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Effects of a simulated thermocline deepening on the biochemical composition of a Mediterranean seagrass

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ABSTRACT

Frequency and severity of marine heatwaves are progressively increasing worldwide due to climate change, causing the deepening of the lower limit of the thermocline, ultimately posing severe threats to habitat-forming and foundation marine species. Among these, seagrasses, especially those dwelling in the deepest part of the photic layer usually below the summer thermocline, like the Mediterranean endemic *Posidonia oceanica*, are at high risk.

We investigated whether and how the biochemical composition of *P. oceanica* leaves varies when the plant is exposed to a simulated thermocline deepening. To do this, we carried out a field manipulation experiment that aimed at investigating changes in protein, water-soluble carbohydrate and lipid contents of *P. oceanica* leaves, as a proxy for either short-term metabolic responses or the leaf fate along the trophic web.

The mechanical and handling stress caused by shoots translocation produced significant effects on almost all the investigated biochemical variables. However, the simulated deepening of the thermocline caused short-term metabolic responses that affected both the leaf biopolymeric composition and the C stored into the leaf biomass. Moreover, changes in the biochemical composition of the seagrass leaves also altered their potential availability to consumers (in terms of caloric content), also suggesting that the effects of the thermocline deepening on *P. oceanica* biochemical composition could extend much further than the metabolism of the seagrass itself, and, altering leaf C storage and caloric content, could exert consequences propagating through the food webs based on its leaves.

1. Introduction

Climate change has increased the likelihood of sea surface temperature (SST) anomalies, which, in turn, have promoted an increase in the duration of marine heatwaves (MHWs) by about 50% over the last century (Oliver et al., 2018; Sen Gupta et al., 2020). The frequency and severity of MHWs will continue surging under the ongoing global warming, with their depth of influence to progressively increase (Frölicher et al., 2018; Oliver et al., 2019, 2021; Juza et al., 2022; Sun et al., 2024; Köhn et al., 2024; Malan et al., 2025).

Extreme SST anomalies associated with marine heatwaves can cause severe impacts on marine ecosystems, including mass mortality events even in sub-superficial water layers (Coma et al., 2009; Garrabou et al., 2009, 2022; Thomson et al., 2015; Wernberg et al., 2016; Hughes et al., 2017; Elzahaby and Schaeffer, 2019; Smale et al., 2019; Goebeler et al., 2022; Bell et al., 2023; Smith et al., 2024). Motile species deepening and mortalities following MHWs track well the rising of heat risk exposure and the displacement in depth of the lower limit of the thermocline (Pinsky et al., 2013; Chu and Fan, 2020; Jordà et al., 2020; Chimienti et al., 2021; Wyatt et al., 2023). Indeed, a deepening of the ocean global thermocline has been observed during the recent decades, and its gradient is currently weakening at a rate of $-2.11 \pm 0.31 \times 10^{-3}$ °C m^{-1} yr^{-1} (Chu and Fan, 2020), exposing benthic communities adapted to colder temperature regimes to progressively warmer temperatures.

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Severe consequences of MHWs, coupled with modified thermocline dynamics, have been globally documented on marine habitats such as coral reefs in tropical areas (Wyatt et al., 2023) and coral and kelp forests at temperate latitudes (Arafeh-Dalmau et al., 2019; Chimienti et al., 2021). Nonetheless, since ecological responses to this phenomenon are likely to be species-specific, further studies are necessary to assess current effects of MHWs and foresee their overall consequences on the future oceans.

Seagrass meadows are among the most important marine habitats worldwide, providing important ecosystem services, such as sediment stabilization, nutrient cycling, carbon sequestration, coast protection from erosion (Duarte et al., 2005; Kennedy et al., 2010; Nordlund et al., 2016; Alongi, 2018; Pergent-Martini et al., 2021; Ascioti et al., 2022). All these services are, in turn, being progressively affected by sea warming and MHWs. For instance, MHWs simulated in mesocosm exerted differential effects on shallow and deep seagrasses, with the former resulting able to acclimate through respiratory homeostasis and activation of photo-protective mechanisms, and the latter experiencing photosynthetic injury and impaired carbon balance (Marín-Guirao et al., 2016). These results led to speculate that "deep" seagrass meadows (normally living below the summer thermocline) could be increasingly stressed, more than the shallower one, in areas most prone to the deepening of the thermocline. Nevertheless, a comprehensive understanding of the extension and the ecological implications of the thermocline deepening on seagrasses is still far from being fully achieved (Jordà et al., 2020). In the Mediterranean Sea, this holds particularly true for the endemic seagrass *Posidonia oceanica* (L.) Delile. Among the slowest growing and longest-lived marine plants (Arnaud-Haond et al., 2012), also *P. oceanica* provides fundamental ecosystem services like blue carbon sink, water oxygenation and biomass production, coastal protection, as well as refuge and breeding habitats for juveniles and adults of marine species of commercial interest (Boudouresque et al., 2009; Pergent-Martini et al., 2021; Campagne et al., 2014; Marbà et al., 2015,). *P. oceanica* is encountered down to 40 m of depth, with the deepest meadow boundary typically observed in the eastern Mediterranean basin and generally positioned well below the summer thermocline (on average ca. 25 m depth).

Due to the strong and seasonally persistent summer water stratification of the Mediterranean water column (Coma et al., 2009), a summer temperature gap of about 10 °C is generated across the top 40 m of the water column (Bennett et al., 2019). The bathymetric distribution of *P. oceanica* implies, therefore, that shallow and deep growing plants are physiologically exposed to different temperature regimes (Dattolo et al., 2014, 2017; Marín-Guirao et al., 2017): shallow plants experience warmer but also more fluctuant temperatures than the deeper ones, although also the latter can experience short periods of high temperatures, especially during the most extreme MHWs. Overall, prolonged temperatures above seagrass optimum can reduce *P. oceanica* photosynthetic rates and leaf productivity, at the same time enhancing respiration rates (Marín-Guirao et al., 2017). High temperature can cause also physiological (e.g. reduced leaf length and growth rate) and morphological (e.g. increased leaf necrosis and bleaching, and reduced leaf area, leaves/shoots number) responses, meadow regression and biomass loss (Boudouresque et al., 2009; Collier et al., 2017; Arias-Ortiz et al., 2018; Beca-Carretero et al., 2018; George et al., 2018; Marín-Guirao et al., 2018; Stipcich et al., 2022a, 2022b, 2025). However, in a field-simulated thermocline deepening, while an initial increase in leaf necrosis has been recorded, a subsequent recovery in leaf photosynthetic area was also detected (Stipcich et al., 2023a). The occurrence of thermal anomalies can also influence the biochemical composition of seagrass leaves, whose changes allow to detect signs of seagrass stress even when the plant morphology appears unaffected (Ceccherelli et al., 2018; Stipcich et al., 2022a). For instance, high temperature associated with MHWs can affect the protein, carbohydrate, saturated and polyunsaturated fatty acids contents, with an overall decrease in polyunsaturated fatty acids and an increase in saturated fatty acids (Beca-Carretero et al., 2018, 2020; Stipcich et al., 2022a, 2023b). Changes in biochemical composition in ecologically pivotal species such as *P. oceanica* can reflect the organism energy intake and storage rates (Parrish, 2009) and modify its nutritional quality, since the nutritional quality of any organic substrate depends on the quality and relative proportions of labile (e.g. proteins and lipids) vs. semi-labile (e.g. carbohydrates) compounds (Tan et al., 2022; Stipcich et al., 2023a). For instance, since the mechanical resistance provided by the seagrass leaf fibers can act as an effective anti-herbivore defense because less attractive for herbivores (de los Santos et al., 2012), sea warming, by modifying the leaf biochemical composition, could expose seagrasses to an enhanced grazing activity, ultimately impacting trophic interactions between producers and consumers (Buñuel et al., 2021).

To the best of our knowledge, whether and how the biochemical composition of *P. oceanica* leaves (in terms of protein, carbohydrate and lipid contents) changes when the plant is exposed to a thermocline deepening is still unknown. To provide insights about this gap of knowledge we carried out a field manipulation experiment that aimed at investigating the effects of a simulated thermocline deepening on the protein, water-soluble carbohydrate and lipid contents of *P. oceanica* leaves. At this aim, cuttings of *P. oceanica* collected from a deep meadow (−31 m) were transplanted at −12 m under a shading net, simulating the conditions of the light irradiance of the origin depth but higher temperature. Short-term changes (7–11 weeks) in the biochemical composition of transplanted cuttings below the shading net (D), simulating the thermocline deepening) were compared with those in deep untouched cuttings (F), deep cuttings translocated at the same depth of origin, exposed to the current thermocline and accounting for the potential effects of translocation (G), and with untouched cuttings at 12 m depth (A).

We hypothesized that the biochemical composition of plants exposed to a deepening thermocline would differ from that of deep-water plants in their natural conditions.

2. Materials and methods

2.1. Study area and experimental set up

The study was carried out in a dense *P. oceanica* meadow, extending from 10 to 31 m depth, located in Konnos Bay, Cyprus (34.98°N; 34.07°E), with the meadow deep front normally being positioned below the summer thermocline.

To simulate the thermocline deepening, six plagiotropic shoots (each with at least five orthotropic shoots) of *P. oceanica* were collected at −31 m (deep meadow) and transplanted at -12 m under a shading net, hence at higher temperature but at the same light irradiance of the depth of origin (D). Cuttings were fixed with cable ties to a metal grid, that was anchored to the substrate (dead matte) with metal pegs. The shading net was tied to a 1 m^2 PVC support, positioned about 60 cm above the bottom. During the whole experiment, the shading net was scrubbed by scuba divers on a weekly basis to remove epiphytes and avoid any sedimentation side effect. Another six plagiotropic shoots were collected from the deep (−31 m) meadow and translocated at the same depth of origin (G), to accounting for possible manipulation effects (Fig. 1). Additional plagiotropic shoots obtained from the untouched shallow meadow at 12 m depth (A) served as controls.

Temperature/light data loggers (HOBO Pendant, 64, Onset Computer Corporation, USA) were deployed in proximity of the experimental treatments to record hourly temperature and light irradiance. During the experiment (July–September 2023), temperature ranged from 24.44 to 29.33 °C at -12 m depth and from 19.39 to 28.87 °C at -31 m depth (Stipcich et al., 2023a). Temperature at -12 m was always higher than that at -31 m, with values below the shading net not significantly different from those in the unshaded meadow (Supplementary Fig. 1). The mean daily irradiance at -12 m was 12.83 ± 9.19 and 5.30 ± 3.38 μmol photons m^{-2} s^{-1} in unshaded and shaded conditions, respectively,

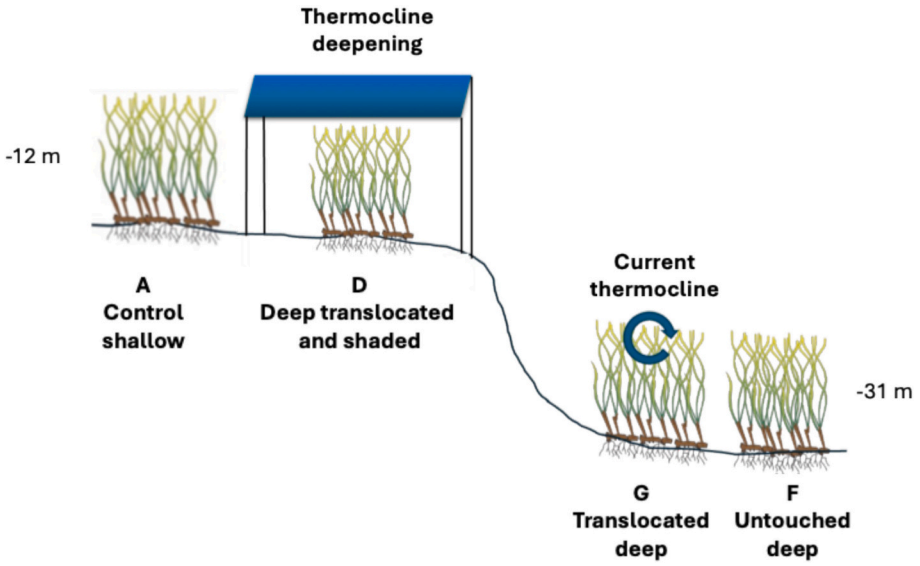

Fig. 1. *P. oceanica* shoots were collected from either a shallow (−12 m) and a deep (−31 m) meadow. Deep shoots were transplanted at −12 m under a shading net simulating the thermocline deepening (D), with the same light intensity as that at -31 m but higher temperature. Other deep shoots were translocated at the same depth (G) of their donor meadows (F, untouched), to serve as controls for manipulation. Shoots from the shallow meadow (at −12 m) were used as control of ongoing changes at the same depth as the shoots translocated to simulate the thermocline deepening.

whereas at -31 m depth it was 6.54 ± 4.79 μmol photons m^{-2} s^{-1} (Stipcich et al., 2023a).

To assess the temporal variation in the biochemical composition of the leaves, six orthotropic shoots were collected at the beginning of the experiment (T_0), after 7 (T_1) and 11 weeks (T_2) in each of the following treatments: control shallow (A), thermocline deepening (D, collected at -31 m and transplanted under the shading net at shallower depth); untouched deep (F); translocated deep (at the same depth of origin, G). The sampling in T_1 (after 7 weeks from the beginning of the experiment) was carried out since a very quick deepening of the thermocline occurred at the end of August (Supplementary Fig. 1). All samples were stored frozen (−20 °C) until analysis in the laboratory.

2.2. Samples preparation

Before the analyses, *P. oceanica* leaves were gently scraped with a clean scalpel to remove epiphytes and epibionts and then rinsed in distilled water to remove any residual epiphyte, sand, and salts, avoiding any damage to the leaf tissues. The cleaned leaves were cut up into small pieces, and the obtained fragments were mixed and grounded, so that the base and the tip of each leaf were uniformly distributed in the material used for the analysis. Cutting and grounding were performed manually maintaining the leaves in an ice bath to prevent heat damage.

2.3. Biochemical analyses and caloric content

Total protein and lipid contents were determined on aliquots (ca. 20–100 mg wet weight) of intact and healthy *P. oceanica* leaves, whereas carbohydrates were determined on water extracts of fresh leaves. The analysis of carbohydrates was limited to water extracts to avoid the detection of structural (dominant) carbohydrates, that have a limited trophic significance due to their mostly refractory nature (Velimirov, 1987).

Protein content, expressed in bovine albumin equivalents, was determined on ca. 20 mg (wet weight) of intact leaves, cut into small pieces with scissors, ground with a mortar and a pestle, maintained in an ice bath to prevent heat damage. The analysis was performed according to the colorimetric method described by Lowry et al. (1951), as modified by Hartree (1972) and Rice (1982) to compensate for phenol interference.

Water soluble carbohydrates were analyzed after extraction from about 50 mg (wet weight) of leaves cut into small pieces with scissors and added with 5 mL of reagent-grade water. The mixture was grounded and homogenized with a mortar and a pestle, while being maintained in an ice bath to prevent heat damage, alternated with 1-min cycles of ultrasonication until a supernatant was obtained. The mixture was then centrifuged (Eppendorf 5804, Eppendorf AG, Germany) at 800*g* for 15 min and 1 mL of the supernatant was used for the analysis. Carbohydrate concentrations, expressed in glucose equivalents, were determined according to the colorimetric assay by Gerchakov and Hatcher (1972) based on the phenol and concentrated sulfuric acid reaction with saccharides (Fabiano et al., 1995).

Total lipid content was determined on 100 mg (wet weight) of leaf cut in small pieces with scissors. Lipid extraction was performed according to Folch et al. (1957) by adding a chloroform-methanol mixture 2:1 v/v. Samples were added with 625 μL of chloroform and 1.25 mL of methanol and vortexed 1 min every 15 min for 1 h at room temperature. Successively, 1.9 mL of chloroform and 450 μL of a 0.2 M KCl solution were added to the mixture. After vortex (1 min) at room temperature samples were centrifuged for 10 min at 4000 rpm. The hydroalcoholic supernatant was eliminated and the remaining fraction, after evaporation in a dry hot bath at 80 to 100 °C for 20 min, was quantified according to the sulfuric acid carbonization procedure (Marsh and Weinstein, 1966). Total lipids were expressed in tripalmitin equivalents (Fabiano et al., 1995).

Protein, carbohydrate, and lipid concentrations were converted into C equivalents using the conversion factors 0.49, 0.40, and 0.75 mgC mg^{-1}, respectively, obtained from the C contents of the respective standard molecule (albumin, glucose and tripalmitin, respectively) and their sum reported as biopolymeric C (Fabiano et al., 1995).

The caloric content of the biopolymeric carbon of leaves was calculated using the following equation (Winberg, 1971; Pusceddu and Fabiano, 1996):

$$\text{kcal g}^{-1} = 0.041\cdot(\%\text{of carbohydrate}) + 0.055\cdot(\%\text{of protein}) + 0.095\cdot(\%\text{of lipid})$$

2.4. Effect size

To visualize and compare the magnitude of the temporal changes in

the biopolymers content of the leaves in each of the four treatments (shallow control, A; thermocline deepening, D; untouched deep, F; translocated deep, G), forest plot representations were prepared based on the effect size metric. This metric quantifies the results of an experimental treatment as the log-proportional change between the mean (X) of experimental treatment replicates (T) and their control (C) value, as follows:

$$R_i = \ln (XT_i/XC_i)$$

In this study, R_i is the log–response ratio for the variable *i* (protein, carbohydrate, lipid and biopolymeric C content), XT_i is the mean value of the variable *i* for each treatment measured at T_1 and T_2, and XTC_i is the mean values of the variable *i* at T_0 or T_1. Based on this metric, forest plots were prepared showing the magnitude and direction of changes in protein, water-soluble carbohydrate, lipid and biopolymeric C contents of *P. oceanica* leaves during the three intervals of time (T_1 vs T_0, T_2 vs T_1, T_2 vs T_0).

2.5. Statistical analyses

First, we assessed temporal variations in protein, water-soluble carbohydrate, lipid and biopolymeric C contents and caloric content of control shallow (A), thermocline deepening (D), untouched deep (F), and translocated deep (G) shoots using two-way ANOVAs, after having checked for assumptions of normality [Shapiro-Wilk test and quantile–quantile (Q–Q) plots] and homogeneity of variance (Levene's test). The analyses included two orthogonal factors: Time (T_0, T_1 and T_2) and Treatment (A, D, F and G). ANOVAs were performed in R (v4.1.3; R Core Team), using the packages *stats*, *car*, and *emmeans*. Since a significant effect of the interaction Time x Treatment was detected for all biopolymers and the caloric content (Supplementary Table 1), Tukey's post hoc comparison tests were also carried out to ascertain differences among treatments at all sampling times (Supporting Table 2A) and across sampling times for each treatment separately (Supplementary Table 2B).

Since the results of these tests provided evidence of generally significant temporal changes in biopolymers contents of *P. oceanica* leaves for all treatments, but translocated deep shoots (Supplementary Table 2B), we tested, also for representation purposes, the differences in the magnitude of temporal change (effect size) of each biopolymer across time intervals (T_0-T_1, T_1-T_2, T_0-T_2) using permutational analyses of variance (PERMANOVA). PERMANOVA is a semiparametric test based on the geometric partitioning of multivariate variation in the space of a chosen dissimilarity measure according to a given ANOVA design, with *p*-values obtained using appropriate distribution-free permutation techniques. Since PERMANOVA on one response variable using Euclidean distance yields the classical univariate F statistic, PERMANOVA can also be used to do univariate ANOVA, but where *p* values are obtained by permutation (Anderson and Millar, 2004), ultimately allowing not to respect the assumption of normality (Anderson, 2017). PERMANOVA tests in the univariate context were carried out on similarity matrixes based on the Euclidean distance of untransformed effect size data, using 9999 random permutations of the appropriate units. The two-way design included the factor "Treatment" (A, D, F, G) orthogonal to the factor "Time" (T_0-T_1, T_1-T_2, T_2–T_0) and their interaction. Pair-wise post-hoc comparisons were then carried out to ascertain, for each of the three intervals of time (T_1-T_0; T_2-T_1; T_2-T_0), the eventual significance of the following contrasts: thermocline deepening vs. translocated (D vs. G); thermocline deepening vs. untouched (D vs. F); thermocline deepening vs. shallow control (D vs. A); translocated vs. untouched (G vs. F).

Furthermore, variations in the biochemical composition (protein, water soluble carbohydrate and lipid C equivalents) of the seagrass leaves in response to translocation and simulated thermocline (translocated and shaded) were assessed using a two-way PERMANOVA on similarity matrixes based on Euclidean distance of normalized data using treatment (shallow control, thermocline deepening, untouched deep, translocated deep) and time (T_1, T_2) as orthogonal fixed factors, using 9999 random permutations of the appropriate units. Since the biochemical analyses on the seagrass leaves at T_0 were carried out on cuttings obtained from the donor meadow only, these data were excluded from the PERMANOVA. Prior to PERMANOVA, a distance-based test for homogeneity of multivariate dispersions (PERMDISP) revealed no significant data dispersions within and between treatments (Supplementary Fig. 2). Moreover, since the main test revealed a significant cross-interaction of the two factors, differences among treatments were investigated separately for each sampling time (T_1 and T_2) with post-hoc tests.

For both univariate and multivariate PERMANOVA analyses, percentages of explained variance by the two factors and their interaction were obtained from the estimated components of variation provided along with the results of the PERMANOVA main tests.

To visualize patterns of differences in the biochemical composition of the seagrass leaves among treatments and across sampling times, a biplot produced after a canonical analysis of principal coordinates (CAP) was prepared.

PERMANOVA and CAP were carried out using the software PRIMER 7+, using the embedded routine package PERMANOVA (Anderson, 2001).

3. Results

Protein, water-soluble carbohydrate, lipid, biopolymeric C and caloric contents were all affected by the Treatment x Time interaction (Supplementary Table 1). The following post-hoc tests revealed that differences among treatments at T_0 occurred only for the lipid content between control shallow leaves and all other treatments (Supplementary Table 2A). Differences between pairs of treatments became significant already at T_1 (though not for all variables) and become more significantly pronounced at T_2, with very few exceptions (Supplementary Table 2A, Supporting Fig. 3). Biopolymers and caloric contents of *P. oceanica* leaves from the different treatments showed variable temporal patterns, with changes that generally, but not consistently for all treatments, appeared already at T_1 and tended to stabilize at T_2 (Supporting Table 2B).

3.1. Magnitude of temporal changes across treatments

To assess and visualize the differences in the magnitude (and direction) of changes among treatments across the time intervals, we calculated the effect size metric. The results of the PERMANOVA tests carried out to ascertain differences among treatments in the magnitude of temporal changes across time intervals for all the investigated variables indicate a consistent effect of the interaction Time x Treatment (Table 1A). The following post-hoc tests revealed that the magnitude of change in all biopolymers varied significantly during the three temporal intervals between all pairs of treatments, with very few exceptions (Table 1B).

During the first seven weeks (T_1-T_0; Fig. 2, left panels) protein content decreased significantly only in control shallow leaves (−23%) whereas did not vary significantly in all other treatments, though slightly decreased in leaves exposed to thermocline deepening (−15%). Carbohydrate contents increased in all treatments, but the increase was significant only in translocated deep plants (+311%). Lipids increased significantly in control shallow plants (+41%) and decreased significantly in all other treatments (−25% on average). Biopolymeric C contents varied much less than the other variables and increased significantly only in translocated deep plants (+35%).

During the following 4 weeks (T_2-T_1) protein content increased significantly in either leaves exposed to the simulated thermocline deepening (+52%) or untouched deep leaves (+38%), whereas did not vary significantly in translocated deep leaves nor in coastal shallow

Table 1
Results of the main PERMANOVA (A) and consequent post-hoc pairwise comparisons (B) testing for significant differences in the temporal effect size among the four treatments (control shallow, A; shaded deep, D; untouched deep, F; translocated deep, G) in protein, water-soluble carbohydrate lipid, biopolymeric C contents of *P. oceanica* shoots, across the three time intervals (T_1 vs T_0, T_2 vs T_1, T_2 vs T_0). Reported are degrees of freedom (df), mean square (MS), Pseudo-F (for the main test), probability level after permutations (P-perm), number of unique permutations, probability level after Montecarlo simulations with 9999 permutations [P(MC)], t (for the post-hoc tests).
* = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$; ns = not significant.

A) Main PERMANOVA test

Variable	Source	df	MS	Pseudo-F	P(perm)	Unique permutations	P(MC)	% of explained variance
Protein	Time (Ti)	2	4.73	634.28	0.0001	9959	0.0001	27.0
	Treatment (Tr)	3	6.29	843.97	0.0001	9948	0.0001	47.9
	Ti × Tr	6	1.08	145.42	0.0001	9961	0.0001	24.6
	Residual	24	0.01					0.5
Carbohydrate	Time (Ti)	2	3.56	122.61	0.0001	9943	0.0001	18.6
	Treatment (Tr)	3	4.20	144.52	0.0001	9947	0.0001	29.2
	Ti × Tr	6	2.43	83.58	0.0001	9947	0.0001	50.4
	Residual	24	0.03					1.8
Lipid	Time (Ti)	2	1.87	32.04	0.0001	9949	0.0001	10.7
	Treatment (Tr)	3	7.79	133.28	0.0001	9958	0.0001	61.0
	Ti × Tr	6	1.08	18.44	0.0001	9954	0.0001	24.1
	Residual	24	0.06					4.1
Biopolymeric C	Time (Ti)	2	1.25	27.69	0.0001	9940	0.0001	5.7
	Treatment (Tr)	3	2.13	47.28	0.0001	9949	0.0001	13.2
	Ti × Tr	6	4.17	92.54	0.0001	9945	0.0001	78.5
	Residual	24	0.05					2.6

B) Post-hoc tests: among treatments

Time interval	Contrast	Protein		Carbohydrate		Lipid		Biopolymeric C	
		t	P(MC)	t	P(MC)	t	P(MC)	t	P(MC)
T_1-T_0	Control shallow vs thermocline deepening	10.696	***	7.571	**	6.093	**	3.154	*
	Control shallow vs untouched deep	12.797	***	1.428	ns	44.515	***	0.677	ns
	Control shallow vs translocated deep	32.465	***	12.869	***	6.954	**	11.379	***
	Thermocline deepening vs untouched deep	9.002	***	0.085	ns	0.852	ns	3.195	*
	Thermocline deepening vs translocated deep	21.665	***	16.480	***	0.391	ns	9.908	***
	Untouched deep vs translocated deep	2.703	ns	7.283	**	1.686	ns	22.736	***
T_2-T_1	Control shallow vs thermocline deepening	26.739	***	11.546	***	2.424	ns	19.030	***
	Control shallow vs untouched deep	49.655	***	23.673	***	40.448	***	0.350	ns
	Control shallow vs translocated deep	3.568	*	21.429	***	30.192	***	3.991	*
	Thermocline deepening vs untouched deep	5.743	**	22.590	***	0.749	ns	12.018	***
	Thermocline deepening vs translocated deep	13.286	***	21.214	***	2.036	ns	12.076	***
	Untouched deep vs translocated deep	11.416	***	4.194	*	18.230	***	3.181	*
T_2-T_0	Control shallow vs thermocline deepening	54.230	***	5.809	**	38.470	***	15.802	***
	Control shallow vs untouched deep	67.746	***	12.027	***	40.909	***	3.119	*
	Control shallow vs translocated deep	43.962	***	2.805	*	38.016	***	0.281	ns
	Thermocline deepening vs untouched deep	14.993	***	18.432	***	7.168	**	18.225	***
	Thermocline deepening vs translocated deep	31.431	***	3.109	*	0.419	ns	15.729	***
	Untouched deep vs translocated deep	51.016	***	15.596	***	7.484	**	3.566	*

leaves. The significant increase in carbohydrate contents in leaves exposed to the simulated thermocline deepening (+211%) was 4 times higher than that in coastal shallow leaves (+43%), whereas in both untouched and translocated deep leaves the carbohydrate content decreased significantly (−34% and − 25%, respectively), though changes in these treatments were not significant. Lipid contents in coastal shallow leaves did not vary significantly (−0.1%) nor in untouched deep leaves (−13%), whereas decreased in both laves exposed to the thermocline deepening (−25%) and translocated deep leaves (−32%). Biopolymeric C of leaves increased significantly only in leaves exposed to the simulated thermocline (+63%).

Considering the entire 11 weeks of the experiment (T_2-T_0) the protein content of control shallow leaves decreased (−25%), whereas increased significantly in both leaves exposed to the thermocline deepening and the untouched deep leaves (+ 29% and + 49, respectively). Carbohydrate contents generally increased in all treatments but significantly only in leaves exposed to thermocline deepening (+320%) and translocated deep leaves (+209%), whereas lipids decreased significantly in all treatments, except for control shallow leaves, by similar rates (−46%, −36%, −47% in leaves exposed to thermocline deepening, untouched and translocated deep leaves, respectively). Biopolymeric C contents varied much less than its constituents, with a significant increased only in leaves exposed to thermocline deepening.

3.2. Changes in the biochemical composition and caloric content of the seagrass leaves

Treatments exerted significant effects on the biochemical composition of the seagrass leaves depending on time, with the latter explaining most (41.8%) of the model variance (Table 2A). The following post-hoc tests revealed that at T_0 the shallow leaves were characterized by a biochemical composition significantly different from that of the deep ones, leading to a biopolymeric content in deep leaves significantly higher (by ca. 36%) than that in shallow ones (Supplementary Fig. 4).

At T_1 the biochemical composition of the leaves differed significantly among all treatments, with exceptions between leaves exposed to the thermocline deepening and control shallow and between untouched deep leaves and those exposed to the thermocline deepening (Table 2B). At T2, significant differences in the biochemical composition of the leaves occurred between all treatments but between leaves exposed to the thermocline deepening exposed and translocated deep leaves (Table 2B).

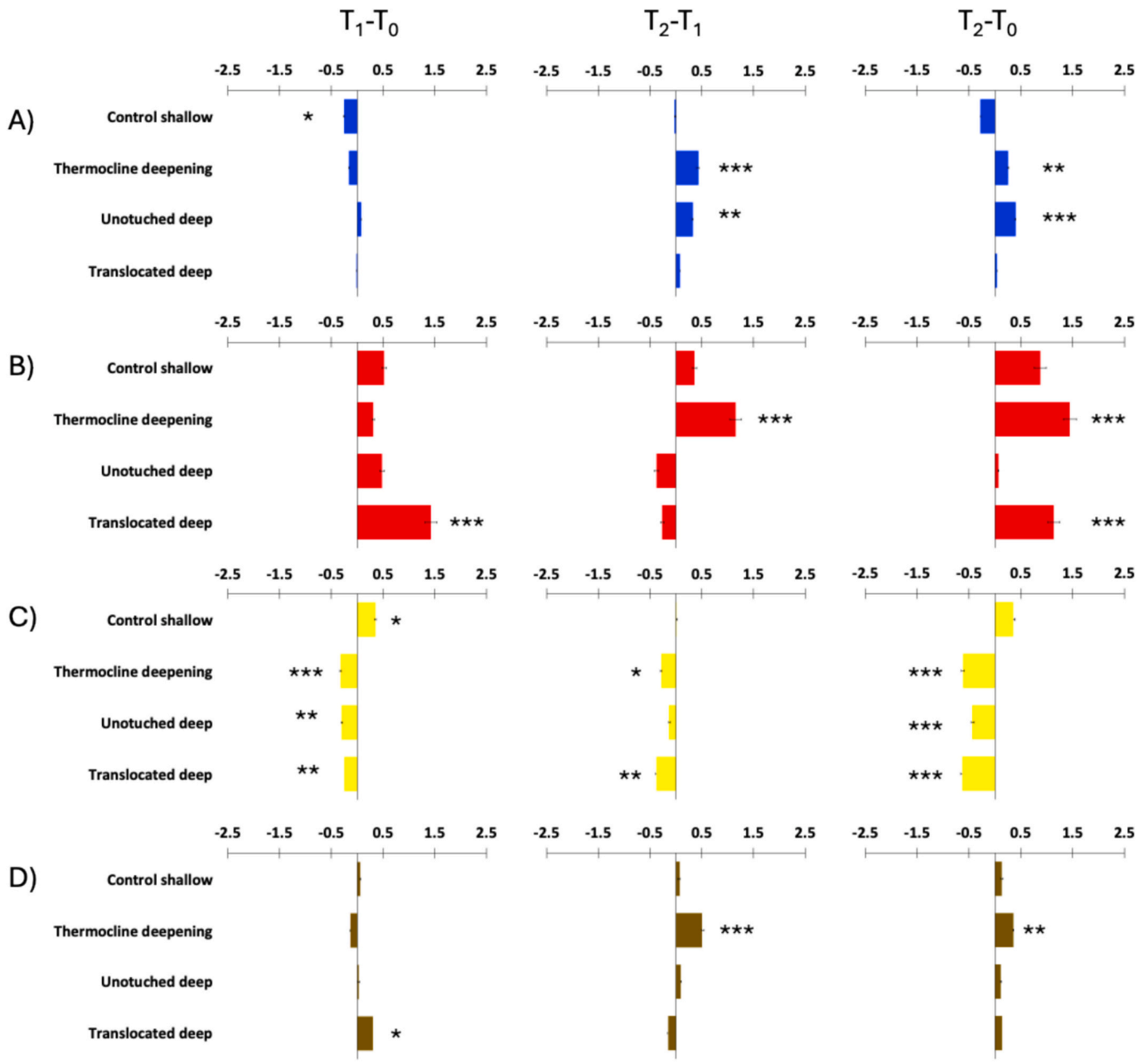

Fig. 2. Temporal effect size in protein, carbohydrate, lipid and biopolymeric C contents of shallow control shallow (A), transplanted and shaded deep (D), untouched deep (F) and translocated deep (G) *P. oceanica* cuttings in the short (T1 vs. T0), intermediate (T2 vs. T1) and long (T2 vs. T0) term. The positive bars denote an increase in the content of the selected variable in the leaves, whereas the negative ones denote a decrease. Asterisks identify significant differences in biopolymers content between times as reported in Supplementary Table 2B. * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$: Unmarked bars indicate not significant changes.

The results of the multivariate post-hoc pairwise tests are mirrored in the biplot produced after the CAP, in which temporal patterns of change are characterized by a general shift in the leaf biochemical composition from T_0 to T_1 and T_2 in all experimental. Overall, changes in control shallow leaves are much more limited than those observed in all other treatments and move in opposite direction (Fig. 3).

At T_1 the carbohydrate fraction of biopolymeric C increased in all treatments, whereas the lipid one increased in control shallow leaves and decreased in all other treatments (Fig. 4). The protein fraction decreased in the control shallow and translocated deep leaves, whereas remained nearly constant in both thermocline deepening-exposed and untouched deep leaves (Fig. 4). At T_2 leaves exposed to the thermocline deepening showed an increase in both the carbohydrate and protein fractions of biopolymeric carbon and a further decrease of the lipid one, whereas control shallow leaves were characterized by a further increase in the carbohydrate fraction compensated by a drop in the lipid one. Untouched and translocated deep leaves were characterized by a decrease in carbohydrate and lipid fractions and an increase in the protein one (Fig. 4).

The caloric content of *P. oceanica* leaves in T_1 decreased in all treatments with exception of control shallow leaves, with a more pronounced drop in the translocated deep leaves (−12.5%; Fig. 5). In T_2 the caloric content of control shallow, translocated and untouched deep leaves remained fairly the same as in T_1, whereas decreased (−19%) in leaves exposed to the thermocline (Fig. 5), resulting the lowest among all treatments at the end of the experiment.

Table 2
Results of the main PERMANOVA (A) and post-hoc (B) tests carried out to ascertain differences in the biochemical composition of *P. oceanica* leaves in response to treatment (control shallow, untouched, translocated, and shaded deep) and sampling time. The results of the post-hoc tests refer to differences in the biochemical composition of *P. oceanica* leaves between couples of treatments at each sampling time. Reported are degrees of freedom (df), mean square (MS), Pseudo-F (for the main test), number of unique permutations, probability level after Montecarlo simulations with 9999 permutations [P(MC)], t (for the post-hoc tests). Post-hoc comparisons among deep shoots were not carried out at T_0, as at the beginning of the experiment the data were obtained from the same donor bed. Not significant contrasts are in bold.

A) Main PERMANOVA test

Factor	df	MS	Pseudo-F	Unique permutations	P(MC)	% explained variance
Time (Ti)	2	13.93	26.546	9939	0.0001	24.9
Treatment (Tr)	3	9.21	17.556	9948	0.0001	21.5
Ti × Tr	6	6.15	11.722	9924	0.0001	41.8
Residual	24	0.52				11.7

B) Post-hoc tests

Time	Contrast	Contrast	t	P(MC)
T_0	A vs D	Control shallow vs. shaded deep	5.18	0.005
	A vs F	Control shadow vs. untouched deep	5.18	0.003
	A vs G	Control shadow vs. translocated deep	5.18	0.004
	D vs F	Shaded deep vs untouched deep		
	D vs G	Shaded deep vs translocated deep		
	F vs G	Untouched vs translocated deep		
T_1	A vs D	Control shallow vs. shaded deep	1.31	**0.242**
	A vs F	Control shadow vs. untouched deep	3.81	0.009
	A vs G	Control shadow vs. translocated deep	3.85	0.010
	D vs F	Shaded deep vs untouched deep	1.81	**0.128**
	D vs G	Shaded deep vs translocated deep	2.95	0.021
	F vs G	Untouched vs translocated deep	2.83	0.018
T_2	A vs D	Control shallow vs. shaded deep	5.07	0.003
	A vs F	Control shadow vs. untouched deep	7.25	0.001
	A vs G	Control shadow vs. translocated deep	3.58	0.007
	D vs F	Shaded deep vs untouched deep	4.22	0.004
	D vs G	Shaded deep vs translocated deep	1.83	**0.128**
	F vs G	Untouched vs translocated deep	4.48	0.003

4. Discussion

4.1. Thermocline deepening effects on biopolymers contents

Overall, changes in biopolymers content of the control shallow leaves were opposite or much narrower than those in all other treatments, suggesting that temporal changes experienced by deep leaves translocated and shaded in shallow thermal conditions mirrored different responses of transplanted shoots to the new environmental conditions. This holds true also for translocated deep shoots. In fact, our results reveal that, at the end of the experiment, the mechanical and handling stress caused by shoots translocation produced a significant increase of the carbohydrate fraction and a significant decrease of the lipid one. Since translocated deep shoots did not experience any environmental change beside the concurrent water warming, shifts in carbohydrate and lipid contents can be reliably interpreted as a peculiar and combined response to warming and the mechanical and handling stress, as previously observed in *P. oceanica* leaves either for carbohydrates (Genot et al., 1994) or lipids (Welti et al., 2007; Aid, 2019). Such interpretation seems upheld also by the morphological observation of a reduced leaf area in translocated shoots compared to the untouched ones (Stipcich et al., 2023b). Since a diminished leaf area of translocated shoots would have led reduced pigment contents and diminished glucide production by photosynthesis (Genot et al., 1994), the positive variation in water-soluble carbohydrate contents, representing the more reactive fraction of the seagrass carbohydrate pool, might possibly be explained by an increased mobilization of stored structural reserves into simple and more reactive sugars (Pirc, 1989). Besides the possible, merely hypothetical, mechanistic explanations for changes in carbohydrate and lipid contents of translocated deep shoots, the positive variation in water-soluble carbohydrates coupled with the decrease of lipids during the first part of the experiment, suggests, anyhow, an active and prompt metabolic response, that on the other hand has not been observed for proteins. This result is unexpectedly in contrast with previous studies that showed a tight relationship of fluctuations in protein content with seagrass responses to different environmental stressors (Kumar et al., 2017; Procaccini et al., 2017; Piro et al., 2015, 2020). On the other hand, the almost nihil change in protein contents of the translocated deep shoots at the end of the experiment was preceded by a minor increase during the second phase of the experiment (T_2). This result suggests that also the protein metabolism was influenced by the translocation in the first period, but also that the translocated deep shoots re-adapted their protein metabolism in a relatively longer term (at the end of the experiment). Despite this apparent homeostasis of the protein metabolism in the longer term, translocated cuttings displayed a clear modification of the carbohydrate and lipid contents that persisted, and even exacerbated, with time. These persisting modifications fit with the recurrently reported loss of cuttings in the early phases of deep *P. oceanica* transplantation activities for restoration purposes, as well as with the reported low survival rate of translocated deep plants (Piazzi et al., 1998, 2021).

The biopolymeric C contents of translocated deep shoots increased only slightly at the end of the experiment, with a rate in the same range of that one observed in the untouched ones. This result suggests that changes in the biochemical composition of translocated cuttings did not affect biopolymeric C storage mechanisms. These findings underline that handling stress associated with translocation can modify biopolymer metabolism and relative contents, but also that these modifications are somehow compensated by the almost invariant (or slightly increasing) biopolymeric C contents.

Although a morphological resilience of deep plants to higher temperature has been detected during the experiment (Stipcich et al., 2023b), the simulated deepening of the thermocline exerted significant effects over the longer-term on all the investigated variables, with patterns of variation resembling those of translocated cuttings. However, the comparative analysis of the effect sizes shows that the responses of *P. oceanica* cuttings exposed to the thermocline deepening and those translocated were quantitatively different. More in details, the effect sizes of thermocline deepening were generally equal or significantly higher than those in both untouched and translocated deep shoots, with the sole exception for proteins. Overall changes in the lipid fraction were consistently negative in thermocline deepening-exposed, untouched and translocated deep shoots, but positive (though not significant) in the control shallow leaves. This result suggests that, on the one hand, the changes in this component were affected by natural temporal variability more than translocation or the exposure to warmer conditions and, on the other one, that the responses of deep shoots differed significantly from those of the control shallow ones. Consequently, this result suggests not to consider total lipid contents of *P. oceanica* leaves a good signature of the effects of either manipulation or thermocline deepening.

The effect of the thermocline deepening and all other treatments with exception of control shallow leaves was initially negative or null on protein contents, which, however, reverted to positive at the end of the experiment, with exception of control shallow leaves. A fast activation of the protein metabolism of seagrasses exposed to thermal stress has already been reported, even though relatively weaker than the one of other bio-compounds (Marín-Guirao et al., 2016, 2017). Nonetheless, this result remains controversial, as other studies did not identify a unique direction of protein change in *P. oceanica* leaves exposed to heating, suggesting that changes in total protein contents also appear a relatively weak indicator of heat stress (Stipcich et al., 2022a).

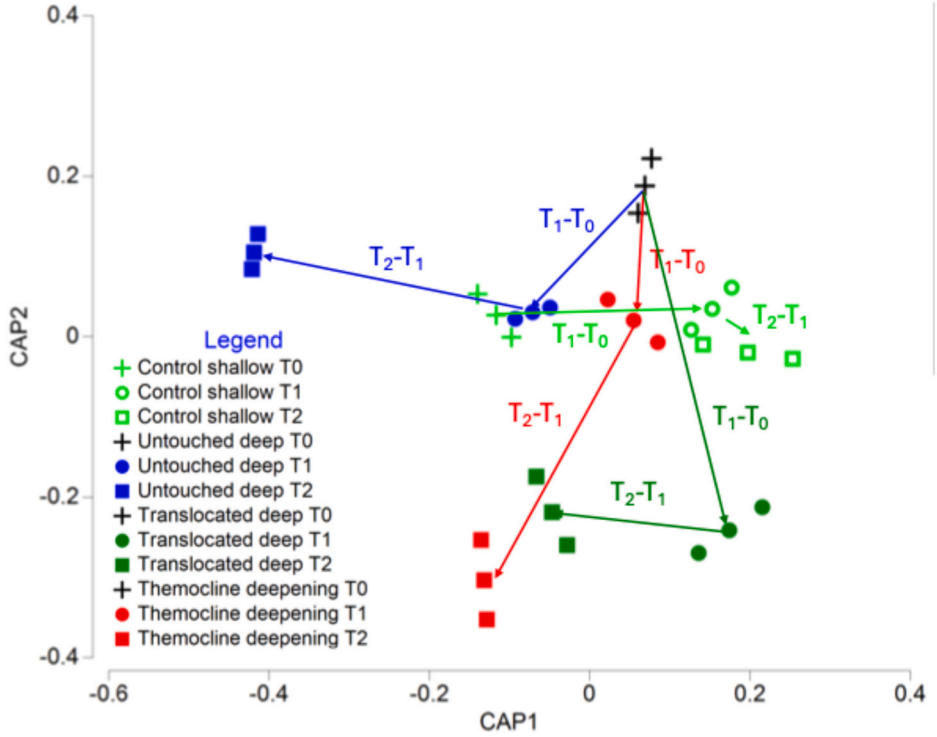

Fig. 3. Biplot after CAP illustrating multivariate differences in the biochemical composition of *P. oceanica* leaves among treatments and sampling times.

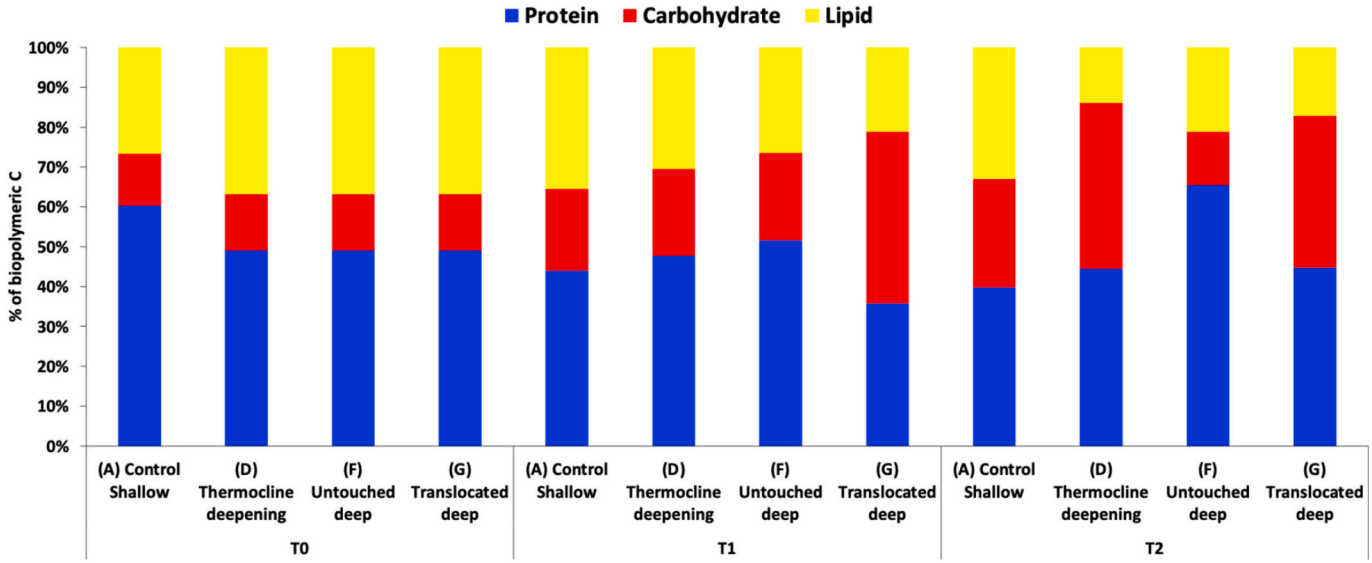

Fig. 4. Changes in the biochemical composition of *P. oceanica* leaves exposed to thermocline deepening, translocation and untouched during the experiment (T0, T1, T2).

Moreover, the magnitude of change in protein contents of leaves exposed to the thermocline deepening was lower than that of untouched deep shoots, suggesting that the thermocline deepening could have buffered the capacity of response of the exposed leaves.

Thermocline deepening did exert the highest effects on water soluble carbohydrates among all treatments, including the control shallow shoots. The positive response of water-soluble carbohydrates to thermal stress has already been observed in previous studies (Stipcich et al., 2022a) and it has been associated with a concurrent necrosis of the leaves (Stipcich et al., 2023b). During the second phase of the experiment (between T_1 and T_2), however, the size of carbohydrate changes in shoots exposed to thermocline deepening and translocated deep shoots was more pronounced than in the preceding phase, though the rate of leaf necrosis did not vary anymore. Such discrepancy suggests that the slight leaf morphological changes were not harbinger of imminent leaf loss (Ceccherelli et al., 2018), rather, together with the observed changes in the biochemical composition, were suggestive of the activation of thermal tolerance mechanisms (Marín-Guirao et al., 2018; Stipcich et al., 2022a). The relatively narrow change in biopolymers content due to the simulated thermocline deepening can be attributed either to the experimental temperature reached, that did not exceed the maximum thermal limit of seagrasses (Ontoria et al., 2019; Marbà et al., 2022), or to the fact that at −31 m depth a deepening of the thermocline concurrently occurred during the experiment (Stipcich et al., 2023b),

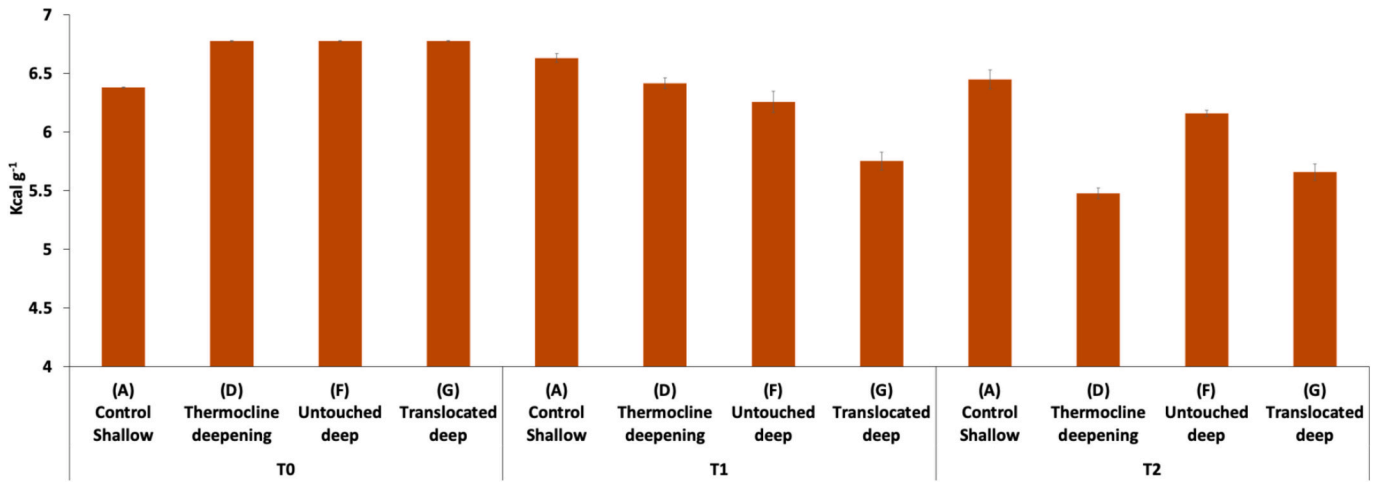

Fig. 5. Mean (±SE) caloric content of *P. oceanica* leaves in the four treatments during the experiment.

thus generating confounding effects that couldn't be disentangled.

4.2. Thermocline deepening effects on seagrass-based trophic webs

The results of our experiment enable us to predict that a future thermocline deepening would modify either the overall leaf biochemical composition (especially in terms of water-soluble carbohydrate contents) or their absolute C storage capacity. *Posidonia oceanica* leaves are used as food by some amphipod crustaceans (*Ampithoe ramondi* and *Gammarella fucicola*), whose feeding preference is influenced by the traits of the seagrass and the nutritional quality of the leaves (Castejón-Silvo et al., 2019). Also, some native (*Sarpa salpa* Linnaeus 1758; *Sparisoma cretense* Linnaeus 1758) and invasive (*Siganus luridus* Rüppel 1828) herbivorous fish, typical of eastern Mediterranean waters can graze on *P. oceanica* leaves (Tomas et al., 2005a; Azzurro et al., 2007; Prado et al., 2007), though they also feed on its epiphytes (Tomas et al., 2005b). The sea urchin *Paracentrotus lividus* is also assigned to be a potential, though debated, grazer of *P. oceanica* leaves and its epiphytes (Tomas et al., 2005a; Prado et al., 2007). In a warmer Mediterranean Sea characterized by a progressively deeper and more persistent summer thermocline, *P. oceanica* grazers, besides the unpredictable intrinsic effects of the thermocline deepening on their physiology, will be exposed to an increase in the quantity of the available resources, whose nutritional value, however, will be progressively lower. We conclude that, according to the optimal foraging theory (Stephens and Krebs, 1986), most important *P. oceanica* grazers could be forced to increase their grazing rates to compensate for the diminished nutritional quality of the leaves. Though the effective consequences of this increase in grazing rates are difficult to predict, our results corroborate the hypothesis that a more persistent and deeper thermocline will have not only direct consequences on the seagrass metabolism, but also on the structure and efficiency of the seagrass-based food web in deeper waters.

4.3. Study limitations and perspectives

The transplantation of *Posidonia oceanica* shoots is a common practice both for investigating its morphological, physiological and compositional responses to different environmental stressors through manipulative experiments (Soru et al., 2022; Stipcich et al., 2022a; Stipcich et al., 2022b; Stipcich et al., 2023a; Boulenger et al., 2026) and for addressing restoration potentialities and limitations (Procaccini and Piazzi, 2001; Balestri et al., 2011; Pansini et al., 2022).

In our study, we hypothesized that a possible summer deepening of the thermocline, driven by both sea surface warming and the occurrence of more intense and persistent marine heatwaves, could alter the plant biochemistry. As it was not possible to identify the occurrence of a deepening thermocline in real time, we artificially simulated it by recreating the same light limitations that occur at the typical depths of the thermocline in the study area. To achieve this, shoots from a meadow at 31 m deep were translocated to a depth of 12 m under a shading net, thus exposing them to the same light intensity as in the donor meadow, but at higher temperature. We assessed the potential effect of the stress caused by shallowly transplanting deep shoots by analyzing both shoots collected from a shallow meadow, a deep meadow and deep shoots translocated to the same depth as the donor meadow.

Although our sampling design was sufficiently robust to isolate the effects of both reduced light intensity and higher temperature, other uncontrolled sources of variance may have contributed to the observed results. For example, as light spectra can affect seagrass photosynthetic traits (Charras-Ferroussier et al., 2026) and carbohydrate metabolism (Ismael et al., 2023), we cannot rule out the possibility that differences in light quality (in terms of spectral composition) between the donor meadow and the artificially shaded area might also have influenced the biochemical composition of the shallowly transplanted shoots or the productivity of the epiphytic autotrophic microbiota (Rotini et al., 2023). This possibility is consistent with the previously reported changes in the pigment composition of the plant under the same experimental conditions considered here (Stipcich et al., 2025). Therefore, since the effects of both varying light spectra and other depth-related factors, such as nutrient availability, could not be included in our study, we acknowledge that further experiments are needed to implement the outcomes of this study. For instance, further experiments are needed to address the consequences of thermocline deepening on the survival of meadows dwelling at the deepest limit where irradiance is minimal, as anomalous temperature can cause unexpected retreat of the deep limit (Mayot et al., 2005).

Furthermore, given the relatively short duration of our experiment, we cannot ascertain whether the biochemical composition of plant shoots exposed to an artificial thermocline would show signs of adaptation or recovery, over a longer period (e.g. the entire summer season). Also, we acknowledge that the hypothesis of a possible optimal foraging effect on seagrass-based trophic webs will need further testing at larger spatial scales. In fact, we cannot exclude that *P. oceanica* plants living at different latitudes and being exposed to different temperature and light regimes, could react to the thermal stress caused by a deeper thermocline in different ways. Such responses, in fact, could depend on their genetic structure and diversity (Procaccini et al., 2001, 2002; Chefaoui et al., 2017) as well as on the high variability of individual thermal tolerance and local adaptation strategies (Pansini et al., 2021; Bennett et al., 2021, 2022; Stipcich et al., 2022a, 2022b).

Although eliminating all the potential biases outlined above would improve our understanding of the effects of thermocline deepening on *P. oceanica*, our research provides additional evidence that climate change could modify its biochemistry, especially of deep-dwelling plants, potentially altering also the seagrass-based trophic webs.

CRediT authorship contribution statement

Patrizia Stipcich: Writing – review & editing, Writing – original draft, Investigation, Conceptualization. **Santina Soru:** Writing – review & editing, Writing – original draft, Formal analysis, Data curation. **Giulia Ceccherelli:** Writing – review & editing, Resources, Conceptualization. **Francesco Palmas:** Formal analysis, Writing – review & editing. **Antonio Pusceddu:** Writing – review & editing, Resources, Funding acquisition, Formal analysis.

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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.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.marpolbul.2026.119441.

Data availability

Data will be made available on request.

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