Depth-induced adjustment of fatty acid and pigment composition suggests high biochemical plasticity in the tropical seagrass *Halophila stipulacea*

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ABSTRACT: *Halophila stipulacea* is the dominant subtidal meadow-forming seagrass in the tropical Gulf of Aqaba (GoA; northern Red Sea), an area characterised by warm and oligotrophic waters. This species occurs across a wide range of environmental conditions, and is considered one of the most deep-growing seagrasses worldwide. This investigation assessed, for the first time, the morphological and biochemical responses of *H. stipulacea* populations adapted to different depths (6–21 m), focussing on total fatty acid (TFA) content and composition, and photosynthetic pigments. *H. stipulacea* leaves (collected July 2016) from greater depths contained 25% more TFA and 22% more photosynthetic pigments than plants from shallower depths. Increases in TFAs were mainly related to higher levels of polyunsaturated fatty acids (PUFAs) and a lower production of saturated fatty acids (SFAs). As PUFAs promote fluidity in chloroplast membranes as well as facilitate electron transport in the photosystems, their observed increase with depth may favour optimal photosynthetic activity under less favourable (e.g. low-irradiance) conditions. Cluster analysis of data on fatty acid composition derived from the literature for other seagrass species across a range of geographic locations highlights the fact that PUFA levels in *H. stipulacea* leaves are more similar to those found in seagrass species inhabiting higher latitudes, and thus colder regions, than in tropical or subtropical species. With *H. stipulacea* successfully spreading into non-native areas, such as the eastern Mediterranean and Caribbean seas, it is critical to understand the eco-physiological mechanisms that allow this species to adapt to a wide range of environmental conditions.

KEY WORDS: Biochemical adaptation · PAR reduction · Essential fatty acids · Polyunsaturated fatty acids · Saturated fatty acids · *Halophila stipulacea* · Gulf of Aqaba · Invasive species · Species comparison

1. INTRODUCTION

Seagrasses are keystone marine macrophytes that provide several ecological services to coastal marine ecosystems, such as high primary productivity, nutrient cycling, shelter and nursery habitat, protection of the coastline and carbon storage, which are estimated at \$2.8 million USD km^{-2} yr^{-1} (Duarte et al. 2006, Costanza et al. 2014). Surprisingly, however, they do not receive the same appreciation as other marine ecosystems such as coral reefs or saltmarshes (Nordlund et al. 2018). The tropical Gulf of Aqaba (GoA) is characterised by warm (21–27°C) and oligotrophic waters (Winters et al. 2017). In this region, *Halophila stipulacea* (Forsk.) Aschers is the most common seagrass species, representing a key com-

ponent of adjacent coral reef systems (Edwards 1987). It is a small, dioecious, tropical species (Nguyen et al. 2018), native to the Red Sea, Persian Gulf and Indian Ocean (Winters et al. 2006, El Shaffai 2011). In the northern GoA, *H. stipulacea* can be found growing from high-light shallow (2 m) to low-light deep (>50 m; Sharon et al. 2011, Winters et al. 2017) environments, and is one of the deepest seagrass reported worldwide (Lipkin et al. 2003, Short et al. 2007, Sharon et al. 2011).

Previous studies have highlighted the marked phenotypic plasticity of *H. stipulacea*, which has allow it to adapt to a wide range of environmental conditions including temperature, irradiance, salinity and nutrient concentrations (Sharon et al. 2009, 2011, Mejia et al. 2016, Oscar et al. 2018). Variations in irradiance and spectral composition have been shown to affect the performance of seagrasses, such as *Halophila* spp., in controlling metabolic processes such as photosynthetic and respiration rates, resulting in local morphological and physiological adaptations (e.g. Beer & Björk 2000, Sharon et al. 2009, 2011, Rotini et al. 2017). In addition, differences in light regimes can affect reproductive effort and seed germination, processes that are associated with the settlement and colonization capacity of seagrass species (Diaz-Almela et al. 2007). Previous studies observed high biometric plasticity in *H. stipulacea*, with the development of larger leaves and production of significantly higher photosynthetic pigment levels at greater depths, but a reduction in percentage cover of the species (Lipkin 1979, Rotini et al. 2017, Winters et al. 2017). Sharon et al. (2011) documented *H. stipulacea* growing at a depth of 48 m, where it received less than 5% of surface photosynthetically active radiation (PAR), demonstrating that this species may extend to very low PAR relative to the surface. Mechanisms related to the exceptional capacity of *H. stipulacea* to tolerate strong gradients of light availability are not completely understood.

Seagrasses, like other marine primary producers, accumulate many essential fatty acids (EFA), such as α-linolenic acid (ALA) and linoleic acid (LA) (Behrens & Kyle 1996, Dalsgaard et al. 2003), which are not biosynthesised by higher trophic levels (Arts et al. 2001). In photosynthetic organisms, lipid metabolism and fatty acid synthesis play an important role in the formation of structural membranes and in energy storage capability (Rabbani et al. 1998, Klyachko-Gurvich et al. 1999, Mendoza et al. 1999). Polyunsaturated fatty acids (PUFAs) are mainly partitioned into structural lipids (glycolipids and phospholipids) that constitute the cellular membranes. In particular, PUFAs promote the fluidity of the thylakoid membranes of chloroplasts, enhance electron transport in the photosystems, and therefore optimise photosynthetic activity (Gombos et al. 1994, Sanina et al. 2004, 2008). Additionally, PUFA synthesis in photosynthetic organisms has been suggested to determine the environmental limits in which they can survive (Iba 2002). On the other hand, saturated fatty acids (SFAs) are mainly partitioned into triacylglycerols (TAGs), which are used as reserve energy compounds in marine primary producers (e.g. Bravo et al. 2001, Pal et al. 2011, Solovchenko 2012), including in seagrass leaves and seeds (Yuvaraj et al. 2012, Pasaribu et al. 2017). In aquatic plants, fatty acid metabolism is strongly regulated by environmental factors such as temperature, irradiance or salinity (Viso et al. 1993, Schmid et al. 2014, Sousa et al. 2017, Beca-Carretero et al. 2018).

Here, we hypothesized that both the morphological and physiological adaptations (photosynthetic pigments, fatty acids) of *H. stipulacea* occur in response to decreasing PAR and changing spectrum along an increasing depth profile. To test this hypothesis, we selected 2 independent transects along a depth gradient within a meadow of *H. stipulacea.* The transects were exposed to similar environmental conditions to ensure that other factors, such as hydrodynamic forces or anthropogenic pressures, did not affect the structural and biochemical responses of the plants. We then assessed (1) changes in leaf biometry (leaf area) and TFA concentration and composition, (2) shifts in photosynthetic pigment levels, (3) any potential correlation between fatty acids and pigments with increasing depth, and (4) compared the contents of PUFAs (% dry weight, DW) and PUFA/SFA ratios in *H. stipulacea* from this study (northern GoA) to other seagrass populations from other sites around the world.

2. MATERIALS AND METHODS

2.1. Study area

The study area was located on the western shore of the northern tip of the GoA, northern Red Sea, Israel (Eilat; Fig. S1 in the Supplement at www. int-res.com/articles/suppl/m608p105_supp.pdf). The meadows that were studied belong to a medium-sized (61 900 m^2) seagrass meadow characterised by high light penetration, a pronounced slope (18°) and coarse-grained sediment, with high hydrodynamics and low anthropogenic pressures (Mejia et al. 2016).
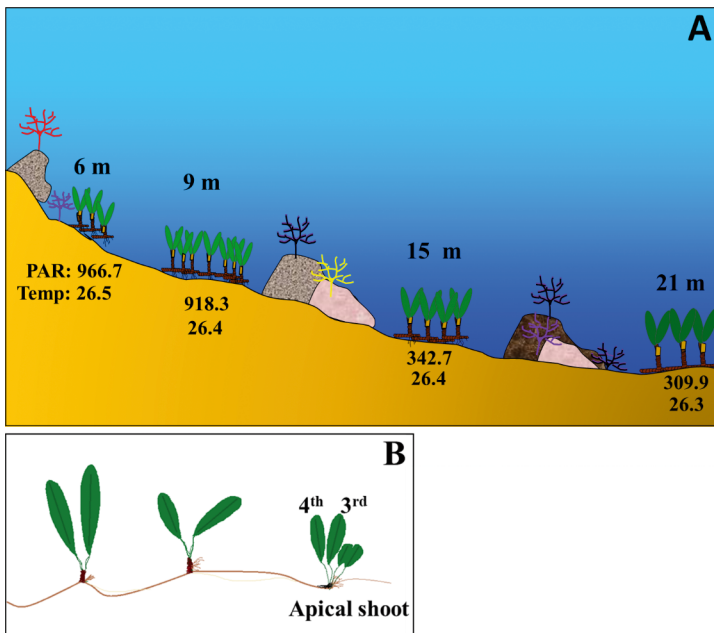
Fig. 1. Schematic drawings representing (A) the sampling design at 3 different depth levels (6 or 9, 15 and 21 m) in the *Halophila stipulacea* meadow, modified from Rotini et al. (2016), and (B) representation of the apical shoot highlighting the 3rd and 4th youngest leaves used for biochemical and morphological analysis. PAR: photosynthetically active radiation (μmol photon $m^{-2} s^{-1}$); Temp: temperature (°C)

H. stipulacea in this meadow co-occurs with hermatypic corals (Rotini et al. 2017, Winters et al. 2017). This study area was chosen because of its relative low human presence compared to other regions in the northern GoA (Mejia et al. 2016, Winters et al. 2017).

2.2. Sample collection

*H. stipulacea s*hoots were collected at 12:00 h from 2 different transects, T-1 (29.497° N, 34.911° E) and T-2 (29.500° N, 34.915° E; Fig. S1) at 3 different depths (6/9, 15 and 21 m) by SCUBA diving on 6 July 2016. Depths of 6 and 9 m represented the shallowest part of the meadow in T-1 and T-2, respectively. Differences in depths in the shallowest edges of the *H. stipulacea* meadow were related to the larger widespread presence of coral reefs at T-2 (9 m) than at T-1 (6 m). In both transects, plants were collected at intervals of 5–10 m along each depth to minimize the likelihood of collecting the same genotypes, as this marine plant is known for being a clonal species. We only selected apical shoots to ensure that the selected leaves were recently produced. *H. stipulacea* has a formation rate of new leaves at intervals of 4–12 d, depending on the season, with an average production of 8.1 d (Wahbeh 1984). Healthy shoots were

transported to the laboratory, where the third and fourth youngest leaves per apical shoot were selected for biochemical analysis (Fig. 1B).

Leaf length (mm) of *H. stipulacea* was measured (±1 mm) using a ruler for 10 freeze-dried leaves from each site and depth, haphazardly selected for subsequent biochemical analyses.

2.3. Biochemical analyses

Selected healthy green leaves were manually cleaned in distilled water and epiphytes were removed. Fresh biomass was hand-dried on blotting paper before freezing at −80°C for 6 h. Subsequently, leaves were freeze-dried for 12 h using a freeze-dryer system before being stored at −20°C (2–3 mo) until analyses were conducted. Selected leaf biomass was then ground to a fine powder using a bead mill homogenizer (Beadmill 4; Fisher Scientific) at 5 m s^{-1} for 3 min.

Seagrass fatty acids were analysed using the protocol described herein, which was previously applied to microalgae, algae and seagrasses (Schmid et al. 2014, Beca-Carretero et al. 2018). Fatty acid methyl esters (FAMEs) were obtained by direct transmethylation of ~20−30 mg of powdered seagrass biomass with dry methanol containing 2% (v/v) H_2SO_4. To avoid oxidation, vials were closed under nitrogen gas and heated at 80°C for 1.5−2 h under continuous stirring. After transmethylation, 1 ml of milli-Q water was added and FAMEs were extracted from the cooled samples using 1 ml of n-hexane. Analysis of FAMEs was performed as described by Beca-Carretero et al. (2018) using an Agilent 7890A/5975C gas chromatograph/mass selective detector (GC/MSD) series (Agilent Technologies) equipped with a flame ionization detector and a fused silica capillary column (DB-WAXETR, 0.25 mm × 30 m × 0.25 μm; Agilent Technologies, catalog no. 122-7332). Identification of FAMEs was achieved by co-chromatography with authentic commercially available FAME standard of fish oil (Menhaden Oil, catalogue no. 47116; Supelco). Total and individual fatty acid contents were quantified by comparison with a known quantity of added pentadecanoic acid 15:0 (99%; Alfa Aesar, catalogue no. A14664-09) as internal standard. The internal standard (10 μl, 5 mg ml^{-1}) was added to the ground seagrass powder prior to direct transmethylation. Results are expressed as

the mean values of 3 replicates (n = 3) for each transect and sampling depth.

Two consecutive pigment extractions with ~20–30 mg of seagrass powder were conducted in 5 ml of 80% acetone each. The 2 extractions were carried out over 20 and 4 h periods, respectively, with continuous stirring in darkness at 4°C. To avoid oxidation, vials with samples were closed under nitrogen gas and kept at 4°C during the extraction time. After the first extraction, the samples were centrifuged and the supernatant (5 ml) was kept in darkness at 4°C; the remaining material was used for the second extraction. Following the second extraction, the supernatants of both extractions were combined to a final volume of 10 ml, which was then used for pigment analysis. Chlorophylls *a* and *b* and carotenoids were quantified by a spectrophotometric absorbance reading (CARY 50 Scan UV-Visible Spectrophotometer), using the equations of Lichtenthaler & Buschmann (1983):

$$\text{Chl } a \text{ (µg ml}^{-1}\text{)} = 12.21E_{663} - 2.81E_{646} \tag{1}$$

$$\text{Chl } b \text{ (µg ml}^{-1}\text{)} = 20.13E_{646} - 5.03E_{663} \tag{2}$$

$$\text{Carotenoids (µg ml}^{-1}\text{)} =$$
$$[1000E_{470} - (3.27\text{chl } a) - (104\text{chl } b)] / 227 \tag{3}$$

where E_{xxx} = absorbance at *xxx* nm − absorbance at 725 nm.

2.4. Biochemical comparison across seagrass populations worldwide

Data of % PUFAs and PUFA/SFA ratios of seagrasses from different locations worldwide were compiled from Beca-Carretero et al. (2018) and Sousa et al. (2017) and were compared with the *H. stipulacea* data that is presented here. SST data were derived from the Bio-ORACLE database (www.bio-oracle.org) (Tyberghein et al. 2012) with a resolution of 0.5 km^2.

2.5. Environmental variables

Long term (2011–2015) salinity, Secchi depth (Sd, in m), sea surface temperature (SST) and surface water contents of NH_4, NO_2 and NO_3 measured at 1 m depth were obtained from Israel's National Monitoring Program at the Gulf of Eilat (September 2016; NMP; http://iui-eilat.ac.il/Research/NMPMeteoData.aspx). An estimation of the maximal level of underwater PAR at different depths was calculated

following the Beer-Lambert Law: $I_D = I_0 e^{-K_d}$, where I_D = light intensity at depth D (measured in Einsteins), I_0 = light intensity at the surface, and K_d = is the light attenuation coefficient. K_d was estimated according to the Duarte (1991) equation: $K_d = 1.7$ Sd. *In situ* data for temperature (°C) and PAR (µmol photon m^{-2} s^{-1}) in adjacent waters (29.493° N, 34.909° E; ~200–400 m from T-1 and T-2, respectively) were collected in July 2016 along a depth gradient (0 to 50 m) by the NMP (Fig. S1).

2.6. Statistical analyses

Prior to performing statistical analyses, data were ln transformed and the assumptions of normality (Kolmogorov-Smirnov test) and homogeneity (Bartlett's test) were tested. A 2-way ANOVA and post hoc Tukey's pairwise test were conducted to evaluate differences between transects and among depths in the biochemical and morphological descriptors. Data treatments and statistical analyses were performed using SPSS Statistics V13.0 (IBM Corporation). All values are reported as means and standard deviation (SD). Moreover, for conducting cluster and similarity profile (SIMPROF; Clarke et al. 2008) analyses, data of PUFA (% DW) and PUFA/SFA ratios from different seagrass species worldwide (n = 20) were first log transformed and standardized. Afterwards, we resembled the data and performed the analysis using Euclidean distance and the group average linkage method. Cluster and SIMPROF analyses were conducted using the statistical programme PRIMER + PERMANOVA 6.

3. RESULTS

3.1. Environmental variables

Estimated midday underwater PAR demonstrated the clarity of the coastal waters in the northern GoA, where values in the shallow parts of the meadows reached 1408.9 ± 87 and 969.8 ± 76 µmol photon m^{-2} s^{-1} at transects T-1 (6 m) and T-2 (9 m), respectively compared with midday PAR levels of 352.8 ± 32 µmol photon m^{-2} s^{-1} in the deeper areas of the meadows (21 m; Table 1). These results were in accordance with *in situ* measurements in adjacent waters obtained in July 2016 (Fig. S2B in the Supplement, Table 1). Temperatures in July 2016 did not vary significantly from 6 m depth (26.5°C) to 2 m depth (26.3°C) (Fig. S2A, Table 1); the northern GoA in par-

Table 1. Environmental variables measured in the study area in July 2011–2015, and in in adjacent waters in July 2016 (Israel's National Monitoring of the Gulf of Eilat; www.meteo-tech.co.il). PAR: photosynthetically active radiation

Environmental descriptors in the study area		
Secchi depth (m)		21.5 ± 3.9
Estimated PAR$_{max}$	At 6 / 9 m	1408.9 ± 87.3 / 969.8 ± 76.7
(µmol photon m^{-2} s^{-1})	At 15 m	661.3 ± 48.2
	At 21 m	352.8 ± 32.3
Surface water temperature (°C)		25.3 ± 0.3
Nutrients	Surface water NH$_4$ (nmol l^{-1})	50.8 ± 32.8
	Surface water NO$_2$ (µmol l^{-1})	0.085 ± 0.03
	Surface water NO$_3$ (µmol l^{-1})	0.84 ± 0.2
Environmental descriptors in adjacent waters		
Depth (m)	Temperature (°C)	PAR (µmol photon m^{-2} s^{-1})
6	26.5	966.7
9	26.4	918.3
15	26.4	342.8
21	26.3	309.9

ticular is characterised by a marked summer thermal stratification of the water column (with a maximum during July–September) (Carlson et al. 2014).

3.2. Biometry

Measurements of leaf lengths (averages of all leaves from all depths along each transect) revealed only small differences between transects (T-1 = 12.2 ± 0.7 mm; T-2 = 12.6 ± 0.7 mm). Leaf length displayed a similar pattern in both transects, with significant increases of 11% in T-1 and 12% in T-2 in leaves from shallow depths compared with their shallower counterparts from the same transect from shallow to

greater depths (ANOVA, p < 0.05) (Fig. 2, see Table 3).

3.3. Biochemical content and composition of *Halophila stipulacea*

Details of total fatty acid (TFA) content and composition of *H. stipulacea* leaves collected from the 2 transects (T-1 and T-2) across a depth gradient (6/9, 15, 21 m) are summarised in Table 2. Average TFA contents in samples from T-1 (1.39 ± 0.2% DW) and T-2 (1.42 ± 0.15% DW) were similar (ANOVA, p > 0.05) (Table 3, Fig. 3). In both transects, PUFAs accounted for the larger percentages of fatty acids at 66.0 ± 1.2%, with ALA (18:3 n-3; 45.4 ± 0.6%) and LA (18:2 n-6, 13.7 ± 0.4%) present as the major PUFAs (Fig. 4, Table 2). SFAs represented 25.1 ± 1.0% of TFAs, with palmitic acid (PAL; 16:0) being the main SFA at 23.4% of TFAs, followed by stearic acid (18:0) which accounted for 2.7% (Fig. 4, Table 2). Finally, monounsaturated fatty acids (MUFAs) accounted for 4.6 ± 0.3% of TFAs; the most abundant fatty acid being oleic acid (OLE; 18:1 n-9) (2.9 ± 0.2% of TFA) (Fig. 4, Table 2).

TFA content of leaves from both transects displayed similar trends, with significant increases from shallow- to deep-adapted plants; there were no marked significant (ANOVA, p > 0.05) differences between transects (Fig. 3). On the other hand, there were slight, but significant, differences in the percentages of both fatty acid groups (PUFAs and SFAs) along the transects (ANOVA, p < 0.01) (Fig. 4, Table 3). In both transects, TFA content increased significantly
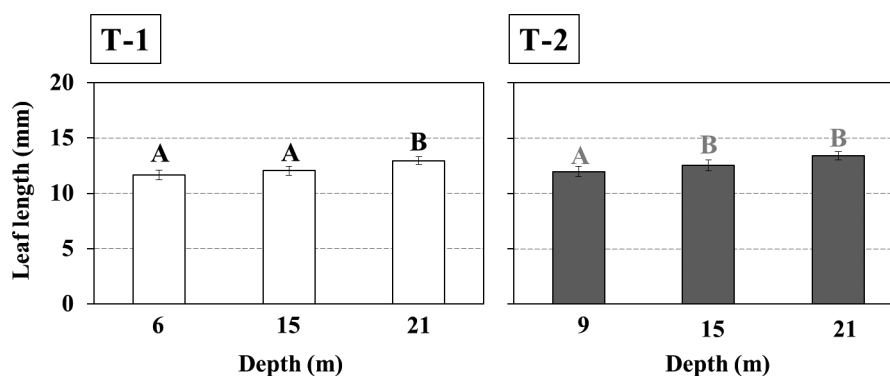

Fig. 2. Mean leaf length (3rd and 4th youngest leaves; see details in Fig. 1) of freeze-dried samples of *Halophila stipulacea* at each transect (T-1 and T-2) with depth. Letters indicate significant differences between depths (where black letters represent T-1; grey letters: T-2) according to the post hoc Tukey's pairwise test. Data are presented as means ± SD (n = 10)

Table 2. Total fatty acid (TFA) content (% dry weight, DW) and composition of saturated fatty acid (SFA), monounsaturated fatty acid (MUFA) and polyunsaturated fatty acid (PUFA) (% TFA) of *Halophila stipulacea* at transects T-1 and T-2 across a depth gradient (6/9, 15 and 21 m). 'Other FAs' represent non-identified fatty acids. Results are expressed as means ± SD (n = 3)

| | T-1 | | | T-2 | | |
	6 m	15 m	21 m	9 m	15 m	21 m
TFA (% DW)	1.16 ± 0.02	1.39 ± 0.02	1.61 ± 0.2	1.23 ± 0.05	1.44 ± 0.01	1.60 ± 0.1
SFA (% of TFA)						
14:0	0.5 ± 0.1	0.9 ± 0.01	0.4 ± 0.01	0.6 ± 0.05	0.5 ± 0.1	0.4 ± 0.1
16:0	23.0 ± 0.2	21.4 ± 0.4	22.3 ± 0.2	23.6 ± 0.2	19.8 ± 0.2	22.7 ± 0.3
18:0	2.0 ± 0.3	2.3 ± 0.1	2.0 ± 0.01	2.7 ± 0.05	2.5 ± 0.3	2.2 ± 0.1
Sum of SFAs	25.5 ± 0.3	24.6 ± 0.3	24.5 ± 0.3	26.8 ± 0.2	25.5 ± 0.3	25.3 ± 0.3
MUFA (% of TFA)						
14:1	0.6 ± 0.1	0.6 ± 0.1	0.7 ± 0.01	0.6 ± 0.05	0.6 ± 0.1	0.6 ± 0.05
16:1 n-7	0.8 ± 0.1	0.6 ± 0.1	0.7 ± 0.1	0.8 ± 0.1	0.8 ± 0.1	0.5 ± 0.3
18:1 n-7	0.5 ± 0.1	0.8 ± 0.1	0.4 ± 0.05	0.6 ± 0.1	0.5 ± 0.1	0.4 ± 0.05
18:1 n-9	3.2 ± 0.1	2.9 ± 0.6	2.4 ± 0.1	2.8 ± 0.05	3.2 ± 0.1	2.9 ± 0.1
Sum of MUFAs	5.0 ± 0.1	4.2 ± 0.2	4.3 ± 0.1	4.8 ± 0.1	5.0 ± 0.1	4.5 ± 0.2
PUFA (% of TFA)						
18:2 n-6	14.9 ± 0.4	12.9 ± 0.4	13 ± 0.2	13.3 ± 0.2	14.8 ± 0.4	13.5 ± 0.2
18:3 n-3	44.6 ± 0.5	47.6 ± 0.4	47.1 ± 0.2	45.1 ± 0.3	44.6 ± 0.4	47.3 ± 0.6
18:3 n-6	0.3 ± 0.05	0.3 ± 0.02	0.2 ± 0.02	0.2 ± 0.05	0.3 ± 0.05	0.2 ± 0.1
18:4 n-3	0.3 ± 0.03	0.4 ± 0.04	0.4 ± 0.1	0.5 ± 0.1	0.3 ± 0.01	0.3 ± 0.1
20:4 n-6	0.6 ± 0.1	1.1 ± 0.2	0.3 ± 0.02	0.7 ± 0.04	0.7 ± 0.5	0.6 ± 0.05
20:5 n-3	0.3 ± 0.05	0.6 ± 0.1	0.5 ± 0.1	0.4 ± 0.05	0.3 ± 0.1	0.4 ± 0.1
Sum of PUFAs	65.1 ± 0.4	67.8 ± 0.4	67.4 ± 0.2	64.0 ± 0.2	65.2 ± 0.3	66.2 ± 0.4
Other FAs (% of TFA)	4.3 ± 0.2	4.2 ± 0.2	4.9 ± 0.2	4.4 ± 0.2	4.3 ± 0.1	4.0 ± 0.3
n-3/n-6 PUFA	2.9 ± 0.2	3.4 ± 0.1	3.5 ± 0.1	3.1 ± 0.1	3.0 ± 0.1	3.3 ± 0.2
PUFA/SFA	2.6 ± 0.1	2.8 ± 0.1	2.8 ± 0.1	2.4 ± 0.1	2.6 ± 0.1	2.6 ± 0.3

with depth, from 1.16 ± 0.02 to 1.61 ± 0.2 % DW and from 1.23 ± 0.05 to 1.60 ± 0.1 % DW, respectively (ANOVA, p < 0.001) (Fig. 3, Tables 2 & 3). These changes were mainly associated with the markedly higher synthesis of PUFAs in plants growing at greater depths (15 and 21 m) (66.6 ± 07 % of TFA) than those from shallow depths (6 and 9 m) (64.6 ± 0.6 % of TFA) (ANOVA, p < 0.05) (Fig. 4, Table 2). For both transects there was a significant increase in the ratio of n-3/n-6 PUFAs from shallow- to deep-adapted plants (T-1 = 21.1 %, T-2 = 11.0 %). Levels of SFAs in *H. stipulacea* leaves were, on average, 6–7 % higher in plants from shallow depths (6 and 9 m), compared to those collected at 15 or 21 m (Fig. 4, Table 2). Finally, the percentages of MUFAs also changed with depth, with largest values observed at 6/9 m (4.9 ± 0.1 % of TFA) and lowest at 21 m (4.4 ± 0.1 % of TFA) (ANOVA, p < 0.01), although there were not marked differences between plants from T-1 and T-2 (Fig. 4, Table 2).

Photosynthetic pigments displayed slight, but significant, differences between T-1 (3.7 ± 0.3 mg g^{-1} DW) and T-2 (4.0 ± 0.3 mg g^{-1} DW) (Fig. 5, Table 3). In both transects, chl *a* was the most abundant photosynthetic pigment (T-1 = 2.2 ± 0.2 mg g^{-1} DW, T-2 =

2.5 ± 0.2 mg g^{-1} DW), followed by chl *b* (T-1 = 1.0 ± 0.1 mg g^{-1} DW, T-2 = 1.0 ± 0.1 mg g^{-1} DW), and finally the carotenoids (T-1 = 0.5 ± 0.1 mg g^{-1} DW, T-2 = 0.5 ± 0.04 mg g^{-1} DW). Overall, total pigment content significantly increased with depth (38 % in T-1, 18 % in T-2) (ANOVA, p < 0.05) (Fig. 5, Table 3). Specifically, chl *a* increased by 27 % on average, chl *b* by 30 % and carotenoids by 23 %.

3.4. Biochemical comparison across seagrass populations worldwide

Data of fatty acid composition compiled from the literature and unpublished data were used to assess similarities between *H. stipulacea* in the GoA and seagrass populations worldwide. Cluster and SIM-PROF analysis of fatty acid composition of 18 studies covering 11 species identified 4 distinct groups of seagrass species (Table S1 in the Supplement). One group, composed of *Thalassia hemprichii*, *Halophila ovalis* and *Posidonia sinuosa*, was characterised by the lowest levels of PUFAs (40.5–50.2 % DW) and low PUFA/SFA ratios (1.2–1.4); the remaining 3 groups were characterised by a higher content of PUFAs and

Table 3. Effect of location (T; transects T-1 and T-2) and depth (D; 6/9, 15 and 21 m) on the synthesis of total fatty acid (TFA) content (% dry weight, DW) and composition (% TFA), and on the production of total pigment level, chlorophyll *a* (chl *a*), chlorophyll *b* (chl *b*) and carotenoids (mg g^{-1} DW) and also on the length (mm shoot^{-1}) of leaves of *Halophila stipulacean*. *F*-values of 2-way ANOVA are shown along with significance levels (*p < 0.05; **p < 0.01, ***p < 0.001). PUFA: polyunsaturated fatty acid; SFA: saturated fatty acid; MUFA: monounsaturated fatty acid

		df	MS	*F*
PUFA	T	1	0.001	25.7**
	D	2	0.002	62.7***
	T × D	2	0.001	6.3*
SFA	T	1	0.01	21.7**
	D	2	0.01	21.4**
	T × D	2	0.00	2.9
MUFA	T	1	0.01	1.2
	D	2	0.03	5.5*
	T × D	2	0.01	1.6
TFA	T	1	0.00	1.0
	D	2	0.10	26.0***
	T × D	2	0.02	5.8*
Chl *a*	T	1	0.10	241.1***
	D	2	0.09	233.5***
	T × D	2	0.03	69.2***
Chl *b*	T	1	0.08	160.2***
	D	2	0.13	256.3***
	T × D	2	0.02	41.4***
Carotenes	T	1	0.12	141.7***
	D	2	0.07	82.5***
	T × D	2	0.04	44.7***
Total pigment	T	1	0.10	213.4***
	D	2	0.10	219.8***
	T × D	2	0.03	60.3***
Leaf length	T	1	0.02	11.9*
	D	2	0.06	45.3***
	T × D	2	0.00	0.1

higher ratios of PUFA/SFA (Fig. 6, Table S1). *H. stipulacea* was encompassed in a subgroup with *Posidonia oceanica* (Spain), *Zostera marina* (Russia, Sea of Japan) and *Cymodocea nodose* (Spain), with intermediate values of PUFAs (65.7–67.6%) and PUFA/SFA ratios (2.6–3.0). Finally, *Phyllospadix iwatensis* and 2 populations of *Z. marina* (Russia, Sea of Japan and China, Yellow Sea) formed another group, characterised by the highest values of PUFAs (72.2–74.3%) and SFA/PUFA ratios (3.8–4.1) (Table S1).

4. DISCUSSION

4.1. TFA content and composition in *Halophila stipulacea*

Pooled averages of TFA contents in samples collected from the 2 transects were similar, which could be expected due to their close proximity (less than 500 m apart; Fig. S1); meadows from both transects are exposed to similar environmental conditions and low anthropogenic pressures (Mejia et al. 2016, Winters et al. 2017). PUFAs were the dominant fatty acid group present in *Halophila stipulacea* leaves, with an average of 66.0 ± 1.2% of TFAs; the nutritionally important ALA and LA were the dominant PUFAs. PUFAs play an essential role in the health of consumers in the next trophic levels (Brett & Müller-Navarra 1997); for example, they regulate optimal physiological state in the reproductive and juvenile stages of several marine species (Tian et al. 2015). In the GoA, *H. stipulacea* biomass represents an important food source for different species, including sea urchins *Tripneustes gratilla* (Hulings & Kirkman 1982) and also some charismatic megafauna such as green turtles *Chelonia mydas* (Turkozan & Durmus 2000, Christianen et al. 2014, 2019).
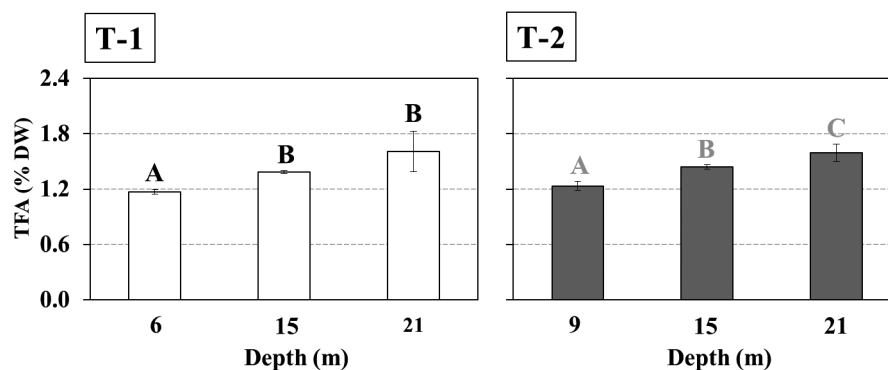

Fig. 3. Total fatty acid (TFA) contents (% dry weight, DW) of *Halophila stipulacea* collected from transects T-1 and T-2 across a depth gradient. Letters indicate significant differences between depths (where black letters represent: T-1; grey letters: T-2) according to the post hoc Tukey's pairwise test. Data are presented as means ± SD (n = 3)
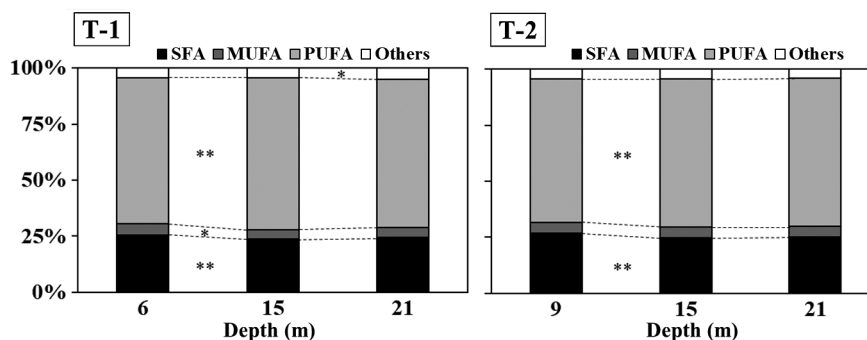
Fig. 4. Proportions of saturated (SFA), monounsaturated (MUFA), polyunsaturated (PUFA) and non-identified (Others) fatty acids (% total fatty acid, TFA) of individuals from transects T-1 and T-2. Differences between depths were evaluated with Student's *t*- test; *p < 0.05–0.01; **p < 0.01 (n = 3)
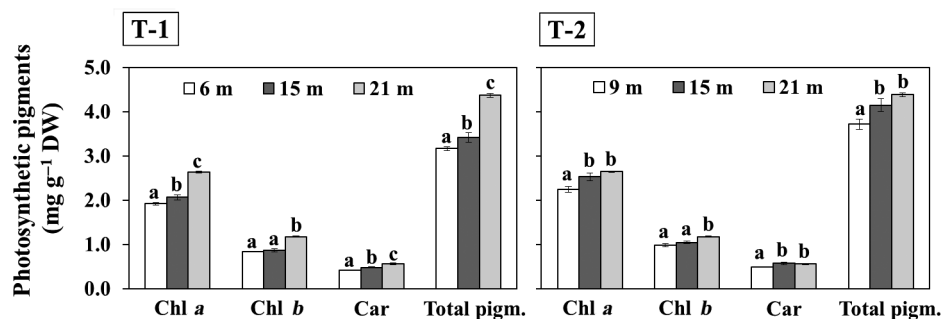

Fig. 5. Total pigment contents, chlorophyll *a* (chl *a*), chlorophyll *b* (chl *b*) and carotenoids (Car) (mg g^{-1} dry weight, DW) of transects T-1 and T-2. Letters above bars: significant differences among depths according to the post hoc Tukey's pairwise test (n = 3)

4.2. Effects of light attenuation on leaf biometry and synthesis of fatty acids

Our results demonstrated a slight but significant increase in leaf length of the *H. stipulacea* plants from shallow to deep areas, which were characterised by a reduction in light availability but not temperature. Previous studies of *H. stipulacea* have also reported this morphological plasticity, and have related it to the ability of the plants to compensate for reductions in PAR with increasing depths (Olesen & Sand-Jensen 1993, Lee et al. 2007, Sharon et al. 2011, Rotini et al. 2017). Results also revealed a high capacity of *H. stipulacea* to adjust fatty acid levels and composition to environmental light conditions at the different depths. In both transects, TFA content increased with depth; such changes were mainly associated with the higher synthesis of PUFAs in plants growing in deeper/reduced PAR habitats (15 and 21 m). Interestingly, the ratio n-3/n-6 PUFAs also increased significantly, pointing towards a major production in the content of fatty acids with a higher level of unsaturation. Differences in the lipid nutritional value of populations adapted to different light regimes could change the susceptibility of the leaves to herbivory depending on their biological or physiological requirements. For instance, previous studies have reported that some fish have higher demands for PUFAs during early life stages and in different

reproductive cycles (Izquierdo et al. 2001, Mazorra et al. 2003). In terrestrial plants, an increase in the synthesis of n-3 PUFAs, mainly ALA (18:3 n-3), was associated with decreases in temperature, increases in salt and pathogen stress (Yaeno et al. 2004, Upchurch 2008). Recent laboratory investigations into the temperate seagrass *Zostera marina* also found similar trends, with slight but marked increases of 8% of TFAs (% DW) and 7% of PUFAs (% TFA) in plants acclimated to low irradiance (60 µmol m^{-2} s^{-1}) compared to plants incubated at saturating (high) irradiance levels (180 µmol m^{-2} s^{-1}) at optimal temperatures for growth (16–20°C) (P. Beca-Carretero unpubl. data). In some macroalgae, a reduction in light availability induced remodelling of the lipids within membrane structures by increasing the content of TFAs, the proportion of PUFAs (e.g. ALA), and reducing the ratio of SFA (Schmid et al. 2014, 2017a,b). Also, under low light conditions PUFA synthesis and production is induced, and therefore phytoplankton species develop larger thylakoid membranes (e.g. Goss & Wilhelm 2010, Guihéneuf & Stengel 2013). This adaptive mechanism is expected, as PUFAs control chloroplast membrane fluidity and facilitate photosynthetic electron transport, thus promoting photosynthetic activity (Gombos et al. 1994, Moon et al. 1995). Taken together, these studies suggest that the regulation of the PUFA composition in photosynthetic structures is an essential mechanism
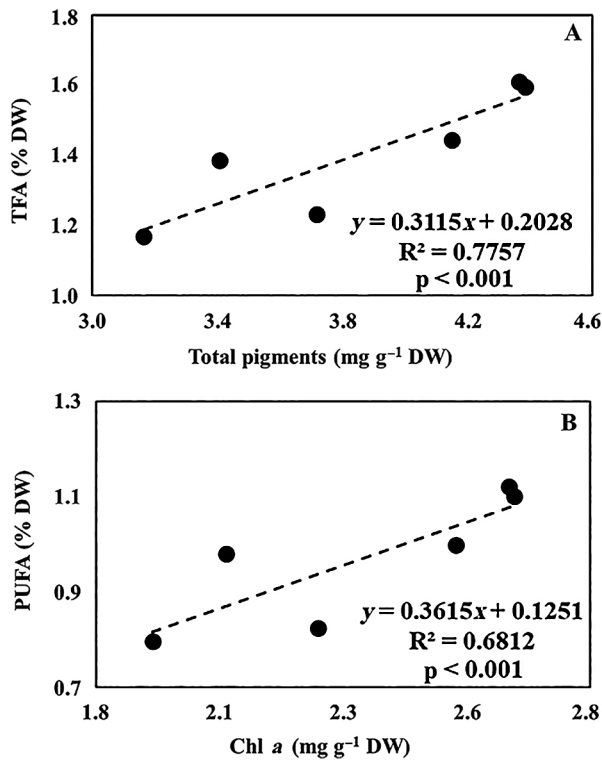
Fig. 6. Linear correlations between (A) total fatty acid (TFA) (% dry weight, DW) and total pigment (mg g^{-1} DW) contents, and (B) polyunsaturated fatty acid PUFA (% DW) and chl *a* (mg g^{-1} DW) contents. Dotted line: linear regression

of photosensitive primary producers to ensure their optimal physiological functioning under different irradiance regimes.

In seagrasses, increases in the production of SFAs were previously associated with their exposure to different environmental stressors, including increases in salinity and elevated temperatures. For instance, exposure of *Posidonia oceanica* and *Cymodocea nodosa* to artificial heat waves increased the synthesis and accumulation of SFAs (Sousa et al. 2017, Beca-Carretero et al. 2018). The production of TAGs, primarily constituted by SFAs, is a typical mechanism of primary producers to store the excess energy generated by photosynthesis, which increases under unfavourable growth conditions (Goncharova et al. 2004, Pal et al. 2011, Solovchenko 2012). Therefore, as *H. stipulacea* in this part of the northern GoA is distributed at depths ranging from 2 to >50 m (Sharon et al. 2011, Winters et al. 2017), the results presented here suggest that *H. stipulacea* plants at shallow depths experience some environmental stress which induces a higher proportion of SFAs. These observations are supported by previous studies in the same region of GoA (South Beach), which reported a

high photoinhibition effect in shallow *H. stipulacea* populations that negatively affected their growth performance (Mejia et al. 2016). The synthesis of SFAs in seagrass leaves may be considered to be readily accessible energy reserves in above-ground structures, as was previously observed in terrestrial plants and other marine primary producers (e.g. Alberdi & Corcuera 1991, Bravo et al. 2001, Pal et al. 2011, Solovchenko 2012).

4.3. Correlation between photosynthetic pigments and fatty acids

Recently, Rotini et al. (2017) reported higher pigment contents (chl *a* and *b*) in *H. stipulacea* as a function of increasing depth. Moreover, field transplantations of *H. stipulacea* to different depths (8 and 33 m and vice versa) revealed a rapid (within <7 d) physiological acclimatization that included an adjustment of photosynthesis (quantum yield efficiency of photosystem II; F_v/F_m) and pigment production, highlighting the high plasticity of this species to rapidly acclimate to changes in light availability (Sharon et al. 2009). The synthesis of higher levels of photosynthetic pigments is a well-described adaptive mechanism of seagrasses and other primary producers to increase photosynthetic efficiency in low-light habitats (Cummings & Zimmerman 2003, Lee et al. 2007).

Both total pigments (mg g^{-1} DW) and chl *a* (mg g^{-1} DW) were significantly correlated (p < 0.001, R^2 = 0.7) with TFA and PUFA contents (% DW), respectively (Fig. 7), as both components increased with depth. Taken together, these results highlight the ability of *H. stipulacea* to adjust its photosynthetic apparatus to optimise the use of the ambient light environments.

4.4. Fatty acid composition comparison across seagrass species

Cluster and SIMPROF analysis were used to compare fatty acid composition of seagrass species from different geographic regions. Our analysis revealed that other seagrass species that are adapted to similar ranges of temperature (21–27°C; Winters et al. 2017), such as species belonging to the same genus, *Halophila ovalis* (48.12% of TFAs, SST = 21.5°C) or *Thallassia hemiprichii* (40.63% of TFAs, SST = 26.1°C) or *Posidonia sinuosa* (50.21%, SST = 21.5°C), contained significantly lower levels of PUFAs than shallow *H. stipulacea* (64.6 ± 0.6% of TFAs) (Nichols & John 1985, Hanson et al. 2010, Beca-Carretero et al. 2018).
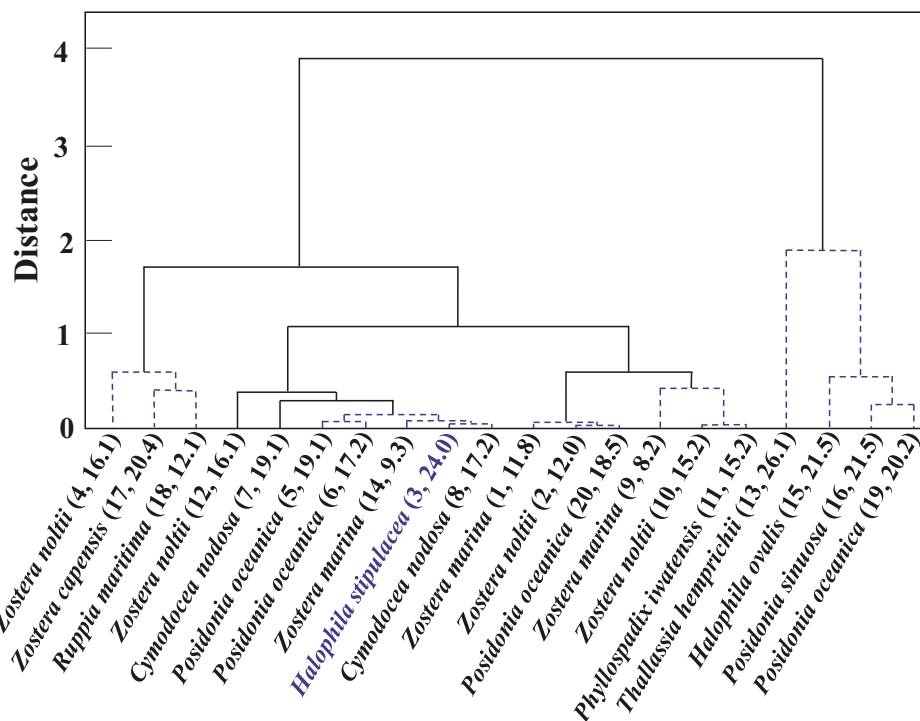
Fig. 7. Cluster and similarity profile (SIMPROF) analysis of 20 seagrass populations (10 species, 5 families) from different latitudinal locations worldwide based on content of polyunsaturated fatty acids (PUFAs; % dry weight, DW) and PUFA/saturated fatty acid (SFA) ratio. Numbers after species names: literature source and annual average sea surface temperature (°C) from each location, respectively (Table S1)

By contrast, PUFA levels and PUFA/SFA ratios of *H. stipulacea* seemed to be equivalent to Mediterranean temperate species such as *P. oceanica* or *C. nodosa* (68.3% of TFAs, SST = 17.5°C), or even to species living at higher latitudes and much colder environments such as *Zostera noltii* (64.8% of TFAs, SST = 16.8°C) (Viso et al. 1993, Coelho et al. 2011, Beca-Carretero et al. 2018). These outcomes suggest that the higher levels of PUFAs (e.g. ALA and LA) and PUFA/SFA ratios observed in *H. stipulacea* renders this species of higher lipid-related nutritional value than other seagrass species adapted to similar temperatures and latitudes, including *Halophila* spp. In terrestrial plants, species with the capacity to biosynthesize higher levels of PUFAs were able to adapt better to colder environments than species which produced lower contents of PUFAs (Alberdi & Corcuera 1991, Routaboul et al. 2000, Iba 2002). It has been widely demonstrated that the different levels of unsaturation in the bio-membranes is the most relevant factor controlling the fluidity of membrane lipids of plants (e.g. Cossins & Sinensky 1984, Sanina et al. 2004, Upchurch 2008) and this is related to their thermal physiological limits for growth and survival (Iba et al. 2002). In seagrasses, the synthesis of PUFA was

also linked to their thermal adaptation (decrease of 2.12% of PUFAs [% of TFA]) per 1°C increase in SST (Beca-Carretero et al. 2018). Thus, the anomalously high levels of PUFAs within *H. stipulacea*, producing higher contents than other tropical or subtropical species, including cohabiting species (i.e. *Halophila ovalis*) (Hulings & Kirkman 1982, Campbell et al. 2015), may partially explain its capacity to spread to colder habitats outside its native range. For example, this species is able to survive winter temperatures in Mediterranean Sea at ~13–16°C (www.bio-oracle.org) (Gambi et al. 2009, Tyberghein et al. 2012, Nguyen et al. 2018), while in native areas winter temperatures reach approximately ~21°C. Moreover, in both native and non-native areas, *H. stipulacea* displays a great capacity to survive in environments with low irradiance levels, having been observed as the seagrass species living at greatest depths or in areas with reduced water transparency and quality such as harbours or polluted areas (e.g. Kenworthy et al. 1993, De Troch et al. 2001, Ruiz & Ballantine 2004, Gambi et al. 2009, Willette et al. 2014). Finally, recent field observations suggest that *H. stipulacea* may be potentially displacing the native *C. nodosa* in the Mediterranean Sea along the Tunisian coasts (Sghaier et al. 2014).

5. CONCLUSIONS

Increases in total fatty acids, PUFA/SFA ratios and n-3/n-6 PUFA ratios were observed with reductions in PAR. Also, our data show that production of PUFAs in seagrass leaves was correlated with photosynthetic pigment production, suggesting that fatty acid production, and particularly PUFAs, facilitate an adaptation to reduced light availability. The biochemical plasticity, regarding both fatty acids and photosynthetic pigments, of *H. stipulacea*, alongside morphological changes, may explain the capacity of this species to colonize an extensive range of light environments. Moreover, our results highlight that the tropical *H. stipulacea* has a considerable ability to produce high PUFA levels equivalent to those in seagrass species inhabiting higher latitudes/colder regions, indicating that high PUFA levels may aid the survival of this species in colder regions. Furthermore, this study suggests that fatty acid composition and content can be used to classify different groups of seagrass species according to their biochemical composition. This work constitutes a new approach to the assessment of seagrasses and a stepping stone in the application of biochemical indicators in a changing environment.

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