Environmental Research 241 (2024) 117629

Contents lists available at ScienceDirect

Environmental Research

journal homepage: www.elsevier.com/locate/envres

Ocean acidification impairs seagrass performance under thermal stress in shallow and deep water

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ARTICLE INFO

Keywords:
Climate change
Light availability
Marine plants
Ocean acidification
Synergistic effects
Thermal stress

ABSTRACT

Despite the effects of ocean acidification (OA) on seagrasses have been widely investigated, predictions of seagrass performance under future climates need to consider multiple environmental factors. Here, we performed a mesocosm study to assess the effects of OA on shallow and deep *Posidonia oceanica* plants. The experiment was run in 2021 and repeated in 2022, a year characterized by a prolonged warm water event, to test how the effects of OA on plants are modulated by thermal stress. The response of *P. oceanica* to experimental conditions was investigated at different levels of biological organization. Under average seawater temperature, there were no effects of OA in both shallow and deep plants, indicating that *P. oceanica* is not limited by current inorganic carbon concentration, regardless of light availability. In contrast, under thermal stress, exposure of plants to OA increased lipid peroxidation and decreased photosynthetic performance, with deep plants displaying higher levels of heat stress, as indicated by the over-expression of stress-related genes and the activation of antioxidant systems. In addition, warming reduced plant growth, regardless of seawater CO_2 and light levels, suggesting that thermal stress may play a fundamental role in the future development of seagrass meadows. Our results suggest that OA may exacerbate the negative effects of future warming on seagrasses.

1. Introduction

Ocean acidification (OA), due to rising atmospheric CO_2, is projected to affect deep water to shallow coastal habitats (Linares et al., 2015; Luo et al., 2016; Nagelkerken and Connell, 2015; Sunday et al., 2017). Although the effects of climate change on marine ecosystems have been extensively investigated over the past few decades, the impact of OA on marine life is still debated (Connell and Leung, 2023; Cornwall et al., 2021; Doney et al., 2011; Genin et al., 2020; Kroeker et al., 2013; Nagelkerken and Connell, 2022; Wernberg et al., 2023). The responses of marine organisms to OA vary broadly, mostly due to differences in the investigated physiological traits and levels of biological organization, hindering predictions of their future performance in combination with other environmental factors (Calosi et al., 2017; Connell et al., 2018; Cornwall et al., 2020; Gao et al., 2012; Koch et al., 2013; Kroeker et al., 2017; Sunday et al., 2017; Zhang et al., 2023).

Seagrasses are commonly considered carbon-limited under current levels of $CO_{2(aq)}$ and an increment of dissolved inorganic carbon (DIC) under future OA scenario could enhance their photosynthetic efficiency, growth rate and biomass (Borum et al., 2016; Koch et al., 2013;

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https://doi.org/10.1016/j.envres.2023.117629
Received 9 August 2023; Received in revised form 21 October 2023; Accepted 7 November 2023
Available online 13 November 2023

Zimmerman, 2021). However, recent studies suggested that seagrass responses to OA are species-specific, depending on variations in their carbon concentration mechanisms and carbon allocation strategies (Borum et al., 2016; Cox et al., 2016; Guerrero-Meseguer et al., 2020; Hassoun et al., 2022; Maberly et al., 2022; Ow et al., 2015). Moreover, local environmental conditions (e.g., light and dissolved inorganic nutrient availability) and other climate-related stressors (e.g., ocean warming) may modify the magnitude and direction of their outcome in unpredictable ways (Alexandre et al., 2012; Collier et al., 2018; Martínez-Crego et al., 2014; Ow et al., 2016; Palacios SL, Zimmerman RC, 2007; Ravaglioli et al., 2017; Viana et al., 2023; Zayas-Santiago et al., 2020; Zhang et al., 2023; Zimmerman et al., 2017).

To date, our understanding of the effects of OA on seagrasses is based on studies performed in shallow habitats (see review by Koch et al., 2013; Zimmerman, 2021), neglecting the relatively wide bathymetric distribution of seagrass meadows (Duarte, 1991). This is at odds with the fact that both light and $CO_{2(aq)}$ availability are key drivers of plant photosynthesis and inorganic carbon acquisition (Celebi-Ergin et al., 2022; Hepburn et al., 2011; Hu et al., 2012; Zimmerman, 2021), suggesting that seagrass responses to OA could also vary with light regimes (Ow et al., 2016; Palacios SL, Zimmerman RC, 2007). Previous studies, testing for the effects of OA and light intensity on shallow plants, found positive effects of elevated $CO_{2(aq)}$ on plant photosynthesis at sub-saturating irradiance (Ow et al., 2016; Schneider et al., 2018). These findings suggested that low light intensity may increase plant reliance on $CO_{2(aq)}$ uptake due to the high energetic requirements of HCO_3^- use, which is overcome by the activation of extrusion proton pumps and external carbonic anhydrase for the conversion of HCO_3^- to CO_2 (Borum et al., 2016; Capó-Bauçà et al., 2022). However, plants from contrasting depths exhibit a wide range of variations, from genetic differentiation to changes in physiological and morphological traits, that can be related to local light conditions (Dattolo et al., 2014, 2017; Olivé et al., 2013; Procaccini et al., 2017; Ralph et al., 2006; Sharon et al., 2011). This may have led to an adaptive differentiation between shallow and deep plants of key metabolic processes, including carbon metabolism and photo-acclimation strategies, to maintain a positive carbon balance under different light environments (Dattolo et al., 2014; Procaccini et al., 2017). Nonetheless, to date, no study has tested how seagrass responses to OA differ according to variations in light availability that characterized shallow and deep habitats.

In addition, despite potential benefits for carbon acquisition, OA could alter other aspects of plant metabolism. Specifically, enhanced $CO_{2(aq)}$ may activate a series of early cellular responses in seagrasses, including the expression of antioxidant and macromolecule damage sensors, and genes coding for proteins that help to adjust cellular physiology and metabolism to avoid cell damage or death (Lauritano et al., 2015; Piro et al., 2020; Ruocco et al., 2017; Zhang et al., 2023). Such changes at the cellular level could affect resource allocation among biological processes, such as increased energy demand for repair and/or defense processes, potentially leading to shifts in plant performance under suboptimal environmental conditions. (Scartazza et al., 2017; Viana et al., 2023; Zhang et al., 2023). For example, thermal stress has been recently shown to outweigh the benefits (if any at all) of elevated $CO_{2(aq)}$ on different seagrass species, leading to a reduction in stored carbon, plant productivity and biomass (Collier et al., 2018; Hendriks et al., 2017; Repolho et al., 2017; Viana et al., 2023; Zhang et al., 2023). Indeed, seawater warming beyond the thermal optimum has been shown to cause plant metabolic impairment due to increased respiration and loss of fixed carbon, oxidative stress and protein degradation (Collier et al., 2017; Koch et al., 2013; Nguyen et al., 2021), potentially leading to the allocation of additional energy to physiological stress response pathways (Nguyen et al., 2021). Nonetheless, to date, there has been little effort to assess how underlying shifts in plant metabolism may drive the whole-plant tolerance to future climate conditions (Viana et al., 2023). Therefore, predictions of the effects of OA on seagrass meadows need to consider different levels of biological organization, from molecular to physiological and morphological levels, as well as appropriate ranges of light and seawater temperature.

Here, using a mesocosm study, we investigated how the response of the seagrass, *Posidonia oceanica*, to OA varied according to different light regimes that characterized shallow and deep meadows. The study was repeated over two years, which were characterized by average seawater temperature (2021) or by a prolonged anomalous warm water event (2022). This provided a unique opportunity to evaluate how a marine heatwave could influence the effects of OA and light availability on *P. oceanica*. We predicted that the effects of OA on *P. oceanica* would vary with light availability and seawater temperature. Specifically, low light conditions may increase seagrass reliance on $CO_{2(aq)}$ due to the higher energetic cost of using HCO_3^- as a carbon substrate for photosynthesis (Hu et al., 2012; Koch et al., 2013; Ow et al., 2016). Thus, under seawater temperatures that were unlikely to cause thermal stress, we hypothesized that enhanced $CO_{2(aq)}$ could provide the greatest benefit to deep plants, by enhancing their productivity and growth. Alternatively, OA may have limited effects on deep compared to shallow plants, if enhanced $CO_{2(aq)}$ availability would exceed the energetic capacity for its assimilation under low light conditions (Zimmerman, 2021). On the other hand, plants under thermal stress were expected to increase the respiratory loss of organic carbon, which would perturb energetic balances and, thus, activate stress response mechanisms (Koch et al., 2013; Marín-Guirao et al., 2016, 2017; Nguyen et al., 2021). This would enhance the allocation of additional energy to defense and/or repair processes (e.g., antioxidant capacity, protein turnover, carbon reserve translocation) expected under OA (Lauritano et al., 2015; Ruocco et al., 2017; Scartazza et al., 2017), reducing plant productivity and growth. The resulting impact could be predicted as more severe in plants from the deep-water meadows, since having evolved under smaller thermal variations and dim light conditions, might be more sensitive to thermal stress (Marín-Guirao et al., 2016, 2017).

2. Materials and methods

2.1. Seagrass collection and experimental treatments

The experiment was run twice at the seasonal peak of plant growth, between the end of May and the beginning of July 2021 and 2022. Satellite-derived sea surface temperature in the study area, the coast off Livorno (NW Mediterranean, 43° 28′ 45.005″ N, 10° 17′ 30.001″ E), indicated the occurrence of a prolonged warming event during May–August 2022 (Fig. 1), which was the hottest summer on record for Europe (https://climate.copernicus.eu/copernicus-2022-was-year-climate-extremes-record-high-temperatures-and-rising-concentrations). The average seawater temperature during the study period (±SE) was 22.2 ± 0.4 °C and 25.4 ± 0.2 °C for the 2021 (from May 28th to July 2nd, n = 36) and 2022 (from June 10th to July 11th, n = 32), respectively. Fragments of *Posidonia oceanica* rhizomes, bearing several vertical shoots (5–20), were collected by divers from well-preserved meadows located off the coast of Livorno, on May 28th, 2021 and June 10th, 2022. At both sampling dates, plant fragments were collected at two different depths (~5 m and ~20 m). Plant material was kept in dark coolers filled with ambient seawater and rapidly transported to the mesocosm facility located at the Livorno Aquarium.

Plant fragments of similar size and vertical shoot number were selected and individually attached to the bottom of eight plastic mesh trays filled with small cobbles (in 2021, 4 and 3 fragments for each tray, bearing 7–18 and 10–20 vertical shoots for shallow and deep conditions, respectively; in 2022, 5 fragments for each tray, bearing 5–14 vertical shoots for both depths). Two trays were then randomly placed in each of the four independent glass aquaria (500 L), with individual water recirculating and filtration systems. Each aquarium was filled with fresh filtered seawater which was thereafter exposed to continuous mechanical filtration and UV sterilization. Aquaria and filters were cleaned every two days to prevent algal blooms and a 50 % filtered seawater was

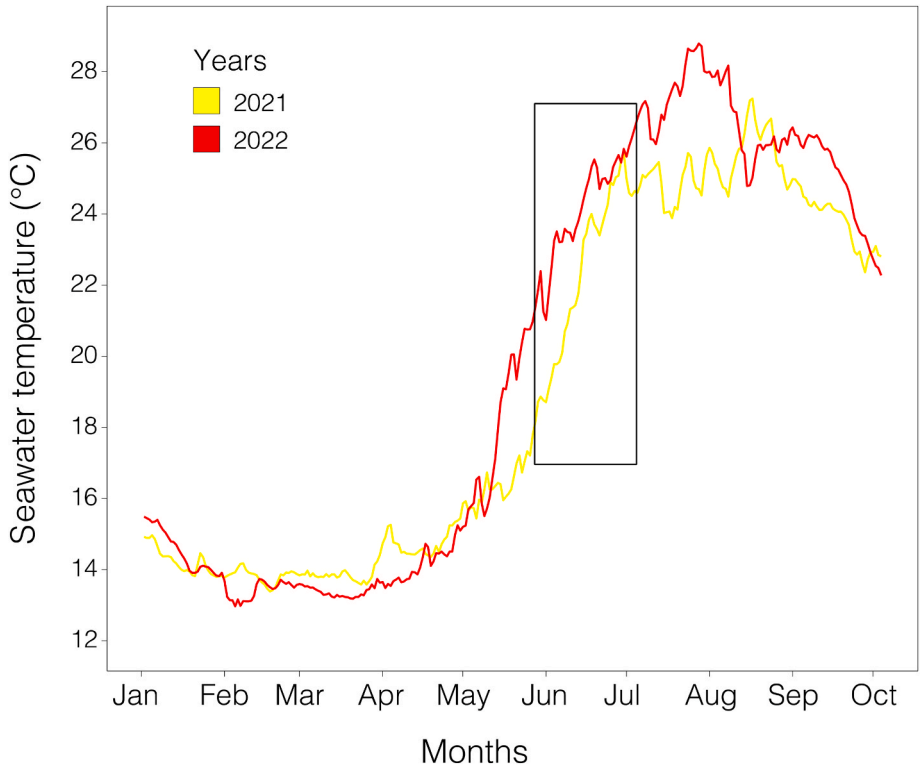

Fig. 1. Daily satellite-derived sea surface temperature at the study site, during January–October 2021 and 2022. Yellow and red lines indicate the satellite-derived sea surface temperature for 2021 and 2022, respectively. The rectangle shows the period of the experiments (from May 28th to July 2nd, 2021 and from June 10th to July 11th, 2022).

also renewed during the experiment runs.

Each aquarium was then randomly assigned to each of the four combinations of seawater [CO_2] (ambient vs. elevated CO_2) and depth (shallow vs. deep) treatments. Although we allocated 6–10 rhizomes bearing several vertical shoots for each tank, we could not have replicate tanks for each combination of [CO_2] and depth conditions. While aware of this shortcoming, the four 500 L mesocosm tanks were placed in a temperature-controlled room, lit by their own illumination system, without other external light sources, and filled in at the start of the experiment using the same source of sand-filtered and UV sterilized seawater, thus minimizing uncontrolled sources of variation among aquaria.

Each aquarium was equipped with three (two Silvermoon Reef Blu 895 and one Silvermoon Marine 895) and two (one Silvermoon Reef Blu 895 and one Silvermoon Marine 895) LED lamps for shallow and deep depth treatments, respectively. The lighting system was also set to simulate the diel fluctuation of light intensity. Irradiance levels in the experimental tanks were adjusted according to the origin of the plants with a peak irradiance of ~300 μmol photons m^{-2} s^{-1} above the canopy for the shallow treatment plants and ~100 μmol photons m^{-2} s^{-1} for deep treatment plants (Dattolo et al., 2014; Marín-Guirao et al., 2016), both with a photoperiod of 14:10 h (light:dark cycle). Seawater temperature was independently controlled in each tank by a chiller (Teco TK 500) and monitored using HOBO data loggers (Hobo pendant Temperature/Light, UA-002-64) along the course of the experiments. Seawater temperature was maintained at a constant level of 19.2 ± 0.01 °C and 22.7 ± 0.003 °C for the 2021 (n = 1459) and 2022 (n = 8695) experiments, respectively, according to observed field values during plant collection. Plants in all aquaria were acclimated for approximately ten days during both experiments (2021 and 2022) to mean prevailing environmental conditions of the study site (2021: temperature = ~19 °C, salinity = ~37.5, pH = ~8.19; 2022: temperature = ~22.5 °C, salinity = ~37, pH = ~8.15). Subsequently, $CO_{2(aq)}$ level was independently increased in the tanks assigned to elevated CO_2 treatment by bubbling the seawater in sumps with CO_2 gas. The pH levels in the tanks were monitored with pH electrodes as a proxy and control for CO_2 addition. The electrodes provided feedbacks to a control system that regulated the pH levels in each experimental tank by bubbling CO_2 gas into the sumps as required. The two CO_2 treatments were established to compare present-day values with those expected by the end of the century according to RCP 8.5, in which anthropogenic CO_2 emissions are highest and without specific climate mitigation strategies (Riahi et al., 2007). Plants were maintained under these experimental conditions for three weeks during both years. In 2021, pH was monitored several times a day throughout the experiment by using a portable pH meter (Hach HQ2100). In 2022, pH was continuously monitored using HOBO pH and temperature data loggers (Onset MX2501) along the course of the experiment. For both years, salinity was measured weekly using a conductivity meter and total alkalinity samples were collected five times from each aquarium throughout the experiment and measured using an automated potentiometric titration with a Methrom 848 Titrino plus system and applying the Gran method to calculate the concentration. Samples were filtered (Sartorius GFF 0.45 μm) and weighed (22.0000 ± 0.0100 g) prior to titration. The *TA* values were calculated in the pH range 3.3–3.8 according to the equation developed by Sass and Ben-Yaakov (1977). The *HCl* titrant (~0.05 mol/L) was calibrated before each session with a seawater Certified Reference Material from A. Dickson's laboratory (Dickson et al., 2003). Samples were measured in triplicates (internal) and the average precision was greater than ±2 μmol kg^{-1}.

Carbonate system variables were then calculated from measured pH, *TA*, temperature and salinity values using CO2SYS program for Excel with constant from Mehrbach et al. (1973) and adjusted by Dickson and Millero (1987). Unfortunately, for the second year, *TA* samples were lost during laboratory procedures and, thus, carbonate system variables were available only for the first year (summarized in Supplementary

Table S1). In particular, in the 2021, mean daily pH values (±SE, n = 377) in the shallow depth tanks were 8.27 ± 0.003 (pCO_2 = 333.53 ± 7.14 μatm, $CO_{2(aq)}$ = 10.77 ± 0.23 μmol kg^{-1}, HCO_3^- = 2019.68 ± 10.62 μmol kg^{-1}) and 7.80 ± 0.004 (pCO_2 = 1136.97 ± 24.05 μatm, $CO_{2(aq)}$ = 36.89 ± 0.77 μmol kg^{-1}, HCO_3^- = 2458.54 ± 7.68 μmol kg^{-1}) for ambient and elevated CO_2 treatments, respectively. In the deep-water treatment tanks, mean pH values (±SE, n = 377) were 8.20 ± 0.003 (pCO_2 = 409.62 ± 10.69 μatm, $CO_{2(aq)}$ = 13.31 ± 0.37 μmol kg^{-1}, HCO_3^- = 2066.31 ± 12.60 μmol kg^{-1}) and 7.70 ± 0.004 (pCO_2 = 1492.44 ± 44.6 μatm, $CO_{2(aq)}$ = 48.75 ± 1.48 μmol kg^{-1}, HCO_3^- = 2573.3 ± 9.73 μmol kg^{-1}) for ambient and elevated CO_2, respectively (Fig. S1). In the 2022, the average pH values (±SE) in the shallow depth treatment tanks were 8.36 ± 0.0003 and 7.78 ± 0.0001 for ambient (n = 17,194) and elevated (n = 25,850) CO_2 treatments, respectively, while those at the deep depth were 8.24 ± 0.0002 and 7.64 ± 0.0002 for ambient (n = 8695) and elevated (n = 25,850) CO_2, respectively (Fig. S2).

The response of both shallow and deep plants to OA was investigated at different levels of biological organization, by combining biochemical (oxidative stress levels and total antioxidant capacity) and physiological (photosynthetic efficiency and leaf content of photosynthetic pigments and secondary metabolites) analyses and plant growth. In addition, in the 2022 experiment, we also assessed the expression of photosynthesis- and stress-related genes (CAB151, CAT, HSP90, OZ_SP) in order to deepen our understanding of the mechanisms underpinning shallow and deep plant responses to combined OA and thermal stress. All the response variables were assessed on intermediate vertical shoots, while shoot-apical meristems were not selected in order to avoid intra-clonal differences in plant responses (Ruocco et al., 2021).

2.2. Gene expression analysis

Gene expression was analyzed for the 2022 experiment. For each aquarium, intermediate leaves were sampled on three randomly selected shoots of different rhizomes. Tissue from each leaf was then rapidly cleared of epiphytes with a razor blade, towel-dried and immediately stored in RNAlater® solution. Samples were then preserved one night at 4 °C and stored at −20 °C until RNA extraction. Total RNA from fully developed leaves of *P. oceanica* was extracted with the Aurum™ Total RNA Mini Kit (BIO-RAD, Hercules, CA, USA), following the manufacturer's protocol. A leaf tissue section of about 7 cm-long (corresponding to about 80–100 mg) was ground to a fine powder with a pestle in a mortar containing liquid nitrogen. Samples were then homogenized through a Mixer Mill MM300 (QIAGEN, Hilden, Germany) and tungsten carbide beads (3 mm) for 3 min at 20.1 Hz. The quality and purity of the total RNA were checked using NanoDrop (ND-1000 UV–Vis spectrophotometer; NanoDrop Technologies, Wilmington, DE, USA) and 1% agarose gel electrophoresis. RNA was used when Abs260 nm/Abs280 nm and Abs260 nm/Abs230 nm ratios were >1.8 and 1.8 < x < 2, respectively. The RNA concentration was accurately determined by the Qubit™ RNA BR Assay kit (Thermo Fisher Scientific, Waltham, MA USA) using the Qubit 2.0 Fluorometer (Thermo Fisher Scientific).

Total RNA (500 ng) from each sample was retro-transcribed into cDNA with the iScript™ cDNA synthesis kit (BIO-RAD), according to the manufacturer's protocol. As genes of interest, a Chlorophyll *a-b* binding protein (CAB151, Mazzuca et al., 2013) and three transcripts related to stress responses and detoxification processes (HSP90, Oz_SP, CAT), already tested in *P. oceanica* under natural seawater acidification conditions (Lauritano et al., 2015), were selected. To normalise target gene expression, we used as reference genes (RG) the 60s ribosomal protein L23a (L23) and the Ribosomal RNA 18S (18S), which have previously displayed a stable expression in *P. oceanica* under a range of different conditions (Lauritano et al., 2015; Serra et al., 2012).

RT-qPCR reactions were carried out as outlined in Lauritano et al. (2015). Briefly, each reaction consisted in 5 μl Fast SYBR® Green Master Mix (Applied Biosystems), 1 μl cDNA (1:5 diluted) template and 4 μl of 0.7 pmol μl^{-1} primers. The thermal profile of the reactions was as follows: 95 °C for 20 s, 40 times 95 °C for 1 s, and 60 °C for 20 s in the Viia7 Real Time PCR System (Applied Biosystem). After normalizing by each primer efficiency, the relative gene expression values were calculated as the negative differences in cycles to cross the threshold value (−ΔCT) between the reference genes and the respective target genes ($-\Delta CT = CT_{reference} - CT_{target}$). Fold expression changes were calculated as: Fold expression change = $\pm 2^{(|(-\Delta CT\ treatment) - (-\Delta CTcontrol)|)}$.

All RT-qPCR reactions were conducted in triplicate, and each assay included three no-template negative controls.

2.3. Lipid peroxidation and total antioxidant capacity determination

At the end of both experiments, six shoots of different rhizomes were randomly sampled in each aquarium for analyses of Lipid Peroxidation (LPO) and Total Antioxidant Capacity (TAC). For the first year, both LPO and TAC were assessed on 5 replicate shoots at deep-water/elevated CO_2 treatments and, for the second year, LPO was assessed on 3 shoots from the shallow-water/ambient CO_2 treatments, due to the loss of 1 and 3 samples, respectively, during laboratory procedures. Once in the laboratory, sample leaves were immediately frozen at −80 °C until biochemical analyses. All samples were carefully checked and specimens colonized by epiphytes were discarded. Malondialdehyde (MDA), a by-product of LPO, extraction and quantification were performed following the Costa et al. (2015) methodology. Briefly, ~300 mg of frozen leaf tissue was powdered and suspended in 5.0 mL of ethanol (80%). The extract was homogenized and then centrifuged at 3000×*g* for 10 min at 4 °C. The supernatant (1.0 mL) was added to 1.0 mL of 20% trichloroacetic acid (TCA) with 0.65% thiobarbituric acid (TBA) and 0.01% butylated hydroxytoluene (BHT) solution. Two blanks were used to verify the correct functioning of the test. All samples were first heated for 25 min at 90 °C, then cooled for 15 min and again centrifuged (3000×*g*, 4 °C, 10 min). The absorbance was measured at 440, 532 and 600 nm (BioTek Synergy HT micro-plate reader) and MDA equivalents were expressed in nmol MDA/g FW (Fresh Weight). The TAC was measured using ferric reducing antioxidant power (FRAP) assay, following the method described by Benzie and Strain (1996) and adapted by Capó et al. (2020). Powdered samples were homogenized in five volumes (w/v) of 50 mM Tris–HCl buffer, 1 mM EDTA, pH 7.5 using a small sample dispersing system (ULTRA-TURRAX®Disperser, IKA). Homogenates were centrifuged at 9000×*g*, 10 min, 4 °C. Then, 20 μL of homogenates were incubated for 30 min with a solution of ferric chloride (2 mM) and 2,4,6-Tris (2-pyridyl)-s-triazine (TPTZ) in acetate buffer pH 3.6. After incubation absorbance was measured at 593 nm and results were expressed as μmol FRAP/L.

2.4. Plant photosynthetic efficiency

In vivo chlorophyll fluorescence measurements were performed on *P. oceanica* leaves with a diving-PAM fluorometer (Walz, Germany). The effective quantum yield, which is an estimate of the photosynthetic efficiency of photosystem II (PSII) in light-adapted plants, was measured between 12:00 and 13:00 at the end of both experiments, by the saturating-light method on leaves. In the 2021 experiment, these measurements were performed on the second youngest leaf of the 8 and 6 shoots in each aquarium, for shallow and deep-water treatments, respectively. In the 2022 experiment, these measurements were performed on 10 different shoots for both depths. The effective quantum yield was expressed as ΔF/Fm' = Fm' – F/Fm', where F is the fluorescence yield of a leaf measured before the application of a saturating light pulse; Fm' is the maximum fluorescence yield induced by a saturating light pulse (Genty et al., 1989).

2.5. Photosynthetic pigments and secondary metabolites determination

At the end of both experiments, intermediate leaves of 6 shoots were

randomly collected from each aquarium to quantify the content of photosynthetic pigments, phenols and flavonoids. In the laboratory, leaves were washed, cleared of epiphytes using a razor blade and stored at − 80 °C until analysis. The frozen leaves were lyophilized and then ground for 15 s in a steel balls mill (Retsch GmbH & Co. KG, Haan, Germany) cooled with dry ice. Leaf powder was stored at − 30 °C until analyses.

The photosynthetic pigments were measured after their extraction from the powdered leaves. Briefly, 2 mg of leaf powder for each sample were extracted with 2 mL of acetone: Tris-HCl 0.5 M, pH 7.8 (80:20, v/v) on a magnetic stirrer for 30 min in a cold room (5 °C). The extract was centrifuged at 13,500 *g* and 5 °C for 10 min. The absorbances of the supernatant were read against a blank made with the extraction solvent at 430, 537, 647, and 663 nm, with a double beam Lambda 25 (PerkinElmer, Milan, Italy) spectrophotometer. The absorbance readings were converted into μmoles/ml following Sims and Gamon (2002) and finally expressed as μmoles/g DW. Each sample extraction was repeated five times.

Total phenols were extracted from the leaf powder following Bolser et al. (1998). An aliquot of 5 mg of leaf powder was extracted with 1 mL of cold 50% methanol in water, on a magnetic stirrer for about 16 h at 5 °C. The extracted total phenols were then centrifuged for 15 min at 13,500 *g* and 5 °C. Each extraction was performed in three replicates.

Polyphenols were assayed based on the Folin-Ciocalteu method (Singleton et al., 1999). Briefly, 100 μl of extract were brought to 625 μl with distilled water and added with 125 μl of Folin-Ciocalteu reagent (Sigma-Aldrich, Milan, Italy). After vortexing, the sample was kept in the dark for 6 min and then 1250 μl of 7% Na_2CO_3 and 1000 μl of distilled water were added. The sample was vortexed and kept in the dark for 90 min. The colored reaction product was read at 760 nm, against a blank reaction mixture, where water replaced the phenolic extract. The amount of total phenols was estimated from a calibration curve made with gallic acid and the results were expressed as mmoles of gallic acid equivalents per gram of DW.

The flavonoid assay was based on the colorimetric method described by Dewanto et al. (2002). Briefly, 200 μl of extract were brought to 1500 μl with distilled water and added with 75 μl of a 5% $NaNO_2$ solution. The mixture was vortexed and kept in the dark for 6 min; afterwards, 150 μl of a 10% $AlCl_3{\cdot}6H_2O$ solution were added. After vortexing and standing for another 5 min in the dark, 500 μl of 1 M NaOH and 275 μl of distilled water were added. The solution was vortexed again and its absorbance was measured immediately at 510 nm against a blank made by replacing the sample with an equal volume of distilled water. The amount of flavonoids was estimated from a calibration curve made with catechin and the results were expressed as mmoles of catechin equivalents per gram of DW. Each extract assay had three replicates for a total of nine replicates per sample.

2.6. Plant growth

Leaf growth was measured using a modified Zieman's method (Zieman, 1974), for both experiments. After the ~10 day acclimation period, shoots of different rhizomes were marked in each aquarium by punching all the leaves together with a needle, just above the ligula of the most external leaf. In the 2021 experiment, plant growth was measured in each aquarium on 8 and 6 replicate shoots for shallow and deep-water treatments, respectively, while, in the 2022 experiment, measurements were performed on 10 replicate shoots for each aquarium. Marked shoots were then collected after 23 and 19 days in the 2021 and 2022 experiments, respectively. In the laboratory, epiphytes were removed with a razor blade and leaf tissue of each shoot was divided into new material (*i.e.*, leaf tissue below the hole) and old material (*i.e.*, leaf tissue above the hole), dried at 60 °C for 24 h and weighed. Leaf growth rate (mg DW $shoot^{-1}$ day^{-1}) was estimated as the weight of new tissue produced divided by the time elapsed between the two sampling events.

2.7. Statistical analyses

The effects of seawater [CO_2], depth and year on biochemical (lipid peroxidation and total antioxidant capacity) and physiological (photosynthetic efficiency and leaf content of photosynthetic pigments and secondary metabolites) plant responses, and leaf growth rate were analyzed using generalized linear mixed models (GLMMs), assuming a gaussian distribution. The factors [CO_2] (ambient vs. elevated CO_2), depth (shallow vs. deep) and year (2021 vs. 2022) were included in the fixed part of the model as predictor variables, while tank was included as a random effect to account for the fact that replicate shoots were grouped within each tank. Expression levels of selected genes of interest (CAB-151, CAT, HPS90 and PZ-SP) were analyzed using GLMMs, including [CO_2] and depth as fixed factors, and tank as a random effect. The GLMMs were run using *glmmTMB* R package. Post-hoc comparisons were performed with R function *emmeans* for the significant interaction terms. Residuals were checked with Q-Q plots and plots of standardized residuals versus expected values, using *DHARMA* R package. The same package was used to assess heteroscedasticity and checked for overdispersion. All statistical analyses were performed in R version 3.6.1.

3. Results

3.1. Expression levels of the genes of interest

There was a significant interaction between [CO_2] and depth on the expression of the stress-related genes, HSP90 and CAT (Table 1). In particular, the heat shock protein gene HSP90 was significantly upregulated in deep plants exposed to elevated CO_2, while there was no change in HSP90 expression between CO_2 treatments in shallow plants (Fig. 2). At elevated CO_2, the antioxidant CAT was upregulated and downregulated in deep and shallow-water treatments, respectively, compared to those maintained at ambient CO_2, although *post hoc* comparisons between CO_2 treatments did not detect significant differences at any depth (Table 1, Fig. 2). Finally, although not statistically significant, both deep and shallow plants tended to increase the expression of the stress-related gene OZ_SP at elevated CO_2 (Table 1, Fig. 2).

Table 1
Results of Generalized Linear Mixed Models (GLMMs) used to assess the effects of seawater [CO_2] (ambient = ACO_2, elevated = ECO_2) and depth (shallow = Shall, deep = Deep) on − ΔCT of target genes at the end of the second experimental run. Coefficients and standard errors (SE) for [CO_2] and depth are reported for the fixed effects, while estimate of variance (σ^2) and standard deviations (SD) for tank are reported for the random effects. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

Effect	HSP90	OZ_SP	CAT	CAB
Fixed effects	***Estimate (SE)***	***Estimate (SE)***	***Estimate (SE)***	***Estimate (SE)***
Intercept	−3.74 (0.46) ***	−5.13 (0.77) ***	−2.76 (0.75) ***	−5.34 (0.80) ***
ECO_2	3.07 (0.64)***	2.03 (1.09)	2.18 (1.06)*	−0.53 (1.13)
Shall	−0.04 (0.64)	0.86 (1.09)	−0.83 (1.06)	0.30 (1.13)
ECO_2 x Shall	−3.27 (0.91) ***	−0.83 (1.54)	−3.49 (1.5)*	−0.37 (1.60)
Random effects	***σ^2 (SD)***	***σ^2 (SD)***	***σ^2 (SD)***	***σ^2 (SD)***
Tank	1.1e-10 (1.1e-05)	5.4e-10 (2.3e-05)	4.8e-10 (2.18e-05)	6.5e-10 (2.5e-05)
Residual	6.2e-01 (7.9e-01)	1.8 (1.3)	1.7 (1.3)	1.9 (1.4)
	Post-hoc contrasts		***Post-hoc contrasts***	
	HSP90 ([CO_2] x depth)		CAT ([CO_2] x depth)	
	Shall: ACO_2 = ECO_2		Shall: ACO_2 = ECO_2	
	Deep: ACO_2 < ECO_2**		Deep: ACO_2 = ECO_2	

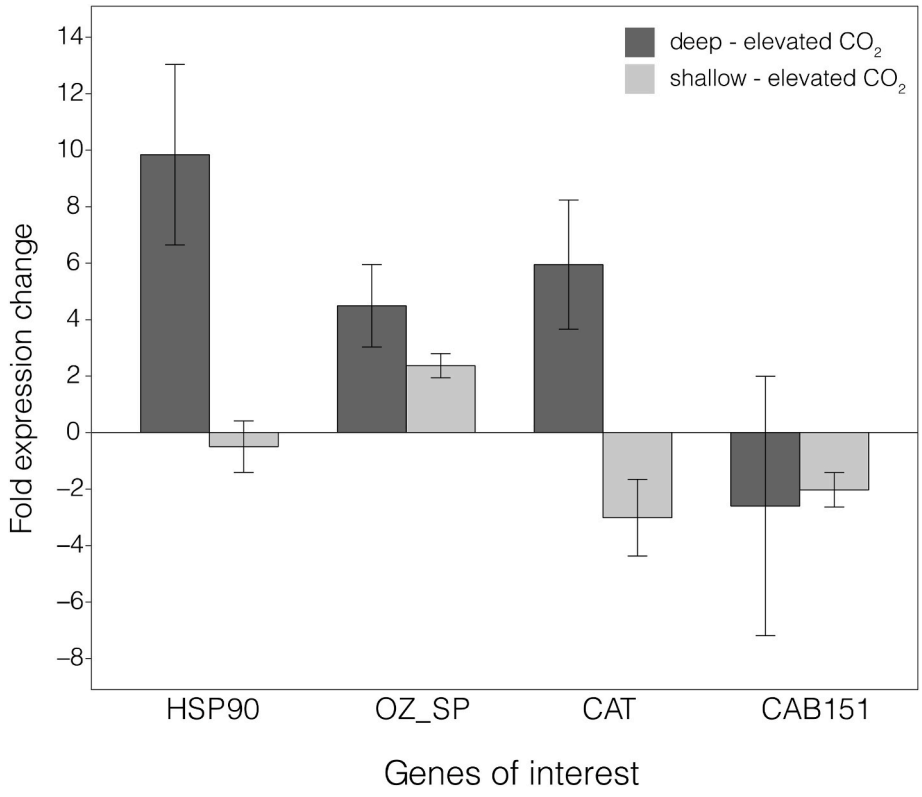

Fig. 2. Relative expression (ΔCT) of stress- and photosynthetic-related genes in deep depth– elevated CO_2, shallow depth – elevated CO_2 vs. control conditions (deep depth – ambient CO_2, shallow depth – ambient CO_2; x-axis) in the 2022 experiment (mean ± SE, n = 3).

The expression of the photosynthesis-related gene (CAB-151) did not show significant changes between CO_2 treatments at both depths, although there was a tendency for CAB expression to be down-regulated under elevated CO_2 for shallow plants (Table 1, Fig. 2).

3.2. Biochemical responses of *P. oceanica* to experimental treatments

There was a significant interaction among [CO_2], depth and year on the lipid peroxidation (LPO) of *P. oceanica* (Table 2). In 2021, there were no differences in LPO between ambient and CO_2 enriched treatments. In contrast, in 2022, which was conducted under warmer conditions, LPO was significantly higher under elevated than ambient CO_2 at both depths and this trend was more pronounced in shallow than in deep-water treatments (Fig. 3a).

There was a significant interaction between depth and year on the total antioxidant capacity (TAC) of *P. oceanica*, regardless of [CO_2]. In 2021, TAC was higher in shallow than in deep plants while, in 2022, TAC was greater in deep than in shallow plants (Table 2, Fig. 3b).

3.3. Physiological responses and leaf growth of *P. oceanica* under experimental treatments

There was a significant interaction between [CO_2] and year on the photosynthetic efficiency of *P. oceanica*, regardless of depth conditions (Table 3). In 2021, there were no differences in the effective quantum yield between CO_2 levels while, in 2022, elevated CO_2 significantly decreased the effective quantum yield (Fig. 4a). In addition, in both years, plant photosynthetic efficiency was significantly higher in deep than shallow-water treatments (Table 3, Fig. 4a).

There was a significant interaction between [CO_2] and year on leaf content of chlorophyll *a*, regardless of depth treatment (Table 3). In 2021, there were no differences in the chlorophyll *a* content between CO_2 treatments, while elevated CO_2 significantly decreased chlorophyll *a* concentration in 2022 (Fig. 4b). Although not statistically significant, the content of chlorophyll *b* and carotenoids tended to be lower at elevated than at ambient CO_2 in 2022 (Table 3, Fig. 4c and d) and were significantly higher in deep than shallow-water treatments in both years (Table 3, Fig. 4c and d). Although not statistically significant, there was also a tendency for chlorophyll *a* concentration to be higher in deep-

Table 2
Results of Generalized Linear Mixed Models (GLMMs) used to assess the effects of seawater [CO_2] (ambient = ACO_2, elevated = ECO_2), depth (shallow = Shall, deep = Deep) and year (Year1–2021, Year2–2022) on lipid peroxidation (LPO) and total antioxidant capacity (TAC). Coefficients and standard errors (SE) for [CO_2], depth and year are reported for the fixed effects, while estimate of variance (σ^2) and standard deviations (SD) for tank are reported for the random effects. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$. [I]Data sqrt (x+1) and [II]Data log (x+1) transformed.

Effect	Lipid peroxidation[II]	Total antioxidant capacity[I]
Fixed effects	***Estimate (SE)***	***Estimate (SE)***
Intercept	0.39 (0.09)***	4.84 (1.67)**
ECO_2	0.03 (0.13)	0.22 (2.48)
Shall	0.24 (0.12)*	9.49 (2.10)**
Year2	1.06 (0.12)***	18.07 (2.36)***
ECO_2 x Shall	−0.10 (0.18)	−2.22 (3.81)
Shall x Year2	−0.71 (0.19)***	−11.15 (3.74)**
ECO_2 x Year2	0.26 (0.18)	−0.09 (3.43)
ECO_2 x Shall x Year2	0.59 (0.26)*	−1.66 (5.07)
Random effects	***σ^2 (SD)***	***σ^2 (SD)***
Tank	1.1e-12 (1.02e-06)	1.2e-08 (0.0001)
Residual	4.6e-02 (2.14e-01)	1.7e+01 (4.0955)
	Post-hoc contrasts	***Post-hoc contrasts***
	([CO_2] x depth x year)	(depth x year)
	Year1 (Shall: $ACO_2 = ECO_2$)	Year1: Shall > Deep***
	Year1 (Deep: $ACO_2 = ECO_2$)	Year2: Shall < Deep*
	Year2 (Shall: $ACO_2 < ECO_2$) ***	
	Year2 (Deep: $ACO_2 < ECO_2$)*	

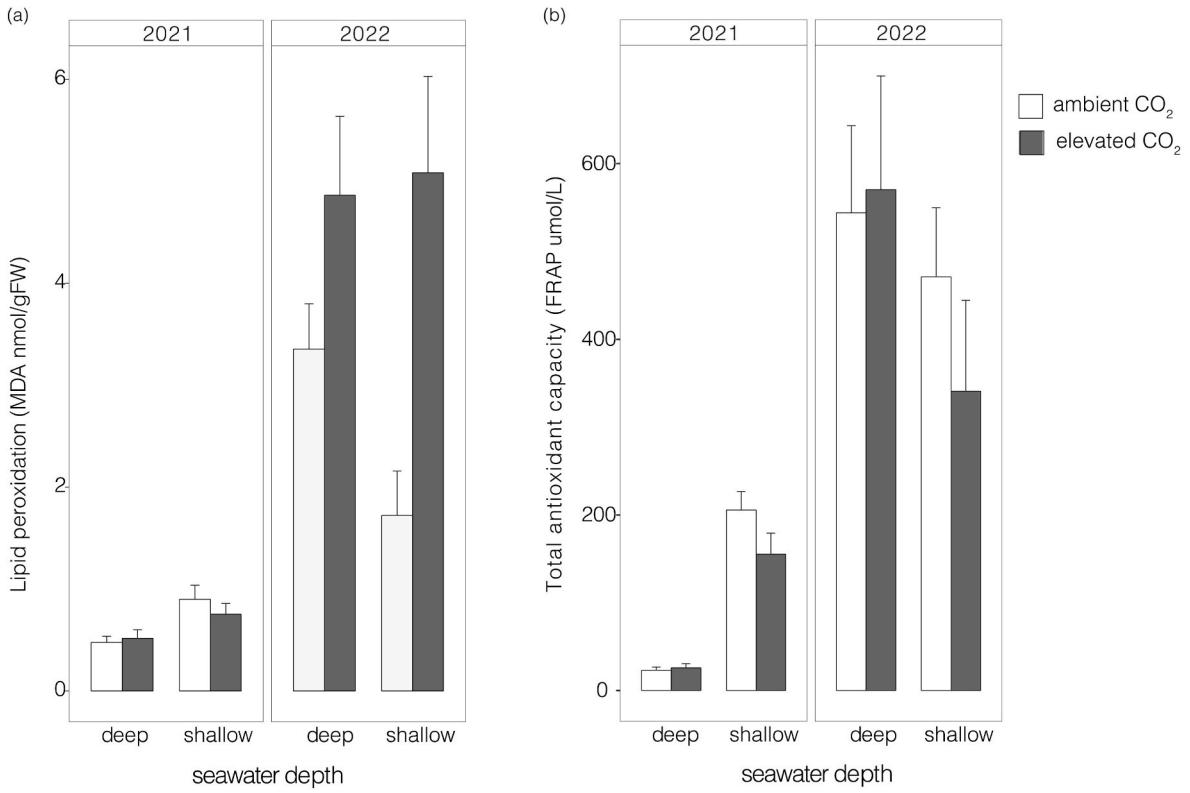

Fig. 3. Mean ± SE levels of (a) lipid peroxidation (MDA, nmol/gFW) and (b) total antioxidant capacity (FRAP, μmol/L) in shallow and deep *P. oceanica* plants under ambient and elevated CO_2 conditions, for both years (n = 6).

water treatments, in both years (Table 3, Fig. 4b).

There was a significant interaction between depth and year on flavonoid and phenol contents in *P. oceanica* leaves, regardless of CO_2 levels (Table 3). In 2021, leaf secondary metabolites were significantly lower in deep than in shallow-water treatments while, in the 2022 experiment, their concentrations were significantly higher in deep than in shallow-water treatments (Fig. 5).

Finally, leaf growth rate in 2022 was significantly lower than 2021, regardless of seawater [CO_2] and depth treatments (Table 4, Fig. 6).

4. Discussion

Thermal stress, due to a marine heatwave during the 2022 experiment, overwhelmed the effects of OA on plant productivity, suggesting that the predicted intensification of extreme heat events may reduce seagrass performance under future climate conditions. Under average seawater temperature conditions, which were unlikely to cause thermal stress, there were no effects of short-term elevation of $CO_{2(aq)}$ on plant performance at both depths. This may indicate that *P. oceanica* is not limited by current seawater inorganic carbon levels, regardless of light availability. In contrast, OA exacerbated thermal stress, causing an increase and a decrease of plant lipid peroxidation and photosynthetic performance, respectively, with deep (low light) plants having a stronger response to heat stress-induced damage (i.e., over-expression of heat shock proteins and activation of antioxidant system).

Seagrasses are generally thought to benefit from OA, as their photosynthetic rates are often C-limited under current DIC levels (Borum et al., 2016; Koch et al., 2013; Zimmerman, 2021). Indeed, despite most seagrass species are able to use HCO_3^- as an inorganic carbon source for photosynthesis (Capó-Bauçà et al., 2022), the processes required for its utilization are more energetically expensive compared to $CO_{2(aq)}$ use (Borum et al., 2016; Zimmerman, 2021). Nonetheless, previous field and mesocosm studies have reported contrasting results, with effects of elevated $CO_{2(aq)}$ on seagrass productivity and biomass ranging from positive to neutral, largely as a consequence of variations in their physiology and life-traits (Campbell and Fourqurean, 2013; Celebi-Ergin et al., 2022; Cox et al., 2015, 2016; Guerrero-Meseguer et al., 2020; Jiang et al., 2010; Maberly et al., 2022; Ow et al., 2015; Zimmerman, 2021).

Here, we found that the effects of elevated $CO_{2(aq)}$ levels on *P. oceanica* plants varied between the two years, which were characterized by markedly different seawater temperature conditions. Under average seawater temperature, during 2021, there were no effects of elevated $CO_{2(aq)}$ on the production and growth of both shallow and deep *P. oceanica*. This is in line with previous short-term mesocosm and field experiments showing no effects of the enhanced $CO_{2(aq)}$ level predicted by the end of the century on the photosynthetic capacity, leaf morphology and growth of shallow *P. oceanica* stands during different seasons (Agawin et al., 2021; Cox et al., 2015, 2016). Similarly, although previous evidence from CO_2 vents indicated that *P. oceanica* meadows may adapt well to future OA, their photosynthetic capacity and metabolism were similar at vents and ambient $CO_{2(aq)}$ sites (Berlinghof et al., 2022; Hall-Spencer et al., 2008; Scartazza et al., 2017). These findings, including our study, may reflect the ability of *P. oceanica* to use bicarbonate as carbon substrate for photosynthesis more efficiently than other seagrass species (Koch et al., 2013; Zimmerman, 2021).

In addition, OA could affect plant metabolism and cellular homeostasis, potentially increasing energy demand or impairing cellular function, as previously reported for different macrophytes (Kumar et al., 2017; Piro et al., 2020; Ruocco et al., 2017; Scartazza et al., 2017; Viana et al., 2023; Zhang et al., 2023). In our study, plants from both depths did not show any sign of cellular stress under OA and average temperature, as lipid peroxidation (as a signal of oxidative damage to cell membranes) and total antioxidant capacity (TAC) did not differ between ambient and elevated $CO_{2(aq)}$, regardless of depth. Such a response is in contrast with the recent findings of Zhang et al. (2023), who found a slight increase and a decrease in the activity of oxidoreductase enzymes and photosynthetic capacity, respectively, of the shallow tropical

Table 3
Results of Generalized Linear Mixed Models (GLMMs) used to assess the effects of seawater [CO_2] (ambient = ACO_2, elevated = ECO_2), depth (shallow = Shall, deep = Deep) and year (Year1–2021, Year2–2022) on the effective quantum yield and leaf content of photosynthetic pigments (chlorophyll *a, b* and carotenoids) and secondary metabolites (flavonoids and phenols). Coefficients and standard errors (SE) for [CO_2], depth and year are reported for the fixed effects, while estimates of variance (σ^2) and standard deviation (SD) per tank are reported for the random effects. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

Effect	Effective quantum yield	Chlorophyll *a*	Chlorophyll *b*
Fixed effects	***Estimate (SE)***	***Estimate (SE)***	***Estimate (SE)***
Intercept	0.64 (0.04)***	4.45 (0.24)***	1.97 (0.12)***
ECO_2	0.03 (0.06)	0.24 (0.34)	0.05 (0.15)
Shall	−0.19 (0.06)***	−0.58 (0.34)	−0.39 (0.15)**
Year2	0.06 (0.05)	−0.23 (0.34)	−0.10 (0.15)
ECO_2 x Shall	−0.01 (0.08)	−0.65 (0.47)	−0.18 (0.21)
Shall x Year2	0.06 (0.07)	−0.02 (0.49)	0.16 (0.21)
ECO_2 x Year2	−0.22 (0.07)**	−1.21 (0.49)*	0.27 (0.21)
ECO_2 x Shall x Year2	0.07 (0.10)	0.49 (0.68)	0.11 (0.29)
Random effects	***σ^2 (SD)***	***σ^2 (SD)***	***σ^2 (SD)***
Tank	1.9e-12 (1.4e-06)	5.6e-11 (7.5e-06)	6.7e-13 (8.2e-07)
Residual	1e-02 (1.02e-01)	3.6e-01 (6e-01)	6.6e-02 2 (.6e-01)
	Post-hoc contrasts	***Post-hoc contrasts***	
	([CO_2] x year)	([CO_2] x year)	
	Year1: ACO_2 = ECO_2	Year1: $ACO_2 = ECO_2$	
	Year2: ACO_2 >ECO_2***	Year2: ACO_2 > ECO_2***	

Effect	Carotenoids	Phenols	Flavonoids
Fixed effects	***Estimate (SE)***	***Estimate (SE)***	***Estimate (SE)***
Intercept	2.81 (0.14)***	43.73 (12.94)***	21.18 (8.64)*
ECO_2	0.03 (0.2)	−16.49 (18.31)	−8.55 (12.22)
Shall	−0.43 (0.2)*	156.55 (18.31)***	110.3 (12.22)***
Year2	−0.03 (0.2)	96.13 (18.31)***	74.85 (12.22)***
ECO_2 x Shall	−0.31 (0.28)	0.5 (25.05)	−10.47 (16.73)
Shall x Year2	0.04 (0.29)	−228.52 (25.88)***	−146.34 (17.28)***
ECO_2 x Year2	−0.28 (0.29)	4.14 (25.88)	2.44 (17.28)
ECO_2 x Shall x Year2	0.42 (0.4)	7.97 (36.02)	5.85 (24.05)
Random effects	***σ^2 (SD)***	***σ^2 (SD)***	***σ^2 (SD)***
Tank	9e-12 (3e-06)	6.9e-16 (8.3e-78)	3.2e-84 (1.8e-42)
Residual	1.2e-01 (3.5e-01)	1e+03 (3.17e+01)	4.5e+02 (2.1e+01)
		Post-hoc contrasts	***Post-hoc contrasts***
		(pH x year)	(pH x year)
		Year1: ACO_2 > ECO_2***	Year1: ACO_2 >ECO_2***
		Year2: ACO_2 < ECO_2***	Year2: ACO_2 <ECO_2***

seagrass, *Thalassia emprichii*, under enhanced $CO_{2(aq)}$. This contrast could be due to variations in energy and resource allocation strategies among seagrass species exposed to OA (Campbell and Fourqurean, 2013; Scartazza et al., 2017; Viana et al., 2023). For instance, previous studies showed that *P. oceanica* decreased and increased stored carbohydrates and nutrient acquisition processes, respectively, to sustain plant production under enhanced $CO_{2(aq)}$ (Agawin et al., 2021; Scartazza et al., 2017), whereas opposite strategies have been reported for other seagrass species (Campbell and Fourqurean, 2013; Egea et al., 2018; Viana et al., 2023). Further studies are, thus, needed to better understand the role played by the below-ground compartments in regulating the whole-plant response to future OA and how these mechanisms could vary among seagrass species characterized by different life-traits.

The lack of an effect of OA found in the deep-water treatment is in contrast with the general view that low light availability may increase plant reliance on $CO_{2(aq)}$ diffusion due to the high energetic requirement of HCO_3^- conversion, accumulation and use for photosynthesis (Koch et al., 2013; Ow et al., 2016; Zimmerman, 2021). Therefore, even species that are able to use HCO_3^- might be expected to positively respond to elevated $CO_{2(aq)}$ at limiting light levels (Koch et al., 2013; Ow et al., 2016). However, previous studies were based on shallow-water plants exposed to different light conditions (Ow et al., 2016), neglecting the fact that deep-water plants, developed under reduced light conditions, might have evolved different molecular and photo-physiological mechanisms to maintain a balance between light harvesting and cellular metabolism (Dattolo et al., 2014, 2017). Under these circumstances, any further increase in seawater CO_2 availability may exceed the energetic capacity for its assimilation (Zimmerman, 2021). To the best of our knowledge, no previous studies have experimentally tested the effects of OA on deep-water plants.

Regardless of $CO_{2(aq)}$ levels, photosynthetic pigment content and efficiency were higher in deep than in shallow *P. oceanica* plants. This may be an acclimation strategy to maintain a positive carbon balance and growth under low irradiance level, as previously reported for other seagrass species at their deeper distribution limits (Beca-Carretero et al., 2019; Sharon et al., 2011). In addition, under average seawater temperature, deep stands of *P. oceanica* did not show any sign of cellular stress, as indicated by lower levels of total antioxidant activity and secondary metabolites (*i.e.*, flavonoids and total phenols), suggesting a successful adaptation to the deep-water light conditions tested in this study. Our results are in agreement with those of Dattolo et al. (2014), who found that shallow *P. oceanica* plants (~5 m depth) activated specific defense mechanisms (e.g., antioxidant enzymes and xanthophyll-cycle related genes) to avoid photo-damage and maintain high photosynthetic performance, in contrast to deep plants (~25 m depth) that appeared to be photo-relaxed. However, a further decrease in light availability (40 μmol photons m^{-2} s^{-1}) has shown to negatively affect vertical shoots of *P. oceanica* at different levels of biological organization, suggesting a possible light shortage limit below which plants show severe stress responses (Ruocco et al., 2021). Moreover, it is worth noting that our results are obtained under laboratory settings in which the complex interactions that characterized natural systems were simplified. Indeed, local environmental conditions (e.g., dissolved inorganic nutrients), as well as biotic interactions (e.g., grazers and epiphytes) may modify deep-water plant responses to enhanced $CO_{2(aq)}$, as previously reported for shallow plants (Egea et al., 2018; Martínez-Crego et al., 2014, 2020; Ravaglioli et al., 2017; Rodríguez et al., 2022). Further experiments are, therefore, warranted to improve our ability to forecast the effects of OA on deep seagrass beds under different environmental conditions.

Importantly, plants from both depths, exposed to a prolonged anomalous warm water event (+3 °C during the period of plant growth) and maintained at increased $CO_{2(aq)}$ levels, showed a significant increase of lipid peroxidation and a tendency in the up-regulation of the stress-related gene (OZ-species), likely signs of physiological stress response pathways under the combination of both stressors. Thermal stress has been shown to cause plant metabolic impairment due to oxidative stress (e.g., accumulation of reactive oxygen species), alteration of membrane fluidity and protein structure (e.g., protein unfolding and/or degradation), and reduction of stored carbohydrates in different seagrass species, including *P. oceanica* (see review by Nguyen et al., 2021). In addition, seagrasses exposed to OA have been recently shown to increase the expression of antioxidant- and stress-related genes, indicating a cellular stress response of plants to elevated $CO_{2(aq)}$ (Lauritano et al., 2015; Piro et al., 2020; Ravaglioli et al., 2017; Ruocco et al., 2017; Zhang et al., 2023). This means that also plants, which are generally highly tolerant to OA (Hall-Spencer et al., 2008; Takahashi et al., 2016), can also be affected by elevated $CO_{2(aq)}$ during prolonged events of positive thermal anomalies. This is supported by recent findings showing that the cumulative effects of increased $CO_{2(aq)}$ and temperature may alter biochemical traits of tropical seagrasses, resulting in a decrease of below-ground stored carbon and an increase in the

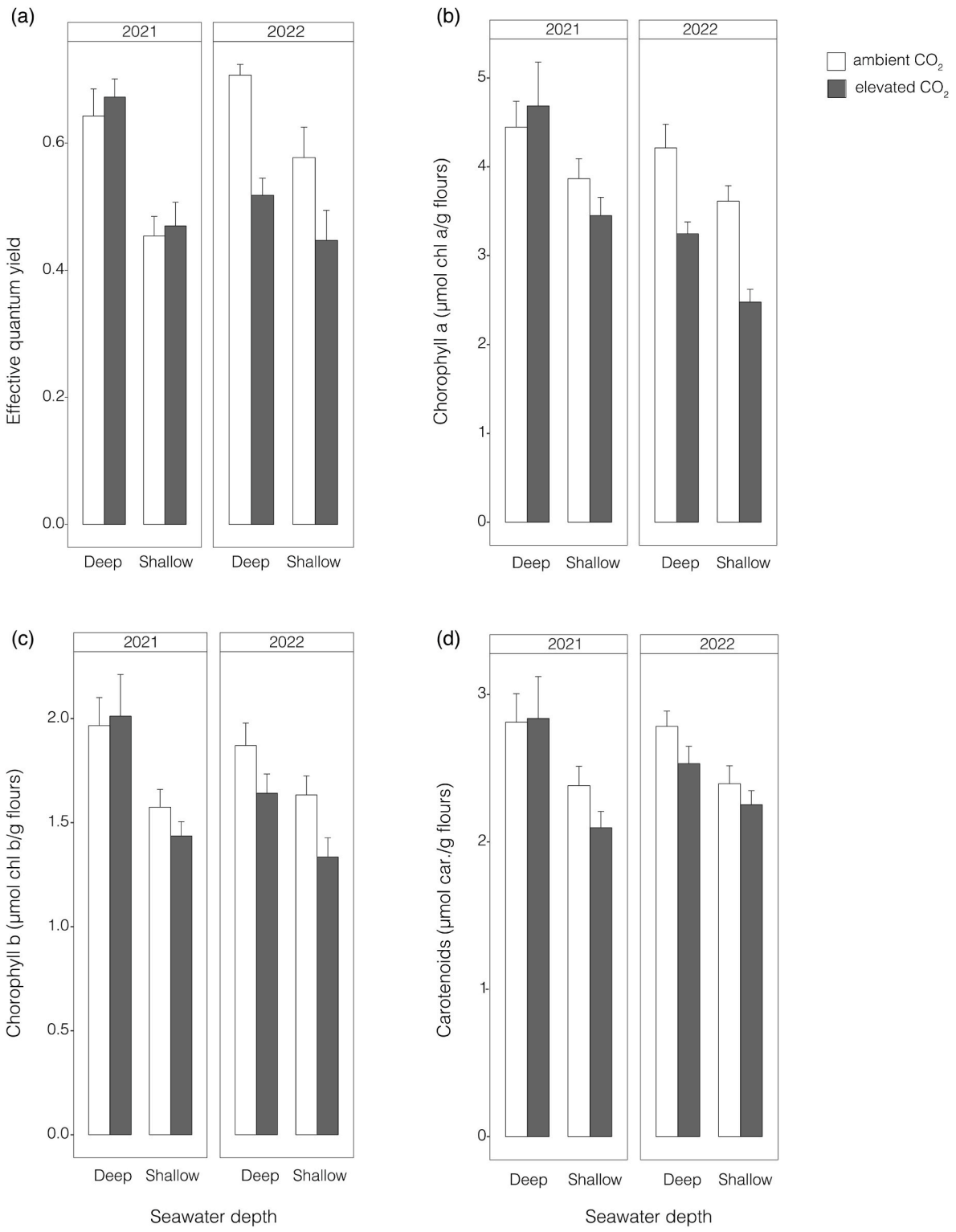

Fig. 4. (a) Photosynthetic efficiency (2021: n = 8 and n = 6 for shallow and deep plants, respectively; 2022: n = 10) and content (µg g^{-1}) of (b) chlorophyll *a*, (c) chlorophyll *b* and (d) carotenoids in leaves of shallow and deep plants under ambient and elevated CO_2 conditions, for the 2021 and 2022 experiments (n = 6). Values are mean ± SE.

activation of different antioxidant enzymes, which function as defensive mechanisms against oxidative stress (Viana et al., 2023; Zhang et al., 2023).

In addition, the combined effect of OA and seawater warming negatively affected the photosynthetic machinery performance of *P. oceanica*, by reducing plant photosynthetic efficiency and leaf pigment content, regardless of depth and, to some extent, the expression of the photosynthesis-related gene (CAB) in shallow plants. These general patterns underwent some variations in the molecular response of deep plants, probably due to a differential response of the target gene to low light regime and stressful conditions. Thermal stress has been shown to adversely affect the functioning of the photosynthetic apparatus of

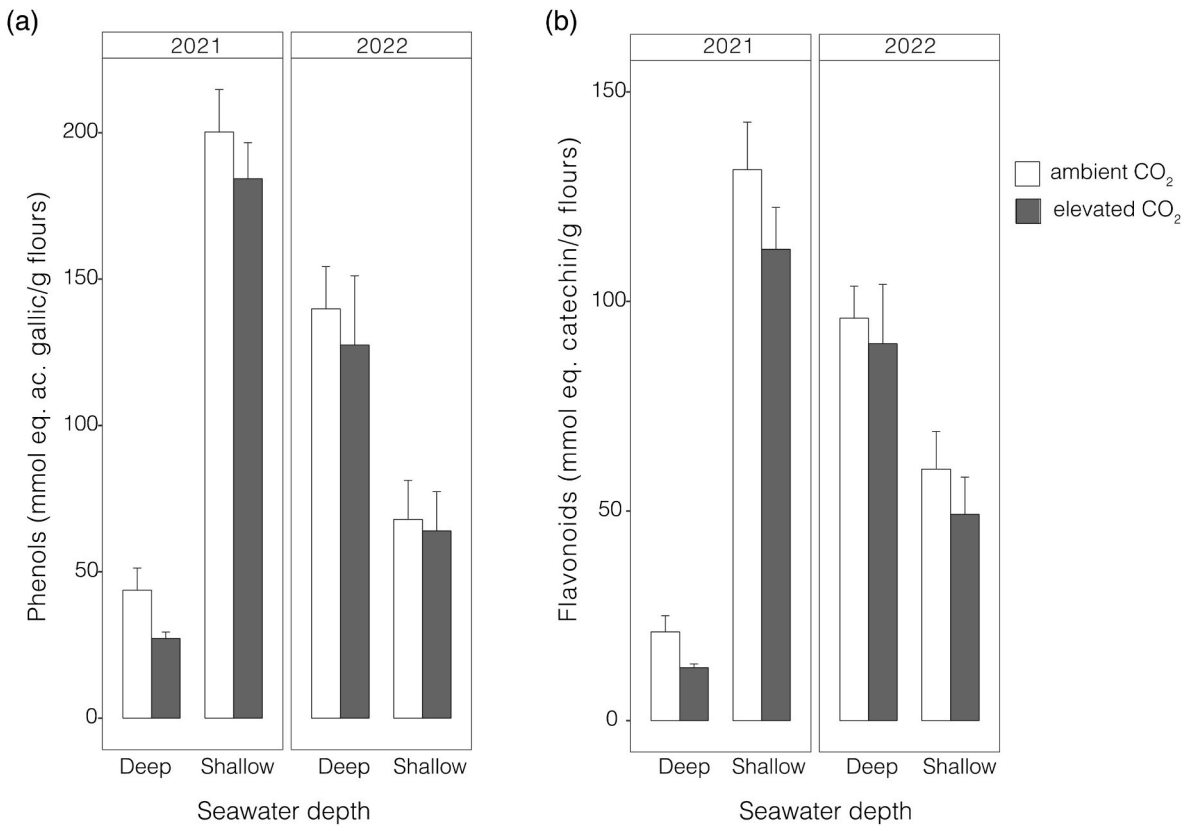

Fig. 5. Mean content of (a) phenols (mmol eq. ac. Gallic g^{-1}) and (b) flavonoids (mmol eq. catechin g^{-1}) in leaves of shallow and deep plants under ambient and elevated CO_2 conditions, for both years (n = 6).

Table 4
Results of Generalized Linear Mixed Models (GLMMs) used to assess the effects of seawater [CO_2] (ambient = ACO_2, elevated = ECO_2), depth (shallow = Shall, deep = Deep) and year (Year1–2021, Year2–2022) on plant growth rate. Coefficients and standard errors (SE) for [CO_2], depth and year are reported for the fixed effects, while estimates of variance (σ^2) and standard deviation (SD) per tank are reported for the random effects. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

Effect	Growth rate
Fixed effects	***Estimate (SE)***
Intercept	4.59 (0.58)***
ECO_2	0.45 (0.82)
Shall	−0.97 (0.77)
Year2	−1.57 (0.77)*
ECO_2 x Shall	0.71 (1.08)
Shall x Year2	1.44 (1.04)
ECO_2 x Year2	−0.32 (1.11)
ECO_2 x Shall x Year2	−1.14 (1.48)
Random effects	**σ^2 (SD)**
Tank	7.7e-10 (2.8e-05)
Residual	2 (1.4)

different seagrass species, through the damaging of PSII activity, inactivation of Rubisco and degradation of photosynthetic pigments (Marín-Guirao et al., 2016; Nguyen et al., 2021). On the other hand, OA could mitigate the negative effects of warming on plant productivity, by enhancing photosynthesis and reducing respiratory loss of fixed carbon (Celebi-Ergin et al., 2022; Egea et al., 2018; Rodríguez et al., 2022; Zayas-Santiago et al., 2020; Zimmerman et al., 2017). Indeed, the few studies that have experimentally tested the cumulative effects of these stressors on seagrass physiology have produced contrasting results, likely due to variations in seagrass responses to increasing $CO_{2(aq)}$

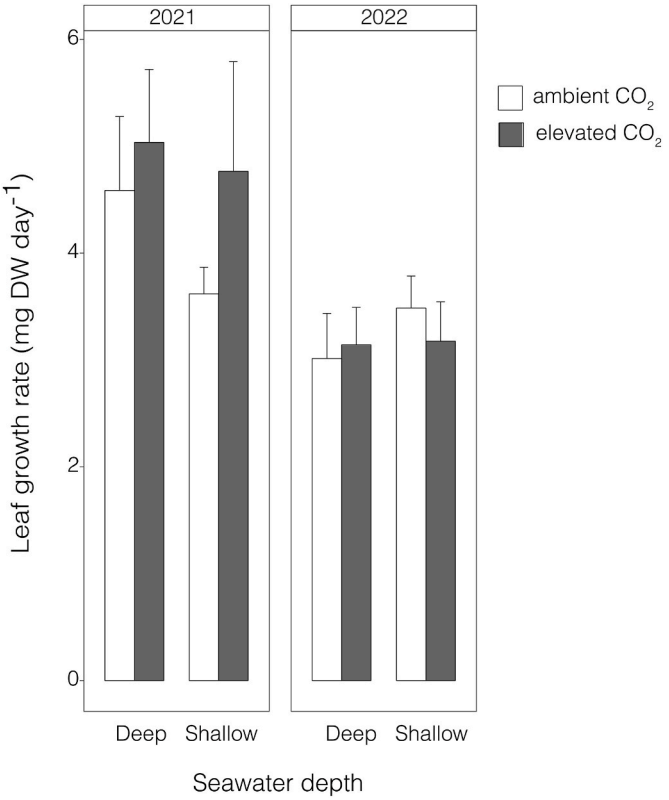

Fig. 6. Leaf growth (mg DW/shoot day, mean ± SE) of shallow and deep plants under ambient and elevated CO_2 conditions, for both years (2021: n = 8 and n = 6 for shallow and deep plants, respectively; 2022: n = 8).

(Collier et al., 2018; Egea et al., 2018; Repolho et al., 2017; Viana et al., 2023; Zhang et al., 2023). In particular, OA could be able to offset thermal stress in species that respond positively to elevated $CO_{2(aq)}$, enhancing light harvesting mechanisms and photosynthetic efficiency (Egea et al., 2018), but not in carbon-saturated species (Collier et al., 2018; Repolho et al., 2017; Viana et al., 2023; Zhang et al., 2023). Our results support previous findings of a limited stimulation of *P. oceanica* production under dissolved CO_2 concentration predicted by the end of the 21st century (Berlinghof et al., 2022; Cox et al., 2015, 2016) and bring new evidence that future OA could exacerbate the negative impacts of thermal stress on this species, as recently found for other tropical seagrasses (Collier et al., 2018; Zhang et al., 2023). The negative impacts of both stressors on *P. oceanica* may be the result of an increased energy allocation to repair and defense processes rather than productivity, due to metabolic impairment, as reported for other photosynthetic organisms under stressful conditions (Briggs and Carpenter, 2019; Gao et al., 2012).

We also found a reduction in the growth rate of *P. oceanica* in the warmer year (2022), regardless of light and CO_2 conditions. This is in line with previous mesocosm experiments that showed a decrease in the growth of *P. oceanica* plants exposed to a marine heatwave, probably due to an increased resource consumption to maintain plant metabolism and activate defense mechanism against heat damage (Marín-Guirao et al., 2018; Traboni et al., 2018). Nonetheless, while thermal stress decreased plant growth at both depths, the expression of heat shock proteins, such as HSP90, was greater in deep than shallow-water plants. Heat shock proteins, including HSP90, play a fundamental role as molecular chaperones to fix non-functional proteins or prevent their accumulation and they are commonly considered an early-warning response of plants exposed to warming and extreme heat events (Marín-Guirao et al., 2016; Nguyen et al., 2021). In agreement with our result, Marín-Guirao et al. (2016) reported a differential molecular response to heat stress among *P. oceanica* plants from contrasting depths (5 m vs. 25 m depths), with deep-water plants increasing the expression of heat shock proteins to much higher levels than shallow-water plants.

Thermal stress also increased the expression of the antioxidant CAT, the total antioxidant capacity (TAC) and the leaf content of secondary metabolites (flavonoids and total phenols) in deep plants, regardless of $CO_{2(aq)}$ levels. The stimulation of antioxidant systems indicated the activation of different defensive mechanisms in deep-water plants in response to stress, likely due to the production of reactive oxygen species (ROS), as previously reported for other aquatic and terrestrial plants exposed to thermal stress (De Silva and Asaeda, 2018; Ruiz-Montoya et al., 2021; Zhang et al., 2023). Likewise, the greater content of phenols and flavonoids in deep-water plants under thermal stress could indicate the stimulation of the antioxidant capacity. Indeed, secondary compounds can be involved in plant protection against a variety of abiotic and biotic stressors (Arnold et al., 2008; Brandt and Koch, 2003; De Silva and Asaeda, 2018; Mannino and Micheli, 2020; Ruiz-Montoya et al., 2021; Vergés et al., 2008) and their accumulation under temperature stress has been reported in different terrestrial plants (Commisso et al., 2016; Mierziak et al., 2014; Wahid, 2007), likely based on their ability to scavenge ROS production (Mierziak et al., 2014). These patterns observed at molecular, biochemical and physiological levels suggest that individuals of *P. oceanica* within the same population, but at different depths, might be thermally adapted to their local conditions (Dattolo et al., 2014; Procaccini et al., 2017), thus resulting in a differential tolerance to heat stress. Our results are in line with previous studies, showing that plants living in more stable and/or colder environments are less tolerant to temperature stress than those from more fluctuating and/or warmer habitats (Franssen et al., 2014; Marín-Guirao et al., 2016, 2018). It is worth noting that the greater variability among replicates observed at molecular and biochemical levels under thermal stress points out the need to better understand the complex interactions of multiple environmental conditions on plant cellular metabolism, taking into account also the role of below-ground compartments. In addition, although we allocated several shoots for each tank and measured plant response to experimental treatments at different levels of biological organization, there were no replicated tanks for each combination of CO_2 and depth conditions. Further experiments are, therefore, warranted to provide a more robust assessment on the mechanisms underpinning shallow- and deep-plant responses to future climate scenario.

5. Conclusion

Both shallow and deep *P. oceanica* plants appear to be unaffected by future OA, when not exposed to temperature stress, suggesting that their photosynthetic rates are not carbon-limited under current seawater CO_2 condition. In contrast, elevated $CO_{2(aq)}$ levels in combination with seawater warming reduced plant performance and productivity, likely by causing a metabolic impairment. The results of our study add to mounting evidence that elevated $CO_{2(aq)}$ concentration will not offset the negative impacts of temperature stress on seagrasses, at least for those species showing limited or no photosynthetic enhancement under elevated $CO_{2(aq)}$ (Collier et al., 2018; Cox et al., 2016; Repolho et al., 2017; Viana et al., 2023; Zhang et al., 2023).

It is important to note that the combined effects of elevated seawater temperature and $CO_{2(aq)}$ levels on *P. oceanica* meadows could vary according to the geographic range of this species, hindering predictions of its vulnerability to climate change. Previous mesocosm experiments showed that cold-adapted populations of *P. oceanica* were more severely affected by thermal stress compared to warm-adapted ones, generally resulting in a reduction of plant production and carbon reserves (Bennett et al., 2022b; Marín-Guirao et al., 2016, 2018). By contrast, recent field studies across the Mediterranean basin, showed that *P. oceanica* performance under thermal stress do not necessary reflect the thermal origin of the species (Bennett et al., 2022a) and populations living close to their warm edge of distribution can be also negatively affected by the fast seawater warming (Litsi-Mizan et al., 2023; Stipcich et al., 2022). Thus, further experiments of multiple populations across a temperature gradient are necessary to predict the response of *P. oceanica* to future climate change.

Finally, seagrasses rank among the most productive ecosystems on Earth, enhance the abundance and diversity of associated organisms (Hughes et al., 2009; Orth et al., 2006; Ralph et al., 2006) and could play a pivotal role in mitigating climate-driven loss of biodiversity (Bulleri et al., 2018; Falkenberg et al., 2021). Indeed, seagrass meadows, like other photosynthetic organisms, can modify seawater chemistry through their metabolic activities (Hendriks et al., 2014; Ricart et al., 2021; Unsworth et al., 2012), potentially providing climatic refugia for OA stress-sensitive species (e.g., calcifying organisms), by either temporally reducing $CO_{2(aq)}$ levels or enhancing the adaptive capacity of the threatened organisms (Falkenberg et al., 2021; Ravaglioli et al., 2024; Semesi et al., 2009; Wahl et al., 2018). Therefore, our results suggest that climate-driven changes in seagrass productivity may challenge the adaptability of associated organisms to elevated $CO_{2(aq)}$ and reduced CO_3^{-2} concentrations and the ability of seagrass meadows to act as future climate refugia. Our findings highlight the importance to raise awareness of the effects of OA on seagrass meadows in combination with other environmental stressors to tune up suitable climate-ready solutions and mitigation measures for preserving biodiversity and ecosystem functioning in the face of climate change.

Credit author statement

FB, CR; GP, ED conceptualization and methodology; CR, ED, LD, DF, MR, SA, JS, GR, CP, FB and GP data curation; CR formal analyses; CR writing – original draft; all the authors review & editing the draft; FB funding acquisition.

Declaration of competing interest

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

Data availability

Data are available on request will be made available on request.

Acknowledgements

We sincerely thank L. Pedicini for help with fieldwork, M. Butenschon for the satellite-derived sea surface temperature data at the study area and I. Olivé and L. Marin-Guirao for their comments and constructive criticism. The authors gratefully acknowledge financial support from the European Union's Horizon 2020 research and innovation program under grant agreement No 869300 FutureMARES. The work of GP and ED was partially supported by the project Marine Hazard (PON03PE_00203_1, MUR, Italian Ministry of University and Research) and by the European Union - NextGenerationEU (National Biodiversity Future Centre -NBFC- Program, MUR, PNRR, Missione 4 Componente 2 Investimento 1.4, Project: CN00000033).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envres.2023.117629.

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