sensor operation. A copper plate and copper mesh package were utilized as anti-fouling devices.

Moore and Jarvis (2008) identified water column DO values of $< 3 \text{ mg O}_2 \text{ L}^{-1}$ to induce tissue degradation in *Z. marina*, thus, we set a threshold of $3 \text{ mg O}_2 \text{ L}^{-1}$.

2.6 Statistical analysis

Differences in the composition of fish assemblages were compared by creating a Bray-Curtis dissimilarity matrix from average relative species-specific encounter rates across individual surveys completed at transplant and donor beds from 2021 – 2023. All species with $< 2\%$ mean encounter rates across all surveys were removed. The dissimilarity matrix was used to create non-metric multi-dimensional scaling plots to visualize changes in fish assemblages across transplant and donor beds. Assemblage differences were tested using permutation multivariate analysis of variance (PERMANOVA) (McCune and Grace, 2002). Differences in group dispersion were tested using the 'betadisper' function in the 'vegan' package and were not significant; therefore, no additional transformations on these data were conducted. To determine species-specific differences in fish assemblage, a similarity percentages (SIMPER) procedure was performed with treatment as a factor. All multivariate statistical approaches were conducted using the 'vegan' package in R (Oksanen et al., 2019).

Environmental data were analyzed and graphed in R (RCore Team, 2021, v. 4.1.2). Data corresponding to sensor malfunction and extreme biofouling were removed during quality control and were excluded from analysis (following Ricart et al., 2021). Standardization of the timeseries date range was applied to environmental data for statistical analysis and was based on the latest data start date and the earliest data end date across all sites for each biophysical parameter. Exposure analysis was conducted utilizing the 'heatwaveR' package in R (Schlegel and Smit, 2018). Exposure calculations were performed based on daily exceedance (or negative exceedance, i.e., below) of established parameter thresholds. Empirical cumulative distribution function (eCDF) plots were employed to differentiate patterns of the environmental metrics among sites (Beheshti et al., 2022). Environmental data lacked variance homogeneity and were therefore analyzed using a non-parametric Kruskal-Wallis test and Dunn's *post hoc* test with a Holm-Bonferroni correction to

critical p-value due to application of multiple tests of significance among sites (Pulido and Borum, 2010; Campbell et al., 2018). A p-value < 0.05 represents a significant difference detected between sites, indicating a rejection of the 'similarity of sites' null hypothesis.

3 Results

3.1 Transplant survivorship

Transplant sites experienced minor loss immediately post-transplanting, followed by a slight expansion in total shoots observed at two of the three sites around 20 days post-transplant, until complete mortality was observed across all sites after day 315 (Figure 2). Survivorship varied across sites, with poor survivorship at Dockweiler (DW), better survivorship at Redondo Canyon (RC), and longest survivorship at Malaga Cove (MC). At DW and RC, 1.9 and 2.1% of material was lost between transplanting and the 24-hour survey respectively, while MC did not lose any transplanted material until day 15. Highlighting site-specific divergence, survivorship at DW was only 35.8% by day 37, while RC and MC experienced 85.4% and 80.4% survivorship respectively. By day 90, DW, RC, and MC had 23.3%, 67.9%, and 68.1% survivorship, but by day 197 complete mortality occurred at DW and RC, and survivorship at MC had declined to 30%.

Further survival disparity was pronounced among transplant methods, with the single shoot method outperforming bundle shoot method across all sites (Figure 2). Methodological survival differences were most prominent at DW, whereby day 37 bundle shoot survivorship was reduced to 23.1%, while single shoot survivorship was substantially higher at 75.4%. Differences in survival among transplant methods were also evident at other sites, whereby day 90 RC single shoot survivorship was 89.3% while bundle shoot survivorship was 60.7%; and at MC single shoot survivorship was 93.1% while bundle shoot survivorship was 59.8%. At MC (the best performing site) 5.4% of single shoots remained on site until after day 315, while bundle shoots suffered prior complete mortality.

3.2 Fish surveys

A total of 3,692 fishes from 26 species were recorded over 139 transects across all five sites. The donor bed sites had greater species richness than transplant sites throughout the study period and greater species encounter rates for all fishes as well (Figure 3). No fish encounters were observed during pre-transplant site selection surveys, and there were no significant increases in encounter rate before and after transplant activities at transplant sites. No meaningful relationships of species richness or encounter rate were detected between the three transplant sites. PERMANOVA results indicated that the composition of fish assemblages observed at donor beds and transplant sites were significantly different from one another ($F_{1,24} = 10.14$, $p = 0.001$), with no overlap in the NMDS plot (Figure 3). According to results from the SIMPER analysis,

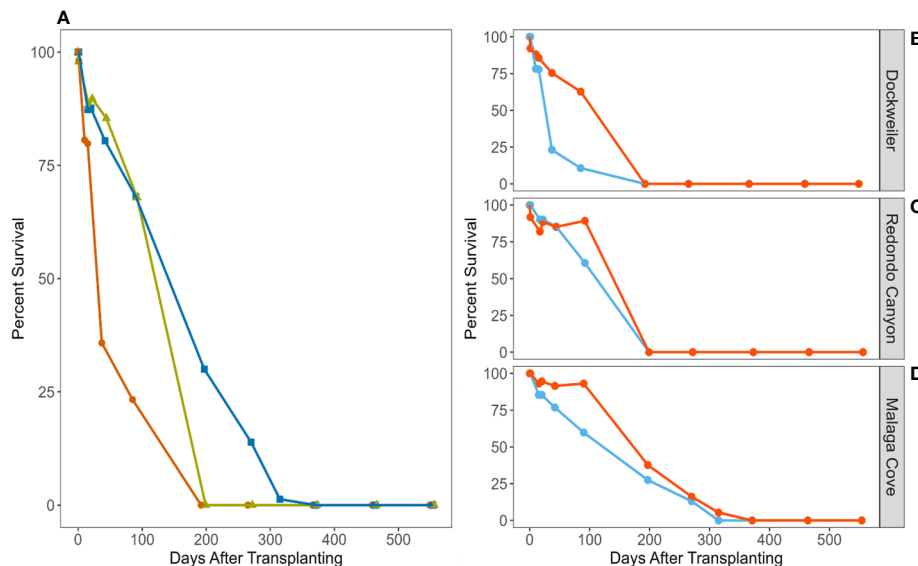


FIGURE 2

Zostera pacifica (wide-leaved eelgrass) transplant survivorship from July 2021 – June 2023, expressed in days after transplanting. Percent survivorship by site (A), color and shape designate site: orange circles (Dockweiler), gold triangles (Redondo Canyon), blue squares (Malaga Cove). Percent survivorship by transplanting method per site (B–D), color designates transplant method: blue (bundle shoot) and orange (single shoot).

differences were driven by greater relative abundance of sanddab (*Citharichthys stigmaeus*) and California halibut (*Paralichthys californicus*) at transplant sites and greater relative abundance of kelp bass (*Paralabrax clathratus*), rock wrasse (*Halichoeres semicinctus*), señorita (*Oxyjulis californica*), and salema (*Xenistius californiensis*) at donor bed sites.

3.3 Temperature exposure

Temperature exposure above the 20°C threshold was not a crucial indicator of transplant performance as values fell well within, and even below, the exposure conditions at the donor bed sites (Figure 4, Tables 1, 2). Transplant site high temperature exposure percentages were DW (2.06%), RC (2.34%), and MC

(3.30%) and were less exposed than the donor bed sites Palisades (PAL) (11.54%) and East End (EE) (17.07%). The longest continual exposure period above the threshold for the sites were 10, 11, 11, 11, and 26 days for DW, RC, MC, PAL, and EE respectively. The highest temperature recorded at the transplant sites was 22.39°C and 23.09°C at the donor bed sites. From August 2021 – January 2023 for transplant sites ($n = 514$ days), DW was significantly different from MC ($p < 0.05$) and MC was significantly different from RC ($p < 0.05$), though DW was not significantly different from RC ($p = 0.76$). For continuity of date range across all transplant and donor sites, from May 2022 – January 2023 ($n = 221$ days) significant differences were detected between comparisons of transplant sites and donor bed sites ($p < 0.05$), though temperatures at the two donor bed sites were not significantly different from one another ($p = 0.28$).

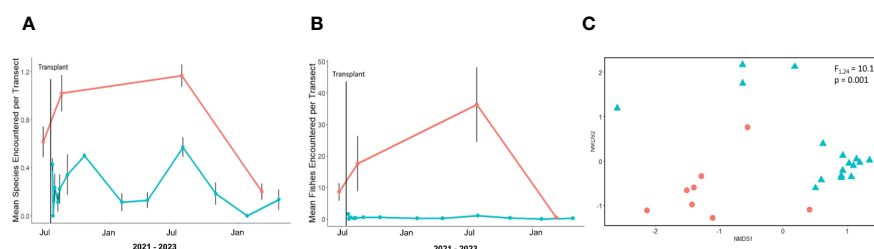


FIGURE 3

Blue color represents transplant sites and red color represents donor bed sites. (A) Species richness at transplant and donor bed sites. Points are averages of all transects at each survey and error bars are standard error. (B) Encounter rates for all fishes at transplant and donor bed sites. Points are averages of all transects at each survey and error bars are standard error. (C) NMDS plot of fish assemblages in transplant and donor bed sites. Each point represents the mean relative encounter rates across all transects during a single survey event at a transplant or donor bed site. Surveys took place from 2021 to 2023. The points remain separate despite passage of time, suggesting relative fish encounters remained different across transplant and donor bed sites. Stress was 0.033. Permutation multivariate analysis of variance (PERMANOVA) results reported on plot.

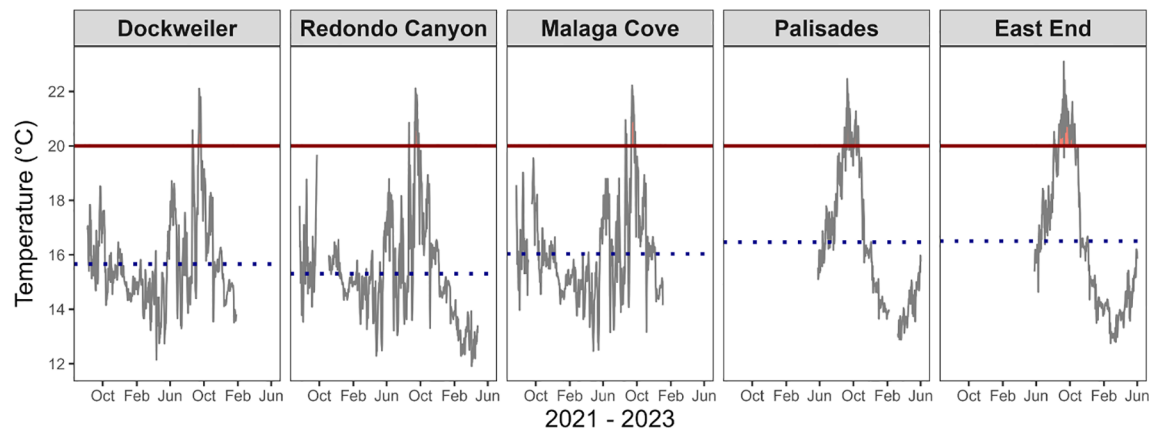


FIGURE 4
Temperature exposure timeseries across all transplant and donor sites from July 2021 – June 2023. Solid black line represents mean daily temperature timeseries, while dotted blue line represents site average across the span of the timeseries. Red horizontal line represents temperature threshold set at 20 °C. Gaps in data correspond to sensor malfunctions. Temperature data was unavailable at donor beds until May 2022. Dockweiler (DW), Redondo Canyon (RC), and Malaga Cove (MC) are transplant sites. Palisades (PAL) and East End (EE) are donor beds. Site average $\pm$ SD in °C: DW (15.66 $\pm$ 1.58), RC (15.30 $\pm$ 1.82), MC (16.03 $\pm$ 1.69), PAL (16.46 $\pm$ 2.41), and EE (16.50 $\pm$ 2.64). See Table 2 for percent time each site was above the threshold.

3.4 Wave exposure

Exposure exceeding the wave thresholds was common from July – August 2021 (Figure 5, Tables 1, 2). Transplant site exposure percentages above the H_{rms} threshold were DW (6.82%), RC (0%), and MC (2.27%), while exposure percentages above the U_{rms} threshold were DW (86.36%), RC (13.64%), and MC (36.36%). The longest continual exposure period above the U_{rms} threshold for

the sites were 28, 3, and 8 days for DW, RC and MC respectively. Over the July – August 2021 deployment (n = 44 days), DW exhibited significantly different H_{rms} values from RC and from MC ($p < 0.05$), while RC and MC were not significantly different ($p = 0.22$). The U_{rms} data were significantly different from one another for all sites ($p < 0.05$).

Based on early results, DW was excluded from the subsequent two deployments, opting rather to monitor better performing transplant

TABLE 2 Exposure summary.

	Dockweiler	Redondo Canyon	Malaga Cove	Palisades	East End
Temperature					
Percent above 20°C	2.06	2.34	3.30	11.54	17.07
Wave					
July-Aug 2021 Deployment					
Percent above H_{rms} threshold*	6.82	0	2.27	-	-
Percent above U_{rms} threshold**	86.36	13.64	36.36	-	-
Sep 2021 - June 2022 Deployment					
Percent above H_{rms} threshold*	-	28.50	31.09	2.56	2.56
Percent above U_{rms} threshold**	-	58.55	83.42	34.36	55.90
PAR					
Percent below 3 mol m ⁻² day ⁻¹	96.13	91.85	87.44	62.72	47.43
Percent above 5 mol m ⁻² day ⁻¹	0	0	0.93	21.01	31.44
DO					
Percent below 3 mg O ₂ L ⁻¹	23.20	1.11	15.35	0	0

* H_{rms} threshold set at 0.59 m.
** U_{rms} threshold set at 0.1 m s⁻¹.
Temperature threshold adapted from Kapsenberg and Hofmann (2016) and Lee et al. (2007). Wave thresholds adapted from Short et al. (2002) and calculated based on donor bed data across all deployments. Photosynthetically active radiation thresholds adapted from Thom et al. (2008). Dissolved oxygen threshold adapted from Moore and Jarvis (2008).
Percent of time above or below a given environmental parameter threshold.

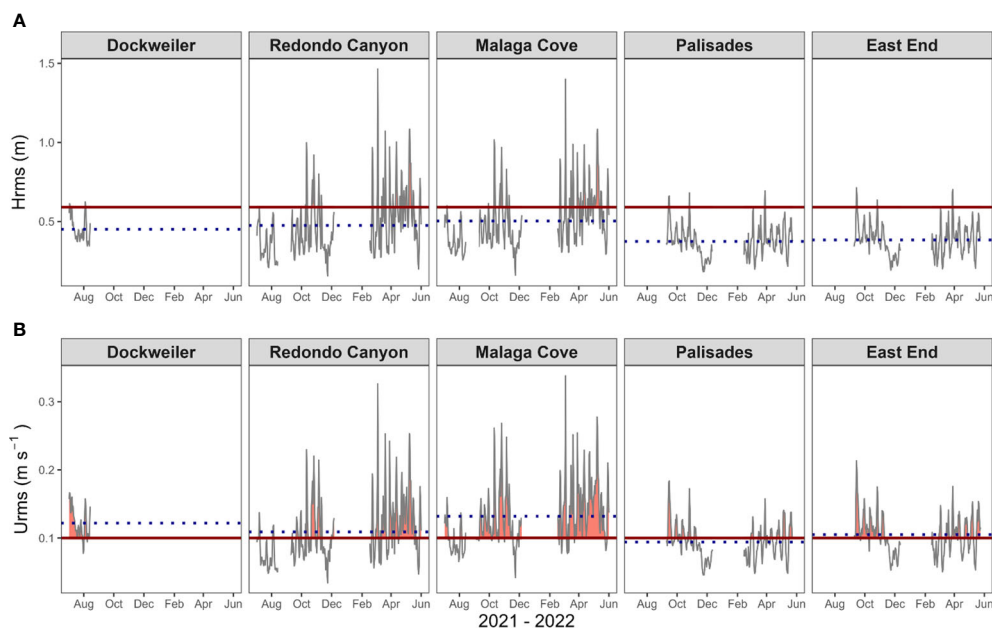


FIGURE 5

Wave characterization, with H_{rms} (A) and U_{rms} (B), exposure timeseries at the three transplant sites: Dockweiler (DW), Redondo Canyon (RC), and Malaga Cove (MC) and two donor beds Palisades (PAL) and East End (EE). Deployments spanned from July – August 2021 ($n = 44$ days), September – December 2021 and February – June 2022 ($n = 195$ days). Note, July – August 2021 sensors were only deployed at transplant sites, and Dockweiler was excluded from the subsequent two deployments while donor beds were added. Solid black line represents daily mean wave data timeseries, while dotted blue line represents site average across the span of the timeseries. Red horizontal line represents H_{rms} threshold of 0.59 m, and U_{rms} threshold of 0.1 $m\ s^{-1}$. H_{rms} site average $\pm$ SD in m: DW (0.45 ± 0.08), RC (0.48 ± 0.20), MC (0.50 ± 0.18), PAL (0.37 ± 0.09), and EE (0.38 ± 0.10). U_{rms} site average $\pm$ SD in $m\ s^{-1}$: DW (0.12 ± 0.02), RC (0.11 ± 0.05), MC (0.13 ± 0.05), PAL (0.09 ± 0.03), and EE (0.11 ± 0.03). See Table 2 for percent time each site was above threshold values.

sites (RC and MC) and donor bed sites (PAL and EE) concurrently. RC, MC, PAL, and EE had continuous *in situ* wave characterizations from September – December 2021 and February – June 2022. These two deployments were aggregated for analysis ($n = 185$ days). Again, it is evident that transplant sites were exposed to higher degrees of H_{rms} and U_{rms} values and for longer durations than donor bed sites (Figure 5, Tables 1, 2). Site exposure percentages above the H_{rms} threshold were RC (28.50%), MC (31.09%), PAL (2.56%), and EE (2.56%), while exposure percentages above the U_{rms} threshold were RC (58.55%), MC (83.42%), PAL (34.36%), and EE (55.90%). The highest daily mean H_{rms} and U_{rms} values recorded at the transplant sites were 1.46 m and 0.34 $m\ s^{-1}$, while the values at the donor beds were 0.71 m and 0.21 $m\ s^{-1}$. H_{rms} values at the transplant sites MC and RC were significantly different ($p = 0.05$), but donor bed sites PAL and EE were not significantly different from one another ($p = 0.44$). All H_{rms} comparisons between transplant sites and donor bed sites were significantly different ($p < 0.05$). The U_{rms} data shows that RC and EE were not significantly different ($p = 0.09$), though all other site comparisons were significantly different from one another ($p < 0.05$).

3.5 Photosynthetically active radiation exposure

PAR exposure below the 3 $mol\ m^{-2}\ day^{-1}$ growth limited, non-survival threshold, as well as exposure (or lack thereof) above the upper survival-supportive threshold of 5 $mol\ m^{-2}\ day^{-1}$ was

prevalent during the project period (Figure 6, Tables 1, 2). Exposure below the growth limited, non-survival threshold is especially prevalent in the late fall and winter months. Transplant site exposure percentages below 3 $mol\ m^{-2}\ day^{-1}$ were DW (96.13%), RC (91.85%), and MC (87.44%) and exhibited greater exposure times than the donor bed sites PAL (62.72%) and EE (47.43%). The longest continual exposure period below the non-survival supportive threshold for the sites were 141, 203, 100, 56, and 75 days for DW, RC, MC, PAL, and EE respectively. Donor bed sites PAL (21.01%) and EE (31.44%) experienced sustained exposure in exceedance of the 5 $mol\ m^{-2}\ day^{-1}$, while MC only had 0.93% and both DW and RC had zero exposure above the survival-supportive threshold. The two donor bed sites each spent considerable time from spring through fall (June 2022 – October 2023) above the survival-supportive threshold (12-day continual exposure at PAL and 29-day continual exposure at EE) and both sites rarely dip below the growth limited, non-survival threshold. The highest daily integrated PAR value recorded at the transplant sites was 6.10 $mol\ m^{-2}\ day^{-1}$ while the highest value at the donor beds was 11.56 $mol\ m^{-2}\ day^{-1}$. Over the timeseries from July 2022 – January 2023 ($n = 159$ days), DW was significantly different from all other transplant and donor bed sites ($p < 0.05$), while RC and MC were not significantly different from each other ($p = 0.88$) but were significantly different from the donor bed sites ($p < 0.05$). Donor bed sites were not significantly different from each other ($p = 0.18$).

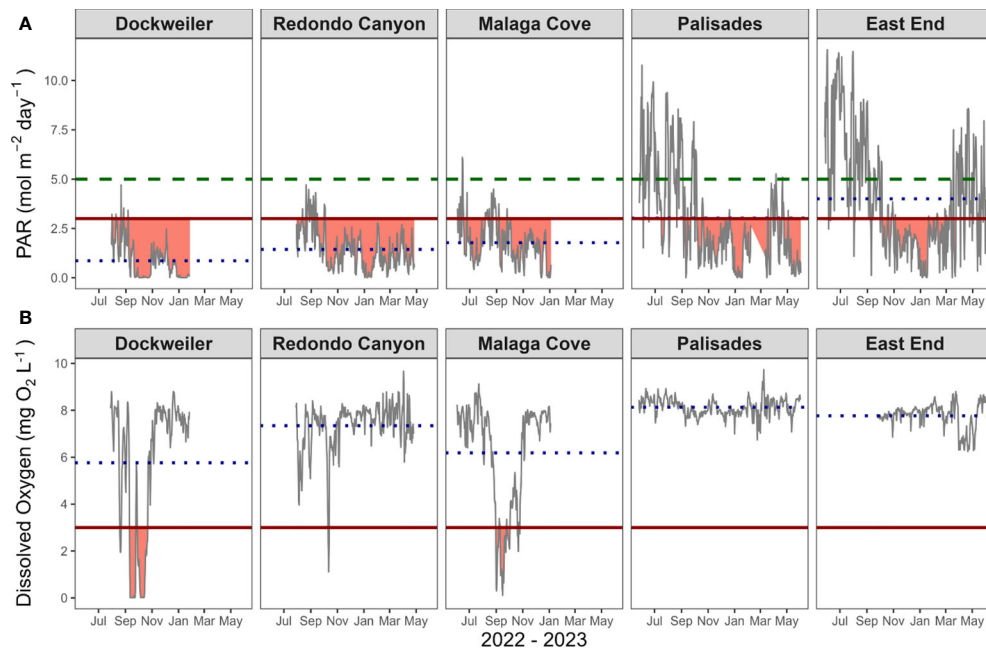


FIGURE 6

Photosynthetically active radiation (PAR) (A) and dissolved oxygen (DO) (B) exposure timeseries across all transplant and donor sites from May 2022 – June 2023. Solid black line represents integrated daily PAR and mean daily DO timeseries. Dotted blue line represents site average. Red horizontal line represents thresholds. For PAR: non-survival threshold of $3 \text{ mol m}^{-2} \text{ day}^{-1}$; green dashed line survival-supportive threshold of $5 \text{ mol m}^{-2} \text{ day}^{-1}$. For DO: tissue degradation threshold of $3 \text{ mg O}_2 \text{ L}^{-1}$. Gaps in data correspond to sensor malfunctions. Dockweiler, Redondo Canyon, and Malaga Cove are transplant sites. Palisades and East End are donor beds. Site average $\pm$ SD for PAR in $\text{mol m}^{-2} \text{ day}^{-1}$: DW (0.87 ± 0.92), RC (1.44 ± 1.00), MC (1.78 ± 1.09), PAL (3.02 ± 2.51), and EE (4.01 ± 2.88). Site average $\pm$ SD for DO in $\text{mg O}_2 \text{ L}^{-1}$: DW (5.77 ± 2.95), RC (7.34 ± 1.04), MC (6.19 ± 2.30), PAL (8.13 ± 0.37), and EE (7.76 ± 0.55). See Table 2 for percent time each site was exposed for each threshold.

3.6 Dissolved oxygen exposure

DO exposure below the $3 \text{ mg O}_2 \text{ L}^{-1}$ tissue degradation threshold occurred (Figure 6, Tables 1, 2). Transplant site exposure percentages below $3 \text{ mg O}_2 \text{ L}^{-1}$ were DW (23.20%), RC (1.11%), and MC (15.35%) and exhibited consequential exposure times compared to the donor bed sites PAL (0%) and EE (0%). The longest continual exposure period below the tissue degradation threshold for the sites were 24, 3, and 14 days for DW, RC and MC respectively, while PAL and EE experienced zero exposure periods. The lowest daily mean DO value recorded at the transplant sites was $0.01 \text{ mg O}_2 \text{ L}^{-1}$ while the lowest value at the donor beds was $6.24 \text{ mg O}_2 \text{ L}^{-1}$. A DO timeseries from September 2022 – January 2023 ($n = 102$ days), indicated transplant sites were not significantly different from one another (DW – RC $p = 0.48$; DW – MC $p = 0.33$; RC – MC $p = 0.11$). Likewise, the oxygen exposure at donor beds were not significantly different ($p = 0.05$). All comparisons between transplant sites and donor bed sites were significantly different ($p < 0.05$).

4 Discussion

4.1 Evaluation of transplant success

This study conducted the first ever mainland *Zostera pacifica* transplant, with methods and results that can allow for further technique development of *Z. pacifica* restoration in the Southern

California Bight (SCB). From the *in situ* biophysical monitoring, this study has developed clear linkages pertaining to *Z. pacifica* environmental constraints, enabling advancements in site selection procedures that may serve as a model for open coast seagrass restoration. At all three sites the majority of transplants initially established, and at two sites, experienced evidence of growth and expansion. However, beyond 315 days there was complete mortality at the coastal transplant sites, most likely due to predominantly unfavorable environmental conditions associated with high wave exposure, low light, and periods of hypoxia. Thus, in the most conspicuous definition of success, or even that from the NOAA California Eelgrass Mitigation Policy (CEMP) (2014) criteria requiring 85% areal coverage two years post transplanting, the transplants were not successful. Transplanting and restoring seagrass beds is notably difficult, and quantification of seagrass transplant performance is far more nuanced and complex than simply considering total survival as the sole metric for success. Contextualizing, in a meta-analysis of 82 seagrass restoration projects on the West Coast of the U.S., 32 – 60% of projects were unsuccessful (Ward and Beheshti, 2023), yet ‘unsuccessful’ projects certainly can still contribute to advancing methods in restoration science (Zhang et al., 2021). As such, other metrics beyond survivorship (i.e., ecosystem services) should also be considered in the evaluation of transplant success (Ward and Beheshti, 2023).

The assessment of ecosystem services (fish community dynamics, carbon stocks, turbidity, etc.) in restored sites compared to donor beds, can serve as valuable performance

metrics and motivations for larger scale restoration projects (Orth et al., 2020; Lange et al., 2022). *Zostera* spp. (eelgrass) beds have high fish biomass and diversity compared to unvegetated sediments (Duffy, 2006), and fish biomass can be a useful comparative metric between transplant and donor beds (Beheshti et al., 2022). Utilizing fish surveys adapted from Obaza et al. (2022), dissimilarity in fish communities is evident between transplant sites and donor bed sites (Figure 3). The small amount of low-density eelgrass followed by bare soft-bottom in the transplant sites as compared with extensive *Z. pacifica* donor beds on Catalina Island is likely the driving factor in fish assemblage differences (Pihl et al., 2006). Older aged, structured seagrass habitat offers more robust ecosystem function (i.e., fish diversity) compared to younger aged seagrass beds (i.e., transplant sites) (McGlathery et al., 2012; Orth et al., 2020; Hardison et al., 2023). The species more commonly encountered at transplant sites were flatfish, as would be expected on soft-bottom habitats in the region (Craig et al., 2004; O'Leary et al., 2021), while those more regularly recorded at donor bed sites are usual inhabitants of open coast *Z. pacifica* (Obaza et al., 2022). Fish surveys associated with this project quantitatively support the conclusion that the eelgrass transplants did not create meaningful structure for nearshore fishes, nor restore that ecosystem function, during the study period.

The only other known *Z. pacifica* transplant occurred in 2002 at a sheltered cove off Anacapa Island where 450 shoots were transplanted to an area of 300 m² and resulted in a successful and stable *Z. pacifica* population on the island (Altstatt et al., 2014). Over 95% mortality was recorded on day 155 in that study, suggesting early mortality may be the norm, even in successful efforts. Altstatt et al. (2014) provides context to our study's ~30% survivorship on day 197 at Malaga Cove (Figure 2) and illustrates the benefit of this effort – affirming the present study represents an advancement on prior restoration efforts for *Z. pacifica*. Studies of open coast seagrass restoration conducted elsewhere globally report similar findings to those in this study, as open coast environments are inherently dynamic, leading to higher degrees of initial transplant mortality (Paulo et al., 2019; Wegoro et al., 2022).

This project highlights insight into the efficacy of exposed mainland coast transplanting methodology, with initial survival differences of the single shoot method over the bundle shoot method (Figure 2). In the exposed mainland coast environment, transplants can be subject to periods of intense wave action and high near bottom velocity (Figure 5; van Katwijk et al., 2009), where the smaller profile single shoots may induce less drag and thus, experience reduced impact compared to bundle shoots (Orth et al., 1999). These insights may contribute to future transplanting efforts, as the bundle shoot method requires a greater degree of harvest investment compared to the single shoot method. Focusing transplanting efforts on the single shoot method allows for a redistribution of the harvest material, spreading the transplanting risk across a greater area and number of 'planting units' (Orth et al., 2006; van Katwijk et al., 2009). The results of this study, in concert with findings from Altstatt et al. (2014), reaffirm that methodological approach is critical (Fonseca et al., 1998), as the single shoot method is linked to higher survivorship along exposed

coastlines in the SCB. However, during the harvesting and transplanting process, the donor material experiences stress (Lange et al., 2022), catalyzing initial mortality or decreased function. Therefore, future studies on the open coast should aim to gauge the effects of transplanting stress and methodology on survival by also transplanting back into donor beds. These efforts may further disentangle the efficacy of transplant methodologies in the absence of environmental conditions that vary from those experienced at the natal donor beds.

4.2 Causative factors of transplant mortality

Stochastic events are prevalent in seagrass beds and can result in adverse transplant performance (Lange et al., 2022). Unpredictable deleterious singular events may have influenced transplant performance, as numerous acute events occurred across transplant sites. The Dockweiler transplant site is located ~2km from the largest wastewater treatment facility in Los Angeles, and from July 2021 – May 2022, 60 effluent limit violations occurred because of an unprecedented > 12-million-gallon raw sewage discharge from a pipe 1.6 km offshore, resulting in numerous algal blooms (LA Water Board, 2023). A large body of literature speaks to the negative impacts of eutrophication on seagrasses (Burkholder et al., 2007; Hughes et al., 2013). Further, during a post-transplanting survey at Redondo Canyon, located at the head of an active submarine canyon, divers observed macroalgae detritus engulfing transplant units. Macroalgae inundation is known to decrease health and survival of *Zostera* spp. by altering light regimes and decreasing photosynthetic capabilities (Huntington and Boyer, 2008), a factor that likely contributed to declines in transplanted *Z. pacifica* survivorship. The pairing of high wave characterizations (H_{rms} and U_{rms} values) and macroalgae inundation at the Redondo Canyon site may have contributed to physical damage – a concept further supported by diver observations of dislodged transplanting units. Hydrodynamical forces and nutrient regimes can negatively impact the biomechanical properties (i.e., shoot strength, elasticity) of seagrasses (Patterson et al., 2001; La Nafe et al., 2013; Risandi et al., 2023), and it is likely that the hydrodynamic conditions and acute eutrophication events impacted the transplant sites, though the quantification of biomechanical properties is outside the scope of this study.

The biophysical characterizations reveal a more compelling and consistent case for deleterious site conditions, as a mosaic of unsuitable environmental conditions occurred at transplant sites. And while biologic controls are undoubtedly important to seagrass systems, regionally along the West Coast of the USA, physical drivers are principally responsible for eelgrass transplant success or decline (Ward and Beheshti, 2023). Yet surprisingly, many *Zostera* spp. restoration projects that offer suspected reasons for transplant success or failure lack quantitative *in situ* environmental data, instead relying on observational data to infer physical drivers (Ward and Beheshti, 2023). In this study, generally, PAR, DO, and

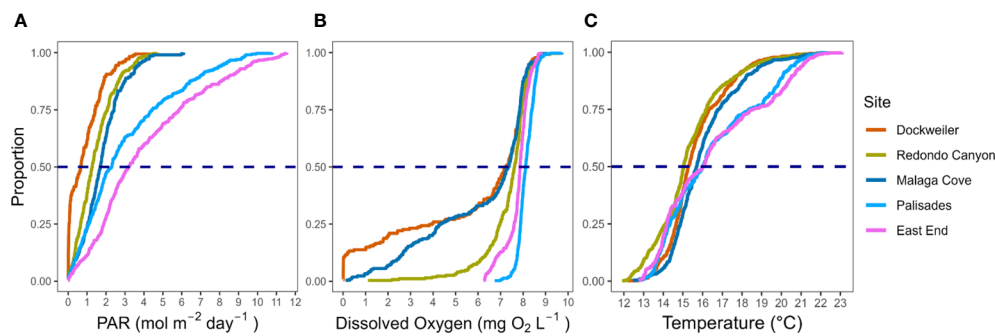


FIGURE 7

Empirical cumulative distribution function (eCDF) curves for (A) photosynthetically active radiation ($\text{mol m}^{-2} \text{ day}^{-1}$), (B) dissolved oxygen ($\text{mg O}_2 \text{ L}^{-1}$), and (C) temperature ($^{\circ}\text{C}$) at transplant and donor sites. Plotted PAR values are daily integrated data, while DO and temperature values are mean daily data. The intersection between eCDF and the horizontal blue dashed line represents median (50%) value at each site. Dockweiler, Redondo Canyon, and Malaga Cove are transplant sites. Palisades and East End are donor beds.

temperature were lower, and wave dynamics (H_{rms} and U_{rms}) higher in transplanted sites compared to donor bed sites (Figures 4–6). Indeed, empirical cumulative distribution function (eCDF) curves of transplant sites compared to donor beds depict a pronounced decoupling between transplant and donor bed sites and show only partial overlap, confirming that conditions at transplanting locations were inapt for *Z. pacifica* long-term survival (Figure 7; e.g., Beheshti et al., 2022). The transplant study approach herein showcases an avenue to discretely monitor *in situ* data and quantify known physical controls on seagrass habitat function.

Minimum colonization depth is often restrained by wave orbital velocity and large wave exposure directly correlates with transplant mortality (Bos and van Katwijk, 2007; de Boer, 2007). Wave characterizations were of paramount importance for transplanting on the exposed mainland coast of the SCB. Inter-site comparisons amongst solely transplant sites from initial nascent stages of the transplant revealed disparities between sites, namely at Dockweiler where exceedances of threshold values corresponded with large drops in survivorship (Figure 5). Coupling survivorship and wave characterizations allow for insights to be gleaned, as periods of similar H_{rms} and U_{rms} values at transplant and donor bed sites reveal stabilization in survivorship (September – October 2021), while deviations above threshold values (November 2021 – June 2022) correspond with transplant mortality (Figures 2, 5). The paucity of wave characterization for exposed mainland coast seagrass beds makes quantitative comparisons challenging. Hansen and Reidenbach (2012) investigated significant wave heights in extant *Z. marina* beds, finding maximum values around 0.18 m – far smaller wave heights than those values reported from extant beds in this study. Further, the wave characterizations of our study are conceptually supported by Hansen and Reidenbach (2013), who investigated near bottom wave velocity inside and outside *Z. marina* beds in lower Chesapeake Bay, finding flows to be significantly higher in the unvegetated site (0.09 m s^{-1}) than within the seagrass site (0.026 m s^{-1}). Additional studies have found, while uncommon in extant beds, maximum velocities of 0.5 m s^{-1} were incurred for brief periods by *Z. marina* (Fonseca et al., 1983; Koch,

2001). Though perhaps the most supportive of our results comes from a study conducted on survival of two western Mediterranean seagrasses at water depths of 12 and 18 m, concluding that near-bottom velocities tolerated were between $0.18 - 0.39 \text{ m s}^{-1}$ (Infantes et al., 2011). These depths and velocities more aptly compare to those experienced in an exposed mainland coastal shelf environment occupied by *Z. pacifica*, evidencing that this species inhabits areas with sustained exposure above values typically tolerated by *Z. marina*, and other seagrasses, in protected environments.

Light availability constrains seagrass growth at the deep edge of the bed, but sufficient quantities are required to maintain health throughout the entirety of the local distribution (Drew, 1979; Dennison, 1987; Duarte et al., 2007). Based on the adapted non-survival light threshold from Thom et al. (2008), transplant sites did not receive sufficient light to satisfy growth requirements. The values ($< 3 \text{ mol m}^{-2} \text{ day}^{-1}$) experienced at the transplant sites, and the ensuing mortality, are supported by results from a field study that found drastic mortality in *Z. marina* transplants exposed to PAR below $3 \text{ mol m}^{-2} \text{ day}^{-1}$ (Moore et al., 1997), as well as by a meta-analysis reporting similar deleterious impacts of values below the established PAR threshold (Dunic and Côté, 2023). Our study also shows that the donor bed sites average below those thresholds from November 2022 – April 2023 and did not induce mortality. During light limiting conditions, *Zostera* spp. can facilitate survival through the metabolization of carbohydrates (sugar and starch content) stored in the rhizomes and leaves (Zimmerman et al., 1995; Burke et al., 1996; Ralph et al., 2007; Vichkovitten et al., 2007; Bertelli and Unsworth, 2018). Research from Baja, Mexico supports this notion that carbohydrate reserves allow *Zostera* spp. to survive in short-term adverse light conditions but noted that mortality occurred when carbohydrate reserves decreased ~85% following three weeks of nearly zero PAR values (Cabello-Pasini et al., 2002). It follows then that lower light limits are not binary, rather eelgrass light requirements are nuanced, requiring sufficient light during growing seasons to build carbohydrate reserves needed to draw down during light limiting conditions encountered when irradiance levels are naturally lower (i.e., winter months) (Govers et al., 2015;

Wong et al., 2020; Wong et al., 2021). The donor bed sites in our study did not experience near zero PAR conditions for an extended duration and did receive sufficient growing season light levels, and thus, *Z. pacifica* at these sites were likely able to draw on ample reserves to survive during brief light limiting conditions. Further, while acclimatization of transplanted *Z. marina* is documented (Krumhansl et al., 2021), the ability to build carbohydrate reserves is diminished under $3 \text{ mol m}^{-2} \text{ day}^{-1}$ light regimes (Eriander, 2017). The transplant sites in our study may have adapted to a new light regime initially, but when PAR values were reduced to $\sim 0 \text{ mol m}^{-2} \text{ day}^{-1}$ for extended periods of time, an insufficient carbohydrate storage likely prevented survival in these adverse light conditions.

Low DO values impact *Z. marina* growth and can accelerate mortality (Holmer and Bondgaard, 2001). Hypoxic values ($< 2 \text{ mg O}_2 \text{ L}^{-1}$) and values nearing anoxia ($< 0.01 \text{ mg O}_2 \text{ L}^{-1}$) are confirmed in other systems containing submerged aquatic vegetation (Tassone et al., 2022). DO values in donor bed sites did not drop below $6.24 \text{ mg O}_2 \text{ L}^{-1}$, but transplant sites, primarily Dockweiler and Malaga Cove, underwent multiple periods of extended hypoxia, and at Dockweiler, even values approaching anoxia (Figure 6). Low dissolved oxygen concentrations are reported as contributors of *Zostera* decline in other systems in the USA, such as the lower Chesapeake Bay (Moore and Jarvis, 2008), an Oregon estuary (Magel et al., 2023), and two separate California estuaries (Walter et al., 2018; Beheshti et al., 2022). A buildup in concentration of sulfides in the rhizome can occur during periods of hypoxia (Goodman et al., 1995), and may have significantly impacted transplant performance, though a quantification of sediment composition in our study is absent. The rapid impact of low DO can manifest in 12 hours and increases with duration, with absolute mortality occurring with anoxic exposure for 36 hours (Pulido and Borum, 2010; Raun and Borum, 2013). Continuous hypoxic conditions for 24 days at Dockweiler and 14 days at Malaga Cove is a salient contributor to transplant failure.

Heat stress induced mortality, decreased photosynthetic efficiency, or reduced growth rate, are common occurrences amongst *Zostera* spp. (Moore et al., 2014; Hammer et al., 2018; Serrano et al., 2021). Our study shows that temperature stress at the transplant sites was not a key variable influencing transplant site performance, as extant *Z. pacifica* donor beds experienced higher overall temperatures and for longer periods of time. The limited temperature threshold exposure in our study is supported by data taken in the proximity of a *Z. pacifica* bed off Santa Cruz Island, California (Kapsenberg and Hofmann, 2016), highlighting that extant open coast *Zostera* spp. proliferate within the range of temperatures reported in our study. Climate change models predict warming ocean temperatures will impact seagrasses (Carr et al., 2012), though the extant *Z. pacifica* beds in our study do not appear to be growing near their physiological temperature tolerances and may be less exposed to climate change related temperature stress compared to other temperate foundational species (i.e., *Macrocystis pyrifera*, see Cavanaugh et al., 2019 and Leichter et al., 2023). Yet, *Zostera* spp. are susceptible to both pulse extreme heat events and episodic heat exposure which can alter local resilience to additional stressors (DuBois et al., 2022),

therefore it follows that the increasing magnitude, frequency, and duration of episodic events (i.e., ENSO) may coincide with baseline climatic warming to induce deleterious impacts on seagrasses in the SCB (Johnson et al., 2003; Echavarría-Heras et al., 2006).

An amalgamation of synergistic stressors, whether short-term stochastic or chronic, ultimately resulted in conditions unfavorable for *Z. pacifica* proliferation at transplant sites. While the survivorship of this project initially performed better than the Anacapa Island transplant conducted by Altstatt et al. (2014), fundamentally, the hydrodynamic data in our study from November 2021 – June 2022, coupled with water quality data from Spring 2022 – Spring 2023 portray an unambiguous unsuitability for long-term *Z. pacifica* survival at these sites. In opposition, the Anacapa transplant site characteristics proved hospitable for *Z. pacifica* expansion following a reduction in grazers responsible for the initial mortality (Altstatt et al., 2014). The present study represents the first quantification of physical parameters within *Z. pacifica* beds on the open coast, distinguishing from the less turbulent conditions of shallow, protected, estuarine *Z. marina* habitat.

5 Conclusion

These data mark a preludial effort to establish *in situ* tolerances and responses for open coast temperate seagrasses along California, and reaffirms the importance of site selection, noting that for hydrodynamically exposed regions, it may be the cynosure factor for transplant consideration, and necessitate discrete pre-transplant environmental sampling efforts. Though the transplant sites in this study did not exhibit sustained survival, as much of the nearshore coastal shelf environment within the SCB consists of the requisite substrate necessitated for *Z. pacifica*, the establishment of a transplanting regime could be of regional significance given the ecological and 'blue carbon' benefits of seagrass restoration (Postlethwaite et al., 2018; Lange et al., 2022; Shayka et al., 2023).

Considering the results of this study, merged with a current scarcity of environmental time-series data from *Z. pacifica* beds, we strongly encourage further research, both *ex situ* and *in situ*, on *Z. pacifica*. Building upon the physical and biological monitoring conducted in this study can spur the expansion of monitoring efforts to other *Z. pacifica* beds in the region, beds that persist on the mainland (as opposed to offshore islands such as the donor beds in this study), which may illuminate ranges of biophysical metrics that more closely reflect transplant site conditions. To facilitate greater efficacy of open coast restoration, a balance must be struck between conservative resource management policies and allocating adequate donor material to overcome critical thresholds of population density needed to induce positive feedback loops and reduce transplant risk on the open coast (Adams et al., 2016; Suykerbuyk et al., 2016; van Katwijk et al., 2016; Paulo et al., 2019; Orth et al., 2020; Valdez et al., 2020). Investigating the use of artificial seagrass structures to baffle strong hydrodynamic conditions (Carus et al., 2022) may reveal applied benefits for transplanting on the open coast. Burgeoning research indicates utilizing seeds may be a

preferred restoration approach (Orth et al., 2020; Gräfnings et al., 2023), and has garnered success in high-energy sites (Unsworth et al., 2019), making it a technique worth investigating along the open coast of California.

Ultimately, the further elucidation of the realized niche *Z. pacifica* inhabits will provide essential information for more targeted open coast site selection and scalable restoration in the SCB. Species-specific research may disentangle long-held assumptions based on shallow protected *Z. marina* habitat, and provide clarity to seagrass resiliency in the region, particularly relevant in the face of climate change and episodic climactic events (i.e., ENSO) which have pervasive impacts on the Pacific Coast of North America (Tegner and Dayton, 1987; Chavez et al., 2002; Fumo et al., 2020). Teasing apart fish community dynamics, species-specific ecosystem services, distributional patterns, and threshold tolerances are fundamental to a holistic comprehension for academics, resource managers, and restoration practitioners alike.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Author contributions

RS: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Supervision, Visualization, Writing – original draft, Validation, Writing – review & editing, Software. AO: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Visualization, Writing – review & editing, Resources, Supervision, Validation. BG: Conceptualization, Data curation, Investigation, Methodology, Writing – review & editing. ML: Data curation, Formal analysis, Visualization, Writing – review & editing, Software, Validation. LM: Formal analysis, Investigation, Methodology, Resources, Software, Supervision, Writing – review & editing, Validation. KE: Conceptualization, Formal analysis, Investigation, Methodology, Resources, Software, Supervision, Writing – review & editing, Data curation, Validation. OC: Conceptualization, Data curation, Investigation, Methodology, Writing – review & editing, Resources, Validation. TF: Conceptualization, Funding acquisition, Investigation, Methodology, Project administration, Writing – review & editing, Resources. JL: Conceptualization, Formal analysis, Funding acquisition,

Investigation, Methodology, Resources, Supervision, Validation, Writing – review & editing.

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Conflict of interest

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

The author(s) declared that they were an editorial board member of Frontiers, at the time of submission. This had no impact on the peer review process and the final decision.

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Characterization of ascorbate-glutathione cycle response in *Zostera marina* seedlings under short-term temperature surge

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The ascorbate-glutathione (AsA-GSH) cycle plays a critical role in scavenging hydrogen peroxide in plants and contributes significantly to plant stress tolerance. This study examines the cycle's response in *Zostera marina* seedlings to warming, specifically under conditions of abnormal sea temperature increase. Three temperature gradients were established: 18°C (control group), 23°C (high-temperature group), and 28°C (abnormally high-temperature group). Results after 7 days of exposure to mild high temperature (23°C) showed decreased MDA content in the HT group, increased AsA/DHA ratio, and enhanced activity of enzymes related to the AsA-GSH cycle. However, exposure to extreme high temperatures resulted in increased oxidative damage and redox imbalance in the AHT group. Initially, enzymes associated with the AsA-GSH cycle, such as APX, MDHAR, GPX, and γ-ECS, increased but significantly decreased later under stress. In contrast, DHAR and GalLDH levels significantly rose on the seventh day. Transcriptome analysis revealed upregulation of APX, MDHAR, DHAR, GR, and γ-ECS genes in the HT group, with a decline in other enzyme gene expressions by the seventh day, except for APX. Under extreme high temperatures, APX expression was downregulated early in the stress period, while DHAR was upregulated, indicating *Z. marina* seedlings can mitigate oxidative damage under short-term high temperatures by activating the AsA-GSH cycle. Conversely, extreme high temperatures may inhibit this cycle, disrupt redox balance, and adversely affect *Z. marina* seedling establishment, potentially leading to their demise.

KEYWORDS

Zostera marina seedlings, ascorbate-glutathione cycle, high temperature stress, reactive oxygen species, transcriptome

1 Introduction

Seagrass beds, recognized as highly productive global ecosystems (Hemming and Duarte, 2000; Unsworth et al., 2018), serve crucial ecological functions in coastal zones (Duarte, 2002) by providing habitats (McDevitt-Irwin et al., 2016) and food sources (Edgar, 1994) for numerous marine organisms and playing significant roles in water purification, sediment stabilization (Orth et al., 2020), and carbon burial (Mtwana Nordlund et al., 2016; Duarte and Dorte, 2017). However, climate change and human disturbances have posed severe threats and accelerated the degradation of these habitats (Waycott et al., 2009). Global warming stands out as a critical factor (Hoegh-Guldberg and Bruno, 2010; Arias-Ortiz et al., 2018; Strydom et al., 2020; Trisos et al., 2020). Ocean temperatures between 2001–2020 were 0.88°C warmer than in 1850–1990 (Jiang et al., 2022). The frequency and duration of extreme heat events, such as summer heatwaves, have increased and are expected to continue rising through the 21st century (Karl and Trenberth, 2003; Christidis et al., 2015; Jiang et al., 2022). These extreme temperatures pose a significant threat, leading to substantial mortality in seagrass beds (Arias-Ortiz et al., 2018; Strydom et al., 2020). Based on global warming projections, the functional extinction of seagrasses could be imminent, emphasizing the urgency for conservation and mitigation efforts (Marbà and Duarte, 2010; Jordà et al., 2012; Chefaoui et al., 2018).

Recent research has highlighted the impact of global warming on the way seagrasses respond to marine events, examining differences at various levels such as growth, physiology, and molecular aspects (Nguyen et al., 2021; Wang et al., 2023; Yan et al., 2023a, b). For instance, severe occurrences like ocean warming or marine heatwaves can restrict the growth of *Zostera marina*, findings that align with transcriptomic and metabolomic studies (Yan et al., 2023a, b). However, the impact of temperature changes, including the frequency and duration of abnormal events, varies on seagrass (Marbà and Duarte, 2010; Kaldy et al., 2017; Kendrick et al., 2019; Magel et al., 2022). Notably, *Z. marina* exhibits different reactions to temperature fluctuations at various stages of its life cycle (Wang et al., 2023; Yan et al., 2023a), with seedlings being particularly temperature-sensitive (Wang et al., 2023).

Marine stress events disrupt the balance of reactive oxygen species (ROS) in seagrasses, challenging their survival. The antioxidant system plays a crucial role in enabling seagrasses to cope with environmental stress, regulating ROS signal transmission and managing adversity (Lee et al., 2016; Olsen et al., 2016). Elevated temperatures have been found to significantly enhance the gene expression of Ascorbate peroxidase (APX), superoxide dismutase (SOD), and catalase (CAT) in *Thalassia hemprichii*, with APX showing the highest increase. This suggests the ascorbate-glutathione cycle is central to the antioxidant system's function (Rakhmad et al., 2019). Additionally, an increase in Mn-SOD gene expression was observed, promoting SOD production (Rakhmad et al., 2019), which breaks down the superoxide anion (O_2^-) produced in excess under high temperatures, thereby maintaining ROS homeostasis (Rakhmad et al., 2019). This mechanism is also evident in *Posidonia oceanica* and *Cymodocea nodosa* (Marín-

Guirao et al., 2016). Other antioxidant enzymes, including glutathione S-transferase, are upregulated in response to high temperatures, as observed in *Z. noltii* and *Z. marina* (Massa et al., 2011; Yan et al., 2019). As mentioned above, the response of some typical antioxidant enzymes to environmental stress has also been studied.

The ascorbate-glutathione cycle (AsA-GSH) is essential for enhancing plant tolerance to prolonged stress and for scavenging ROS in plants (Alscher et al., 1997), clearing H_2O_2 and facilitating the regeneration of AsA and GSH (Kang et al., 2013; Bartoli et al., 2017). *Paenonia ostii* T. has been shown to increase the levels of AsA, GSH, and related enzyme activities under treatment with multi-walled carbon nanotubes (MWCNTs), mitigating the effects of high temperatures (Zhao et al., 2021). Similarly, the presence of endogenous nitric oxide assists salicylic acid in enhancing *Capsicum annuum* L.'s tolerance to salt by upregulating activities of enzymes related to the AsA-GSH cycle (Kaya et al., 2019). Externally applied AsA and GSH can regulate the AsA-GSH cycle and reduce cold damage in stored postharvest bell pepper fruits (Yao et al., 2021). While much of the research on the AsA-GSH cycle has focused on terrestrial plants, its role in seagrasses, especially in response to temperature changes, remains underexplored. Understanding the AsA-GSH cycle in seagrasses is vital for comprehending their resilience against oxidative damage caused by environmental stresses (Zhao et al., 2021; Kaya et al., 2019; Karam et al., 2017; Yao et al., 2021).

Given the importance of understanding the vulnerability of seagrass seedlings, this study focuses on the response of the typical temperate seagrass, *Zostera marina*, to increased temperatures through the lens of the AsA-GSH cycle. Utilizing a mesoscale experimental approach as outlined by Yan et al. (Yan et al., 2023a, b) and Wang et al. (2023), the study integrates physiology and transcriptomics to elucidate the AsA-GSH cycle's behavior under short-term, extreme high temperature. This study aims to provide theoretical support for the protection and restoration of seagrass beds.

2 Materials and methods

2.1 Experimental site and materials

Zostera marina reproduces through both sexual and asexual means. The population survey of Tangshan *Z. marina* shows that the seed banks typically germinate in the spring, and the seedlings are established from April to May (Xu, 2021). In our study, *Z. marina* seedlings were collected on May 2023 from Caoheidian, Tangshan, Hebei (39°5'40.45" N, 118°40'57.15" E). Upon collection, 100 plants exhibiting healthy growth and of approximately equal lengths were promptly transported to the laboratory within 30 minutes. These plants were meticulously cleaned with a fine-bristle brush to remove debris and organisms attached to the leaves, stolons, and root system. Subsequently, four plants were randomly grouped together and placed in an acrylic isolation box measuring 15 cm × 15 cm × 15 cm, with stolons secured using ceramic grains.

Five such groups were accommodated in each mesoscale incubator. The incubators, each 1.5 m in length, 1 m in width, and 1 m in height, featured a recirculation system that introduced natural seawater to simulate the *Z. marina*'s natural habitat accurately. Real-time monitoring of temperature and pH was conducted, maintaining a temperature of $\pm 1^\circ\text{C}$ with a heating rod and a pH of ± 0.05 using a carbon dioxide enrichment device, as detailed by Yan et al. (Yan et al., 2023a, b). Before starting the main experiments, all plants underwent a 7-day acclimatization phase under specific conditions: 18°C , a light intensity of $240\ \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$, a 12 D: 12 L light cycle, a pH of 8.2 ± 0.05 , and a salinity level of 32, mirroring the natural seawater conditions in Tangshan.

2.2 Experimental design

To investigate how *Z. marina* seedlings respond to ocean warming and particularly to abnormal Sea Surface Temperature (SST) events, this study established three temperature conditions: 18°C (control group, representing the midday mean SST of the sampling month), 23°C (the peak temperature during the sampling month), and 28°C (an abnormally high temperature). A growth box containing a single *Z. marina* specimen exhibiting consistent, healthy development was placed in an incubator. For each temperature condition, three replicates were set up, with each replicate comprising nine plants, totaling 81 *Z. marina* seedlings across all groups. The seedlings were exposed to their respective temperatures for 14 days, with continuous monitoring of temperature and pH levels. To prevent rhizome hypoxia, the ceramic support was rotated daily. Leaf samples were collected at specified intervals throughout the experiment (0 d, 1/4 d, 1/2 d, 3/4 d, 1 d, 1.5 d, 2 d, 3 d, 4 d, and 7 d) to minimize variations due to sampling time and individual plant differences. Care was taken to exclude damaged leaves from sampling to avoid the impact of physical damage. Each sampled group was quickly frozen in liquid nitrogen and preserved at -80°C .

2.3 Physiological indexes measurement

For the analysis, *Z. marina* seedling leaf samples were prepared using reagent kits appropriate for plant tissue, focusing on indicators of oxidative damage and the levels of enzymes and substances linked to ASA and GSH metabolism. The samples were processed with a fast preparation instrument (MP/FastPrep-24, USA) using Lysing Matrix D, containing 1.4 mm ceramic beads, with a parameter of 6 m/s and a cracking time of 40 s) and then centrifuged in a benchtop freezer centrifuge (SIGMA-3K15, Germany). The experiments were conducted with the following parameters: 4°C , 10,000 rpm, and 10 minutes. These parameters were adjusted as per the instructions provided by the reagent kits. The assays were performed using a full-wavelength enzyme labeler (MD VERSAmax, USA), with the instrument's wavelength being adjusted according to the reagent kits' guidelines. Additionally, GPX family genes displayed various trends throughout the stress period, associated with the roles of different GPX family members

(Islam et al., 2015). To assess the extent of oxidative damage following high temperature stress, various assay kits were employed: the Plant ROS enzyme-linked immunoassay kit (MLBIO, Shanghai, China) for reactive oxygen species (ROS), the hydrogen peroxide (H_2O_2) content assay kit (Beyotime, Shanghai, China), and the lipid oxidation assay kit (Beyotime, Shanghai, China) for measuring malondialdehyde (MDA) levels. Additionally, the total antioxidant capacity (TAC) was determined using a TAC assay kit (ABTS method, S0116, Beyotime, Shanghai, China) (Buege and Aust, 1978; Jung et al., 2019).

Redox status, characterized by ASA/DHA and GSH/GSSG ratios, was assessed using assay kits for ASA/total ASA (Beyotime, Shanghai, China) and for GSH and GSSG contents (Solarbio, Beijing, China). The key enzymes in the ASA-GSH cycle, including ascorbate peroxidase (APX), dehydroascorbate reductase (DHAR), monodehydroascorbate reductase (MDHAR), and glutathione reductase (GR), were measured using activity assay kits for APX, DHAR, and MDHAR (Solarbio, Beijing, China), drawing on methodologies from Groden and Beck (1979), Chew et al. (2003), and Foyer and Noctor (2011). Additionally, Glutathione Peroxidase (GPX), which uses GSH produced by GR to eliminate H_2O_2 , was measured with activity assay kits for GPX (Solarbio, Beijing, China). This measurement indirectly reflects the GR content of key enzymes in the AsA-GSH cycle. The content of other critical enzymes in the ASA-GSH cycle, L-1,4-galactonolactone dehydrogenase (GalLDH) and γ -glutamylcysteine synthetase (γ -ECS), was determined using assay kits for GalLDH (Solarbio, Beijing, China) and γ -ECS (D&B, Shanghai, China), based on findings by Wheeler et al. (1998), Linster and Clarke (2008), Oppenheimer et al. (1979), and Noctor and Foyer (1998).

2.4 Transcriptome analysis

Seedlings exposed to temperatures of 18°C , 23°C , and 28°C for 1 day (early stage of stress), 4 days (mid stage of stress), and 7 days (late stage of stress) were selected for transcriptomic analysis. From the total RNA extracted from all samples, rRNA was removed, and mRNA was enriched and amplified by PCR after reverse transcription, end repair, and splicing. The data underwent quality checks using agarose gel electrophoresis (for DNA contamination), a NanoPhotometer spectrophotometer (for RNA purity), a Qubit 2.0 Fluorometer (for RNA concentration), and an Agilent 2100 bioanalyzer (for RNA integrity), ensuring the quality of library construction. Low-quality data and base quality were analyzed using fastq (Chen et al., 2018). Further rRNA removal was performed by ribosome comparison (Langmead and Salzberg, 2012), and comparison with the reference genome (Zosma_marina.v.2.1) was carried out (Olsen et al., 2016). Genetic analysis included calculating and counting sample expression using RSEM (Li and Dewey, 2011; Pertea et al., 2015) to identify related genes and their expression changes. Outlier samples were excluded through sample relationship analysis. Intergroup differences in read counts were analyzed using DESeq software (Love et al., 2014), identifying significantly differentially

expressed genes (DEGs) with an FDR < 0.05. DEGs underwent cluster analysis, Gene Ontology (GO) enrichment analysis, and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis to elucidate the biochemical metabolism and signaling pathways related to the ASA-GSH cycle.

2.5 Statistical analyses

Microsoft Excel 2019 was utilized for statistical analysis of the collected data, focusing on physiological indices as detailed in Section 2.3. Data were presented as mean $\pm$ standard deviation (SD). For graphical representation, Origin 2021 facilitated the creation of double Y-axis histograms. IBM SPSS Statistics version 27.0 enabled the execution of normality assessments, variance homogeneity tests, and one-way analysis of variance (ANOVA) on the experimental data. Further analyses included multiple comparisons using LSD, Tamhane, and Dunnett methods, with Duncan's test identifying homogeneous subsets. Significant differences were noted with p -values < 0.05. In transcriptome analysis, Pearson correlation coefficient was applied to identify differentially expressed genes (DEGs) based on p -values and FDR < 0.05. Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analyses were conducted on these DEGs.

3 Results

3.1 Oxidative damage and total antioxidant capacity

Relative to the Control group, reactive oxygen species (ROS), hydrogen peroxide (H₂O₂), and malondialdehyde (MDA) levels were markedly elevated in both HT and AHT groups, as shown in Figure 1. Specifically, ROS levels in the HT group surged significantly at 0.25 days, 1.5 days, 2 days, and 3 days, registering increases of 40.23%, 13.73%, 27.56%, and 19.22% over the Control group, respectively (p < 0.05), with a 7.35% rise at 7 days (Figure 1A). H₂O₂ levels showed significant upticks at 0.5 days, 1 day, and 1.5 days, with increases of 45.68%, 65.49%, and 45.48% above the Control, respectively (p < 0.05), and a 5.17% increase at 7 days (Figure 1B). MDA levels also significantly rose at 0.25 days and 1 day, up by 35.22% and 27.56% over the Control, respectively (p < 0.05), but were 13.15% lower at 7 days (Figure 1C). While no significant difference was observed in total antioxidant capacity (TAC), a trend of initial decrease followed by an increase was noted at 0.25 days, 0.5 days, and 0.75 days (Figure 1D). Compared to the Control group, TAC dropped by 5.67%, 9.53%, and 4.06%, but increased by 6.20% and 4.37% at 4 days and 7 days, respectively (Figure 1D).

In the AHT group, ROS levels significantly exceeded those in the Control group at 0.5 days, 0.75 days, 2 days, and 7 days, by 8.21%, 27.75%, 27.56%, 19.22%, and 18.57%, respectively (p < 0.05; Figure 1A). H₂O₂ levels were considerably higher at 0.5 days, 0.75

days, 1 day, 1.5 days, and 7 days, exceeding Control levels by 62.82%, 84.48%, 43.41%, 50.59%, and 65.59%, respectively (p < 0.05; Figure 1B). MDA levels, higher than those in the Control on day 0, 1 day, and 7 days, were notably increased on 0.25 days, 0.5 days, 0.75 days, 1 day, and 7 days by 5.26%, 19.54%, 43.90%, 12.84%, and 19.56%, with significant hikes observed at 0.75 days and 7 days (p < 0.05; Figure 1C). TAC showed a significant reduction of 15.41% at 0.25 days (p < 0.05), and decreases of 4.22% and 4.35% at 1 day and 3 days compared to the Control, with a 6.78% increase at 7 days (Figure 1D).

3.2 Redox status

Compared with the Control group, the HT group exhibited three increases (0.25 d, 0.5 d, and 3 d-7 d) and two decreases (0.5 d and 2 d) in AsA content by the 7th day. The AsA content was significantly higher by 217.24% at 0.25 d (p < 0.05), significantly lower by 74.60% at 2 d, and 59.26% higher at 7 d (Figure 2A). GSH content showed significant differences only at 1.5 d and 3 d, by 11.59% and 18.09% respectively, with no significant difference at 7 d (Figure 2B). DHA content significantly varied (p < 0.05) at 2 d and 3 d, showing decreases of 65.01% and 47.24% respectively; the difference was not significant at 7 d (Figure 2C). GSSG content differences were all non-significant, being slightly higher than the Control group by 1.32% and 2.24% at 1.5 d and 3 d (Figure 2D). The AsA/DHA ratio was higher than the Control group, except at 0.5 d and 2 d, with significant increases at 0.25 d, 1 d, 3 d, and 7 d by 145.26%, 56.80%, 137.29%, and 65.03%, respectively (Figure 2E). The GSH/GSSG ratio was significantly higher than the Control group at 1.5 d and 3 d, by 10.13% and 15.51%, respectively (Figure 2F).

3.3 Key enzymes for ASA-GSH cycle

In group AHT, AsA content was higher than in the Control group from 0 d-4 d, including a significant increase of 124.66% at 0.5 d (p < 0.05) and a decrease of 25.70% at 7 d (Figure 2A). GSH content significantly decreased throughout the entire experiment (p < 0.05), with a 33.36% decrease observed at 7 d (Figure 2B). DHA content experienced two decreases (0 d-1.5 d and 4 d-7 d) and one increase (1.5 d-4 d), with significant increases of 43.90%, 49.98%, and 84.23% (p < 0.05) at 0.25 d, 3 d, and 4 d, respectively, a significant decrease of 37.47% at 1.5 d (p < 0.05), and no significant difference at 7 d (Figure 2C). GSSG content increased by 5.16% at 7 d (Figure 2D). The AsA/DHA ratio was significantly higher at 0.5 d and 1.5 d (p < 0.05), by 97.90% and 113.50% respectively, and decreased by 32.54% and 24.93% at 4 d and 7 d (Figure 2E). The GSH/GSSG ratio significantly decreased (p < 0.05), with a 36.63% decrease at 7 d (Figure 2F). Key enzymes for ASA-GSH cycle

Compared to the Control group, the APX enzyme activity in the HT group significantly increased at 0.5 days, 1 day, and 1.5 days by 202.63%, 81.08%, and 77.78%, respectively. It significantly decreased by 65.22% at 0.75 days and showed no significant difference at 7 days (Figure 3A). MDHAR enzyme activity tripled,

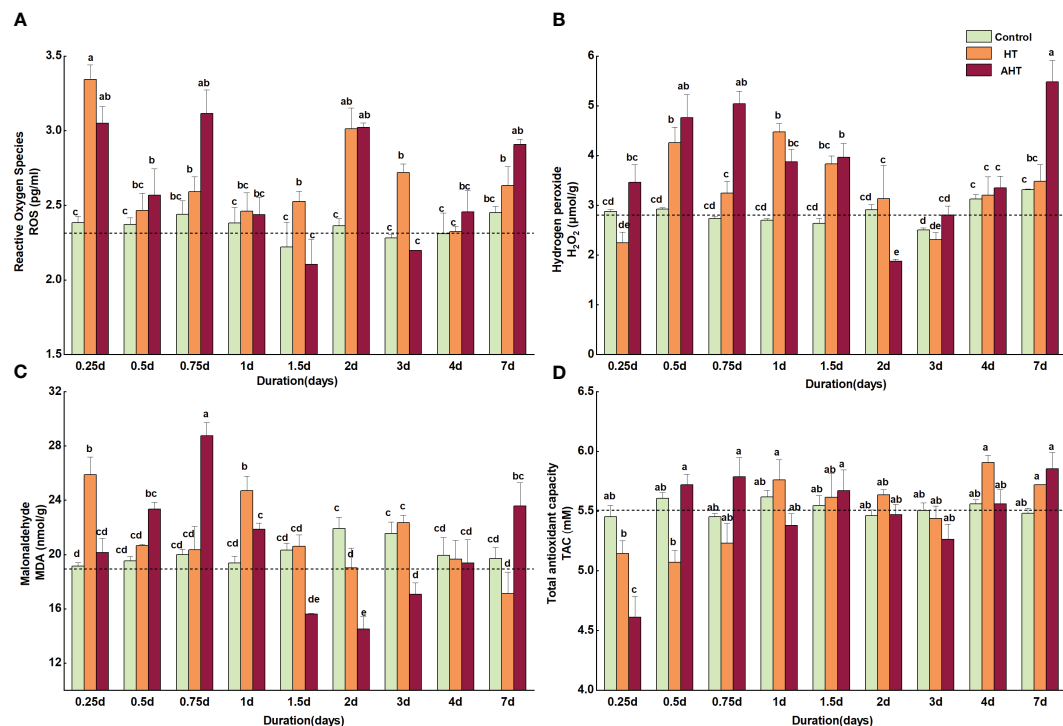


FIGURE 1

Effect of high temperature on reactive oxygen species (ROS), hydrogen peroxide (H_2O_2), malonaldehyde (MDA), and total antioxidant capacity (TAC) for *Zostera marina* seedlings. The data notation is as follows: (A) denotes ROS content; (B) denotes H_2O_2 content; (C) denotes MDA content; and (D) denotes TAC in the seedling leaves. The experiment involved three groups: the Control group, maintained at 18°C; the High Temperature (HT) group, maintained at 23°C; and the Advanced High Temperature (AHT) group, maintained at 28°C. Lowercase letters (a, b, c, and d) indicate statistically significant differences, with identical letters signifying no significant difference among values. Dashed lines in the graphs represent the baseline levels of these indicators at the onset of stress ($t=0$).

significantly increasing at 0.25 days, 1 day, and 7 days by 54.38%, 84.43%, and 122.48%, respectively ($p < 0.05$), but significantly decreased by 71.80% at 4 days ($p < 0.05$; Figure 3B). DHAR enzyme activity initially increased, then decreased, peaking between 1.5 days and 2 days (Figure 3C). GPX enzyme activity significantly increased at 1.5 days, 3 days, and 7 days, surpassing the Control group by 88.70%, 108.71%, and 157.93%, respectively ($p < 0.05$; Figure 3D). GalLDH enzyme activity significantly increased at 1.5 days, 2 days, and 7 days, respectively exceeding the Control group by 40.58%, 48.20%, and 36.92% ($p < 0.05$; Figure 3E). γ -ECS enzyme activity significantly increased by 30.13% at 0.75 days ($p < 0.05$), and was lower at all other times except for 0.25 days and 0.75 days (Figure 3F).

In the AHT group, APX enzyme activity increased by 51.02%, 71.05%, and 13.33% at 0.25 days, 0.5 days, and 1.5 days; the lowest levels were at 0.75 days and 4 days, 28.26% and 53.19% lower than the Control group, respectively (Figure 3A). MDHAR enzyme activity at 0.25 days and 1 day was 47.09% and 56.58% higher than the Control group ($p < 0.05$) but was lower at other times (Figure 3B). DHAR enzyme activity was significantly higher than the Control, increasing by 31.81%, 122.63%, 195.52%, and 106.04% at 0.5 days, 1 day, 2 days, and 7 days, respectively ($p < 0.05$; Figure 3C). GPX enzyme activity was higher than the Control group by 13.36%, 4.05%, 38.74%, and 35.78% at 0.25 days, 1.5 days, 2 days, and 4 days, respectively, and declined by 8.49% at 7 days

(Figure 3D). GalLDH enzyme activity increased by 41.18%, 23.91%, and 29.84% compared to the Control at 0.25 days, 1.5 days, and 7 days ($p < 0.05$), and was significantly lower by 41.31% at 0.75 days ($p < 0.05$; Figure 3E). γ -ECS enzyme activity was higher than the Control by 10.93%, 9.69%, and 14.16% at 0.25 days, 0.5 days, and 0.75 days, respectively, then decreased (Figure 3F).

3.4 Transcriptome results

Samples from the 18°C (Control), 23°C (HT), and 28°C (AHT) groups were selected for transcriptome sequencing at 1 day, 4 days, and 7 days, totaling 27 samples across the three groups. A total of 1,177,247,620 clean reads, representing 99.60% of the raw reads, were obtained. The Q30 base distribution ranged from 93.07% to 95.42%, with an average GC content of 44.87%. Comparison with the reference genome showed an average matching rate of 94.81%, indicating the sequencing data's high integrity and quality.

Correlation analysis reveals that as temperature and stress duration increase, the Pearson correlation coefficient tends to decrease, with the differences in correlation becoming more pronounced. Specifically, the difference in correlation between Group AHT and the Control group was larger than between the Control and HT groups. The Pearson correlation coefficients

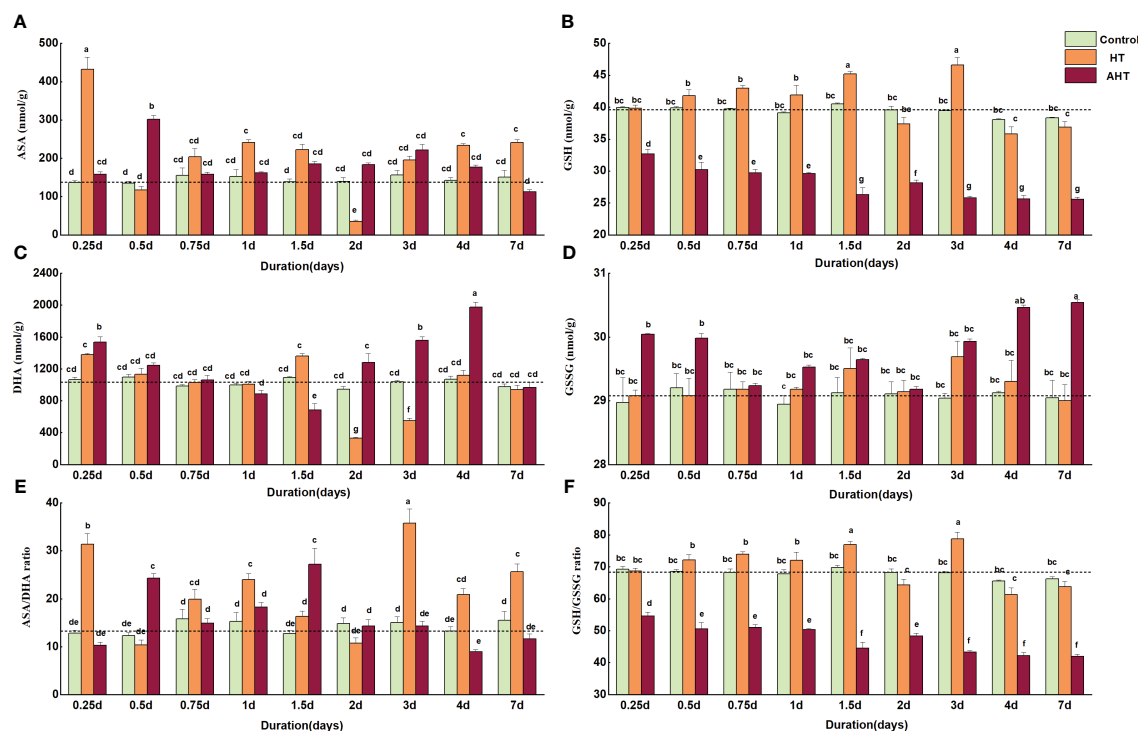


FIGURE 2

Effect of high temperature on redox status (AsA/DHA, GSH/GSSG) for *Zostera marina* seedlings. (A): Content of ascorbate (AsA); (B): Content of glutathione (GSH); (C): Content of DHA; (D) Content of GSSG; (E): Ratio of AsA/DHA; and (F): Ratio of GSH/GSSG. Similar to the first note, the Control group was kept at 18°C; the HT group at 23°C; and the AHT group at 28°C. The significance of differences between groups is indicated by lowercase letters (a, b, c, and d), with identical letters denoting no significant difference. The initial levels of these biomarkers at the beginning of the stress period (t=0) are depicted by dashed lines.

decreased as stress duration increased, with the largest correlation difference observed after 7 days.

In Group HT, the numbers of differentially expressed genes (DEGs) at 1 day, 4 days, and 7 days compared to the Control group were 41, 140, and 204, respectively. The proportion of up-regulated DEGs increased with stress duration, being 48.78%, 67.14%, and 76.96%, respectively. In Group AHT, the numbers of DEGs at 1 day, 4 days, and 7 days compared to the Control group were 3024, 1032, and 2255, respectively. The number of DEGs first increased, then decreased, and finally increased again with the duration of stress. The proportion of up-regulated DEGs decreased as stress duration increased, with percentages of 69.35%, 64.73%, and 63.73%, respectively. This indicates that the number of DEGs in *Z. marina* seedlings rose with increasing temperature, with Group AHT showing ten times more DEGs than Group HT after 7 days.

Key enzyme genes of the AsA-GSH cycle were analyzed to produce a heatmap (Figure 4). Compared to the Control group, the APX gene in Group HT was up-regulated during 7 days of stress, peaking on the 7th day, with its expression positively correlating with the stress duration. Genes MDHAR, DHAR, GR, GaILDH, and γ -ECS were up-regulated, showing a pattern of initial increase followed by a decrease, with the highest expression on the 4th day, although γ -ECS's expression was lower than that of MDHAR, DHAR, and GR. In Group AHT, the APX gene was down-regulated initially before increasing again. MDHAR gene expression was up-regulated but less than in Group HT, peaking on the 4th day.

DHAR, GaILDH, GR, and γ -ECS gene expressions were higher than those in the Control and AHT groups. The expressions of DHAR and GaILDH peaked on the 7th day, positively correlating with stress duration. GR and γ -ECS expressions initially increased then decreased, reaching their maximum on the 4th and 1st days, respectively. Additionally, GPX family genes displayed various trends throughout the stress period, associated with the roles of different GPX family members. GPX6 was notably up-regulated early in the stress period and down-regulated afterwards, while GPX1, GPX3, and GPX7 were up-regulated in the middle to late stages of stress, with the highest expression levels on the 4th day.

4 Discussion

The phenomenon of global warming, particularly the occurrence of extreme high temperatures, presents a significant risk to the survival of seagrass beds. Such temperature stresses may compromise plant viability by elevating respiration rates in seagrasses, thereby generating excessive Reactive Oxygen Species (ROS) and causing metabolic imbalances (Lee et al., 2007; Koch et al., 2013). Alternatively, high temperatures might directly impair the seagrass's photosynthetic system (PSII) or trigger non-photochemical quenching, leading to a sustained reduction in photosynthetic activity. This could constrain seagrass growth or even result in death (Collier and Waycott, 2014; Yan et al., 2023a, b). Our prior studies have demonstrated that

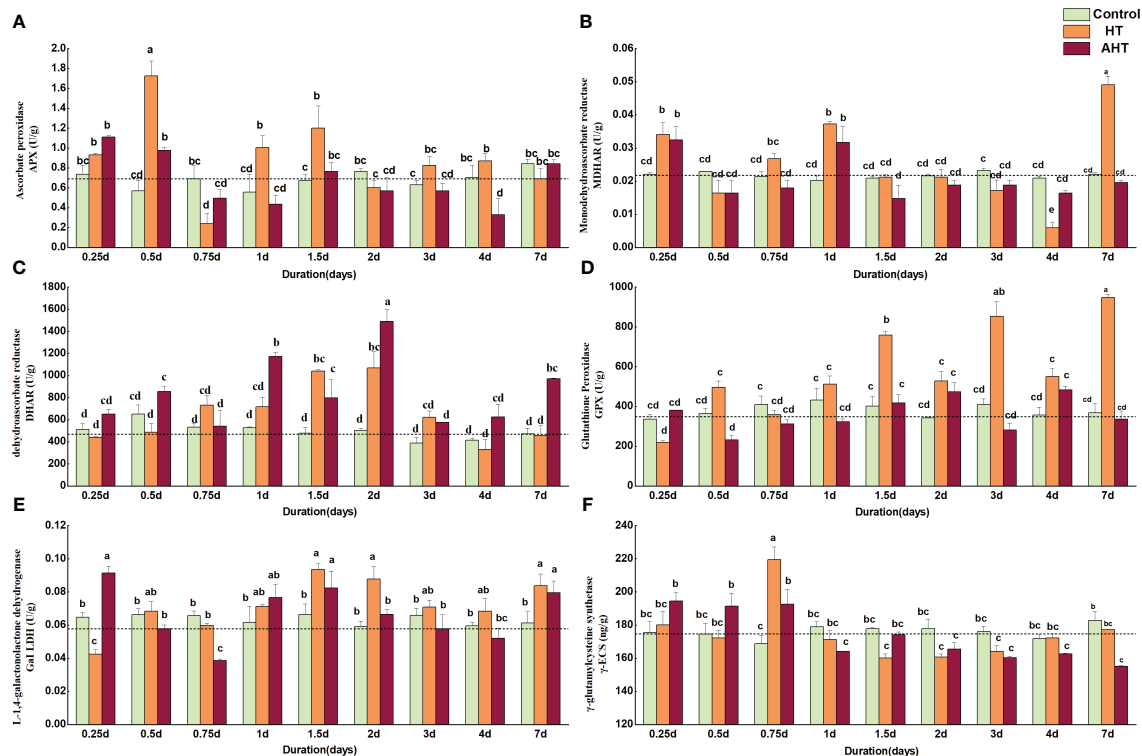


FIGURE 3

The effect of high temperature on AsA-GSH cycle related enzymes (APX, MDHAR, DHAR and GPX) and key enzymes for AsA and GSH synthesis (GalLDH and γ -ECS) in *Zostera marina*. (A): Content of ascorbate peroxidase (APX); (B): Content of monodehydroascorbate reductase (MDHAR); (C): Content of dehydroascorbate reductase (DHAR); (D) Content of glutathione peroxidase (GPX); (E): Content of L-1,4-galactonolactone dehydrogenase (GalLDH); and (F): Content of γ -glutamylcysteine synthetase (γ -ECS). The study grouped seedlings into three temperature conditions: Control at 18°C, HT at 23°C, and AHT at 28°C. Significant differences in enzyme content are denoted by lowercase letters (a, b, c, and d), with identical letters indicating no significant difference. Dashed lines illustrate the initial content levels of these indicators at the start of stress exposure (t=0).

seasonal temperature rises negatively affect the growth of *Z. marina* seedlings, with notable reductions in leaf size under thermal stress (Yan et al., 2023a, b). Building on this background, the current study investigates the role of the AsA-GSH antioxidant system in mitigating high-temperature stress, including conditions of extreme heat, in *Z. marina* seedlings.

The research findings indicate that, in response to high temperatures, *Z. marina* seedlings enhance the gene expression and activity levels of key enzymes within the AsA-GSH cycle. This adjustment increases enzyme content and the AsA/DHA ratio, enabling the seedlings to counteract high-temperature stress effectively by neutralizing excess ROS through the AsA-GSH cycle and GPX. However, under conditions of extreme high temperature, the AsA-GSH cycle's effectiveness is compromised; the concentration of essential substances and enzymes declines, disrupting the redox balance (Figure 5). While moderate temperature elevations can activate the AsA-GSH cycle, bolstering the heat tolerance of *Z. marina* seedlings and minimizing oxidative harm, it proves insufficient under extreme high temperatures, leading to significant oxidative damage.

Reactive oxygen species (ROS) are unintended by-products of aerobic metabolism in plants, potentially causing oxidative harm when they accumulate excessively (Sharma et al., 2012; Tutar et al., 2017). The antioxidant system serves as a crucial defensive

mechanism against environmental stress, playing a vital role in protecting seagrass from temperature-induced stress (Sharma et al., 2012; Tutar et al., 2017). In this study, exposure to short-term high temperature resulted in elevated levels of ROS, H_2O_2 , and MDA in *Z. marina* seedlings. However, seedlings subjected to mild high temperatures (HT group) managed to revert to normal levels after 7 days, with MDA levels even dropping below control levels. In contrast, seedlings exposed to extreme high temperatures (AHT group) exhibited significantly higher levels of ROS, H_2O_2 , and MDA compared to the control (Figure 1). Additionally, the Total Antioxidant Capacity (TAC) of both HT and AHT groups initially fell, then rose, and finally declined again during the stress period, peaking on the 4th and 7th day, respectively (Figure 1D). These observations suggest that the antioxidant system is initially activated to combat oxidative damage in *Z. marina* seedlings under high temperature stress. While the system is effective against mild high temperatures, it becomes overwhelmed and inadequate under extreme heat, leading to reduced TAC and potentially inhibiting the AsA-GSH cycle's ability to prevent oxidative damage.

The two crucial non-enzymatic antioxidants, ascorbic acid (AsA) and glutathione (GSH), within the AsA-GSH cycle play pivotal roles in both directly and indirectly scavenging reactive oxygen species (ROS) through the antioxidant system and participating in redox signaling. The balance between their

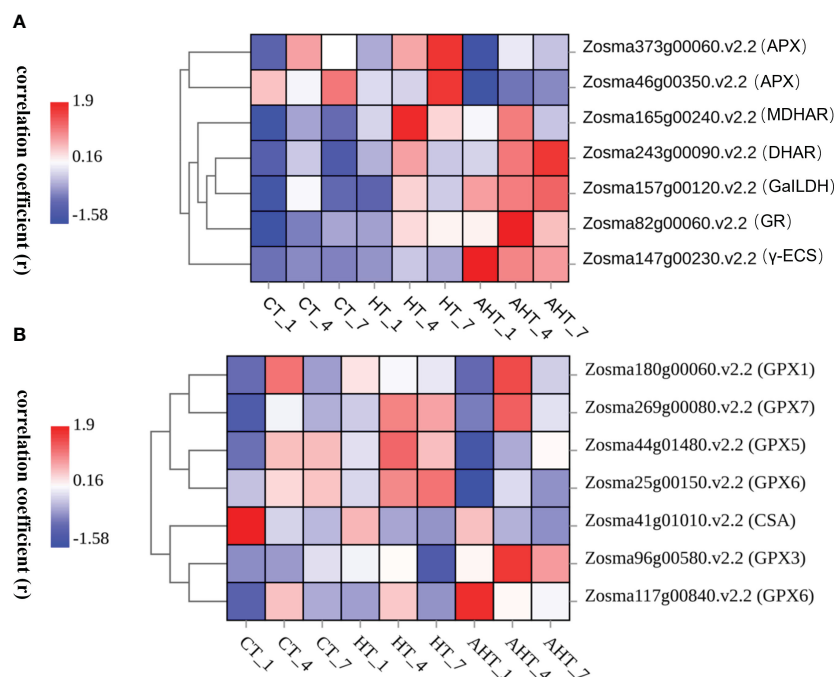


FIGURE 4

Heatmap of Pearson's correlation coefficient (r) for APX, MDHAR, DHAR, GR, γ -ECS (A) and GPX (B) of *Z. marina* seedlings. The color coding within the study—red for high and blue for low—illustrates the Pearson's correlation coefficient (r), indicating the abundance of target gene expression. Specifically, a redder hue signifies higher abundance, whereas a bluer hue indicates lower abundance. The samples from days 1, 4, and 7 of stress exposure are marked as “_1”, “_4”, and “_7” respectively.

reduced and oxidized forms is key in maintaining cellular redox balance and activating various defense mechanisms (Chew et al., 2003; Fotopoulos et al., 2010). This study observed that under high temperature, the reduced AsA levels in eelgrass seedlings increased ($p > 0.05$), while reduced GSH, oxidized DHA, and GSSG levels tended to stabilize by the seventh day after initial fluctuations, with the AsA/DHA ratio increasing and the GSH/GSSG ratio normalizing (Figure 2). However, under extreme high temperatures, while reduced AsA and oxidized DHA contents, as well as AsA/DHA ratios, tended to stabilize, reduced GSH content and GSH/GSSG ratio significantly decreased, and oxidized GSSG increased during the stress period (Figure 2). This indicates that under high temperatures, eelgrass seedlings can maintain redox balance by modulating AsA and GSH metabolism to scavenge ROS, but extreme temperatures severely inhibited GSH metabolism, disrupting the redox state.

The AsA-GSH cycle is the primary pathway for hydrogen peroxide (H_2O_2) metabolism in plants. It involves the decomposition of H_2O_2 into water and monodehydroascorbate (MDHA) by ascorbate peroxidase (APX), the reduction of MDHA to ascorbate (AsA) or its disproportionation to dehydroascorbate (DHA) by MDHA reductase (MDHAR), and the reduction of DHA back to AsA by dehydroascorbate reductase (DHAR) using glutathione (GSH). Glutathione disulfide (GSSG), which is produced in this process, can be reduced back to GSH via a NADPH-dependent glutathione reductase (GR; Escudero-Feliu et al., 2023). This cycle not only decomposes H_2O_2 but also regenerates two critical antioxidants, AsA and

GSH, aiding in the maintenance of reactive oxygen species (ROS) and redox balance in plants (Chew et al., 2003; Escudero-Feliu et al., 2023, 2023). In this study, under high temperatures, the contents of APX and DHAR initially fluctuated before normalizing, whereas MDHAR and glutathione peroxidase (GPX) enzyme levels significantly increased, particularly MDHAR at day 7 during the stress period (Figure 3). The variations in APX, MDHAR, DHAR, GR, gamma-glutamylcysteine synthetase (γ -ECS), and glutamate-ligase (GaILDH) levels were in line with gene expression upregulation. With the exception of APX, the expression of these cycle-related enzymes decreased later in the stress period (Figure 5). At extremely high temperatures, however, APX and MDHAR levels dropped, DHAR significantly increased, and GPX levels were substantially lower than those in the high-temperature (HT) group (Figure 3). Gene expression also proves the accuracy of the trend of enzyme content changes. The APX gene expression is downregulated in the early stages of stress, and upregulation of MDHAR and GR is also lower than that of the HT group. DHAR and GaILDH are significantly upregulated, while the gene expression level of MDHAR and γ -ECS decreases in the later stage of stress (Figure 5). The study indicates that high temperature stress activates AsA-GSH cycle enzymes in eelgrass seedlings, with the cycle prioritizing the consumption of reduced GSH to ensure the production of reduced AsA and excess H_2O_2 scavenging, aided by APX. The increase in GPX content, along with a rise in reduced GSH and GR content, suggests a robust GSH pool, essential for the AsA-GSH cycle's operation and effective H_2O_2 scavenging (Bela et al., 2015; Rajput et al., 2021). The decrease in

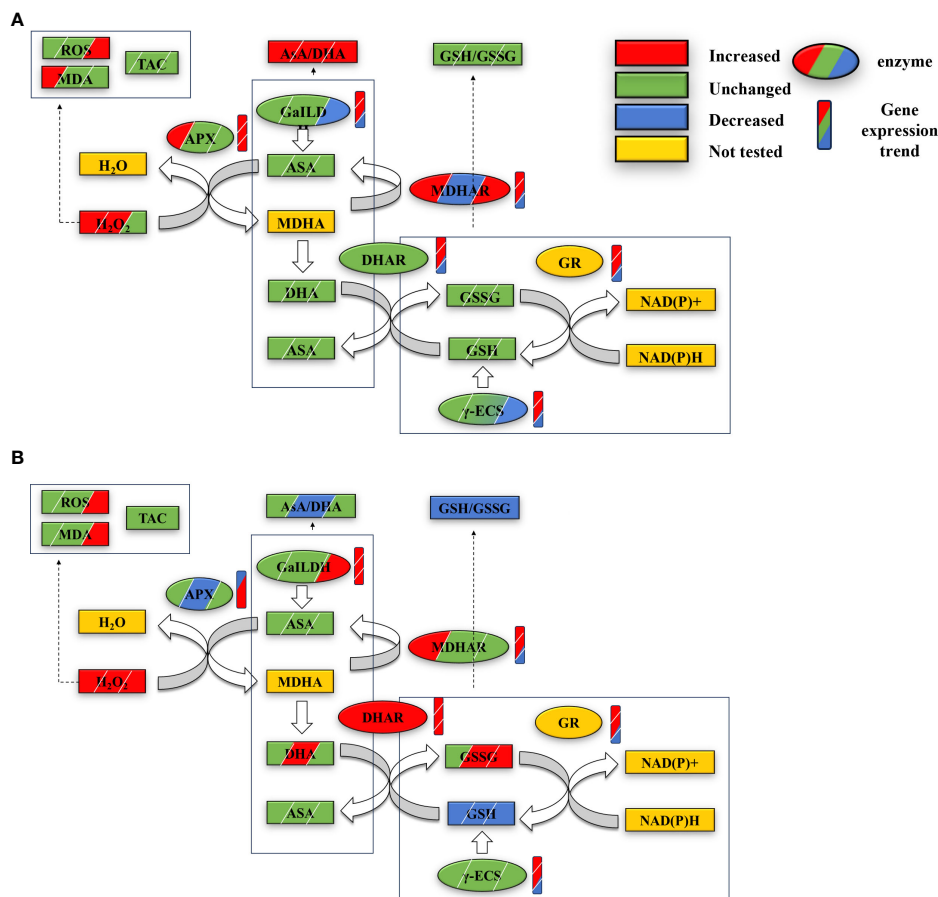


FIGURE 5

Changes in contents and gene expressions of key substances and enzymes of the AsA-GSH cycle in eelgrass seedlings at 1st, 4th and 7th day in HT group (A) and AHT group (B) under high temperature stress. From left to right or top to bottom represents the changes at days 1, 4, and 7; the graphs are labeled with the changes in oxidative stress, antioxidant capacity, and redox pairs of AsA/DHA and GSH/GSSG within the AsA-GSH cycle in seedlings from the HT group (A) and AHT group (B) across days 1, 4, and 7 under high-temperature stress. These graphics also depict changes in the content of key substances, enzymes, and gene expressions related to the AsA-GSH cycle, including modifications in enzyme content and gene expression (marked by rectangles for the cycle changes and ellipses for enzyme and gene changes). Key terms defined include ROS (reactive oxygen species), MDA (malondialdehyde), TAC (total antioxidant capacity), AsA (ascorbate, reduced form), DHA (dehydroascorbate, oxidized form), MDHA (monodehydroascorbate), GSH (glutathione, reduced form), GSSG (glutathione disulfide, oxidized form), along with the enzymes APX, MDHAR, DHAR, GR (glutathione reductase), GaLLDH (L-galactono-1, 4-lactone dehydrogenase), and γ-ECS. NADPH and NADP⁺ (reduced and oxidized forms of nicotinamide adenine dinucleotide phosphate, respectively) are also included in the study's scope.

gene expression of key enzymes and the reduction of H₂O₂ and malondialdehyde (MDA) content towards the late stage of stress suggest the AsA-GSH cycle's role in enhancing heat resistance in eelgrass seedlings. Yet, at extremely high temperatures, a significant increase in DHA content and the substantial depletion of reduced GSH led to a redox imbalance, as evidenced by GSH, GSSG, and the GSH/GSSG ratio (Figure 2), aligning with transcriptomic data (Figure 3). Under extreme temperatures, early stress period downregulation of APX genes, alongside continuous upregulation of GaLLDH and DHAR genes, highlights the rapid consumption of reductive AsA and production of oxidative DHA. However, the downregulation of MDHAR, GR, and γ-ECS genes by day 7 suggests reduced GSH production inhibition and GSH/GSSG redox balance disruption. This imbalance indicates that AsA reduction and H₂O₂ scavenging become unsustainable, leading to a hypothesis of prolonged stress causing complete redox imbalance and eventual death of eelgrass seedlings—a hypothesis confirmed by the

complete death of these seedlings after 14 days of stress. Previous research by the group also observed significant decreases in physiological indicators such as wet weight, and leaf dimensions under high temperature stress, aligning with these findings (Yan et al., 2023a, b). Moreover, the significant increase in GaLLDH content under high temperature and the decrease in key enzymes for GSH synthesis under extreme temperatures (Figure 3) suggest an inhibition of GSH synthesis, impacting the AsA-GSH cycle's operation and forcing a larger reliance on the AsA ab initio pathway for reductonal AsA generation.

GO and KEGG analysis reveals that the DEGs in the HT and AHT groups, under the biological processes category, predominantly encompass cellular processes, metabolic processes, single-organism processes, responses to stimuli, cellular component organization or biogenesis, biological regulation, localization, and regulation of biological processes. Within the cellular components category, enrichment is notably seen in cells, cell walls, organelle

parts, membrane parts, and macromolecular complexes. For molecular functions, enrichment is primarily in binding, catalytic activity, and transporter activity. The DEGs in the HT and Control groups are chiefly enriched in metabolic pathways and biosynthesis of secondary metabolites, whereas those in the AHT and Control groups focus on the biosynthesis of secondary metabolites. Specifically, the DEGs in the AHT and CT groups are enriched in the biosynthesis of amino acids, carbon metabolism, ribosome biogenesis in eukaryotes, DNA replication, starch and sucrose metabolism, carbon fixation in photosynthetic organisms, and nucleocytoplasmic transport. This suggests that the amino acid and carbon metabolism of *Zostera marina* seedlings are significantly impacted by high temperature stress, aligning with studies on the effects of ocean warming and acidification on *Zostera marina* (Yan et al., 2023b). Additionally, transcriptome analysis indicates significant enrichment in glutathione metabolism, arachidonic acid metabolism, and ascorbate and aldarate metabolism under high-temperature stress, highlighting the crucial role of the AsA-GSH cycle in the heat stress response of eelgrass seedlings. The analysis also reveals significant up-regulation of HSP70 and HSP90 related genes, consistent with findings by Marín-Guirao et al. (2016).

In summary, high-temperature conditions trigger the activation of the AsA-GSH cycle in eelgrass seedlings, utilizing AsA to reduce H_2O_2 and maintain the AsA/DHA redox balance via reduced GSH. This process also involves up-regulating various enzymes within the cycle to regulate H_2O_2 clearance and redox balance. However, at extreme high temperatures, the suppression of circulating APX enzymes leads to rapid H_2O_2 accumulation, resulting in the oxidation of AsA to DHA and a swift depletion of the GSH pool. This disrupts the GSH/GSSG equilibrium and affects the AsA/DHA balance, exacerbating oxidative damage over time, potentially leading to death. After 14 days of stress at extreme high temperatures, all eelgrass seedlings died, affirming the severe impact of prolonged high-temperature stress. Thus, while high-temperature stress activates the AsA-GSH cycle to enhance heat tolerance in eelgrass seedlings, it is insufficient to maintain redox balance under extreme conditions, leading to significant oxidative damage.

5 Conclusion

In this study, we demonstrated that under high-temperature stress (23°C), *Z. marina* seedlings could increase the expression of AsA-GSH cycle-related enzymes and their synthetase genes to maintain redox homeostasis. This adaptation potentially enhances heat resistance and prevents oxidative damage. However, at extremely high temperatures (28°C), the AsA-GSH cycle was insufficient to preserve the redox balance in eelgrass seedlings. The DHAR content significantly rose after the 7th day of stress, yet the redox state was disrupted due to a substantial accumulation of GSSG. Consequently, the AsA-GSH cycle nearly ceased, leading to death after 14 days of stress. Thus, extreme high temperatures significantly impair the growth of seedlings. With the increasing frequency of such temperatures, seagrass beds, including their

establishment and expansion, will be severely impacted. This research is valuable for further exploration and identification of heat-resistant genes in seagrass. It also provides theoretical support for the restoration of *Z. marina* seagrass beds.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: The transcriptome data can be found in Bioproject accession number: PRJNA1069623. Further inquiries can be directed to the corresponding authors.

Author contributions

YP: Data curation, Formal analysis, Methodology, Visualization, Writing – original draft, Writing – review & editing. ZW: Data curation, Formal analysis, Methodology, Writing – original draft. WY: Conceptualization, Funding acquisition, Supervision, Writing – original draft, Writing – review & editing. BZ: Conceptualization, Funding acquisition, Supervision, Writing – review & editing.

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Conflict of interest

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Reduced seagrass resilience due to environmental and anthropogenic effects may lead to future die-off events in Florida Bay

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Coastal systems often depend on foundation species such as seagrasses that are supported by self-facilitation. Seagrass meadows, however, are threatened worldwide due to climate change and local human impact, disrupting self-facilitation leading to system instability. Florida Bay is a large seagrass dominated coastal ecosystem that suffered from multiple seagrass mortality events over the last half century driven by hypoxia, high water temperatures, hypersalinity, and high biological oxygen demand. These conditions reduce the amount of photosynthetically-derived oxygen in the plant causing sulphide intrusion into meristematic tissues. Using a bay-wide sampling design and long-term monitoring trends of seagrass condition, we investigated the current state of the meadows, sediment characteristics (e.g., organic matter, sulphide, nutrients) and discuss how climate stressors interact with plant and sediment oxygen dynamics. Our survey revealed that at sites where seagrass had been previously denuded by die-off, the dominant seagrass *Thalassia testudinum* had not recovered, while the pioneering seagrass *Halodule wrightii* recolonized the impacted areas. Organic matter and sulphide levels were higher at the impacted sites, apparently a persistent characteristic of the formerly dense *T. testudinum* meadows in central and western Florida Bay. These sediment conditions promote sulphide intrusion of *T. testudinum* belowground tissue under anoxic conditions. Plant oxidation initially buffers sulphide intrusion, but disruption of this oxidation mechanism due to changing environmental conditions results in widespread mortality and seagrass community collapse. Climate change cannot be fully mitigated by local management, however, attempts can be made to control critical salinity and oxygen levels by increasing freshwater input, reducing hypersalinity and aiming to keep the internal seagrass oxidation feedback intact. Our study

shows that the Florida Bay seagrass ecosystem is still recovering four years post die-off and continues to be susceptible to future climate change and system degradation.

KEYWORDS

Florida bay, climate change, hypoxia, sulphide toxicity, seagrass die-off, turtlegrass *Thalassia testudinum*

Introduction

Seagrass meadows are a key ecological component of many coastal ecosystems. They are essential foundation species due to their habitat-formation, the high biodiversity they support and their socio-economic importance; they serve as carbon and nutrient sinks, control coastal erosion and contribute to secondary production (Heck et al., 2003; Orth et al., 2006; Waycott et al., 2009). However, seagrass meadows are rapidly declining due to human impacts such as eutrophication, coastal development, and climate change, resulting in the loss of valuable ecosystem services (Costanza et al., 1997, 2008; Waycott et al., 2009). Increased severity and frequency of drought and heat waves are expected to drive further decline through hypersalinity, desiccation, hypoxia, and heat stress, all of which can disrupt internal processes important for seagrass ecosystem resilience. For example, drought was recently shown to cause desiccation stress in a large intertidal seagrass-dominated system in Africa, disrupting system stabilizing mutualism, leading to a systemwide die-off event (de Fouw et al., 2016, 2018). In 2010, Shark Bay in Australia was exposed to a severe marine heat wave that led to a widespread loss of seagrass (Fraser et al., 2014; Strydom et al., 2020). Increasing fluctuations in coastal salinity levels due to climate change are expected to affect seagrass survival and distribution (Durako and Howarth, 2017; Sandoval-Gil et al., 2023). This illustrates how extreme meteorological and climatic events can disrupt otherwise stable, long-lived foundation species adapted to dynamic environmental conditions.

Florida Bay is a large seagrass-dominated coastal ecosystem that has suffered from multiple seagrass mortality events over the last half century (Zieman et al., 1989; Robblee et al., 1991; Schmidt, 2002; Hall et al., 2016). Between 1974–1975, 1987–1991 and again in 2015, high water temperatures, hypersalinity, and/or high biological oxygen demand all contributed to the periods of hypoxia and sulphide toxicity that primarily affected the climax seagrass species, *Thalassia testudinum* (Zieman, 1997; Zieman et al., 1999; Koch et al., 2007d; Hall et al., 2016). After the 1987 event, exposed sediments, loss of *T. testudinum* as a stable nutrient sink and liberated nutrients from sediments and seagrass detritus led to cyanobacterial blooms and sediment resuspension events that persisted for nearly a decade. Hence, this contributed to widespread light limitation and secondary losses to a wider range of seagrass species (e.g., *Halodule wrightii* and *Syringodium*

filiforme) (Robblee et al., 1991; Fourqurean and Robblee, 1999; Rodemann et al., 2021). System recovery from die-off events lasted 17–23 years (Hall et al., 2021).

In addition to largescale die-off events, sulphide-driven *T. testudinum* mortality occurs annually in Florida Bay, typically at the end of summer and in early autumn when plant biomass, salinity and water temperatures are highest and daylength begins to shorten, reducing the amount of photosynthetically-derived oxygen in the plant and the meadow canopy (Hall et al., 1999; Koch et al., 2007d). Hypersaline conditions in Florida Bay are most frequent and severe in the west-central bay (Fourqurean et al., 1992; Hall et al., 2016) where 40–50 PSU often occurs at the end of the dry season (April–May) and at times 70 PSU has been recorded (Fourqurean and Robblee, 1999; Nuttle et al., 2000; Hall et al., 2016). Combined with high temperature (>33°C) leading to reduced photosynthetic efficiency in seagrasses (>60 PSU) (Koch et al., 2007a, c; Johnson et al., 2020) and widespread bottom-water anoxia, all capable of increasing oxygen imbalance in the plant, particularly in the densest stands of *T. testudinum* (Hall et al., 2021). Simultaneously, high water temperatures increase anaerobic decomposition by sulphate-reducing bacteria, producing sulphide as waste product (Jørgensen, 1982; Lamers et al., 2013). Finally, these external stressors lead to sulphide intrusion in the plant and is thought to be the proximate cause of mortality (Borum et al., 2005; Koch et al., 2007c; Johnson et al., 2018).

Under more benign environmental conditions, seagrasses typically facilitate their own growing conditions through porewater oxygenation and by increasing water clarity, by suspended particle trapping and sediment stabilization (van der Heide et al., 2007; Fourqurean et al., 2012; Hansen and Reidenbach, 2012). A consequence of this self-facilitation process is enhanced organic matter accumulation, however, stimulating anaerobic microbial activity resulting in increased sulphide concentrations (Jørgensen, 1982; Lamers et al., 2013). Organic matter sufficient to initiate sulphide-driven seagrass mortality events may routinely occur in Florida Bay sediments, as evidenced by patchy, small-scale mortality observed in west-central Florida Bay. This area of the bay is where die-off is most pronounced and has been the locus for all major events documented thus far. Due to extensive water management modifications in the Everglades and infrastructure development of the Florida Keys over the last century, it is currently the least-well flushed region of the bay, receiving relatively low influx of freshwater from the Everglades and has limited hydrologic

connectivity to either the Gulf of Mexico or the Atlantic Ocean (Lee et al., 2016).

During the day, seagrasses photosynthetically produce oxygen which is released into the water within the phyllosphere and through their roots into the surrounding rhizosphere (radial oxygen loss). At night, oxygen gradients across the sediment water interface help to drive oxygen from the water column to sediment porewater through roots and rhizomes (Figure 1) (Koch et al., 2022). Oxygen can detoxify the sediment by converting free sulphides to sulphate (Pedersen et al., 2004; Borum et al., 2005). However, this plant detoxification mechanism can be disrupted by high water temperatures and hypersalinity that can compromise the photosynthetic capacity of *T. testudinum*, reducing oxygen concentrations in the bottom water and limiting downward

diffusive flux of oxygen into the sediment at night (Johnson et al., 2020). In such cases, sulphide can outpace oxygen, particularly under warmer temperature conditions when sulphide production and metabolic demand increases. This situation may cause higher sulphide intrusion in the plant meristem increasing plant mortality (Koch et al., 2022).

To get insight in the current state of the seagrass meadows and understand how external stressors interact with plant dynamics and sediment conditions, we conducted a landscape-level examination of edaphic condition (e.g., organic matter, sulphide, nutrients), sediment morphology and seagrass condition in Florida Bay. Four years after the most recent die-off event in 2015, we conducted a bay-wide study using a gridded sampling design comprising 104 stations. At each station we investigated sediment variables, porewater sulphide concentrations and seagrass status. For long term trends of seagrass condition 15 permanent transects across the bay were used. We hypothesized that 1) based on recovery trajectories of past die-off events, sites impacted would not have fully recovered to pre-die-off conditions; 2) impacted sites would differ in seagrass species and biomass from unimpacted sites and 3) impacted sites would exhibit higher sediment organic matter and sulphide levels than unimpacted sites a legacy from the die-off event. We discuss what these findings mean for the plant oxidation feedback and how the system may be more vulnerable to external environmental stressors due to the greater potential of sulphide build-up.

Methods

Study area

Florida Bay is located at the southern end of the Florida peninsula, bordering the Everglades in the north, the Florida Keys in the east and south, and the Gulf of Mexico in the west (Supplementary Figure S1). Historically the bay was covered with a heterogeneous macrophyte community of seagrass species like *Halodule wrightii*, *Ruppia maritima* and *T. testudinum* (Fourqurean and Robblee, 1999). For more than a century, development and water management have reduced freshwater flows to the bay from the Everglades. Freshwater flows into Florida Bay were 2-3 times greater and salinities averaged 3-9 PSU lower prior to intense drainage and water diversion in the upstream Everglades in the 20th century (Marshall et al., 2014, 2020), with a lower and more variable salinity regime supporting the mixed macrophyte assemblages (Fourqurean et al., 2003). The reduction of freshwater input changed a formerly heterogeneous macrophyte community to a *T. testudinum* dominated system (Zieman et al., 1989), with high organic biomass that can precede mortality events (Hall et al., 2021). This is one of the largest populations of *T. testudinum* in the world covering about 2,000 km² (Fourqurean and Robblee, 1999). The construction of roads and railways between the Florida Keys islands also changed the input of saltwater from the Atlantic Ocean. The combined fresh- and saltwater input changes from the north and the east increased the residence time of the water in the bay considerably (Madden,

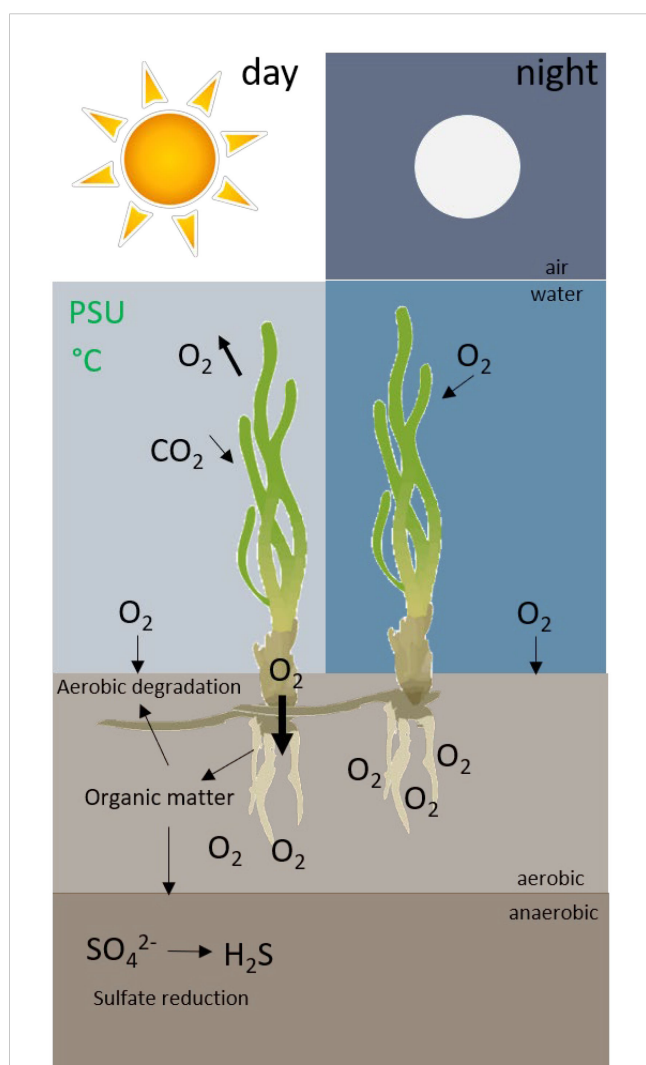


FIGURE 1
Conceptual model of the photosynthetically-derived oxygen sulphide detoxification mechanism in *Thalassia testudinum*. During the day, seagrasses photosynthetically produce oxygen which is released into the water within the canopy layer and through their roots into the surrounding rhizosphere in a process called radial oxygen loss (ROL). At night, oxygen gradients across the sediment water interface help to drive oxygen from the water column to sediment porewater through roots and rhizomes. Oxygen can detoxify the sediment by converting free sulphides to sulphate.

2013). In addition, Florida Bay consists of more than 50 shallow basins (0.5 to 3.0 m depth) separated by a network of shallow mudbanks (Lee et al., 2016). This complex geomorphology results in limited water exchange between basins and large differences in water residence times within basins, ranging from a month to a year (Boyer et al., 1999; Lee et al., 2006, 2008, 2016).

Spatio-temporal transect sampling

Temporal trends of aboveground seagrass biomass were collected yearly by the Florida Fish Habitat Assessment Program (FHAP) in 50-meter transects in 15 basins since 2006. Five basins were impacted by the die-off event in 2015 and others were unimpacted (see: Fredley et al., 2019; Peñalver et al., 2020; Hall et al., 2021). In 2016, visual surveys were conducted by FHAP to determine impacted sites and converted to polygon maps (Supplementary Figure S1) (Hall et al., 2016). Biomass was sampled with sediment cores (ϕ : 15 cm) from three haphazardly selected locations adjacent of each transect line, and dry mass was determined accordingly (see below). We used mean aboveground dry mass per basin per year (2006 to 2021) in autumn for historical trends.

Gridded spatial sampling

Seagrass biomass and sediment characteristics were collected in a large monitoring campaign between 2 October and 20 October in 2019 covering the entirety of Florida Bay. The gridded sampling design consisted of sites taken at 5 km intervals and several additional sites targeting the die-off areas (Supplementary Figure S1). In total 16 stations at impacted sites and 86 stations at unimpacted areas were visited. At each sample location sediment depth was measured with a calibrated pole (220 cm) inserted into the sediment until contact with the solid carbonate platform below. Water depth was similarly measured from the surface to the sediment-water interface. Seagrass was collected with sediment cores (ϕ : 15 cm) to a depth of about 20 cm and then sieved in 1-mm mesh bags. Live seagrass was separated from shell debris and detritus and sorted by species in the laboratory. Aboveground and belowground biomass was separated, and dry weight was determined after drying for 48 hours at 60°C.

To measure porewater sulphide concentrations and sediment parameters, a separate small sediment core (ϕ 10 cm) was taken to a depth of 15 cm. The intact sediment core was immediately transferred to small buckets (ϕ 10 cm) and covered with a lid to prevent oxygen contamination. In the laboratory the sulphide was measured by anaerobically collecting sediment pore water samples in 60-ml vacuumed syringes connected to 10-cm rhizon samplers (Eijkelkamp Agrisearch Equipment, Giesbeek, the Netherlands). Total dissolved sulphide concentration in the pore water was measured immediately after sampling, in a mixture of 50% sample and 50% Sulphide Anti-Oxidation Buffer (SAOB) using an ion-specific silver-sulphide electrode (van der Heide et al., 2012; de Fouw et al., 2016). Sediment samples were taken with a small core (ϕ 1.5 cm) to a depth of 5 cm from the sediment in the buckets.

Sediment samples were freeze-dried in the laboratory and grainsize by using a particle size analyzer with auto sampler (Coulter LS 13 320), and organic matter as loss on ignition (LOI; 5 hrs. at 550°C) were determined. Total phosphorus of the sediment was determined after digestion of dried sediment with nitric acid and analyzed using an inductively coupled plasma emission spectrophotometer (ICP-OES iCAP 6000; Thermo Fisher Scientific, Waltham, MA, USA). Sediment total nitrogen was determined of dried sediment on an elemental analyzer (Thermo Scientific).

Statistics

Differences in organic matter content, porewater sulphide, sediment depth, water depth, sediment phosphorus and -nitrogen between sites impacted and unimpacted by the die-off were tested within one-way analyses of variance. Difference of mean seagrass biomass of the transects before 2015 and after the die-off impact were also tested in a two-way analyses of variance, and Tukey HSD for *post-hoc* comparisons. We tested whether the independent variables had a significant effect at a significance level of $P=0.05$. Values are reported as means $\pm$ standard deviation. We investigated the occurrence of impacted or unimpacted in relation to current environmental variables (organic matter content, porewater sulphide, sediment depth, water depth, sediment phosphorus and -nitrogen) with logistic regression models (i.e., binary linear mixed models). By using a logistic regression model with a stepwise backward elimination of variables procedure we predicted the occurrence of the die-off. To detect multicollinearity between explanatory variables, variance inflation factors (VIF) were computed with the *car* R package (Fox and Weisberg, 2019) in combination with Pearson correlation coefficient. Sediment nitrogen content was highly correlated with organic matter content (Pearson $\rho=0.93$) and sediment depth and water depth were also correlated (Pearson $\rho=0.72$), therefore nitrogen content and water depth were dropped before starting the stepwise procedure. This resulted in a VIF of 1.2, indicating that we successfully reduced collinearity (Zuur et al., 2009). All statistical analysis were performed with R statistical and programming environment (R Core Team version 4.1.1, 2021).

Results

At 73% of the gridded stations *T. testudinum* was observed with an average aboveground biomass of $41.10 \pm 58.32 \text{ g m}^{-2}$. Aboveground biomass between die-off impacted and unimpacted sites did not differ (Figure 2, $F_{1,100} = 0.07$, $P=0.79$). However, highest aboveground biomass of *H. wrightii* was found at the impacted area in the north-west part of the bay (Figure 2, $F_{1,100} = 10.97$, $P<0.01$). Die-off impacted sites had 1.6 times significantly higher organic matter (Figure 3, $F_{1,100} = 27.5$, $P<0.001$) and porewater sulphide concentrations where 2.6 times higher compared to unimpacted sites (Figure 3, $F_{1,100} = 17.89$, $P<0.001$). Impacted sites were also characterized by shallower water depths (1.25 m vs 1.85 m; Supplementary Figure S2, $F_{1,100} = 10.85$, $P<0.01$).

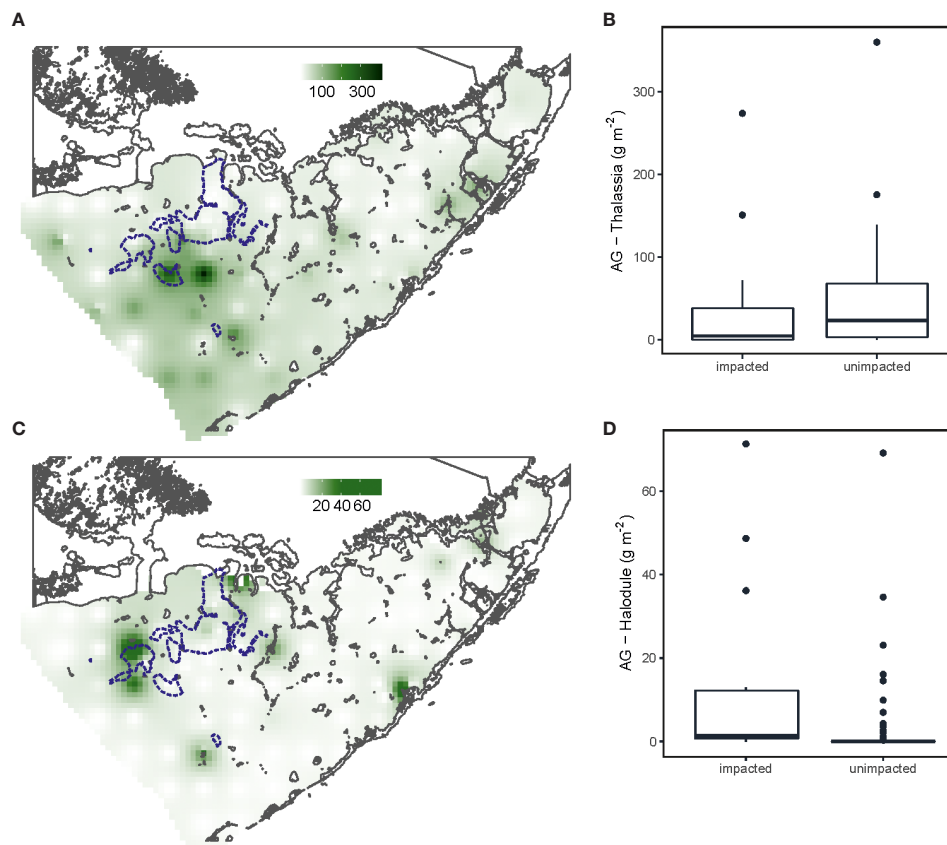


FIGURE 2

Thalassia testudinum aboveground biomass across the Florida Bay in sampling grid (A) and in impacted and unimpacted die-off areas (B). *Halodule wrightii* aboveground biomass across the Florida Bay (C) and in impacted and unimpacted die-off areas (D). Die-off area from visual surveys conducted in 2016 by FHAP to determine impacted sites (blue lines).

and sediment depths were twice greater (1.45 m vs 0.75 m; [Supplementary Figure S2](#), $F_{1,100} = 6.75$, $P < 0.05$). Sediment nutrient concentrations of phosphorus ($F_{1,100} = 11.59$, $P < 0.001$) and nitrogen ($F_{1,100} = 23.50$, $P < 0.001$) were both higher at impacted sites than unimpacted sites ([Supplementary Figure S3](#)).

When explanatory variables were included in the stepwise backward logistic regression procedure, the occurrence of the die-off was best explained by sediment organic matter ($\chi^2 = 21.55$, $P < 0.001$; [Figure 4A](#)). From our temporal analysis, there was a significant decrease in mean aboveground *T. testudinum* biomass before and after the die-off at basins that were impacted (134.2 versus 47.7 g m⁻²), but no difference in basins that were not impacted ([Figure 4B](#)). Consequently, we detected a significant two-way interaction between whether a site was impacted/unimpacted and moment before/after the die-off event ($F_{1,234} = 54.64$, $P < 0.001$).

Discussion

Our field study provides recent insights into local conditions – sediment characteristics in particular – in relation to seagrass standing stock following a large die-off event in Florida Bay. Sites impacted by the die-off in 2015 have higher organic matter content and sulphide

concentrations than unimpacted areas. In areas where *T. testudinum* biomass was reduced by die-off, the pioneering seagrass *H. wrightii* had recolonized the impacted areas and had higher biomass at those sites than elsewhere in the bay. *T. testudinum* standing stock in the gridded sampling data did not differ between impacted and unimpacted sites. However, our temporal transect analysis showed that *T. testudinum* biomass was higher pre die-off at the impacted sites, indicating that high biomass was a precursor of die-off, and that impacted sites had not yet fully recovered four years after the event. The higher organic matter and sulphide levels at the impacted sites suggests that these sites are vulnerable to unfavorable environmental perturbations (e.g. high temperatures and hypersalinity) leading to hypoxia, resulting in seagrass degradation.

Organic matter was the most important predictor explaining the occurrence of the die-off in our analyses. The origin of the higher sediment organic matter content at the impacted sites is a combination of processes. Water quality measurements show that the die-off areas in the central bay have the highest concentration of total organic carbon, -nitrogen and -phosphorus in the water column of any region of the bay and high import of organic matter from the upstream marsh and mangrove zone ([Childers et al., 2006](#)). As such in the central bay there is a strong self-facilitating internal feedback, leading to two effects: a more nutrient-rich substrate in which seagrass can grow to potentially

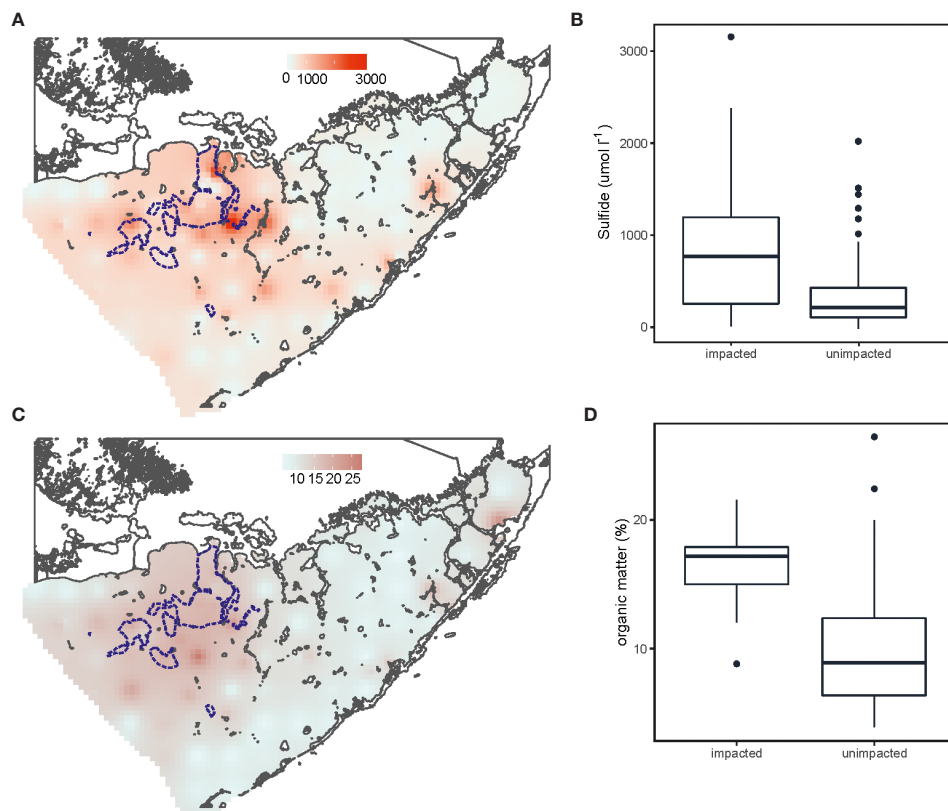


FIGURE 3

Sediment organic matter (A, B) and sulphide concentrations (C, D) across the Florida Bay and in impacted and unimpacted die-off areas. Die-off area from visual surveys conducted in 2016 by FHAP to determine impacted sites (blue lines).

high densities (Fourqurean and Robblee, 1999); and inputs of labile organic material from both internal seagrass production and external import that increases respiratory demand and encourages anaerobic processes leading to sulphide production (Koch et al., 2007d, 2022). Both of these processes are self-reinforcing, making the community more vulnerable to system degradation via

unfavorable environmental conditions. When system degradation occurs, seagrass die-off causes a spike in organic material and nutrients in the sediment matrix. Thus, the build-up of sediment organic matter due to the combination of internal dynamics and die-off event, creates ideal circumstances for sulphide concentrations to increase. After the die-off, seagrass biomass is

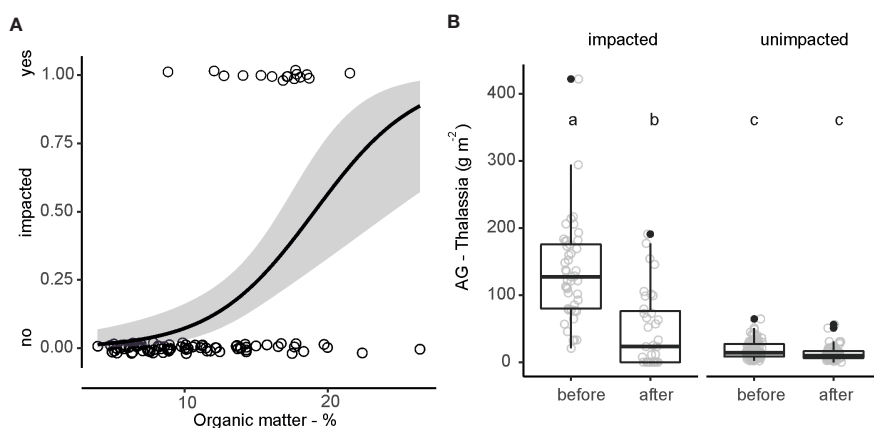


FIGURE 4

The occurrence of impacted (1) and unimpacted sites (0) plotted against organic matter with prediction of logistic regression with 95% confidence interval (A) and aboveground *T. testudinum* biomass at impacted and unimpacted sites before (2006–2014) and after the die-off (2015–2021). Letters above the plots depict Tukey post hoc outcome (B).

low or absent, causing organic matter and sulphide to decrease again, allowing seagrass to recover over time. Overall, this combination of processes may result in cyclic collapse-and-recovery dynamics.

Porewater sulphide concentrations were considerably higher in impacted sites four year post die-off, similar as what was found at post-die-off sites in Florida Bay in the past (Carlson et al., 1994; Kavanagh, 2016). Under favorable environmental seagrass conditions, concentrations found in our study (max 3.2 mM) are not directly toxic for *T. testudinum* (Koch and Erskine, 2001). Under stress conditions, increased temperature (35°C) and salinity (55 PSU), sulphide concentrations of 6 mM increased *T. testudinum* mortality (Koch and Erskine, 2001). The dense *T. testudinum* canopies at the west central site allow for oxygen saturated water retained in the phyllo-sphere and radial oxygen loss through roots to keep a positive oxygen balance preventing sulphide intrusion. When environmental conditions deteriorates, increase of water temperature (>33°C) and salinity (>55 PSU), the risk of disruption of internal plant oxidation increases, and compromise oxygen balance and contributed to anoxia and sulphide intrusion and plant mortality (Figure 5B) (Johnson et al., 2020; Koch et al., 2022; MacLeod et al., 2023).

In central and western Florida Bay, cycles of periods of increasing *T. testudinum* density were punctuated by large die-offs and followed by seagrass succession and recovery of healthy *T. testudinum* meadows. High organic matter and sulphide is a characteristic of the dense *T. testudinum* meadows in central and western Florida Bay and may

create a ‘time bomb effect’ ready to flood the *T. testudinum* meristems with toxic sulphide when conditions permit. This is similar to cyclic shifts in shallow lakes in Europe where accumulation of phosphorus creates an unsustainable condition (time bomb) due to slow internal eutrophication (van Nes et al., 2007). A recent study in a tropical intertidal seagrass system showed similar dynamics. Here a facultative lucinid-seagrass mutualism buffers sulphide toxicity by excessive accumulation of organic matter leading to slow-fast cycles, characterized by a long-term degraded- and seagrass dominated state, with fast transitions in between (de Fouw et al., 2018). In the Florida Bay system, the internal plant oxidation initially buffers sulphide intrusion (Figure 5A), but disruption of the internal oxidation feedback due to environmental perturbation eventually leads to bottom anoxia during the night and increased sulphide production by high organic matter, ending in sulphide intrusion and plant mortality (Figure 5B). Furthermore, seagrass mortality increases turbidity and decreases water clarity through destabilization and re-suspension of sediments in the water column, as well as release of nutrients that can support algal blooms and further reduce irradiance (Figure 5C). Which explains the slow recovery trajectory at impacted sites.

A specific goal of the Comprehensive Everglades Restoration Plan (CERP) by the South Florida Water Management District (SFWMD) and the U.S. Army Corps of Engineers is supplying additional water from the Everglades to Florida Bay via management of Everglades hydrology toward the regime historically present prior to Everglades drainage (see Madden et al. 2009). This is intended to increase seasonal

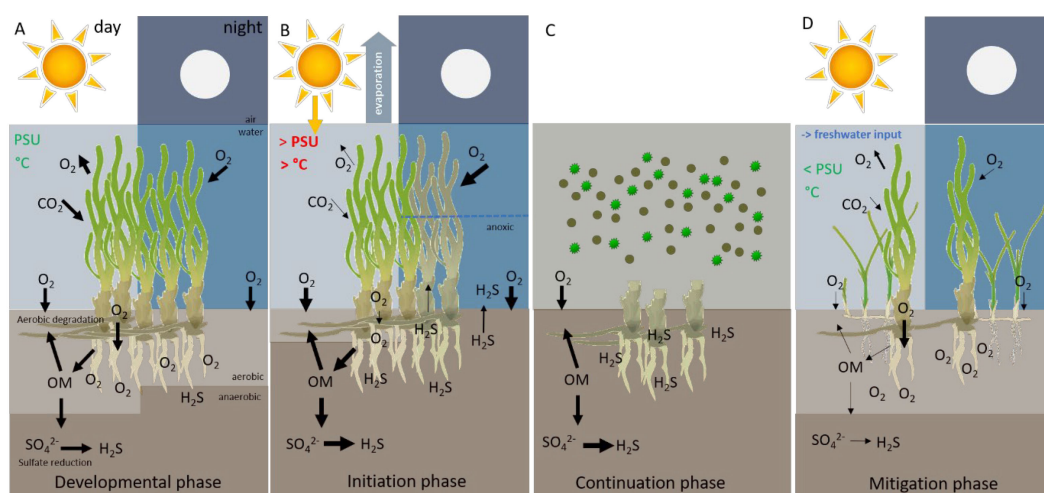


FIGURE 5

High light and high sediment nutrient concentrations create good growth conditions and dense meadows develop, leading to increased photosynthetically-derived oxygen that pressurizes aerenchymal tissues and supersaturates bottom waters, which drives ROL during the day and night. Dense meadows also increase labile organic material from both internal seagrass production and external import that encourages sulphide production and increases respiratory demand (A). The plant detoxification mechanism can be disrupted by high water temperatures, hypersalinity and/or stratification that can compromise photosynthetic capacity, reduce oxygen concentrations in the bottom water and limit downward diffusive flux of oxygen into the sediment at night. In such cases, sulphide can outpace oxygen and causes higher sulphide intrusion in the plant meristem increasing plant mortality, leading to large die-off events (B). After the initial die-off, seagrass mortality increases turbidity and decreases water clarity through destabilization and re-suspension of sediments in the water column, as well as release of nutrients that can support algal blooms and further reduce irradiance (C). Restoration of historic increasing seasonal freshwater flow and flushing rates, enhance toxic conditions, limit hypersalinity and provide a more natural hydrologic and estuarine gradient into north-central Florida Bay. Potentially leading to less stress for macrophyte community, increase oxygen in the water column, leading to a lower risk of sulphide intrusion and promote a more heterogeneous macrophyte community with more *H. wrightii* and *S. filiforme* are believed to be less susceptible to environmental perturbations than communities dominated by monotypic *T. testudinum* (D) (Based on Zieman, 1997; Koch et al., 2007d).

freshwater flow and flushing rates, enhance oxic conditions, limit hypersalinity and provide a more natural hydrologic and estuarine gradient in north-central Florida Bay. This may lead to less stress for the benthic macrophyte community, increase oxygen in the water column, lower the risk of sulphide intrusion (Koch et al., 2007b; Fredley et al., 2019). For the upstream mangrove community, reduced saltwater intrusion should help maintain intact substrate (peat) and prevent nutrient and organic matter export to the downstream seagrass meadows. For the downstream seagrass community, restoration will promote a more heterogeneous macrophyte community (Fourqurean et al., 2003; Madden et al., 2009). Such mixed-species communities with more *H. wrightii* and *S. filiforme* are believed to be less susceptible to environmental perturbations than communities dominated by monotypic stands of *T. testudinum* (Figure 5D). In addition to altered hydrology, historic loss of megafaunal grazers such as green turtles and manatees (*Chelonia mydas* and *Trichechus manatus*) has been suggested to allow development of monospecific *T. testudinum* meadows with canopy high biomass in Florida Bay and the Caribbean (Jackson, 1997; Jackson et al., 2001). Very dense *T. testudinum* meadows with high ratio of below- to above-ground biomass have a very high oxygen demand and are less resilient to hypoxia than a heterogeneous macrophyte community (Fourqurean et al., 2003; Fredley et al., 2019).

Apart from the human-altered hydrology, sea level rise has also increased saltwater intrusion into the Everglades freshwater ecosystems, altering the availability and distribution of nutrients and carbon (Charles et al., 2019; Servais et al., 2019). Historically, freshwater from the Everglades is not likely to increase nutrients in Florida Bay significantly, as N and P input from the Gulf of Mexico far exceeds Everglades input (Rudnick et al., 1999). However, as sea level rise, more organic material is exported from upstream freshwater marshes as saltwater intrusion cause substrate breakdown and peat collapse (Wilson et al., 2018). Further, as hurricane intensity and frequency has increased, peat collapse in coastal mangroves has resulted in turbidity plumes and additional nutrient mobility in the lower Everglades (Rodemann et al., 2021). This can affect the composition and concentration of nutrients reaching Florida Bay, potentially increasing algal blooms (Shangguan et al., 2017), reducing subsurface irradiance impairing the internal dynamics in *T. testudinum* meadows. Overall, sea level rise and hurricanes may thus play a significant role in shaping nutrient dynamics and ecosystem health in the transition from the Everglades to Florida Bay.

Increasing temperature due to climate change is another important challenge facing Florida Bay. In the summer of 2023 water temperatures in Florida Bay exceeded 38°C for an extended period (unpub. data C. Madden), well above the 33°C threshold for thermal breakdown of *T. testudinum* and *H. wrightii* physiological processes (Koch et al., 2007a). Temperature projections for the next 50 years for South Florida region are in the range of zero to +2.5°C, however precipitations projections show no clear pattern in the sign of change and range from -20 to +30% (Obeysekera et al., 2015). Climate change will probably increase water temperature and subsequently salinity levels due to increased evaporation, which will put more pressure on the internal dynamics of dense *T. testudinum* meadows. The potentially cumulative increased organic matter load in the western central part of

the bay due to die-off events makes the meadows less resistant to climate change. Thus, restoration of the historic greater freshwater input from the Everglades may be imperative to maintain healthy seagrass meadows in the bay and help to reduce the risk of sudden die-offs.

Our study shows that the Florida Bay seagrass ecosystem continues to be susceptible to die-off. Local anthropogenic impact of the last decades has significantly changed the bay and in the combination with climate change, recurrent and severe die-offs events have been a direct result of this (Hall et al., 2016, 2021; Rodemann et al., 2021). There is now increasing evidence that coastal macrophyte communities rely on positive interactions (e.g. facilitation and mutualism), to reduce environmental stress (Bruno et al., 2003; Maxwell et al., 2017; Valdez et al., 2020; van der Heide et al., 2021). Recent studies have shown the importance of a facultative mutualistic interactions between bivalves and macrophytes in seagrass meadows and salt marshes during drought and increasing temperatures (Angelini et al., 2016; de Fouw et al., 2016, 2018). At the same time, however, disruption of positive interactions can exacerbate the coastal ecosystem degradation by compromising its ability to reduce stress (de Fouw et al., 2016; Strydom et al., 2020). Due to global change, disruption of plant oxidation mechanism may therefore become a more common phenomenon for the Florida Bay seagrass communities. Freshwater input, which favors a heterogeneous macrophyte community, may reduce that risk. To better track progress toward this goal and to constrain estimates of critical thresholds, we suggest that monitoring programs consider additional *in situ* measurements such as primary production rates, porewater sulphide concentrations, bottom water temperatures, bottom- and pore-water salinities and diurnally resolved dissolved oxygen estimates to better understand, predict and prevent future community or even ecosystem collapse.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Author contributions

JD: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Software, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing. CM: Conceptualization, Data curation, Investigation, Methodology, Project administration, Resources, Supervision, Validation, Writing – review & editing. BF: Conceptualization, Data curation, Investigation, Methodology, Project administration, Resources, Validation, Writing – review & editing. MH: Data curation, Investigation, Resources, Validation, Writing – review & editing. YV: Investigation, Methodology, Resources, Writing – review & editing. SH: Investigation, Methodology, Resources, Writing – review & editing. TF: Conceptualization, Investigation, Methodology, Resources, Writing – review & editing. TS: Investigation, Resources, Writing – review & editing. MB: Investigation, Resources, Writing – review & editing. TV: Conceptualization, Funding acquisition,

Investigation, Methodology, Resources, Supervision, Validation, Writing – review & editing.

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Supplementary material

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Short-term effects of a research-scale oyster cage aquaculture system on sediment transport, water quality, and seagrass meadow health in Copano Bay, TX, USA

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Competing uses for nearshore coastal space, such as aquaculture, have complex environmental and ecological interactions with surrounding seagrass meadows. Oyster aquaculture is among the fastest-growing aquaculture segments in the United States, and it brings concerns such as increased sedimentation from farm maintenance operations and altered water quality through oyster filtration. Changes in seagrass coverage and growth are common indicators of ecological health used to determine anthropogenic impacts on nearshore environments. This study characterized the effects of a research-scale adjustable long-line oyster aquaculture system on the health of adjacent seagrass meadows in Copano Bay, TX. Four *Halodule wrightii* meadows were identified at various distances from the research site: 100 m upstream (upstream), directly adjacent (0 m), 30 m downstream (30 m), and 60 m downstream (60 m). Sites were monitored for 1) seagrass health, 2) water quality parameters, and 3) sediment deposition. Over the 18-week sampling period, no significant differences ($p > 0.05$) were found for water quality variables or sediment grain-size analysis using a one-way ANOVA. A linear mixed-effects model was used for repeated measures of seagrass data, with no effect of the site found on mean seagrass length, coverage, or maximum length ($p > 0.05$). These results suggest that seagrass health indicators were unaffected by proximity to the oyster system. It was concluded that an oyster research aquaculture system of the type and size at the project location had no major negative or positive impact on seagrass meadow health due to high background variability and the overall minor footprint of the oyster farm across a comparatively large spatial extent.

KEYWORDS

seagrass, oyster aquaculture, *Crassostrea virginica*, *Halodule wrightii*, Gulf of Mexico

1 Introduction

Seagrasses are keystone species vital to estuary and marine habitats' health and ecosystem function (Hemminga and Duarte, 2000; Dawes et al., 2004). Seagrass meadows offer shelter and habitat for marine food webs necessary for biodiversity (Duffy, 2006), recreational and commercial fisheries (Saenger et al., 2013; Williams et al., 2016; Nordlund et al., 2018; Unsworth et al., 2019), and other keystone species such as sea urchins (Prado et al., 2009) and sea stars (Pinn et al., 2014; Galasso et al., 2015). Other ecosystem services provided by seagrasses include the stabilization of coastal sediment (De Boer, 2007; Meysick et al., 2022), nutrient cycling (Cuddy and Dunton, 2023), and carbon sequestration (Bedulli et al., 2020; Ricart et al., 2020). With favorable environmental conditions, healthy seagrass will generally have robust root systems, long leaf lengths, and dense coverage (Dunton, 1994; Wood and Lavery, 2000; Congdon et al., 2023). Seagrasses can propagate sexually via seed production or asexually through clonal growth. Increased sexual reproduction has been well documented to occur in response to severe environmental disturbances, presumably as a mechanism to withstand harsh conditions that may be temporary (Alexandre et al., 2005; Cabaço et al., 2009; Cabaço and Santos, 2012; Paulo et al., 2019; Meling-López et al., 2021). Anthropogenic stressors, however, such as eutrophication, runoff, dredging, or shading, have altered hydrodynamics and chemical water quality, inhibiting seagrass health and reproduction (Dunton, 1994; Fitzpatrick and Kirkman, 1995; Roca et al., 2016; Fraser and Kendrick, 2017).

A variety of indicators have been used to measure the morphology and health of seagrass beds, such as above and belowground biomass ratios (Lee and Dunton, 2000; Kim et al., 2013), biomass-density relationships (Cabaço et al., 2007; Vieira et al., 2018, 2022), efficiency of space occupation (Vieira et al., 2018, 2022), and the use of molecular and biochemical metrics coupled with multivariate analysis (Romero et al., 2007; Cabaço et al., 2009; Garcia-Marín et al., 2013). Worldwide, seagrass coverage has been decreasing throughout the 20th century. Between 1940 and 1990, the total overall seagrass coverage decreased annually by about 1.5 percent, with that rate rising to a 7 percent annual decrease after 1990 (Waycott et al., 2009). Between 1880 and 2021, seagrass in 547 sites worldwide decreased on average by 19.1 percent, with the majority of this decrease happening in non-tropical areas (Duníc et al., 2021). Human development and changes to coastal land use and hydrology, such as sediment loading, can kill off seagrass meadows by decreasing light availability (Lee and Dunton, 1997; Waycott et al., 2009; Kim et al., 2015; Saunders et al., 2017; Mehrubeoglu et al., 2024). Other stressors result from nutrient over-enrichment, which can lead to increased epiphyte and macroalgae bloom that compete for space and nutrients with seagrasses or potentially smother them entirely (Burkholder et al., 1992, 2007; Van Katwijk et al., 2011; Han and Liu, 2014).

In some cases, seagrasses are showing resilience to human impacts. In Tampa Bay, total nitrogen input was reduced by 50% in the 1970s, leading to an increase in seagrass coverage of more than 65 percent since the 1980s (Greening et al., 2018). In another

study, it was found that all seagrass meadows in a heavily impacted area of the U.K. have increased in size over the last 10-15 years, with most populations appearing robust and healthy despite the high levels of nutrients and turbidity in the water column (Bertelli et al., 2018). Seagrass meadows in various regions affected by human activities have demonstrated resilience when anthropogenic pressures are reduced, leading to a slower rate of decline compared to the 20th century (de Los Santos et al., 2019; Burdick et al., 2020; Orth et al., 2020). These studies show that seagrass meadows have expanded in some areas despite human impacts like nitrification and sedimentation. This suggests that seagrass can thrive with mild nutrient and sediment loads, restoring the ecosystem and improving habitat health (Crawford et al., 2003; Orth et al., 2020; Vieira et al., 2022; Hodgson and Bucher, 2023). Although some limited geographic areas have documented increases in seagrass coverage, density, and biomass, the majority of seagrass beds adjacent to human populations worldwide are negatively affected by anthropogenic impacts. In addition to changes in seagrass meadow coverage, stress to seagrass can also result in morphological changes that may be harder to detect and that have the potential to lead to decreased resilience to ensuing stress events (Olivé et al., 2007; McMahon et al., 2013; Barry et al., 2018).

Commercially farmed oysters are commonly spawned in hatcheries and then placed in the water, where they are grown using different methods specific to the farming region. In areas with hard, rocky substrates, on-bottom culture or bottom cages are typically used (Lu, 2015). For off-bottom culture, methods consisting of cages, rack and bag culture, tray culture, floating cages, and suspended culture are typically utilized (Walton et al., 2013; Lu, 2015). Adjustable long-line systems (ALS) designs are commonly used in soft substrate, in which oysters are stocked in cages suspended mid-water by a series of lines and pilings (Lavoie, 2005). The advantages of such suspended or floating aquaculture methods are lower mortality as cages are situated farther from benthic predation and have access to higher food concentrations (Lu, 2015). The use of "off-bottom" oyster aquaculture can also reduce disruptions of benthic organisms, such as seagrass beds (Dumbauld and McCoy, 2015), and can also reduce shading as compared to "on-bottom" cultivation methods by being situated high in the water column and moving with the wind and tides. However, some disruptions occur as workers may have to access cages on foot. Because of the impact some methods of oyster aquaculture can have on benthic habitats, substantial regulatory concern exists for how oyster aquaculture could affect seagrass, primarily with a focus on entrainment of sediment from the farm to adjacent native seagrass beds and shading of the benthic environment (Handley et al., 2007; Dunton et al., 2011).

Since the recent legalization of off-bottom oyster aquaculture in Texas (2019), the impacts of oyster aquaculture on native ecosystems in potential growing areas have become a more novel and emerging topic of discussion. Ecosystems such as seagrass (Kowalski et al., 2009; Dunton and Wilson, 2010; Larkin et al., 2017; Congdon et al., 2019) and oyster reefs (Stunz et al., 2010; Blomberg et al., 2018) play a key role in providing essential habitat to a variety of marine species.

Due to the strong potential for oysters to be cultured in areas overlapping seagrass ecosystems, a knowledge base must be built describing how aquaculture operations could affect them. Some studies have found oyster aquaculture to negatively impact seagrass by reducing light availability to seagrasses either by directly blocking the light with oyster aquaculture gear (Skinner et al., 2013) or indirectly through waste, leading to higher phytoplankton and increasing turbidity (Pietros and Rice, 2003). However, other studies have found that oyster aquaculture in well-mixed bodies of water has minimal effect on nearby water quality parameters (Crawford et al., 2003; Turner et al., 2019) or that using oyster aquaculture and seagrasses grown together may offer the most sustainable outcomes for both humans and ecological function (Hori et al., 2019; Cravo et al., 2022). To better understand the anthropogenic effects of off-bottom oyster cultivation, this study evaluated the short-term impact of ALS oyster culture on the health status of established *Halodule wrightii* (Aschers) meadows in the immediate project area. The study was conducted during the summer growing season (July) through the onset of fall (November). This period was chosen because it closely aligned with the timeline that the oyster system was set up and because analysis during this season would highlight any above-ground biomass changes caused by the oyster system. Research like this study could, over time, help inform rules and regulations related to oyster aquaculture, seagrass sensitivity, and day-to-day management activities associated with oyster cage culture operations.

2 Materials and methods

2.1 Copano bay research site

Copano Bay is a 180-km² secondary bay system and estuary located within the Mission-Aransas National Estuarine Research Reserve (NERR), the eastern portion of which connects to Aransas Bay. Due to its shallow depth and NW/SE orientation, it is well mixed by prevailing southeast winds. Copano Bay has an average depth of 2m (Mooney and McClelland, 2012) with typically low freshwater inflow and high winds (Spalt et al., 2020). There is a prevailing wind direction between 105 and 165 degrees in Copano Bay during the summer, with a shift to an NE wind during the colder months (NOAA, 2023). Three primary freshwater sources enter the bay: Copano Creek, the Mission River, and the Aransas River. Copano Bay salinity varies drastically between seasons, ranging from near zero to greater than 40ppt, depending on rain, hurricane, and other conditions (Turner et al., 2016; Bugica et al., 2020). Because of this, ephemeral seagrass coverage has been extraordinarily variable throughout the years, with ecosystems shifting with the changing salinity (Kowalski and DeYoe, 2016; Congdon et al., 2019). It is also possible that species composition changes over time as changing water parameters become more or less tolerant to certain species (Darnell et al., 2021). The surrounding terrestrial landscape forming Copano Bay is primarily agricultural, consisting of scrubland (31.57%), pasture/hay (24.49%), and cultivated crops (22.74%) (Wagner and Moench, 2009).

2.2 Oyster farm description and study design

On June 25th, 2020, oysters (*Crassostrea virginica*) (Gmelin, 1971) were stocked directly in Copano Bay using three parallel 25 m ALS lines. There were 76 cages at a density of 80 adult oysters per cage (typically 7.6 cm in shell height) for a total of 6,080 adult oysters (Figure 1). The orientation of the research system was approximately 100 m offshore and parallel to the shoreline. Adult oysters were stocked to simulate a mature culture and to amplify potential impacts that the research-scale farm might have. Losses of individual oysters due to mortality were periodically replaced throughout the study to maintain consistent system biomass. Cages within the research system were adjusted to a depth of approximately 1-1.5 m and periodically desiccated for the removal of biofouling. Four sites were selected parallel to the shoreline, with seagrass at each of the four sites. One site (0 m) (28.063214, -97.1872) was adjacent to the oyster farm. Two sites were downstream of the oyster farm at 30 m (28.0633475, -97.1875568) and 60 m (28.063517, -97.187867). The final site (upstream) was 100 m (28.062792, -97.186144) upstream of the oyster farm. The four sites together create a transect parallel with the coastline going in the direction of the prevailing current.

2.3 Seagrass surveys

Preliminary surveys in April and May 2020 showed that seasonal, ephemeral seagrass meadows were beginning to sprout along the coast in the project area, providing an opportunity to initiate this study. All seagrass was identified as *Halodule wrightii* at the project site (Staugler, 2019) at a depth of 1-1.5 m accessible by wading from the shoreline without scuba equipment. To identify areas of seagrass at the project site that were both downstream and upstream of the oyster cages, an initial visual survey was conducted, followed by GPS recording. It was also noted that during warmer months, the prevailing current direction at the site is southeast (Mott and Lehman, 2005) and wind-driven, creating a calmer environment where these ephemeral seagrass patches can thrive. This was verified with data from wind direction collected from nearby Copano East and Copano West weather stations in the bay (NERR, n.d.) visualized with the “openair” R package (Carslaw and Ropkins, 2009) (Figure 2). Because currents in this bay are primarily wind-driven, the prevailing current direction was observed to typically align with the prevailing wind direction (southeast) along the coast. The hydrodynamic model TxBLEND also confirmed the current direction (Guthrie et al., 2010; Schoenbaechler et al., 2011). Once the predominant current direction was established and seagrass test meadows were identified, visual surveys were conducted on clear-water days.

Seagrass meadows in close proximity to the cages (within 2 m), 30 m downstream, 60 m downstream, and 100 m upstream from the system were marked and named for ease of identification (0 m, 30 m, 60 m, and Upstream). Seagrass sampling began on August 6th, 2020, and ended on October 22nd, 2020. The test meadow 100 m upstream from the system was chosen as an “untreated” control. The original research design included Before-after-control-impact

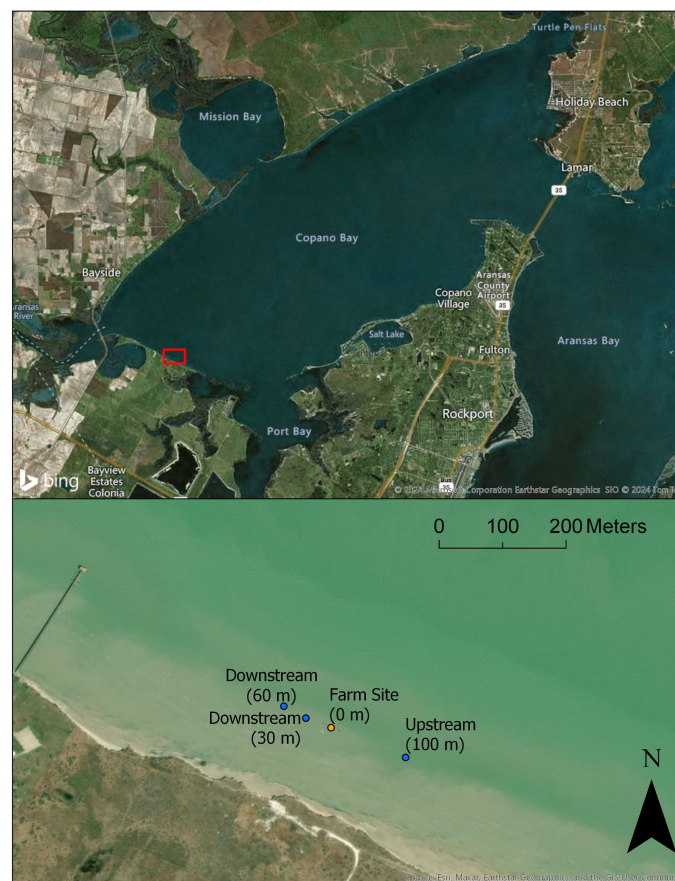


FIGURE 1

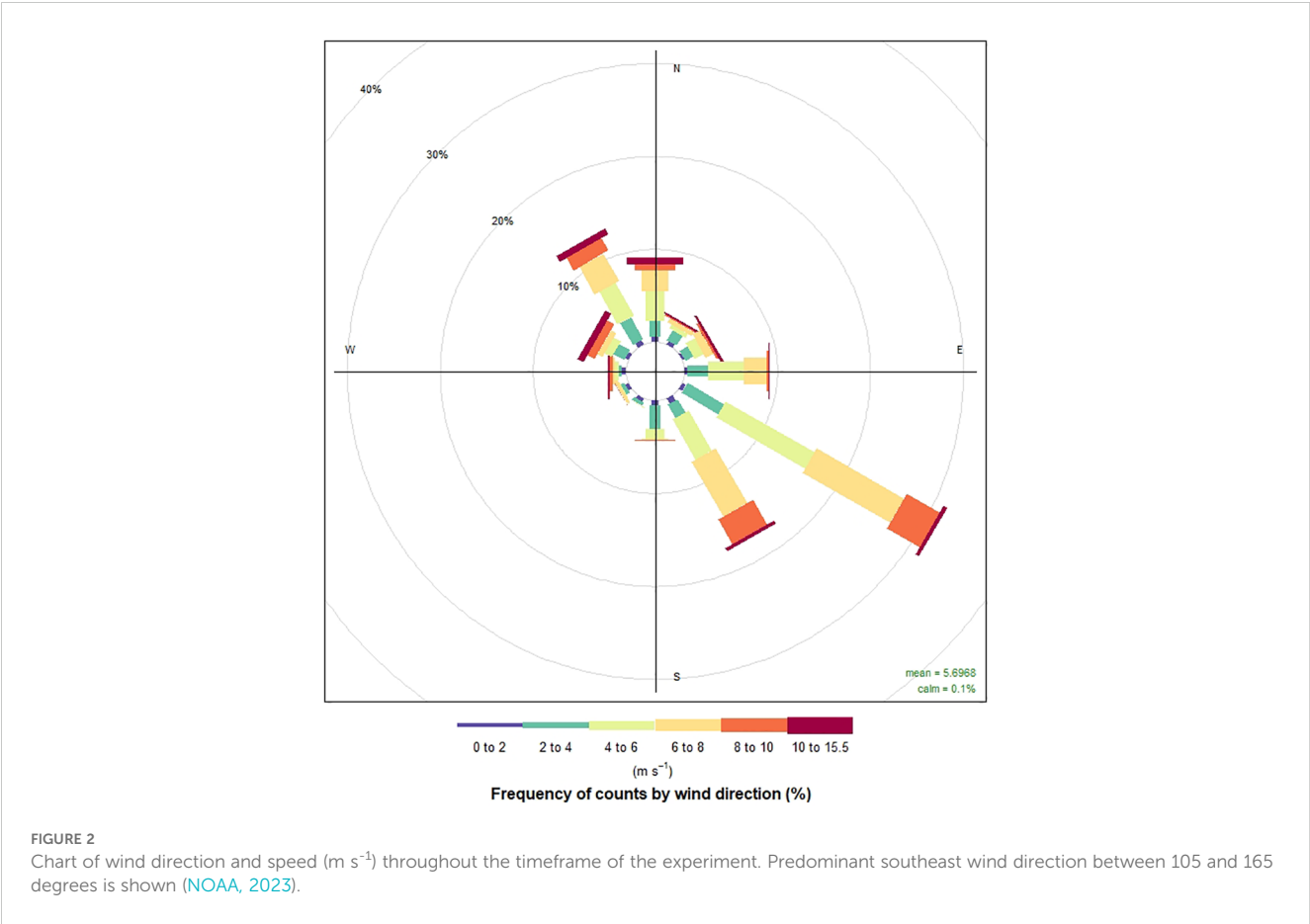
Copano Bay is a secondary bay system connected to Aransas Bay, with satellite imagery of each site (from left to right: 60m downstream, 30m downstream, 0m (orange), and 100m upstream).

(BACI), but due to factors not controllable by this study, sampling of seagrass sites began after the farm had been fully installed and stocked, as opposed to before the construction of the farm would be preferable. All test meadows were chosen to have similar depths and marked with transect lines mounted on two PVC posts per site. The PVC posts act as a visual indicator to ensure that trampling of seagrass beds does not occur or otherwise influence the study. Indicators of seagrass health at sampling sites included mean leaf length, coverage, and maximum leaf length (Heidelbaugh and Nelson, 1996). A 0.25 m by 0.25 m PVC quadrat divided into nine sections was used to determine the coverage (visually estimated) of test meadows following the methods of Dunton et al. (2011). Four random distance (but within 3m) placements of the quadrats in each respective seagrass test meadow were performed weekly along the transect line.

2.4 Water quality

Water quality parameters (e.g., temperature, salinity, dissolved oxygen (DO), and turbidity) were determined weekly beginning on August 6th, 2020, and ending on October 22nd, 2020 (n = 1 per sample

seagrass meadow site) using a YSI Pro DSS data sonde (Yellow Spring Instruments, Yellow Springs, OH) (Table 1). Light intensity and temperature readings were taken every hour using a HOBO Pendant[®] Temperature/Light 8K Data Logger (Onset Computer Corporation, Bourne, MA) placed just above the seagrass surface, at the same depth, facing upright, at each of the four meadow sites and were regularly cleaned of biofouling. With Copano Bay's minimal tidal range and strong winds, the tidal effect on depth here was negligible. The 0 m site loggers were placed beneath the oyster cage lines. Dissolved inorganic nutrients (e.g., total ammonia-nitrogen, nitrite plus nitrate-nitrogen, and phosphate) were determined on a twice-monthly basis by Woods Hole Oceanographic Institute using the Seal Analytical AutoAnalyzer Method G-171-96 (nitrate plus nitrite and ammonia) and Seal Analytical Continuous-Flow Analyzers Method G-297-03 (phosphate) from water samples taken (Seal Analytical, Mequon, WI) Data from the water samples (data sonde and grab-type) were acquired in 200ml bottles, 100 m upstream from the cage study and at 0 m, 30 m, and 60 m intervals downstream within the test seagrass meadows. All samples were filtered and frozen, then overnighted for processing. The sampling schedule for water samples coincided with another project and, therefore, differed slightly from the seagrass sampling schedule.



2.5 Sediment sampling

To evaluate the impact of sediment from routine farming activities on seagrass health, sediment traps (Eadie et al., 1991; Evans et al., 1998; Muzzi and Eadie, 2002) were deployed at the

TABLE 1 Sampling schedule and periodicity throughout the study.

Variables	Frequency
Seagrass Health	
Mean Leaf Length	Weekly
Maximum coverage	Monthly
Maximum leaf Length	Monthly
Water Quality	
Nutrients (DIN, OP)	Biweekly
Relative Light Intensity	Hourly
Temperature	Hourly
Turbidity	Biweekly
Salinity	Biweekly
D.O.	Biweekly
Sediment Loading	
Sediment Trap Collection	Biweekly

oyster culture system, 30 m downstream, 60 m downstream, and 100 m upstream within the test seagrass meadows with sampling between July 28th, 2020 through October 22nd, 2020. The sediment traps were constructed of 7.5-cm diameter PVC pipe fitted with a 7.5-cm diameter funnel inserted and glued into the opening to baffle wave action inside the trap and to prevent waves from washing out any collected sediment. Glass jars were inserted underneath the funnel to act as removable collection containers. The sediment content of these traps was analyzed at least twice monthly, more frequently, to account for weather events. The glass collection jars were removed, capped, bagged, and returned to the laboratory for immediate analysis. Contents were placed in tared ceramic drying pans and dried at 60°C for 72 hrs. The total weight of dried trap sediment was then determined. Dried sediment was sieved through sizes 5, 10, 35, 60, 120, and 230 sieves, and weights were recorded for each category. This data was reported as a percentage of total sediment weight by particle size.

2.6 Statistical analysis

Two statistical analyses were conducted in this study. First, a series of one-way ANOVAs was used to evaluate differences in water quality and sediment loading variables across four sites. Specifically, ten one-way ANOVAs were conducted for each dependent variable related to water quality, while seven additional ANOVAs were performed for sediment variables. All data were

assessed for normality and homoscedasticity prior to analysis. The one-way ANOVA was performed using the “glht” function in the “multcomp” package in R (Hothorn et al., 2008; Pinheiro, 2009; Pinheiro et al., 2012). *Post hoc* comparisons between site means were made using Tukey’s HSD test and pairwise *t*-tests with Bonferroni correction. To analyze repeated measures data on seagrass health (mean leaf length, coverage, and maximum leaf length) at each site, a linear mixed-effects model was employed using the “nlme” package (Bates et al., 2015). This approach allowed the assessment of site effects on each seagrass health metric. All statistical analyses were performed in R (Version 4.0.3; Team, 2020), with significance set at $\alpha = 0.05$.

3 Results

3.1 Water quality

There were no significant differences in mean turbidity, dissolved oxygen saturation, dissolved oxygen concentration, salinity, incident light, water temperature, nitrate + nitrite, ammonium, or phosphate among the four sampling sites regardless of proximity or orientation with the model oyster aquaculture site (Table 2).

3.2 Seagrass

Seagrass meadow health was evaluated using mean leaf length, coverage, and maximum leaf length. Mean leaf lengths were similar among sites, at 13.72, 14.12, 13.73, and 16.00 cm at the 100m upstream site, 0 m, 30 m, and 60 m sites, respectively (Table 3). Mean leaf length and seagrass coverage generally increased at most sites from early August to mid-September but sharply decreased after two major storms (September 19–22, mid-October) (Figures 3, 4). The linear mixed-effect model was used to detect whether the sites impacted each of the seagrass variables across repeated measures metrics. The results of the linear mixed-effects model

for mean seagrass length ($p = 0.397$), coverage ($p = 0.138$), and maximum leaf length ($p = 0.238$) were not statistically significant, indicating that the site did not significantly influence any of these metrics ($p > 0.05$) (Table 4).

3.3 Sediment

The mean sediment deposition rate ranged from 9.15 to 11.42 g/day (Figure 5). More than half of the sediment (50.1–57.4%) was collected in the 250- μ m to 125- μ m size range across all sites (Figure 6). No significant differences in sediment particle size distribution were found among sites ($p > 0.05$). However, ANOVA revealed a significant difference in overall sediment deposition among sites ($p = 0.0377$) (Table 5). Despite this, *post hoc* analyses using Tukey’s HSD and pairwise *t*-tests with Bonferroni correction found no statistically significant pairwise differences in sediment deposition between any of the four sites (all *p*-values > 0.05) (Supplementary Tables A4, A5).

4 Discussion

4.1 Wind-driven vertical mixing and sediment loading

The present study focused on the potential effects of ALS cage farming of oysters on seagrass meadows. In the immediate area (maximum 100 m) from the research oyster operation, there were no significant differences in turbidity, temperature, light penetration, salinity, dissolved oxygen concentration, and percent saturation during the 18-week study. These findings indicate that human activities associated with access and management of the research system had no measurable effect on these factors. Due to the shallow water depth (1.0 – 1.5 m) and high turbidity driven by fetch (Cho, 2007), wind-driven vertical mixing and substantial longshore-oriented current were likely to offset these factors

TABLE 2 Results of water quality ANOVA between four sites.

Parameter	Df	Sum Sq	Mean Sq	F Value	P-Value
Turbidity (NTU)	3	3762	1254	.75	.528
Do (mg/l)	3	72.7	24.23	1.578	.226
Do (%)	3	225	85	.182	.908
Light (lum/ft ²)	3	2.383 x10 ⁻⁶	794493	1.94	1.21
Temp (C)	3	9	3.109	.201	.896
Salinity (PPT)	3	6.18	2.059	.343	.795
Nitrite+Nitrate (μ mol/L)	3	.849	.2831	.959	.431
Phosphate (μ mol/L)	3	.0390	.01299	.699	.564
Silicate (μ mol/L)	3	2686	895.4	1.042	.395
Ammonium (μ mol/L)	3	.124	.0414	.084	.968

TABLE 3 Seagrass data.

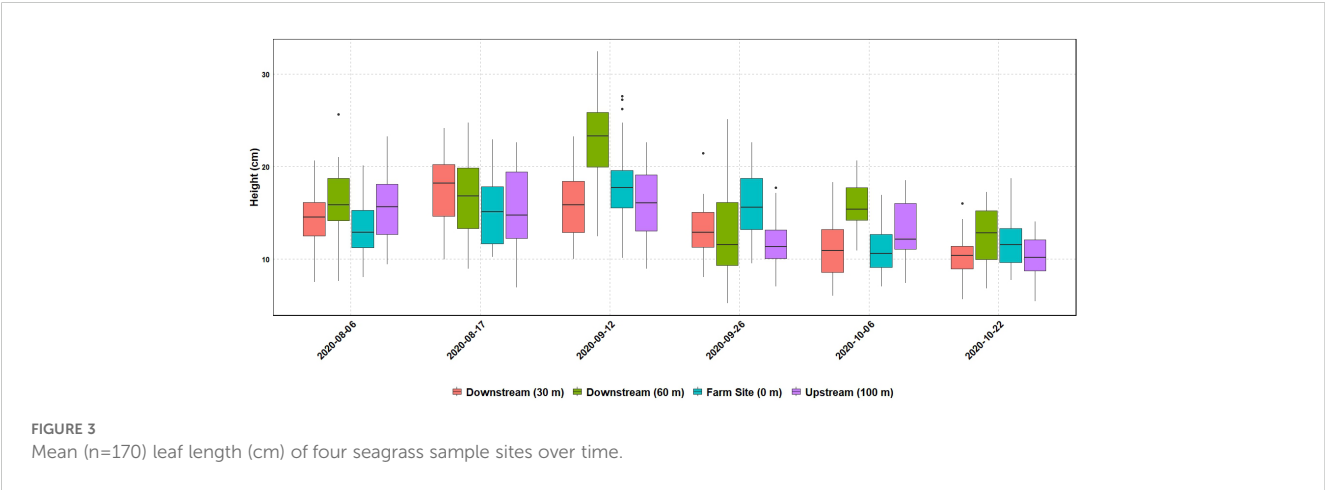
Factor	Sample Site	Mean	Standard Deviation	Range
Leaf Length (n = 170) (cm)	Upstream	13.72	4.13	5.40 - 23.20
	0 m	14.12	4.25	7.00 - 27.60
	30 m	13.73	4.33	5.60 - 24.10
	60 m	16.00	5.26	5.20 - 32.40
Percent Coverage	Upstream	62.79	13.16	44.73 - 72.08
	0 m	64.15	22.89	30.15 - 86.65
	30 m	72.42	14.25	61.09 - 83.03
	60 m	78.61	13.62	61.11 - 95.70
Max Leaf Length (cm)	Upstream	19.77	3.66	14.00 - 23.20
	0 m	21.47	3.78	16.90 - 27.60
	30 m	20.60	3.04	16.00 - 24.10
	60 m	24.38	4.95	17.90 - 32.40

Including mean, standard deviation, and range, by variable, at each of four sample sites (Upstream, 0 m, 30 m, 60 m). Leaf length, percent coverage, and maximum leaf length are all shown.

(Soria et al., 2021). Excess sediment loading is known to be detrimental to seagrass habitats through many studies of intensive anthropogenic activity (Dunton et al., 2003; De Boer, 2007; Van Katwijk et al., 2011; Saunders et al., 2017). Studies directly measuring sediment loading as a result of bivalve aquaculture typically occur at a much smaller scale compared to other anthropogenic activities such as dredging or construction (Onuf, 1994; Erftemeijer and Lewis, 2006). Impacts of bivalve aquaculture typically exhibit minor impacts on the benthic environment, although these results are highly dependent on experiment size and local ecosystem conditions (Bertin and Chaumillon, 2006; Mallet et al., 2006; Comeau et al., 2014; Testa et al., 2015). Stress to seagrass due to sedimentation from oyster farming is more likely to occur on larger farms at high densities, the use of more mechanized equipment involved with infrastructures and pilings, in systems with lower flushing potential, and dependence on gear type (Turner et al., 2019; Muething et al., 2020; Gadeken et al., 2021).

4.2 Nutrient impacts to seagrass meadows

Excess nutrients in the water column can directly influence growth patterns and morphometrics (Lee and Dunton, 2000) or lead to algal bloom or epiphyte shading that impacts seagrass resilience (Valiela et al., 1997). Eutrophication can further escalate drastic changes in the community composition of seagrasses and associated species (Deegan et al., 2002; Gil et al., 2006; Eklöf et al., 2009; Pérez-Ruzafa et al., 2012). Oysters are well-documented buffers against excess nutrient loading, consuming nitrogen and phosphorus-containing phytoplankton through the water column and sequestering it into tissue and shell (Kellogg et al., 2014; Bricker et al., 2014, 2018; Cubillo et al., 2023). On the other hand, oysters produce waste (feces, pseudofeces, and urea) that can harm seagrasses in high concentrations (Buzin et al., 2015; Pietros and Rice, 2003). However, this waste is rapidly absorbed by phytoplankton, algae, and bacteria, contributing to nutrient cycling (Pietros and Rice, 2003; Buzin et al., 2015; Moreno-Marín



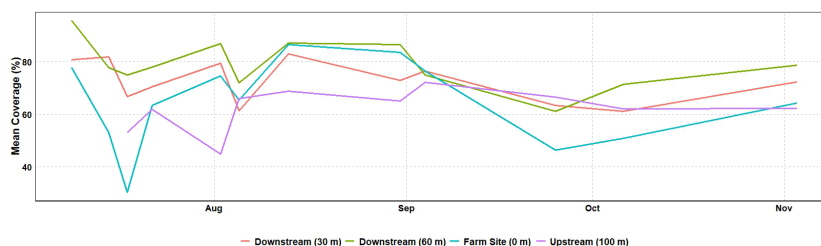


FIGURE 4
Mean (n=45) percent coverage throughout the study at the four seagrass sites.

et al., 2016; Gray et al., 2022). Nutrient levels were not significantly or noticeably different among the four sampling sites, with the exception of nitrate plus nitrite, which was detectable at the 0 m site and barely detectable at the 60 m site but undetectable at the other sites. As nutrients and other water quality factors were similar, there were no significant differences in seagrass biomass or coverage between the sites.

Within this study, seagrass damage from storms had a more considerable impact than any impact from the model oyster farm. Seagrass damage from severe weather in the Gulf of Mexico is a common occurrence, which has accelerated from climate changes influencing the El Niño-Southern Oscillation (ENSO) atmospheric patterns (Fodrie et al., 2010; Congdon et al., 2019, 2023). Subsequent decreases in leaf length can be seen from Tropical Storm Beta (September 19–22) and an unnamed storm towards the end of October (Figure 3). Severe weather has been observed to damage seagrasses through physical wave energy, destroying shoots directly or via sediment erosion, leaving seagrasses prone to uprooting (Congdon et al., 2019; Wilson et al., 2020). Sediment

and seagrass biomass were not measured in this study, but future studies should aim to quantify sediment nutrient composition changes over time that are associated with commercial oyster aquaculture systems.

4.3 Balancing human needs and ecological preservation

Scholars have identified several pathways through which oyster aquaculture can influence seagrass habitats both positively and negatively, although much of the research findings include negligible or minor impacts (Crawford et al., 2003; Wagner et al., 2012; Smith et al., 2018; Turner et al., 2019). Individual studies typically provide valuable yet highly localized information that can be affected by seasonal patterns or site-specific factors. However, these studies can also be synthesized in a broader approach to identify techniques that minimize aquaculture effects on native seagrasses. Some identified practices to lessen the impact of

TABLE 4 Results of repeated measures linear mixed-effects models fit by REML on mean seagrass length, coverage, and maximum leaf length.

Parameter	Mean Seagrass Length	Coverage	Maximum Leaf Length
Model Fit			
AIC	3908.46	363.11	145.05
BIC	3926.44	370.25	149.41
Log-Likelihood	-1950.23	-177.56	-68.52
Fixed Effects			
Intercept (β)	14.53 (SE = 0.59, $p < 0.001$)	69.65 (SE = 2.36, $p < 0.001$)	21.61 (SE = 0.80, $p < 0.001$)
Predictor Effect (β)	0.010 (SE = 0.0097, $p = 0.397$)	0.096 (SE = 0.040, $p = 0.138$)	0.022 (SE = 0.013, $p = 0.238$)
Random Effects			
Intercept SD (Site)	1.12	3.35	0.0014
Residual SD	4.51	11.26	3.92
Observations	664	46	24
Groups (Sites)	4	4	4

No significant effects were found from the predictor variable (i.e., site) for mean seagrass length ($p = 0.937$), coverage ($p = 0.138$), or maximum leaf length ($p = 0.238$).

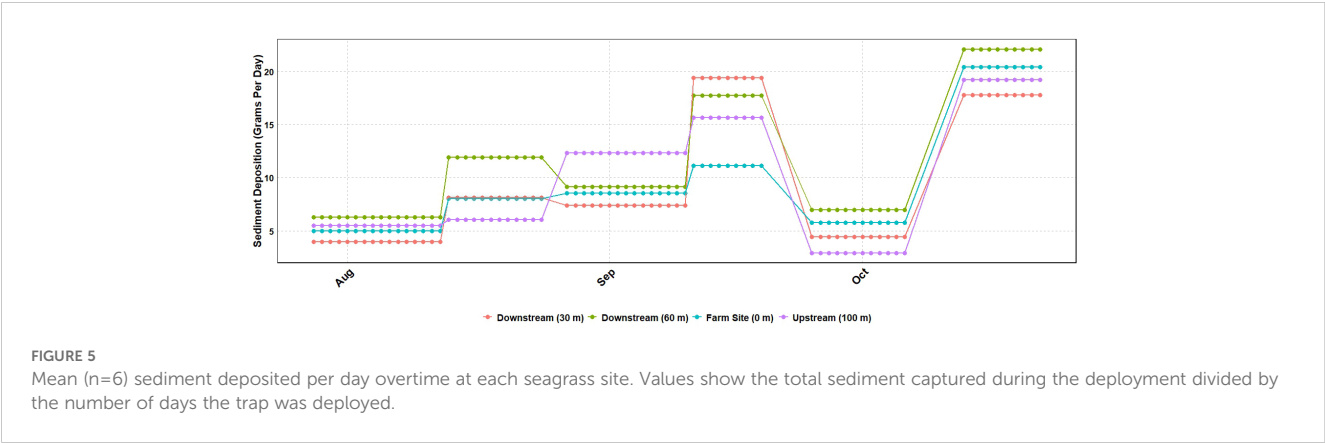


FIGURE 5
Mean (n=6) sediment deposited per day overtime at each seagrass site. Values show the total sediment captured during the deployment divided by the number of days the trap was deployed.

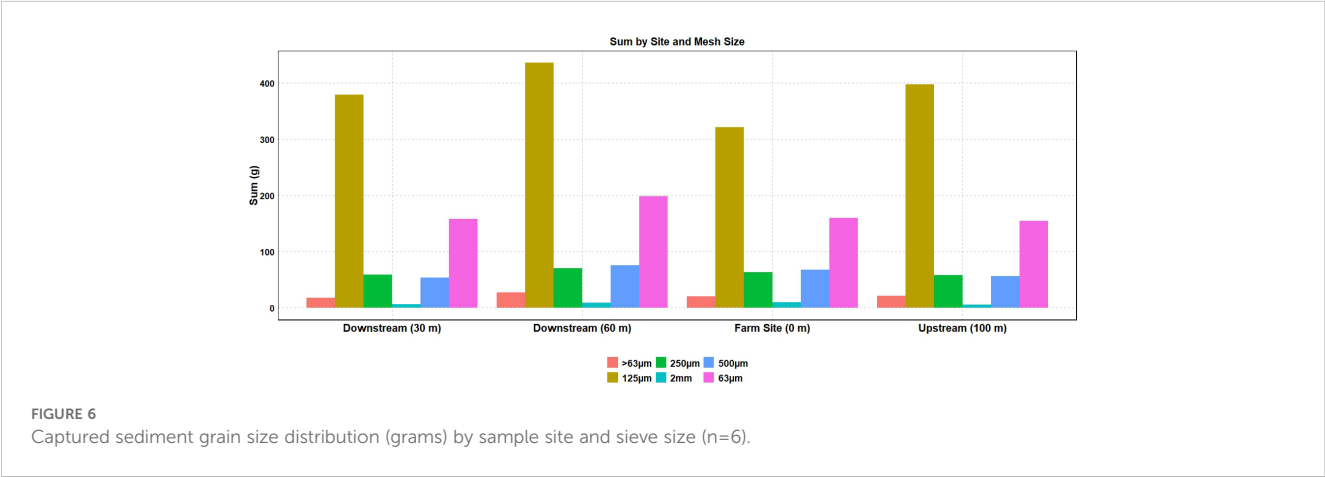


FIGURE 6
Captured sediment grain size distribution (grams) by sample site and sieve size (n=6).

commercial oyster aquaculture include site selection to avoid sensitive seagrass habitats, the use of off-bottom culture methods to help protect benthic habitats, and appropriate densities of cultured species to reduce competition and other environmental impacts (Booth and Heck, 2009; Tallis et al., 2009; Dumbauld et al., 2009; Dumbauld and McCoy, 2015; Ferretto et al., 2022).

In addition to the spatial expansion of aquaculture as a matter of food security and economic activity, other concepts of sustainability are also advancing bivalve aquaculture. Additional emphasis on expanding bivalve aquaculture exists through the

growing attention to sustainable “no feed” aquaculture systems, generally more sustainable than other forms of fed aquaculture (Tacon and Metian, 2015; Cottrell et al., 2020). The utilization of bivalves as a method for the enhancement of water quality has also been championed as a prominent ecosystem function of the cultured species (Bricker et al., 2018, 2020; Ayvazian et al., 2021). These aspects, among others, provide an added level of intricacy when balancing multiple approaches to sustainability within limited and highly valuable estuarine environments.

4.4 Future study design considerations

One limitation of the present study was the spatial scale of the oyster culture system. As the culture system was originally designed as a research farm to evaluate whether farming oysters could be successful in Copano Bay, it was significantly smaller than a commercial-scale oyster farm and its culture systems. Any impact that the system and farm activities had on surrounding waters and seagrass meadows was smaller and more challenging to detect than at a commercial-scale farm. This farm had a total of 76 cages with a total of 6,080 adult oysters. A commercial farm might have over 500,000 oysters of various sizes in the same area. Adult oysters were used for the project to increase the system’s bio-load to make any impacts that the system might have had on seagrass more noticeable. In a commercial farm, there would be many more

TABLE 5 ANOVA of sediment deposition variables, where deposition (.0377) was found to be significant among sites.

	Df	Sum Sq	Mean Sq	F value	P-value
Deposition (g/day)	3	256	58.26	2.85	.0377 *
2 mm	3	2.11	.7032	.51	.68
500µm	3	51.6	17.19	.387	.763
250 µm	3	17.2	5.717	.213	.886
125 µm	3	1147	832.4	.217	.884
63 µm	3	212	70.63	.434	.731
<63 µm	3	8.2	2.733	.45	.72

An asterisk (*) indicates significant values (p<.05).

oysters ranging in size from seed (about 6–10 mm) to market size (about 65 mm), and there would be many more pilings, cages, and lines depending on the style of the oyster farm. In the future, as more commercial-scale farms are established in Texas, similar studies should be repeated in much larger areas and over longer timescales. These longer, larger studies would highlight slight differences over smaller areas and time scales. Changes to seagrass health, water quality, or sedimentation may take several months or years to show significant measurable change, and additional studies across longer spatial scales can aid future management and conservations of seagrass when their habitats are shared with cultivated species, in particular aquaculture where large populations can be introduced quickly.

Additionally, other studies have used oysters in closer proximity over seagrass, which could be used to show greater relationships between the effects of both organisms on each other and the impacts of equipment on sediment transport and local hydrology (Booth and Heck, 2009). Smaller estuarine systems with less background variability and mixing (i.e., lagoons, saltwater ponds, or other waterbodies with low mixing) may be used to highlight ecological interactions. Extending the length of the study across multiple repeated seasons (i.e., multiple summers) may also highlight any long-term changes commonly seen in belowground, perennial biomass. Due to the ephemeral nature of seagrass beds in Copano Bay, long-term studies are still needed to characterize effects on seagrass beds via monitoring over multiple seasons with high and low environmental conditions. Future studies should also begin collecting baseline results far in advance of the addition of oyster aquaculture systems to better assess changes caused by the aquaculture system. This was not possible in this study but would provide extremely valuable information in future studies. Belowground biomass should also be considered in future works, as this would elucidate any changes or stress responses relating to anthropogenic impacts on belowground biomass that this current study was unable to observe.

5 Conclusion

The oyster culture system at the study site did not significantly affect seagrass health for the 18-week project duration. The seagrass presence appeared to be impacted more by aeolian forces and their influence on current and sediment transport. At present, in Texas, location of oyster farms is not permitted within 60 m of seagrass beds. The results of this study set up a first look at some of the effects that oyster aquaculture could have on seagrass ecosystems and highlight the research needs that future studies should focus on. Although the results could be interpreted as supportive of the conclusion that oyster farming's potential impacts on seagrass could be avoided, or at least reduced, by situating farms in shallow, wind-driven bays, more research is needed across different and larger spatial areas, and using longer timescales to substantiate this conclusion. This study also shows that seagrass in slightly more sedimented areas can grow and

thrive in high-wave action bays such as Copano Bay. Based on the results of the present study, it appears that any effects from the research oyster culture system were not noticeable in the sample sites. Therefore, the current 60 m buffer zone may be considered adequate in situating small, research-scale oyster farms where no effects (either positive or negative) will be detectable on nearby natural seagrass. Conducting a similar study with similar independent and dependent variables, but at a commercial scale, could further elucidate the relationship between commercial oyster farming in ALS-type cages and seagrass health. It is also recommended that similar studies be conducted with farming other gear types, gear such as on-bottom and floating cages, and within regions containing other species of seagrass. Seagrass has the potential to be resilient to various anthropogenic impacts; therefore, identifying, understanding, and mitigating the impacts of aquaculture activities on seagrasses is critical to support balancing human and ecological needs.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding author.

Author contributions

RR: Conceptualization, Data curation, Formal analysis, Methodology, Project administration, Software, Validation, Writing – original draft, Writing – review & editing. AL: Data curation, Project administration, Software, Supervision, Visualization, Writing – original draft, Writing – review & editing. JF: Conceptualization, Funding acquisition, Investigation, Methodology, Project administration, Resources, Supervision, Writing – review & editing.

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

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Supplementary material

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fmars.2024.1382153/full#supplementary-material>

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Seagrass restoration in the greater Southeast Asia region: techniques, species, survival and comparisons among investigations

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The greater Southeast Asian region contains the largest global extent of tropical seagrass; however, anthropogenic degradation is estimated to be greater than 7% per year. Although the areal extent of seagrass is presently 36,765 km², Fortes group estimates that 50% of the original seagrass has been degraded from a variety of impacts. One set of solutions to degradation is to restore tropical seagrass successfully, for which information from past results is needed to avoid failures. Van Katwijk, Thorhaug and others provided a global seagrass restoration review of 1,786 trials, but did not include the full Southeast Asian regional information. Thus, we review findings from 228 trials in the greater Southeast Asian region, involving 305,807 restored units with an extent of 372,649 m². Seagrasses planted with varying successes include 13 tropical species and five subtropical or near-subtemperate species. We compare methodologies as well as key factors of light level, energetics, and depth. This review demonstrates the highest survival in seagrass restoration employing sprigs or plugs at medium depths (2–4 m) with adequate light levels in medium to low energetics planting one to several dominant species. Substrate anchors improved successful

establishment. Information gaps occur in quantified monitoring of seagrass services reassembled with tropical-seagrass restoration; thus, fisheries' nursery potentials are not provided. Future actions need national seagrass restoration policies and plans to restore degraded seagrasses. At present, such policies and plans are non-existent in most greater Southeast Asian regional nations, with the exceptions of Australia and the Philippines, although some nations have national plans for restoring corals or mangroves.

KEYWORDS

seagrass restoration, seagrass restoration in Southeast Asian region, seagrass restoration *Enhalus acoroides*, seagrass restoration *Thalassia hemprechii*, seagrass restoration southeast Asia survival and success, seagrass restoration *Halophila ovalis*

1 Introduction

We review the investigations of restoration of seagrasses within the greater Southeast Asian region as one of the sets of important solutions to maintain and bolster seagrass resources after seagrass degradation. Regional seagrass degradation level is presently estimated to be approximately 50% of the original seagrass extent (Ooi et al., 2011; Arias-Ortiz et al., 2018; Fortes et al., 2018). These authors indicate that anthropogenic impacts within the coastal zone have been an important cause of degradation. The seagrass importance in the greater Southeast Asian region is stated by these authors and others as based on containing the most seagrass species plus the largest seagrass biomass. The region has also been identified as a key global area for carbon sequestered by seagrass productivity (Orth et al., 2006; Alongi et al., 2016; Gallagher et al., 2019; Macreadie et al., 2019; Thorhaug et al., 2020a, 2020c), as well as an important global marine biodiversity region (Ooi et al., 2011; Nakaoka et al., 2014; Fortes et al., 2018; Langlois et al., 2023).

1.1 Southeast Asian seagrass present extent and degradation of extent

A dismal future for Southeast Asian nations has been predicted if seagrass degradation is not reversed. Ooi et al. (2011); Fortes et al. (2018) estimate a 30%–50% loss. The present extent of 36,765 km², estimated by Fortes et al. (2018) and McKensie et al. (2020), is considered to be an underestimate, but did not include regional areas of Papua New Guinea (PNG) or western Australia. Green and Short (2003) estimated that the Indonesian seagrass extent alone is 30,000 km². Indonesian mangroves, for comparison, in carefully documented measurements from satellite mapping, lost 250,000 ha in the last decade (Spalding et al., 2010; Giri et al., 2011). Clearly, anthropogenic impacts in coastal Indonesia are considerable (Giri et al., 2011), which implies impacts on the seagrasses adjacent to the mangroves. Langlois et al. (2020) estimated the extent of 235,261

km² for the tropical Indo-Pacific (which includes a far greater area with the inclusion of the Indian Ocean and parts of Oceania as well as the Southeast Asian region). McKensie et al. (2024) emphasize the substantial ongoing extent debate due to deep seagrass being difficult to ascertain by aerial imagery.

This review raises the question, “Can the loss of a maximum of 7% per year of seagrass ecosystem services, predicted by Waycott et al. (2009), be tolerated socially (values for nutrition and fisheries employment) and economically in the Southeast Asian region”?

1.2 Objectives of this review

Our hypothesis is that various seagrass species can be restored with moderate survival in the greater Southeast Asian region. We analyze this by comparing a series of seagrass restoration efforts in various regional nations to delineate the state of seagrass restoration in the Southeast Asian region.

2 Methods

2.1 The hydrological setting of the review

The Southeast Asian region is rich in seagrasses (Figure 1), due to the underlying physical–chemical environment. Convergent equatorial ocean currents form a complex pattern, termed the “through flow” (Wyrski, 1987) (Figure 2), flowing from the Equatorial Pacific past the Southeast Asian region southwestward into the Indian Ocean Waters. Partially driven by a hydrostatic head of 30 cm between the Equatorial Pacific and the Indian Oceans (Sprintall et al., 2009), oceans water pour over the underlying Sunda Plate, through island archipelagos. The Sunda Plate (Figure 3) consists of a large shallow shelf and lies between volcanically formed island archipelagos and the Asian mainland forming multiple estuaries and coastlines sheltering large seagrass meadows. Upwellings of nutrient-rich deeper water fertilizing

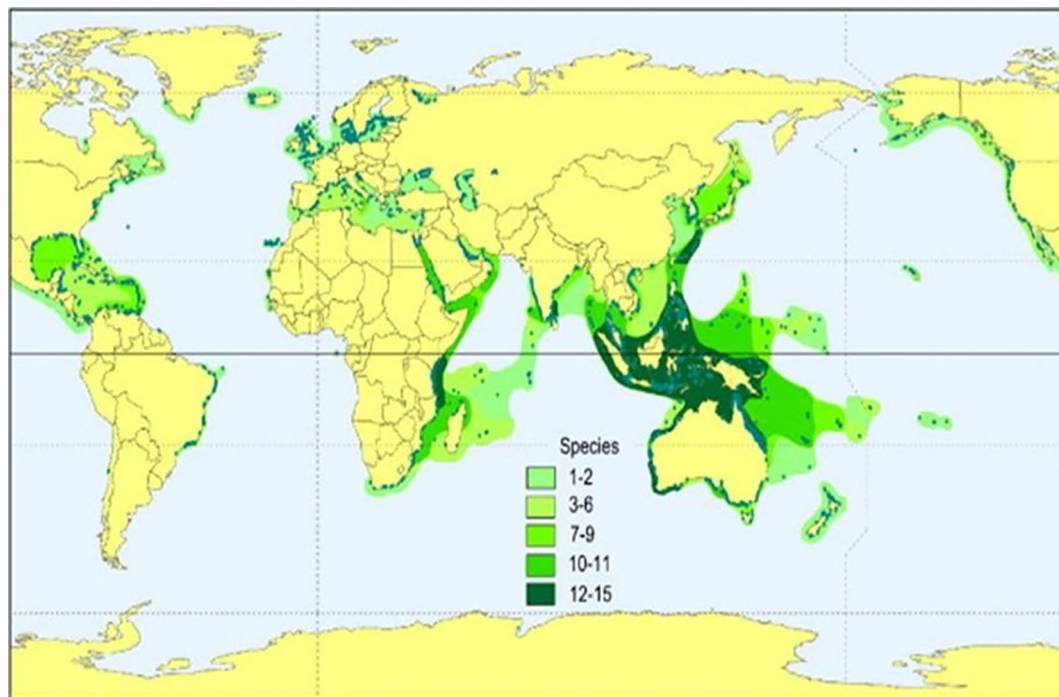


FIGURE 1

Map illustrating the distribution and diversity of seagrass species in coastal regions worldwide. Areas are shaded in varying green tones, indicating species diversity: 1–2, 3–6, 7–9, 10–11, and 12–15 species. The highest diversity is in Southeast Asia and Oceania (from Short 2017).

upper layers occasionally punctuate coastal shelves near periodic deep trenches (Figures 2, 3). The northeast Indian Ocean water flow modulated by trade winds and the Equatorial Indian Ocean current patterns also have effects on the western nations of the greater Southeast Asian region (Conservation Biology Institute, 2010). Ashton (2015) points out that volcanic soils are particularly nutrient-rich, stimulating tropical forests' growth rates. Our operational definition of "Greater Southeast Asia" begins at the Philippines' northeastern corner running westward across the top of the Gulf of Tonkin to the Myanmar continental shelf in the Andaman Sea. From Myanmar, our perimeter moves southwest of Perth, Australia, with our southwestern corner facing the Indian Ocean (see Figure 4). The perimeter moves on a diagonal to the northeast from Perth, including the Northern Australia shoreline including estuaries to the southeastern shelf edge of PNG. From the southeast shelf of PNG, the perimeter goes north to the northeastern side of the Philippines.

2.2 Methods of review

We first recognized the need for this summary of results in tropical Southeast Asia at our Seagrass Restoration workshop during the World Seagrass Association Conference and Workshops in Singapore. We generally followed the same protocol we had used in van Katwijk et al. (2016). We compiled data from restoration trials conducted from published articles listed in Web of Science. A trial consists of one or more shoots or seeds

that have the same "treatment", i.e., they are planted at the same location, with similar techniques and treatments in the same year and season, using the same species and plant material. The study is not a traditional meta-analysis (e.g., Harrison and Kaufman, 2011); first, we aimed not to exclude any reported trial (resulting in many missing values of factors key to growth and survival); second, since the recorded characteristics frequently had no controls, effect sizes can only be estimated relatively between categories (as an example, plant material has the following categories: seeds, sods, rhizome fragments, seedlings, or plugs); and third, the data did not allow for assignment of a nesting factor like sources.

van Katwijk et al.'s (2016) global review included 1,786 trials. Our review herein replicated less than seven from van Katwijk's Southeast Asian locations in our almost 40 investigations. The conceptual approach of van Katwijk et al. (2016) used each specific site per species as one trial with a single survival rate for that trial, disregarding the number of planting units (PUs) carried out at that site, whereas, throughout this review, we refer to each PU (the singular unit that was planted at each site) as the fundamental unit for calculating survival rate. Hence, the trial data sets are handled separately for the survival of individual PUs using the total number of PUs planted as the basis for "survival". Thus, our method allots large-scale plantings with more weight reflected as percentages of PUs being larger than small-scale plantings. In various trials, the PU consisted of one of the following donor material: a seed; a germinated seed (termed a seedling); a sprig (containing at least one rhizome fragment with roots, a meristem, and blades); a sod (large plant mass containing roots, rhizomes,

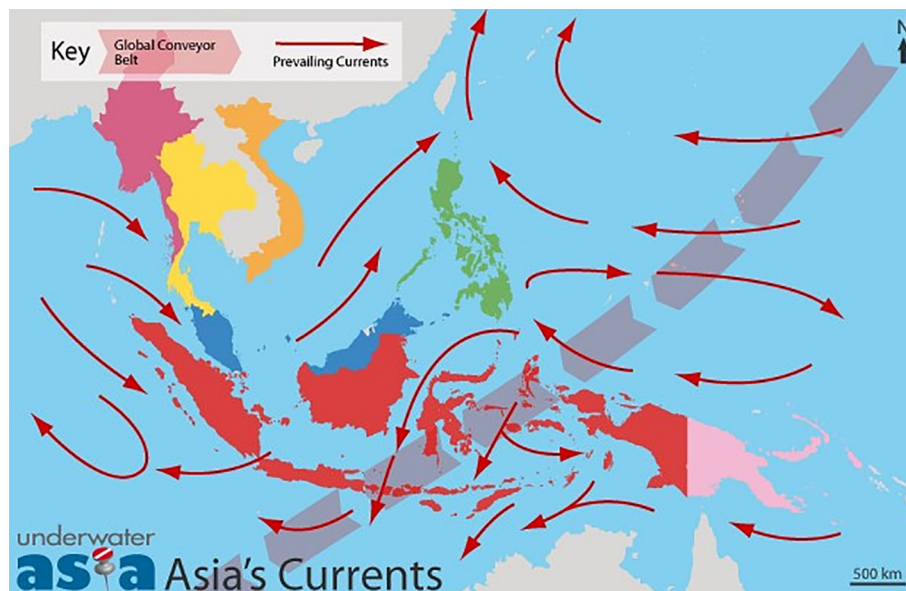


FIGURE 2

Map highlighting ocean currents in Asia, showing the Global Conveyor Belt and prevailing currents with red arrows. Countries are color-coded with a distance scale of 500 kilometers. (from KGR Oceans,2009).

sediment, stems, and blades with some underlying sediment, which could measure to a meter square); or a plug (like a sod, but much smaller, usually approximately 8–14 cm in diameter).

Environmental factors were generally recorded by investigators either descriptively or quantitatively to include the following: planting date, published citation date, geo-reference, planting season, plot size, energy level at site, estimated light level, depth, and salinity. All data did not have complete environmental and biological factors for each

trial and site. In some studies, the investigators could estimate data from ongoing corollary studies at the same site. The biological factors monitored included species, number of PUs, survival percent, interval of monitoring in months, final density of donor site (blades m^{-2}), and planting methodology. Other factors included depth, approximate light level split into high, medium, and low defined under the table itself from surface light levels, approximate energetic level, general sediment composition, anchor types, and use of growth substances.

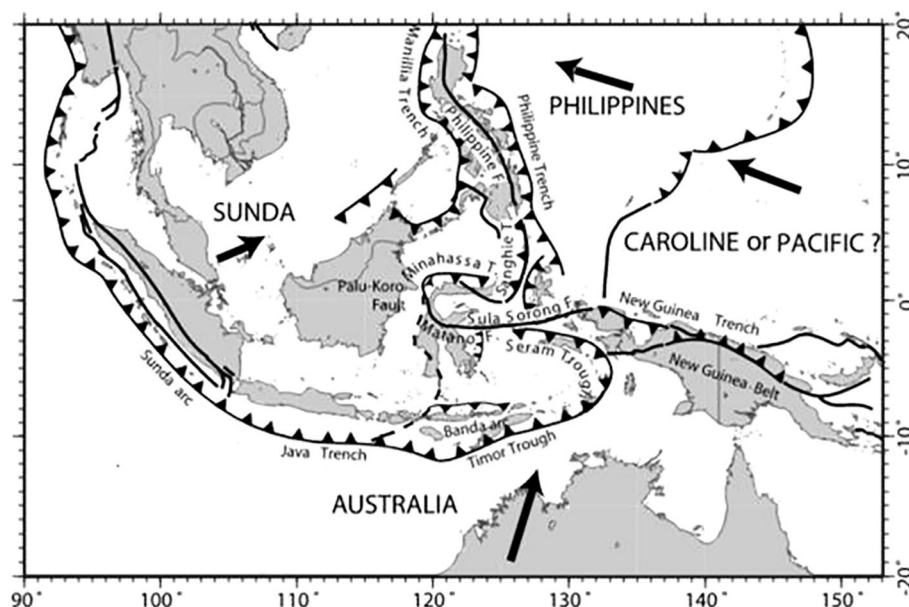


FIGURE 3

Map depicting tectonic plates and trenches in Southeast Asia. Labels include Sunda, Philippines, and Australia. Key features are the Sunda Arc, Java Trench, Philippine Trench, and Timor Trough. Arrows indicate plate movements (from Baroux et al, 1998).

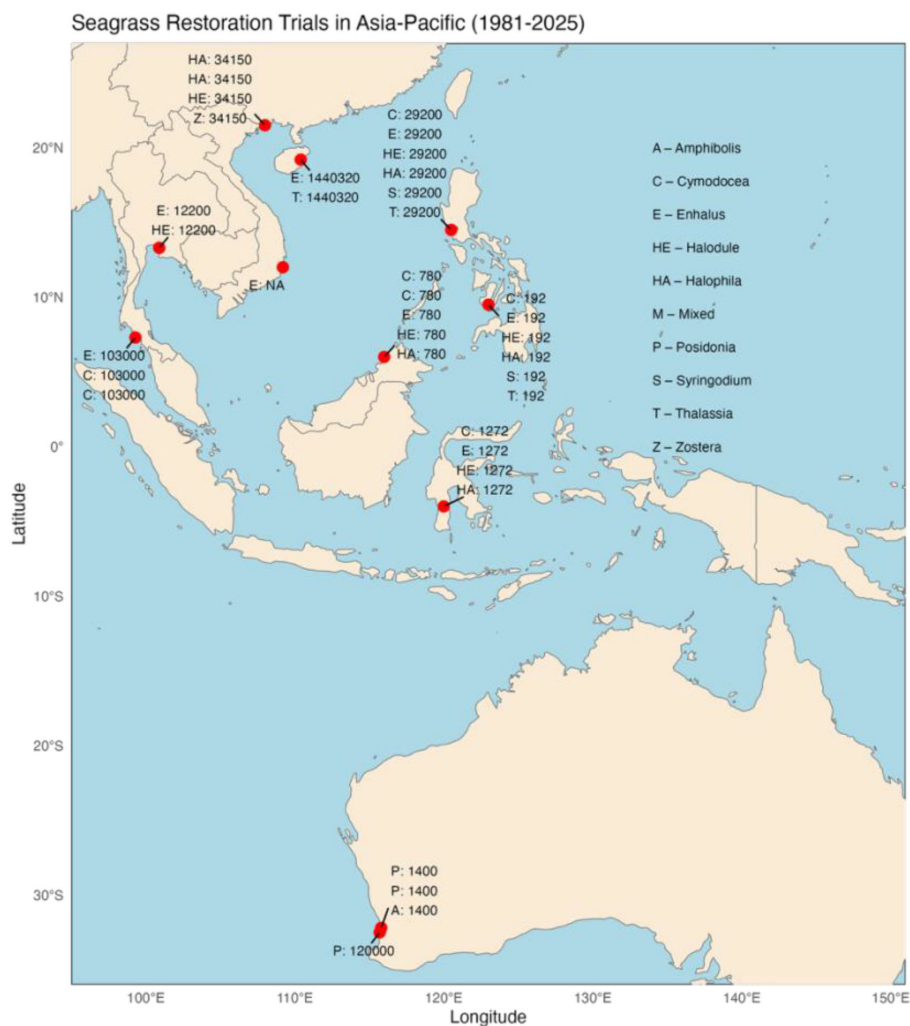


FIGURE 4

Map of the Asia-Pacific region showing seagrass restoration trials from 1981 to 2025. Red dots indicate various locations with annotations for seagrass species, such as *Amphibolis*, *Cymodocea*, and *Enhalus*. Latitude and longitude are marked, with specific codes and numbers denoting trial data for each site (composed by SBZ from earlier fig. by TMY).

Diverse monitoring techniques occurred among the numerous investigators. The restoration investigator team was always the monitoring investigator. We compare metrics for various techniques, species, and treatments of PUs to produce data that characterize the survival of restored plants. We chose to compare PU survival as a function of time as the success metric. A monitoring discrepancy among the 228 trials occurred due to investigator choice of amount of PU measured. Some studies measured all units at all planted areas and some measured only sub-sections of planted areas. The conceptual error may occur when just a sub-section is monitored and if a segment of the planted areas did not survive and the reporter did not take the non-surviving segment into account as zeros for missing PUs. (Note: this affected larger plantings only; in very small plantings, all PUs were consistently measured.) This conceptual error of taking a percentage from surviving PUs and not using zeros for non-surviving PUs would skew results with elevated percentage survival rates. For this reason, we report the subsample population

of PUs from which the survival was calculated as well as the original number of PUs.

2.3 Methods for comparison: are the data adequate for a quantitative analysis?

Upon examination of data, it was found that simple summing, percentages, and means could fulfill the general objectives to compare results among studies in a variety of the greater Southeast Asian locations with multiple species tested, multiple methodologies, and key environmental factors all affecting the survival rates. The list with the investigators' per nation and per author is found in Table 1. Original data are found in an array of publications and reports listed in Table 1 and the literature cited (publications in multiple languages, but the majority in English).

TABLE 1 Overview of seagrass restoration baseline data in Southeast Asia 1974–2019 listed in order of date of trials seen in columns: Investigators and citation date; Nation and site names; Seagrass species; Method of planting; Number of planting units; Survival in year 1 & percentage & remarks.

Investigators/ Citation date	Nation/Site	Seagrass species (planted in the trials)	Planting units		Survival % - reported 1 year or more
			Method planting	Number	
Thorhaug and Cruz (1988)	Philippines: Bataan Peninsula (3 sites) ; Manila Bay (2 sites); Marinduque Island (3 sites)	<i>Cymodocea rotundata</i> , <i>Enhalus acoroides</i> , <i>Halodule uninervis</i> , <i>Halophila ovalis</i> , <i>Syringodium isoetifolium</i> , <i>Thalassia hemprichii</i>	Plug Seed Sprig	29,200	Variable: depending on technique and pollution type at site. Range from 96% to 0%
Calumpong et al. (1996)	Philippines: Bais Ba; Batangan Beach; El Oriente Beach; Negros Island	<i>C. rotundata</i> , <i>E. acoroides</i> , <i>H. uninervis</i> <i>H. ovalis</i> , <i>H. isoetifolium</i> , <i>T. hemprichii</i>	Plug	192	65.5%
Saleh et al. (2020)	Malaysia: Gaya Island, Sabah	<i>C. rotundata</i> , <i>C. serrulata</i> , <i>E. acoroides</i> , <i>H. uninervis</i> , <i>H. ovalis</i>	Plug Sprig	780	24.6%
Tri (2008)	S. Vietnam: Khanh Hoa	<i>E. acoroides</i>	Seedling Sprig	–	–
Kiswara et al., 2010a	Indonesia: Terate, Banten Bay	<i>E. acoroides</i>	Seedling	9	Variable: depending on rhizome length - 10 cm = 51.11% ± 25.58% 5 cm (17.78% ± 18.59%)
Kiswara et al., 2010b	Indonesia: Kuala Pasar, Banten Bay	<i>E. acoroides</i>	Seedling	12	35.0%
Kiswara and UluMuddin, 2010	Indonesia: Pari Island, Jakarta Bay	<i>E. acoroides</i>	Seedling	40	–
Kiswara (2013)	Indonesia: Pari island, Jakarta Bay	<i>E. acoroides</i>	Seedling	6	97.9%
Williams et al. (2017)	Indonesia: Pulau Badi Island, Spermonde Archipelago, South Sulawesi	<i>C. rotundata</i> , <i>E. acoroides</i> , <i>H. uninervis</i> , <i>H. ovalis</i>	Sprig	1,272	32.9%
Lanuru (2011)	Indonesia: Lae-lae Island Spermonde Archipelago, SW Sulawesi; Labakkang, South Sulawesi	<i>E. acoroides</i>	Shoots	8	–
Asriani et al. (2019)	Indonesia : Nugraha-Bintan Isld. Spermonde Isl.S. Sulawesi	<i>E. acoroides</i>	seedlings	x	20 to 93.33%
Wismar et al., 2023	Indonesia: Panjang Island, Jepara	<i>E. acoroides</i>	Seedlings	25	88.3% seedlings 96.7% anchors
Puruhito et al., 2024	Indonesia: Dompok Island, Tanjungpinang	<i>E. acoroides</i>	Seedlings	593	66.7%, 97.8%
Ambo-Rappe (2022)		<i>E. acoroides</i>	Seedlings		

(Continued)

TABLE 1 Continued

Investigators/ Citation date	Nation/Site	Seagrass species (planted in the trials)	Planting units		Survival % - reported 1 year or more
			Method planting	Number	
Cohen-Shachan (2016)	Review of SE Asia	-	-	-	-
Qiu et al. (2014)	China: Guangxi, Gulf of Tonkin	<i>Halophila beccarii</i> , <i>H. ovalis</i> , <i>H. uninervis</i> , <i>Zostera japonica</i>	Plug	34,150	-
Shuo et al. (2019)	China: Li'an Lagoon, Hainan Island	<i>E. acoroides</i>	Seed		
Shen et al. (2023)	CHINA: Hainan island.	<i>T. hemprichii</i> <i>Enhalus acoroides</i>	sprigs	1440, 320	88.8%, 90.6%
Talbot and Wilkinson (2001)	SINGAPORE:	<i>Australian Inst Mar Sci</i> <i>Coral reef, seagrass s</i> <i>mangroves. A handbook.</i>	overview	na	overview
Reid et al. (2019)	Cambodia: Kep archipelago	<i>Status of coral reefs and</i> <i>seagrass Vincent: Cambodian</i> <i>J. natural history 2019(1)</i> <i>124-139</i>	List of animals in natural seagrass.	na	Animal inventory
DMCR (Pansert et al., 2016)	Thailand: Paklok Bay (Phuket); Koh Tiap, Chumphon	<i>E. acoroides</i>	Seedling Plug	-	-
Vichkovitten et al. (2016)	Thailand: Sriracha Bay (Chonburi)	<i>E. acoroides</i>	Sprig	7,200	-
Royal Thai Navy, Ministry of Defense (2017)	Thailand: Sikao (Trang)	<i>E. acoroides</i> <i>C. rotundata</i> , <i>Cymodocea serrulata</i>	Sprig	-	-
Metropolitan Electricity Authority (MEA) of Trang (2008)	Thailand: Palian (Trang)	<i>E. acoroides</i>	Sprig	-	-
Provincial Electricity Authority (PEA) (2016)	Thailand: Sikao (Trang)	<i>E. acoroides</i>	Seedling	103,000	0%
Pfizer Foundation & World Vision Foundation of Thailand	Thailand: Pak Meng (Trang)	<i>E. acoroides</i>	-	-	-
Action Chemical Company (2018)	Thailand: Udom Bay (Chonburi)	<i>Halodule pinifolia</i>	-	5,000	-
Kirkman (1999)	Australia: Garden Island, Western Australia (WA)	<i>Amphibolis Antarctica</i>	Seedling	216	9.3%
Paling et al. (1998, 1999)	Australia: Cockburn Sound, WA	<i>Posidonia sinuosa</i> , <i>Posidonia coriacea</i> , <i>A. griffithii</i>	Sod	1,400	69.4%
Verduin et al 2025 under review	Australia: Cockburn Sound, WA	<i>Posidonia Australis</i>	Sprig	835	78.0%
Paling et al. (2001a)	Australia: Success Bay, WA	<i>A. griffithii</i> , <i>P. coriacea</i>	Sod	1,000	27.0%
van Keulen et al. (2003)	Australia: Carnac Island, WA	<i>A. griffithii</i> <i>P. sinuosa</i>	Sod Sprig	1,320	21.4%
Paling et al. (2001b)	Australia: Success Bay, WA	<i>A. griffithsii</i> <i>P. coriacea</i>	Sod	280	70.0%

(Continued)

TABLE 1 Continued

Investigators/ Citation date	Nation/Site	Seagrass species (planted in the trials)	Planting units		Survival % - reported 1 year or more
			Method planting	Number	
Paling et al. (2003)	Australia: Success Bay, WA	<i>Amphibolis griffithii</i> , <i>P. coriacea</i>	Sod	144	25.0%
Paling et al. (2007)	Australia: Cockburn Sound, WA	<i>P. sinuosa</i>	Sod Sprig	560	27.0%
Horn et al. (2009)	Australia: Cockburn Sound, WA	<i>P. sinuosa</i>	Sod	40	–
Verduin et al. (2010)	Australia: Southern Flats, WA	<i>P. Australis</i>	Sprig	120,000	63.33%
Verduin et al. (2025)	Australia: Success Bank, WA	<i>P. Australis</i> , <i>P. sinuosa</i>	Plug	850	–
Verduin et al. (2025)	Australia: Owen Anchorage, WA	<i>P. Australis</i>	Sprig	1500	–

Seagrass plants, planting data, and methodologies are organized by factor in Table 2 and by species in Table 3.

3 Results

3.1 Overall results

In 10 of 14 nations, namely, the Philippines, Malaysia, Vietnam, Indonesia, tropical China, Australia, Cambodia, Vietnam, Singapore, and Thailand (Table 1), 38 investigator groups worked on seagrass restoration. Many of the 228 trials were small studies (tens to hundreds of PUs). These investigators placed a total of 305,807 PUs into a space of 372,649 m² (Table 1). Table 1 shows that most large-scale plantings were executed by knowledgeable seagrass scientists (e.g., Paling, Verduin, van Keulen, Kiswara, Calumpang, Phillips, Williams, Ambo-Rappe, Huang, Thorhaug, and Cruz). However, some trials were carried out by unskilled community volunteers, as indicated in Table 1, which generally showed far less survival.

3.2 Successful results with various planting techniques

The survival rate differed among the four major planting techniques. We rank them from the highest to the least: sprigs (35.5% survival of 150,678 PUs within 81 trials); sods (25.0% survival of 3,824 PUs with many fewer PU plantings, with 57 trials); plugs (24.6% survival of 41,942 PUs within 56 trials); and seedlings¹ [0.7% if all seedlings planted were considered but with far higher survival (11%) if the PEA (Thailand Electric company) planting was excluded from the 105,713 PUs within 25 trials] (Table 2). A gap in some subsets of data exists, since the monitoring period of some trials ended after the first year. We consider 1 year as an inadequate monitoring period to evaluate long-term success. Table 2 shows that the amount of usage of various restoration techniques is the following at 12 months post-planting: 35.5% of trials utilized sprigs; 25.6%, sods; 24.6%, plugs; 11.0%, seedlings; and 3.9%, seeds.² The most frequently monitored time was 12 months. Note that three-fourths of the sites were measured additionally at 24 months.

3.3 Results of the effect of depth on restoration success

The majority, almost 56.5% of PUs (176,816), were planted at depths of 2 m or less seen in Table 2. At medium depths of 2–4 m, there were 121,863 PUs from 45 trials (but predominantly—120,835 PUs—from the Australian planting). At depths of over 4 m, Australian investigators planted a total of 13,132 PUs using scuba gear. The medium-depth plantings result in higher survival (64.2%) than shallower (6.2% survival) or deeper (43.4% survival) plantings (Table 2). The lowest survival rate occurred as a subset within the shallow (0–2 m) depth group, which was the “very” shallow cohort (planted at or less than 0.3 m). Intertidal plantings had little to no success in the results reported herein. In a comparative depth planting investigation globally reported by Verduin et al. (2010),

¹ Seedling data in this Southeast Asian region were skewed by a single failed trial by a Provincial Electricity Authority in Thailand, where *Enhalus acoroides* was planted without expert guidance, using the seedling technique to plant 103,000 PUs. These were apparently planted in very shallow depths just before a monsoon by community participation, not by knowledgeable scientists.

² The four types of seagrass seeds (Kuo and den Hartog, 2006) are the following: (1) hard-exterior seeds that bury in sediment and germinate later; (2) fleshy fruit that floats during dispersal, then sink to the bottom (e.g., *Enhalus* and *Thalassia*); (3) viviparous seedlings (e.g., *Syringodium*) dispersed by flotation of fruit or fruit-bearing blades; and (4) floating seeds (*Ruppia*).

TABLE 2 For seagrass restoration in the greater Southeast Asia region, variables of restoration planted units measured for total planted units from all cited investigators. (Each table includes Bold Font which indicates average of the PU's finally monitored).

A. Techniques	Plug	Seed	Seedling	Sod	Sprig	Totals
Trials	56	9	19	57	79	220
% of total trials	25.6%	4.1%	8.7%	25.6%	36.1%	–
Units planted (PU)	41,942	3,650	103,272	3,824	149,238	302,376
Subset units monitored	5,392	3,000	222	3,384	136,615	148,613
Subset units survived	1,964	744	26	1,893	80,673	85,300
% survival monitored	36.4%	24.8%	11.7%	55.9%	59.1%	57.4%

B. Depth	Shallow 0-2m	Medium 2-4m	Deep 4-6m	Total
Trials per depth	141	43	67	243
% of total trials	56.2%	17.1%	26.7%	–
Units planted (PU)	176,816	121,863	13,132	311,811
Subset units monitored	24,826	121,863	4,152	150,453
Subset units survived	7,848	76,571	1,804	86,222
% survival monitored	31.6%	63.0%	43.4%	57.3%

C. Light	Low	Medium	High	Total
trials at that light level	24	89	30	143
% of total trials	16.8%	62.2%	21.0%	–
units planted (PU)	7,996	52,656	133,714	194,366
Subset units monitored	6,296	10,176	8,614	25,086
Subset units survived	1,517	3,449	2,626	7,592
% survival monitored	24.1%	33.9%	30.5%	30.2%

D. Energetics/hydrodynamics	Low	Medium	High	Total
Trials at each energy level	46	65	28	139
% of total trials	31.1%	43.9%	25.0%	–
Units planted (PU)	6,094	171,280	121,180	298,554
Subset units monitored	4,246	132,248	8,400	144,894
Subset units survived	1,620	79,228	2,614	83,462
% survival monitored	38.2%	59.9%	31.1%	–

E. Anchor and fertilizer	With anchor	Without anchor	With fertilizer	Without fertilizer
Total trials	228	228	188	188
Trials with anchor/fertilizer	61	167	6	182
% of total trials	26.8%	73.2%	3.2%	96.8%

(Continued)

Continued

E. Anchor and fertilizer	With anchor	Without anchor	With fertilizer	Without fertilizer
Units planted (PU)	124,525	182,272	30	305,377
Subset units monitored	122,903	26,550	0	147,573
Subset units survived	77,089	8,439	0	85,095
% survival monitored	62.7%	31.8%	n/a	57.7%

Each table includes number of trials, factors of trials, percentage of total trials each variable represents, number of units planted (PU), subset of planted units monitored, number of subset monitored which survived, percentage of subset monitored which survived. (A). Planting Techniques; (B). Depth of planting; (C). General light level of planting; (D). Energetics generally at planted site; (E). Use of anchor and/or use of fertilizer. See percentage survival of Planting Units (PU's) monitored for percentage surviving after 1 year result. Bold indicates the survival percentage at each category of those PU finally monitored.

plantings at 2 m showed substantially higher survival (70%) than at 4 m or deeper (37%).

3.4 Results of light intensity on restoration success

Depth is related to both light intensity and light quality in shallow marine and estuarine environments. Restoration survival percentages of planted units were slightly greater in medium light (33.9%) than in high light intensity (30.5%), or in low light intensity (24.1%) (Table 2). These results did not show as great a difference among light levels as survival for depth results. The large numbers planted in Australia in high light may be a factor here. Obviously, multiple simultaneous factors were influencing the seagrass light requirement such as energetics, or perhaps pulses of turbidity lessening light intensity. The number of planted units in trials showed that plantings in medium light (63%) were more abundant than at high light (23.8%) or at low light (15.9%) (Table 2).³ Importantly, no measurements of the duration of low light intensity were included, or of other light changes such as caused by diurnal pulses or variation of riverine turbidity. In a number of trials, the light data were by investigators' estimates or by Secchi-disc measurements, not direct photometer measurements. Thus, these light data do not comprise a set of statistically accurate metrics. Some detailed light measurements with photometers are given in seagrass-restoration studies by Thorhaug and Cruz (1987); Paling et al. (1998; 1999), Verduin et al. (2010); Kendrick and Verduin, (2025), and Williams et al. (2017).

³ The terms "low light" is $\leq 15\%$ of surface light, "medium light" is $16\% - 50\%$ of surface light, and "high light" is above 50% surface light.

⁴ Our use of qualitative energy terms was as follows: Low-energy sites are highly protected, experiencing calm water, with exposure to low currents and low tidal flow. Medium-energy sites are associated with occasional strong waves and currents during storms and moderately protected from prevailing or occasional high storm winds. High-energy sites are far less protected from prevailing winds and can experience the effect of fetch, open ocean waves, and occasionally major currents.

3.5 Results of energetics such as wave energy and currents on restoration success

Clearly, in Table 2, the low-energy plantings (38.2%) and medium-energy plantings (60.6%) were greater than survival rates of higher-energy sites (27.8%).⁴ Estuarine energy regimes can be relatively calm and experience periods during which the PU roots attach themselves into the sediment structure. High wave energy within seasonal monsoon events (rather than daily) are present in many Southeast Asian sites. In the Intertropical Convergence Zone (ITCZ), monsoon winds occur far less than in other Southeast Asian regional nations such as the Philippines. Experienced investigators related that they chose not to plant on the seaward side of barrier islands to avoid the disruptive effects of waves. Likewise, some did not plant in high energetic seasons. Some investigators stated in their multiple test studies that they attempt to find a balance between sufficient depth to overcome disruptive energetics and sufficient light penetration for seagrass growth requirements.

3.6 Results of anchors on planting success

To improve survival, investigators of 61 trials used anchors to stabilize planted units, mostly within medium- and higher-energy regimes (Table 2). They anchored 125,285 PUs in 61 trials. Anchored PUs showed higher survival (62.7%) than the 167 non-anchored PUs (30%) (Table 2). Anchor types varied: hand-made bamboo frames onto which sprigs were tied (Kiswara, 2018); clips; stakes; and metal frames pinned and buried in the sediment with sprigs firmly attached (Verduin et al., 2010) (Table 2). As an example, the anchors of Verduin and Sinclair (2013) were the most complex and had high survival rates. *Thalassia*, *Cymodocea*, *Enhalus*, and *Halodule* sprigs, sods, and plugs were established without anchors with moderate to high survival at medium- to low-energy sites.

3.7 Results of fertilizers and growth-stimulating additives on success

Only a small number (6) of trials in Southeast Asia used growth stimulators such as fertilizers for improved initial growth (Table 2).

TABLE 3 For seagrass restoration in greater Southeast Asia region, number of total planted units per genera and species, planted units, survival number, percentage survival of planted units. Bold font indicates survival percentage of each category of those PU was finally monitored.

Tropical genera	#PU	#PU survived	% survival
<i>Cymodocea rotundata</i>	3,832	442	11.5%
<i>Enhalus acoroides</i> **	6,666	2,335	35.0%
<i>Halodule uninervis</i>	3,032	668	22.0%
<i>Halophila ovalis</i>	1,864	761	40.8%
<i>Syringodium isoetifforme</i>	3,448	1,089	31.6%
<i>Thalassia hemprichii</i>	4,632	1,716	37.0%
Subtemperate/ subtropical genera (in Western Central Australia)	#PU	#PU survived	% survival
<i>Amphibolis antarctica</i>	216	20	9.3%
<i>Amphibolis griffithii</i>	1,260	321	25.5%
<i>Posidonia Australis</i>	120,835	76,647	63.4%
<i>Posidonia coriacea</i>	300	247	82.3%
<i>Posidonia sinuosa</i>	1,320	559	42.3%

The table is separated into two parts: tropical genera plantings and subtropical to subtemperate (carried out in Western Australia only). **Exception to table is the survival of 103,000 Planted Units of *Enhalus* seeds (from Thailand PEA) which is not included due to skewing of data by non-scientists community group attempting their first restoration for the power industry equalling zero success. Inclusion of PEA data would have offset all other data done by scientific investigators due to PEA large planting unit numbers in the *Enhalus* seed trials. Bold indicates the survival percentage at each category of those PU finally monitored.

The larger-scale trials did not use any growth stimulants. Without any growth additives, the large-scale survival rate was 57.7%.

3.8 Results of species employed as restoration material

Southeast Asian seagrass restoration trials used 13 species (Tables 3, 1). Eight tropical species (of 21 regional tropical species) and five subtemperate species were planted. From 305,807 PUs, the most intensively planted were the following: *Enhalus acoroides* (105,438 PUs), *Posidonia australis* (122,995 PUs), and two species of *Halodule* (*H. uninervis* and *H. pinifolia*; 18,207 PUs in total). The group of medium numbers of planted seagrasses included the following: *Halophila* (*H. ovalis* and *H. becarii*) (3,023 PUs in total); *Thalassia hemprichii* (5,264 PUs), two species of *Cymodocea* (*C. rotundata* and *C. serrulata*; 6,072 PUs), and *Syringodium isoetifolium* (3,832 PUs).

3.9 Results of various seagrass species survival in restoration trials

Over the entire Southeast Asian region, the restoration survival rates of plantings in multiple locations by various methods were as follows: *E.*

acoroides, 35.0% (this percentage does not include the PEA plantings); *Halophila ovalis*, 40.8%; *T. hemprichii*, 49.3%; *Syringodium isoetifforme*, 31.6%; *H. uninervis*, 22.4%; and *Cymodocea rotundata*, 11.7% (Tables 3, 1; Figure 4) (Wismar et al., 2023). In western Australia, in the subtropical/subtemperate part of the region, the highest survival rate was seen in large-scale restoration where the genus *Posidonia coriacea* demonstrated higher survival (82.3%) than the other species planted as demonstrated in the following results: *P. coriacea*, 82.3%; *P. australis*, 63.4% (2 ha at 80% plus 1 ha at 30%), plus *P. sinuosa* at 42.3%. Other species that showed lower survival were *Amphibolis griffithii* (25.5%) and *A. antarctica* (9.3%) planted within the same area of western Australia. Thus, summing from the total results of the almost 40 investigations, the seagrasses species demonstrating the highest survival were *Enhalus acoroides*, *T. hemprichii*, *H. ovalis*, and three species of *Posidonia*: *P. coriacea*, *P. australis*, and *P. sinuosa*.

The success criterion for species included survival and lateral growth. Investigators generally ascertained from test plots or observational knowledge the most locally appropriate species for the trials. Control sites comprised proximate naturally occurring seagrass area, which investigators compared to restored seagrass in terms of blade density, blade characteristics, etc (Verduin et al., 2012).

3.10 Results of stated objectives for seagrass planting and monitoring actions

The highest trial numbers were stated by the authors to be restored for “experimental” purposes, in which investigators sought information about the factors allowing successfully seagrass growth at a multiple given sites and which method and seagrass species was most suited in various sites. “Mitigation” purposes comprised the largest volume of PU deployment (particularly in Australia, Thailand, and the Philippines where government mitigation policies were in effect) (Table 1; Figure 4).

4 General discussion

4.1 Summary of main findings

For the greater Southeast Asian region of tropical and subtropical to subtemperate seagrass restoration investigations, we assembled an updated, unbiased review supplementing the larger global seagrass restoration (1,786 trials) review of van Katwijk et al. (2016) where only seven southeast Asian investigations were used. The summary results of almost 40 trials from 10 of the 14 regional nations are seen in the conclusions below. We have attempted to enumerate environmental and biological factors plus methodologies allowing various survival. Below, we point out gaps in the data. In these attempts to restore, investigators found a wide variety of results. This set of varying survival metrics (ranging from 0% to 83%) can be compared to van Katwijk et al.’s (2016) global study that found a global average of 37% overall survival for 1,738 investigations when combining small- and large-scale plantings. van Katwijk et al. (2016) attributed this apparently moderate

survival percentage to many small efforts that lowered survival rates compared to the larger-scale plantings. The general results for the greater Southeast Asian region demonstrate that some tropical seagrass species were more successfully restored, including *E. acoroides* near 40% and *T. hemprichii* at 49.3%, while other species demonstrated an overall lower survival rate (*C. rotundata* at 11%). For the subtropical/subtemperate regions, *Posidonia* had species survival differences with *P. coriacea* at 82.3% and *P. australis* at 63.4%, with other *Posidonia* species far lower. Our recommended techniques and species (discussed below), when well executed, should be useful in initial large-scale attempts to restore and mitigate such seagrass regional losses as reported by Fortes et al. (2018). The entire region is in urgent need of seagrass restoration from large-scale projects such as those executed by Verduin and Paling and their groups. These Australian large-scale plantings serve as examples to move forward in planning and execution for governments at multiple levels, philanthropic foundations, and NGOs throughout the region (Buelow et al., 2022). Large-scale plans, securing funding, and prodigious work should form the future of seagrass restoration in the greater Southeast Asian region.

The summary of the best survival results indicates the following: (1) species in tropical areas (*E. acoroides*, *T. hemprichii*, *Halophila* sp., and *H. uninervis*) and in subtemperate/subtropical regions like Australia (*P. coriacea* and *P. australis*); (2) planting by methods of sprigs or plugs; (3) planting at medium depth (2–4 m) in moderate-to low-energy areas of sufficient light, possibly with anchoring devices; (4) growth stimulants do not appear to be needed; and (5) site selection needs to be carefully carried out by a knowledgeable seagrass scientist to produce the best survival results.

Our hypothesis that seagrass restoration is viable for multiple seagrass species in the Southeast Asian region has been supported by these reviewed data.

Within this region, there are 14 nations, each with differing environmental assets, histories, legal systems, and government attitudes toward environmental conservation of coastal resources, resulting in differing environmental policies. Most of these nations have extensive seagrass resources. Natural resource management policies, regulations, and enforcement have created a patchwork of seagrass habitats with extensive seagrass loss (Ooi et al., 2011; Fortes et al., 2018), which needs enhancing. Restored seagrasses were found to be sustainable over many decades of continual growth in other parts of the world (van Katwijk et al., 2016; Nordlund et al., 2017; Thorhaug et al., 2020c; Seraphim et al., 2020; Kiswara, 2018; Kendrick et al., 2025).

4.2 Fundamental concepts and problems

This review suggests that the ecological and physical conditions can be managed for large-scale seagrass transplantation survival in the greater Southeast Asian region. The prime example of large-scale success is the approach applied in subtropical to subtemperate regions of western central Australia (Paling et al., 2007; Verduin et al., 2010; Verduin and Sinclair, 2013, and Kendrick et al., 2025). This Australian Indian Ocean study bears resemblance to large-scale projects that have

been carried out in other areas of the world in terms of survival results (60%–85%). These large-scale efforts by Verduin and Paling groups were built on a series of trials showing survival of species and methods that could be applied to a degraded estuary to restore a partial seagrass meadow, the area of which ameliorated the meadow's long-term absence post-degradation. The total efforts were monitored over 8 years (Verduin et al., 2025), demonstrating longevity in the species of restored seagrass there.

Generally, in the reviewed trials, many regionally diverse groups found higher success when sprigs were affixed to buried frames as the use of anchors or other materials. Our review demonstrated key environmental factors influencing the best survival with a combination of adequate light for photosynthesis and adequate depth to avoid uprooting. Approximate light level split into high, medium and low (defined under the table itself from surface light levels), (Kendrick et al., 2025) were higher survival.

A major problem encountered by most seagrass restoration regional practitioners included inadequate funding to carry out longer-term monitoring for survival and growth over multiple years. This included funds to obtain instrumentation to measure environmental factors such as light intensity, oxygen and dissolved carbon dioxide water content, and animal recolonization rates. They also lacked funds to monitor services provided by restored seagrasses over time scales.

A second important problem encountered was the lack of awareness of many governments to the importance of ecosystem services provided by seagrass as compared to those provided by coral reefs or mangroves. This led to governments not assessing and managing the social benefits of seagrass restoration to their citizens. It also created a low number of policies and/or regulations, leading to greater seagrass protection and enhancement.

A third problem with the review is that the restoration investigations were chiefly small-scale, not large-scale studies. This did not allow the type of large-scale process discussed in van Katwijk et al. (2016) to occur so as to influence the survival percentage of the total projects. In the van Katwijk review, the average survival of transplanted units was 37%, and in our review, the average survival was in this same range. The large-scale survival rate was far higher in both reviews. The van Katwijk review had many large-scale projects from multiple ocean basins, mainly the Atlantic Ocean, although our review had fewer than 20% of almost 40 studies.

The overlap in studies cited by both van Katwijk et al. (2016) and this review was chiefly some of the larger studies: Paling et al. (1998, 2001), Verduin et al. (2010), Thorhaug and Cruz, (1987, 1990), Calumpong et al. (1996), and Qui (2014). The repeated use of these studies is definitely important since they confirmed the feasibility of large-scale restoration and showed species tolerance to various types of degradation generally found regionally.

4.3 Different schools of thought or concepts

Most seagrass investigators agree on the Southeast Asian regional degradation (Todd et al., 2010; Ooi et al., 2011; Nakaoka

et al., 2014; Fortes et al., 2018; Thorhaug et al., 2020a, 2020b). Fortes et al. (2018) estimated that at least 50% of the original seagrass have been decimated from a variety of impacts. One school of thought is that degrading activities must be corrected first, prior to restoring seagrass. The second school of thought is that some level of the degrading effects can be physiologically tolerated by various dominant species of seagrasses at variable distances from the degradation source. Usually, these species' tolerances are ascertained after pilot testing, which has shown some dominant seagrass species having higher tolerances to diverse pollutants than others. [For example, this principle is long-established in the Atlantic tropics/subtropics with the dominant habitat species *Thalassia testudinum* and *Halodule wrightii* being far more tolerant of multiple degrading factors than *Syringodium filiforme* (Thorhaug et al., 1985; Thorhaug, 1985, 1987, 2001)]. This second school of thought is widespread among the "Restore America's Estuaries" group of 1,500 members and 300,000 volunteers in 800 scientific and government management projects, holding biannual meetings of thousands of restoration practitioners over the past 25 years, all working to restore coastal vegetation in damaged estuaries. The basic estuarine problems still remain, although effluents are sometimes reduced substantially. A number of investigations report on restoring specific types of areas of degradation. In the greater Southeast Asian region, survival in damaged estuaries and subsequent growth of seagrass have also been tested in the face of various types of degradation. A variety of seagrass species were tested in areas degraded by urban waste, dredge channels and artificial land fill, thermal effluents, mining wastes, and non-degraded controls. The focus was to find types of degraded habitats that could be restored by some species. Results clearly showed some dominant species (*E. acoroides*, *T. hemprichii*, *H. ovalis*, *H. uninervis*, *P. australis*, *P. sinuosa*, and *A. griffithii*) that tolerated a variety of impacts (in historical order, Thorhaug and Cruz, 1987, 1988; Calumpong et al., 1996; Paling et al., 1998, 2001a, 2001b, 2003; Verduin et al., 2010, 2024). That various seagrass species have a range of tolerance to degraded habitat has been shown in over a wide range of multiple seagrass restorations in the Atlantic, such as Chesapeake Bay (Orth et al., 2020), and in Atlantic tropical areas such as Jamaica (Thorhaug et al., 1985) and Biscayne Bay, Florida (Thorhaug and Hixon, 1975; Thorhaug, 1985), Texas coastal waters (Thorhaug, 2001; Thorhaug et al., 2020c), as well as in the Pacific, in Vancouver Bay, British Columbia, Canada (Durance, 2001).

4.4 Gaps and limitations in the studies reviewed

In the almost 40 studies and 288 trials reviewed, there were some notable gaps. First, many studies did not use two controls to ascertain seagrass transplantation survival: (1) naturally occurring seagrasses and (2) areas barren of seagrass to compare transplantation survival and growth. Both controls are essential to understand natural changes that occur over time in estuarine or coastal sites. Usually, the changes are discovered in time-sequential monitoring. Second, there was no

single monitoring methodology, especially for environmental data and for biological data on lateral growth and density of blades of transplanted seagrass. The data measured were dependent on the capability of the investigators, who frequently measured survival only, without a standard environmental monitoring protocol. This gap created difficulties in making statistical comparisons among trials.

Third, few investigators measured seagrass ecosystem services over the period of maturation of seagrass transplantation, including increased biodiversity (fish, invertebrates, reptiles, mammals, and, at some sites, endangered species), improved fish nursery habitat, sublittoral sediment stability, shoreline stability, water clarity, and organic carbon sequestration in sediment under the seagrass. Exceptions are studies on restored-seagrass services including Marbà et al. (2015) on sedimentary organic carbon under restored seagrass in western Australia and Ambo-Rappe (2022) on recolonization of invertebrates in restored *Enhalus* sites in northern Indonesia.

Fourth, planting attempts by the non-scientific community stand out as having very different results from plantings led by experienced seagrass scientists. Seagrass restorations at very shallow sites had already been demonstrated to be disrupted by wave energy compared to plantings deeper than 2 m in the 1,786 trials reviewed by van Katwijk et al. (2016).⁵ While we appreciate the PEA Thailand community groups' enthusiasm and concern for the environment, they failed completely. It is our opinion that community efforts should be led by someone with scientific seagrass experience. Data from community plantings created negative results due to their large numbers of PUs not surviving. This lowered the overall survival rate for that species and methodology. Our intuition tells us that community plantings were carried out in very shallow waters, because the groups thought that the depths for mangrove planting would also be appropriate for seagrass planting. Their methods may have followed previous community mangrove planting methods that included planting in dry intertidal areas or in very shallow depths where non-water-skilled community members would be comfortable working. These depths are known to produce very low survival areas for seagrass transplantation, clearly discussed in van Katwijk et al. (2016).

4.5 Recommendations for future seagrass restoration

To improve future success in larger-scale seagrass restoration, based on this review, recommendations for future investigations include the following: (1) Monitoring duration should last at least 3–4 years post-restoration; (2) monitoring should include key environmental factors (listed above), seagrass survival, lateral rhizomal growth expansion, blade length and density conducted across pre- and post-restoration sites, and the nearby naturally

⁵ We offer the caveat that seagrass restoration is more difficult than mangrove or marsh restoration and should not be thought of as using similar methods. Those experienced in seagrass restoration have repeatedly found that it does not have similar success rates as mangrove or marsh restoration.

occurring seagrass and control areas barren of seagrass; (3) it is optimal to measure animal communities for restoration studies including barren controls [McLaughlin et al. (1983) is an example]; (4) it is highly advisable for materials and funding to be made available to measure organic carbon in sediments at 5-, 10-, and 15-cm depths in restored seagrass at 1 and 2 years and after to build a global database on carbon sequestration associated with seagrass restoration; this can add into an “offset” revenue stream; (5) to prepare for large-scale projects, dominant species in each nation or general coastal region should be tested for tolerance limits to ambient degrading factors at the site such as urban wastes, dredging and filling, agricultural run-off, and other pollutants; and (6) large-scale projects should be funded and carefully managed, planned, and executed as demonstrations to governments, citizens, and organizations that may finance large-scale funding.

4.6 Final concepts

On a much broader scale, this review raises the question, “Can the maximum loss of 7% per year of seagrass ecosystems and their services, predicted by Waycott et al. (2009), be allowed socially (health-wise) and economically (for village employment for planting and enhanced fisheries) in the greater Southeast Asian region?” If not, should governments at the national and international scale as well as other philanthropic groups now begin to conserve and restore seagrasses in large scale?

For those who doubt restoration can catch up with degradation, an example of how rapidly a new technology can be spread throughout the greater Southeast Asian region is the increased mariculture of seaweed and its resulting employment and production in villages throughout Southeast Asia. The type of large-scale restoration we suggest will require national policies focusing on seagrass and coastal habitats to mitigate and restore seagrass. Funding from government agencies and other philanthropic sources as well as training courses are needed.

5 Conclusions

Southeast Asia is estimated to presently have 36,762.6 km² of seagrass (Fortes et al., 2018), and the greater Southeast Asian region includes additional seagrass extents if PNG, tropical to subtemperate west Australia, and south China are included. The largest global tropical seagrass region is found in this greater Southeast Asian region (Fortes et al., 2018). Minimal estimates are that 50% of the seagrass stock has been degraded over the last century (Ooi et al., 2011; Alongi et al., 2016; Fortes et al., 2018). These losses highlight the challenging need for seagrass restoration. In our review of 228 trials in almost 40 investigations, we found that seagrass was restored with 305,807 PUs, covering an extent of 372,649 m². Restoration investigations have led to survival in large restoration projects in central western Australia with smaller projects in Indonesia, the Philippines, Thailand, Malaysia, Vietnam, and tropical China (Figure 4). For nine tropical species, *E. acoroides* at 35% and *T. hemprichii* at 49.3% survived at higher levels

than the more moderate survival of *H. ovalis* and *H. uninervis*. For four subtemperate or subtropical species, high to medium survival occurred [*P. coriacea* (82.3%) and *P. australis* (63.4%) were the most successful]. Few seagrass services were reported from the restorations: (1) sedimentary carbon measurement under restored seagrass in west Australia (Marbà et al., 2015) and (2) recolonization of invertebrates in restored *Enhalus* sites in northern Indonesia (Ambo-Rappe, 2022). Other seagrass services were not included in the restoration reports we examined, so we cannot draw actionable conclusions. However, based on comparative data with the Atlantic subtropical and tropical zones, there are indications that restored seagrass meadows appear to be important for the return of lost ecosystem services. This must be fully investigated.

Author contributions

AT: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing. JV: Writing – review & editing, Data curation, Investigation, Methodology, Validation, Visualization. WK: Writing – review & editing, Data curation, Formal analysis, Investigation, Methodology, Visualization. AP: Writing – review & editing, Data curation, Methodology, Validation. XH: Writing – review & editing, Data curation, Formal analysis, Investigation, Methodology, Validation, Visualization. JG: Writing – review & editing, Formal analysis, Investigation, Methodology, Validation, Visualization, Conceptualization, Data curation, Writing – original draft. T-KY: Writing – review & editing, Conceptualization, Data curation, Investigation, Validation, Visualization, Methodology. RA-R: Writing – review & editing, Data curation, Investigation, Methodology, Resources, Validation, Visualization. SD: Writing – review & editing, Data curation, Formal analysis, Methodology, Validation, Visualization. AS: Writing – review & editing, Data curation, Investigation, Methodology, Validation, Visualization, Supervision, Writing – original draft. GB: Writing – review & editing, Data curation, Validation, Visualization, Methodology, Writing – original draft.

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Conflict of interest

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

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