Predicted warming intensifies the negative effects of nutrient increase on tropical seagrass: A physiological and fatty acid approach

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ABSTRACT

The Gulf of Aqaba (GoA; northern Red Sea) supports extensive seagrass meadows, dominated by the small tropical seagrass species, *Halophila stipulacea*. Due to its semi-closed structure, in the GoA, regional nutrient loading and global warming are considered the biggest threats to local seagrass meadows, and their combination can potentially amplify their negative impacts. Using a seagrass-dedicated mesocosm, we exposed two seagrass populations with different local "nutrient history" to control (27 °C) and simulated warming (31 °C), with and without nutrients (20 μM DIN). Following four weeks of these treatments ("stress phase"), all plants were returned to control conditions ("recovery phase") for another three weeks. Results showed that exposure to only thermal stress favoured growth, compared to exposure to only nutrient increase that reduced F_v/F_m and growth but favoured algae proliferation. Exposure to the combined thermal and nutrient stress, negatively affected seagrass performance resulting in high mortality observed after four weeks of combined exposure. The negative effects of combined stressors were stronger in populations with low "nutrient history". Additionally, we propose two novel fatty acid (FA) biomarkers, one based on FA unsaturation, 16:3n-3/16:2n-6, and the other on FA elongation processes, 18:2n-6/16:2n-6. Fatty acid analyses showed a significant decrease in 16:3n-3/16:2n-6 and 18:3n-3/18:2n-6 with increases in temperature and nutrients; a more drastic decline was found under the interaction of both stressors. Our results point out that C16 PUFAs, that are synthesized within the "prokaryotic pathway", are more sensitive to thermal and the combined thermal + nutrients stressors than C18 PUFAs, which are synthesized within the "eukaryotic pathway". In general, following a month of control conditions, a clear recovery of most of the seagrass descriptors was observed, highlighting the great capability of *Halophila stipulacea* to recover from stress conditions. Our results have important ecological and management implications to the seagrass meadows in the GoA and elsewhere. For seagrasses to survive climate change, managers must put efforts into limiting other stressors such as eutrophication that would potentially reduce the seagrass resilience to climate change.

1. 1. Introdction

Global change derived from anthropogenic activity has rapidly increased greenhouse gases resulting in changes to the global environment (i.e. Steffen et al., 2006). Consequently, the planet is rapidly warming at unprecedented rates that are expected to accelerate through the 21st century (Intergovernmental Panel on Climate Change [IPCC], 2014]). Warming represents an ongoing threat to marine ecosystems, causing a major loss of their functionality as climate change progresses during this century (Jordà et al., 2012). Growing anthropogenic

pressures on nearshore ecosystems entail increases in coastal nutrient inputs that can alter biogeochemical cycles and, ultimately, generate eutrophication events (Burkholder et al., 2007). Combined effects of global (warming) and local (eutrophication) stressors can result in synergetic pressures on marine ecosystems leading to species distribution shifts, species extinctions and ecosystem collapses, generating critical losses of their associated goods and ecological services (Yang and Rudolf, 2010; Doney et al., 2012).

Seagrasses are marine flowering plants (angiosperms) that colonize shallow coastal waters across the planet (Orth et al., 2006). Seagrass meadows provide several ecological services such as enriching the surrounding water with oxygen, sequestration of large amounts of atmospheric CO_2 (Blue Carbon) (Duarte et al., 2010; Fourqurean et al., 2012; Lamb et al., 2017, reduction of nutrients and pathogens from the water, providing shelter and nursery grounds to several marine species, protection from erosion. Due to their proximity to coastal developments, and their sometimes shallow depth, seagrass meadows are strongly impacted by the combined effects of nutrient increase and global stressors such as warming that have caused an increasing rates of seagrass loss in the last decades (Short and Neckles, 1999; Waycott et al., 2009).

Temperature and the plant's capacity to take up nutrients, have been identified as some of the most relevant factors controlling seagrass physiological and growth responses (i.e. Lee et al., 2007; Artika et al., 2020). While moderate increases in temperature may favour seagrass physiological processes and productivity, temperature increases above optimal or sub-optimal levels can generate negative effects on seagrass performance. The vulnerability of seagrasses to warming depends on the species' thermal range, local temperature regimes and population-specific capacity to adapt (Reusch, 2014; Short et al., 2016).

Tropical and subtropical seagrass ecosystems are usually exposed to nutrient-limited (oligotrophic) environments, thus moderate inputs of nutrients can favour seagrass physiology and growth performance (Duarte, 1992; Cardini et al., 2017). However, beyond these moderate nutrient levels, availability of excess nutrients in the environment, can generate toxicity in seagrass plants resulting in physiological stress and inhibition of growth (Van Katwijk et al., 1997; Brun et al., 2002). Nutrient-enriched conditions can also promote algae growth, leading to reduction in light quality and oxygen reduction in the water column (hypoxia) and resulting in seagrass stress and potential habitat decline (reviewed in Lee et al., 2007; Mascaro et al., 2009). Recent studies reported that warming enhances the negative effects of nutrient increases in seagrasses by decreasing meadow cover, plants productivity, photosynthetic efficiency and energetic reserves in their tissues (Ontoria et al., 2019a; Pazzaglia et al., 2020; Viana et al., 2020; Helber et al., 2021a; Helber et al., 2021b; *reviewed in* Nguyen et al., 2021).

Halophila stipulacea is a small dioecious marine angiosperm native to the Red Sea, Indian Ocean, and the Persian Gulf where it is distributed in subtidal marine areas. *H. stipulacea* is capable of acclimatizing to a wide range of environmental gradients including temperature, salinity, irradiance, and nutrient concentration (Sharon et al., 2011; Oscar et al., 2018; Beca-Carretero et al., 2019; Winters et al., 2020). Soon after opening the Suez Canal, *H. stipulacea* became a Lessepsian migrant, invading most of the eastern Mediterranean Sea (Lipkin et al., 2003; Winters et al., 2020). As climate change progresses during this century, it is expected that *H. stipulacea* will expand its distribution to colonize large coastal areas in the central and western parts of the Mediterranean Sea basin (Gamliel et al., 2020; Beca-Carretero et al., 2020a; Thibaut et al., 2022). In 2002, these seagrass species were observed for the first time in the Caribbean Sea, and since then it has spread to most Caribbean Island nations (Willette et al., 2014; Winters et al., 2020).

Over the last number of decades, different plant characteristics associated with diverse biological levels from genes to populations have been applied as indicators to assess seagrass physiological stress or vulnerability (Martínez-Crego et al., 2008; Marbà et al., 2013). Fatty acid (FA) responses can represent sensitive biomarkers to study the

ecological status of seagrasses since FAs form part of the structure of the membranes of the thylakoids (Beca-Carretero et al., 2020c; Franzitta et al., 2021). They play a key role in the photosynthetic activity and, therefore, are highly responsive to changes in environmental conditions. In terms of FA composition, seagrasses are classified as 16:3 plants because they have the capacity to synthetize FAs by the "prokaryotic" and simultaneously by the "eukaryotic" pathways, a combination which is the most common process to synthetize FA in terrestrial plants (Mongrand et al., 1998; Duarte et al., 2018; Beca-Carretero et al., 2019). Previous studies with terrestrial primary producers showed that the specific FA pathway used varies according to environmental conditions and physiological stress of primary producers (Browse et al., 1986; Franzitta et al., 2021).

With these considerations in mind, we aimed to 1) understand the responses of two populations of *H. stipulacea* exposed to different *in situ* ambient nutrient levels to the individual and the combined effects of increases in water temperatures (+4 °C above summer average water temperatures) predicted to reach the GoA by the end of 2100 and increased nutrient levels, and 2) compare "classic" indicators (e.g., physiological, biochemical, morphological and photophysiological) with FA analyses to detect stress in *H. stipulacea*. Lastly, (iii) we evaluated whether *H. stipulacea* can recover from stress conditions. We hypothesized that predicted warming will favor *H. stipulacea* physiological and growth responses as this tropical species has a wide thermal niche, and GoA does not represent its thermal extreme within its temperature range (Winters et al., 2020). However, we expected that the potential negative effects of nutrient stress will be intensified by warming events. Additionally, we theorized that fatty acid (FA) responses will likely reflect the physiological state of the seagrass plants, and will show clear patterns of both effects of temperature and nutrient stress. Here, we propose two additional and novel FA biomarkers, one based on FA unsaturation processes, 16:3n-3/16:2n-6, and the other biomarker related with FA length elongation, 18:2n-6/16:2n-6, as potential indicators of thermal and nutrient stress.

2. Materials and methods

2.1. Plant collection sites

Plants were collected from two sites in the northern GoA (Eilat, Israel), from the South Beach (SB; 29.4928215°N, 34.9059872°E) and Tur Yam (TY; 49.72529°N, 34.9141850°E)The seagrass meadow at TY has an extent of 67,365 m^2 and is characterized by medium-sized sediments, low bathymetric slope (5.3°), some turbidity in the water column, low presence of corals, and is affected by medium–high anthropogenic pressures (an oil terminal that is seldom used and a small marina onsite). In contrast, the meadow at the SB site is slightly smaller (61,900 m^2), is characterized by coarse grain sediments, a steep bathymetric slope (17.92°), clear waters, and a high cover of corals, and is considered a near-pristine area that enables seagrass to grow to depths of over 50 m (Mejia et al., 2016; Rotini et al., 2017; Winters et al., 2017; Beca-Carretero et al., 2020b; Azcárate-García et al., 2020).

From each site, a total of 120–150*H. stipulacea* plant fragments bearing 6–8 shoots were carefully collected at a depth of 10 m by SCUBA-diving (June 2019). Irradiance levels at 10 m depth were measured using the Diving PAM's photosynthetic active radiation (PAR) sensor (Walz, Germany) along a 50 m transect (~250 μmol photons m^{-2} s^{-1}) to set similar irradiance levels used in the mesocosm (detailed below). Shoots were collected every 5–10 m from each other to minimize sampling of the same clones. *In situ* water temperatures at the time of plant collection ranged between 25 and 26 °C. Plants were collected one month before the maximum annual sea surface temperatures (SSTs) of 27–28 °C were reached (in July-August; Winters et al., 2017) to match the time of exposure in the experiment.

Collected shoots were transported to the seagrass mesocosm facility (detailed below; *also see* Nguyen et al., 2020; Oscar et al., 2018;

Szitenberg et al., 2022) in zip-lock plastic bags filled with seawater to avoid the damage and dehydration of the shoots; bags were placed in cooler boxes to ensure that temperature was similar during the transportation (2 h driving) to the laboratory. Once there, harvested shoots were carefully cleaned (removing epiphytic organisms) and dead plant tissues were removed from the shoots. This was followed by "sterilizing" shoots by dipping them in freshwater for 2 min to potentially kill associated pathogens. We selected *H. stipulacea* plants with similar size and number of shoots (4–5). In each aquarium, we planted 16 to 18 plants from each population (SB and TY). All planted shoots were acclimatized for 4–5 weeks at 27 ℃ prior to the onset of treatments (detailed below).

2.2. Warming and nutrient experiment set-up

We carried out a mesocosm-controlled experiment on *H. stipulacea* plants from two locations, Tur Yam (TY) and South Beach (SB), exposed to control (27℃) or increased (31℃) water temperature, with and without nutrients treatments for 1 month followed by a recovery phase of another three weeks (Fig. 1).

Plants were incubated in a total of 20 aquaria (5 aquaria per treatment), arranged in four temperature baths, with each bath containing five replicated aquaria and subjected to one of the four different treatments described below. Each aquarium (60 L in volume) contained 50 L of artificially made seawater (https://www.redseafish.com/red-sea-salts/red-sea-salt/) and was layered with a ~ 8 cm layer of natural sediment. Each aquarium was partially divided (the divider reached up to only 12 cm above the sediment; insert in Fig. 1), creating a divided area of SB and TY, allowing plants from both populations to be exposed to the exact same conditions and grow side by side. Irradiance was provided by three stripes of LED Lamps, two stripes that included mostly blue and red LEDs connected to a dimmer which controlled the intensity separately for the blue and red channels, and one central stripe of white LEDs to increase the general amount of PAR (Parus, Holland).

Halophila stipulacea shoots were incubated at irradiances of 250 μmol photons m^{-2} s^{-1} (measured on the leaves of the shoots using the DIVING-PAM's PAR sensor) in a light (L): dark (D) cycle of 12 h: 12 h (similar to irradiance levels during the time of collection). Salinity was maintained at 40 PSU (partial salinity units, equivalent to the average salinity of the Red Sea's water during time of collection) and was monitored daily using a WTW-340i conductivity meter (WTW, Germany). High salinity created by evaporation was corrected daily by adding distilled water. 10 % of the seawater in all aquaria was renewed weekly by emptying 5 L and refilling the aquaria with newly made seawater of the same amount. Additionally, 100 mL of seawater from the GoA collected in SB and TY was added weekly in each aquarium to potentially inoculate the holobiont community in the aquaria during the acclimation period (4 weeks) (Szitenberg et al. 2022).

The *H. stipulacea* shoots collected from the two different populations (SB and TY) were exposed to four different experimental conditions: (i) Control conditions simulating current summer conditions in the GoA, i.e., 27 ℃ and low nutrient levels (~1.8 μM of DIN; measured at control conditions); (ii) natural summer conditions (27 ℃) with increased nutrients levels (20 μM of DIN), simulating eutrophication levels 10x of the maximal nutrient levels experienced in the northern GoA's shallow water at present (Beca-Carretero et al., 2020b); (iii) increased water temperature conditions (31 ℃; 4 ℃ above the current average summer temperature), simulating the predicted temperature increase in the northern GoA by the end of this century (IPCC, 2014, RCP 8.5; Fine et al., 2013; Nguyen et al., 2020) and low (i.e, control) nutrient levels; and finally, (iv) increased water temperature conditions (31 ℃) combined with increased nutrients levels (20 μM of DIN), simulating a combination of predicted water temperatures, together with increased nutrient inputs.

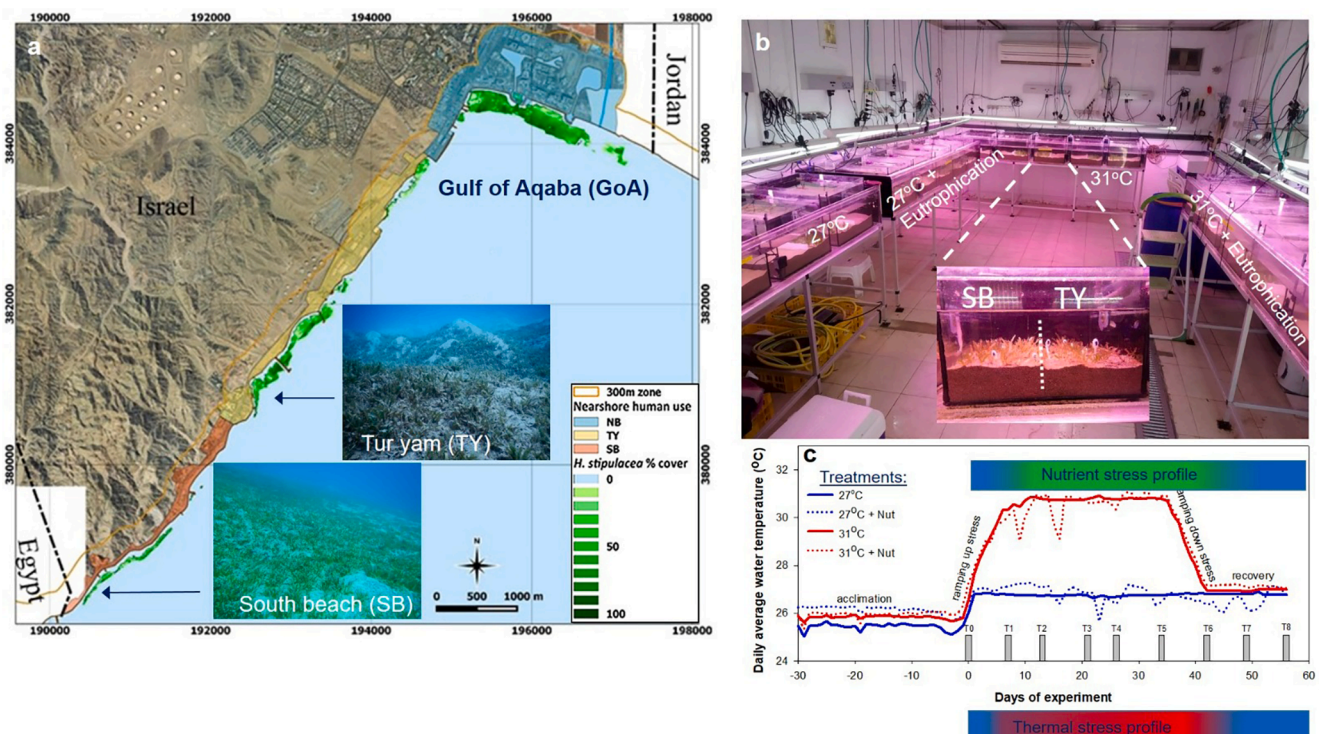For simulating high nutrient conditions, we added nutrient-enriched

Fig. 1. Map of study area (Panel a), northern Gulf of Aqaba (GoA), Red Sea, Israel, with the locations of the two study sites marked by arrows: Tur Yam (TY) and South Beach (SB). *Halophila stipulacea* distribution in the region is represented in green [adapted from Winters et al. (2017)]. The mesocosm set up used in the experimental design (Panel b). *Halophila stipulacea* plants growing in control conditions (27 ℃); 27 ℃ and increased nutrient levels (20 μM of DIN; 27C + nut); warming condition (31 ℃); 31 ℃ and increased nutrient levels (20 μM of DIN; 31C + nut) (Panel c). Five aquaria (n = 5) containing 16–20 seagrass plants each. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

(20 μM of DIN) solutions based on the volume (60 L) of the aquaria using the slow-release fertilizer pellets (Osmocote, 17:11:10 N:P:K) (Helber et al., 2021b). The NPK pellets were grounded into a fine powder and dissolved in water' later they were gradually added three times per week on alternative days to the nutrient enriched dedicated aquaria.Additionally, 50 mL of aquaria-water were weeklysampled weekly from four replicated aquaria from each treatment and were filtered through 0.022-μm syringe filters, frozen and kept for measuring nutrients in the water (detailed below). Water salinity and temperature in all experimental aquaria were monitored (and if needed, corrected) daily using the WTW Multi 340i portable meter.

Water samples were collected every week (T_0 to T_8) from each aquarium using acid-rinsed plastic syringes (approximately 30 mL) at 10 cm above the seagrass plants. Water samples were immediately filtered into HDPE vials using sterile syringe filters (LABSOLUTE R; cellulose acetate; 0.45 μm pore size) and stored at −80 °C. Nutrient analyses were carried out spectrophotometrically with a TECAN plate reader (Infinite 200 Pro microplate reader; Switzerland) according to Laskov et al. (2007). The detection limits were established in 0.08, 0.32, 0.7, and 0.022 μM for $NO^{2−}$, NO_x ($NO^{3−}$ and $NO^{2−}$), NH_4^+, and $PO_4^{3−}$, respectively. The NO_x ($NO^{3−}$ and $NO^{2−}$) and NH_4^+ concentrations sum up to dissolved inorganic nitrogen (DIN). The coefficient of variation was always < 3.4 % (Helber et al., 2021b).

Following the 4 weeks of the acclimation period, the temperature was gradually increased during one week from 27 to 31 °C (+0.65 °C/day; "ramping up" stage; Fig. 1c, 2a) in the high temperature treatment. Upon reaching 31 °C (at T_1), water temperature was maintained at this temperature for another 4 weeks (from T_1 to T_5), i.e., allowing *H. stipulacea* plants to be exposed to warming with/without nutrient increase. After the exposure period (T_5), we began to gradually lower the temperature of the heated aquaria back to 27 °C (- 0.65 °C/day; "ramping down" stage), and no more nutrients were added. Finally, plants from the four water baths were exposed for three weeks (between T_6 and T_8) to control pre stress conditions (27 °C and no nutrients). The experiment finished at T_8 (Fig. 1c and 2a).

2.3. Seagrass responses

2.3.1. Growth rates and mortality rate

To measure growth parameters, two plants per aquarium were selected and marked with plastic cable ties at the 1st internode following the methodology described in Azcárate-García et al. (2020). Only plants with an apical shoot bearing two full-grown leaves were selected. Rhizome elongation rates (cm plant^{-1} day^{-1}) were calculated by measuring the length of the newly produced rhizomes over time and dividing this by the number of days between measurements. Similarly, shoot formation rates (shoot plant^{-1} day^{-1}) were determined by counting the number of new shoots produced between weekly measurements (T_1 to T_8). Mortality rates were calculated by counting the number of dead plants relative to original number of plants on each sampling period of the experiment (T_0 to T_8) and later normalized to the number of days between measurements.

2.3.2. Physiological measurements

Photo-physiological effects of the exposure to increased water temperature with/without nutrient additions were assessed by measuring the maximal quantum yield of photosystem II (PSII) (F_v/F_m; Winters et al., 2011) using the Diving-PAM chlorophyll fluorometer (Walz, Germany). All measurements were performed underwater 1 h before lights were switched on (i.e., under darkness) in order to maximize the frequency of open photosystem II reaction centres (cf. Winters et al., 2003, 2011). The PAM's fibre-optical probe was kept at a fixed distance (1.5 cm) and angle (70°) from each sample using the dedicated leaf clip. Leaves were always cleaned from epiphytes prior to performing the measurements. Within each aquarium, measurements were made on the base of the 3rd youngest shoot of two shoots from each population

(shoots were randomly chosen, marked at T0 and remeasured at each time point). Measurements from the same population and aquarium were averaged (n = 5).

2.4. Biochemical responses

For biochemical analysis, only healthy leaves (and avoiding epiphytes or damaged parts) of the 2nd and 3rd youngest shoots were selected each week (T_0 to T_8) from aquaria incubated under different combined temperature and nutrient conditions. Collected leaves were cleaned with distilled water prior to processing and immediately frozen at −80C.

2.4.1. Fatty acid analysis

Fatty acid content and composition of *H. stipulacea* leaf biomass was determined by applying the protocol previously used for seagrasses (Beca-Carretero et al., 2020c). Fatty acid methyl esters (FAMEs) were obtained by direct transmethylation of ∼ 5–10 mg of powdered freeze dried leaf biomass with dry methanol containing 2 % (v/v) H_2SO_4. To prevent oxidation, vials were closed with nitrogen gas before being heated at 80 °C for 2 h under continuous stirring conditions. After transmethylation, we added 1 mL of Milli-Q water and later extracted the FAME using 0.25 mL of *n*-hexane. Analysis of FAME was conducted using a Clarus 500 Gas Chromatograph (Perkin Elmer Instruments, USA) equipped with a flame ionization detector and a fused silica capillary column (SP-2330, 0.25 mm × 30 m × 0.2 μm, Supelco, catalog No.: 24019). Identification of FAME was achieved by co-chromatography with commercially available FAME certified standard material (Supelco 37 Component FAME Mix, catalog no. CRM47885). Total and individual fatty acid contents were quantified by comparisons with a known quantity of the standard Nonadecanoic acid, 19:0 (≥98, catalog no. N5252, Sigma Aldrich, St Louis, USA) as an internal standard. We added the 19:0 standard (20 μl, 0,8 mg mL^{-1}) before starting the direct transmethylation and expressed the results as the mean values of 3 biological replicates (n = 3) for each treatment, population and T_0, T_2, T_5 and T_8 time points.

2.4.2. Nitrogen and carbon content

Nitrogen (N) and carbon (C) contents of leaf tissues of *H. stipulacea* plants were analyzed following the methodology described in Viana et al. (2020). Freeze-dried leaf tissues were ground to a fine powder with mortar and pestle, and 1–2 mg DW of tissue were weighed with a microbalance analytical scale, inserted into tin capsules and analyzed for C and N content as the % of DW using an Euro EA3000 Elemental Analyzer (EuroVector).

2.5. Epiphytes cover

Epiphyte cover was calculated by taking a picture of each aquarium with Tur Yam and South Beach populations (Fig. S1) and treatment (n = 5) using a water-proof camera SJCAM sj4000. To estimate the percentage of cover a grid of 10 × 10 (=100) points was overlaid onto each photograph, and points enclosed in the grid with the presence of epiphytes were counted.

2.6. Statistical analysis

Data of morphology, growth rates, population descriptors and biochemical compounds were log-transformed and were later checked for homogeneity of variance using Levene's test and for normality by applying the Shapiro-Wilk test. Since our data did not meet the test's criteria, the non-parametric test PERMANOVA analyses based on a similarity matrix created from the Euclidean distances was implemented to test differences in seagrass descriptors responses to temperature (27 and 31 °C) and nutrient enrichment (control and enriched with 20 μM DIN). We used "treatment", "time" and "population" as fixed factors.

This was followed by applying a post hoc pairwise test to identify the treatments that differed significantly (p < 0.05). All statistical analyses were performed using the PRIMER and PERMANOVA 6 statistical packages (Anderson et al., 2008). Seagrass descriptors values are reported as means ± standard errors (±SE).

In order to evaluate the potential correlation between physiological (Fv/Fm) and growth responses versus specific fatty acids indicators

based on unsaturation indexes (16:3n-3/16:2n-6 and 18:3n-3/18:2n-6) and FA elongation indexes (16:3n-3/18:3n-3 and 18:2n-6/16:2n-6) we performed Deming correlation analysis.
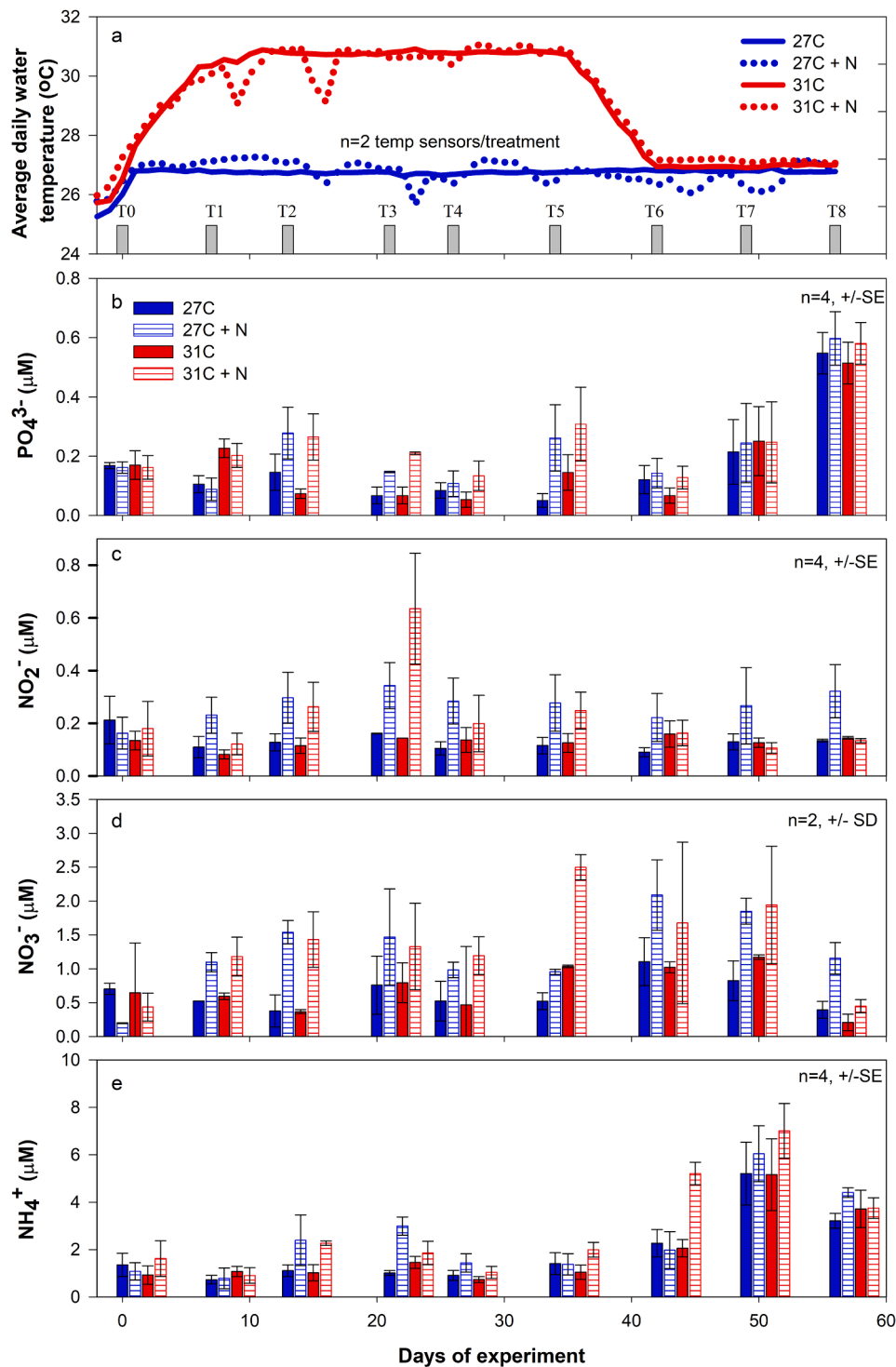
Fig. 2. Daily average of water temperatures (°C) (Panel a); Weekly measurements of nutrient concentrations (μM) of phosphate (PO_4^{3-}) (Panel b); nitrite (NO_2^-) (Panel c); nitrate (NO_3^-) (Panel d); ammonium (NH_4^+) (Panel e). Measurements from T_0 to T_8 in different treatments (27 °C; 27 °C and nutrients; 31 °C; 31 °C and nutrients). Results are expressed as mean ± SE (n = 4) in panel b, c and e, and mean ± SD (n = 2) for panel d.

3. Results

3.1. Nutrient measurements

Analysis of water samples showed that nutrient enrichment was successful in creating higher levels of nutrients (DIN and PO_3^-) in the water column during the exposure periods ($T_1 – T_5$, Fig. 2b-e). However, unlike the immediate return of higher water temperatures to control levels at the end of the ramping down phase ($T_5 – T_6$), nutrient levels remained high even after we stopped enriching them (after T_5) and

continued to be elevated during the recovery phase ($T_7 – T_8$) in the treatments previously exposed to simulated eutrophication. Salinity remained constant at ~ 39–40 PSU throughout the experiment, simulating natural conditions from the GoA (Beca-Carretero et al., 2020b).

3.2. Fatty acid content and composition

Average values of TFA (total fatty acids) were 1.2 ± 0.1 and 1.3 ± 0.2 of DW in Tur Yam and South Beach populations, respectively (Tables S3 and S4). The most common FAs were polyunsaturated fatty
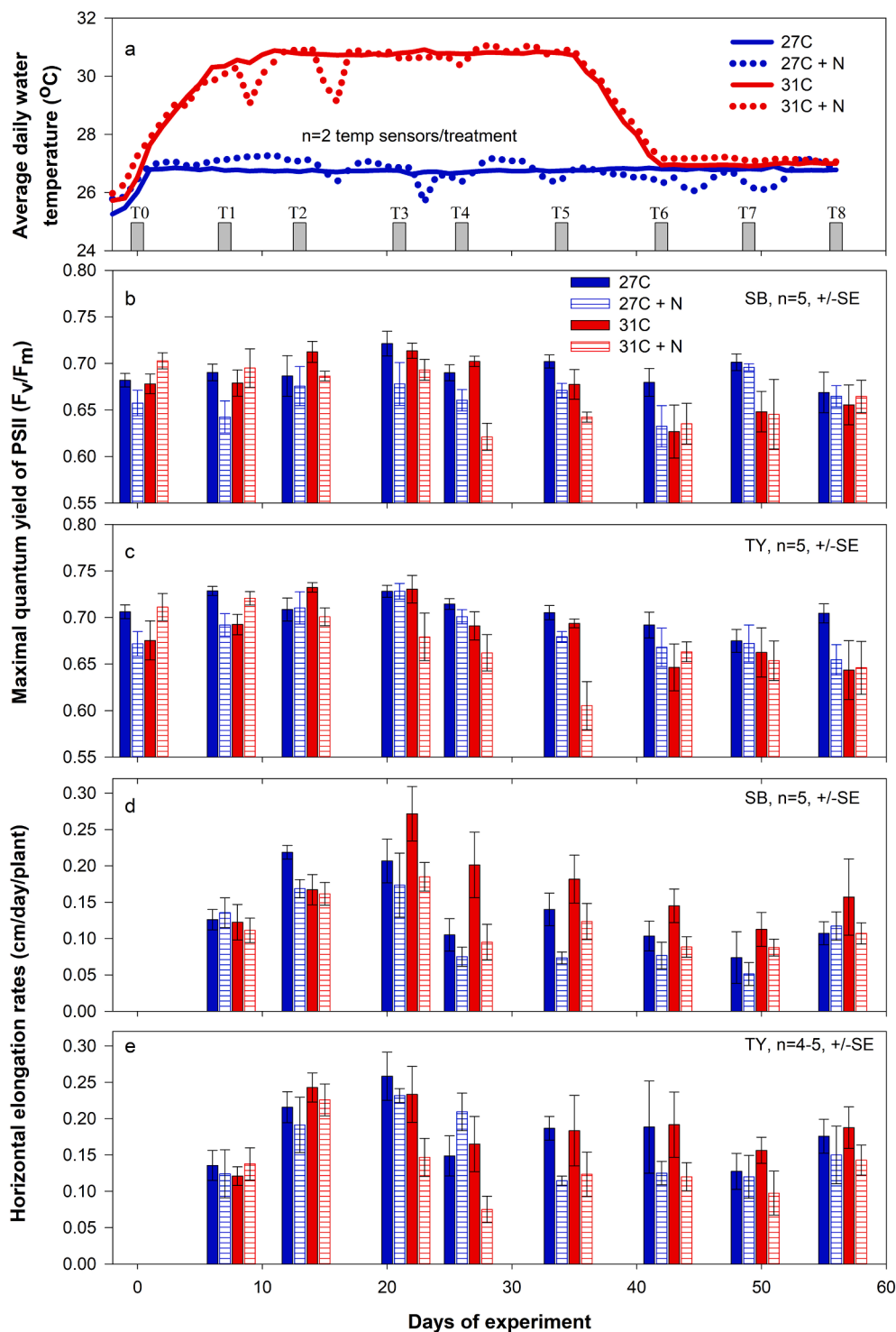
Fig. 3. Daily average of water temperatures (°C) (Panel a); Maximal quantum yield (Fv/Fm) (Panel b, c) and rhizome horizontal elongation (Panel d, e) of *Halophila stipulacea* plants measured in the different treatments (27 °C; 27 °C and nutrients; 31 °C; 31 °C and nutrients) and time points (T_0, T_1, T_2, T_3, T_4, T_5, T_6, T_7 and T_8) of South Beach (Panel b, d) and Tur Yam (Panel c, e) populations. Letters indicate significant differences among treatments based on pairwise tests. Results are expressed as mean ± SE (n = 5).

acids (PUFAs), which accounted for an average of 60.9 ± 2.4 % of TFA across all treatments and time points. The most abundant PUFA was α-linolenic acid (ALA, 18:3n-3 [44.0 ± 2.4 %]), followed by linoleic acid (LA, 18:2n-6 [14.0 ± 0.9 %]) and hexadecatrienoic acid (HTA, 16:3n-3 [2.2 ± 0.3 %]). The second most abundant group of FAs were saturated fatty acids (SFAs) which accounted for an average value of 29.7 ± 2.0 % of TFA, where palmitic acid (16:0 [24.4 ± 1.5 %]) was the most common SFA, followed by stearic acid (18:0). Finally, monounsaturated fatty acids (MUFAs) accounted for an average of 9.4 ± 0.7 % of TFA (Tables S3 and S4).

3.3. Responses to temperature increase

Overall, exposure to increased water temperature affected the seagrass descriptors differently. We found a significant effect in the interaction "treatment × time" in F_v/F_m (Fig. 3b-c). Already at the early stages of increased temperature (T_2), measured F_v/F_m values were 4 % significantly higher in the warming treatment (31 °C) in comparison with the control one (27 °C) in both SB and TY populations (Fig. 3b-c, Table 1). However, by the end of the warming period (T_5), there were no significant differences of F_v/F_m values across temperature treatments. Horizontal elongation rates (Fig. 3d-e) and shoot production (Fig. S2) were not affected at the early (T_2) or later (T_5) stages of the exposure to increased temperature in both populations. Higher mortality rates were observed at T_5 in both populations under warming conditions (31C, 0.07 ± 0.05 % plant^{-1}d^{-1}; Fig. 4b-c) in comparison with plants exposed to control temperatures (27C, 0.03 ± 0.03 %plant plant^{-1}d^{-1}). Temperature also affected N content at T_5, with significantly higher N contents at 31C compared to 27 °C (PERMANOVA, $p < 0.05$) (Table 2).

Temperature increases significantly reduced the accumulation of polyunsaturated fatty acids reduction in the ratio n-3/ n-6 PUFA (PERMANOVA, $p < 0.05$) (Tables S3 and S4). These reductions were stronger after a long-term exposure to thermal stress (T_5) compared with samples collected after a relatively short exposure to this stress (T_2) (PERMANOVA, $p < 5$). More specifically, levels of the unsaturation indexes 16:3n-3/16:2n-6 and 16:3n-3/18:3n-3 were found to be significantly lower with increases in temperature (Fig. 5). For instance, the 16:3n-3/16:2n-6 showed a fast reduction of 42 % from 27 to 31 °C at T_2, and 62 % at T_5 for plants from TY, and a reduction of 43 and 52 % for SB plants, respectively (Tables S3 and S4). The 18:3n-3/18:2n-6 was less sensitive to warming and showed a slight but significant reduction of 16 % at T_2 and 12 % at T5 in SB plants (PERMANOVA, $p < 0.05$) (Table S7). On the other hand, thermal stress significantly increased the levels of the FA elongation indexes (18:2n-6/16:2n-6 and 16:3n-3/18:3n-3) of both populations at T5 in comparison with control plants (PERMANOVA, $p < 5$) (Fig. 6; Table 1). These changes were mostly explained by changes in C16 PUFAs in comparison with C18 PUFAs (Table S3 and S4).

3.4. Responses to nutrient increase

Results showed that nutrient increase affected seagrass descriptors in different ways. On the one hand, F_v/F_m and horizontal elongation rates showed significant reductions from control to nutrient-enriched conditions, which was more accentuated at prolonged exposure to eutrophication (T_5) than at early stages (T_2) in both populations (PERMANOVA, $p < 0.5$, Table 1). At T_5, plants exposed to nutrients grew 52 % and 60 % slower in SB and TY populations, respectively than control conditions (SB = 0.14 cm d^{-1}; TY = 0.19 cm d^{-1}) (Fig. 3d-e). Higher mortality rates were observed in plants exposed to higher nutrient levels in comparison with control treatments, although, these differences were only observed in T_5 in SB (Fig. 4b). In addition, we found a significant increase in epiphytes and macroalgae in the aquaria growing under simulated eutrophication conditions (Table 3; Fig. S1); particularly visible at T_5 in comparison with T_2 (PERMANOVA, $p < 0.05$). N content of seagrass leaves were generally significantly higher in nutrient treatments (PERMANOVA, $p < 0.05$) in both SB and TY populations at T3; while at T8, in

the recovery phase, there no differences among treatments (Table 1 and 2). N contents in the SB population were less affected by treatments in earlier time points, however, they were significantly higher by T8 in the nutrient treatments. Carbon (C) content did not differ among treatments but was significantly lower by T8 in both populations.

Nutrient enrichment favored the accumulation of PUFA and reduced the levels of SFA; these patterns were more evident at T_5 than at earlier stages of exposure to the nutrients (T_2) (PERMANOVA, $p < 0.05$, Table 1). Increases in PUFA were explained by the significant increases in n-6 PUFA relative to n-3 PUFA, and in the reduction of in 18:3n-3/ 18:2n-6, particularly evident at T_5 (Figure 5 and S5). 18:3n-3/18:2n-6 displayed a significant and clear reduction at T_5 from 7.02 ± 0.8 % down to 5.75 ± 0.9 % in SB seagrasses, while, this trend was lower in TY plants, indicating the observed significant interaction between treatment and population (PERMANOVA, $p > 0.05$) (Table S6 and S7). However, the unsaturation indicator 16:3n-3/16:2n-6 was not affected by increases in nutrient treatments (Fig. 5). The FA elongation index18:3n-3/16:3n-3 showed a significant increase in both populations with increases in nutrient concentrations at the early state (T_2), and they were even more accentuated at T_5 (Fig. 6).

3.5. Combined effects

Physiological and mortality responses showed a synergetic response at T_5 to combined effects (Figs. 3 and 4). The combination of warming and nutrient increase generated a stronger negative response than individual effects of temperature or nutrient increase. For instance, at T_5, SB and TY plants exposed to the combined stresses, suffered a significant reduction of 9 and 14 % respectively in their photosynthetic efficiency (F_v/F_m) compared to their respective controls (Fig. 3b-c; Table 1). At T_5, mortality rates under combined stressors reached maximum values observed during the experiment from both SB (0.20 ± 0.08 % plant^{-1} d^{-1}) and TY (0.10 ± 0.09 % plant^{-1} d^{-1}) (Fig. 4b-c). In contrast, horizontal growth and shoot production pointed out an additive effect to combined stressors, as there were no significant differences between nutrient treatment at 27 °C and at 31 °C in both T_2 and T_5 (Fig. 3d-e and S2).

We found a synergetic effect of warming and nutrient increase in most of the FA descriptors (Figs. 5, 6 and S5). Combined effects significantly increased SFA levels and reduced n-3 PUFA contents (PERMANOVA, $p < 0.05$) (Table 1). Particularly, at T_2, the unsaturation index 18:3n-3/18:2n-6 showed a robust trend, with reductions of 12.3 and 11.8 % under combined stress conditions in comparison with control conditions in TY and SB, respectively (Fig. 5; Tables S3 and S4). In T_5 these differences were even higher with significant reductions in 18:3n-3/18:2n-6 of 17 and 21 % in *H. stipulacea* plants from TY and SB, respectively. The unsaturation index16:3n-3/16:2n-6 reported significant differences between control and plants exposed to combined stressors, however, these responses were not synergetic (Fig. 5). Interestingly, the index18:3n-3/18:2n-6 positively and significantly correlated with photosynthetic efficiency measurements (F_v/F_m) (Deming regression, n = 32, R = 0.62, $p < 0.05$) and growth rates (Deming regression, n = 24, R = 0.61, $p < 0.05$) (Fig. S8). However, we did not find any correlations between 16:3n-3/16:2n-6 and F_v/F_m or growth rates. The FA elongation index 18:3n-3/16:3n-3 showed synergetic effects with a marked increase in T_2, but only in TY, while at T_5, there were observed synergetic significant increases between treatments in SB and TY (Fig. 6). 18:2n-6/16:2n-6 reported significant differences between control and plants exposed to combined stressors, however, these responses were not synergetic (Fig. 6).

Overall, most of seagrass descriptors including growth rates, shoot production, mortality rates and biochemical composition demonstrated a strong recovery at the end of the experiment (T_8), with no significant differences between plants exposed to control conditions and plants exposed to thermal stress, nutrient stress or its combined effects (Figs. 3-6).

Table 1

Results of PERMANOVA analyses assessing the effects of population (P), treatment (TR) and time (TI) on seagrass traits including F_v/F_m, horizontal elongation, number of new apical shoots, polyunsaturated fatty acids (PUFA), saturated FA, PUFA/SFA, 18:3n-3/18:2n-6 and 16:3n-3/16:2n-6 ratios. Bold letters indicate significant differences. Pseudo F-values of three-way ANOVA are shown along with significance levels (*p < 0.05; **p < 0.01; ***p < 0.001).

	df	MS	*Pseudo-F*	df	MS	*Pseudo-F*
	Fv/Fm			**SFA**		
Population (PO)	1	1.40	1.94	1	4.35	**6.30 ***
Treatment (TR)	3	3.21	**4.46 ****	3	4.48	**6.48 ****
Time (TI)	3	6.90	**9.58 ****	3	2.66	**3.85 ***
POxTR	3	0.93	1.29	3	0.28	0.40
POxTI	3	0.84	1.17	3	0.27	0.39
TRxTI	9	2.92	**4.06 ****	9	1.85	**2.68 ***
POxTRxTI	9	0.39	0.54	9	0.75	1.09
Res	128	0.72		64	0.69	
	Horizontal elongation			**PUFA/SFA**		
Population (PO)	1	7.91	**10.37 ****	1	5.39	**7.06 ****
Treatment (TR)	3	2.86	**3.75 ***	3	3.80	**4.98 ****
Time (TI)	3	9.79	**12.83 ****	3	1.46	1.91
POxTR	3	0.06	0.08	3	0.12	0.16
POxTI	3	0.72	0.95	3	0.21	0.28
TRxTI	9	0.91	1.19	9	1.88	**2.46 ***
POxTRxTI	9	0.56	0.73	9	0.80	1.04
Res	128	0.76		64	0.76	
	N° of new apical			**PUFA**		
Population (PO)	1	12.72	**14.33 ****	1	7.18	**8.51 ****
Treatment (TR)	3	0.25	0.28	3	2.2	**2.61 ***
Time (TI)	3	2.27	2.56	3	1.83	2.17
POxTR	3	0.41	0.47	3	0.27	0.31
POxTI	3	1.89	2.13	3	0.6	0.71
TRxTI	9	1.45	1.63	9	1.21	1.43
POxTRxTI	9	0.58	0.65	9	0.91	1.08
Res	128	0.89		64	0.84	
	18:3 n-3/18:2 n-6			**16:3 n-3/16:2 n-6**		
Population (PO)	1	7.18	**8.51****	1	0.02	0.05
Treatment (TR)	3	2.2	**2.61***	3	11.08	**29.78****
Time (TI)	3	1.83	2.17	3	3.18	**8.56****
POxTR	3	0.27	0.31	3	0.33	0.89
POxTI	3	0.6	0.71	3	0.08	0.23
TRxTI	9	1.21	1.43	9	2.74	**7.37****
POxTRxTI	9	0.91	1.08	9	0.28	0.74
Res	64	0.84		64	0.37	
	18:2 n-6/16:2 n-6			**18:3 n-3/16:3 n-3**		
Population (PO)	1	7.67	**15.06****	1	2.76	**6.36***
Treatment (TR)	3	6.09	**11.96****	3	6.53	**15.04****
Time (TI)	3	3.28	**6.44****	3	3.87	**8.91****
POxTR	3	0.22	0.44	3	0.12	0.27
POxTI	3	1.38	2.70	3	1.02	2.35
TRxTI	9	2.13	**4.18****	9	2.98	**6.86****
POxTRxTI	9	0.62	1.22	9	0.40	0.91
Res	64	0.51		64	0.43	
	N			**C**		
Population (PO)	1	0.04	0.09	1	0.03	0.36
Treatment (TR)	3	4.03	**9.33****	3	0.11	1.29
Time (TI)	2	17.1	**39.61****	2	41.3	**484.4****
POxTR	3	0.19	0.46	3	0.18	2.10
POxTI	2	0.35	0.83	2	0.26	3.04
TRxTI	6	2.46	**5.71****	6	0.25	**2.96***
POxTRxTI	6	0.24	0.56	6	0.27	3.19
Res	70			70	0.09	
	C:N					
Population (PO)	1	0.2	0.8			
Treatment (TR)	3	**3.4**	**12.1****			
Time (TI)	2	**23.1**	**82.9****			
POxTR	3	0.6	2.0			
POxTI	2	0.1	0.4			
TRxTI	6	**2.0**	**7.3***			
POxTRxTI	6	0.1	0.5			
Res	70	0.3				
	Epiphytes cover					

(continued on next page)

Table 1 (*continued*)

Treatment (TR)	3	1.9	8.6**
Time (TI)	2	18.5	82.5**
TRxTI	6	0.9	
Res	48	0.2	0.2
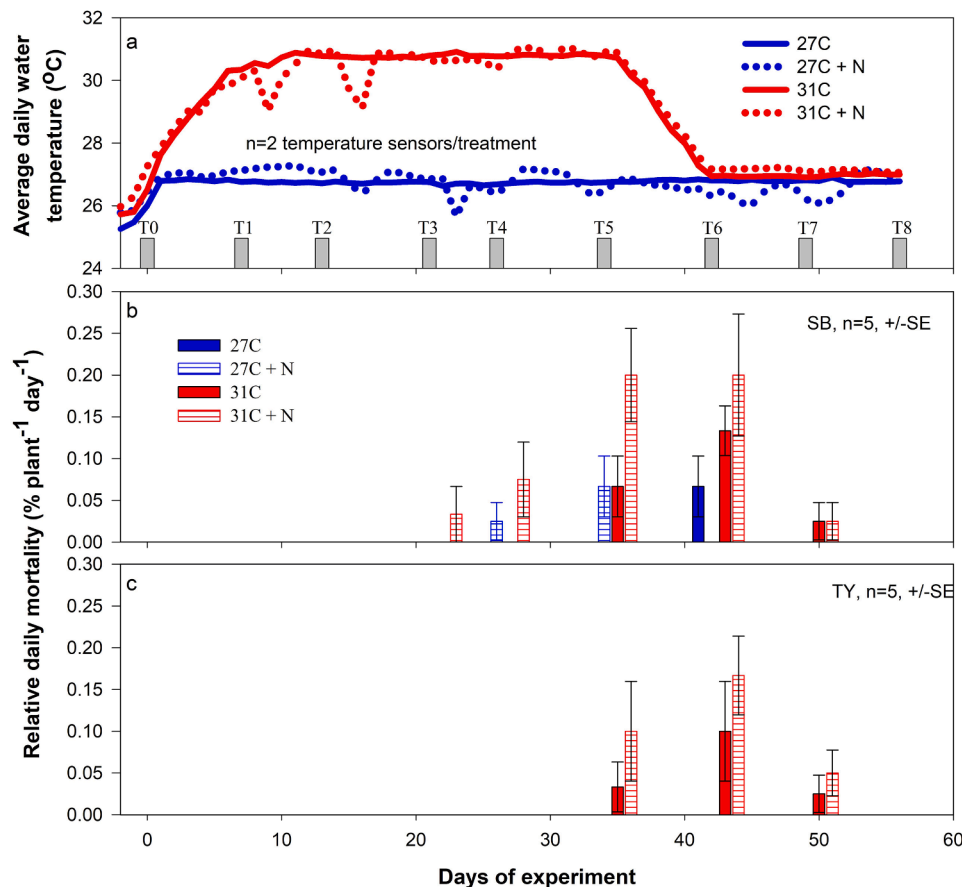
Fig. 4. Relative daily mortality of *Halophila stipulacea* plants measured for all the treatments (27 °C; 27 °C and nutrients; 31 °C; 31 °C and nutrients) and time points (T_0, T_1, T_2, T_3, T_4, T_5, T_6, T_7 and T_8) of South beach (Panel B) and Tur Yam (Panel C) populations. Missing bars indicates no leaf mortality present. Results are expressed as mean ± SE (n = 5).

4. Discussion

4.1. Effects of warming

The expected warming of 4 °C in the GoA did not demonstrate negative effects on most of the seagrass traits, indicating that their warming threshold was not exceeded in this experiment, and potentially will not be exceeded during this century based on IPCC predictions under the climate scenario of higher greenhouse gas emission (RCP 8.5) or on local climate change studies (Fine et al., 2013). Indeed, native H. stipulacea populations from the Persian Gulf and the Indian Ocean, are regularly exposed to temperatures of 30–31 °C, often reaching a maximum of 34 °C in summer (Tyberghein et al., 2012; Naser, 2014; Campbell et al., 2015). Recent studies comparing native plants from GoA and invasive from the Mediterranean Sea reported negative photo-physiological and morphological responses of native plants to experimental temperatures of 30–32 °C, however, invasive plants from the Mediterranean Sea and native from the eastern coast of Africa showed to be un-affected/or even favoured by warming (Georgiou et al., 2016; Nguyen et al., 2020; Viana et al., 2020). These results pointed towards a population-specific response modulated by local temperature regimes, as was also reported in other seagrass species such as *Z. marina*,

C. nodosa and *P. oceanica* (Winters et al., 2011; Beca-Carretero et al., 2018a, b; Bennett et al., 2021). Noticeably, growth rates observed at control conditions (27 °C) matched those reported for *in situ* measurements of the same seagrass meadows (TY and SB) in the summertime when plants were exposed to 26–27 °C (Azcárate-Garcia et al., 2020). These results confirm the robustness of our experimental design and conclusions of this study.

Increases in temperature favoured the accumulation of SFA relative to PUFA, indicating a clear reduction in unsaturation levels in photosynthetic structures of *H. stipulacea*. Particularly, warming enhanced increases in the Palmitic acid (16:0), which were also observed with *Z. noltei* specimens exposed to thermal and acidification stress (Franzitta et al., 2021). This SFA is known to play a key functioning role in the photosystem II where it provides stability to the thylakoid membrane (Gounaris and Barber, 1985; Duarte et al., 2018). In addition, both unsaturation biomarkers (16:3n-3/16:2n-6 and 18:3n-3/18:2n-6) showed a marked reduction in their values with increases in temperature, as was previously observed in marine and terrestrial primary producers (i.e. Sanina et al., 2008; Garcia et al., 2016). Noteworthy, responses of 16:3n-3/16:2n-6 were clearly higher than in 18:3n-3/18:2n-6. These outcomes can be explained because warming reduces the requirement of unsaturation levels in the membrane of the

Table 2

Nitrogen (N) (% of DW), Carbon (C) (% of DW) and C:N of *Halophila stipulacea* leaves measured for all the treatments (27 °C; 27 °C and nutrients; 31 °C; 31 °C and nutrients) and time points (T_1, T_3 and T_8) of South Beach (Panel B) and Tur Yam (Panel C) populations. Results are expressed as mean ± SE (n = 4).

Population	Time	Treatment	(N) %	(C) %	C:N
TY	1	27 °C	0.77 ± 0.1	26.56 ± 1.5	34.6 ± 2.1
	1	27 °C + Nut	0.93 ± 0.1	28.08 ± 1.0	30.4 ± 2.6
	1	31 °C	1.05 ± 0.1	27.93 ± 0.3	26.8 ± 2.5
	1	31 °C + Nut	1.02 ± 0.1	27.11 ± 0.3	27.0 ± 2.9
	3	27 °C	0.95 ± 0.1	26.86 ± 0.4	28.5 ± 2.6
	3	27 °C + Nut	1.25 ± 0.2	26.51 ± 0.4	21.7 ± 3.0
	3	31 °C	1.40 ± 0.2	27.10 ± 0.3	19.6 ± 2.1
	3	31 °C + Nut	1.22 ± 0.2	26.09 ± 0.8	22.0 ± 3.6
	8	27 °C	0.83 ± 0.2	18.49 ± 2.1	22.8 ± 2.1
	8	27 °C + Nut	0.93 ± 0.1	17.08 ± 0.7	18.5 ± 1.5
	8	31 °C	0.65 ± 0.1	16.08 ± 1.7	24.8 ± 0.4
	8	31 °C + Nut	1.05 ± 0.1	18.49 ± 0.7	17.9 ± 1.9
SB	1	27 °C	0.8 ± 0.2	26.6 ± 0.8	34.2 ± 6.1
	1	27 °C + Nut	0.9 ± 0.1	27.5 ± 0.6	29.3 ± 2.2
	1	31 °C	0.9 ± 0.1	26.9 ± 0.8	28.5 ± 1.5
	1	31 °C + Nut	0.8 ± 0.1	25.8 ± 2.0	32.3 ± 3.0
	3	27 °C	1.0 ± 0.1	27.1 ± 0.3	27.4 ± 3.1
	3	27 °C + Nut	1.3 ± 0.1	27.4 ± 0.5	21.6 ± 1.2
	3	31 °C	1.4 ± 0.2	27.5 ± 0.7	20.5 ± 2.8
	3	31 °C + Nut	1.2 ± 0.1	27.2 ± 0.3	23.5 ± 2.3
	8	27 °C	0.8 ± 0.1	15.5 ± 2.1	15.5 ± 2.1
	8	27 °C + Nut	1.0 ± 0.2	17.4 ± 1.1	17.7 ± 2.7
	8	31 °C	0.7 ± 0.1	19.3 ± 2.1	27.8 ± 2.1
	8	31 °C + Nut	1.2 ± 0.2	20.4 ± 1.6	18.0 ± 2.0
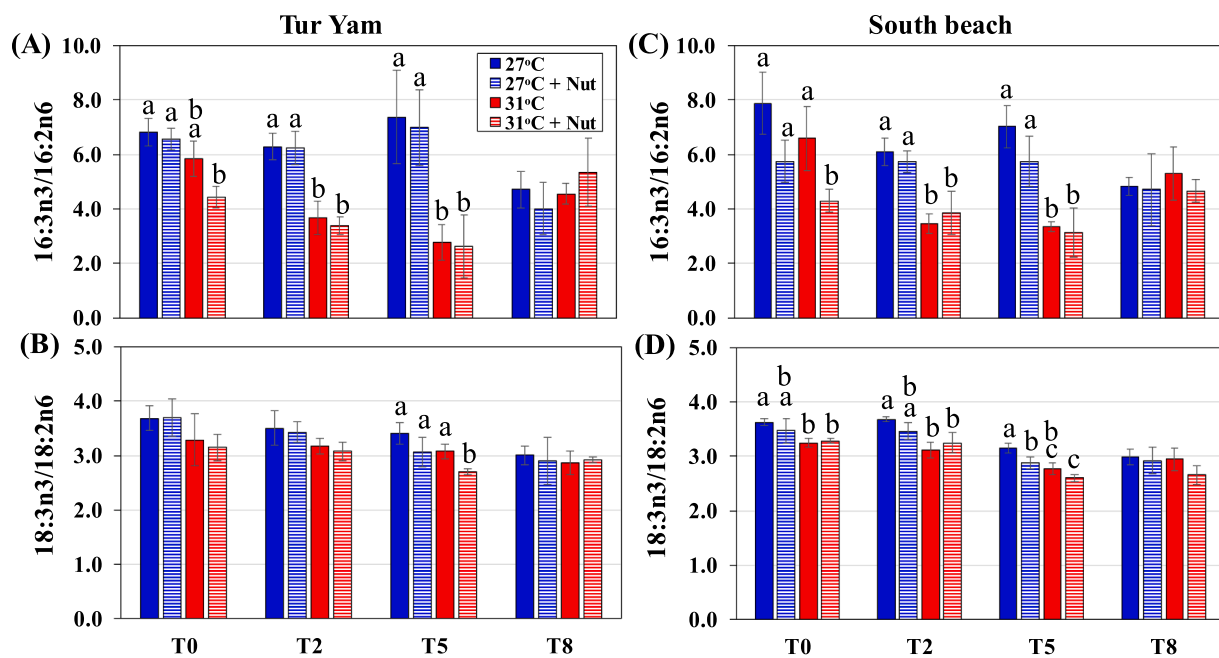
Fig. 5. Unsaturation indexes: 16:3n3/16:2n6 (Panel A and C) and 18:3n-3/18:2n-6 (Panel B and D) of leaves of *Halophila stipulacea* from all the treatments (27 °C; 27 °C and nutrients; 31 °C; 31 °C and nutrients) and time points (T_0, T_2, T_5 and T_8) of Tur Yam (Panel A and B) and South Beach (Panel C and D). Results are expressed as mean ± SE (n = 3). Letters indicate significant differences between treatments according to the PERMANOVA.

thylakoids in the chloroplasts to achieve optimal membrane fluidity and, photosynthetic and integral membrane' proteins functioning (i.e. Upchurch, 2008; Hixson and Arts, 2016). More specifically, plant FA desaturase (FAD) enzymes are involved in heat-stress responses due to their role in adjusting the unsaturation levels of membrane lipids (Martinière et al., 2011; Niu and Xiang, 2018). Desaturases FAD 6, 7, and 8 modulate their activity in response to physiological demands to favour the synthesis of (i) dienoic FAs (family n-6), which are more involved in the photosynthesis machinery, or the synthesis of (ii) trienoic FAs (family n-3), which are more related to the biogenesis and maintenance of the membrane of the chloroplasts (McConn and Browse,

1998; Routaboul et al., 2000). In line with our results, terrestrial plants and algae are capable of changing the unsaturation levels in membrane structures in response to abiotic stress by the specific enzymes, the FADs (Los and Murata, 1998). Particularly, FADS gene expression is involved in the acclimation of primary producers to thermal gradients and nutrient stress (Los et al., 2013; Horváth et al., 2012). For instance, in green algae, the expression of n-3 PUFA desaturase (ω-3 FAD1, ω-3 FAD2) and omega-6 fatty acid desaturase (ω-6 FAD) was favoured under phosphate stress conditions (Anne-Marie et al., 2020).

In relation to the carbon elongation indexes, 18:2n-6/16:2n-6 demonstrated significant negative responses to thermal stress,
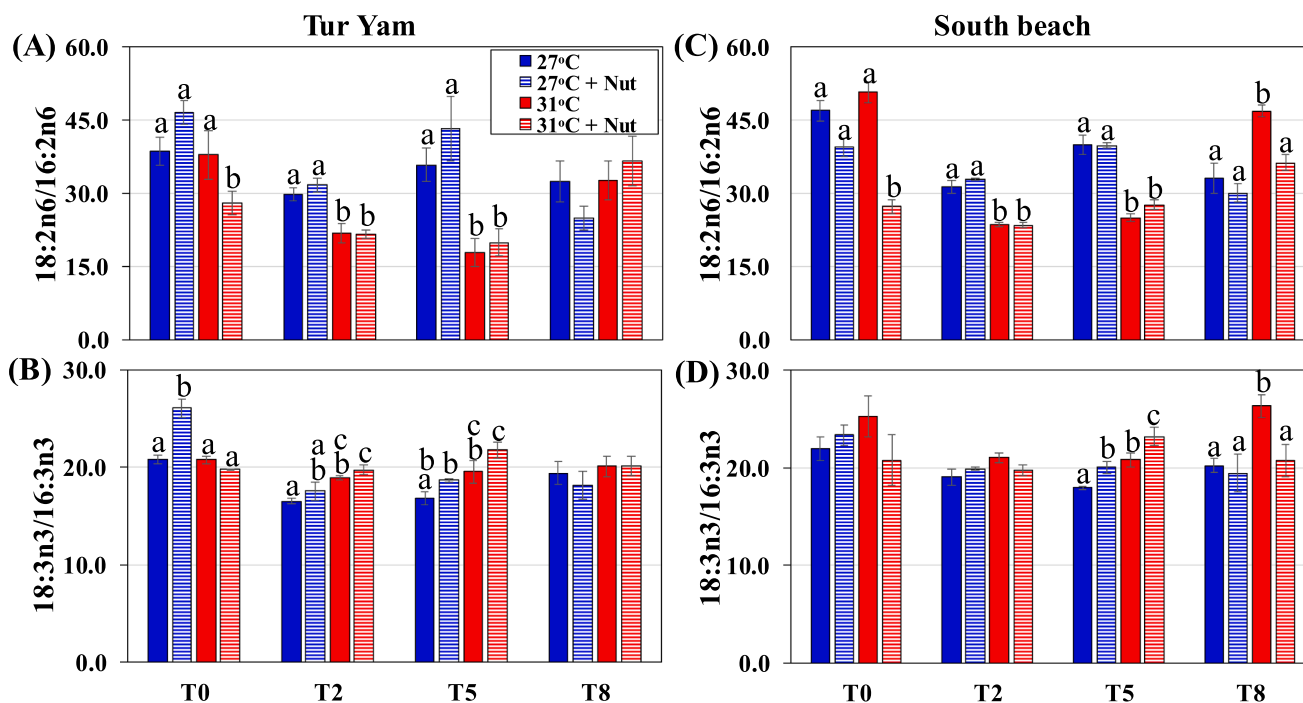
Fig. 6. Carbon elongation indexes: 16:2n-6/18:2n-6 (Panel A and C) and 16:3n-3/18:3n-3 (Panel B and D) of leaves of *Halophila stipulacea* from all the treatments (27 °C; 27 °C and nutrients; 31 °C; 31 °C and nutrients) and time points (T$_0$, T$_2$, T$_5$ and T$_8$) of Tur Yam (Panel A and B) and South Beach (Panel C and D). Results are expressed as mean ± SE (n = 3). Letters indicate significant differences between treatments according to the PERMANOVA.

TABLE 3

Percentage of cover of epiphytes measured in each aquarium for all the treatments (27 °C; 27 °C and nutrients; 31 °C; 31 °C and nutrients) and time points (T$_0$, T$_2$ and T$_8$) of South Beach (Panel B) and Tur Yam (Panel C) populations. Results are expressed as mean + SD (n = 5).

| Treatment	Time	Epiphytes (% of cover)
27 °C	0	3.4 ± 2.8
	5	22.2 ± 6.8
	8	32.4 ± 7.5
27 °C + Nut	0	4.7 ± 3.4
	5	54.5 ± 17.4
	8	45.0 ± 13.4
31 °C	0	2.2 ± 1.8
	5	45.1 ± 8.9
	8	32.3 ± 7.5
31 °C + Nut	0	4.1 ± 3.3
	5	44.7 ± 4.9
	8	35.1 ± 8.9

however, 18:3n-3/16:3n-3 showed significant increases with warming. Changes in both indicators were mainly explained by variations in C16 PUFAs in comparisons with C18 PUFAs, reinforcing the same finding observed in unsaturation biomarkers. Collectively, these results pointed out that C16 PUFAs, which are synthesized by the "prokaryotic pathway", are more sensitive to thermal stress than C18 PUFAs, which are synthesized by the "eukaryotic pathway" (Fig. 7). These results were consistent in both populations (TY and SB). Similar responses, but with less pronounced patterns, were reported by Franzitta et al. 2021 with the seagrass *Z. noltei* exposed to heat stress treatments. These results can be explained because 16:3 PUFAs are only synthesized and found in the membranes of the thylakoid in the chloroplasts where photosynthesis takes place (Mongrand et al., 1998), while, C 18:3 PUFAs are synthesized in both the chloroplast (prokaryotic pathway) and the extra chloroplast membranes (eukaryotic pathway) and are essential components of any cellular membrane, including non-photosynthetic structures (Browse et al., 1986; Wallis et al., 2002). The synthesis and mobilization of C16 PUFAs require lower energy than C18 PUFAs,

therefore, we can hypothesize that "16:3 plants (prokaryotic pathway)" favour less energetic processes to remodel the membrane of the thylakoids than "18:3 plants (eukaryotic pathway)", and thus, C16 PUFAs are more prone to suffer early modulation by environmental stress than C18 PUFAs. Nevertheless, this assumption was not tested in this study, therefore, additional investigations are needed to corroborate this hypothesis. In line with our results, studies with green microalgae observed that under nutrient stress (*N*-starvation) conditions and stationary phases a decrease in the ratio 18:2n-6/16:2n-6 was observed, indicating that the prokaryotic pathways predominate in relation to the eukaryotic pathway under less favourable conditions for growth (Cohen and Khozin-Goldberg, 2010).

4.2. Effects of nutrients increase

The generated nutrient enrichment was successfully implemented during the exposure phase (T$_2$-T$_5$) (Fig. 2b-e). However, an overall increase of nutrients in the seawater was observed in all aquaria (including the controls that were not enriched) during the recovery phase (T$_7$ and T$_8$). These patterns can be explained by the maturity of the associated microorganisms involved in marine nutrient cycles that can modify nitrogen and phosphorus structures and release them into the environment as nitrate, ammonium or phosphate (Pajares and Ramos, 2019; DyhrMan et al., 2007). Nevertheless, these hypotheses were not tested in this experiment.

At control conditions, levels of N (% of DW) in seagrass tissues were always lower than 1.2 indicating *N*-limitation in seagrasses (Duarte, 1992) as was also reported in plants of *H. stipulacea* sampled from natural meadows within the northern GoA (Beca-Carretero et al., 2019). In this study, the higher levels of N in seagrass leaves under DIN enriched conditions can be explained by the larger availability of N in the enriched aquaria. Increases in nutrient contents damaged the photosynthetic efficiency as was demonstrated by significant depletions in Fv/Fm levels from control (0.71 ± 0.01) to nutrient (0.67 ± 0.01) treatments. In support of this, the high exposure to ammonium was reported to constrain the synthesis of the ATP needed for the electron transport

Chloroplast

Prokaryotic pathway

Endoplasmatic Reticulum

Eukaryotic pathway
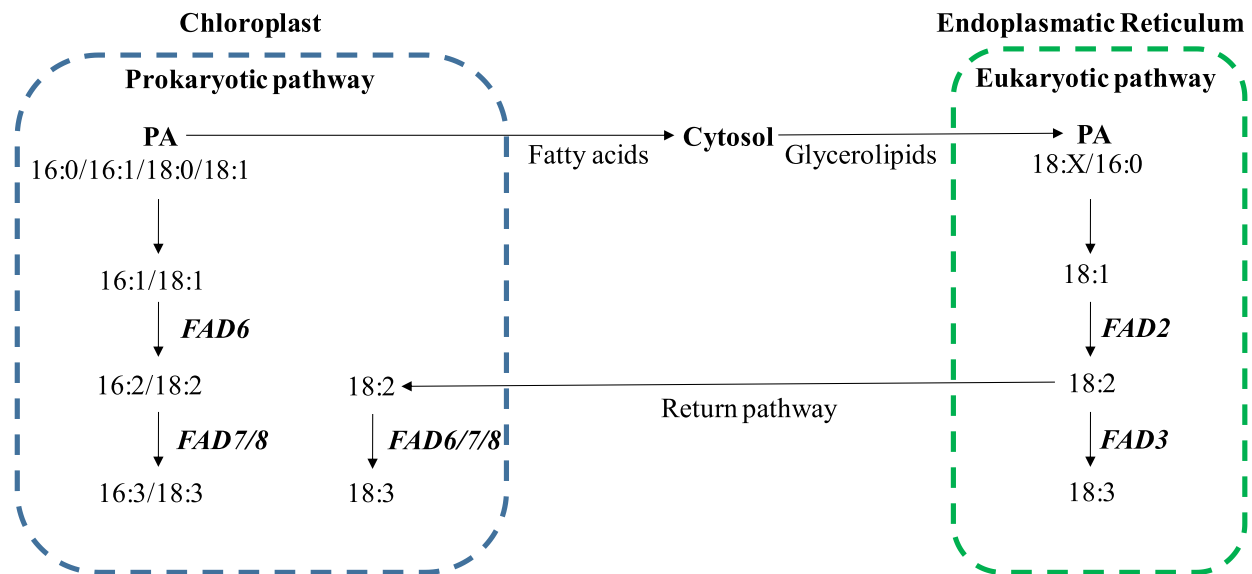
Fig. 7. Simplified scheme of fatty acid (FA) synthesis (based on He et al. 2020) in seagrasses. De novo FA synthesis occurs in the plastid (chloroplast), where the main products of FA biosynthesis are 16:0 and 18:1. A proportion of FAs remain in the chloroplast 'prokaryotic pathway'. The rest of FAs are exported into the cytosol and later to the endoplasmic reticulum to continue with the 'eukaryotic pathway'.

during the photosynthesis process (*reviewed in* Britto and Kronzucker, 2002). Similar detrimental trends were previously observed under experimental eutrophication conditions for *H. stipulacea* and other seagrass species (i.e. Campbell et al., 2006; Ontoria et al., 2019b). The observed reductions in growth rates can be explained by less efficient photosynthesis activity decreasing the ability to generate an excess of energy that can be later transformed into carbon (C) budgets (Alcoverro et al., 2001). It was evident that the higher nutrient treatment at 27 °C entailed significant proliferations of macroalgae (Table 3; Figure S1). Blooms of macroalgae and epiphytes on seagrasses have been usually correlated with increases in nutrient availability in the water column and have been reported to drive massive seagrass losses and habitat collapses (Dennison et al., 1989; Brodersen et al., 2015).

Increases in nutrient levels significantly enhanced the accumulation of PUFA, mostly explained by increments in n-6 PUFA in detriment to the accumulation of n-3 PUFA, that was accompanied by reductions in SFA levels. The unsaturation biomarker 18:3n-3/18:2n-6 showed a robust negative trend under nutrient increase simulations, however, the other unsaturation biomarker 16:3n-3/16:2n-6 did not report any significant pattern. The carbon elongation biomarker 18:3n-3/16:3n-3 displayed a significant and positive pattern with increases in nutrient concentrations, while for the 18:2n-6/16:2n-6 indexes, a negative trend with nutrient concentration was observed. These changes were primarily associated with changes in 18:3n-3, while 16:3n-3 remained more stable, indicating that 18:3 PUFAs from seagrass leaves are more sensitive to nutrient stress than 16:3 PUFAs, which were otherwise more sensitive to temperature changes. Similar trends were found in Arabidopsis plants and green microalgae under N starvations conditions and this was explained by (i) specific co-ordination of target FA groups in thylakoids and plastoglobules of chloroplasts (Gaude et al., 2007) and by (ii) the predominance of the prokaryotic pathway over the eukaryotic pathway under nutrient stress (Cohen and Khozin-Goldberg, 2010). 18:2n-6/16:2n-6 indexes, a negative trend with nutrient concentration was observed. Again, this trend was mainly attributed to higher increases of 16:2n-6 than increment of 18:2n-6 with nutrient enrichment, indicating the fundamental role of 16:2 PUFAs regulating the responses of seagrasses to nutrient stress.

4.3. Combined effects of warming and simulated eutrophication

Our results demonstrated that warming enhances the negative effects

of nutrient increase in most of the seagrass indicators including photosynthetic efficiency, growth, biochemical composition and mortality rates. In this direction, some studies reported that the harmful synergetic effects of temperature and nutrient inputs significantly reduced photosynthetic capacity and increased necrotic tissues and mortality in the temperate seagrass species such as *Cymodocea nodosa, Zostera Capensis, Z. marina* and *P. oceanica* (Moreno-Marín et al., 2018; Mvungi and Pillay, 2019; Ontoria et al., 2019a, b; Pazzaglia et al., 2020; Helber et al., 2021a; *reviewed in* Nguyen et al., 2021). On the other hand, recent studies did not observe a synergetic response of temperature and nutrient increase in *Z. marina* and in seedlings of the tropical species *Enahlus acoroides* (Kaldy, 2014; Viana et al., 2020), suggesting a species-specific response. In this study, F_v/F_m values of plants exposed to combined stress treatments reached the lowest values (~0.62) during the experiment alongside the highest mortality rates. In addition, TY performed better and had lower mortality rates than SB when exposed to combined stress conditions. This might be associated with a higher pre-existing anthropogenic pressure at TY in comparison with the low level of anthropological stress in the near-pristine area of SB (Mejia et al., 2016). These population-specific responses may be attributed to the "ecological stress memory" as was previously suggested by Helber et al. 2021b. Ecological stress memory is defined as any response of a single plant after a stress experience that modifies the response of the plant towards future stress events including the mode of interaction with other ecological units (Walter et al., 2013). In this support, additional results from this study suggested an environmental microbial fingerprint, which may reflect *in situ* environmental conditions, and may add a holobiont level of plasticity to seagrasses, favouring their acclimation to nutrient and thermal stress (Szitenberg et al. 2022).

The unsaturation biomarker 18:3n-3/18:2n-6 reported clear synergetic detrimental patterns under combined treatments in comparison with control conditions. This finding can be explained because decreases in ALA (18:3n-3) were related with negative increases in reactive oxygen species (ROS) quenching and membrane remodelling in seagrass leaves (Franzitta et al., 2021). It was shown that the damage of the photosynthetic machinery (indicated by the reductions in Fv/Fm values) and lower growth rates correlated with reductions in 18:3n-3/18:2n-6, indicating that this biomarker can reflect the eco-physiological state of seagrasses. Further examining this biomarker, plants from control conditions were found to contain 18:3n-3/18:2n-6 that were equal or higher than 3, while under thermal and nutrient stress conditions, these ratios

were always lower than 3, with the lowest values reported in T_5 (2.6 and 2.7 for SB and TY, respectively). Thus, values of the 18:3n-3/18:2n-6 that were lower than 3.0 might indicate some degree of stress or/and vulnerability of seagrasses induced by anomalous temperature or nutrient increase. In support of this, a recent study with *H. stipulacea* specimens from the GoA reported 18:3n-3/18:2n-6 ranging from 3.0 to 3.7 in plants collected at depths ranging from 6 to 21 m in semi-pristine areas (Beca-Carretero et al., 2019). In addition, the proposed biomarker based on carbon elongation 18:3n-3/16:3n-3 reported synergetic positive responses to both thermal and nutrient stress, again, mostly explained by changes in C16 PUFAs in comparison with C18 PUFAs. Previous studies in primary producers highlighted specific functional and structural responses of FA groups as a function of their carbon length (Bach and Faure, 2010).

Overall, growth, physiological and FA responses to increases in temperature, nutrient and their combination, were more accentuated at T_5 than at T_2. These trends can be explained because the ecophysiological state of seagrasses varies according to the length of time of exposure to stress conditions and the nature and intensity of such stresses (Alcoverro et al., 2001; Gerendás et al., 1997; Bittsánszkyet al., 2015; Reszczyńska and Hanaka, 2020). Noteworthy, in this study a clear recovery of most of the seagrass descriptors was observed, highlighting the great capability of this species to recover from stress conditions. These results further demonstrate the high resilience of this species to cope with such stress, which might partially explain its high success to colonize new habitats, in addition to being able to persist in disturbed habitats (Winters et al., 2020). Lastly, our results indicate that increases of temperature or nutrients and their combination, might limit the capacity of seagrasses to produce the nutritionally important n-3 PUFA, which could have negative effects on its associated trophic food web (Pazzaglia et al., 2022; Beca-Carretero et al., 2021).

This study highlights the fast and specific response of the FA indicators used here in comparison with "classic" (e.g., physiological [Fv/Fm], biochemical [photosynthetic pigments, C and N], morphological and production) indicators (Roca et al., 2016). Our outcomes suggest the existence of potential FA thresholds based on FAs unsaturation and carbon elongation levels that would hint to the mechanism behind the physiological stress response of seagrasses.

4.4. Management considerations

Our outcomes demonstrate the vulnerability of seagrasses to the combined effect of thermal and nutrient stress. Supporting our results, previous seagrass studies investigating the combined effects of warming and other stressors such as sulphide, anoxia or light reduction, have also suggested synergetic negative responses on seagrass ecosystems (Koch et al., 2007; York et al., 2013). Recent studies have shown that the rates of warming in the Gulf's water are actually faster than the average of world's coastal warming trends (Fine et al., 2013, Nguyen et al., 2020). While it might be difficult to directly manage the effects of global warming, sources of eutrophication are usually on local scales. In the northern GoA for example, much of the eutrophication is attributed to anthropogenic activity such as coastal development and terrestrial runoffs from local agricultural fields (reviewed in Winters et al., 2017). It should be noted that the results provided here are based on mesocosm experiments, therefore caution is necessary to interpret and extrapolate these results to real conditions and future predictions. However, these mesocosm experiment send a clear message: For seagrasses to be able to survive climate change, managers must put efforts on limiting other stressors such as nutrient increase.

CRediT authorship contribution statement

Pedro Beca-Carretero: Conceptualization, Data curation, Formal analysis, Investigation, Writing – original draft, Writing – review & editing. **Tomás Azcárate-García:** Data curation, Methodology,

Investigation, Writing – original draft. **Mirta Teichberg:** Data curation, Methodology, Investigation, Writing – original draft. **Priyanka Patra:** Data curation, Methodology. **Farhan Feroze:** Data curation, Methodology. **Maria J. González:** Data curation, Methodology, Investigation, Writing – original draft. **Isabel Medina:** Conceptualization, Data curation, Methodology, Supervision, Writing – original draft, Writing – review & editing. **Gidon Winters:** Conceptualization, Data curation, Methodology, Writing – original draft, Writing – review & editing.

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 will be made available on request.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ecolind.2022.109184.

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